COMPARATIVE RESPONSES OF CATTLE, SHEEP, AND GOATS TO LARVAE OF THE EMERGING TREMATODE, *DICROCOELIUM DENDRITICUM*, AND DEVELOPMENT OF A DIAGNOSTIC TOOL

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Abstract

Parasitic infections caused by species of gastrointestinal ‘worms’ constrain efficient livestock production through the negative effects they have on individual hosts. *Dicrocoelium dendriticum* is a host generalist trematode that infects grazing mammals and is an introduced species in Alberta. Reducing the impact of emerging, generalist parasites such as this one requires sensitive and specific diagnosis of individual hosts as well as an understanding of relative host responses to infection. I found high interspecific variation in fluke intensity, fluke recruitment, fecal egg counts, and antibody responses in experimentally infected cattle, sheep, and goats. Fluke intensity was significantly higher in goats (x=257±38) than in sheep (x=132±80) or cattle (x=111±103). Anti-fluke IgG and IgM antibody concentrations were high in sheep sera, but undetectable in cattle sera. A coproantigen ELISA detected *D. dendriticum* protein in cattle and sheep feces at 9 days post-infection. However, assay sensitivities were poor in sheep (x=45%) and cattle (x=23%).

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Table of Contents

Thesis Examination Committee………………………………………….……………..ii
Abstract……………………………………………………………………...……..iii
Acknowledgments………………………………………….....………………..iv
Table of Contents……………………………………...……………………v
List of Tables………………………………...……………..…………………vii
List of Figures……………………………………...……………………..viii
1.2 The Model System: *Dicrocoelium dendriticum*..6
1.3 Objectives 9
1.4 Literature Cited 10

Chapter 2. Comparative recruitment, fecal egg production, and humoral immune responses of cattle, sheep, and goats to larvae of the generalist liver fluke, *Dicrocoelium dendriticum* 16
2.1 Abstract 16
2.2 Introduction 17
2.3 Methods 19
2.3.1 Source of Hosts 19
2.3.2 Experimental Infections 20
2.3.3 Fluke Recovery 21
2.3.4 Fecal Sample Collection and Egg Counts 21
2.3.5 Serological ELISA Development 22
2.3.6 Data Analyses 24
2.4 Results 25
2.4.1 Worm Counts 25
2.4.2 Fecal Egg Counts 26
2.4.3 Serological ELISA 27
2.5 Discussion 29
2.6 Literature Cited 33

Chapter 3. Development of a coproantigen ELISA to detect *Dicrocoelium dendriticum* proteins in feces of experimentally infected sheep and cattle 42
3.1 Abstract 42
3.2 Introduction 43
3.3 Methods 47
3.3.1 Source of Hosts 47
3.3.2 Experimental Infections 48
3.3.3 Fecal Extract Preparation 48
3.3.4 Polyclonal Antibody Production and Purification 49
3.3.5 Coproantigen ELISA Development 50
3.3.6 Coproantigen ELISA Protocol 53
3.3.7 Data Analyses 54
3.4 Results 55
3.4.1 Pre-infection Fecal Egg Analyses 55
3.4.2 Limits of Detection 56
3.4.3 Fluke Coproantigens Detection 56
3.5 Discussion 57
3.6 Literature Cited 61

Chapter 4. General Conclusions 73

4.1 Literature Cited 77

List of Tables

2.1. Summary of host species, ages, sexes, and the number of metacercariae administered for experimental infections in 2011, 2013, and 2014……………………………………….……...…38

2.2. Summary of recoveries of *D. dendriticum* in experimentally-exposed beef cattle, sheep, and goats……………………………………………………………………………………....38

2.3. Summary fecal egg count (e.p.g.) data for *D. dendriticum* in experimentally exposed cattle, sheep, and goats. Data expressed in days post infection (d.p.i.)……………………..39

2.4. Summary of one-way repeated measures ANOVA to determine the effect of time and host species on IgG and IgM antibody response to experimental infection with *D. dendriticum*……………………………………………………………………….………39

3.1. Summary of host species, ages, sexes, and the number of metacercariae administered for experimental infections in 2012, 2013, and 2014………………………………………….…67

3.2. Experimentally infected cattle and their fluke burdens, days post-infection of positive coproantigen ELISA results, and the highest concentration of *D. dendriticum* protein detected in their fecal samples…………………………………………………….…….67

3.3. Experimentally infected sheep and their fluke burdens, days post-infection of positive coproantigen ELISA results, and the highest concentration of *D. dendriticum* protein detected in their fecal samples…………………………………………………….……….68

List of Figures

2.1. Mean optical densities ± s.e.m. from serological ELISAs measuring IgG antibody response to experimental infection with *D. dendriticum* over time in sheep, cattle, and goats. Each point represents an average of adjusted OD measurements from all serum samples falling within the coordinating d.p.i. range……………………………………………………………………….40
2.2. Mean optical densities ± s.e.m. from serological ELISAs measuring IgM antibody response to experimental infection with *D. dendriticum* over time in sheep, cattle, and goats. Each point represents an average of adjusted OD measurements from all serum samples falling within the coordinating d.p.i. range……………………………………………………………………….41
3.1. Standard curve of *D. dendriticum* ELISA in carbonate buffer……………………………....69
3.2. Standard curve of *D. dendriticum* ELISA in sheep fecal extract (negative control)……..… .69
3.3 Standard curve of *D. dendriticum* ELISA in cattle fecal extract (negative control)………....70
3.4. ELISA's standard curves in cattle fecal extracts diluted 1:1, 1:5, 1:10 and 1:25 in CBS........71
3.5. ELISA's standard curves in sheep fecal extracts diluted 1:1, 1:5, 1:10 and 1:25 in CBS…...71
3.6. Positive coproantigen ELISA samples from cattle and sheep, as measured by log transformed optical density, and their corresponding day post infection……………………………………...72
3.7. Positive coproantigen ELISA samples from cattle and sheep, as measured by log transformed optical density, and their corresponding fluke burden…………………………………………72

Chapter 1. General Introduction

Parasites and diseases of livestock are major impediments to efficient food production (Fitzpatrick 2013). This is problematic given that the world population is expected to rise from seven to eight billion over the next 15 years (UNPD 2008, Fitzpatrick 2013). As a result, 50% more food, in the form of protein, will be needed by 2030 (FAO 2008, Fitzpatrick 2013). In the UK, animal diseases are estimated to cause a 17% loss of production (Flint and Woolliams 2008). Developing countries are more susceptible to production losses due to animal diseases, with an estimated 35-50% loss of production (Flint and Woolliams 2008). Furthermore, the development of resistance to the main chemical treatments used for parasite and disease control in livestock is a major issue worldwide that threatens the sustainability of livestock operations (Besier 2007, Kaplan and Vidyashankar 2012, Fitzpatrick 2013). These issues highlight the need for early diagnosis of parasites and diseases.

Parasitic infections caused by gastrointestinal 'worms' or helminths (nematodes, cestodes, and trematodes) constrain efficient livestock production through the negative effects they have on individual hosts (van Dijk et al. 2010, Fitzpatrick 2013, Charlier et al. 2014). Comparisons of performance between healthy and infected hosts provide evidence of the potential negative consequences of infection of livestock with worms. Domestic calves experimentally infected with the gastrointestinal (GI) nematode, *Ostertagia ostertagi*, experience weight loss, diarrhea, and abomasal lesions (Ritchie et al. 1966). Studies of the trematodes *Fasciola gigantica* and *Fasciola hepatica* have shown that infected sheep can experience reduced weight gain within two weeks of exposure when compared to healthy sheep (Raadsma et al. 2007). *Fasciola gigantica*- infected

sheep had elevated levels of the hepatic enzyme gamma glutamyl transferase (GGT), indicating epithelial damage in the bile ducts (Raadsma et al. 2007). Similarly, goats infected with *F. hepatica* also experienced elevated GGT levels, and severe liver damage that led to death in some animals (Martinez-Moreno et al. 1999). Sheep naturally infected with the lancet liver fluke, *Dicrocoelium dendriticum,* experience weight loss and tissue necrosis and inflammation as a result of hepatogenous photosensitization due to bile duct blockage (Sargison et al. 2012). These results show that reducing the impact of GI helminths is an important component in maximizing production efficiency in livestock (Fitzpatrick 2013).

Despite evidence of direct effects on livestock health, establishing helminths' precise economic impact on livestock production remains difficult due to the numerous indirect effects that are often associated with infection (Charlier et al. 2014). One example involves the link between liver fluke and certain bacterial infections in cattle (Charlier et al. 2014). Similarly, helminth infections can cause changes to a host's intestinal microbiota that in turn, can influence host response to infection (Cervi et al. 2004, Kane et al. 2004, van Riet et al. 2007). Pregnant ewes experimentally infected with trematodes *D. dendriticum* and *F. hepatica* developed mastitis during *post-partum* lactation significantly more than the control group (Mavrogianni et al. 2014). Katsoulos et al. (2011) analyzed 254 sheep livers and found that the percentage of cholelithiasis (gall stones) was significantly higher in livers parasitized with *F. hepatica* and/or *D. dendriticum*. These indirect effects highlight the complexity of the outcomes of helminth infections and the difficulty in determining their economic impact.

However, estimates of costs incurred directly due to parasitism are substantial, even without accounting for indirect effects. Schweizer et al. (2005) estimated that *F. hepatica* caused losses of $78 million (CAD) annually in Switzerland alone, which represents a loss of $450 per animal. In Alberta (Canada), undiagnosed parasitism in livestock is thought to cost the industry tens of millions of dollars each year (Bauck et al. 1989, Jim et al. 1992, Schunicht et al. 2000). While some losses are related to production, additional losses come from the high cost of treating livestock with anthelmintic drugs (Perry and Randolph 1999).

A further problem that arises with the use of anthelmintic drugs is the development of drug resistance, which stems from common practices like blanket treatment of entire herds (Wolstenholme et al. 2004). The over-use of anthelmintic drugs leads to anthelmintic resistance, which threatens the sustainability of livestock operations (Kaplan 2004, van Dijk et al. 2010). Hoste et al. (2002b) presented a solution to reduce the progression of anthelmintic resistance within worm populations and reduce the high costs of blanket treatment. They found that targeted application of anthelmintics could help better control trichostrongyle nematodes within groups of goats and could also prevent the establishment and spread of drug resistance. The key shortcoming of this approach is that it requires the distinction between uninfected hosts within a population with those that contain various numbers of worms (Hoste et al. 2002a, Hoste et al. 2002c).

Diagnosing helminth infections often relies on fecal egg enumeration, which involves centrifugation and microscopy to detect parasite eggs in fecal samples (Rehbein et al. 1999, Rojo-Vázquez et al. 2012). This method of diagnosis is notoriously unreliable

due to intermittent and highly variable egg shedding patterns by individual worms and in the difficulty in detecting eggs that are often small and cryptic (Braun et al. 1995, Jithendran and Bhat 1996, 1999, Rinaldi et al. 2011). Another common method of diagnosis involves the detection of parasite-specific serum antibodies using enzyme-linked immunosorbent assay (ELISA). In some cases, anti-helminth antibodies are detected more consistently and earlier than parasite eggs in the feces (Vercruysse and Claerebout 2001). Serological ELISAs are useful for assessing the relative levels of host immune responses but they are not ideal for diagnosis since antibodies often persist in the blood post-infection and therefore may not necessarily reveal current infection status (Vercruysse and Claerebout 2001, Andersen et al. 2013). Considering the downfalls of egg counts and serology for diagnosing current infection status in individual hosts, it is clear that they offer limited promise as sensitive and specific diagnostic tools.

Another type of diagnostic tool relies on the detection of worm antigens in feces (Fraser and Craig 1997, Andersen et al. 2013). Coproantigens typically refers to parasite-derived proteins (e.g. excretory/secretory products) that are transported through the gastrointestinal tract into the feces (Charlier et al. 2014). Coproantigens are measurable by ELISAs (Fraser and Craig 1997, Andersen et al. 2013). The key advantages of this method are two-fold: detection of parasite antigens represents true infection status of an individual host, and antigen concentrations measured in fecal samples can indicate worm intensity (Mezo et al. 2004, Skotarek et al. 2010, Demerdash et al. 2011, Brockwell et al. 2013, Watwiengkam et al. 2013, Elsemore et al. 2014). For example, Skotarek et al. (2010) used polyclonal antibodies developed against the cestode *Anoplocephala perfoliata* and fecal samples from horses in a coproantigen ELISA. They were able to

detect *A. perfoliata* proteins in fecal samples from infected horses and found a positive correlation between antigen concentration and tapeworm intensity. Watwiengkam et al. (2013) used monoclonal antibodies to detect *Opisthorchis viverrini* in human fecal samples. They too found a correlation between antigen concentration detected and worm intensity. Coproantigen ELISAs have been developed for *Fasciola gigantica* and *Fasciola hepatica* in cattle with success in correlating antigen concentration with fluke intensity (Demerdash et al. 2011, Brockwell et al. 2013). These results show that current infection status can be determined within hosts via coproantigen ELISAs. They also show that coproantigen ELISAs can potentially indicate worm intensity in individual hosts. Coproantigen ELISAs could therefore be sensitive and specific diagnostic assays for other parasites of livestock.

While diagnostic tools can help manage parasites of livestock, it is also important to understand host immune responses to infections especially with generalist parasites that can infect multiple host species. Protective immune responses can develop with multiple exposures to parasites (van der Ree and Mutapi 2015). Gastrointestinal nematodes often elicit an effective immune response in ruminant hosts, which can lead to protection to subsequent exposure (Knox et al. 2012). Thus, host immune responses influence the development of parasitic infections, can help protect against their associated negative effects, and can influence overall rates of transmission of eggs and other larval stages onto pasture. The strength and overall importance of host immune responses vary between and within species of host (Colditz 2002). For example, Beck et al. (2015) found that a population of beef cattle on a co-grazed pasture were responsible for transmitting approximately 80% of the 300 billion *D. dendriticum* eggs that are estimated to

contaminate pasture each year in Cypress Hills Provincial Park in Alberta, Canada. By contrast, sub-populations of calf and yearling deer and elk were responsible for a much smaller proportion of the eggs found on pasture. This disparity between cattle and wildlife contribution to eggs on pasture emphasizes the interspecific differences in host responses to infection. Despite knowing that interspecific differences exist, they are not well documented. Filling this knowledge gap for host generalist parasites could help determine relative species susceptibility and their contribution to disease transmission via eggs shedding onto shared pasture. Such knowledge would help inform management decisions and could help reduce the economic impact of such species.

