THE USE OF *DUDDINGTONIA FLAGRANS* FOR GASTROINTESTINAL PARASITIC NEMATODE CONTROL IN FECES OF EXOTIC ARTIODACTYLIDS AT DISNEY'S ANIMAL KINGDOM®

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Dedicated to

Joey Cavalier

The Terry Family

The Cavalier Family

My Friends and Classmates

My Wacky Cats, Lazy Dragons, and Stella

"Let me tell you something you already know. The world ain't all sunshine and rainbows. It's a very mean and nasty place and I don't care how tough you are it will beat you to your knees and keep you there permanently if you let it. You, me, or nobody is gonna hit as hard as life. But it ain't about how hard ya hit. It's about how hard you can get hit and keep moving forward. How much you can take and keep moving forward. That's how winning is done!"
-Rocky Balboa

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ABSTRACT

Gastrointestinal nematodes (GIN) are parasites of major concern for domestic and exotic ruminant species around the world. In the past, zoological facilities used anthelmintics as their primary control method. Challenges in accurate dosing and administration of anthelmintics to exotic hoofstock contributed to the development of resistant nematode populations in zoological settings. The historic dependency on anthelmintics to control GIN populations is no longer an option. Biological alternatives are urgently needed, in both exotic and domestic ruminants, in the war against resistance. One such alternative is the use of the nematophagous fungus, *Duddingtonia flagrans*. Three independent studies were conducted: A nine day study in the spring of 2010, a nine day study in the spring of 2011, and a 12 week study in the summer of 2011. The first study evaluated the efficacy of *D. flagrans* chlamydospores, as a suspension mixed into feed, in reducing infective GIN in feces at a dose of 500,000 chlamydospores per kg/BW administered for 4 consecutive days to giraffe and antelope. The second and third studies evaluated the efficacy of a powdered mixture containing *D. flagrans* chlamydospores incorporated into feed in reducing infective GIN in feces at a dose of 30,000 chlamydospores per kg/BW administered for 4 consecutive days and 8 weeks, respectively, in giraffe, antelope, and gerenuk (study 3 only). For studies 1 and 2, fecal samples were collected daily to monitor fecal egg count and percent reduction of infective larvae (L3) in fecal cultures. For study 3, samples were collected on a weekly basis. Results from all 3 studies indicated that *D. flagrans* was effective in reducing L3 in the feces during the period of feeding, The results from these studies demonstrated that the use of *D. flagrans* in exotic artiodactylids infected with GIN could be a long term prophylactic tool to reduce forage infectivity. Used in conjunction with other control methods, *D. flagrans* could be part of the future of GIN parasite control in zoological facilities.

CHAPTER 1
INTRODUCTION

Gastrointestinal nematodes (GIN) are parasites of major concern for many species of ruminants around the world. Sheep and goats, as well as other livestock, are some of the domestic ruminant species that are greatly affected by GIN. Production losses as well as decreased animal health are the greatest concerns with these parasites. Diarrhea, anemia, weight loss, decreased reproduction, decreased production (e.g. wool quality, milk production, etc.), and increased morbidity and mortality are just some of the devastating losses that GIN can create (Pugh and Baird, 2011). In the domestic small ruminant industry worldwide, there is an estimated production loss extending into the millions of dollars each year. Exact values are difficult to obtain due to the many factors that affect loss which include management, nutrition, environmental factors and stress, genetics, and concurrent disease (Vlassoff et al., 2001).

Exotic or nondomestic ruminant species such as giraffe, roan antelope, deer, oryx, sable antelope, blackbuck, bongo, okapi, and wildebeest are a few of the many species of artiodactylids that also exhibit GIN infections. Gastrointestinal nematode infections are not as severe and numerous in wild artiodactylids in their natural habitats as they are in captive artiodactylids in zoological facilities. Reasons for increased numbers of GIN and severity of infection in captive artiodactylids are due to stress, the lack of browse in enclosures, increased stocking rates, irrigation, and the inability to close exhibits. Stressful conditions caused by captivity can decrease the function of the immune system and increase the body's exposure to parasites and diseases (Fagiolini et al., 2010). In zoological facilities, guests must be able to view animals in their enclosures. Due to this reason, there is a decreased availability of browse which allows animals that are natural browsers in the wild, such as giraffe, to graze instead.

Grazing on contaminated savannahs or pastures drastically increases the chance that an animal will be infected with infective stage larvae of GIN. Increased stocking rates allow for high levels of savannah or pasture fecal contamination, less area to move away from contaminated feces, and thus induces a high density infective parasite area. Irrigation is used to enhance vegetation for aesthetic purposes as well as increase food supply for animals. The increased moisture allows for optimal environmental conditions for GIN to thrive. The inability to close enclosures to rest for rotation also poses a problem in zoological facilities because it allows for the vegetation to be eaten at very short lengths where the larvae climb, and it does not allow for the existing larvae to die off (Young et al., 2000).

Losses and clinical disease are similar in both domestic and exotic ruminants. The major concern with exotic ruminants is a decrease in animal health and/or death. Many zoological facilities are concerned with animal conservation, and a loss due to parasitism caused by GIN can be a costly one. The average cost of a mature female giraffe is estimated to be around $30,000.00.

Within the nematode Superfamily Trichostrongyloidea, there are several species that affect both domestic and nondomestic ruminants, and the five major species are *Haemonchus* spp., *Teladorsagia* spp., *Ostertagia* spp., *Cooperia* spp., and *Trichostrongylus* spp. Of these parasitic species, *H. contortus,* a parasite of the abomasum, is the most important with respect to economic impact and clinical disease because it is a blood feeding parasite that can cause severe anemia in animals. This particular parasite is endemic to the Southeastern United States as well as other areas that have climates of high temperature and humidity (Soli et al., 2010).

Traditionally, the three classes of anthelmintic drugs are the benzimidazoles, the nicotinic antagonists, and the macrocyclic lactones. They have been relied on to decrease or eliminate

GIN burdens in domestic ruminants, such as sheep and goats. The same practice of using anthelmintic drugs was also established in zoological facilities for exotic ruminants. Zoological parasite control programs were based on an empirical, rotational drug program (Fontenot and Miller, 2011). Accurate dosing and administration of anthelmintic drugs proved to be a challenge for veterinarians and keepers in zoological facilities due to varying compliance of orally administered medications, estimated body weights as well as unknown pharmacokinetic data (Fontenot et al., 2008). For these reasons, anthelmintic resistance has become the most recent problem for the nondomestic/exotic ruminant zoo industry. Similar reasons, such as overuse of anthelmintics, subtherapeutic doses, and poor pasture management, have caused resistant GIN populations in domestic ruminants as well. The first published case of resistant *H. contortus* to all three drug classes available in the United States was reported by Terrill et al. (2001).

Resistance to anthelmintics has been reported across the globe and includes, but is not limited to Australia, New Zealand, Africa, and Europe. The first documented case of resistant strains of *H. contortus* dates back as early as 1964 in the United States (Conway, 1964). Other anthelmintic resistant GIN includes *Teladorsagia*. Of the anthelmintic resistant GIN, *H. contortus* is the most important due to its high prevalence in the United States and its blood feeding. Resistance is the product of Mendelian genetics. Resistant worms that survive anthelmintic treatment reproduce, which passes the resistant genes onto the next generation of worms. The worm population eventually saturates with resistant genes and leads to drug failure (Sangster, 1999). Cross resistance and multiple resistance have made matters even more complicated in regard to GIN control. Anthelmintics are no longer an inexpensive tool to

increase animal production and decrease GIN. They must now be viewed as very valuable chemical resources that should be used only in a selective manner (Kaplan, 2006).

The problem of resistant GIN across the globe has resulted in an urgent need for other control methods. Nonchemical biological alternatives to anthelmintics are becoming increasingly popular as a method to help control GIN. The most investigated of these biological alternatives are the nematode-trapping fungus *Duddingtonia flagrans*, copper oxide wire particles (COWP), and condensed tannins. These alternatives used in combination with integrated strategies can help to decrease the heavy reliance on anthelmintics.

Duddingtonia flagrans is a nematode trapping fungus that traps the free living stages of larvae in feces. *D. flagrans* has an advantage over other nematode trapping fungi because of its ability to produce large amounts of thick-walled chlamydospores (Mendoza de Gives et al., 1998). These thick-walled chlamydospores have the ability to pass through and survive the digestive tract of animals, including ruminants. Once the chlamydospores inoculate the feces, they form a network of hyphae which serve as sticky traps that inhibit the free living stage of larvae from completing its lifecycle (Fontentot et al., 2003). This method of nonchemical biological control reduces pasture contamination and therefore should reduce the amount of viable infective larvae that are able to complete their life cycle within animals.

The objectives of this study were 1) to determine the efficacy of chlamydospores of a Mexican isolate of *D. flagrans* at a dose of approximately 500,000 chlamydospores/kg of body weight of exotic artiodactylids in reducing infective GIN larvae (L3) in feces, and 2) to determine the efficacy of chlamydospores of a dry, powdered Australian isolate of *D. flagrans* at a dose of approximately 30,000 chlamydospores/kg of body weight of exotic artiodactylids in reducing L3 in feces.

CHAPTER 2
LITERATURE REVIEW

2.1. Trichostrongyle Type Parasites

The Superfamily Trichostrongyloidea is the most important group of parasites in the ruminant industry. These bursate nematodes cause significant production losses as well as a decrease in overall animal health and even death. Members of the trichostrongyle type parasite group include *Haemonchus* spp., *Cooperia* spp., *Trichostrongylus* spp., *Telardorsagia* spp., and *Ostertagia* spp. These GIN have a direct life cycle, meaning that no intermediate host is required for the completion of the life cycle.

Of these parasites, *H. contortus* causes the most pathology within animals and they are prolific egg layers. The L3 of *H. contortus*, after ingested by the animal, migrate to the lumen of the abomasum and penetrates the mucosa. After a molt, fourth stage larvae (L4) return to the lumen and mature to adults. Both L4 and adults are blood feeders and ingest the seeping blood from the mucosal disruption these stages cause (Ballweber, 2001). This can cause many problems in its host, but most importantly it results in anemia. For this reason, *Haemonchus* spp. are considered the most important of the bursate GIN. This parasite can also cause decreased growth, edema (bottle jaw), emaciation, poor production, weight loss, intestinal disturbances, and even death resulting from the loss of blood and hemolytic proteins that are injected into the host's system (Roberts and Janovy Jr., 2005).

Haemonchus spp. thrive in subtropical and tropical environments. They can also survive in more temperate regions where animals are housed during milder climates (Pugh et al., 1998). The southeast portion of the United States serves as an optimal environment for these parasites. *Haemonchus* spp. are large nematodes reaching a length of 10 to 30 mm in their adult stage (Love and Hutchinson, 2003). *Haemonchus* spp. are commonly referred to as the barberpole

worm because of the appearance of the female's white ovaries twisting around its red intestines. This parasite is most commonly found in small ruminants such as sheep and goats, but also has an affinity for cattle as well as nondomestic/exotic artiodactylids. For this reason, this nematode not only has a significant negative impact on the domestic ruminant industry, but it also negatively affects game farms, wildlife reserves, and zoological facilities. Other members of the Superfamily Trichostrongyloidea can also infect nondomestic/exotic artiodactylids as well as domestic ruminants.

2.2. Life Cycle of Trichostrongyle Nematodes

The life cycle of all bursate nematodes are very similar. The direct life cycles of these parasites require no intermediate host. Female worms in the GI tract of the ruminant produce eggs which pass through the remaining GI tract and are deposited into the feces. *Haemonchus* spp. females are known for their high fecundity and produce an estimated 5,000-10,000 eggs per day (Nolan, 2006). Once the eggs are deposited into the feces, they hatch, given the appropriate environmental climate. The first stage larvae (L1) molt into second stage larvae (L2) and then into infective third stage larvae (L3). The L1 and L2 larvae feed on organic matter in the feces whereas the L3 larvae are unable to feed due to the retention of the cuticle from the L2 stage. This cuticle serves as a protective sheath from extreme environmental conditions and can extend the survival of the larvae until optimal conditions are present for further development. (Pugh et al., 1998).

When proper moisture, via rain, dew, or irrigation, is available, the L3 migrate out of the feces and onto surrounding vegetation. The ruminant becomes infected by consuming the L3 while grazing on pasture. Once ingested, the L3 travel to the rumen and exsheath the retained cuticle. The L3 of *Haemonchus* spp., *Ostertagia* spp., *Teladorsagia* spp., and *Trichostrongylus*

axei move into the abomasum of the animal via the ingesta. In the case of *Cooperia* spp. and other *Trichostrongylus* spp., this takes place in the small intestine (Roberts and Janovy Jr., 2005). The L3 larvae then penetrate into the mucosa of their preferred GI organ. Within the time frame of a few days, the L3 molt to L4, then migrate back into the lumen (Pugh et al., 1998). These larvae are able to feed on blood because they now have a small buccal capsule (Roberts and Janovy Jr., 2005). The L4 larvae then molt into immature adults given the proper environmental conditions. If harsh conditions are present, the L4 larvae are able to go into arrested development (also known as hypobiosis) in the mucosa and remain there for up to 4 months. Once proper climate conditions are present, development of the L4 larvae resume (Pugh et al., 1998). The immature adults then develop into mature reproducing adults which produce eggs that deposit in the feces. The lifecycle of the trichostrongyle type nematode, which takes approximately 30-42 days, is now complete.

2.3. Anthelmintic Control of Gastrointestinal Nematodes

Before the 1940s, natural substances were used to help reduce GIN burdens in animals. These natural substances, such as tobacco, arsenic, and mercury, not only killed the worms but also were toxic to the animals in many cases. In the mid 1900's, broad spectrum anthelmintics were introduced as a way to control GIN burdens (Howell et al., 2008). Anthelmintics are drugs that target helminths in the animal's body and are selectively toxic to the worms. This is usually achieved by exposing the nematodes to higher concentrations of anthelmintics without exposing the host cells to high concentrations and by inhibiting vital metabolic processes of the nematodes hence paralyzing and/or starving the worms (Kahn, 2005). Ideally, anthelmintics should be easy to administer, be cost effective for the producer, have a broad spectrum of activity against larval

and adult stages of parasites, have a post prophylactic effect, be compatible with other compounds, and have little to no withdrawal period due to drug residues (Kahn, 2005).

Anthelmintics, also referred to as dewormers, are divided into 3 major chemical classes which are based upon the drug's mode of action and chemical structure. The three classes of anthelmintics are the benzimidazoles, the nicotinic agonists, and the macrocyclic lactones. Two new anthelmintic classes, the amino-acetonitrile derivatives [AAD] which contains the drug monepantel (Zolvix®), and the spiroindoles [SI], are currently being introduced in other countries but have not yet been released in the United States (George et al., 2012).

2.3.1. Benzimidazoles

The first class of broad spectrum anthelmintics introduced was the benzimidazoles. Benzimidazole is formed by the fusion of benzene and imidazole. Thiabendazole (TBZ®), the first drug in this class, was introduced in the early 1960's (Bogan and Armour, 1987). Other drugs in this class include fenbendazole (Panacur®, Safeguard®), albendazole (Valbazen®), oxfendazole (Synanthic®), mebendazole, flubendazol, oxibendazole, albendazole sulfoxide, thiophanate, febantel, netobimin, and triclabendazole (Kahn, 2005). Benzimidazoles have wide safety margins, meaning that the doses at which adverse effects are seen are much higher than the recommended dose.

Currently, thiabendazole (TBZ®) and albendazole (Valbazen®) are approved anthelmintics for use in sheep by the Food and Drug Administration (FDA), however thiabendazole is no longer available due to lack of efficacy against modern resistant GIN (Schoenian, 2012). Fenbendazole (Safeguard®) is FDA-approved for use in goats, but is not approved for use in sheep. Fenbendazole and oxfendazole are sometimes used "extra-label" in sheep because of the current problem of resistant GIN (Rook, 2009).

The benzimidazoles, also called the white dewormers, work by impairing nematode cell structure and metabolism. This class of anthelmintic inhibits polymerization of β-tubulin, a protein, by binding to it. The polymerization of β-tubulin is vital for cell structure, cellular transport, and energy metabolism (Kahn, 2005). The inhibition of tubulin polymerization at the positive pole leads to microtubule depolymerization at the negative pole by essentially capping the microtubules thus decreasing their total length (Martin, 1997). The binding to β-tubulin and consequent inhibition of microtubule formation in the intestinal cells of the parasite decreases cell transport, decreases glucose uptake, and causes stored glycogen to be utilized faster (Martin, 1997). This essentially starves the worm and causes an inhibition of egg production.

Benzimidazoles are considered to be true "broad spectrum" anthelmintics. Members of this class are effective against adult, immature adults, larval stages, and inhibited larval stages of GIN (Bogan and Armour, 1987). Benzimidazoles also exhibit some activity against immature and mature stages of *Fasciola hepatica* (liver flukes) as well as *Moniezia* spp (tapeworms) in sheep and cattle (Kahn, 2005).

This class of anthelmintic is most effective if received directly into the rumen, rather than the abomasum via the esophageal groove. Bypass of the rumen, which serves as a holding reservoir, via the esophageal groove and deposition directly into the abomasum shortens drug absorption time and increases the rate at which it is excreted from the body therefore reducing efficacy of the drug (Kahn, 2005). To increase the efficacy of this anthelmintic class, pretreatment fasting is suggested 24 hours before dosing the animal. The flow rate of ingesta is reduced allowing the anthelmintic to stay in the GI system longer which increases contact time between the drug and worms (Hennessy, 1997). Additionally, repeated deworming (2-3 times) with full doses given 12 hours apart is suggested to prolong contact time (Kahn, 2005).

