2017

ANALYSIS OF HUMORAL IMMUNE RESPONSES IN HORSES WITH EQUINE PROTOZOAL MYELOENCEPHALITIS

Catherine-Jane Angwin
University of Kentucky, katyjane.angwin@uky.edu
Digital Object Identifier: https://doi.org/10.13023/ETD.2017.239

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Catherine-Jane Angwin, Student

Dr. Daniel K. Howe, Major Professor

Dr. Daniel K. Howe, Director of Graduate Studies

ANALYSIS OF HUMORAL IMMUNE RESPONSES IN HORSES WITH EQUINE PROTOZOAL MYELOENCEPHALITIS.

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science
in the College of Agriculture, Food and Environment
at the University of Kentucky

By

Catherine-Jane Angwin

Lexington, Kentucky

Director: Dr. Daniel K. Howe, Professor of Molecular Parasitology

Lexington, Kentucky

2017

ABSTRACT OF THESIS

ANALYSIS OF HUMORAL IMMUNE RESPONSES IN HORSES WITH EQUINE PROTOZOAL MYELOENCEPHALITIS.

Equine protozoal myeloencephalitis (EPM), caused by the protozoan parasite *Sarcocystis neurona,* is one of the most important neurological diseases of horses in the Americas. While seroprevalence of *S. neurona* in horses is high, clinical manifestation of EPM occurs in less than 1% of infected horses. Factors governing the occurrence and severity of EPM are largely unknown, although horse immunity might play an important role in clinical outcome. We hypothesize that EPM occurs due to an aberrant immune response, which will be discernable in the equine IgG subisotypes a, b, and (T) that recognize *S. neurona* in infected diseased horses versus infected but clinically healthy horses. Based on previously-established serum antibody concentrations for IgG subisotypes in healthy horses, standard curves were generated and served to establish the concentration of antigen-specific IgG subisotypes in equine serum and CSF in infected diseased and infected normal horses. The subisotype concentrations and ratios between subisotypes were analyzed to assess whether neurological disease is associated with detectable differences in the antibody response elicited by infection. Results indicate a type I biased immune response in infected diseased horses, implicating the role of immunity in the development of EPM.

KEYWORDS: Equine Protozoal Myeloencephalitis; *Sarcocystis neurona*; immunopathology; immunoglobulin; horse

CATHERINE-JANE ANGWIN

JUNE, 2017

ANALYSIS OF HUMORAL IMMUNE RESPONSES IN HORSES WITH EQUINE PROTOZOAL MYELOENCEPHALITIS.

By

Catherine-Jane Angwin

DR. DANIEL K. HOWE
Director of Thesis

DR. DANIEL K. HOWE
Director of Graduate Studies

JUNE, 2017

ACKNOWLEDGMENTS

The following thesis, while an individual work, benefited from the insights and direction of several people: First, my Thesis Chair, Dr. Dan Howe, and committee members, Dr. David Horohov and Dr. Steve Reed, who provided timely and instructive comments and evaluation at every stage of the thesis process. I would also like to thank Michelle Yeargan in the Molecular Parasitology Laboratory at the University of Kentucky for technical and laboratory assistance. I thank Dr. A. Estellar-Vico and Dr. A. Adams from the Department of Veterinary Science, University of Kentucky, for providing reagents required for this study; Equine Diagnostic Solutions, LLC, for providing serum and CSF samples; and Dr. B. Goff from Plant and Soils Science, University of Kentucky, for his assistance with statistical analysis. This research was supported by funds from the Amerman Family Equine Research Endowment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: The Parasite

1.1 Protozoal Parasites

Protozoa are single celled eukaryotic organisms. Despite their small size, protozoa are responsible for causing major disease worldwide. Malaria, caused by Plasmodium spp., is a severe disease causing fever, and sometimes death, with 3.2 billion people living in at-risk areas globally. Toxoplasmosis, caused by *Toxoplasma gondii*, is a human pathogen well-known to cause disease in immunocompromised patients and those suffering from acquired immune deficiency syndrome (AIDS), and can be transmitted transplacentally in pregnant women. African sleeping sickness, caused by *Trypanosoma brucei*, is a disease prevalent in sub-Saharan African, resulting in neurological disease and death, if untreated. Cryptosporidiosis, caused by Cryptosporidium spp., is the leading cause of waterborne disease in the United States. Protozoan diseases have also been well characterized in the horse: Equine piroplasmosis is a tick-borne disease caused by *Babesia equi/Theileria caballi*, affecting all equids. Dourine is a sexually transmitted disease caused by *Trypanosoma equiperdum*, with high mortality in acutely infected breeding animals. Surra, caused by *Trypanosoma evansi*, is of great global importance due to economic losses from decreased productivity in working animals and reproductive losses. Equine Protozoal Myeloencephalitis (EPM), caused by *Sarcocystis neurona*, is an important neurological disease of horses in the Americas. It is EPM, its etiologic agent, and its host that will be the focus of this paper.

1.2 Phylum Apicomplexa

EPM is largely caused by the protozoa *Sarcocystis neurona*, which is a member of the phylum Apicomplexa. Apicomplexa includes over 5,000 protozoa, of which almost all are obligate intracellular pathogens, infecting vertebrate and invertebrate species (Blader et al., 2015). Also in this phylum are the aforementioned genera Plasmodium, Babesia, Toxoplasma, and Cryptosporidium. Sarcocystis species are closely related to both *Toxoplasma gondii* and Neospora species. Due to its ease of use, the availability of genetic tools and excellent animal models, and because of its importance as a human pathogen, *T. gondii* has been the focus of much research within the phylum Apicomplexa (Dubey et al., 2015). It is somewhat fortunate for those working on Sarcocystis species that much of the research applied to Sarcocystis can be drawn from that conducted in Toxoplasma. This transfer of research tools across genera is applicable due to the genetic similarities between Toxoplasma and Sarcocystis. However, it must be noted that there are known inherent differences between these two parasites, and not all biological mechanisms and host responses can be assumed to be identical.