1.2 The Model System: *Dicrocoelium dendriticum*

Dicrocoelium dendriticum (Plagiorchiida: Dicrocoelidae), also known as the small liver fluke and the lancet liver fluke, is a parasite of grazing animals. Lancet liver flukes are host generalists living in the bile ducts of infected hosts. The lancet liver fluke can parasitize ruminants like cattle, sheep, goats, lamas, elk, and deer (Manga-González and González-Lanza 2005, Klein et al. 2012, Beck et al. 2014). Before reaching its final host, *D.dendriticum* infects two intermediate hosts – snails and ants (Otranto and Traversa 2003). Adult worms (8-14 mm in length and 2-3 mm in width) shed eggs, which are released in the feces of the final host (Euzeby 1971). Terrestrial snails ingest the eggs, within which a miracidium develops. Thousands of daughter sporocysts are produced via asexual reproduction within the snails' digestive gland, which then release cercariae. Cercariae are expelled from the respiratory chamber in aqueous slime balls released into the environment, which are in turn ingested by ants. Metacercariae develop within the haemocoel of infected ants. The presence of a single metacercaria in an ant's

subesophageal ganglion causes the ant to clamp its jaw onto the tips of vegetation at temperatures below 15 °C (Otranto and Traversa 2002). This altered behaviour results in ants that are more likely to be ingested by grazing animals. Following excystation within the stomach, metacercariae migrate to the bile ducts where they mature into gravid adults (Otranto and Traversa 2002).

In addition to having a complex life cycle, *D. dendriticum* has broad and diverse host and geographic ranges in almost every continent (Otranto and Traversa 2003, Goater and Colwell 2007, Klein et al. 2012). The presence of *D. dendriticum* in North America is of particular concern because it is known to infect wild ungulates and livestock (Goater and Colwell 2007). The prevalence of *D. dendriticum* in the Cypress Hills Provincial Park of Alberta, Canada is 60-90% in deer, elk, and beef cattle populations, and several high intensity infections of 1000 flukes or more have been reported (Goater and Colwell 2007).

The effects of *D. dendriticum* in sheep are well documented. Katsoulos et al. (2011) found that infection with *D. dendriticum* was a significant risk factor for the presence of gallstones in sheep. Similarly, pregnant ewes infected with *D. dendriticum* have increased occurrences of mastitis (Mavrogianni et al. 2014). Sargison et al. (2012) examined lambs experiencing weight loss and hepatogenous photosensitization, resulting in tissue necrosis and inflammation, and identified dicrocoeliosis as the cause. The lack of research on *D. dendriticum* in cattle is a concern for Alberta's livestock industry, which consists of 5 million head of cattle (Statistics Canada, 2013).

Dicrocoelium dendriticum infections are often diagnosed by enumerating eggs in host feces (Rehbein et al. 1999, Rojo-Vázquez et al. 2012). Broglia et al. (2009)

experimentally infected sheep with *D. dendriticum* and developed a serological ELISA using serum samples collected throughout the course of infection. They were able to detect anti-fluke antibodies at 30 days post-infection, a remarkable improvement from the 58 days post-infection detection via fecal egg counts. Experimentally infected sheep produced IgG antibodies against *D. dendriticum* starting at approximately 30 d.p.i. and their levels remained high until at least 180 d.p.i. (Gonzalez-Lanza et al. 2000, Broglia et al. 2009).

Colwell and Goater (2010) performed Western blots and serological ELISAs using serum from naturally infected cattle and determined that cattle produced IgG1 and IgM antibodies against some *D. dendriticum* antigens. However, it is unclear whether cattle produced these antibodies at 30 d.p.i., like experimentally infected sheep.

A coproantigen ELISA has not been developed yet for *D. dendriticum*. Given the previous results of coproantigen ELISAs for other parasites, it could be a diagnostic tool for the small liver fluke as well (Skotarek et al. 2010, Demerdash et al. 2011, Brockwell et al. 2013, Watwiengkam et al. 2013, Elsemore et al. 2014). The coproantigen ELISA could detect the presence of the small liver fluke, allowing for epidemiological studies of the parasite in wildlife while helping with disease management in livestock herds. Accurate and early detection of *D. dendriticum* could ultimately reduce its occurrence and minimize the development of anthelmintic resistance.

Immune responses against *D. dendriticum* in various ungulates have not been compared. Experimental infections with *D. dendriticum* have not been carried out in cattle or goats.

1.3 Objectives

The two objectives of my research project were firstly to compare the course of infection and the host responses of three host species to infection with *D. dendriticum*, and secondly to develop a *D. dendriticum*-specific coproantigen assay.

To meet these objectives, cattle, sheep, and goats were experimentally infected with *D. dendriticum*. Blood and fecal samples were collected throughout the infection process; blood serum was used in a serological assay to characterize the animals' immune responses (Chapter 2), while fecal material was tested by coproantigen ELISA to determine the presence of *D. dendriticum* (Chapter 3).

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Chapter 2. Comparative recruitment, fecal egg production, and humoral immune responses of cattle, sheep, and goats to larvae of the generalist liver fluke, *Dicrocoelium dendriticum*

2.1 Abstract

Helminths are an important group of livestock parasites that are often associated with lower productivity of wool, meat, and milk, in addition to reduced fertility in individual hosts. Host immune responses can contribute to the reduction of long-term productivity losses in individual hosts and can help reduce the economic impact of these types of infections. For helminths that are host generalists, it is important to characterize and understand factors leading to interspecific variation in host response and to overall host compatibility. This is a challenging task when it involves generalists that infect a wide range of sympatric hosts, both domestic and wild. To address this shortcoming, beef cattle, sheep, and goats were infected with metacercariae of *Dicrocoelium dendriticum*, a generalist liver fluke with a cosmopolitan distribution. Fluke intensities and percent fluke recoveries were evaluated at slaughter, and levels of blood serum IgG and IgM antibody classes against *D. dendriticum* were evaluated at various intervals post-infection. Results showed that worm recovery was highest in goats, followed by sheep and cattle. Anti-*D. dendriticum* antibodies in cattle sera remained undetectable, whereas IgG antibodies in sheep increased nine fold throughout the infection and goat IgG antibodies increased by 1.2-fold. Anti-fluke IgM antibodies for sheep and goats peaked at 43-56 d.p.i. with sheep increasing three-fold, and goat 1.4-fold. These results demonstrated a high variation in immune responses of beef cattle, sheep, and goats following *D. dendriticum* infection.

2.2 Introduction

Parasites of livestock can impose financial burdens for livestock production worldwide. In Alberta, Canada, undiagnosed parasites in livestock cost the industry tens of millions of dollars each year (Bauck et al. 1989, Jim et al. 1992, Schunicht et al. 2000). The parasitic worms, or helminths (nematodes, cestodes, trematodes), are known to cause pathological effects in infected animals including reduced meat, wool, and milk production, reduced feed conversion efficiency, and reduced fertility (Fitzpatrick 2013, Charlier et al. 2014).

Given the impact that this group of parasites has on livestock production, it is necessary to understand the nature of the host immune response following exposure. Typically, when a host becomes infected with a helminth, a Th2-type immune response is activated (Harris 2011, van der Ree and Mutapi 2015). Antibodies are up-regulated, with the IgG_1 class recognizing most of the parasite antigens (Flynn et al. 2010, van der Ree and Mutapi 2015). Multiple exposures to helminths can lead to a protective immune response by the host (Gomez-Munoz et al. 1999, van der Ree and Mutapi 2015). Thus, host immune responses influence the development of parasitic infections in individual hosts.

Other factors such as diet, breed, host age, and temperature, can cause variation in immune responses resulting from parasitic infections. For example, Shakya et al. (2011) artificially infected two breeds of lambs with the nematode, *Haemonchus contortus* and evaluated serum antibody concentrations and the densities of other effector cells (eosinophils, neutrophils, and mast cells). They concluded that one breed had a significantly stronger and more long-lasting immune response to this worm than the other

breed. Beck et al. (2014) found a clear age-related pattern in elk infected with the lancet liver fluke, *D. dendriticum*, with peak fluke burdens at two years of age, followed by a decrease. However, beef cattle that co-grazed the same pasture did not demonstrate an age-related decline. Mice and rats display interspecific variation in their immune responses, exhibiting different reactions to excretory-secretory antigens of lung-stage larvae of *Schistosoma mansoni* for example (Badr et al. 2015). Because many helminths are host generalists, the relative responses of different host species to helminth infections are important to know so that the treatment and management of these parasites can be tailored to the host type. However, the role of host immunity is unclear in multi-host parasites, in part due to the high interspecific variation and also to the context-dependent nature of parasite-induced negative effects. By understanding how different species respond to a given parasite, we can determine which species contribute most to egg-to-pasture transmission (Beck et al. 2015).

The small liver fluke, *D. dendriticum*, is a trematode with a broad host and geographical range (Otranto and Traversa 2002). The fluke resides in its host's bile ducts and gall bladder, thereby causing liver damage, which in turn can affect milk and meat production (Otranto and Traversa 2002). Following emergence in the mid-1990's, this trematode is now common in Cypress Hills Provincial Park (CHP), Alberta where it has been found to infect over 60% of resident elk, mule deer, white-tailed deer, and beef cattle (Goater and Colwell 2007, Beck et al. 2014).

Immune responses of sheep and cattle to *D. dendriticum* have been studied previously. Colwell and Goater (2010) obtained serum from naturally infected cattle, which was analyzed for IgG1, IgG2, and IgM anti-fluke antibodies. Cattle produced IgG1

and IgM antibodies against *D. dendriticum*, but IgG2 antibodies were not detectable. Experimentally infected sheep produced IgG antibodies against *D. dendriticum,* which were detected starting at 30 days post-infection (d.p.i.) (Broglia et al. 2009). At 60 d.p.i., Ferreras-Estrada et al. (2007) found a peak in IgG antibodies in experimentally infected sheep, which remained high until the animals were slaughtered at 180 d.p.i. Taken together, these results show that cattle and sheep produce antibodies against the small liver fluke, but their immunity and response to infection relative to each other is unknown.

In this study, we evaluated relative rates of *D. dendriticum* recovery and immune responses in sheep, goats, and beef cattle exposed to known doses of fluke larvae.

2.3 Methods

2.3.1 Source of Hosts

In the summers of 2011, 2013, and 2014, a total of 18 steers and 18 lambs were isolated in cattle and sheep barns at the Lethbridge Research and Development Centre (LeRDC) of Agriculture and Agri-Food Canada in Lethbridge, Alberta. In 2014, five yearling female feeder goats were purchased from a producer near Lethbridge and isolated in addition to the steers and lambs. Holstein steers were between eight months and one and a half years of age (Table 2.1) and were purchased from producers near Lethbridge. In 2011, yearling ewes were isolated from the LeRDC flock, while in 2013 and 2014 three-month-old ram lambs were utilized from the same flock (Table 2.1). It should be noted that animals were never exposed to an environment with a *D. dendriticum* presence. Steers were housed in an outdoor feed yard at LeRDC and fed hay and barley/corn silage. Goats were housed in an outdoor feed yard at LeRDC and fed hay.

Sheep were kept in an indoor barn at LeRDC and fed lamb grower ration, hay, and alfalfa pellets. All animals were given *ad libitum* access to salt and minerals. Animals had no previous exposure to *D. dendriticum* and fecal analysis was done on each animal to confirm their negative infection status prior to experimental infection.

2.3.2 Experimental Infections

Previous and ongoing studies in the Goater lab have indicated that all 'zombie' ants that 'cling' tightly to vegetation in CHP contain 1-148 metacercariae (Goater, unpublished observations). To infect animals with *D. dendriticum* in the spring of each year, large numbers of infected ants 'clinging' to vegetation were collected in Cypress Hills Provincial Park, Alberta (CHP). Live ants were brought back to the lab, placed in saline, and dissected to isolate the metacercariae from the ants' abdomens (Schuster 1991, Manga-González and González-Lanza 2005). Using a dissecting microscope, the metacercariae were counted, added to porcine hard gelatin capsules (size #000, Torpac®, Fairfield, New Jersey, USA), and any excess liquid was removed. Two small filled capsules were placed inside one large porcine hard gelatin capsule (size #12EL, 7.5ml, Torpac®, Fairfield, New Jersey, USA). One large capsule per animal was administered orally using a balling gun within 15 minutes of capsule assembly (Broglia et al. 2009), while two cattle and two sheep were isolated as negative controls. The numbers of metacercariae administered to the 16 cattle, 16 sheep, and 5 goats are summarized in Table 2.1. Numbers of metacercariae were chosen based on methods of Broglia et al. (2009).

2.3.3 Fluke Recovery

Animals were slaughtered between 93 and 179 days post infection (d.p.i.) and their livers dissected to evaluate worm intensity. The necropsy techniques described in Goater and Colwell (2007) were followed. Each liver was thinly sliced (0.5-1.0 cm) and placed in trays containing 95% saline. Liver slices were palpated by hand to dislodge flukes from hepatic and bile ducts. Adult worms were removed and transferred to dishes containing saline. Once liver slices were processed, the trays of saline were examined under a dissecting microscope for the enumeration of individual flukes.

2.3.4 Fecal Sample Collection and Egg Counts

To estimate rates of parasite development and fecundity in the different host species, fecal samples were collected approximately bi-weekly between 0-179 d.p.i. The samples were removed from the animals' rectum and frozen at -20°C (Rinaldi et al. 2011) Samples were thawed and then a modified Wisconsin Fecal Flotation with zinc sulphate was used to determine egg counts following methods in Schunicht et al. (2000). Five grams of feces was mixed with 20 ml of saturated zinc sulphate solution ($ZnS0_4$) and poured through 3 layers of cheesecloth. The fecal solution was divided into two 15 ml tubes (Cellstar® Polypropylene Tubes, Greiner Bio-One North America Inc., Monroe, North Carolina, USA). Additional $ZnS0_4$ was added to fill the tubes until a meniscus formed on each. A 22 mm x 22 mm cover slip was placed on the tops of each tube, followed by centrifuging at 1500 rpm for 10 minutes. Cover slips were removed and placed on glass slides, allowing two cover slips for each slide. Slides were examined for the presence of worm eggs under 4-10X magnification.