2.3.2. Nicotinic Agonists

The second class of anthelmintics are nicotinic agonists and includes the imidazothiazole and tetrahydropyrimidine groups. The imidazothiazoles group includes levamisole (Prohibit®, Levasol®, and Tramisol®) and the tetrahydropyrimidine group includes morantel (Rumatel®,and Nematel®) and pyrantel (Strongid®) (Schoenian, 2012).

Currently, levamisole is the only FDA-approved anthelmintic in this class for use in sheep, and morantel is the only FDA-approved drug in this class for use in goats (Schoenian, 2012). Pyrantel, in the tetrahydropyrimidine group, is often used "extra-label" in sheep due to high prevalence of GIN (Rook, 2009). Levamisole has a narrower margin of safety than anthelmintics in the benzimidazole class.

The nicotinic agonist class works by acting as agonists at synaptic and extrasynaptic nicotinic acetylcholine receptors on the muscle cells of GIN (Martin, 1997). Nicotinic agonists mimic the paralytic effects of excessive amounts of acetylcholine, a natural neurotransmitter; nicotine can also initiate similar paralytic effects (Riviere and Papich, 2009). Nicotinic agonists have a similar mode of action as nicotine; hence the anthelmintics' effects are described as nicotine-like.

Levamisole, the sole member of the imidathiazole group, acts as a ganglion stimulant, and is known as a cholinergic agonist. The stimulant activity of levamisole excites ganglion-like structures in muscle cells of the nematode by selectively gating acetylcholine receptor ion channels which creates sustained muscle contractions (Riviere and Papich, 2009). This leads to a neuromuscular depolarizing blockade, which is followed by paralysis and eventually death of the nematode (Kahn, 2005). The tetrahydropyrimidine group has a similar mode of action as the imidathiazole group. These anthelmintics act as agonists at excitatory nicotine acetylcholine

(nACh) receptors in nematode muscle cells and induce contractions and spastic paralysis in the nematode (Riviere and Papich, 2009).

The nicotinic agonists are effective in ruminants primarily against GIN. Levamisole also has some efficacy against lungworms. Levamisole exhibits activity against adult and larval stages, but lacks efficacy against hypobiosed larvae. The tetrahydropyrimidine group exhibits activity against primarily adult GIN (Kahn, 2005).

Levamisole is usually administered orally or subcutaneous, and the efficacy is usually equivalent regardless of the route of administration. The tetrahydropyrimidine group is usually administered orally and efficacy of aqueous solutions depends on exposure to light (Kahn, 2005).

2.3.3. Macrocyclic Lactones

The third class of anthelmintics, the macrocyclic lactones, was introduced in the early 1980's (Holden-Dye and Walker, 2007). This class is comprised of two chemical groups, avermectins and milbemycins. The avermectin group includes ivermectin (Ivomec® and Primectin®), eprinomectin (Eprinex®), and doramectin (Dectomax®). The milbemycin group includes the drug moxidectin (Cydectin® and Quest®). Macrocyclic lactones generally have a wide margin of safety (Schoenian, 2012). Ivomec, Primectin, and Cydectin are FDA-approved for use in sheep. Others are used "extra-label" due to resistance issues.

The macrocyclic lactones act against GIN by binding to glutamate-gated chloride channel receptors which are located in the nerve cells of the parasite; host animals do not possess this ion channel (Martin, 1997). The glutamate-gated chloride channel opens and allows an influx of chloride ions. This is followed by paralysis of the pharynx which inhibits pharyngeal pumping that is vital for feeding. Additional effects include paralysis of the body wall and uterine muscles which inhibits motility and reproduction of the nematode. Macrocyclic lactone

concentrations affect the interval of time that nematodes are immobilized, as mobility may be regained as concentrations decreases, but generally the pharynx of the nematode is more sensitive to these drugs and exhibits extended periods of paralysis which causes death due to inhibition of feeding (Kahn, 2005). Macrocyclic lactones have been shown to have more than one site of action as they also inhibit GABA-activated channels (Martin, 1997).

Macrocyclic lactones are also called endectocides because they are effective against a broad range of endoparasites as well as ectoparasites. The macrocyclic lactones are effective against all stages of GIN, including hypobiosed larvae, but are ineffective against cestodes and trematodes (Junquera, 2013).

The routes of administration for the macrocyclic lactones include oral, subcutaneous, and pour on. Macrocyclic lactones are known to have prolonged efficacy which is important for protection from reinfection. All routes of administration are well absorbed and are distributed throughout the body with a particular concentration in adipose tissue; however, body condition of the animal may have an effect on the time of persistence if administered subcutaneous (Kahn, 2005). An increase in efficacy can be achieved by practicing feed withdrawal before oral administration.

2.3.4. Amino-Acetonitrile Derivatives (Monepantel)

A new class of anthelmintics, the amino acetonitrile derivatives (AAD), was discovered in the year 2000. The first drug is this class, monepantel (Zolvix®), was developed by Novartis Animal Health after testing over 700 AAD products to find a product that could demonstrate optimal efficacy, safety, and fight against resistant and multi-resistant nematodes (Novartis Animal Health, 2010). The drug became available for use in sheep in New Zealand in 2009 (Leathwick, 2012).

Zolvix® works by binding to a newly discovered receptor, Hco-MPTL-1, present only in nematodes (Novartis Animal Health, 2010). This causes paralysis and death in the nematodes. Zolvix® is considered to have a wide margin of safety (over 10 times the maximum recommended dose) in sheep; it also provides no apparent risk to humans or the environment because the newly discovered receptors are only in nematodes (Novartis Animal Health, 2010).

Zolvix® is effective against adult nematodes, fourth stage larvae, and hypobiosed 4th stage larvae (Stein et al., 2010). This newly discovered anthelmintic has been found to be effective against nematodes that are currently resistant to benzimidazoles, nicotinic agonists, and macrocyclic lactones because of its mode of action (Stein et al., 2010). Zolvix® is labeled as a ready-to-use oral solution (Novartis Animal Health, 2010). Zolvix® is not currently available for use in the United States.

2.3.5. Spiroindoles

The most recent class of anthelmintics, the spiroindoles (SI), was released in the year 2010. Currently, there is only one drug in this class, derquantel. Derquantel is used in combination with abermectin, a member of the macrocyclic lactone class, in an oral drench; this provides a spectrum of anthelmintic activity including activity against resistant and multiresistant nematodes (Sargison, 2012).

Derquantel works by binding to acetycholine receptors, and causing paralysis of the nematodes' muscles and rapid death of the nematodes (Ruiz-Lancheros et al., 2011). The derquantel and abermectin combination oral drench is effective against all adult nematodes, L4, lungworms, nasal botflies, and itch-mites (Winter and Clarkson, 2012). Currently, the spiroindole class of anthelmintics is only available for use in New Zealand.

2.4. Anthelmintic Resistance

The most important health problem plaguing the small ruminant industry is GIN. In the past, GIN have been managed by the administration of anthelmintics (Kaplan, 2006). The past and current reliance on anthelmintics has caused a problem of GIN resistance to anthelmintics. Resistance is generally defined as a decrease in anthelmintic efficiency against parasites that are usually susceptible to the anthelmintic (Sangster and Gill, 1999).

Many factors contribute to resistance, with the frequent and overuse of anthelmintics being a primary contributor. A lack of refugia, and the underdosing of animals with anthelmintics have also contributed to the growing problem of resistance. The overuse of anthelmintics was partly due to the mentality that anthelmintics should be used to maximize animal productivity and health. The frequent use of anthelmintics resulted in a population of resistant GIN. These resistant GIN passed their genes on to the next generation therefore making their offspring resistant to anthelmintics as well. The persistent use of the same anthelmintic used against nematodes that already exhibited resistance to that anthelmintic resulted in a larger population of nematodes that were resistant to that class of anthelmintic (Pugh et al., 1998). The lack of refugia, or portion of the nematode population that is not selected for by anthelmintic treatment, creates a pool of resistant genes. Maintaining a pool of susceptible genes to dilute out resistant genes on pasture is vital in the battle against drug resistance (Kahn, 2005).

Resistance of GIN to anthelmintics is a global problem. Some regions of the world are more likely to exhibit resistance than others, with subtropical and tropical climates being more probable for resistance (Pugh et al., 1998). The southeastern portion of the United States is a prime climate for a parasite notorious not only for its detrimental effects on ruminants but for its resistance to anthelmintics, *H. contortus*.

The first case of resistance was reported in the United States in 1964 (Conway, 1964). The blood feeding parasite, *H. contortus*, was shown to be resistant to thiabendazole (Waller, 1994). Shortly after, reports of resistance began surfacing in Australia. Since then, resistance has spread across nematode phyla and across the major chemical classes of anthelmintics. Resistance to all anthelmintics in the benzimidazole class has been documented in the United States, Australia, New Zealand, South Africa, and England. Resistance to the nicotinic agonist class has also been recognized worldwide as well as resistance to the macrocyclic lactones (Waghorn et al., 2006).

Anthelmintics within a drug class usually exhibit similar modes of action. Resistance of GIN to one anthelmintic in a class can carry over to other anthelmintics in the same class, creating cross-resistance (Kahn, 2005). Multiple resistance is also a common occurrence. This occurs when a nematode develops resistance to multiple anthelmintic classes after exposure. The first case of multiple-drug resistant *H. contortus* was published in 2001 (Kaplan, 2006b).

The two most recent classes of anthelmintics, the amino-acetonitrile derivatives and spiroindoles, are used to treat GIN that exhibit resistance to the older anthelmintic classes. However, these new anthelmintics, developed nearly 25 years after the macrocyclic lactones emerged, are only available in certain countries, and their use is limited as to not perpetuate the issue of growing resistance (Leathwick, 2012). Strategic use of these anthelmintics, in conjunction with other anthelmintics that are effective, is imperative in the fight against resistant GIN (Papadopoulos et al., 2012). Alternatives to anthelmintics, such as biological alternatives and vaccines, are currently being explored to aid in the fight against GIN in ruminants.

2.5. Artiodactylids

The order Artiodactyla is composed of even-toed ungulates (hoofed animals) and includes such animals as pigs, hippopotamuses, deer, camels, sheep, goats, cattle, giraffe and antelope. Artiodactyla is divided into three suborders: Suina (pig group), Tylopoda (camel group), and Ruminantia (goat and cattle group) (Huffman, 2011). The suborder Ruminantia is divided into families which include ruminant species such as giraffe, okapi, deer, antelope, goats, and sheep.

The continent of Africa is home to many species of exotic artiodactylids that belong to the suborder Ruminantia. These animals feeding habits are categorized into three general categories, similar to domestic ruminants, which include the grazers, browsers, and intermediate feeders. Animals that graze, such as sheep, usually eat vegetation (mostly grass) that is at or near ground level whereas animals that browse eat leaves, bark, twigs, and stems from plants. Giraffe tend to be exclusively browsers by nature. Their height gives them a distinct advantage for accessing taller plants and shrubs (National Geographic Book of Mammals, 1998). Their diets consist mainly of acacia trees, but in the winter months they eat less palatable evergreen trees. Gerenuks, also known as Waller's gazelles, are exclusive browsers as well. Intermediate feeders, such as sable antelope and roan antelope, are intermediate feeders that have diets that are comprised of mostly grass but 5-15% of their intake is browse (Wildlife Ranching, 2009a; Wildlife Ranching, 2009b). There are certain advantages and disadvantages associated with both browsing and grazing such as accessibility, resource limitations because of environmental conditions (snow, drought, etc.), varying nutritional values of grasses and plants, and the potential of vegetation to harbor infectious GIN L3.

2.5.1. Captive Artiodactylids

Exotic animals have always held the attention of human interest for thousands of years. The manner in which these animals have captivated us has caused us to take them from their natural habitats, move them across the globe, and confine them for our own enjoyment and convenience. Historically, the standard to house exotic animals in zoological facilities was by confining animals to cages which often had concrete floors. This type of confinement did not allow roaming, grazing, and other natural activities which initiated controversy concerning animal welfare and animal health. Recently, the standard has changed from cages to expansive enclosures which often times are suitable for mixed species. The mentality has changed as well; formerly keeping exotic animals captive for our own tourism has evolved into species conservation because of illegal poaching and the natural habitats of these animals being destroyed at alarming rates. Though the transformation from cages to large enclosures has its benefits, there are also some consequences associated with this, one of which is potential heavy GIN burdens, especially in ruminant artiodactylids (Young et al., 2000).

2.5.2. Gastrointestinal Nematodes in Artiodactylids

The harboring of GIN by exotic artiodactylids in their native environment is not an uncommon occurrence. Many of the GIN species that infect domestic small ruminants also infect exotic artiodactylids (Preston et al., 1979). According to Sachs et al. (1979), three species of *Haemonchus* are found in East African giraffe, and other studies showed that giraffe residing in national parks in Africa were infected with *Haemonchus* spp. (Young et al., 2000). GIN burdens of exotic artiodactylids in the wild are generally manageable and the levels of infection usually do not have any deleterious effects (Nalubamba and Mudenda, 2012).

The burden of GIN in captive artiodactylids tends to be much higher than artiodactylids residing in the wild. A survey conducted in North American zoos indicated that 91 of 99 facilities experienced problems with internal parasite infections in their exotic hoofstock (Isaza et al., 1990). Mortality rates, in zoological facilities and parks, from GIN induced gastroenteritis ranged from 5-17% (Goosens et al., 2005). There are many reasons for the increase in GIN infection including higher stocking rates, irrigation of vegetation, decreased immune response caused by stress of captivity, insufficient nutrition, inability to close enclosures, and lack of browse. Higher stocking rates allows for high levels of GIN on pasture/savannah, less area to move away from contaminated feces, and thus induces a high density L3 environment (Kaplan, 2006a). For aesthetic purposes, irrigation is used to enhance vegetation in enclosures and also to increase the food supply for animals. Increased moisture allows for optimal environmental conditions for GIN to thrive. Stressful conditions caused by being in captivity can compromise function of the immune system thus reducing an animal's ability to fight off GIN infection (Fagiolini et al., 2010). Insufficient nutrition, in the form of reduced protein intake, has been correlated to decreased resilience and resistance to GIN infection (Ezenwa, 2004). The inability to rest/close enclosures in zoological facilities allows for vegetation to be eaten down to very short lengths where L3 are readily available (3-5 inches) on the shorter forage (Kaplan, 2006a). Feeding behaviors of animals sometimes change when they are brought into captivity. In zoological facilities, guests must be able to view animals in their enclosures. Browsers, such as giraffe and gerenuk, often times graze in zoological facilities because of the lack of browse (trees and bushes) even though most zoos provide complete diets and browse in designated raised feeders. Grazing on contaminated savannahs/pastures increases the chance that an animal will be infected with GIN L3.

2.5.3. Control of Gastrointestinal Nematodes in Captive Artiodactylids

In the past, management of GIN infections in captive exotic hoofstock resembled that of controlled management programs used in small ruminant production. Protocols relied primarily on an "empirical, rotational anthelmintic program" which encompassed all three classes of anthelmintics (Fontenot et al., 2008). Accurate dosing and administration of anthelmintics proved to be a challenge for veterinarians and zoo keepers due to varying compliance of orally administered anthelmintics, estimated body weights as well as unknown pharmacokinetic data as to what the animals' bodies do to the anthelmintic which can in turn effect the efficacy (Fontenot and Miller, 2011). Therefore, subtherapeutic doses may be one of the contributing factors of anthelmintic resistance now being observed in exotic artiodactylids in zoological institutions. The frequent use of anthelmintics has also contributed to the resistance problem. Garretson et al. (2009) reported that a young male giraffe in Lion Country Safari in Florida demonstrated resistance of *H. contortus* to all 3 classes of anthelmintics.

Anthelmintics alone are no longer a reliable tool to control GIN infections in both the small ruminant industry and in zoological institutions. With the problem of resistance and lack of new anthelmintic options, a different approach is needed to combat GIN infection. In zoological facilities, proper anthelmintic treatment, animal management, GIN monitoring strategies, environmental control, and biological alternatives should be used. Anthelmintics should be used conservatively rather than on a rotational basis and smart drenching should be implemented (Van Wyk et al., 2006). Smart drenching is an approach that considers the pharmacokinetics of anthelmintics, host physiology, parasite biology, genetic selection for resistance, and resistance status of GIN to create strategies that decrease the selection for resistance and maximize anthelmintic efficacy (Kaplan, 2006b). Using the FAMACHA system to detect anemia before

treatment is administered would be ideal, but presents challenges because it would require correlation of conjunctiva color to anemia, standardization among species, and a large population data set (Fontenot and Miller, 2011); and trying to check the lower eyelid color of exotic hoofstock would be a challenge. Animal management by mixing species on an enclosure to increase refugia could introduce susceptible genes to dilute out resistant genes and thus reduce the number of resistant GIN. Parasite monitoring strategies such as fecal egg counts (FEC), larval development assays (LDA), fecal larval cultures (FLC), as well as FEC reduction tests (FECRT) could prove valuable in regards to monitoring and controlling GIN infections. FEC are used in monitoring patterns of infection and success of GIN management. LDA help identify nematode population and levels of resistance (Kaplan, 2006b). FLC help to identify species, individual, enclosure, and seasonal variations of GIN populations (Fontenot and Miller, 2011). The FECRT is the most definitive way to determine if anthelmintic resistance is present but is labor intensive and can be expensive (Kaplan, 2006b). Environmental control, via lower stocking rates, limiting exposure time on exhibit to less than 12 hours per day, tillage of exhibits before replanting, feces removal by staff, minimizing irrigation schedules, and water control, could also help to reduce GIN infection by lowering the number of L3 on forage (Fontenot and Miller, 2011).

With the lack of availability of new anthelmintics, other alternatives must be explored. The use of copper oxide wire particles, condensed tannin containing plants, nematophagous fungi, and other biological alternatives in conjunction with the implementation of the strategic use of anthelmintics, animal management, GIN monitoring strategies, and proper environmental control could be beneficial in the battle against resistant GIN in both the small ruminant industry and in zoological facilities.