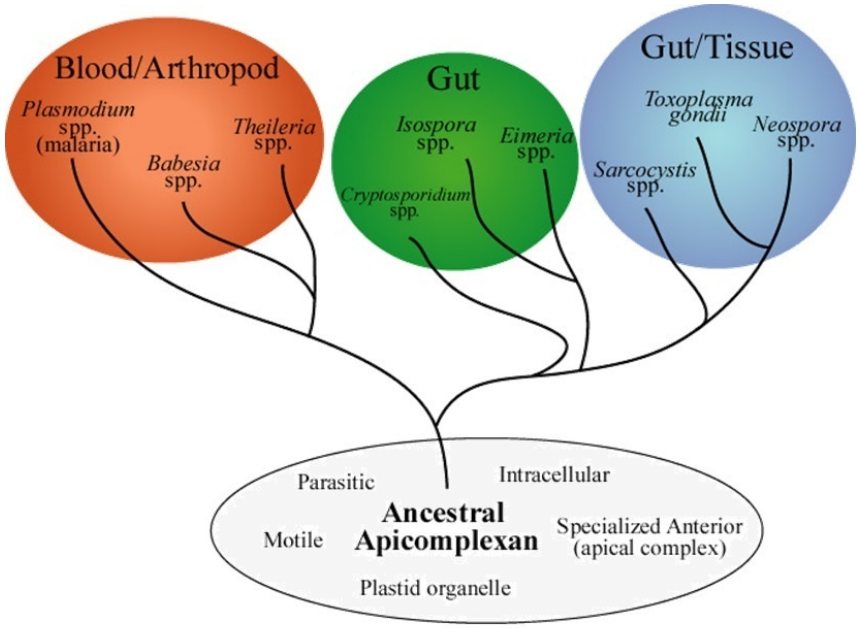

Figure 1.2.1 Phylogenetic tree of the phylum Apicomplexa, showing relative relatedness of Apicomplexan genera found in the blood, gut, and tissue of host animals.

1.3 Sarcocystis species

Sarcocystis is arguably the most successful protozoan parasite genus in nature, with the potential to infect all vertebrates, including fish, reptiles, birds, and mammals (Dubey et al., 2015). In general, they convey low pathogenicity, and infections are well tolerated (Dubey et al., 2016), although this is not always the case. Within the phylum Apicomplexa, Sarcocystis spp. are obligate intracellular parasites that are capable of causing disease in humans and animals, and can be found in muscles and the central nervous system (CNS) (Dubey et al., 2016). The life cycle is obligatorily two host, with asexual reproduction occurring in the intermediate host, and sexual reproduction and the expulsion of sporocysts occurring in the definitive host (Dubey et al., 2016).

Sarcocystis spp. are well known in the livestock industry, with *Sarcocystis cruzi*, *Sarcocystis tenella*, and *Sarcocystis capracanis* causing decreased weight gain, and occasionally abortion and death, in cattle, sheep, and goats, respectively (Dubey et al., 2016), and *Sarcocystis aucheniae* forming cysts in the muscle of camelids, resulting in the condemnation of carcass meat and economic loss for producers (Dubey et al., 2016).

1.4 Economic Losses due to Sarcocystis Infections

Globally, the main economic costs due to Sarcocystis infection come from the condemnation and downgrading of meat containing visible sarcocysts otherwise designated for consumption. This cost is estimated to be in the millions of dollars. However, the true economic losses are challenging to calculate as no monetary values can be placed on poor feed efficiency, reduced milk or wool production, and reproductive problems. Clinical disease due to Sarcocystis can be difficult to diagnose, and therefore the true cost of treatment is also ambiguous (Dubey et al., 2016). Estimates of the annual

cost due to *S. neurona* infection in the horses were calculated in 1998 by the Animal and Plant Health Inspection Service, and are addressed in the following chapter.

1.5 Life Cycle of *Sarcocystis neurona*

Like most members of the Phylum Apicomplexan, all Sarcocystis species have an obligate intracellular heteroxenous life cycle involving both sexual and asexual stages of reproduction, with each stage of reproduction occurring in one or more different host animal. The sexual stage of reproduction occurs in the gastrointestinal tract of the meat eating definitive host, which for *S. neurona* is the metatherian North and South American opossums (*Didelphis virginiana* and *Didelphis albiventris*, respectively) (Dubey et al., 2015). Whether other species of South American opossum are definitive hosts is not yet known (Dubey et al., 2015). Between 6%-31% of opossums tested in the United States were found to harbor sporocysts (described later in this section) (Dubey et al., 2016).

In the definitive host, the bradyzoite stage of the parasite actively moves to penetrate the lining of the small intestine. Here parasites differentiate into micro (male) and macro (female) gamonts in preparation for sexual reproduction. Through the process of fertilization, microgametes move to the periphery of the macrogamont, where their membranes fuse and the nucleus of the microgamont is transferred into the macrogamont to create a zygote. A wall develops around the zygote, and an oocyst, or sporocyst, is formed (Dubey et al., 2016). Sporocysts are released into the lumen of the intestine and are then shed into the environment through opossum feces, where they can be picked up by the intermediate hosts.

The asexual stage of reproduction occurs in the intermediate host, of which there are many for *S. neurona*. The opossum is the only known definitive host for *S. neurona*, but unlike other Sarcocystis spp. *S. neurona* has numerous intermediate hosts, including skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), armadillos (*Dasypus novemcinctus*), and cats (*Felis catus*) (Dubey et al., 2015). Intermediate hosts become infected with *S. neurona* sporocysts by ingesting food or water contaminated by infected opossum feces (Dubey et al., 2016; Fenger et al., 1995). In the intermediate host, sporozoites enter intestinal epithelial cells, where they undergo the first stages of asexual reproduction (Fenger et al., 1995). Upon entering the host epithelial cells, the sporozoites transform into oval schizonts. Unlike other intracellular stages of Sarcocystis spp., schizonts are located free within the cytoplasm of the host cell, and are not surrounded by a parasitophorous vacuole (distinguishing it from *T. gondii*) (Dubey et al., 2016). Schizonts undergo endopolygamy, and release 64 merozoites into the cell. The host cell is unable to sustain 64 merozoites, and dies, releasing the parasites into the blood stream. Merozoites spread throughout the body, and are usually controlled by a potent immune response (Blader et al., 2015). It is possible that it is this immune response, in combination with the microenvironment of myocytes that trigger the differentiation of *S. neurona* merozoites into bradyzoites.

Sarcocyst development starts when merozoites enter a muscle or neural cell (Dubey et al., 2016). The intracellular merozoite is surrounded by a parasitophorous vacuole, which becomes the external wall of the sarcocyst. After multiple divisions, the sarcocyst is filled with the bradyzoite, or slow-growing, form of the parasite, which ultimately become the infective stage for the definitive host (Dubey et al., 2016). Because they contain the infectious bradyzoites, sarcocysts are essential for the completion of the parasite's life cycle, and have been found in intermediate host skeletal muscles, cardiac muscles, and brain (Dubey et al., 2015). Sarcocysts usually persist in the body of the intermediate host for the life of the host, although rupture of sarcocysts can occur, resulting in the release of bradyzoites, but does not initiate new development of sarcocysts (Dubey et al., 2016). Bradyzoites are non-infective to the intermediate host, or between intermediate hosts (Dubey et al., 2016). When the intermediate hosts die, they are scavenged by the definitive host. The definitive host becomes infected when it ingests muscular or neural tissue from an intermediate host containing mature sarcocysts. The sarcocysts are encysted in the intestines of the opossum, where they restart their life cycle.