2.3.5 Serological ELISA Development

In each year of artificial infections, 7 ml blood samples were collected from each animals' jugular vein before exposure and then weekly or biweekly between 0-182 d.p.i., similar to previous studies (Broglia et al. 2009, Brockwell et al. 2013). Blood samples were centrifuged (accuSpin 3R, Fisher Scientific, Waltham, Massachusetts, USA) at 2500 rpm for 20 minutes after being collected in sterile Vacutainers®, and then placed at 4°C overnight. The following day, the samples were centrifuged again. Serum was transferred to 1 ml tubes (Titertube® Micro Test Tubes, Bio-Rad, Hercules, California, USA), and then stored at -20°C. Two ml of crude *D. dendriticum* protein extract was prepared following methods in Colwell and Baron (1990). In brief, 2 ml of fluke tissue was pulverized with a glass rod after the addition of 1 ml of phosphate buffered saline (PBS; 0.01M Na_2HPO_4, 0.01M $NaH_2PO_4 \cdot H_2O$, 0.15 NaCl, pH 7.2) and 10 μl of Phenylmethanesulfonyl fluoride solution (Sigma Life Science, lot #BCBB3651, Oakville, Ontario, Canada). Next, the solution containing fluke tissue was mixed using a homogenizer (Model PT 10/35, 110 volts, 6 Amps, 60Hz, Brinkmann Instruments Co., Waltham, Massachusetts, USA). The homogenized flukes were then sonicated three times at 40%, on power 4 for 15 seconds (Vibra cell, Sonics and Materials Inc., Danbury, Connecticut, USA). The sonicated material was divided into three 1.5 ml microfuge tubes (Axygen, Corning, New York, USA) and centrifuged (Micromax, International Equipment Company, Chattanooga, Tennessee, USA) at 3000 rpm at 4°C for 30 minutes. The resulting supernatant of *D.dendriticum* crude antigen was pipetted into Spectra/Por® molecularporous membrane tubing, MWCO: 3, 500 (VWR Scientific, Radnor, Pennsylvania, USA), sealed, and dialyzed for 4 hours in double distilled water (ddH_20) at

4°C. The dialyzed antigen was separated into two 15 ml tubes (Cellstar® Polypropylene Tubes, Greiner Bio-One North America Inc., Monroe, North Carolina, USA) and frozen at -80°C for 30 minutes. The tubes' lids were replaced with punctured Parafilm M®. The tubes of frozen crude antigen were placed in a freeze dryer (FreeZone 2.5 Liter Benchtop Freeze Dry System, Labconco®, Kansas City, Missouri, USA) for two days at -80°C. Freeze-dried *D.dendriticum* crude antigen was reconstituted with 1 ml of ddH_20. Protein analysis indicated a concentration of 45.0 mg/ml.

The serological ELISA was developed using methods modified from Skotarek et al. (2010) to detect the cestode *Anoplocephala perfoliata* in horses. *Dicrocoelium dendriticum* crude antigen was diluted to 7.5 μg/ml in CBS (carbonate buffer, 0.1M, pH 9.0) (Liu et al. 2013). All wells of 96-well flat bottom plates (MaxiSorp, Non Sterile, PS, lot #1110936, Thermo Scientific Nunc, Waltham, Massachusetts, USA) were coated with 100 μl of the diluted antigen. After an incubation of two hours at 37°C, plates were washed twice in PBS-T (PBS containing 0.05% Tween 20, pH 7.2) using a plate washer (Model 1575 ImmunoWash, Bio-Rad, Hercules, California, USA). For ovine and bovine serum samples, goat milk diluted 1:1 in distilled water was used to block wells, while goat serum samples used 3% Milk (Blotting Grade Blocker Non-Fat Dry Milk, Bio-Rad, Hercules, California, USA) in CBS. All wells were blocked with 200 μl of milk and left to incubate at room temperature on a shaker for 15 minutes. Plates were washed as above, before the addition of 100 μl/well of serum diluted 1:100 in CBS. Time series serum samples from all animals and years were added to duplicate wells on a randomly generated template. Two columns in the plate were left for positive and negative pooled control serum for each species to be added. Negative pooled serum was obtained from 5-8

animals of similar age to experimental animals and combined. Positive pooled serum was obtained from 6-12 infected animals of similar ages and combined. Plates were sealed and incubated for 45 minutes at 37°C. After being washed, 1:1000 diluted secondary antibody conjugated to horseradish peroxidase (Hrp) was added at 50 μl/well and incubated for 45 minutes at 37°C. All samples were analyzed for both IgG and IgM antibodies, using appropriate secondary antibodies for each species. For bovine serum samples, Goat Anti-Bovine IgG (H+L) Hrp Affinity purified (Jackson Immunologicals, West Grove, Pennsylvania, USA) and Sheep Anti-Bovine IgM Hrp (Bethyl Laboratories, Montgomery, Texas, USA) were used. Ovine serum samples used Rabbit Anti Sheep IgG (H+L) Hrp (Jackson Immunologicals, West Grove, Pennsylvania, USA) and Rabbit Anti Sheep IgM Hrp (Bethyl Laboratories, Montgomery, Texas, USA). For goat serum samples, Rabbit Anti Goat IgG (H+L) Hrp (rb) (Miles Scientific, Elkhart, Indiana, USA) and Rabbit Anti Goat IgM Hrp (AbD Serotec, Raleigh, North Carolina, USA) were used. Plates were washed and 100 μl/well of an ELISA Hrp Substrate (ABTS® Peroxidase Substrate System, KPL, Gaithersburg, Maryland, USA) was added. After a 30-minute incubation in the dark, absorbance at 405 nm was determined using a spectrophotometer (Spectra Max 340PC plate reader, Molecular Devices, Sunnyvale, California, USA).

2.3.6 Data Analyses

'Time to first egg detection' was assessed as the first fecal sample collected at a given d.p.i. that eggs were detected in each individual host. Mean eggs per gram (e.p.g.) was calculated for each host species at 77-90 and 91-105 d.p.i. Analyses of difference in worm intensity, percent recovery, and e.p.g. used medians and were analyzed using non-parametric Kruskal-Wallis tests.

Serological ELISA optical densities (ODs) were recorded and adjusted using a plate-specific value. This value was an average from the pooled negative control serum wells plus three standard deviations (Richardson et al. 1983). Averages of two wells from each sample were used to estimate OD. The coefficient of variation was less than 20% for each sample (Reed et al. 2002). For each plate, average ODs were subtracted by the negative control OD value from their respective plates. The adjusted OD values were sorted by d.p.i. and grouped together in bins starting at 0 d.p.i., followed by 1-14 d.p.i., and increasing at two-week intervals. The bins were averaged and standard error was calculated for animals sampled at each time period. Absolute OD data were first tested for normality. To determine the role of host species and time elapsed since infection on mean OD for IgG and IgM, respectively, I used a one-way repeated measures ANOVA. Due to diminishing sample collection over time, only the first nine time points were included from those animals that had been repeatedly assessed from 0 d.p.i. to 99-112 d.p.i. The Bonferroni multiple comparisons test was applied when ANOVA revealed significant differences ($p<0.05$) in order to reduce the chances of making type I errors by adjusting the P values (Field 2005). P values were adjusted by dividing the by the number of comparisons being made (Field 2005). Due to small sample sizes, data derived from goats could not be normalized. Data analysis used non-parametric Kruskal Wallace tests with median OD values for both IgG and IgM.

2.4 Results

2.4.1 Worm Counts

The numbers of adult *D. dendriticum* worms varied extensively among livers from cattle, sheep, and goats (Table 2.2). Median worm counts were significantly different

between the three species (Kruskal-Wallis Tests, $\chi^2(2)=8.64$, $p=0.013$, mean rank intensity = 15, 19, and 32 for cattle, sheep, and goats, respectively). Intensities in sheep and cattle were not significantly different (Kruskal-Wallis Tests, $\chi^2(1)=1.20$, $p=0.274$), but intensities in sheep and cattle were significantly lower than those in goats (Kruskal-Wallis Tests, $\chi^2(1)=7.43$, $p=0.006$; $\chi^2(1)=6.14$, $p=0.013$).

The proportion of worms recovered also varied extensively within and between species. Median percent recoveries were significantly different between sheep, cattle, and goats (Kruskal-Wallis Tests, $\chi^2(2)=14.1$, $p=0.001$), with a mean rank percent recovery of 19% for sheep, 14% for cattle, and 35% for goats. Sheep and cattle had significantly lower percent recovery than goats (Kruskal-Wallis Tests, $\chi^2(1)=10.9$, $p=0.001$; $\chi^2(1)=10.9$, $p=0.001$, respectively). Percent worm recovery was not significantly different between cattle and sheep (Kruskal-Wallis Tests, $\chi^2(1)=1.95$, $p=0.163$).

2.4.2 Fecal Egg Counts

'Times to first egg detection' in sheep, cattle, and goats were similar with the first fecal sample collected containing eggs ranging from 47-49 days for all three species (Table 2.3). The first day of egg output ranged between 47-75 d.p.i. in sheep. First egg output after infection ranged from 49-68 d.p.i. in goats. Cattle had the largest range of first egg output, with eggs first detected from 48-118 d.p.i. Overall, the three host species had significantly different mean first days of egg output (Kruskal-Wallis Tests, $\chi^2(2)=8.80$, $p=0.012$), with sheep having a mean rank of 13 days, 24 for cattle, and 19 for goats. Sheep had significantly earlier first egg outputs than cattle (Kruskal-Wallis, $\chi^2(1)=8.36$, $p=0.004$). Cattle and goats' first egg outputs were not significantly different (Kruskal-Wallis, $\chi^2(1)=1.03$, $p=0.31$), nor were sheep and goats' (Kruskal-Wallis,

$\chi^2(1)=1.69$, $p=0.194$). When comparing differences in median e.p.g. between the three species at 77-90 d.p.i. and 91-105 d.p.i., a significant difference was found (Kruskal-Wallis, $\chi^2(2)=12.8$, $p=0.002$; $\chi^2(2)=18.6$, $p<0.0001$, respectively). Cattle had significantly lower egg counts at 77-90 d.p.i. compared to sheep and goats (Kruskal-Wallis, $\chi^2(1)=8.59$, $p=0.003$; $\chi^2(1)=8.08$, $p=0.004$, respectively). Similarly, cattle had significantly lower egg counts from 91-105 d.p.i. than sheep and goats (Kruskal-Wallis, $\chi^2(1)=12.5$, $p<0.0001$; $\chi^2(1)=11.0$, $p=0.001$, respectively).

2.4.3 Serological ELISA

Mauchly's Test of Sphericity was used to determine if variances of the differences between all conditions were equal (Field 2005). The test demonstrated that the assumption of sphericity was violated for IgG and IgM responses over time ($\chi^2(35)=243$, $p<0.001$ and $\chi^2(35)=61$, $p=0.006$, respectively) and therefore significant differences between the variances of differences were present (Field 2005). As a result, a Greenhouse-Geisser correction was used to correct the degrees of freedom of the F-distribution (Field 2005, Schurer et al. 2014). There was a significant effect of time and host species on the IgG antibody response (Table 2.4). In general, there was no detectable increase in antigen-specific IgG antibody in the blood of any of the hosts until approximately day 29, when OD values in sheep were first detected at levels higher than the negative control (Fig. 2.1). The interaction between time and host species was also significant, indicating that the pattern of IgG antibody expression over time depended on host species. Thus, the IgG response in sheep that started at approximately day 29 continued to rise approximately nine-fold up to the end of the experiment over four months later, with a maximum adjusted OD of 1.5 ± 0.07 (Fig. 2.1). In contrast, cattle

demonstrated no detectable IgG response (Fig. 2.1). The Bonferroni correction of the P values showed that the sheep IgG response was significantly different from both cattle and goats ($p<0.0001$ and $p=0.003$, respectively). However, cattle and goats' responses were not significantly different ($p=0.252$). The IgG response of goats was intermediate between sheep and cattle, with a significant 1.2-fold rise from zero starting at approximately day 43 and a maximum OD of 0.2 ± 0.05 (Fig. 2.1), followed thereafter by a plateau up to 4 months later.

Time and host species had significant effects on IgM antibody responses according to the repeated measures ANOVA (Table 2.4). Bonferroni corrections revealed that sheep had significantly different IgM responses from cattle and goats ($p<0.0001$). However, cattle and goats' IgM responses were not significantly different ($p=0.819$). Sheep and goat IgM antibodies were first detected at day 29 (Fig. 2.2). Sheep IgM antibodies underwent a three-fold increase from 0 d.p.i. to the 43-56 d.p.i. range after which a decrease was observed (Fig. 2.2). This peak occurs at an OD of 0.9 ± 0.1. Similarly, this peak was seen in goat IgM response with a 1.4-fold increase from 0 d.p.i. to the 43-56 d.p.i. range with a maximum OD of 0.3± 0.1 (Fig. 2.2). Despite not being significantly different from cattle's IgM response, which remained below zero for the entirety of the experiment (Fig. 2.2), goats did have a significant increase from zero at 43-56 and 57-70 d.p.i. (Kruskal-Wallis, $\chi^2(1)=5.77$, $p=0.016$; $\chi^2(1)=4.81$, $p=0.028$, respectively). The negative control cattle and sheep had adjusted ODs for both IgG and IgM below zero throughout the infection.

2.5 Discussion

Host-parasite interactions for generalist parasites are poorly understood. Thus, the relative performance of generalist parasites in different hosts and relative host responses to the parasite are unknown. Results showed that estimates of *per capita* worm performance differed between species. Although worms reached maturity in all three species, rates of recovery, development, and egg production tended to be lowest in cattle compared to the other two species of host. My findings also indicate that cattle, sheep, and goats respond differently to *D. dendriticum* infection. Sheep had significantly greater IgG and IgM production and produced both antibodies for a longer period of time compared to cattle and goats whose responses were not significantly different from each other. These results indicate that for this generalist parasite, host responses and performance in different hosts will play a key role in determining overall rates of pasture contamination with eggs.

Fecal egg counts provide one indication of rates of adult worm development. Cattle had significantly later dates of first egg detection and lower mean e.p.g. than sheep and goats. The low e.p.g. are consistent with results of Colwell and Goater (2010) who found that naturally infected cattle had low mean e.p.g., ranging from 1 to 4. In contrast, egg counts in sheep and goats exposed to similar numbers of metacercariae were approximately 5 times higher. Results from a similar study involving sheep exposed to approximately the same number of metacercariae, produced 33-800 eggs within 49 to 79 d.p.i. (Campo et al. 2000). Hoste et al. (2010) reviewed differences between goats and sheep responses to gastrointestinal nematodes and reported that when both species are co-grazing, goats have a significantly higher worm burden and egg production than sheep

(Le Jambre and Royal 1976, Pomroy et al. 1986, Jallow et al. 1994, Huntley et al. 1995). Taken together, these results indicate that worm performance tends to be higher in goats and sheep than cattle. Furthermore, goats surpass sheep in fecal egg counts, worm burdens and recovery.