2.6. Nonchemical Alternatives

The alarming rates by which GIN resistance to anthelmintics is growing is raising great concerns about current and future control in the domestic ruminant industry and in facilities maintaining exotic hoofstock. Transitioning from the frequent use of anthelmintics to biological alternatives, while using anthelmintics conservatively, seems to potentially be a promising strategy. Alternatives such as condensed tannin containing plants, vaccines, copper oxide wire particles and nematode trapping fungi in combination with proper pasture management, breeding for resistance, animal nutrition, the FAMACHA© system, and a smart drenching system could ultimately reduce mortality and morbidity.

2.6.1. Condensed Tannins

Plants containing condensed tannins, compounds that bind proteins and other molecules, are currently being used as a biological alternative to chemical anthelmintics. Tannins are divided into two groups, hydrolyzable tannins (HTs) and condensed tannins (CTs) (Coffey et al., 2007). HTs are tannins that are easily water soluble and are found in oak (*Quercus* spp) and many tropical tree legumes. HTs are potentially toxic to animals, but ruminants can adapt to eat these tannins by decreasing the excretion of degradation products in the urine (Chafton, 2006). However, high levels of HTs can potentially cause hemorrhagic gastroenteritis, necrosis of the liver, and kidney damage in ruminants; in a study conducted with sheep and cattle that were fed oak and other plants containing HTs, levels of HTs of more than 20% caused high mortality and morbidity (Cannas, n.d.). HTs have not been linked to a decrease in GIN infection (SCSRPC).

Of the two types of tannins, CTs are the most abundant. CTs are found in forage legumes, browse, and other plants worldwide. The effects of CTs are dependent on the plant, concentration of CT, and the animal consuming it. Negative effects include decreased

palatability causing reduced intake and digestion which directly affects productivity in a negative way. These effects are usually observed when CT concentration is high (Coffey et al., 2007). Benefits of CT intake includes reduction of bloat, increased production of milk, increased wool growth and growth rate, increased liveweight gain, increased amounts of bypass proteins, and higher ovulation rates (Terrill, n.d.). The most important benefit of CTs is their effects on GIN infection. CTs have been shown to reduce GIN infection, especially *H. contortus,* as well as reduce female fecundity thus reducing egg output (Pugh and Baird, 2011). A decrease of GIN egg hatchability and larval development in feces are additional benefits. The most studied CTs, in regards to reducing GIN infection, are sulla, big trefoil, sanfoin, and sericea lespedeza (Coffey et al., 2007).

Of the CT containing plants that affect GIN infection, sericea lespedeza (SL, *Lespedeza cuneata*) has the highest level, 4.6-15.2% DM, of CT content (Coffey et al., 2007). SL is a perennial legume found in warm and hot climates, especially in the Eastern and Southeastern United States. It is drought tolerant and can acclimate to low fertility and acidic soils (SCSRPC). It has been used to stabilize depleted and eroded soils and has been used to create habitats for wildlife; however, some states consider SL a noxious and invasive weed (Chafton, 2006).

SL can be offered to ruminants in multiple forms, each having its own pros and cons, which include forage, hay, and pellets. One problem with offering SL as forage is the issue of decreased palatability because of the bitter taste. Allowing animals to graze SL has management benefits which include less exposure to GIN since the plant grows off the ground, and the potential to increase resistance and resilience due to high levels of protein (Min et al., 2005). The feeding of SL as hay has little to no palatability issues, but the loss of active plant material during the haying process, due to fragile SL leaves, can be problematic (Terrill et al., 2006).

Pelleted SL has no palatability issues and is effective in controlling GIN infection. The feeding of pelleted SL and SL hay has been shown to significantly reduce FEC.

The exact mechanism of action is not known, but it is suggested that CTs, such as those contained in SL, directly impact nematodes through cuticle disruption which causes distress to the nematode. Decreases in egg hatchability and larval development have also been noted in animals fed SL; this is most likely due to CTs binding to larvae, feed nutrients, and bacterial growth in the feces (which serve as a food source for larvae) may be limited (Coffey et al., 2007). CT containing forages are also thought to benefit animal health by way of increased amino acid absorption, protein nutrition, and immune system function (Min et al., 2005). Nearly all research done on the impact of CTs (in various forms) on GIN infection was conducted in domestic ruminants, but studies are currently underway using pelleted SL in exotic artiodactylids.

2.6.2. Vaccines

The development of vaccines effective against GIN has been evaluated as an alternative to anthelmintics. Vaccines have been developed for other parasites, including lungworms in cattle and tapeworms in sheep, but currently there are no vaccines against GIN for ruminants that are commercially available (Bain and Urquhart, 1988; Lightowlers, 2000). In the last 25 years, significant progress has been made in identifying vaccine antigens for GIN species in grazing ruminants (Smith and Zarlenga, 2006). Gut and natural antigens have both been studied for use in vaccines.

Gut antigens, also known as hidden antigens, are antigens containing gut membrane proteins from a blood-feeding nematode. Once the host is vaccinated with the antigen, an antibody response is initiated. When the nematode feeds on blood, it ingests antibodies. The

antibodies bind with the nematode's intestinal antigens (proteins) which cause a disruption in the digestive processes (Smith and Zarlenga, 2006). This ultimately leads to starvation, a decrease in fecundity, and death. The antigens are "hidden" in the worm's gut and are not detected by the host's immune system. For this reason, multiple vaccinations are needed to maintain high antibody levels in the host's body (Smith and Taylor, 2009). The gut antigen based vaccine approach against *H. contortus* shows promise because multiple candidate vaccine antigens, such as H11 and H-gal-GP, have been isolated (Kabagambe et al., 2000). Other gut antigens used to vaccinate kid and yearling goats resulted in reduced FEC and nematode burden (Jasmer and McGuire, 1991). Considerations to take into account in regards to producing gut antigen vaccines are the cost and time that would be needed to extract natural antigens from nematode gut cells on a large scale basis (Smith and Zarlenga, 2006). New technologies are being developed to increase the extraction of natural antigens since recombinant antigens have not yet been successful (Smith and Taylor, 2009).

Other natural antigens have been used for vaccines against both blood-feeding and non-blood-feeding GIN. In contrast to hidden antigens, these other natural antigens are recognized by the host immune system during infection (Sutherland and Scott, 2010). These antigens are obtained from somatic and excretory/secretory products of various adult stages and L3 (including irradiated larvae) of GIN (Smith and Zarlinga, 2006). These antigens initiate an acquired immune response. Vaccines using these natural antigens against *H. contortus* offer limited protection. These vaccines can help reduce pasture contamination, but may not provide sufficient protection from haemonchosis in lambs (Getachew et al., 2007).

Currently, there are no vaccines on the market to protect against GIN infection in ruminants. While significant progress has been made in many areas, such as the identification of

key vaccine antigens, other hurtles still exist before a viable vaccine can be commercially produced. The prospect of a multivalent vaccine is even more distant. Cost effective, stable recombinant vaccines and identification of correct adjuvants for administration makes the production and success of vaccines elusive (Smith and Zarlenga, 2006). Vaccines protecting against GIN infection have not yet been studied in exotic artiodactylids.

2.6.3. Copper Oxide Wire Particles

Copper has been used for many years to treat copper deficiency in the grazing ruminant industry. It has also been used, in various forms, as an anthelmintic since the early 1900's (SCSRPC). Copper oxide wire particles (COWP) have been shown to be effective against nematodes, specifically *H. contortus,* by lowering burdens and reducing FEC (Burke et al., 2004; Burke et al., 2007; Soli et al., 2010). After ingestion, COWP move with ingesta to the abomasum where the particles adhere to the mucosal folds (Vatta et al., 2009). The lodged particles dissolve, over a period of several weeks, in the acidic environment and slowly release free copper which increases soluble copper concentrations. Excess copper is eventually stored in the liver. The exact mode of action is not yet known, but researchers have speculated that copper changes the ideal abomasal environment for the nematodes causing death and/or expulsion (Burke et al., 2004). Subsequent decreases in FEC as well as increases in blood packed-cell volume (PCV) have been observed after COWP administration. This is most likely due to the effect of COWP on the adult *H. contortus* (Burke et al., 2004). COWP have not been observed to be effective against intestinal parasites (Burke et al., 2007). Fecal larval cultures are generally recommended, before treatment with COWP, to determine if the predominant population is *H. contortus* (Fontenot and Miller, 2011).

COWP have been shown to be effective in reducing nematode burdens and FEC in both sheep and goats (Soli et al., 2010). COWP boluses (Copasure©) are available (12.5 and 25 g bolus) for use in copper deficient cattle, but smaller doses (0.5 to 2 g) have been repackaged for use in sheep and goats (Schoenian, 2008). Sheep are very sensitive to high levels of copper and toxicity may develop. Although COWP are released slowly, caution should still be used when administering COWP. Other factors that contribute to an increased risk of copper toxicity include breed, deficiencies of other minerals such as molybdenum, exposure to poultry litter, age, and health status (Hale et al., 2007).

The use of COWP in exotic artiodactylids has been investigated at Disney's Animal Kingdom® and Disney's Animal Kingdom® Lodge. Four species of artiodactylids, including scimitar horned oryx, roan antelope, blackbuck, and blesbock, were used in the study. The results indicated a more than 90% reduction in FEC by 7 days post treatment in three out of the four species. COWP doses administered were based on the manufacturer's recommendation of 12.5 grams for cattle weighing less than 227 kg (Fontenot et al., 2008). Before implementing COWP in an exotic hoofstock GIN control program, considerations must be made regarding species differences, preexisting liver disease, copper status of animals, mineral interaction and deficiencies, and supplemented copper. Investigations of COWP on reproduction, copper accumulation, and species differences in toxicity should also be made before using COWP as part of an integrated control program (Fontenot and Miller, 2011).

2.6.4. *Duddingtonia flagrans*

Much research has been done using nematode-destroying fungi as a biological control agent to reduce levels of GIN L3 in feces. These fungi are found worldwide and occur naturally in soil and other environments that are rich in organic matter (Jackson and Miller, 2006). The

fungi range in feeding habits from saprophytic fungi to fungi classified as obligate parasites (Nordbring-Hertz, 2006). Nematode-destroying fungi are a group of microfungi composed of more than 150 species with the majority being nematode-trapping fungi (Grønvold et al., 1993). Nematode-destroying fungi are generally divided into three groups: endoparasitic fungi, egg-parasitic fungi, and nematode trapping fungi (nematophagous fungi). Endoparasitic fungi spores invade nematodes by cuticle penetration and/or ingestion of spores. The fungi then begin to germinate which results in the digestion of the parasite (De and Sanyal, 2009). This type of fungi experiences no hyphal development outside the parasite except for hyphae that release spores; for this reason, endoparasitic fungi are classified as obligate parasites (Grønvold et al., 1993). Egg-parasitic fungi invade nematode eggs by penetration of the egg shell with specialized vegetative structures called appressoria and penetration hyphae (Deacon, 1984). The appresoria anchors the fungus to the egg while the penetration hypha invades and kills the developing juveniles in the egg (Deacon, 1984; Nordbring-Hertz et al., 2006). Nematode-trapping fungi, also known as predatory fungi, trap and destroy nematodes by producing a variety of trapping structures such as constricting rings, non-constricting rings, adhesive knobs, adhesive hyphae, adhesive branches, and adhesive networks on the mycelium (bundle of hyphae) (Grønvold et al., 1993). Trapping structures formed are dependent on the species and the environment, which includes both abiotic and biotic factors. The most important biotic factor is the presence of nematodes because they can induce trap formation by touching the mycelium and they serve as a food source (Nordbring-Hertz et al., 2006). Some members of the predatory fungi group form traps spontaneously whereas others are dependent upon environmental factors (such as nematode presence) to induce trap formulation. This is sometimes done by a change in morphology as seen in *Arthrobotrys* spp., which tend to be more saprophytic in nature

(Nordbring-Hertz et al., 2006). Once a nematode is anchored by the trapping structure(s), penetration of the cuticle occurs. Tropic hyphae grow, enter and fill the body of the nematode, and digest it (Grønvold et al., 1996a).

Nematode-trapping fungi are the most abundant of the nematode-destroying fungi. The use of nematode-trapping fungi to reduce GIN L3 in the feces of ruminants has been documented with success. The most commonly used route of spore deployment is by feed additive or administration by oral suspension (Waller et al., 2004). Once ingested, the spores pass through the animal's GI tract and are deposited in the feces along with GIN eggs. In the feces, the fungi germinate and form trapping structures that are able to immobilize L3 as they migrate through the fecal mass. The larvae are eventually killed by digestion using specialized enzymes. Many species of fungi have been studied in regards to reducing levels of GIN L3 in feces which would then reduce levels on pasture. However, the spores of most species do not have high survivability rates when passing through the ruminant GI tract. One species of nematode-trapping fungi, *D. flagrans*, has been shown to survive the harsh environment of the ruminant GI tract as compared to other members of this group (Fontenot et al., 2003).

Duddingtonia flagrans belongs to the artificial group Deuteromycetes which is commonly referred to as Fungi Imperfecti because no known sexual reproduction occurs; only asexual reproduction has been observed (Nordbring-Hertz et al., 2006). *D. flagrans* has many distinct advantages over other fungi when used to reduce L3 on pasture. The primary advantage offered by *D. flagrans* is the ability of its thick-walled chlamydospores (resting spores) to survive passage through the ruminant GI tract, as well as its ability to maintain viability which enables colonization (Hoste and Torres-Acosta, 2011). Although GI tract survivability is much higher with this fungus compared to others, losses are still eminent. It has been reported that the

number of chlamydospores lost in rumen digestion (in vitro) were negligible, but the number of chlamydospores lost when subject to abomasum digestion (in vitro) was nearly 36% (Ojeda-Robertos et al., 2009). Some studies suggest even higher levels of chlamydospore loss using in vivo studies (Ojeda-Robertos et al., 2009; Ojeda-Robertos et al., 2008a) *Duddingtonia flagrans* is also capable of producing limited numbers of another type of spore called conidia. Conidia, thin-walled spores, are produced in young fungi and do not possess the ability to resist digestion in the GI tract of ruminants (Grønvald et al., 1996b). Another advantage of *D. flagrans* is its rapid growth rate and its affinity for trapping and digesting nematodes (Waller et al., 2004). Maximum trap formation rates of 700-800 traps/cm^2/2 days were achieved at the optimum temperature of 30°C when induced with 20 L3 of *Ostertagia ostertagi* per cm^2 (Grønvold et al.,1996b). This same study also revealed that trap formation occurred at temperatures ranging between 10°C to 35°C, with trap formation gradually dropping off in a period of 2 to 3 weeks at temperatures of 20°C and 30°C, and as trap induction slowed with mycelial age, chlamydospore production increased (Grønvold et al., 1996b).

Duddingtonia flagrans uses traps called networks to capture GIN L3 in the feces. After chlamydospores are ingested by the animal, they are deposited in the feces and begin to germinate and form vegetative hyphae. Lateral branches begin to form, grow out, and loop back around to the parental vegetative hyphae; more branches begin to form from the already existing loop (primary loop) which then creates a sticky three dimensional network (Grønvold et al., 1996b). The process of producing sticky networks happens concomitantly with the hatching and development of GIN larvae in the feces (Ketzis et al., 2006). Movement and physical contact from migrating L3 induces formation of networks (traps). Once the L3 are trapped, they are unable to migrate out of the fecal mass and up the grass blades to be consumed by the ruminant

host. They will soon be digested by the penetrating fungal hypha. As the fungus ages in the feces, chlamydospores are formed by the enlargement of hyphal cells (Grønvald et al., 1996b). As the chlamydospores grow, they are released from their casings. The main goal of using *D. flagrans* chlamydospores is for long term reduction of pasture contamination (Ketzis et al., 2006). The fungus has no anthelmintic effect on adult GIN in the animal.

A number of studies have been conducted using *D. flagrans* chlamydospores in oral suspensions and in feeding supplements administered to sheep, goat, and cattle of varying life stages with single species infections and mixed species infections of GIN (Larsen et al., 1996; Knox and Faedo, 2001; Waller et al.,2001; Fontenot et al., 2003; Dimander et al.,2003; Terrill et al., 2004; Waller et al., 2006; Mendoza de gives et al., 1998; Peña et al., 2002; Waghorn et al., 2003; Ojeda-Robertos et al., 2008). The efficacy of *D. flagrans* is determined by a reduction in L3 in feces (or around feces for plot studies) and by a reduction in infection in tracer animals (Ketzis et al., 2006). Mendoza de Gives et al. (1998) reported that a single dose of 11,350,000 chlamydospores given to young sheep resulted in a reduction of 88% of *H. contortus* L3 in the feces, and the effect of the fungus continued for 4-5 days post treatment. In a study by Waller et al. (2001), *Trichostrongylus columbrifortis* L3 numbers in the feces of young sheep were virtually eliminated by offering 5 grams of barley grain, containing 4×10^6 *D. flagrans* chlamydospores, per day for 5 consecutive days. Grains with adhering fungal chlamydospores were also incorporated into block grains which exhibited similar efficacy. Peña et al. (2002) reported that a chlamydospore dose of 10^5, fed for 7 consecutive days, was effective in reducing L3 in feces of lambs by 82.8 to 99.7%. Fontenot et al. (2003) reported a dose of 5×10^5 chlamydospores per kg of body weight, in a granular product form, fed for 18 weeks in grazing ewes resulted in a reduction of L3 in feces, as well as reduced pasture infectivity, and reduced

nematode burdens of 96.8% in tracer animals. Waghorn et al. (2003) reported that lambs and kids fed fungal doses of 250,000 or 500,000 chlamydospores per kg/liveweight in a suspension for two consecutive days resulted in an efficacy of approximately 78%. The fungus did not exhibit bias to parasite species or host animal. Another study using Spanish meat goats with mixed species infections revealed that when fed daily as a feed additive, *D. flagrans* significantly reduced the development of L3 in feces (Terrill et al., 2004). Inconsistent L3 reduction was also observed with intermittent feeding of the chlamydospores. A 3 year plot study conducted with cattle (0.5-1.0 x 10^6 chlamydospores per kg/BW per day) reduced L3 availability on herbage during grazing seasons and between grazing seasons; however, there was no effect on the reduction of overwintered L3 (Dimander et al., 2003).