The only known means by which a definitive host can acquire a *S. neurona* infection is through the ingestion of mature sarcocysts containing the infectious bradyzoite form of the parasite. Immature sarcocysts and schizonts are not infectious for the definitive host (Dubey et al., 2016). Sarcocystis spp. do not generally cause illness in their definitive hosts (Dubey et al., 2016). Opossums are opportunistic scavengers, and rely heavily on dead animals for their diet, making the natural *S. neurona* life cycle highly efficient (Furr and Reed, 2015).

It is only the sporozoite-containing sporocyst shed through opossum feces that is infectious to the intermediate host. Intermediate hosts do not shed parasites into the environment, and can therefore not pass the parasite from one animal to another.

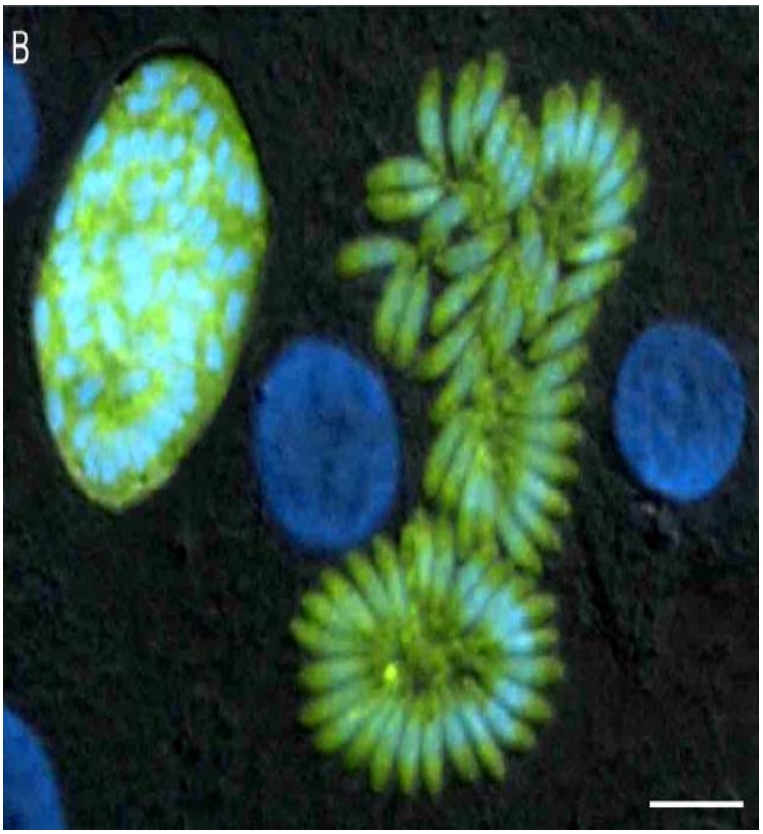

Figure 1.5.1 (left) Fluorescence images of *S. neurona*. Transgenic clone of *Sarcocystis neurona* expressing yellow fluorescent protein. Differential interference contrast image with epifluorescence image overlay showing a bovine turbinate cell monolayer containing a late-stage schizonts and a mature schizont of a clone of *S. neurona* that stably expressed YFP. Host cell and parasite nuclei were stained with DAPI (blue). Bar-10µm. (Dubey et al., 2015).

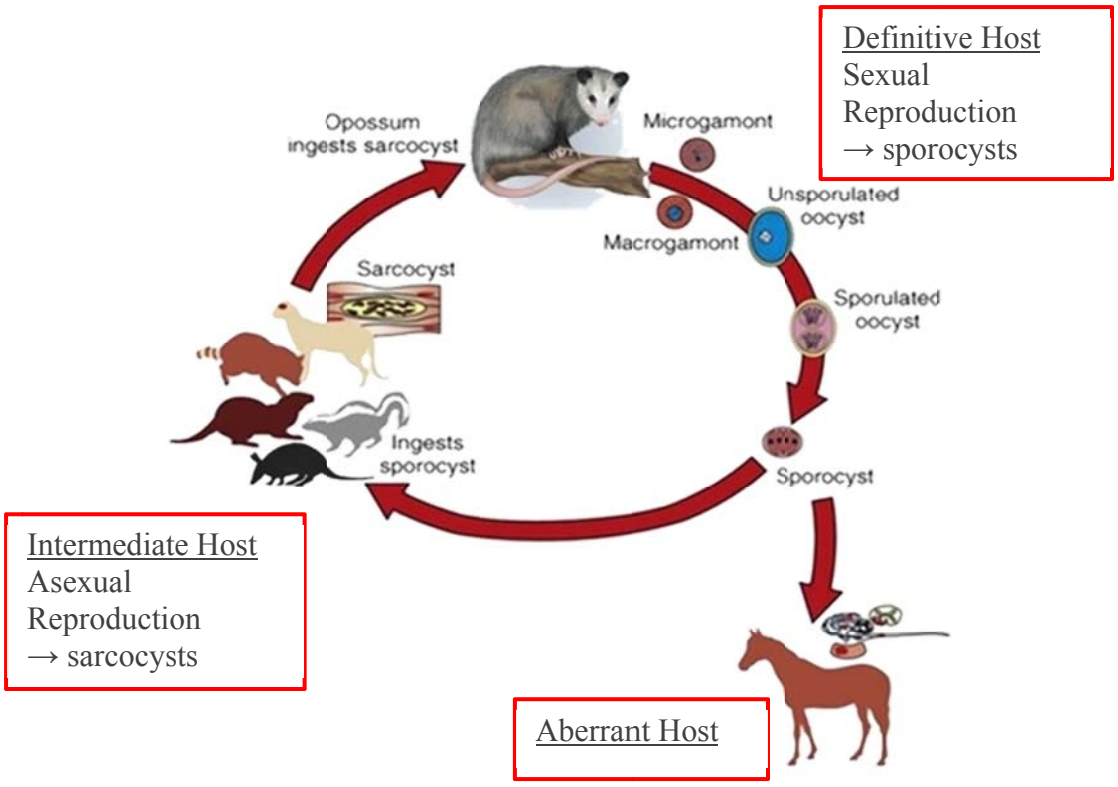

Figure 1.5.2 Life cycle of *S. neurona*, with sexual replication occurring the definitive host and asexual replication occurring in the intermediate host. Also showing the horse as the aberrant host.