Previous studies have shown that anti-*D. dendriticum* IgG antibodies first appear around 30 d.p.i. in experimentally infected sheep (Broglia et al. 2009), peak at 60 d.p.i., and then continue to increase throughout infection up until 180 d.p.i. (Ferreras-Estrada et al. 2007). Similarly, Phiri et al. (2006) found that IgG antibodies dominated over other classes during experimental infections with *Fasciola spp*. in cattle and sheep. My results are similar, showing detection of IgG antibodies at 29-42 d.p.i., maxima at 57-70 d.p.i., and a steady increase to over 150 days. In contrast, patterns of IgM production were subdued and short-lived, a pattern consistent with the results of other studies involving parasites of livestock (Frontera et al. 2003, Paweska et al. 2003, Phiri et al. 2006). Although the general pattern of IgG and IgM expression was similar between goats and sheep, the former's response was more subdued. Goats' immune responses are less efficiently acquired and expressed compared to sheep (Hoste et al. 2010). These results show that sheep and goats respond to *D. dendriticum* metacercariae by producing strong and consistent IgM and IgG antibodies. Follow-up studies should focus on determining the extent to which these strong responses are protective under pasture conditions of continuous exposure.

In contrast to the pattern seen in sheep and goats, anti-*D. dendriticum* IgG and IgM antibodies were not detectable in *D. dendriticum*-infected cattle exposed in this study. Although other studies have detected IgG and IgM in cattle infected with liver

flukes, *Fasciola spp*. (Phiri et al. 2006) and *D. dendriticum* (Colwell and Goater 2010), there were some key differences from this study. In experimentally infected cattle and sheep, Phiri et al. (2006) found that cattle did produce IgG but at a later date than sheep who produced it two weeks after infection. Colwell and Goater (2010) tested naturally infected cattle that were likely chronically exposed to *D. dendriticum* metacercariae. My study using experimentally infected cattle only followed their immune responses for a maximum of 182 days. Further, in the study by Colwell and Goater (2010), intensity of infection was close to 1000 worms/host whereas in my study cattle harboured at most 324 worms. It is possible that these differences translated into discrepancies in their immune responses to *D. dendriticum*. Because cattle have a larger body size than sheep or goats, it is also possible that worm intensity used in this study was not sufficient to elicit an immune response from cattle. One way to determine if the lack of IgG and IgM responses was due to lower intensities would be to increase the number of metacercariae administered to cattle.

Interspecific differences in host responses to parasitic infections are well documented in the literature. Hosts of different species are expected to have unique life histories and therefore diverse costs and trade-offs associated with immunity (Zuk and Stoehr 2002). The "costs of immunity" hypothesis states that the optimal immune response is not always the maximum immune response (Zuk and Stoehr 2002). Because each species has different internal and external demands on their energy, it might not always be beneficial to expend energy on immune responses to parasitic infections. Nussey et al. (2014) illustrated this hypothesis in wild Soay sheep. A positive correlation between anti-helminth IgG antibodies produced in the summer and winter survival in

Soay sheep was observed. Therefore, the interspecific differences seen in my study could be explained by different life histories and trade-offs of cattle, sheep, and goats. For cattle, it may not be beneficial to mount an antibody response to *D. dendriticum*, while for sheep it may be a priority. Goats may benefit from mounting modest antibody responses to *D. dendriticum*.

Another potential explanation for interspecific differences in host responses to *D. dendriticum* infections is that cattle, sheep, and goats have different innate susceptibilities. Susceptibility to the nematode, *Haemonchus contortus*, differs between breeds of sheep (Shakya et al. 2011). Nussey et al. (2014) also showed that immune responses were protective in wild Soay sheep. Antibody responses and therefore resistance to *D. dendriticum* is highest in sheep, followed by goats and then cattle in this study.

Relative transmission rates have important implications for multi-host parasites like *D. dendriticum* because they can reveal which species are dominant with regards to eggs shedding onto pasture. The results of this study show that goats have the highest mean e.p.g. and therefore have the potential to transmit eggs at the highest rates, followed by sheep and then cattle. However, overall transmission rates will also be determined by host density on pasture (Beck et al. 2015). Despite cattle having relatively low egg outputs, in CHP this population of hosts is responsible for transmitting approximately 80% of the 300 billion eggs that contaminate pasture each year (Beck et al. 2015). This demonstrates that although a given host species might have relatively low parasite egg output, they can still contribute significantly to infection spread if they are present at high densities.

With generalist parasites, it is important to know which hosts are infected, how they respond to infection, and the degree to which they contribute to subsequent infections. Immunoresponsiveness to infection and population density need to be considered. By determining relative rates of transmission from pasture into definitive hosts and the relative host susceptibilities of co-grazing hosts, we can identify which species most require treatment to reduce parasite spread and reduce the impact of an emerging parasite. This study compared immune responses of three host species to experimental infection with *D. dendriticum*. By understanding how different domestic hosts respond to this infection, we can improve management of livestock diseases and diminish the economic impact of parasitism.

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Table 2.1. Summary of host species, ages, sexes, and the number of metacercariae administered for experimental infections in 2011, 2013, and 2014.

Host	Sample Size	Year	Sex	Age	# Metacercariae administered
Cattle	4	2011	Male	2 years	625-650
Cattle	6	2013	Male	6 months	987-1498
Cattle	6	2014	Male	8 months- 1 ½ years	425-583
Sheep	4	2011	Female	1 year	625-650
Sheep	6	2013	Male	3 months	949-1498
Sheep	6	2014	Male	3 months	482-712
Goats	5	2014	Female	1 year	472-584

Table 2.2. Summary of recoveries of *D. dendriticum* in experimentally exposed beef cattle, sheep, and goats.

Host	sample size	mean ± s.d. worm intensity	range of worm intensity	median worm intensity	mean ± s.d. percent worm recovery	range of percent worm recovery	median percent worm recovery
Cattle	16	111± 103	1-324	84	13.2 ± 9.0	0.2-24.5	12.8
Sheep	16	132 ± 80	31-283	132	17.5 ± 8.5	4.2-26.6	20.3
Goats	5	257± 38	205-299	249	50.4 ± 8.6	41.8-59.8	47.7

Table 2.3. Summary of fecal egg count (e.p.g.) for *D. dendriticum* in experimentally exposed cattle, sheep, and goats. Data expressed in days post infection (d.p.i.).

Host	time to first egg detection (days)	range of first egg detection (days)	mean ± s.d. time to first egg detection (days)	mean ± s.d. e.p.g. at 77-90 days	mean ± s.d. e.p.g. at 91-105 days
Cattle	48	48-118	72 ± 15.9	5.0 ± 6.1	3.7 ± 3.8
Sheep	47	47-75	59 ± 7.99	25.9 ± 30.2	39.0 ± 23.4
Goats	49	49-68	61 ± 7.16	35.2 ± 18.3	55.4 ± 24.7

Table 2.4. Summary of one-way repeated measures ANOVA to determine the effect of time and host species on IgG and IgM antibody response to experimental infection with *D. dendriticum*.

Source of Variation	df	MS	F	p-Value
		IgG		
Time	1.6	2.9	27	<0.0001
Time x Host	3.1	1.4	13	<0.0001
Error (Time)	28	0.17	-	-
		IgM		
Time	3.5	0.77	21	<0.0001
Time x Host	7.0	0.32	8.8	<0.0001
Error (Time)	63	0.036	-	-

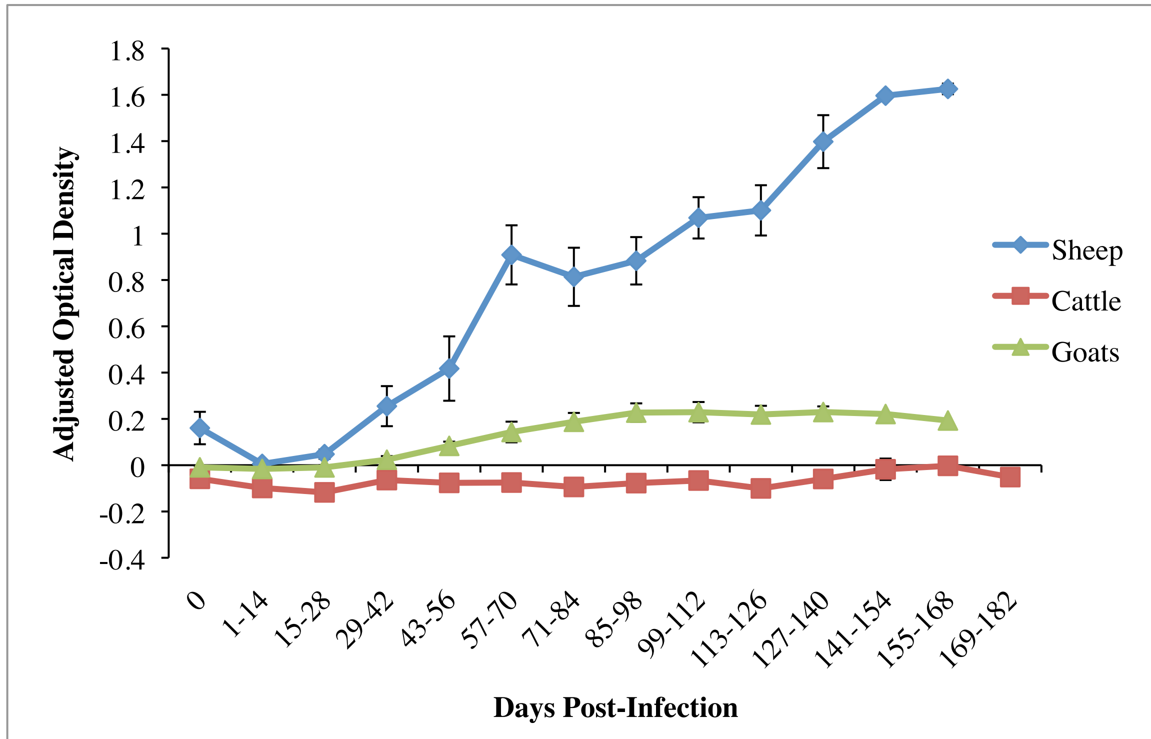

Figure 2.1. Mean optical densities ± s.e.m. from serological ELISAs measuring IgG antibody response to experimental infection with *D. dendriticum* over time in sheep, cattle, and goats. Each point represents an average of adjusted OD measurements from all serum samples falling within the coordinating d.p.i. range.

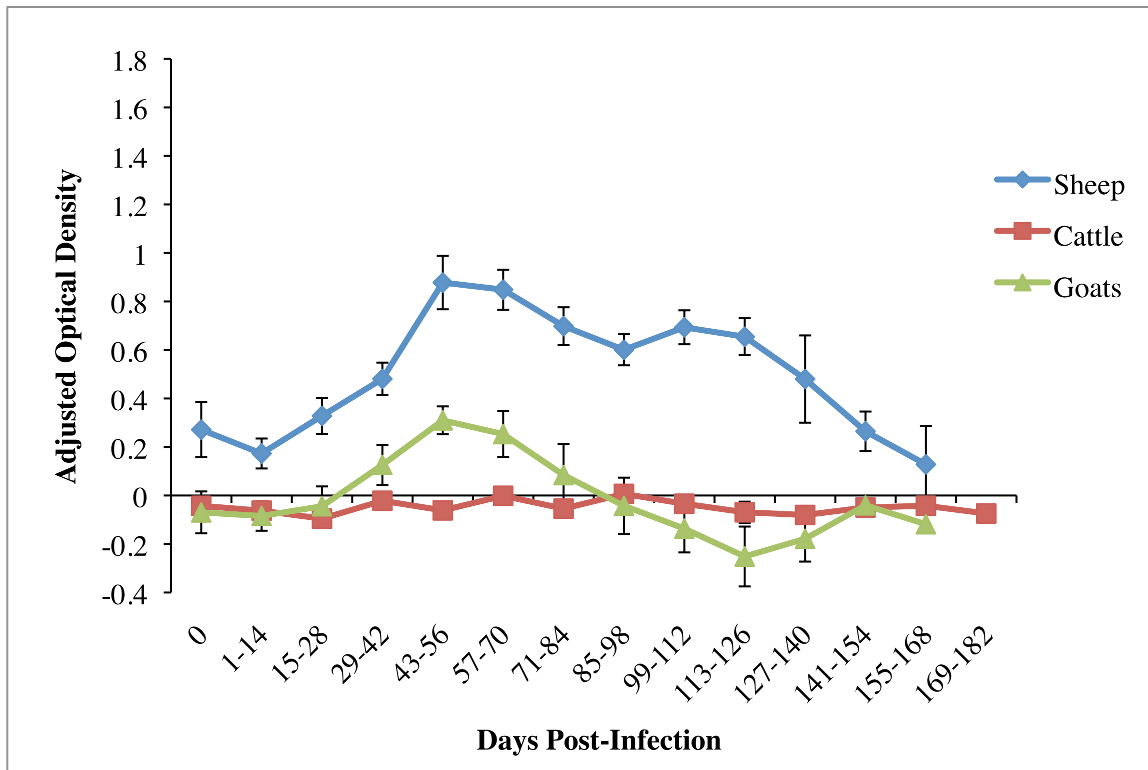

Figure 2.2. Mean optical densities ± s.e.m. from serological ELISAs measuring IgM antibody response to experimental infection with *D*. *dendriticum* over time in sheep, cattle, and goats. Each point represents an average of adjusted OD measurements from all serum samples falling within the coordinating d.p.i. range.

Chapter 3. Development of a coproantigen ELISA to detect *Dicrocoelium dendriticum* proteins in feces of experimentally infected sheep and cattle

3.1 Abstract

Parasitic helminths of livestock can reduce meat, wool, and milk production of their hosts, and can also reduce their feed conversion efficiency and fertility. Current diagnostic techniques for these parasites have important shortcomings relative to their accuracy at detecting the presence of worms and the levels of infection in individual hosts. In this study, cattle and sheep were infected with the emerging, generalist liver fluke *Dicrocoelium dendriticum*. Prevalence of this parasite in beef cattle, deer, and elk in Cypress Hills Provincial Park in Alberta, Canada has reached 60-90% since its first documentation in the mid 1990's. I developed a coproantigen ELISA using polyclonal antibodies to detect *D. dendriticum* proteins in serially collected fecal samples from cattle (n=8) and sheep (n=15) that were experimentally infected with metacercariae. The indirect coproantigen ELISA detected *D. dendriticum* proteins in cattle and sheep, but not consistently, suggesting that protein levels in fecal samples fluctuate over time. Fluke proteins were detected as early as 9 days post-infection (d.p.i.) in both species, with limits of detection of 49 and 98 ng/ml in sheep and cattle fecal extracts, respectively. Sensitivities (true positives) of the assays were 45% and 23% in sheep and cattle, respectively. Results were not correlated with infection duration or with fluke intensity in either cattle or sheep. These results show that a coproantigen ELISA for *D. dendriticum* has potential to be an early detection tool, however low sensitivities indicate that further research is needed to optimize it as a reliable diagnostic method.