The general idea surrounding the use of *D. flagrans* is that the administration of large enough doses of chlamydospores will result in a large amount of excreted chlamydospores in the feces to achieve the desired reduction of L3. The McMaster technique was used for quantifying FEC to quantify chlamydospores per gram (CPG) in hopes of predicting a dose dependent effect (Ojeda-Robertos et al., 2008a). The CPG resulted in a clear dose dependent effect with higher doses of *D. flagrans* chlamydospores producing higher CPG; however, above certain levels, higher doses did not produce higher CPG. Ojeda-Robertos et al. (2008b) also described the relationship between CPG of *D. flagrans* and EPG of *H. contortus* on reducing L3 (2008b). The results suggested that a feasible chlamydospore dose could be calculated based on EPG, with a ratio of 5-10 CPG to 1 EPG being recommended and digestibility of chlamydospores, estimated to be around 10%, should be taken into account when calculating chlamydospore dose (Ojeda-Robertos et al., 2008a.). To achieve the full benefit of *D. flagrans*, chlamydospores should be fed daily for a period of several weeks during which chlamydospores would be continuously shed in

the feces at the same time GIN eggs are shed (Waller et al., 2004). Based on study data, the recommended doses for sheep and goats is 250,000-500,000 chlamydospores/kg BW and the recommended dose for cattle is 1,000,000 chlamydospores/kg BW (Ketzis et al., 2006).

Larval reduction using *D. flagrans* usually occurs 7 to 14 days after beginning the treatment, and the effects can last up to 4-5 days post treatment (Burke et al., 2005; Mendoza de Gives et al., 1998). The shelf life of the chlamydospores varies according to environment. Moist environments enable germination which renders chlamydospores vulnerable when passing through the gastrointestinal tract. This can shorten shelf life to less than one week (Larsen, 2006). In contrast, air dried chlamydospores have a shelf life of more than 20 months (Grønvald et al., 1996b).

It is known that copper is an active fungicide and if COWP are used to treat *H. contortus* in the animal, there may be an effect on viability of the fungus in the feces. This possible interaction was evaluated, and there were no adverse effects of COWP on *D. flagrans* L3 reduction (Burke et al., 2005). A beneficial effect was noted in that there was reduced L3 due to the reduction of nematode burdens from COWP, and the additional larval reduction by *D. flagrans*. This gives promise to using both of these biological alternatives together in a control strategy to reduce GIN infection (Burke et al., 2005).

Although *D. flagrans* offers many benefits, one concern is the manual daily dosing of animals with chlamydospores. Possible solutions include delivering spores through a sustained release system and feeding blocks (Ketzis et al., 2006; Waller et al., 2004). Another concern is the lack of a commercial source of spores (SCSRPC). Currently, there is a company in Australia investigating ways to produce large amounts of chlamydospores and incorporate them into a feed additive form that is sustainable for use in the domestic ruminant industry (Fontenot and Miller,

2011). Differentiation in efficacy between isolates of *D. flagrans* has also been expressed as a concern (Waller et al., 2004). Genetic similarity of *D. flagrans* isolates found all over the world has been established making it ubiquitous (Waller, 2003). An additional concern is the potential adverse effects of *D. flagrans* on the environment, particularly beneficial soil nematodes. Knox et al. (2002) reported that free-living soil nematodes and microarthropods were not affected by *D. flagrans*. In addition, there were no negative effects of *D. flagrans* on other nematode-trapping fungi (Knox et al., 2002). *D. flagrans* should be used in combination with other control methods as part of an integrated control strategy.

The use of *D. flagrans* chlamydospores to reduce numbers of infective larvae in the feces of exotic artiodactylids species housed at Disney's Animal Kingdom® and Disney's Animal Kingdom® Lodge will be discussed in upcoming chapters of this document.

CHAPTER 3
MATERIALS AND METHODS

3.1. Location

All 3 studies were conducted at Disney's Animal Kingdom® and/or Disney's Animal Kingdom® Lodge located in Bay Lake, Florida. Disney's Animal Kingdom® was further divided into the Trails and the West Savannah locations. Disney's Animal Kingdom® Lodge was also further divided into the Sunset Savannah, the Uzima Savannah, and the Arusha Savannah locations. All of these locations experienced GIN infections.

3.2. Animals

All 3 studies consisted of mixed species of adult exotic artiodactylids: reticulated giraffe, masai giraffe, sable antelope, roan antelope, and gerenuk. Both male and female animals were included. Average bodyweight (BW) of giraffe, antelope, and gerenuk were 900 kg, 300 kg, and 50 kg, respectively. Weights were used to calculate chlamydospore doses.

3.3. Infection

All of the animals were naturally infected by grazing on savannahs where *H. contortus* was identified to be the primary (>90%) GIN. Secondary GIN included *Cooperia* spp. and *Trichostrongylus* spp..

3.4. Sources of *Duddingtonia flagrans*

The chlamydospores of *D. flagrans* used in Study 1 were obtained from National Center for Veterinary Parasitology, Jiutepec, Morelos, Mexico. Chlamydospores were supplied in a suspension. The chlamydospores used in studies 2 and 3 were obtained from International Animal Health Products, Huntingwood NSW, Australia. Chlamydospores were supplied in a dry powder supplement feed form.

3.5. Experimental Design

3.5.1. Study 1

This study included 4 adult sable antelope and 5 adult reticulated giraffe. All giraffe were located at Disney's Animal Kingdom® Lodge's Sunset Savannah and all sable antelope were located at Disney's Animal Kingdom's® West Savannah location. Animals were 'on show' for the majority of the day and savannahs provided forage and browse in feeders. Animals had access to water at all times. Animals were housed individually for feeding (approximately 2 hours) in compartmentalized facilities. The duration of the study was nine days (5/23/10-5/31/10). Both species were randomly allocated, based on FEC, into 2 groups, treatment (3 giraffe and 2 sable antelope) and control (2 giraffe and 2 sable antelope). The aim of this study was to evaluate the efficacy of *D. flagrans,* in reducing the number of L3 in the feces using a dose of 500,000 chlamydospores per kg/BW. The chlamydospores were fed daily for 4 days (5/24/10-5/27/10). Suspensions of chlamydospores were kept refrigerated until use to prevent premature germination. Chlamydospores, upon use, were shaken, mixed into feed thoroughly, and immediately fed to treatment animals. Control animals were offered feed only. Consumption patterns were monitored by weighing feed before and after consumption. Each day, individual animal fecal samples were collected off the ground (in the stall or on the savannah) immediately after deposition. After collection, feces were separated into FEC and culture bags, labeled, stored, and shipped (in refrigerated packages) to the Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University to be processed and cultured. The McMaster technique was used to determine FEC, reported as eggs per gram (EPG). Percent hatch and percent reduction calculations were based on FEC and fecal culture results.

3.5.2. Study 2

This study included 1 roan antelope, 2 sable antelope, and 3 reticulated giraffe. All animals in this trial were located at Disney's Animal Kingdom® Lodge. The roan antelope, sable antelope, and giraffe were maintained on Arusha, Uzima, and Sunset Savannah, respectively. Savannahs provided forage, artificially placed browse, and water was available at all times. Animals were housed individually for feeding (approximately 2 hours), in compartmentalized facilities. The duration of this study was 9 days (5/29/13-6/6/11). For both antelope and giraffe, animals were randomly allocated, based on FEC, into treatment (n=2) and control (n=1) groups. The aim of this study was to evaluate the efficacy of *D. flagrans*, incorporated into a supplement feed, in reducing the number of L3 in the feces at a dose of 30,000 chlamydospores per kg/BW. Chlamydospore product was kept at room temperature and was incorporated into each individual's feed mix (species specific) containing grain, sweet feed, and wet beet pulp. This feed mix was weighed before and after feeding in order to monitor consumption patterns. Chlamydospores were fed daily for 4 days (5/30/11-6/2/11). Control group animals were offered feed containing no chlamydospores. Each day, individual animal fecal samples were collected off the ground (in the stall or on the savannah) immediately after deposition. After collection, feces were separated into FEC and culture bags, labeled, stored, and shipped (in refrigerated packages) to the Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University to be processed and cultured. The McMaster technique was used to determine FEC, reported as eggs per gram (EPG). Percent hatch and percent reduction calculations were based on FEC and fecal culture results

3.5.3. Study 3

This study was an extended version of Study 2 with an increased study span of 74 days (7/1/11-9/12/11) and increased sample sizes (25 animals total). Animals in this trial were located in various savannahs at Disney's Animal Kingdom® and Disney's Animal Kingdom® Lodge (Table 1). This study included 4 gerenuk, 3 roan antelope, 7 sable antelope, 6 reticulated giraffe, and 5 masai giraffe. For statistical purposes, roan and sable antelope were grouped together and reticulated and masai giraffe were grouped together.

Table 1. Location of animals included in Study 3 at Disney's Animal Kingdom®. Treatment and control animals are indicated by (T) or (C), respectively.

Species	Location (Savannah)				
	Trails	West Savannah	Sunset	Uzima	Arusha
Gerenuk	4 Gerenuk (2T,2C)	-	-	-	-
Antelope	-	5 Sable (2T,3C)		2 Sable (1T,1C)	3 Roan (2T,1C)
Giraffe	-	5 Masai (2T,3C)	4 Reticulated (2T,2C)	2 Reticulated (2T)	-

Savannahs provided forage, artificially placed browse, and water was available at all times. All animals were housed individually for feeding, in a compartmentalized facility (approximately 2 hours). The time of day at which feeding took place varied by location. The three groups of animals (gerenuk, antelope, and giraffe) were randomly allocated, based on FEC, into treatment and control groups. The numbers of animals that received treatment were: 2 gerenuk, 5 antelope (2 roan and 3 sable), and 6 giraffe (4 reticulated, 2 masai). The remaining animals were part of the control groups. The aim of this study was to evaluate the efficacy of the Australian isolate of *D. flagrans* in reducing the number of L3 in the feces at a dose of

30,000 chlamydospores per kg/BW over an extended interval of time. The chlamydospore product was kept at room temperature and was incorporated into each individual's feed mix (species specific) containing grain, sweet feed, and wet beet pulp. This feed mix was weighed before and after feeding in order to monitor consumption patterns. Control group animals were offered feed containing no chlamydospores. Chlamydospores were fed daily for 58 days (7/3/11-8/29/11). It was noted that, for some animals, feeding was missed occasionally due to issues with compliance or not coming in. Each week, individual animal fecal samples were collected off the ground (in the stall or on the savannah) immediately after deposition. After collection, feces were separated into FEC and culture bags, labeled, stored, and shipped (in refrigerated packages) to the Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University to be processed and cultured. The McMaster technique was used to determine FEC, reported as eggs per gram (EPG). Percent hatch and percent reduction calculations were based on FEC and fecal culture results.

3.6. Techniques

3.6.1. Fecal Egg Count

Upon receipt, the FEC samples were immediately stored in the refrigerator until processed. FEC were determined using the modified McMaster technique. In this technique, 2 grams of feces were broken up in a 125 ml cup using a tongue depressor. Thirty ml of a saturated salt solution (737 g of iodized salt dissolved in 3000 ml of tap water) was added and the solution was mixed manually with a tongue depressor. An electric drink mixer (Drinkmaster® Drink Mixer, Hamilton Beach Brands, Inc., Glen Allen, NC) was then used to further disrupt and mix the feces in solution thoroughly. Immediately after mixing, a sample of the solution was extracted using a pipette and inserted into one chamber of a McMaster slide.

This was repeated to fill the second chamber of the McMaster slide. Trichostrongyle type eggs were counted using the 10x objective of the microscope. Total eggs counted in both chambers were multiplied by 50 to get EPG.

3.6.2. Fecal Larval Cultures

Fecal larval cultures, in conjunction with the baermann technique, were used to allow GIN eggs to hatch and develop to L3 for recovery, enumeration, and identification. For all studies, multiple fecal cultures were made for each individual sample and the addition of vermiculite to cultures varied. In Study 1, four cultures were made per sample with two of the cultures containing vermiculite. In Study 2, two cultures were made per sample with no vermiculite added. In Study 3, three cultures were made per sample with one culture containing vermiculite. Cups with holes in the bottle were used for cultures. Feces were weighed, divided into 125 ml plastic cups, and broken up using a tongue depressor. For Study 1, 7.5 grams of feces were used for each culture. For Studies 2 and Trial 3, total feces were weighed and divided into equal parts. For cultures containing vermiculite, feces and vermiculite were combined in approximately equal amounts. Water was added to all cultures until a crumbly moist consistency was achieved. Cheesecloth and rubber bands were used to cover the top of each culture cup. Culture cups were then inverted (not submerged) into a larger 250 ml tricorner plastic cup containing 70 ml of water. Cultures were incubated at room temperature (approximately 27 ˚C). After a 2 week incubation period, culture cups were submerged into the tricorner cups containing water. Additional amounts of water were added to fill the tricorner cups to the rim (to saturate culture cups). Cultures were allowed to sit, fully submerged, for one day to allow L3 to migrate out of the culture mass and accumulate in the bottom of the tricorner cups. The culture cups were removed, and the contents of the tricorner cups were carefully (not to disturb the sediment)

vacuumed down to a volume of 50 ml. The sediment was then resuspended in the cups and transferred to 50 ml centrifuge tubes. Tubes were allowed to sit for at least 2 hours to allow L3 to settle to the bottom. The supernatant was then vacuumed to obtain a final volume of approximately 14 ml per sample. Samples were resuspended and then transferred to 15 ml centrifuge tubes and one ml of 10% formalin was added to each tube for preservation purposes.

3.6.3. Larval Counts and Identification

The supernatant in the 15 ml tubes were vacuumed to 1 ml. A pipette was used to resuspend larvae and draw off a 100 µl aliquot which was placed on a microscope slide. A drop of iodine was mixed into the aliquot, and a slip cover was placed on top of the slide. L3 were then enumerated using a microscope and counter. The first 100 or total (if the total was less than 100) L3 were identified to genus to approximate population percentages.

3.7. Calculations

3.7.1. Fecal Egg Count

The formula used to calculate FEC was number of eggs counted x 50, expressed as EPG.

3.7.2. Larvae per Gram

The formula used to calculate LPG was (L3/tube)/wt. of feces cultured

3.7.3. Percent Hatch

Percent hatch was calculated (LPG/EPG) x 100

3.7.4. Percent Reduction

Percent hatch was used to calculate percent reduction. Percent reduction was [(control mean % hatch – treatment mean % hatch)/(control mean % hatch)] x 100

When results were negative, the percent reduction was considered zero.

3.8. Statistical Analysis

Data were analyzed using SAS® (version 9.3) as a repeated measures analysis of variance using PROC MIXED. Variance components were used as the covariance structure. The response variable was percent hatch. Fixed effects included treatment and time. When overall differences were found, post hoc comparisons were conducted with pairwise t-tests of least-squares means. All differences were considered significant at $p \leq 0.05$.

CHAPTER 4
RESULTS

4.1 Study 1

4.1.1. Giraffe

Overall mean FEC of the control and treated groups was 250-800 and 867-1317, respectively (Figure 1). Mean FEC of the control group was consistently lower than the treated group.

Mean percent hatch was similar ($p > 0.05$) for both groups on day 0. Subsequent to the start of chlamydospore feeding (day 1), the treatment group mean percent hatch was consistently lower than the control group, through day 6 (Figure 2). The only significant difference ($p \leq 0.05$) was on day 3. By days 7 and 8, the treatment group mean percent hatch increased and was similar to that of the control group.

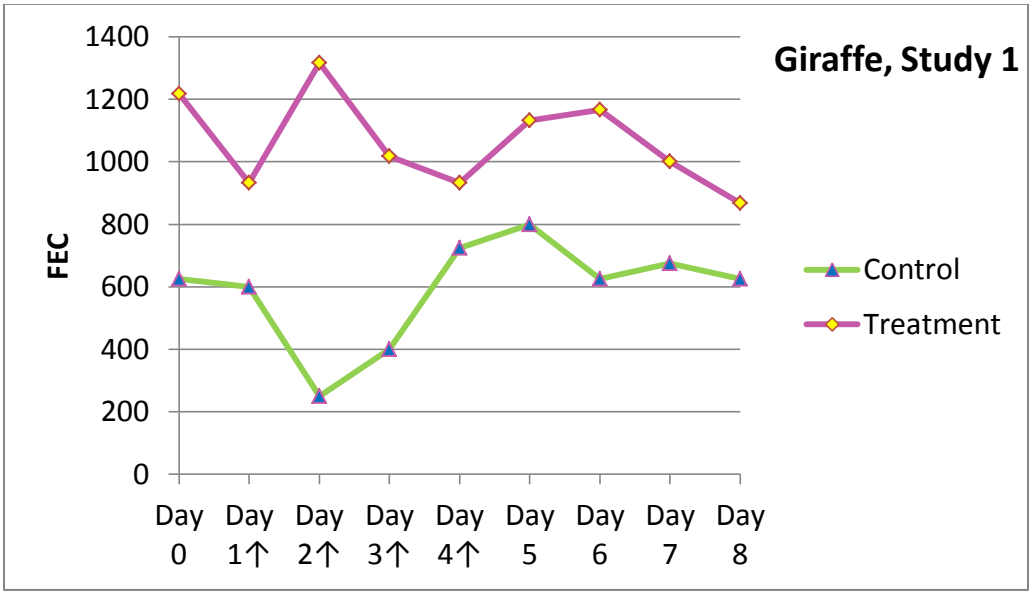

Figure 1. Study 1. Mean fecal egg count for treatment (n=3) and control (n=2) giraffe that were fed *Duddingtonia flagrans* chlamydospores (500,000 per kg/BW). Arrows indicate chlamydospore feeding period.