1.6 *Sarcocystis neurona* in the Horse

S. neurona reaches the equine host in the same way it reaches intermediate hosts, through contamination of the environment by opossum feces containing sporocysts. As with intermediate hosts, sporozoites are the only infective form of the parasite to the horse. However, unlike in intermediate hosts, the parasite does not develop to form bradyzoite-containing muscle cysts. The parasites invade through the intestinal epithelial cells and form schizonts, which divide into the merozoite form of the parasite. Merozoites and schizonts are the only stages found in horses, and they are predominantly confined to the CNS (Fenger et al., 1995). In most horses, the immune system successfully controls merozoite infection, and eliminates the parasite from the host animal. Horses can become infected multiple times, without development of clinical signs. Merozoites found in the horse are not infectious to either the definitive or intermediate host, and are not shed into the environment. Because these parasites are not shed into the environment, horses cannot pass *S. neurona* infections between each other. Should the host animal die and become scavenged by other animals, the parasites it harbors are still not infectious, as they do not exist in either in the sporozoite or bradyzoite form in the horse. Because horses do not contain a form of the parasite that is infectious to either the intermediate or definitive host, the parasite is unable to continue its lifecycle once it has infected the

horse. There is seemingly no benefit to the parasite to infect the horse, and horses are therefore considered to be aberrant, accidental, or dead-end natural hosts of *Sarcocystis neurona* (Fenger et al., 1995).

Although transplacental infection of Sarcocystis spp. has been documented in some host animals (most notably cattle and sheep), it is uncommon, and has not been reported in the horse (Dubey et al., 2016).

1.7 Distribution

The distribution of *Sarcocystis neurona* is dependent on its definitive host, *Didelphis virginiana* and *Didelphis albiventris* in North and South America, respectively. This makes it largely a disease of the Americas, although there have been reports of horses that have been exposed to the parasite while in the Americas and go on to develop clinical disease when in another country (Dubey et al., 2015). Within the United States, there is a high prevalence of antibodies to *S. neurona*, with seroprevalence being lower outside of the habitat range of the opossum (such as the intermountain west) and reaching almost 90% in some areas of high opossum densities (such as Oklahoma) (Dubey et al., 2015). In Central and South America, studies have shown that horses often have regionally high exposure rates (Dangoudoubiyam et al., 2011; Hoane et al., 2006).

Chapter 2: Equine Protozoal Myeloencephalitis

2.1 Disease Description

Equine Protozoal Myeloencephalitis (EPM) is most commonly caused by the etiologic agent *Sarcocystis neurona*, and is one of the most important neurological diseases of horses in the Americas. EPM was originally referred to as "segmental myelitis," and was first described by Rooney et al. in 1970 (Rooney et al., 1970). It was discovered to be of protozoan etiology in 1974, although it was not known to be caused by *S. neurona* until 1991 (Dubey et al., 2016). Although there are many neurologic disorders that affect the horse, EPM is one of the most commonly diagnosed (Dubey et al., 2015).

While the seroprevalence of *S. neurona* in horses is high, clinical manifestation of EPM occurs in less than 1% of the infected horses (MacKay et al., 2000; NAHMS, 2001). It is therefore apparent that neurological disease is not the outcome of every *S. neurona* infection.

It is likely that clinical disease due to *S. neurona* is rare because pathology is caused by the merozoite stage of the parasite attempting to invade cells, and this stage is typically well-controlled by the host immune response (Blader et al., 2015).

EPM is rare in non-horse equids, but, as mentioned later in this chapter, can affect animals other than the horse, and is a common cause of mortality in marine mammals (Dubey et al., 2015).

2.2 Clinical Signs

Although EPM is usually a progressively debilitating disease with a gradual onset, this varies widely from horse to horse. The disease may stabilize in some horses, remaining static for periods of time, but in others it can have a rapid onset with focal or multifocal signs of neurologic disease (Dubey et al., 2015). Early clinical signs of disease can include stumbling and are often confused with hind end lameness (Dubey et al., 2015). Gradual progression of clinical signs includes ataxia, asymmetric weakness, abnormal upper airway function, and hypoalgesia. Neurological symptoms can include head tilting, facial nerve paralysis, seizures, and, in more severe cases, difficulty swallowing. The location and severity of clinical signs depends on the localization of the damage in the central nervous system (CNS), and damage can occur in the brain, brainstem, spinal cord, or any combination of the areas of CNS (Dubey et al., 2015). For example, behavioral changes or seizures are caused by damage to the cerebrum, lesions in the brainstem and spinal cord can cause gait abnormalities and incoordination; and damage to gray matter in the brain, which controls the nerves running to limb muscles, produces weakness and atrophy of innervated muscles. This is particularly true of the hind end gluteal or temporalis muscles, which are often atrophied asymmetrically (Dubey et al., 2015).

2.3 Lesions

Upon necropsy, in some EPM cases parasites can be numerous and easily found in the CNS. However, in other cases, even those with severe lesions, parasites are sparse or non-detectable. This implies that pathogenesis is, at least in part, immune-based (Dubey et al., 2015; Furr and Reed, 2015)

It is therefore likely that it is not the parasite itself that causes damage within the CNS, but rather the host immune response to the parasite. When a cell is invaded, a trigger is given to the immune response to kill the infected cell. When this occurs, it is not this singular cell that is destroyed, but also surrounding cells are subject to collateral damage. Thereby, this immune response often results in lesions, and therefore CNS damage. Lesions due to *S. neurona* infection do not always occur, but when they do, they are confined to the CNS (Dubey et al., 2015). The brainstem is the most commonly affected region of the brain, yet lesions are more frequently seen in the spinal cord (Dubey et al., 2015). These lesions often result in the previously described gait abnormalities typically seen in clinical EPM cases.

Both gross and/or microscopic lesions can occur. Gross lesions typically consist of areas of hemorrhage, sometimes with mild swelling. Microscopic lesions predominantly contain lymphocytes and macrophages, but eosinophils are commonly present, and are often accompanied by inflammation and tissue degeneration (Dubey et al., 2015).