3.2 Introduction

Parasitism can have a significant economic impact on the livestock industry due to the high cost of treatment with anti-parasite drugs and health problems associated with infection (Perry and Randolph 1999). It is estimated that tens of millions of dollars are lost each year in the livestock industry due to undiagnosed parasitism in Alberta (Canada) alone (Bauck et al. 1989, Jim et al. 1992, Schunicht et al. 2000). On a global scale, parasites of livestock are economically important, particularly with helminths (i.e., the 'worms') (Charlier et al. 2014). These parasites reduce weight gain, production of milk, meat, and wool as well as fertility in infected animals (Charlier et al. 2014). For example, the trematode *Fasciola hepatica* is estimated to cause losses of up to $78 million (CAD) in Switzerland each year, which corresponds roughly to $450 loss per infected animal (Schweizer et al. 2005). Gastrointestinal nematodes are estimated to cost the UK ranching industry $174 million (CAD) per annum (Nieuwhof and Bishop 2005).

To prevent or reduce productivity losses, livestock producers tend to treat all of their animals with anthelmintic drugs, regardless of their infection status (Wolstenholme et al. 2004). This blanket treatment can lead to widespread anthelmintic drug resistance, which threatens the sustainability of livestock operations (Kaplan 2004, van Dijk et al. 2010). Furthermore, the overuse of anthelmintic drugs can be a financial burden for livestock producers (van Dijk et al. 2010). To reduce the risk of anthelmintic drug resistance development, the targeted administration of anti-helmintic drugs to animals harbouring a minimum threshold number of parasites has been proposed (Hoste et al. 2002a). Such targeted application of anthelmintics helped control trichostrongyles in goats and prevented the development of drug resistance (Hoste et al. 2002a, Hoste et al.

2002b, Kaplan 2004, Stear et al. 2007, Molento 2009, Epe and Kaminsky 2013, McMahon et al. 2013). However, to support such a targeted approach, producers must be able to discern between infected and uninfected animals, and ideally, between animals that differ in worm intensity. To meet this goal, sensitive and specific diagnostic tools are required.

Current diagnostic methods for helminths include detection of parasite eggs in feces using a combination of standard centrifugation and flotation techniques (Rehbein et al. 1999, Rojo-Vázquez et al. 2012, Roeber et al. 2013). However, data collected with this method is frequently inaccurate since eggs are shed intermittently by individual worms and inconsistently by hosts (Braun et al. 1995, Jithendran and Bhat 1996, 1999, Rinaldi et al. 2011). Thus, an egg-negative result does not necessarily translate to negative infection status. Further, this lack of sensitivity is often further compounded by the difficulty in correctly identifying parasite eggs within fecal samples (Campo et al. 2000). Other methods of diagnosis involve detecting parasite-specific serum antibodies via enzyme-linked immunosorbent assay (ELISA). Often, serological antibodies can be detected more consistently and earlier than parasite eggs in the feces (Broglia et al. 2009, Colwell and Goater 2010, Roeber et al. 2013). Despite serological ELISAs often being more sensitive and specific than fecal egg counts, they also suffer shortcomings (Colwell and Goater 2010). Since antibodies can persist in the blood after an infection is cleared, true infection status is not always revealed (Roeber et al. 2013). Additionally, serum collection is an invasive process and is not feasible for large herds of livestock or wildlife. Collecting fecal samples, however, is non-invasive and can be easily performed for a large number of animals. Improving upon serological ELISAs are PCR-based diagnostic tools, which

can be highly sensitive and specific (Gasser 2006, Roeber et al. 2013). For example, Nielsen et al. (2008) developed a highly sensitive and specific real-time quantitative PCR assay that was able to detect *Strongylus vulgaris* eggs in fecal samples from horses. However, a major drawback of PCR is that it requires expensive instrumentation and reagents and can also be time consuming and training-intensive when extensive optimization is required (Gasser 2006, Roeber et al. 2013). These requirements limit the use of PCR in field diagnostics.

Parasitologists use the term 'coproantigens' to describe proteins released by parasites that travel through the gastrointestinal tract to end up in the feces (Charlier et al. 2014). These antigens are measurable using ELISAs (Jas et al. 2010, Skotarek et al. 2010, Demerdash et al. 2011, Brockwell et al. 2013, Watwiengkam et al. 2013, Elsemore et al. 2014, Kajugu et al. 2015). Skotarek et al. (2010) used a coproantigen ELISA for the detection of a 12-13 kDa protein from the cestode, *Anoplocephala perfoliata*, in horse fecal samples. They found a positive correlation between coproantigen concentration and tapeworm infection intensity. Similarly, Watwiengkam et al. (2013) used monoclonal antibodies to detect proteins originating from the trematode, *Opisthorchis viverrini*, in human fecal samples. Coproantigen ELISAs have also been developed for both *Fasciola gigantica* and *F. hepatica* in cattle (Demerdash et al. 2011, Brockwell et al. 2013). These authors found a significant positive correlation between antigen concentration and worm intensity. Thus, coproantigen ELISAs have potential to be more accurate than fecal egg enumeration, involve more feasible sample collection compared to serological diagnosis, and be more practical than PCR.

Dicrocoelium dendriticum, also referred to as the small liver fluke, is a cosmopolitan, host generalist trematode that emerged in the Cypress Hills Provincial Park (CHP), Alberta in the mid 1990's (Goater and Colwell 2007, Beck et al. 2014). It is found in 60-90% of beef cattle, elk and deer in CHP (Goater and Colwell 2007). The small liver fluke has a complex, three-host life cycle that ends with adult flukes in the bile ducts and gall bladder of grazing hosts like cattle, sheep, goats, elk, and deer (Otranto and Traversa 2002). Grazing hosts have been documented to experience liver damage, and studies in sheep have demonstrated correlations between *D. dendriticum* intensity, gall stone formation, and photosensitization (Katsoulos et al. 2011, Sargison et al. 2012, Charlier et al. 2014). Although the small liver fluke's impact on cattle is not well documented, it is known that other helminths reduce the production of milk, meat, and wool, and decrease fertility of infected hosts (Charlier et al. 2014).

The most common method for detecting *D. dendriticum* in individual hosts is fecal egg detection and enumeration (Rehbein et al. 1999, Rinaldi et al. 2011). However, fecal egg counts are notoriously inaccurate for this parasite, partly due to intermittent egg shedding by gravid adults (Campo et al. 2000). Eggs are not shed until 49-79 days post-infection (d.p.i.), when flukes have matured to adults (Campo et al. 2000, Rojo-Vázquez et al. 2012). Other diagnostic methods for *D. dendriticum* rely on the detection of *D. dendriticum*-specific antibodies in blood serum (Broglia et al. 2009, Colwell and Goater 2010). Anti-fluke IgG antibodies can be detected at 30 d.p.i., allowing for earlier detection than fecal egg counts (Broglia et al. 2009). The downfalls of the serum-based method are the persistence of antibodies in the blood post- infection, and the invasive nature of blood collection (Colwell and Goater 2010). The coproantigen ELISAs could

improve on current diagnostic methods and circumvent the problems of intermittent egg shedding and antibody persistence in the serum. Wildlife fecal samples could also be collected and analyzed. A coproantigen ELISA has not yet been developed to detect *D. dendriticum*.

The high prevalence of *D. dendriticum* in Alberta, a province with a significant portion of Canada's livestock production, calls for an improved diagnostic tool. First, an accurate diagnostic tool will allow researchers to monitor the spread of this emerging parasite to other regions. Second, it would allow researchers to identify high fluke protein shedders within a population of infected hosts, including wildlife that could then be targeted for treatment. This study aims to develop a coproantigen ELISA for detecting *D. dendriticum* proteins in cattle and sheep feces.

3.3 Methods

3.3.1 Source of Hosts

In 2012-2014, 8 Holstein steers and 15 sheep were isolated from their respective herds at Lethbridge Research and Development Centre (LeRDC) of Agriculture and Agri-Food Canada in Lethbridge, Alberta. Sheep were isolated from the LeRDC flock while cattle were purchased from a nearby producer. All cattle were age-matched males, while sheep differed in age and sex (Table 3.1). Steers were housed in an outdoor feed yard at LeRDC and fed hay and barley/corn silage. Sheep were kept in an indoor barn at LeRDC and fed lamb grower ration, hay, and alfalfa pellets. All animals were provided *ad libitum* access to water, salt and minerals. Animals had no previous exposure to *D. dendriticum*. Fecal egg counts were performed using methods described in Chapter 2 for each animal

to confirm their negative status for the presence of *D. dendriticum* and to identify any other parasite(s) present prior to experimental infections.

3.3.2 Experimental Infections

The infection protocol used to expose individual cattle and sheep to *D. dendriticum* was described in Chapter 2. Animals were slaughtered at 93-179 d.p.i. and then their livers were dissected to assess worm intensity. Fecal samples were taken directly from the animals' rectum and frozen at -20 °C. Fecal samples were obtained from each host bi-weekly throughout the infection for fecal egg counts in Chapter 2. Remaining samples from Chapter 2 were frozen until use in this assay development. The remainder of the fecal samples were not consistent bi-weekly samples, and ranged from bi-weekly to 103 days between samples. Additional fecal samples were collected from sheep (n=10) and cattle (n=7) that were not exposed to *D. dendriticum* metacercariae. These latter fecal samples were pooled for cattle and sheep separately to serve as negative control samples for the coproantigen assay.

3.3.3 Fecal Extract Preparation

To prepare fecal extracts, feces from 15 sheep, 8 cattle, the pooled negative sheep and the pooled negative cattle samples were thawed and measured by volume in 15 ml or 50 ml tubes (Cellstar® Polypropylene Tubes, Greiner Bio-One, North America, Inc.). Two to six fecal samples were collected from each animal throughout the infection period. Phosphate buffered saline (PBS; 0.01M Na_2HPO_4, 0.01M $NaH_2PO_4 \cdot H_2O$, 0.15 NaCl, pH 7.2) was added to each sample at a volume-to-volume ratio of 1:6 of feces to buffer (Watwiengkam et al. 2013). After being thoroughly mixed via hand shaking, samples were centrifuged at 805 x g at 4 °C for 30 minutes (Centrifuge 5810 R,

Eppendorf, Hamburg, Germany). Resulting supernatants were transferred to new tubes that were then centrifuged at 3220 x g at 4 °C for 10 minutes.

Earlier studies have shown that the manner in which fecal samples are processed has an impact on the detection of coproantigens (Brockwell et al. 2013, Watwiengkam et al. 2013). Therefore, I selected and compared four methods of fecal extraction, each of which was repeated three times following the initial PBS extraction (Watwiengkam et al. 2013). I tested the effect of a heat treatment and the addition of trichloroacetic acid (TCA) but found that the addition of carbonate buffer (CBS, 0.1M, pH 9.0) to the PBS fecal supernatant provided the lowest non-specific binding. Following each extraction in PBS, supernatants were poured into new tubes and an equal volume of CBS was added to each sample (Kahama et al. 1998, Watwiengkam et al. 2013). The samples were then stored at 4 °C until ready for use in the assay. The purpose of testing these alternative extraction methods was to improve the extraction efficiency and the ELISA's consistency with its ability to detect fluke protein in the fecal extract.

3.3.4 Polyclonal Antibody Production and Purification

Polyclonal antibodies were produced by ImmunoPrecise Antibodies Ltd., Victoria, British Columbia, Canada. Rabbits were inoculated with *D. dendriticum* whole fluke protein extract produced in Chapter 2. Antibody production against *D. dendriticum* was verified by ELISA, and blood serum containing antibodies was collected from the rabbits. The serum was sent to our laboratory and the polyclonal IgG antibodies isolated using an antibody purification kit (AbSelect G Serum Antibody Purification System, Innova Biosciences, Cambridge, United Kingdom) according to manufacturer's instructions. The resulting purified polyclonal IgG antibodies were quantified by

measuring absorbance at 280 nm using a spectrophotometer (Spectra Max 340PC plate reader, Molecular Devices, Sunnyvale, California, USA) (Layne 1957, Stoscheck 1990).

3.3.5 Coproantigen ELISA Development

First, *D. dendriticum* protein extract was diluted to 2 μg/ml in CBS and added in replicates of three to the first two columns of 96 well plates (MaxiSorp, Non Sterile, PS, lot #1110936, Thermo Scientific Nunc, Waltham, Massachusetts, USA). Carbonate buffer was added to columns 2-12, and one in ten serial dilutions were carried out, leaving the last column as a negative control. After two hours incubating at 37 °C, wells were washed twice in PBS-T (phosphate buffered saline containing 0.05% Tween 20, pH 7.2) using a plate washer (Model 1575 ImmunoWash, Bio-Rad, Hercules, California, USA), and blocked for 15 minutes at room temperature with goat milk diluted in an equal volume of distilled water. Wells were washed again, and purified polyclonal antibodies diluted to 10 μg/ml in CBS were added at 100 μl/well. After a 45 minute incubation at 37 °C, wells were washed and Goat Anti-Rabbit IgG (H+L) conjugated to HRP (Cedarlane Laboratories, Burlington, Ontario, Canada) was diluted 1:1000 in CBS and added at 50 μl/well. The plate was incubated and washed again, and ELISA Hrp Substrate (ABTS® Peroxidase Substrate System, KPL, Gaithersburg, Maryland, USA) was added at 100 μl/well. After 30-minute incubation in a dark drawer, optical densities (OD) were read at 405 nm using a spectrophotometer (Spectra Max 340PC plate reader, Molecular Devices, Sunnyvale, California, USA). Concentrations and corresponding averaged OD values were graphed in Microsoft Excel (Fig. 3.1). The LOD was calculated by taking the mean OD value of the blank plus two standard deviations (Dixit et al. 2010, Demerdash et al. 2011). The resulting OD value was used to determine the LOD.