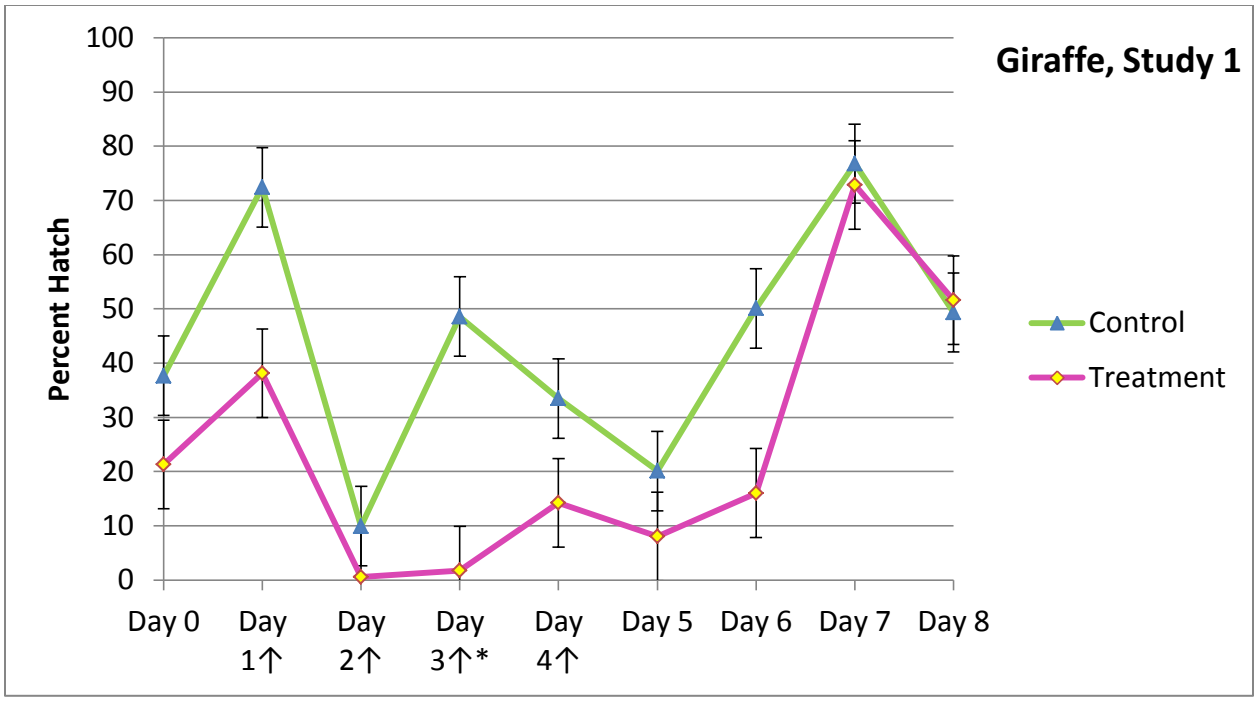

Figure 2. Study 1. Mean percent hatch and development to L3 in feces for treatment (n=3) and control (n=2) giraffe that were fed *Duddingtonia flagrans* chlamydospores (500,000 per kg/ BW). Arrows indicate chlamydospore feeding period.
*****Indicates statistical significance of $p \leq 0.05$.

The mean percent reduction of L3 steadily increased for the treated group from day 0 to day 3, reaching a maximum percent reduction of 96.5% (Figure 3). Mean percent reduction decreased to 57.6% on day 4 followed by an increase on days 5 and 6. This was 2 days after stopping chlamydospore feeding which showed a residual effect of activity. Subsequently, mean percent reductions decreased to 5.1% and 0% on days 7 and 8, respectively.

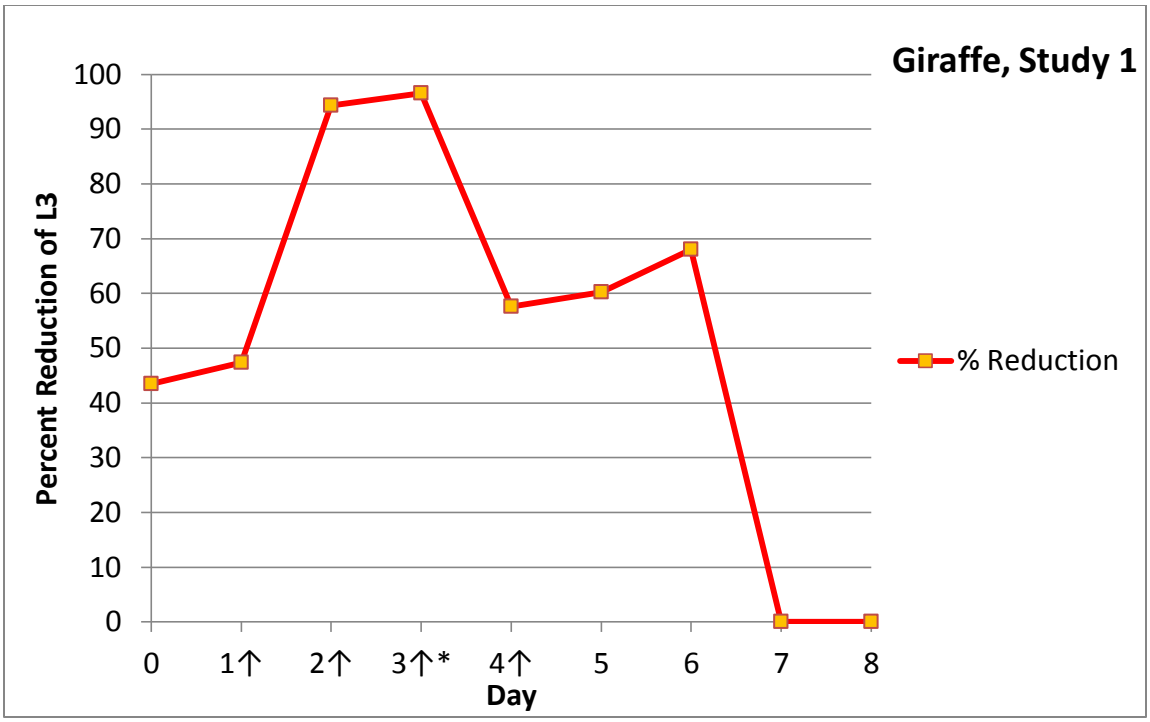

Figure 3. Study 1. Mean percent reduction of L3 comparing treated (n=3) and control (n=2) giraffe that were fed *Duddingtonia flagrans* chlamydospores (500,000 per kg/BW). Arrows indicate chlamydospore feeding period.
*Indicates significant reduction ($p \leq 0.05$).

4.1.2. Antelope

Overall mean FEC of the control and treated groups was 1175-4550 and 1775-4975, respectively (Figure 4). Mean FEC was similar for both groups.

Mean percent hatch was similar ($p > 0.05$) for both groups on day 0. Subsequent to the start of chlamydospore feeding (day 1), the treatment group mean percent hatch was consistently lower than the control group through day 5 (Figure 5). The only significant difference ($p \leq 0.05$) was on day 5. Percent hatch values were not able to be calculated for day 6 due to multiple missing samples. By days 7 and 8, the treatment group mean percent hatch increased and was similar to that of the control group.

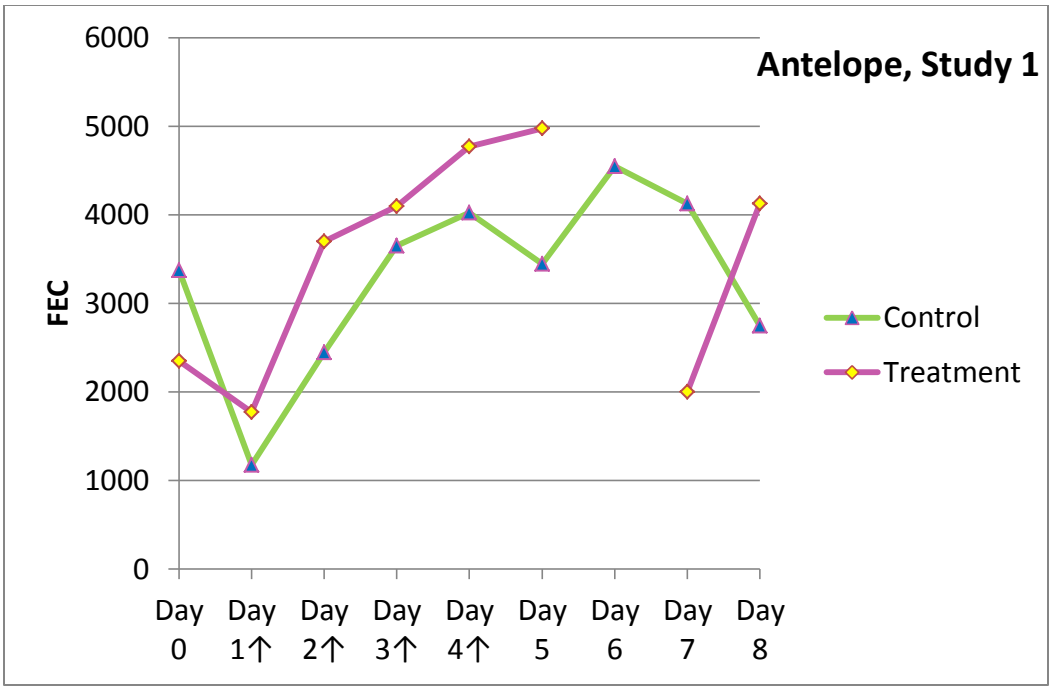

Figure 4. Study 1. Mean fecal egg count for treatment (n=2) and control (n=2) antelope that were fed *Duddingtonia flagrans* chlamydospores (500,000 per kg/BW). Arrows indicate chlamydospore feeding period.

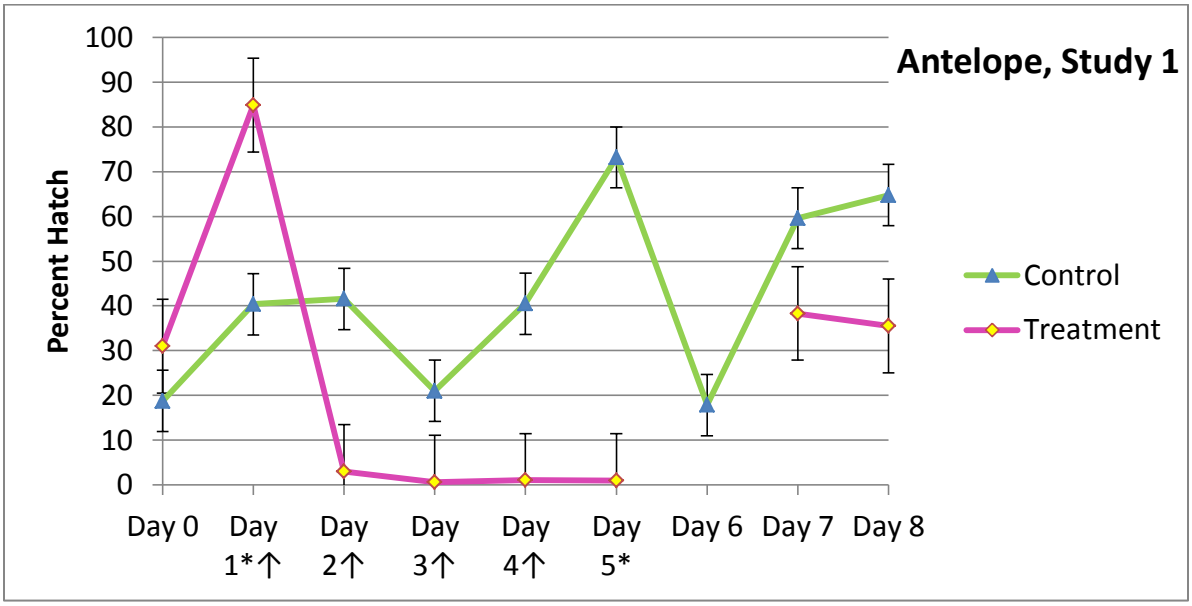

Figure 5. Study 1. Mean percent hatch and development to L3 in feces for treatment (n=2) and control (n=2) antelope that were fed *Duddingtonia flagrans* chlamydospores (500,000 per kg/BW). Arrows indicate chlamydospore feeding period.
*****Indicates statistical significance of $p \leq 0.05$.

Mean percent reduction was zero on days 0 and 1 (Figure 6) then increased to above 90% through day 5. Percent reduction was not able to be calculated for day 6 due to missing data. Subsequent to stopping chlamydospore feeding, mean percent reduction then decreased to 35.7% and 45.1% on days 7 and 8, respectively.

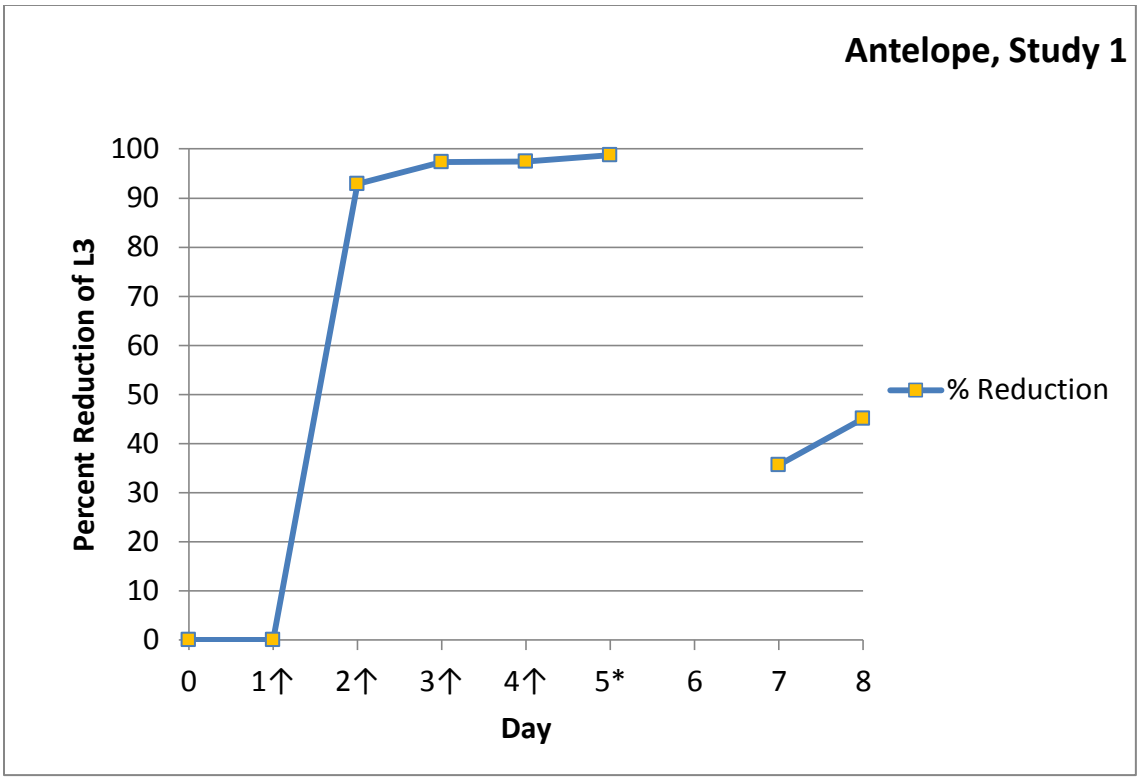

Figure 6. Study 1. Mean percent reduction of L3 comparing treated (n=2) and control (n=2) antelope that were fed *Duddingtonia flagrans* chlamydospores (500,000 per kg/BW). Arrows indicate chlamydospore feeding period.
*Indicates significant reduction ($p \leq 0.05$).

4.2 Study 2

4.2.1. Giraffe

Overall mean FEC of the control and treated groups was 300-950 and 100-500, respectively (Figure 7). Mean FEC of the treated group was consistently lower than the control group from day 2 on.

Mean percent hatch was higher for the control giraffe on days 0 and 1. Subsequent to the start of chlamydospore feeding (day 1), the treatment group mean percent hatch was consistently lower than the control giraffe through day 5 (Figure 8). By day 7 and 8, the treatment group's mean percent hatch increased and was similar to that of the control.