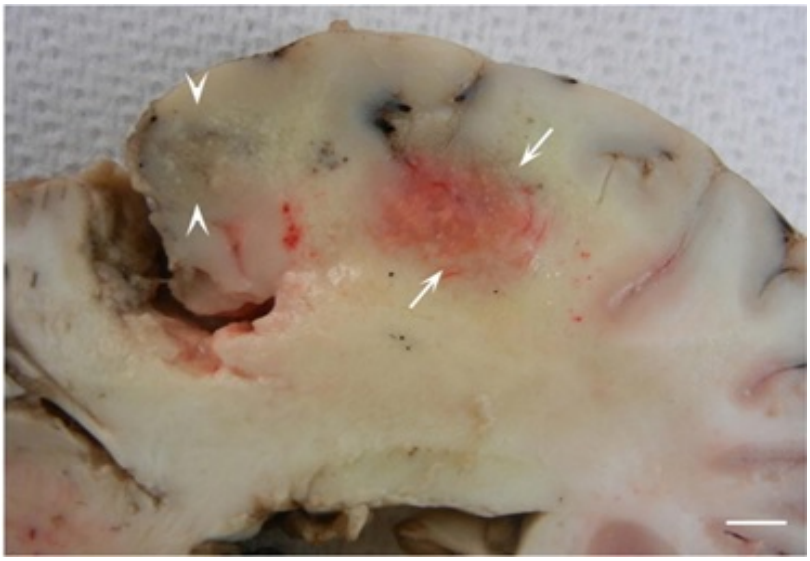

Figure 2.3.1 Cut view of cerebrum of a 20-year-old Paint horse with histologically and PCR confirmed EPM. The horse had a six-day history of muscle fasciculations, bruxism, difficulty eating and drinking, and circling to the left with head pressing. Note hemorrhagic and yellow discolored areas indicative of necrosis. Bar = 5mm (Dubey et al., 2015).

2.4 Infectivity and Central Nervous System Access

Parasites with a two-host life cycle are thought to manipulate physiological responses in the intermediate host, in order to more effectively and efficiently reach the definitive host, where they can complete their life cycle through sexual reproduction (Berdoy et al., 2000). In order to enter and exit the cell at will, and obtain nutrients, intracellular pathogens require interactions with the host cell, yet simultaneously avoid setting off signals that would result in their elimination, and must therefore manipulate innate immune mechanisms (Sibley, 2011).

In horses with EPM, *Sarcocystis neurona* parasites are able to parasitize all regions of the CNS, and may be found in neurons, mononuclear cells, and glial cells (Sellon et al., 2004). Because the horse acquires the parasites through contaminated food or water sources, the parasites must therefore reach the CNS from the gastrointestinal tract. As previously mentioned, *S. neurona* invades intestinal epithelial cells and disseminates into the blood. However, parasitemia in horses is rarely noted in naturally infected horses (Dubey, 2001; Sellon et al., 2004), and in studies conducted in mice, parasites were found to be in the CNS after 13 days of infection (Dubey, 2001). Therefore, to reach the CNS cells, the parasite must not only evade the immune system, but also gain access to the highly protected CNS. As mentioned later in this document, the blood-brain barrier is a barrier largely composed of tightly-associated brain endothelial cells. There are three ways to cross the blood-brain barrier: through the endothelial cells (transcellularly), between the endothelial cells (paracellularly), or inside another cell that is actively transported across the endothelial cells (Kim, 2008). The etiologic agents of African sleeping sickness, Protozoan parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, gain access to the CNS paracellularly or transcellularly (Kim, 2008). With Apicomplexan parasites, the relationship with endothelial cells can vary widely – from direct invasion and lysis (as seen in *T. gondii*), initial replication within these cells (as seen with some avian and reptilian Plasmodium spp.), to the adhesion of infected erythrocytes allowing for parasite sequestration (as seen with *P. falciparum* and Babesia spp.) (Konradt et al., 2016). Each of these interactions reflects different strategies that have arisen through evolution of the parasite-host relationship for immune evasion and to facilitate transmission into an immune privileged site (Konradt et al., 2016).

Research in into *T. gondii* has demonstrated two broad mechanisms of gaining entry: the "Trojan horse" theory, and the direct invasion theory. It is likely that these are not mutually exclusive and that parasites in fact employ a combination of both methods. Due to the similarity of *S. neurona* to *T. gondii*, it is not unreasonable to assume that *S. neurona* employs similar mechanisms of entry.

"Trojan horse" theory

T. gondii is thought to exploit host monocytic or dendritic cells to allow for transportation across the BBB, in a "Trojan Horse" fashion (Lachenmaier et al., 2011). *T. gondii* infection results in upregulation of the transcriptome, which, increases the cytokines,

chemokines, and the adhesion molecule ICAM-1 expressed by endothelial cells. Proinflammatory cytokines increase permeability of the BBB, and ICAM-1 enhances the attachment and extravasation of leukocytes, allowing for increased transmigration into the CNS (Lachenmaier et al., 2011).

However, additional research was unable to show infected cells crossing the blood-brain barrier, and rather the alternative explanation that free *T. gondii* parasites in the blood directly infect the endothelial cells of the blood brain barrier, where they replicate and lyse the host cells, thereby gaining access to the CNS (Konradt et al., 2016).

Direct invasion theory

The direct invasion theory proposes that *T. gondii* parasites are found free in the blood, and are able to invade, replicate in, and lyse endothelial cells of the blood brain barrier, and thereby gain direct access to the CNS (Konradt et al., 2016). This occurs in areas of low shear stress, that have sufficiently "stagnant" blood flow velocities to allow for *T. gondii* to adhere and invade epithelial cells (Konradt et al., 2016).

2.5 Economic impact with horses

In 1998, the Animal and Plant Health Inspection Service estimated the annual cost of EPM to the equine industry to be $28 million, with the largest cost incurred by lost use of the animal. Lost use can be interpreted as loss of earnings through competition, work use, and/or breeding.

Costs in subsequent years have not been calculated, but given the lack of an effective vaccine for EPM and no way of determining which animals will fall victim to disease, it is reasonable to assume similar costs still exist today.