Methods used for the preparation of the whole fluke protein were described in Chapter 2. The coproantigen ELISA was developed using methods described in Skotarek et al. (2010) for the detection of the cestode, *Anoplocephala perfoliata*, in horse feces. First, a checkerboard ELISA was carried out to determine the optimal concentration of polyclonal antibodies to use in the coproantigen ELISA. This was done by adding 25 μg/ml of whole fluke protein diluted in negative control fecal extract to the first two rows of 96-well flat bottom plates at 100 μl/well (MaxiSorp, Non Sterile, PS, lot #1110936, Thermo Scientific Nunc, Waltham, Massachusetts, USA) (Skotarek et al. 2010). Negative fecal extract was added at 100 μl/well to rows 2-8. One in two serial dilutions of protein were carried out from rows 2-7, leaving the eighth without protein. The plate was sealed and incubated at 37 °C for two hours. The plate was washed twice in PBS-T (phosphate buffered saline containing 0.05% Tween 20, pH 7.2) using a plate washer (Model 1575 ImmunoWash, Bio-Rad, Hercules, California, USA). All wells were blocked with 200 μl of goat milk diluted 1:1 in distilled water and left to incubate at room temperature on a shaker for 15 minutes. Plates were washed as above. Next, 20 μg/ml of polyclonal antibody diluted in CBS was added to the first two columns of the plate at 100 μl/well. Carbonate buffer was added to columns 2-12, at 100 μl/well. One in two serial dilutions were carried out from columns 2-11, leaving the 12th without polyclonal antibodies added. The plate was sealed and incubated at 37 °C for 45 minutes, then washed twice as before. Goat Anti-Rabbit IgG (H+L) conjugated to Hrp (Cedarlane Laboratories, Burlington, Ontario, Canada) was diluted 1:1000 in CBS and added at 50 μl/well to all wells. After 45-minute incubation at 37 °C, the plate was washed. Lastly, 100 μl/well of an ELISA Hrp Substrate (ABTS® Peroxidase Substrate System, KPL, Gaithersburg, Maryland,

USA) was added. After a 30-minute incubation in a dark drawer, plates were read at 405 nm using a spectrophotometer (Spectra Max 340PC plate reader, Molecular Devices, Sunnyvale, California, USA) and an optimal concentration of polyclonal antibody was selected.

Four different dilutions of fecal extracts (sheep, cattle) were tested to determine an optimal dilution for the coproantigen ELISA. Undiluted, 1:5, 1:10, and 1:25 dilutions of fecal extract in CBS were tested. For each dilution, the following was done in replicates of three to account for variation between samples: spiked extract with 25 μg/ml of whole fluke protein and added to the first two columns of a 96-well flat bottom plate (MaxiSorp, Non Sterile, PS, lot #1110936, Thermo Scientific Nunc, Waltham, Massachusetts, USA). Fecal extract, without fluke protein added, was pipetted into columns 2-9. One in two serial dilutions were carried through the columns 2-8. Plates were sealed and incubated for two hours at 37 °C followed by two washes with PBS-T using the plate washer. All wells were blocked for 15 minutes at room temperature with 200 μl of goat milk diluted 1:1 in distilled water. After plates were washed again, 100 μl/well of polyclonal antibody was added at 10 μg/ml (optimal concentration by checkerboard) diluted in CBS. Plates were incubated 45 minutes at 37 °C followed by washing in PBS-T. Goat Anti-Rabbit IgG (H+L) conjugated to HRP (Cedarlane Laboratories, Burlington, Ontario, Canada) was diluted 1:1000 in CBS and added at 50 μl/well. After 45 minutes in a 37 °C incubator, plates were washed and ELISA Hrp Substrate (ABTS® Peroxidase Substrate System, KPL, Gaithersburg, Maryland, USA) was added at 100 μl/well. After 30-minute incubation in a dark drawer, optical densities (OD) were read at 405 nm using the spectrophotometer. Resulting OD values were adjusted by subtracting the average

negative control OD and divided by the 25 μg/ml protein concentration value to allow for data to be compared on the same scale.

3.3.6 Coproantigen ELISA Protocol

For the coproantigen ELISA to detect *D. dendriticum* proteins in fecal samples, the fecal extract samples prepared from experimentally infected cattle and sheep were first diluted 1:10 in CBS. Next, 100 μl/well of diluted fecal extract samples were added to 96 well plates (MaxiSorp, Non Sterile, PS, lot #1110936, Thermo Scientific Nunc, Waltham, Massachusetts, USA). Each sample had three replicates to control for variation between sample runs, and each plate had negative control fecal extract samples and positive control fecal extract samples. Each plate had a standard curve, which consisted of 100 μl/well of whole fluke protein diluted in negative control fecal extract starting at 3.125 μg/ml and one in two serially diluted down seven wells, in replicates of three. Plates were incubated for two hours at 37 °C, followed by two washes with PBS-T (phosphate buffered saline containing 0.05% Tween 20, pH 7.2) using a plate washer (Model 1575 ImmunoWash, Bio-Rad, Hercules, California, USA). Next, wells were blocked for 15 minutes at room temperature with 200 μl/well of goat milk diluted 1:1 in distilled water. Plates were washed twice with PBS-T using the plate washer. Polyclonal antibodies were diluted to 10 μg/ml in CBS and added at 100 μl/well. After 45 minutes incubating at 37 °C, plates were washed again, as above. The secondary antibody, Goat Anti-Rabbit IgG (H+L) conjugated to HRP (Cedarlane Laboratories, Burlington, Ontario, Canada) was diluted 1:1000 in CBS and added at 50 μl/well. Plates were incubated at 37 °C for 45 minutes and then washed as done previously. Lastly an ELISA Hrp Substrate (ABTS® Peroxidase Substrate System, KPL, Gaithersburg, Maryland, USA) was added at

100 μl/well and plates were incubated in the dark for 30 minutes. Plates were read at 405 nm using a spectrophotometer (Spectra Max 340PC plate reader, Molecular Devices, Sunnyvale, California, USA).

To determine the limit of detection of the coproantigen ELISA in cattle and sheep fecal extract, the OD values for the standard curve on each plate were averaged and graphed against their corresponding concentrations (Fig. 3.2 and 3.3). The resulting curves for cattle and sheep were analyzed using linear regression from the lowest average OD on the curve to the highest. The lowest standard concentration before the OD value of the y-intercept was considered the limit of detection (Dixit et al. 2010, Demerdash et al. 2011).

3.3.7 Data Analyses

The average of each sample's three replicates was taken to estimate mean absorbance, with all samples' coefficients of variation being under 20% (Reed et al. 2002). Each plate's OD values were adjusted by subtracting plate-specific negative control OD values to allow different plates to be compared with each other. The cut-off value for each plate was calculated according to standard procedure, by taking the mean of the negative ODs plus two standard deviations (Demerdash et al. 2011). Standard curves for each plate were graphed and the corresponding formula (y=mx+b) was used to calculate the concentration of fluke protein in each sample. Each sample's average OD (y) was used in the formula to solve for the concentration (x) (©EnCor Biotechnology Inc. 2015). Samples with fluke protein concentrations less than the limit of detection value were considered negative, and those equal to or greater were considered positive.

The sensitivity for the coproantigen ELISA for sheep and cattle was calculated using the formula DI(+)= (true positives x 100) / (true positives + false negatives), where DI(+) represents the detectability index of positives (Colwell and Baron 1990). Specificity for sheep and cattle was calculated using the formula DI(-)= (true negatives x 100) / (true negatives + false positives) where DI(-) represents the detectability index of negatives (Colwell and Baron 1990). Sensitivity represents the detectability index of positives, while specificity represents the detectability index of negatives (Colwell and Baron 1990). True positives are animals infected with *D. dendriticum* that also tested positive in the coproantigen ELISA. True negatives are uninfected (control) animals that tested negative in the assay. False positives are uninfected animals that tested positive, while false negatives are animals that are infected, but tested positive. For the positive samples, protein concentrations were $\log_{10}$ transformed to normalize their distribution. To test for correlations between concentration, d.p.i., and fluke intensity, I used Pearson correlation analyses.

3.4 Results

3.4.1 Pre-infection Fecal Egg Analyses

Each of the 8 Holsteins contained coccidia oocysts in their pre-infection fecal samples. Two Holsteins contained eggs of the nematode *Trichuris* spp. and one had eggs of the nematode *Nematodirus* spp. Nine sheep contained coccidia oocysts in their pre-infection fecal samples.

3.4.2 Limits of Detection

Purified polyclonal antibodies were able to detect concentrations of fluke protein as low as 0.2 ng in CBS (Fig. 3.1). The coproantigen assay in sheep fecal extract could reliably detect fluke protein at a concentration of 49 ng/ml and higher (Fig. 3.2). In cattle fecal extract, the assay could reliably detect fluke protein beginning at a concentration of 98 ng/ml (Fig. 3.3).

3.4.3 Fluke Coproantigens Detection

The optimal standard curve for detection of whole fluke protein was determined to be the result of the 1:10 diluted fecal extract (Fig. 3.4 and 3.5).

Dicrocoelium dendriticum crude protein was detected in fecal samples of experimentally infected hosts as early as 9 d.p.i. in both cattle and sheep (Tables 3.2 and 3.3). For infected sheep, the ELISA detected fluke protein in 13 out of 15 animals and in 23 out of 51 fecal samples from the 15 sheep. The range of d.p.i. for protein detected in sheep feces was 9-153 d.p.i. For cattle, the ELISA detected fluke protein in 5 out of 8 animals and 6 out of 26 fecal samples from the 8 cattle. Fluke protein was detected in cattle from 9-172 d.p.i. In animals that tested positive for *D. dendriticum*, worm burdens ranged from 31-350 in sheep, and 23-242 in cattle. Positive concentrations of fluke protein detected in sheep feces ranged from 0.053 to 0.837 μg/ml (Table 3.3). Positive fluke protein concentrations detected in cattle feces ranged from 0.098 to 0.243 μg/ml (Table 3.2).

The assay had sensitivities of 45% and 23% in sheep and cattle, respectively. Specificity was 100% for both species. Protein concentration was not correlated with age of infection in cattle or sheep (Fig.3.6; $r(6)= 0.065$, $p>0.05$; $r(23)=0.357$, $p>0.05$,

respectively). Protein concentration was not correlated with fluke intensity in cattle nor in sheep (Fig.3.7; $r(6)=0.067$, $p>0.05$; $r(23)=0.192$, $p>0.05$, respectively).

3.5 Discussion

To my knowledge, this is the first coproantigen ELISA developed for *D. dendriticum*. Using polyclonal antibodies against crude fluke proteins, I was able to detect *D. dendriticum* protein in the majority of experimentally infected cattle and sheep feces. However, the assay was more sensitive for sheep than for cattle. In a single steer and a single sheep, coproantigens were detected at 9 d.p.i., which is 21 days earlier than Broglia et al.'s (2009) serological ELISA for *D. dendriticum* and approximately 20 days earlier than the serological ELISA described in Chapter 2. Antigen levels fluctuated throughout the infection period. This level of variability in coproantigen concentration throughout infection is consistent with the results of other studies (Brockwell et al. 2013). Brockwell et al. (2013) first detected *Fasciola hepatica* coproantigens in cattle feces 33 days later than my *D. dendriticum* coproantigen ELISA. Despite achieving early detection, I was unable to correlate coproantigen concentration with fluke intensity or with age of infection, a result that contrasts with the result of other studies (Skotarek et al. 2010, Demerdash et al. 2011, Brockwell et al. 2013).

Current diagnostic methods for *D. dendriticum* can detect infection as early as 30 d.p.i. using serological ELISAs (Broglia et al. 2009) or 49 d.p.i. using fecal egg counts (Campo et al., 2000; Chapter 2). Fluke protein detection at 9 d.p.i. using coproantigen methodology was unexpected because Brockwell et al.'s (2013) coproantigen ELISAs for *Fasciola hepatica* detection in cattle measured protein as soon as 42 d.p.i. Although such early detection is uncommon in other species of trematodes, a coproantigen ELISA has

not yet been developed for *D. dendriticum*. Morphew et al. (2007) mentioned this kind of early detection where the developmental pattern of excysted *F. hepatica* metacercariae emerging in the duodenum can lead to the release of excretory-secretory (ES) proteins.

A key difference between my study and others involving coproantigen ELISAs is the type of parasite antigen used to produce polyclonal or monoclonal antibodies. I used whole fluke crude protein to develop polyclonal antibodies. Other studies have used more specific protein extracts, such as selected protein bands from Excretory/Secretory (E/S) proteins (Skotarek et al. 2010, Demerdash et al. 2011, Brockwell et al. 2013, Elsemore et al. 2014, Shalaby et al. 2014). The early detection in this study could be due to the polyclonal antibodies I used that were specific to a whole range of fluke proteins rather than more specific isolated E/S proteins. Early proteins released by immature flukes could be found among whole fluke proteins but not in certain adult E/S products. After *D. dendriticum* metacercariae enter the host body, they migrate to the bile ducts and undergo rapid growth and maturation (Otranto and Traversa 2002). By 49-79 d.p.i., eggs are detected in host feces, corresponding to the maturation period of juvenile flukes (Campo et al., 2000; Chapter 2). Since my study detected protein at 9 d.p.i. for cattle and sheep, it is likely that these proteins originated from the rapidly developing immature flukes or from deceased immature flukes that failed to survive. My use of polyclonal antibodies against whole fluke protein might have been more useful in detecting the presence of juvenile flukes at early stages of infection. On the other hand, I could have been detecting protein from dying flukes.

Significant correlations between parasite intensity and optical densities in coproantigen ELISAs have been found previously (Skotarek et al. 2010, Brockwell et al.

2013). Skotarek et al. (2010) detected *Anoplocephala perfoliata* in horses with as few as five tapeworms and correlated worm intensity with the OD associated with a 12-13 kDa E/S antigen. Similarly, large liver flukes, *Fasciola hepatica*, were detected by coproantigen ELISA and coproantigen concentration also correlated positively with fluke intensity (Brockwell et al. 2013). Differences in the overall size of individual *D. dendriticum* compared to *A. perfoliata* and *F. hepatica* may explain these divergent results. Based on size differences, I would expect to see much lower parasite protein concentrations in *D. dendriticum*'s host feces than in *F. hepatica* or *A. perfoliata*'s, depending on their infection intensities. Therefore, it is possible that my inability to correlate coproantigen concentration or OD with fluke intensity was caused by the small size of these flukes and the reduced protein available within infected animals' feces.

It is thought that large liver flukes such as *Fasciola hepatica*, constantly shed their tegument in order to evade host immunity (Hanna 1980). However, it is not known how often *D. dendriticum* actually sheds tegument, if the tegument is present in the feces of their hosts, and if the protein they release is evenly distributed throughout the feces. The detection of fluke protein in host feces in my study is thought to originate from their tegumental shedding. Intermittent fluke protein detection and a lack of correlation between infection intensity and OD in my study could partly be a result of fluke tegument being shed at different intervals or protein being unevenly distributed in the feces. Thus, my results could be explained by the absence of shed protein in some fecal samples or concentrations below a detection limit. In other words, if small liver flukes are not frequently shedding their tegument, then intermittent detection of fluke coproantigens

could be explained by an irregular presence of fluke protein in host feces. This pattern of protein secretion would result in a coproantigen detection tool with low sensitivity.