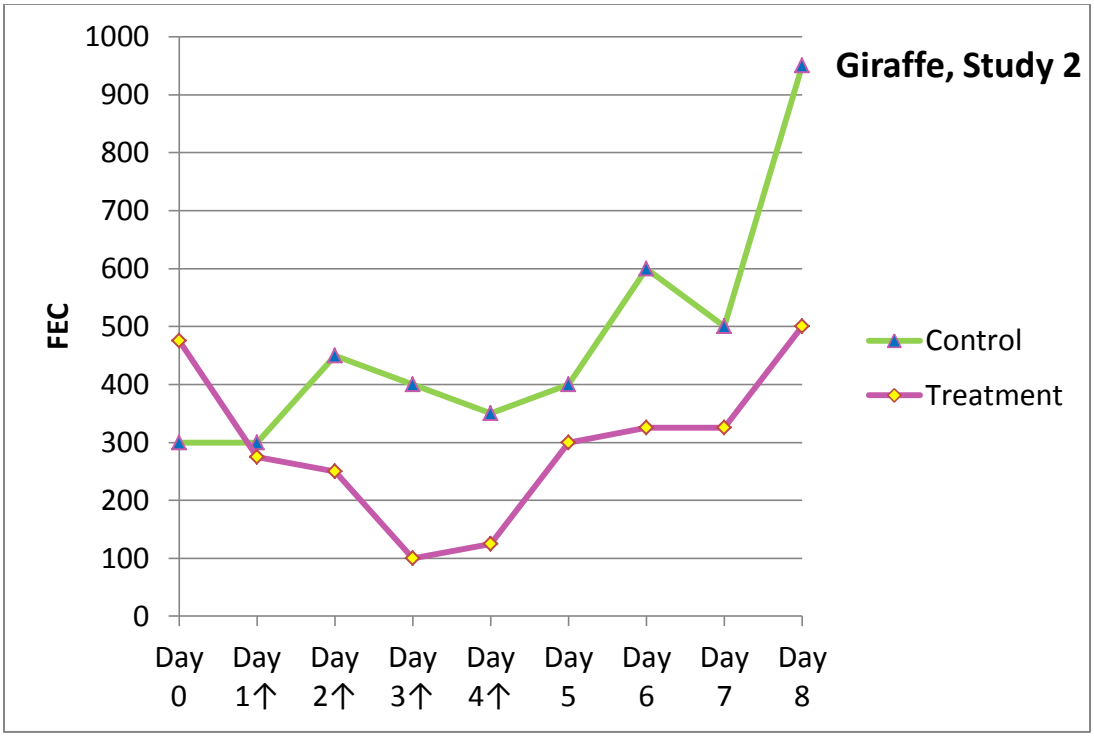

Figure 7. Study 2. Mean fecal egg count for treatment (n=2) and control (n=1) giraffe that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

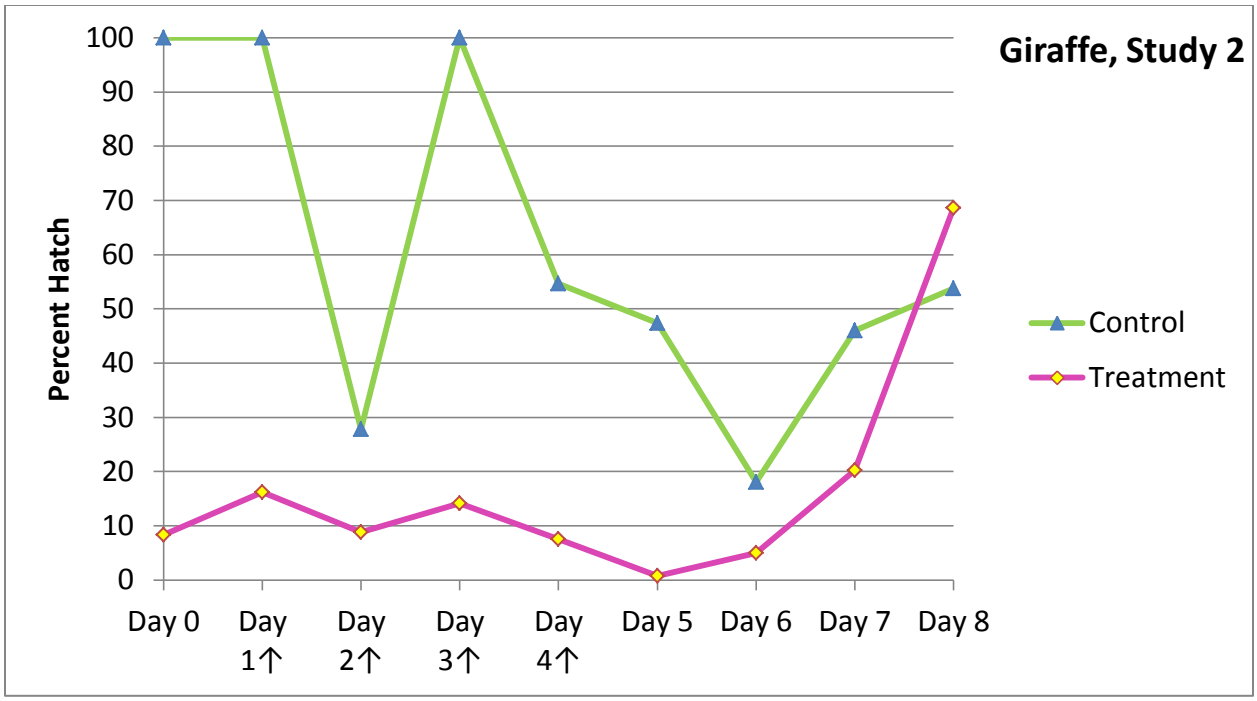

Figure 8. Study 2. Mean percent hatch and development to L3 in feces for treatment (n=2) and control (n=1) giraffe that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/ BW). Arrows indicate chlamydospore feeding period.

The mean percent reduction of L3 was consistently high from day 0 to 5, reaching a maximum percent reduction of 98.5% on day 5 (Figure 9). Mean percent reduction then decreased steadily to 0% on day 8.

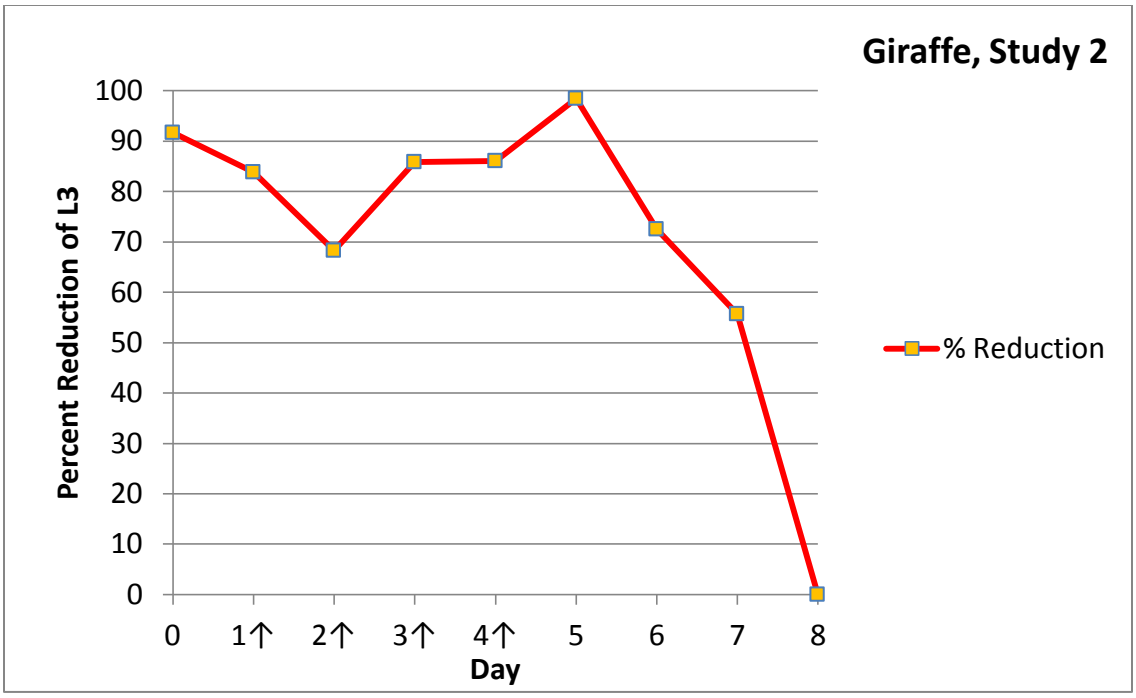

Figure 9. Study 2. Mean percent reduction of L3 comparing treated (n=2) and control (n=1) giraffe that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

4.2.2. Antelope

Overall mean FEC of the control and treated groups was 150-950 and 950-2350, respectively (Figure 10). Mean FEC of the control group was consistently lower than the treated group.

Mean percent hatch was similar for both groups on day 0. Subsequent to the start of chlamydospore feeding (day 1), the treatment group's mean percent hatch was consistently lower than the control group through day 6 (Figure 11). By days 7 and 8, the treatment group's mean percent hatch increased and was similar to that of the control group.

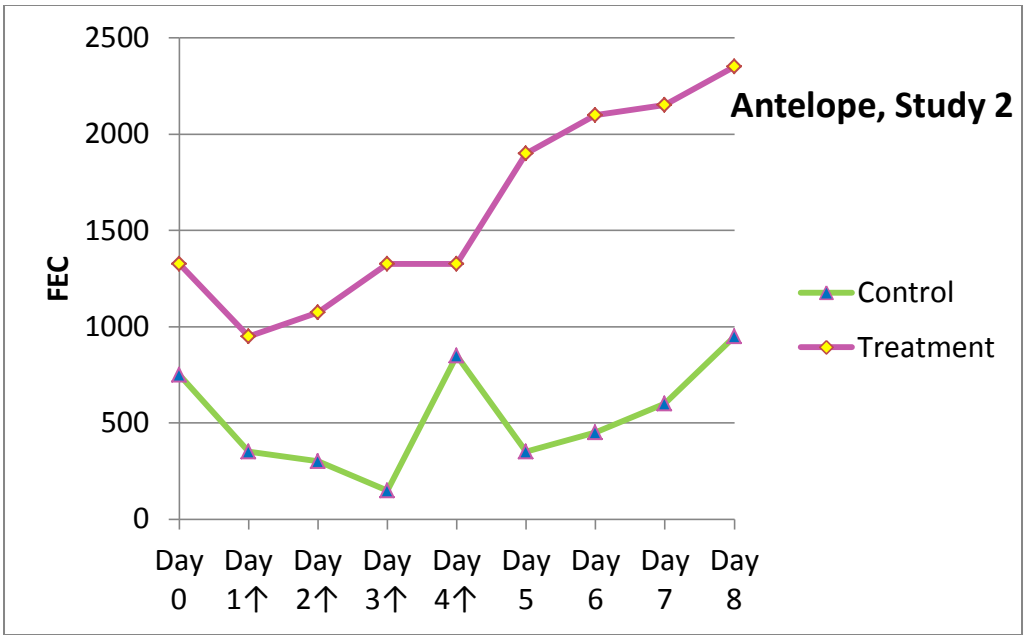

Figure 10. Study 2. Mean fecal egg count for treatment (n=2) and control (n=1) antelope that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

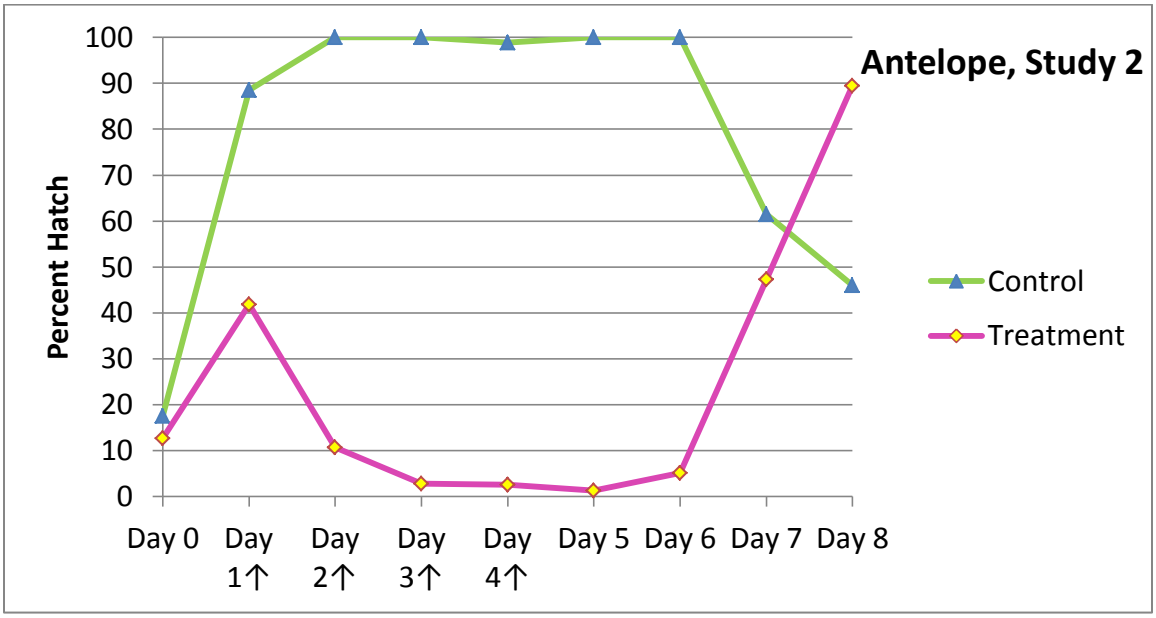

Figure 11. Study 2. Mean percent hatch and development to L3 in feces for treatment (n=2) and control (n=1) antelope that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/ BW). Arrows indicate chlamydospore feeding period.

Mean percent reduction was low on days 0 and 1 (Figure 12) and increased to above 90% through day 6. Subsequent to stopping chlamydospore feeding, mean percent reduction then decreased to 23.1% and 0% on days 7 and 8, respectively.

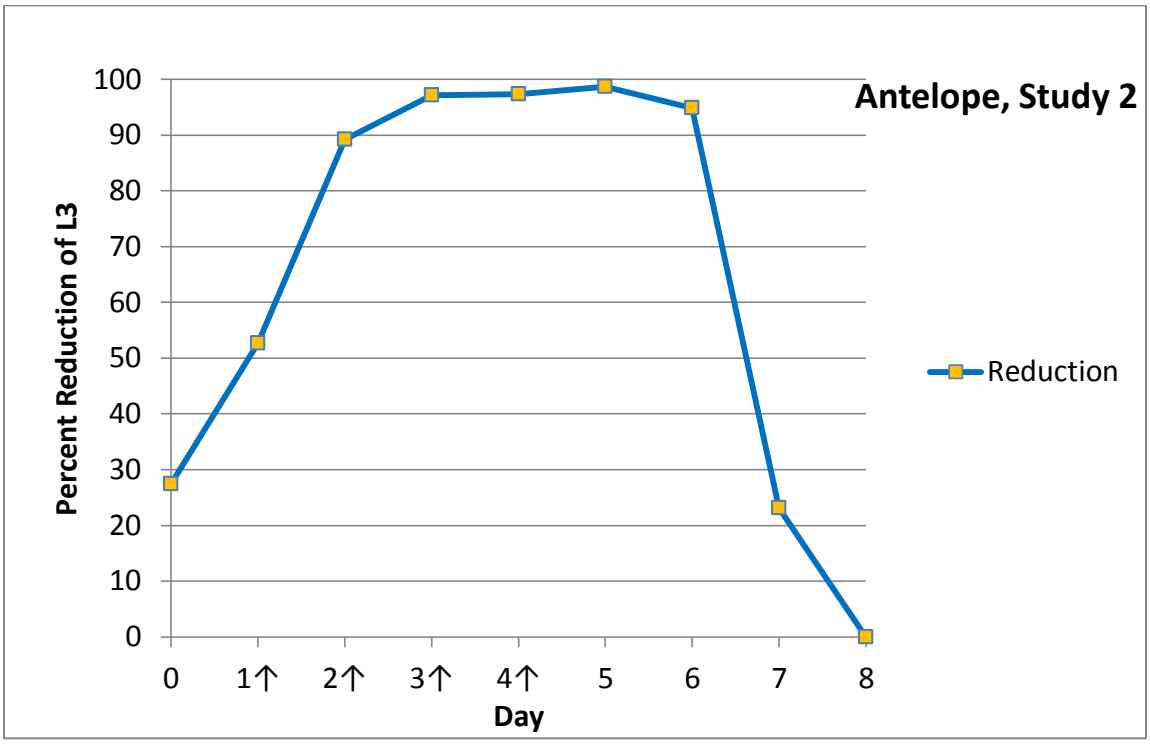

Figure 12. Study 2. Mean percent reduction of L3 comparing treated (n=2) and control (n=1) antelope that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

4.3 Study 3

4.3.1. Giraffe

Overall mean FEC of the control and treated groups was 20-760 and 208-2042, respectively (Figure 13). Mean FEC of the control group was consistently lower than the treated group through week 6. Subsequently, FEC were similar.

Mean percent hatch was similar ($p > 0.05$) for both groups on week 1 (Figure 14). Subsequent to the start of chlamydospore feeding (week 1), the treatment group mean percent hatch was consistently lower than the control group through week 9. By weeks 10 and 11, the treatment group mean percent hatch increased and was similar to that of the control.