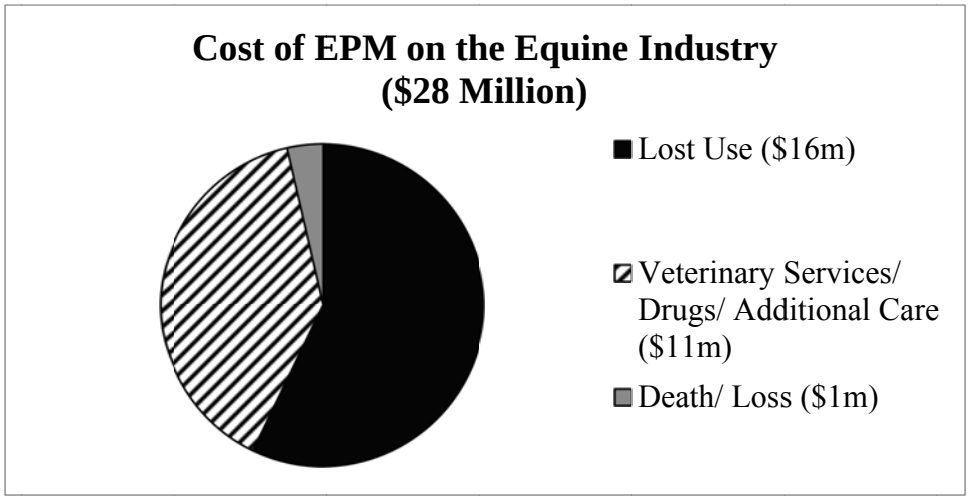

Figure 2.5.1 Pie chart showing estimated annual cost of Equine Protozoal Myeloencephalitis on the Equine Industry. Total annual cost was estimated at $28 million, divided into three parts: lost use ($16 million); Veterinary Services, drugs, and additional rehabilitative care ($11 million); and Death/Loss ($1 million) (NAHMS, 2001).

2.6 Risk Factors for Disease / Epidemiology

Seroprevalence to *S. neurona* across the United States is high, and increases with age (Saville et al., 1997). Yet less than 1% of these animals go on to develop clinical EPM (NAHMS, 2001). As yet, it is not clear what the risk factors are that lead to the occurrence and severity of EPM in this small percentage of horses and not others. Additionally, because these risk factors are largely unknown, it has been difficult to reliably induce disease experimentally in horses (Dubey et al., 2015).

EPM typically occurs in individual cases, and not in outbreaks, although there is a regional ecology associated with the disease, with more cases occurring where there are higher populations of opossums (Furr and Reed, 2015; Morley et al., 2008). Sporocysts shed into the environment are resistant to freezing (Dubey et al., 1978) and to disinfectants (McKenna and Charleston, 1992), meaning they can survive on a pasture over winter or in a feed storage area for months, even when opossums may not be seen to be active (Savini et al., 1996).

The average age of horses affected with EPM is approximately 3.5 to 4.5 years old, although all horses are believed to be susceptible to disease development (Boy et al., 1990; Dubey et al., 2015). However, there are no confirmed reports of clinical EPM in horses under 6 months of age (Dubey et al., 2015). There are also no documented cases of congenital transmission of *S. neurona* infection in land mammals (Dubey et al., 2015).

Stress, notably in the form of pregnancy, lactation and weather, is known to have effect on the susceptibility to infection and severity of disease in animals infected by other Sarcocystis species (Dubey et al., 2016). Research into the effects of transportation stress on horses has led the conclusion that stress can induce clinical disease in the horse. Horses transported by road for 55 hours were dosed with *S. neurona* sporocysts as the horses were unloaded at their destination. The transported horses seroconverted to become *S. neurona* positive sooner than non-transported horses, and exhibited more severe clinical signs (Saville et al., 2001). This is likely because stress leads to immune suppression, which is commonly implicated in protozoan parasite infections (Furr and Reed, 2015). However, a second study placing horses under a second round of transportation-induced stress did not see any difference in the severity of clinical signs (Saville et al., 2004).

Non-transport-induced stress was tested in horses experimentally treated with immunosuppressant drugs. There was no difference in the CNS histopathology between immunosuppressed and non-immunosuppressed horses, although the treated horses were found to have more severe clinical signs (Cutler et al., 2001).

While the role of stress on clinical outcome of *S. neurona* infection cannot be ruled out, it is clear this is a complex relationship, further complicated by limited understanding of the equine immune system.

The outcome of most Sarcocystis infections is dependent on the dose, and is species dependent (Dubey et al., 2016). However, studies in the horse found that clinical outcome was not dose dependent, with disease arising from infection by as few as 100 sporocysts (Sofaly et al., 2002).

Neither nutritional status nor concurrent infection seems to be associated with clinical EPM (Dubey et al., 2015). It is reasonable to assume that any modulation of immune responses by other pathogens may play a role in how the host responds to *S. neurona* infection, but this warrants further research and is beyond the scope of this document.

Therefore, risk factors assumed to be governing the development of clinical EPM include: age, stress, and exposure to opossums and their feces (Morley et al., 2008). However, the only risk factor for EPM that can be claimed with any certainty is the last - living, or having lived in, the geographic range of the definitive host, making EPM a disease of the Americas. Therefore, horses in other countries should have no exposure to *S. neurona* (Dubey et al., 2015), and most horses that test positive for the parasite outside of the endemic Americas have spent time within those regions (Furr and Reed, 2015).

Testing for *S. neurona* has come back positive in a group of horses from France and Spain (Arias et al., 2012; Pitel et al., 2002), and this is likely due to cross-reactivity with another *Sarcocystis* species found to be endemic to Europe (Dubey et al., 2015).

Horse immunity is considered to play an important role in clinical outcome, although at present, little research has been conducted into the host immune response to *S. neurona* (Dubey et al., 2015). This will be further addressed later in this document.

2.7 Diagnosis of EPM

Antemortem diagnosis of EPM has proven challenging for numerous reasons. Actual pathogenesis of the disease is not clear, and *S. neurona* parasites have not been found in affected muscles (Dubey et al., 2015). Parasitemia is rare in infected horses, making it almost impossible to detect parasites microscopically in serum, in contrast to diseases as malaria, where blood stages of Plasmodium spp. are clearly visible.

Because seroprevalence to *S. neurona* is variably high across the Americas, detecting parasite-specific serum antibodies is of little diagnostic use, as it is clear that infection does not equal disease (Dubey et al., 2015; Furr et al., 2002). Although analysis of parasite-specific cerebrospinal fluid antibodies may have more diagnostic use, as high levels of CNS antibodies are often indicative of CNS infection and intrathecal antibody production, it is not usually diagnostically conclusive. In the healthy horse, antibodies transfer passively across the blood-brain barrier at low levels. If the blood-brain barrier has been compromised by some other type of infection, it is also possible that antibodies will transfer across in higher numbers. Blood contamination during CSF collection may also confuse diagnostic potential (Furr et al., 2002).