The coproantigen ELISA that I developed for detecting *D. dendriticum* is imperfect. The low sensitivities of the assay in sheep and cattle as well as the intermittent detection of fluke protein make this tool unreliable relative to serological ELISAs that consistently detect anti-fluke antibodies despite their other drawbacks (Broglia et al. 2009).

Cross-reactivity with other parasites in this ELISA was not tested in this study, but will be required. At this point in its development, we cannot rely on an accurate diagnosis when using this coproantigen ELISA. However, this diagnostic assay holds potential as it has been demonstrated to detect fluke protein earlier than other existing diagnostic methods. The majority of animals did have fluke protein detected in their fecal samples at some point during infection. For this assay to be effective, more fecal samples taken over time will increase the chances of a positive detection if the animal is infected with *D. dendriticum*. Additionally, the assay was more effective in sheep than in cattle, with a greater proportion of the sheep having positive detections than cattle. As a result, the sensitivity of the assay in sheep was almost twice that in cattle. This difference is likely a result of interspecific differences in diet and digestion which lead to differences in feces composition (Huston et al. 1986).

A coproantigen ELISA that is able to detect *D. dendriticum* using sheep and cattle fecal samples represents a useful diagnostic tool that is non-invasive. This is particularly important for tracking current infection spread in a biome like CHP where domestic and wild animals are both infected with a generalist parasite. A diagnostic tool that can detect

infection without requiring the host's presence allows for the inclusion of wildlife. This is important when attempting to map a parasite's epidemiology and reduce its spread. This coproantigen ELISA holds potential for early detection within the first week of infection. By identifying infected animals early in infection with the small liver fluke, they can be treated quickly and the associated productivity losses could be reduced. This would be an improvement in diagnosis, especially for Alberta where the livestock industry has over 5 million head of cattle (Statistics Canada, 2013). Early diagnosis allows livestock producers to improve their treatment and management of parasitic infections, which can reduce the economic impact of parasitism. To reach the point where this tool can benefit livestock producers, it is important to include fecal samples from naturally infected hosts rather than only experimentally infected animals as was done in this study.

To improve the sensitivity of this assay, some key questions must be answered. How often do flukes' teguments end up in host feces? Does fluke protein in host feces fluctuate throughout the day or from day to day? Can the fluke protein in the feces be characterized and have monoclonal or polyclonal antibodies produced against it? Can fecal extraction methods be improved? With further research to answer these questions, this coproantigen ELISA could be improved to be an effective diagnostic tool for *D. dendriticum* and benefit livestock producers worldwide.

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Table 3.1. Summary of host species, ages, sexes, and the number of metacercariae administered for experimental infections in 2012, 2013, and 2014.

Host	Sample Size	Year	Sex	Age	# Metacercaria administered
Cattle	2	2013	Male	6 months	987-1498
Cattle	6	2014	Male	8 months- 1 ½ years	425-583
Sheep	6	2012	Female	3-9 years	1008-1087
Sheep	3	2013	Male	3 months	949-1498
Sheep	6	2014	Male	3 months	482-712

Table 3.2. Experimentally infected cattle and their fluke burdens, days post-infection of positive coproantigen ELISA results, and the highest concentration of *D. dendriticum* protein detected in their fecal samples.

Animal ID	Fluke Burden	Earliest Sample (d.p.i.)	Earliest Positive Detection (d.p.i.)	Highest Positive Concentration Detected (μg/ml)
548	26	9	9	0.098
550	68	37	37	0.167
551	64	6	69	0.116
1	139	37	-	-
3	23	9	9	0.243
2	100	42	167	0.170
348	125	118	-	-
543	242	118	-	-

Table 3.3. Experimentally infected sheep and their fluke burdens, days post-infection of positive coproantigen ELISA results, and the highest concentration of *D. dendriticum* protein detected in their fecal samples.

Animal ID	**Fluke Burden**	**Earliest Sample (d.p.i.)**	**Earliest Positive Detection (d.p.i.)**	**Highest Concentration Detected (μg/ml)**
147	31	53	53	0.140
80	42	104	-	-
154	45	9	9	0.837
118	63	35	91	0.111
93	100	20	77	0.414
83	112	33	33	0.422
108	120	34	34	0.714
138	132	40	40	0.309
203	154	34	34	0.476
33	155	49	49	0.268
145	175	37	-	-
64	237	104	-	-
17	256	104	104	0.053
8	261	33	33	0.322
37	350	72	72	0.335

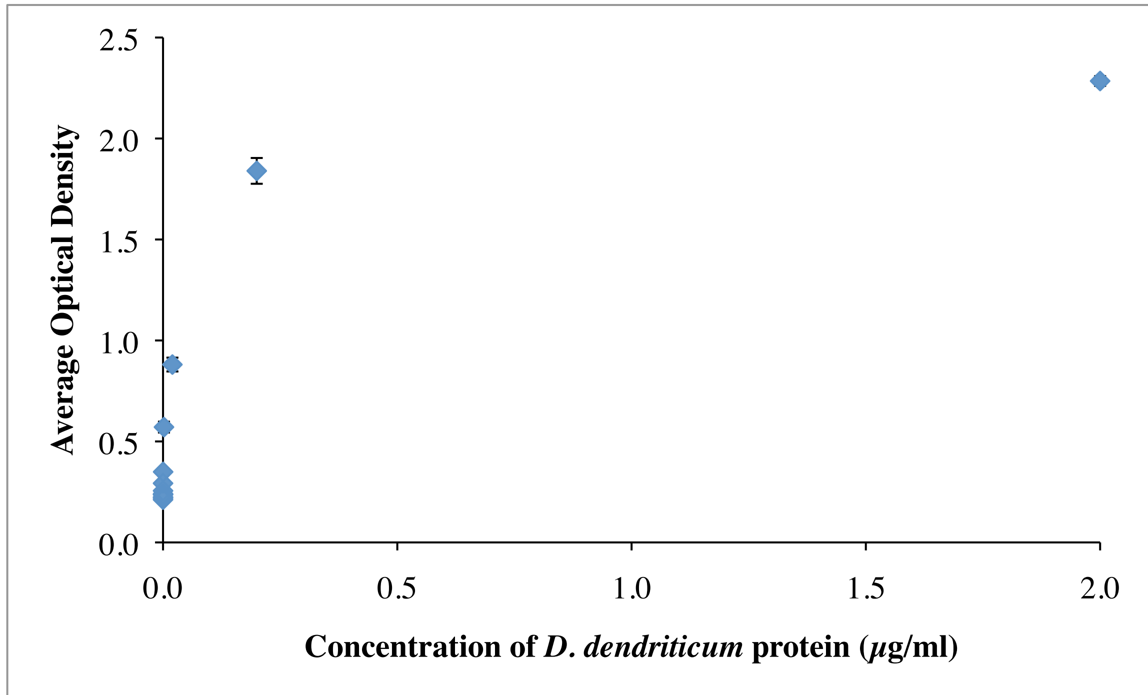

Figure 3.1. Standard curve of *D. dendriticum* ELISA in carbonate buffer.

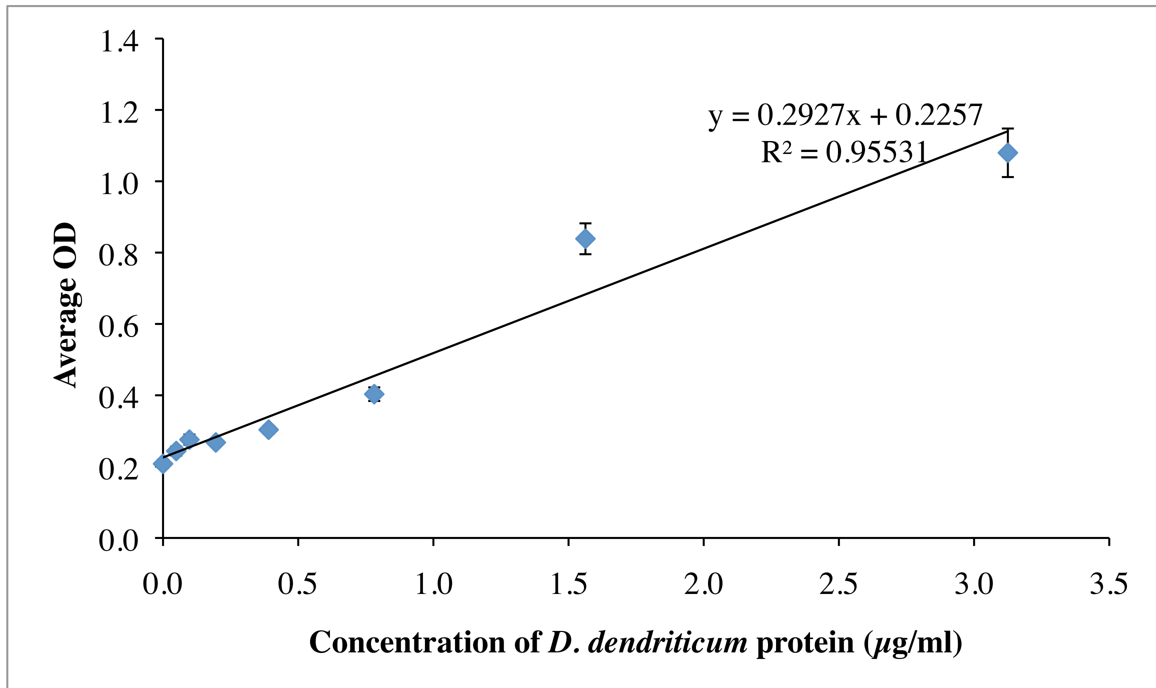

Figure 3.2. Standard curve of *D. dendriticum* ELISA in sheep fecal extract (negative control).

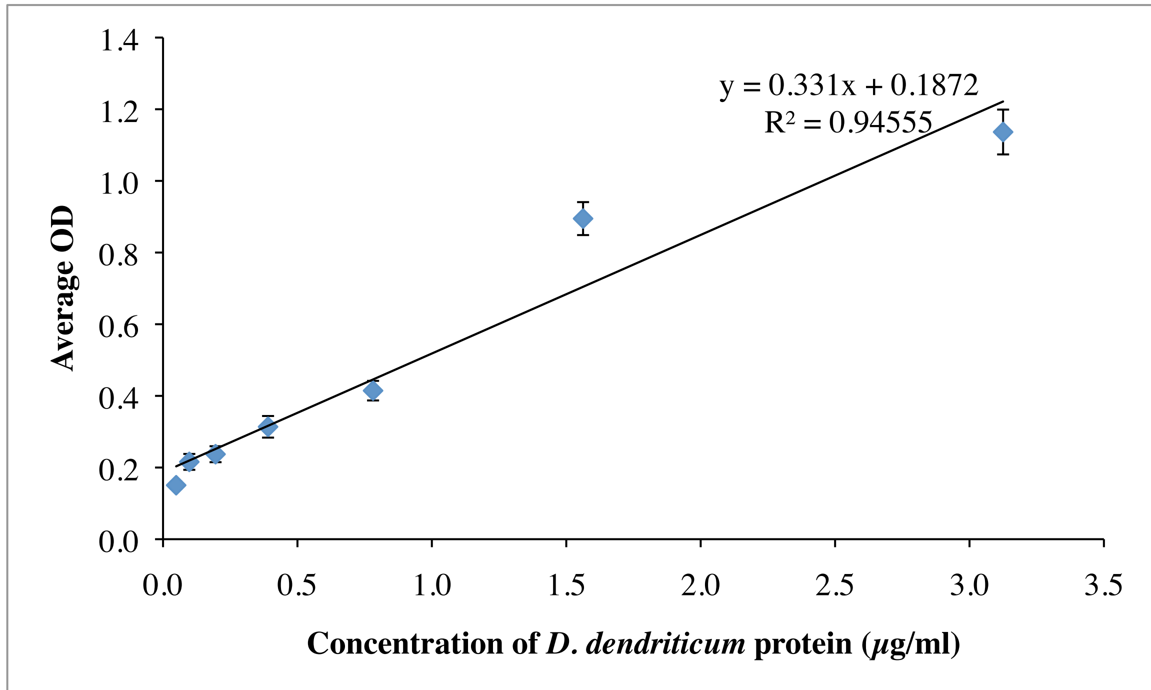

Figure 3.3. Standard curve of *D. dendriticum* ELISA in cattle fecal extract (negative control)**.**

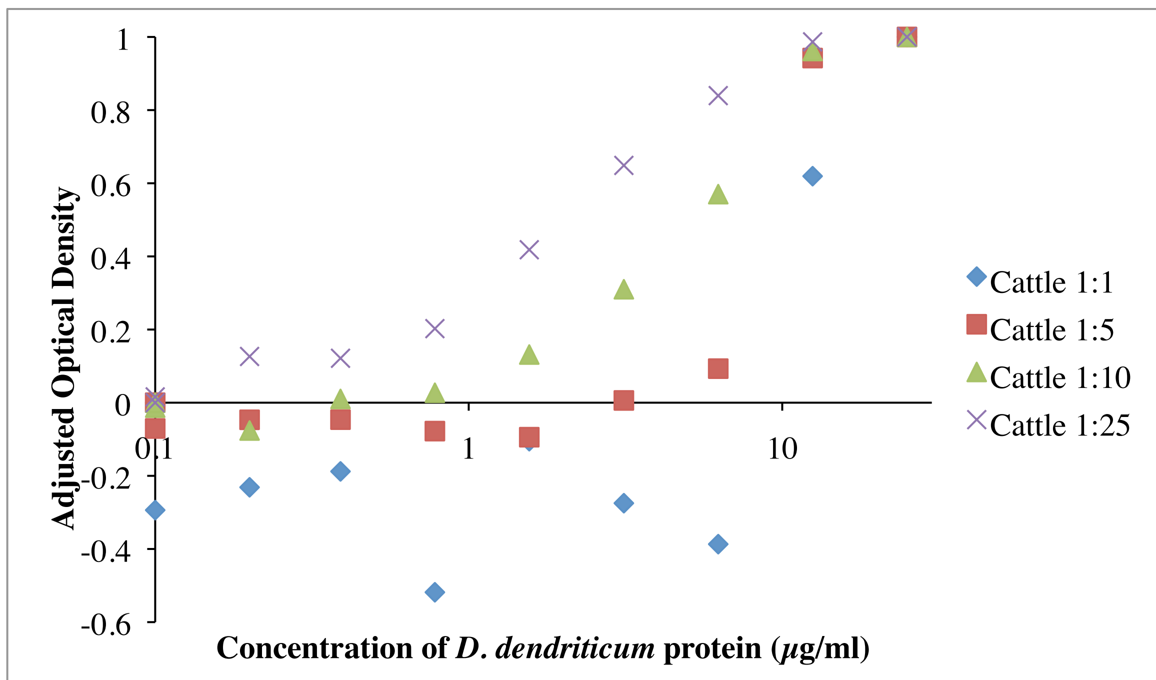

Figure 3.4. ELISA's standard curves in cattle fecal extracts diluted 1:1, 1:5, 1:10 and 1:25 in CBS.