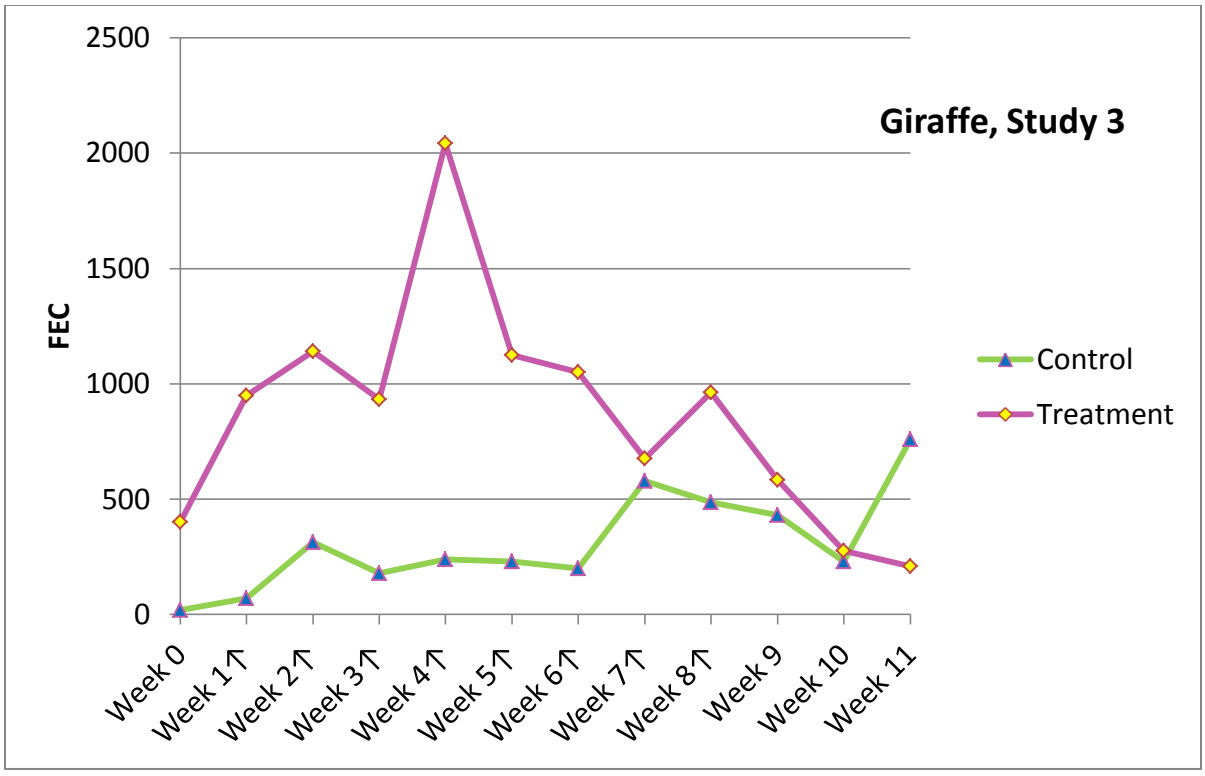

Figure 13. Study 3. Mean fecal egg count for treatment (n=6) and control (n=5) giraffe that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

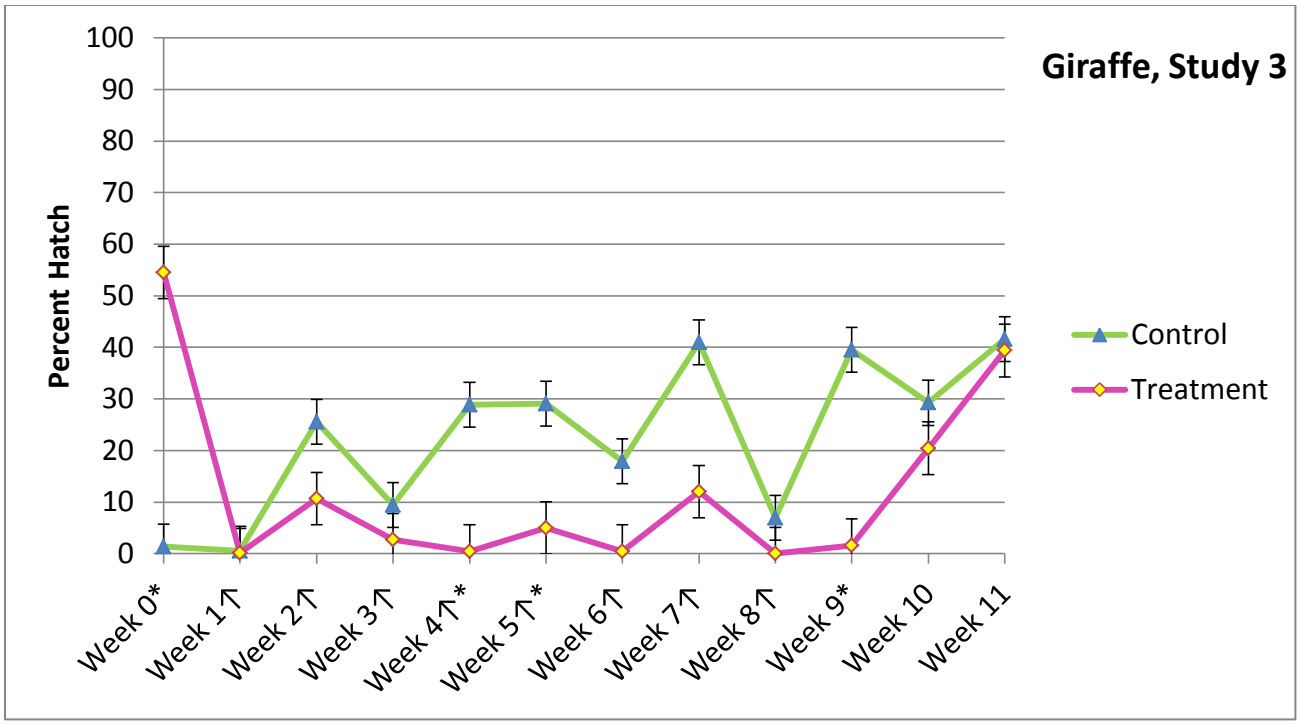

Figure 14. Study 3. Mean percent hatch and development to L3 in feces for treatment (n=6) and control (n=5) giraffe that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/ BW). Arrows indicate chlamydospore feeding period.
*****Indicates statistical significance of $p \leq 0.05$.

Mean percent reduction was zero on week 0 (Figure 15) then increased to above 80% from weeks 4 through 9 (except for week 7). Subsequent to stopping chlamydospore feeding, mean percent reduction then decreased to 30.1% and 5.3% on weeks 10 and 11.

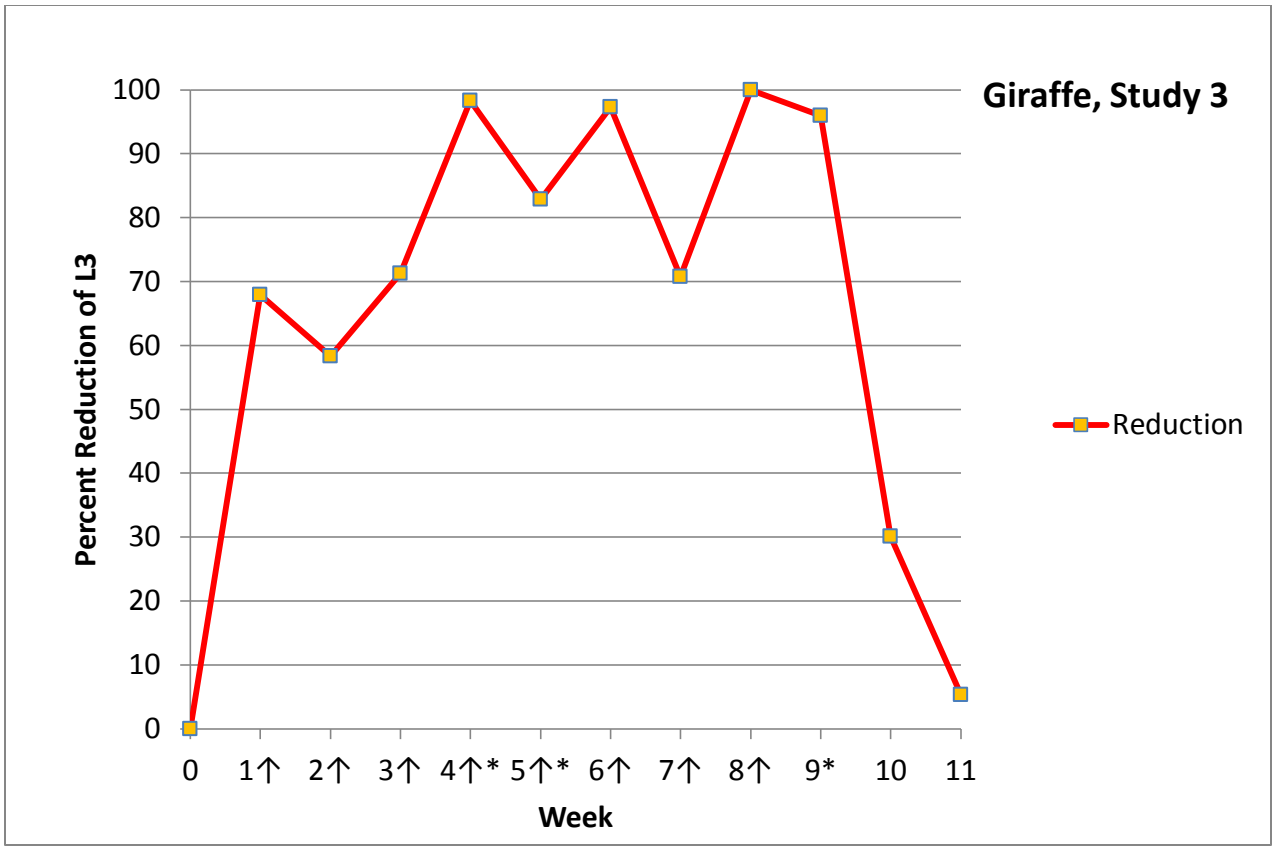

Figure 15. Study 3. Mean percent reduction of L3 comparing treated (n=6) and control (n=5) giraffe that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.
*Indicates significant reduction ($p \leq 0.05$).

4.3.2. Antelope

Overall mean FEC of the control and treated groups was 450-1930 and 200-4650, respectively (Figure 16). Mean FEC was similar for both groups.

Mean percent hatch was similar ($p > 0.05$) for both groups on weeks 0 and 1 (Figure 17). Subsequent to the start of chlamydospore feeding (week 1), the treatment group mean percent hatch was consistently lower than the control group through week 9. By week 10 and 11, the treatment group mean percent hatch increased and was similar to that of the control group.

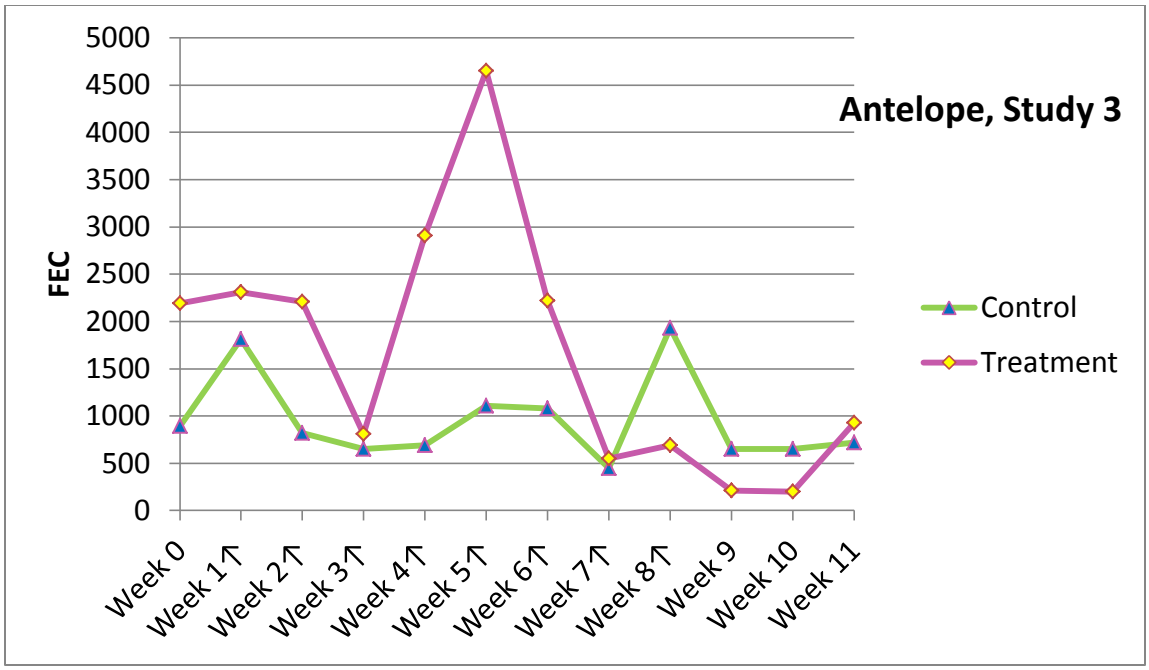

Figure 16. Study 3. Mean fecal egg count for treatment (n=5) and control (n=5) antelope that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

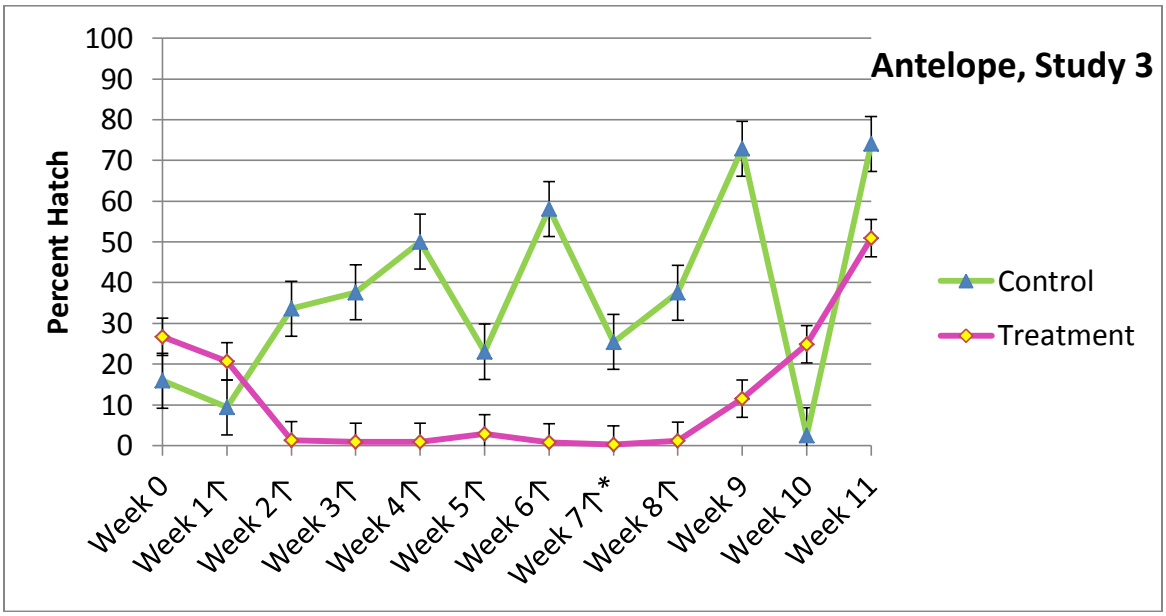

Figure 17. Study 3. Mean percent hatch and development to L3 in feces for treatment (n=5) and control (n=5) antelope that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/ BW). Arrows indicate chlamydospore feeding period.
*****Indicates statistical significance of $p \leq 0.05$.

Mean percent reduction was zero on weeks 0 and 1 (Figure 18) and increased to above 90% through week 9 (except for week 5). Subsequent to stopping chlamydospore feeding, mean percent reduction then decreased to 0% and 31.2% on weeks 10 and 11, respectively.

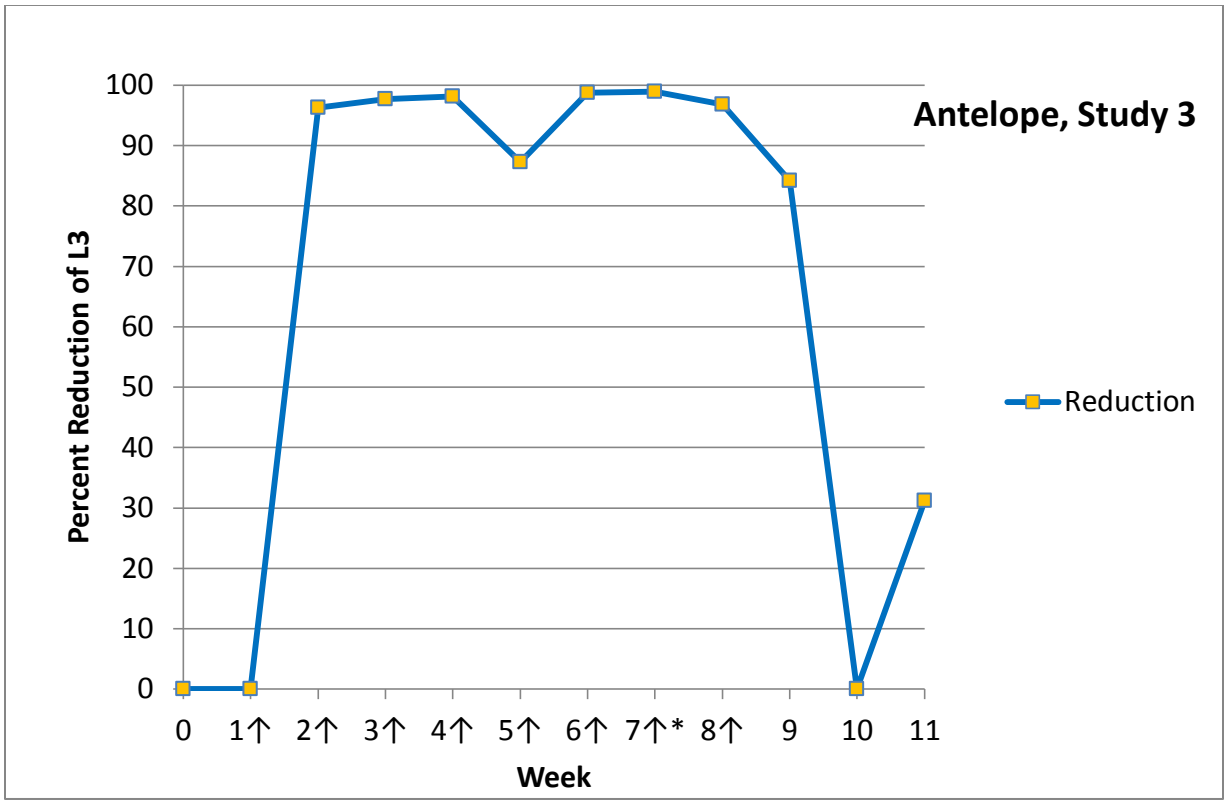

Figure 18. Study 3. Mean percent reduction of L3 comparing treated (n=5) and control (n=5) antelope that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.
*Indicates significant reduction ($p \leq 0.05$).

4.3.3. Gerenuk

Overall mean FEC of the control and treated groups was 0-2075 and 25-1975, respectively (Figure 19). Mean FEC was similar for both groups.