The early clinical signs of EPM can easily be confused with lameness issues, which add to the ambiguity, particularly as lameness issues are far more prevalent than neurological

disorders. To diagnose EPM with confidence, a full clinical veterinary evaluation must be conducted, including radiographs, serology, CSF collection, a lameness exam, serum chemistry profiling, and historical information. If EPM is suspected, a veterinarian must be consulted for the following three steps. 1) Conducting a full neurologic exam. The veterinarian will look for the three A's of atrophy, asymmetry, and ataxia (Furr and Reed, 2015). These are signs that are classical of neurologic disease, rather than a disease of the musculoskeletal system. 2) If there is cause to believe neurological damage has occurred, other potential causes for disease should be eliminated to help direct a full diagnostic evaluation. Other diseases to be ruled out include viral or bacterial neurological disease, particularly if an outbreak is known to have occurred in the area (Furr and Reed, 2015). Both serum and CSF should be evaluated. If all such testing is negative, the probability of EPM increases, which leads to step three. 3) immunodiagnostic testing to demonstrate the presence of antibodies against *S. neurona* in serum and CSF to assess intrathecal production (Furr and Reed, 2015). Neither serum nor CSF alone are good indicators of EPM, because horses can have a high antibody titer in serum, and therefore also in CSF due to passive transfer of lymphocytes (addressed in Chapter 3). Therefore, it is important to have both samples for accurate diagnostic testing. To obtain a CSF sample, a spinal tap must be performed by an experienced veterinarian. Although the steps are listed chronologically, they are usually carried out during the same veterinary consult.

2.8 Diagnostic Assays

More accurate diagnosis of EPM became possible with the development of the serological assay, the Western blot (immunoblot) assay. Since its development, several serological assays have been developed and are commercially available in laboratories across the Americas. The development of semi-quantitative assays allows for the detection of intrathecal antibody production against *S. neurona*, which can be compared to serum derived antibody production, for the accurate identification of EPM horses (Furr and Reed, 2015).

Western Blot (Immunoblot)

This assay was first developed for use in EPM diagnosis in 1993, and has therefore now been available for over two decades. Although it continues to be offered by several diagnostic testing laboratories, primarily a research tool that is fairly laborious and requires significant expertise to interpret accurately. It has lower sensitivity and specificity than subsequently developed diagnostic assays (Dubey et al., 2015).

Enzyme-Linked Immunosorbent Assays (ELISAs)

Sarcocystis neurona merozoites express a family of surface antigens (SnSAGs) that are abundant and immunogenic. SnSAGs can be produced in recombinant form for use in diagnostic tools, such as the ELISA.

SnSAGs elicit a strong humoral immune response in infected animals, and are therefore used as a target molecule in immunological serum assays for the detection of antibodies

against *S. neurona* (Dubey et al., 2016). Not all strains of *S. neurona* express the same surface antigen, with SnSAGs 1, 5, and 6 being mutually exclusive in strains occurring in nature. SnSAGs 2, 3, and 4, however, are located on the surface of the extracellular merozoite stage, and are present throughout the intracellular development of the schizont as well (Dubey et al., 2016).

Different strains of the *S. neurona* parasite display antigenic diversity, meaning that not all SnSAGs occur in all strains of *S. neurona* (Dubey et al., 2016). Using just one SnSAG, or a combination that will not cover the range of diversity, will be of little diagnostic value. SnSAG2, SnSAG3, and SnSAG4 are highly conserved proteins that exhibit little or no sequence polymorphism across parasite strains. SnSAG3 and SnSAG4 have been fused into one chimeric protein, facilitating diagnostic assays. This chimeric protein is used as part of a two-ELISA commercially available test (with the other ELISA testing for SnSAG2) for EPM (Dubey et al., 2015). More recently, the trivalent protein rSnSAG2/4/3 has been developed, showing ample specificity and sensitivity in detecting *S. neurona* antibodies (Yeargan et al., 2015). The diagnostic accuracy of this assay is 93% sensitivity and 83% specificity (Reed et al., 2013). By using either of these assays to test serum and CSF samples, and comparing the two, thus determining intrathecal antibody production (discussed later), EPM can be efficiently and effectively diagnosed.

Immunohistochemistry (IHC)

In tissues collected post-mortem, *S. neurona* schizonts and merozoites, the two parasite stages found in the horse, are stained with either polyclonal or monoclonal antibodies specifically against culture-derived *S. neurona* merozoites. Not all *S. neurona* organisms in EPM cases will stain by immunohistochemistry, and it must be remembered that parasites are often sparse or undetectable in the tissues of EPM horses (Dubey et al., 2015).

Immunofluorescent Antibody Test (IFAT)

The Indirect Fluorescent Antibody Test (IFAT) for EPM was optimized and validated at the University of California, Davis. This test requires a fluorescence microscope, which is specialized equipment requiring expertise in interpretation of parasite fluorescence. When conducted by individuals with proper training and expertise, results from this type of test are accurate. It is available for commercial use at the University of California Veterinary Diagnostic Laboratory (Dubey et al., 2015) and the University of Kentucky Diagnostic Laboratory. As with any other serological assay for EPM, confirmation of a serum antibody titer alone is of little diagnostic value. However, when used to test both serum and CSF antibody titers and compare the ratio to determine intrathecal antigen-specific antibody production, the IFAT can be a useful diagnostic tool.

The Goldman-Witmer coefficient (C-value)

The C-value and the antigen-specific antibody index (AI) are algorithms used to determine whether there is a greater concentration of pathogen-specific antibody in the

CSF than would normally be present through passive transfer of the blood-brain barrier of a healthy animal. These studies have been corroborated with the SnSAG ELISAs, and have mostly been abandoned in favor of the latter, which are inexpensive and less cumbersome to perform (Dubey et al., 2015).

2.9 Treatment of EPM

Because much of the damage to the equine CNS is due to the inflammatory response, treatment of horses suspected to have EPM must be done as quickly as possible. If administered promptly upon diagnosis, treatment results in successful recovery of 70-75% of horses (Dubey et al., 2001). The most common treatments are antiprotozoal drugs, of which there are three approved by the Food and Drug Administration on the market: ponazuril, diclazuril, and a sulfadiazine/pyrimethamine combination, under respective brand names. Bioavailability of the anti-protozoals ponazuril and diclazuril has been tested, and they have been found to cross the blood-brain barrier.

Ponazuril

The oral paste ponazuril (Marquis®, Bayer) was the first FDA-approved medication for EPM. Ponazuril is thought to act as a coccidiostat by inhibiting apicoplast and/or mitochondrial function in the *S. neurona* parasite (Mitchell et al., 2005). Toxicity studies have found ponazuril to be very safe, with no systemic toxicity, even at doses six times higher than recommended (Kennedy et al., 2001).