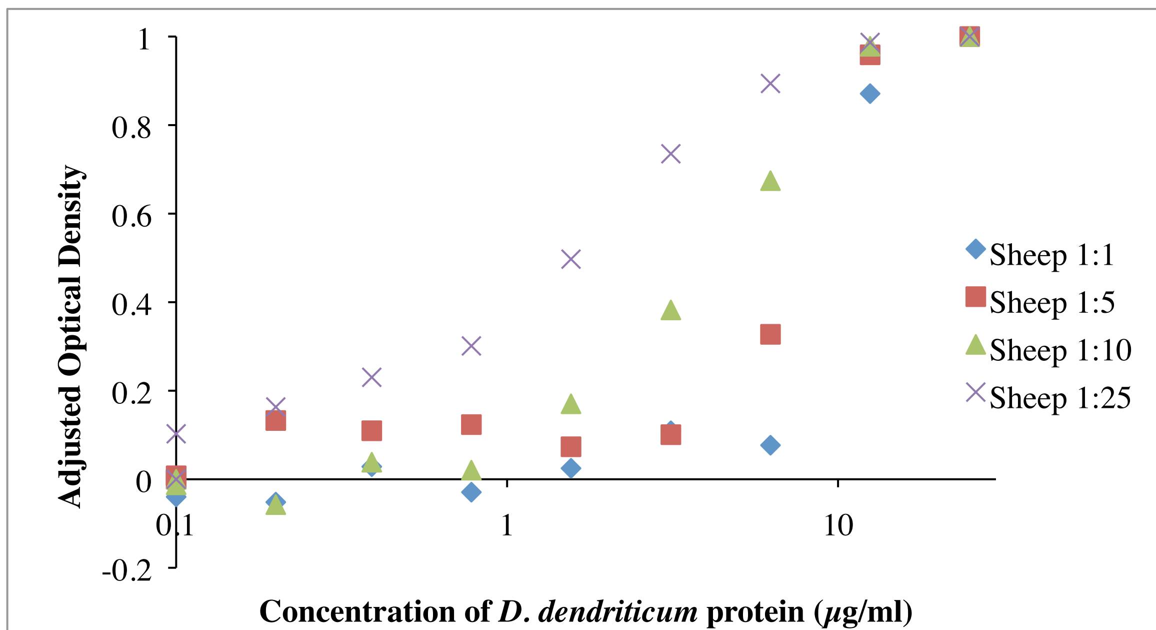

Figure 3.5. ELISA's standard curves in sheep fecal extracts diluted 1:1, 1:5, 1:10 and 1:25 in CBS.

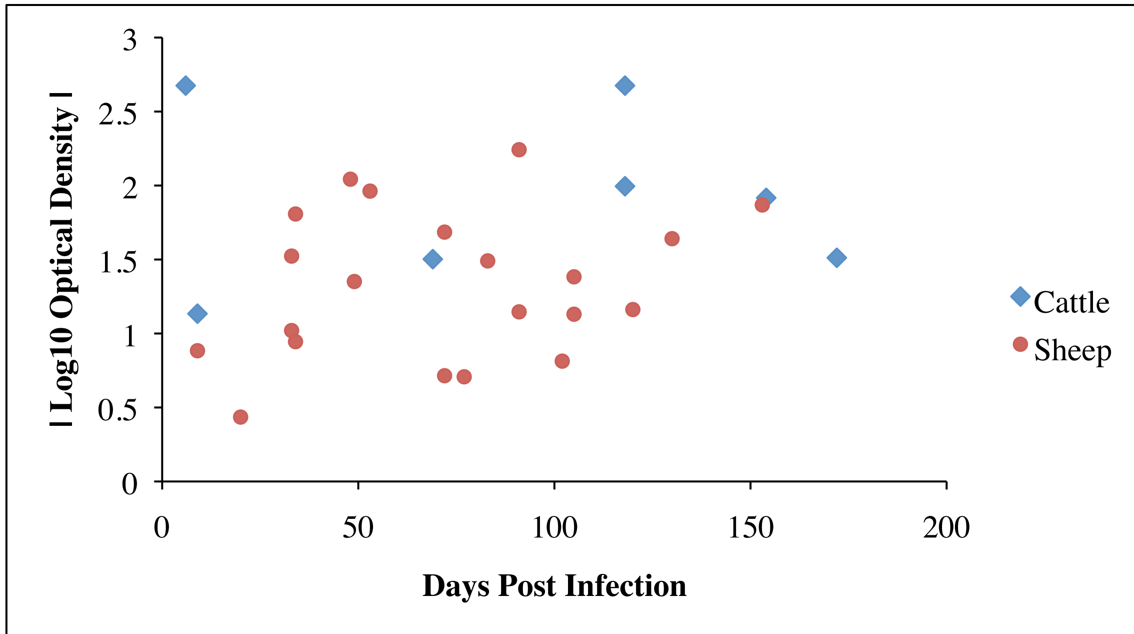

Figure 3.6. Positive coproantigen ELISA results from cattle and sheep, as measured by log transformed optical density, and their corresponding day post infection.

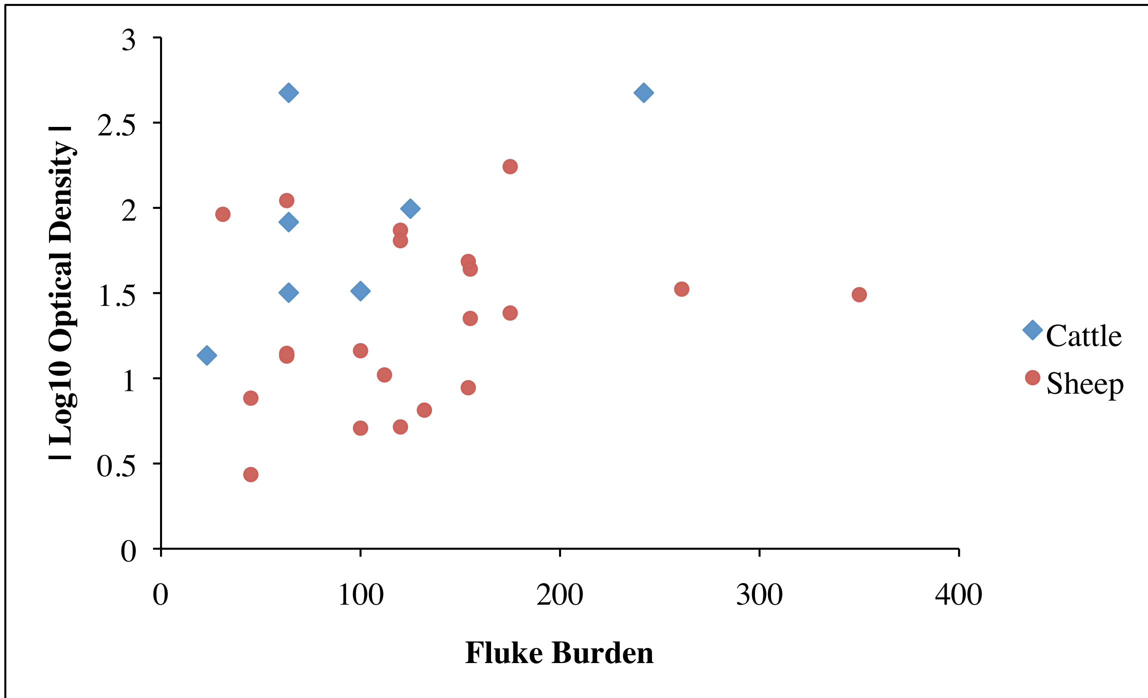

Figure 3.7. Positive coproantigen ELISA results from cattle and sheep, as measured by log transformed optical density, and their corresponding fluke burden.

Chapter 4. General Conclusions

My results in Chapter 2 are consistent with other experimental studies reporting strong differences in immune defences and in parasite performance between and within host species exposed to helminths (Shakya et al. 2011, Beck et al. 2014, Badr et al. 2015).

There are many factors that could underlie the high interspecific variation that I observed in *D. dendriticum* intensity, percent recovery, fecal eggs per gram, and host antibody responses. Although I attempted to control for as many potential confounding factors as possible, there were unavoidable differences in experimental conditions between the exposure trials into sheep, goats, and cattle. Variation in the quality and quantity of the diets that were provided to the three species is one possibility. While all individuals were provided *ad libitum* hay, cattle were also fed barley/corn silage, and lambs were supplemented with grower ration and alfalfa pellets. The effects of host diet on immuno-competency (Lazzaro and Little 2009, Ponton et al. 2013) and especially on individual parasite performance (Ponton et al. 2013) are well known. For example, Vagenas et al. (2007) used an in silico mathematical model to determine the effect of nutrition on the performance of a gastrointestinal nematode, *Teladorsagia circumcincta* in lambs. They found that diet had a significant effect on worm burdens in infected hosts.

Variation in intrinsic host factors such as body size, host sex, and differences in features such as host age might also have contributed to variation in immunity and worm performance. Since equivalent-aged cattle are much larger than sheep and goats, their lack of antibody responses could be attributed to low worm intensity relative to their body size. Additionally, architectural, physiological, and biochemical differences in the

gastrointestinal tracts between species (Kararli 1995) might also lead to differences in rates of excystation and/or migration of juvenile worms to the bile ducts.

My data indicates high interspecific variation in serological IgG and IgM concentrations. Similar results have been demonstrated in a wide range of helminth/host interactions (Shakya et al. 2011, Badr et al. 2015). The "costs of immunity" hypothesis provides an explanation for these differences by stressing that each species has different internal and external demands on their energy (Zuk and Stoehr 2002). As a result, it may not always be optimal for hosts to mount a strong immune response, due to energetic costs and also to immunopathology. According to this hypothesis, in my study sheep, cattle, and goats may have different energy demands that result in varying IgG and IgM antibody responses to *D. dendriticum*.

One shortcoming of my study is that hosts were exposed to fluke metacercariae once, while in nature they are likely exposed numerous times while grazing and unintentionally consuming infected ants. More data is required to understand the nature of the host immune response to repeated exposures. It is also possible that the numbers of metacercariae administered to hosts in my study were not sufficient compared to what grazing animals would be exposed to in nature. Another shortcoming of this research is that we do not know if IgG and IgM responses are protective in multiple exposures to *D. dendriticum*. In addition, this research does not consider other important immune effector cells that are part of the immune response such as macrophages and eosinophils. Although cattle had no detectable IgG and IgM antibodies against *D. dendriticum*, it is possible that they produced an immune response using other effector cells, like mast cells

and eosinophils (e.g. Shakya et al. (2011)) and other components of typical Th-1 responses.

My results in Chapter 3 represent the first detection of *D. dendriticum* proteins in host feces using a coproantigen ELISA. Not only were fluke proteins detected in fecal samples at concentrations as low as 98 ng/ml in cattle and 49 ng/ml in sheep, they were found as early as 9 d.p.i. in both species. This represents the earliest detection of *D. dendriticum* infection in experimentally infected animals, to our knowledge. Campo et al. (2000) detected fluke infections in sheep as early as 49 d.p.i. through fecal egg detection, while Broglia et al. (2009) used serological ELISAs to detect infections in sheep as early as 30 d.p.i.

The coproantigen ELISA developed in this study holds potential to be an excellent, early diagnostic tool for *D. dendriticum* in ungulates. Unlike serological ELISAs, which do not always indicate current infection status due to antibody persistence in the blood (Opsteegh et al. 2011), coproantigen detection relies on parasite protein in host feces (Charlier et al. 2014). As a result, coproantigen ELISAs can be more reliable at indicating current infection status in hosts. An important implication of this tool is the potential for it to be used in wildlife species since animals need not be present in order for fecal samples to be collected and tested. Wildlife is important in parasite transmission as they share many of their parasites with domestic animals (Walker and Morgan 2014). A sensitive coproantigen assay could allow for infections in wildlife to be monitored and would help map the range and spread of invasive parasites such as *D. dendriticum* in Cypress Hills Provincial Park, which is an important step for parasite control and in epidemiological studies of emerging pathogens.

There are a few shortcomings of my study in Chapter 3. Firstly, the fecal samples from experimentally infected cattle and sheep were not consistently sampled during the experimental infections. As a result there is not a clear picture of fluke protein levels in feces throughout infection. Another issue with my study is the poor sensitivity of the coproantigen ELISA, with only 45% and 23% of positive sheep and cattle fecal samples being correctly identified. These low sensitivities make it an unreliable diagnostic tool at this point.

Future research should apply my methods in Chapter 2 for assessing host species' responses to other economically important helminths. By doing this, producers could use a more holistic approach to livestock management by taking multiple parasites' effects on species into consideration. Despite having reasonable sample size for cattle and sheep, there were only five goats included in this study. To extend the results in Chapter 2, future research should repeat my study with more animals of all three species, especially goats. For the coproantigen ELISA, future studies should focus on improving the poor sensitivities of the assays in cattle and sheep. Once a satisfactory sensitivity has been achieved for these domestic hosts, the assay should be tested for its reliability in samples of feces from wildlife. To achieve this, it is important to first determine if coproantigens' presence in feces truly fluctuates over time or if the inconsistency in detection is due to the fecal extraction and assay methods, or an uneven distribution of protein in fecal samples.

This project has demonstrated that interspecific differences in hosts of generalist parasites are important to consider when managing and diagnosing parasitic infections in livestock due to the variation found among cattle, sheep, and goats infected with *D.*

dendriticum. Host species should be evaluated when attempting to reduce the impact of parasitism in livestock. Lastly, I have described a diagnostic tool for *D. dendriticum* that has potential to map infection spread in domestic and wild host species using fecal samples. The combination of understanding host species responses to *D. dendriticum* and a potentially effective diagnostic tool contributes to the ultimate goal of improving the efficiency of livestock production and curbing the impact of this and other parasites.

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