Mean percent hatch was similar ($p > 0.05$) for both groups throughout the study (Figure 20), but the treatment group was consistently lower than the control group weeks 6 through 10. By week 11, treatment group mean percent hatch increased and was similar to that of the control group.

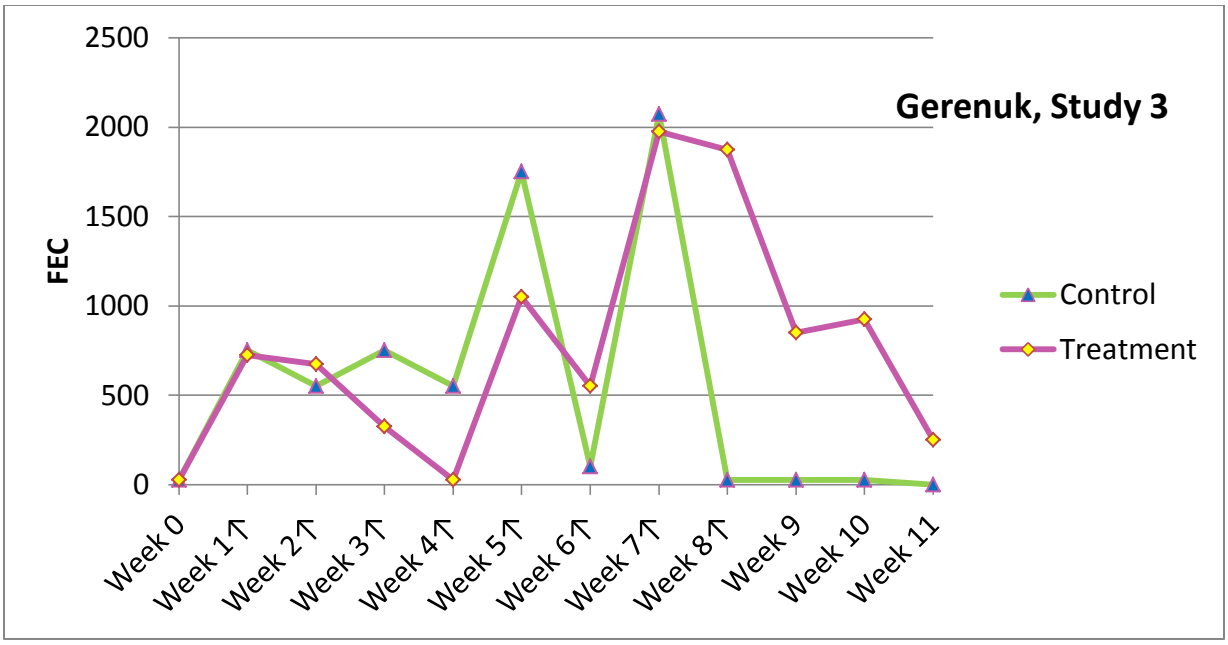

Figure 19. Study 3. Mean fecal egg count for treatment (n=2) and control (n=2) gerenuk that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

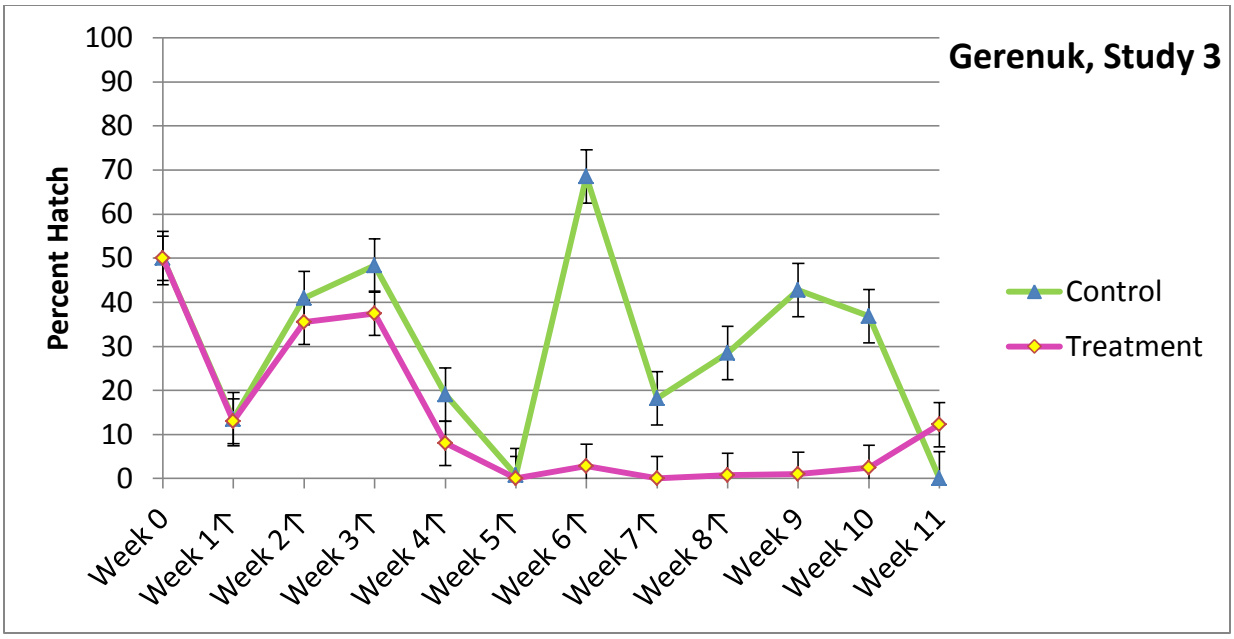

Figure 20. Study 3. Mean percent hatch and development to L3 in feces for treatment (n=2) and control (n=2) gerenuk that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/ BW). Arrows indicate chlamydospore feeding period.

Mean percent reduction was zero at week 0, steadily increased and consistently was above 90% from weeks 5 through week 10 (Figure 21). Subsequent to stopping chlamydospore feeding, mean percent reduction then decreased to 0% on week 11.

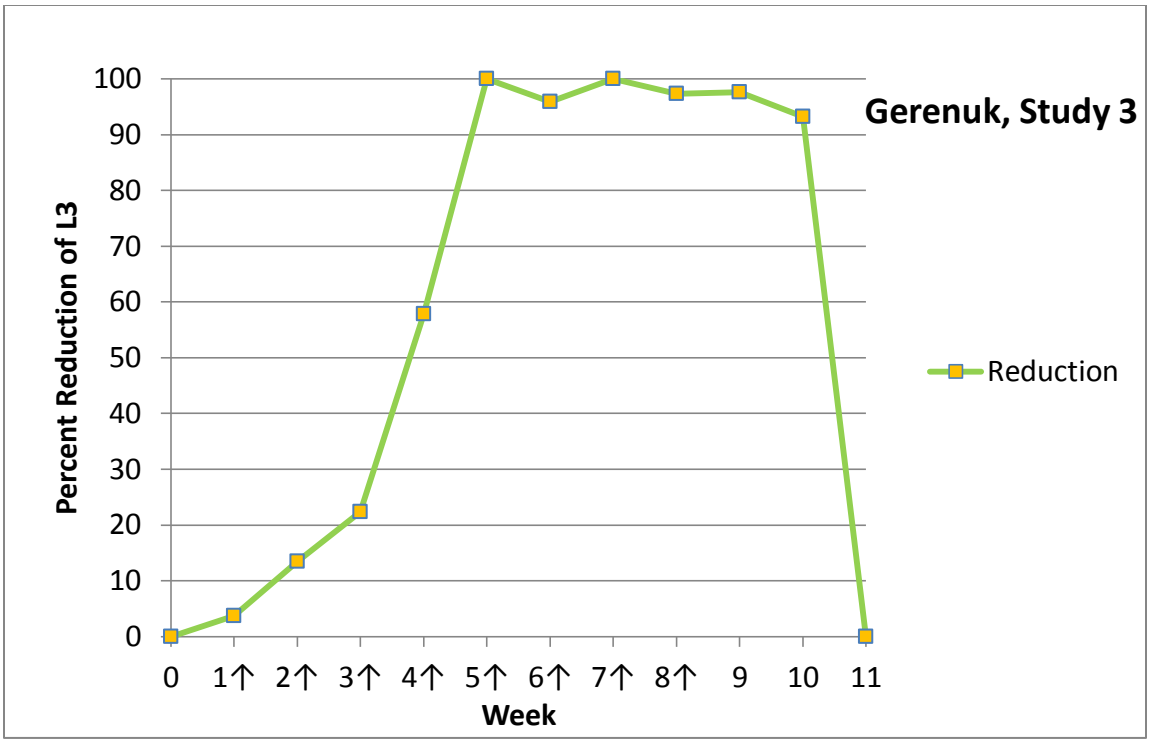

Figure 21. Study 3. Mean percent reduction of L3 comparing treated (n=2) and control (n=2) gerenuk that were fed *Duddingtonia flagrans* chlamydospores (30,000 per kg/BW). Arrows indicate chlamydospore feeding period.

CHAPTER 5
DISCUSSION AND CONCLUSION

Although many studies have been done illustrating the positive effects of *D. flagrans* in reducing L3 in feces of domestic ruminants, no studies, until now, have examined the use of *D. flagrans,* as a biological alternative, in exotic artiodactylids. The present studies revealed that the nematophagous fungus, *Duddingtonia flagrans*, is effective in reducing L3 of trichostrongyle type GIN in the feces of exotic artiodactylids in captivity. The results of these studies concur with studies done in sheep that showed that doses ranging from 5×10^5 to 10^6 chlamydospores per kg/BW per day had >80% reduction in L3 that developed from GIN eggs in feces (Larsen et al., 1998; Fontenot et al., 2003). Similar results were also reported in a study by Peña et al. (2002) which indicated that the use of five different doses of chlamydospores, ranging from 2.5×10^4 to 5×10^5 per kg/BW resulted in reductions ranging from 76.6-100%.

The objective of study 1 was to evaluate the effect of a Mexican isolate of *D. flagrans* at a dose of 500,000 chlamydospores per kg/BW in reducing the number of L3 in the feces of giraffe and antelope. For giraffe, the percent reduction was low prior to chlamydospore feeding and was high for days 2-3 and decreased on day 4. This decrease could be attributed to consumption issues with treatment animals. Days 5 and 6 showed a residual effect after feeding was discontinued. This effect subsequently dissipated by days 7 and 8 (3-4 days post feeding). This residual effect was also observed by Mendoza de Gives et al., (1998) and Peña et al. (2002) where residual activity was seen for 2 days post treatment in Suffolk ewes. For antelope, percent reduction was similar to that of the giraffe with high reduction from days 2-5 which subsequently decreased after feeding was discontinued (days 7 and 8). The residual effect was present on day 5 but since day 6 data was not available, it was not possible to demonstrate any residual effect on that day. Overall, percent reduction for both the giraffe and antelope indicated that *D. flagrans*

was effective in reducing the amount of L3 in the feces during the period of feeding and up to 2 days post-feeding. Sample size was a limitation in regards to establishing significant statistical inferences. Increasing sample size numbers in each group should reduce variability in each group and may create significant differences detectable by statistical evaluation.

In studies 2 and 3, a substantially lower dose (30,000 chlamydospores per kg/BW) of an Australian isolate of *D. flagrans* was administered to animals for 4 days and 8 weeks, respectively, to evaluate reducing L3 in the feces of giraffe, antelope, and gerenuk (trial 3 only). In study 2, percent reduction for the giraffe started off at a high value and kept a high level of reduction until day 5. This high reduction during the pre-feeding period and fluctuation of reduction during the feeding created a challenge when trying to evaluate the effect. This was probably due to only having one control animal and the lack of consistency in percent hatch drastically affecting the trend on reduction. As seen in study 1, reduction remained high on days 5 and 6 after feeding was discontinued and then decreased by day 7 and 8. Percent reduction for the antelope started off low and increased to over 90% for days 3-6 which, again, coincided with the fungal feeding period and residual effect time frame. The decreased reduction on days 7 and 8 again indicated that the effect of the fungus had dissipated.

In study 3, weekly sampling instead of daily sampling was done over a 12 week span. The fungus was fed daily to every other day for 8 weeks with a 3 week post-feeding period. The percent reduction for giraffe was low on week 0 and high from week 1 through week 8 which, again, coincided with the feeding period. Reduction remained high on week 9 which was a week after feeding was discontinued. This was not expected as it was more than the 2 days observed in the other studies. There is no explanation for this extended period of activity. Then, again, as previously observed, reduction dissipated by weeks 10 and 11 which indicated the

effect was gone. The percent reduction for antelope was low on weeks 0 and 1. The low reduction on week 1 was not expected, and could be attributed to non-compliance issues with feeding during that first week of feeding. Subsequently, percent reduction was high from weeks 2 through 8 during the feeding period. As with the giraffe, reduction remained high on week 9 with no explanation. The percent reduction for gerenuk started out low and increased steadily until reaching a high level on week 5. This slow increase could be attributed to non-compliance issues as noted for the antelope, with non-compliance being more of a problem. An acclimation period may have been needed for these animals to adjust to the method of administering the chlamydospores. Reduction was then high during the feeding period (weeks 5 through 8). Again, there was an observed residual effect, but this time it was for 2 weeks (9 and 10) after feeding was discontinued. Reduction then decreased on week 11 as expected. The residual effect observed in trial 3 was substantially longer than that observed in trials 1 and 2. This extension of the residual period was consistent for all 3 species and the reason for this is unknown.

The results from all 3 studies suggest that *D. flagrans* is effective in reducing mixed species of GIN L3 in the feces of 3 exotic artiodactylids at doses of 30,000 and 500,000 chlamydospores per kg/BW. A study done by Waghorn et al. (2003) using *D. flagrans* in 2 different hosts, sheep and goats, had similar results and indicated that the fungus did not discriminate with broad spectrum activity against 3 GIN L3 with no preference over parasite species.

The chlamydospores in trial 1 were in suspension form before mixing into feedstuff whereas the chlamydospores used in trials 2 and 3 were in a powdered mixture that was added into feed. *Duddingtonia flagrans* not only has the ability to survive the ruminant digestive tract, but studies have shown the chlamydospores remain viable in suspension, on barley grains, and

when incorporated into feed blocks (using a manufacturing process). Efficacy of activity was not reduced in any of these means of deployment as they all resulted in high percent reductions of L3 (Mendoza de Gives et al., 1998; Waller et al., 2001). Another factor to consider is activity of different isolates of *D. flagrans* which could have different effects. Both isolates (Mexican and Australian) used in these studies had essentially the same efficacy. Isolates of *D. flagrans* have been found to be genetically similar which make them similar in structure and ability (Waller, 2003). Therefore, activity might be expected to be similar, which they were. The fact that *D. flagrans* is effective at a large range of doses, exhibits no discrimination between GIN L3 species, has high survivability rates in the ruminant digestive tract as well as viability when incorporated into different methods of deployment, is ubiquitous, and isolates are genetically similar gives this fungus a huge advantage over other fungi. These advantages make this fungus an asset in integrated parasite control programs as a method to reduce habitat infectivity in zoological settings.

For optimal results in L3 reduction, the fungus should be fed in the early grazing season, and daily feeding should be maintained for at least 6-10 weeks as to reduce the number of L3 on pasture during peak parasite season (Ketzis et al., 2006). The span of trials 1 and 2 were very short with 9 days being the total study time. Although results seemed promising in all the trials in reducing L3 and increasing percent reduction in fecal cultures, prolonged feeding of the fungus over several weeks must be sustained before overall levels of pasture infectivity decreases. The ultimate goal of the fungus treatment is to decrease the number of L3 on herbage therefore eventually decreasing worm burdens in animals. It is not used as an anthelmintic; it is used to help clean up pastures.

Maintaining exotic artiodactylids in captive zoological settings has its consequences with high levels of parasitism being a primary issue. The cause of high parasite burdens in these animals include increased stocking rates, lack of browse as to not obstruct guest views, stress from captivity, irrigation (to enhance vegetation), and the inability to close exhibits. The traditional use of anthelmintics to reduce GIN burdens is no longer effective in the zoological setting. Estimated body weights, varying compliance, and unknown pharmacokinetic data have resulted in resistance of GIN in exotic artiodactylids to traditional anthelmintic classes. In Disney's Animal Kingdom®, 100% benzimidazole and ivermectin resistance was reported as well as a low grade resistance to levamisole (D. Fontenot, personal communication, July 23, 2012). Drugs with no resistance reported as of yet are being used on a very limited basis. Environmental control strategies have been implemented by zoos and include feces removal programs, maintaining mixed species exhibits with animals that don't share mutual parasites, rotating herds thus limiting exposure time, and the adding of browse in elevated feeders. Even with these control measures, resistant GIN burdens are still a huge problem. The exploration of biological alternatives is essential to help control resistant GIN.

In the present studies, *D. flagrans* was shown to be effective in reducing L3 in feces. Results were variable at times because of varying compliance and missing samples from animals. A possible solution to the compliance issue could be implementation of *D. flagrans* in a sustained release form that would last at least 60 days. A major limitation of these studies was the small sample size numbers. This is unavoidable when working with limited populations of exotic animals in restricted enclosures, especially when samples are missing or unexpected discrepancies in FEC or larval cultures occur, all of which can result in skewed results. Significance could be affected by the results of a single animal or single data point. Another

limitation in regards to these studies was the large number of people involved in collection, shipping, and processing which increases the chance of errors.

The results of these trials indicated that the inclusion of *D. flagrans* in zoological parasite control programs could be a useful tool in reducing infective L3 of GIN in enclosures. *Duddingtonia flagrans,* used in combination with proper exhibit management, proper animal management, parasite monitoring programs, other biological alternatives such as COWP, and the smart use of anthelmintics could be a valuable asset in the overall control of GIN. Further studies involving the use of *D. flagrans* in exotic artiodactylids should be conducted investigating effects of long term use on pasture and on subsequent animal re-infection along with effective administration strategies to achieve maximum compliance.

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VITA

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