Diclazuril

Diclazuril (Protazil®, Merck Animal Health) is chemically similar to ponazuril, but instead of an oral paste, is administered through alfalfa-based pellets that are top dressed in the daily grain ration (Dubey et al., 2015). Diclazuril has also been shown to be an EPM preventative in a study conducted on foals fed medicated pellets daily from 1-12 months. Naturally exposed foals fed diclazuril had significantly reduced seroconversion to *S. neurona* than foals without medicated pellets (Pusterla et al., 2015).

Sulfadiazine/Pyrimethamine

The combination of sulfonamide and pyrimethamine (S/P) has been widely used over the past four decades, and has been known to treat to cerebral toxoplasmosis in humans. In humans, the drug can cross the blood-brain barrier, making it bioavailable. Although this has not been tested in the horse, it is proposed to have the same capabilities. A pre-mixed S/P combination is commercially available as the oral suspension ReBalance® (PRN Pharmacal). The cooperative anti-folate effects of sulfadiazine and pyrimethamine block nucleic acid and amino acid synthesis, ultimately leading to parasite death (Dubey et al., 2015). Although less expensive than the coccidiostats, this compound requires administration for a prolonged period, and has been known to have toxic side effects, including anemia, leukopenia, fetal loss, and fetal abnormalities (Dubey et al., 2015).

Immunostimulants

Immunostimulants and immunomodulators have been proposed as a disease treatment on the presumption that immunosuppression is a component of EPM pathophysiology. However, to date, there has been no conclusive evidence as to their success, and there are no such treatments FDA approved or commercially available for use in EPM horses.

There is currently no commercially available vaccine for EPM, and undoubtedly the best treatment for EPM is prevention. As previously mentioned, up to 31% of opossums harbor *S. neurona* sporocysts, and a single opossum can shed millions of sporocysts in their feces for months (Dubey et al., 2016). Sporocysts are resistant to most environmental influences, and special measures must be taken to kill them, such as heating at 60C for one minute, heating at 55°C for 15 mins, or using 100% ammonium hydroxide (Dubey et al., 2016; Dubey et al., 2015). Therefore, reducing opossum access to feed and water sources is the best method of prevention.

2.10 Relapse

Horses can relapse with clinical EPM, with clinical signs similar to previous episode/s, even after prolonged therapy. It is currently not clear why relapse occurs. In *Toxoplasma gondii* infections, the bradyzoite containing cysts harbor latent infection without generating disease. Immunosuppression can result in these bradyzoites converting back to the tachyzoite stage of the parasite, which is responsible for the tissue destruction resulting in clinical disease (Blader et al., 2015). However, sarcocysts do not form in equine tissue. It is unknown whether schizonts can remain dormant in equine tissues (Dubey et al., 2015).

2.11 *Sarcocystis neurona* and Marine Mammals

Although not the focus of this particular study, it should be mentioned that *S. neurona* is also known to infect marine mammals. As well as caused by *Toxoplasma gondii*, protozoal encephalitis due to *S. neurona* is an important cause or mortality in sea otters on the western coast of the United States, which is consistent with the distribution of opossums (Dubey et al., 2015). Most notably, a large epizootic occurred during a 3-week period in 2004, resulting in the death of approximately 2% of the federally listed threatened southern sea otter (*Enhydra lutris neresis*) population (Miller et al., 2010). *S. neurona* caused encephalitis has also been reported in pacific harbor seals (*Phoca vitulina richardsi*) and pacific harbor porpoises (*Phocoena phocoena*). As in the case of the horse, infection with the *S. neurona* parasite does not necessarily mean clinical disease. Infection, although not necessarily clinical signs, have been found in a wide variety of other marine mammals, including the California sea lion (*Zalophus califonianus*), Guadalupe fur seals (*Arcocepahlus townsendi*), Steller sea lions (*Eumatopias jubatus*), Northern fur seals (*Callorhinus ursinus*), Northern elephant seals (*Mirounga angustirostris*), a Pacific white sided dolphin (*Langenorthynchus obliquidens*), a killer whale (*Orcinus orca*), and a pygmy sperm whale (*Kogia breviceps*) (Barbosa et al., 2015).

2.12 *Neospora caninum* and *Neospora hughesi*

Neospora caninum and *Neospora hughesi* infections may also cause neurologic disease that is clinically indistinguishable from that caused by *S. neurona*. However, infections by *S. neurona* appear to be a much more frequent cause of EPM (Morley et al. 2008). When discussing EPM in the context of this document, the focus will be on *S. neurona* as the etiologic agent of disease.

Chapter 3: Immune System and Central Nervous System

Although it is now possible to confidently diagnose a horse suffering from EPM, this gives no indication as to why less than 1% of horses go on to develop clinical disease from infection and others can become infected yet remain clinically healthy.

Because it is not known why some horses are able to control *S. neurona* infections and other go on to develop clinical disease, and the immune system is implicated in this differential, it is valuable to examine in further detail the equine immune system and its various components.

This section will focus on the mammalian immune system in general, and form the basis of our understanding for subsequent chapters concerning the immune system in response to disease. It is not intended to be a comprehensive list of all components at play in the immune system as a whole, but merely to address components that could be of importance in the response to *Sarcocystis neurona* infection, based on our knowledge of immune responses to related parasites, and of the equine immune system.

3.1 Overview

The immune system is a collection of cells and their physiological interactions that defend the body from invasion or infection by foreign bodies (Immunisation Advisory Centre, 2012; Parham, 2015). The mammalian immune system is comprised of three parts main parts: physical barriers, innate immunity, and adaptive immunity.

Foreign invaders contain antigens on the cell surface, which are recognized by the innate immune system, serving as the first line of defense. The innate immune system mounts an immediate response that is short lived, non-specific, but capable of eliminating the majority of infections (Parham, 2015). More severe infections may overcome the innate immunity defense systems. In this case, phagocytic cells, such as the dendritic cells and macrophages, present pathogenic antigens on major histocompatibility complexes on their cell surface. These receptors are recognized by the adaptive immune system, which is able to mount a targeted, antigen specific immune response (Parham, 2015). The adaptive immune response is mainly comprised of B cells, which produce antibodies, and T cells, which can perform a number of effector functions depending on the type of pathogen. T cells are particularly effective at eliminating intracellular threats, such as viruses and intracellular bacteria and parasites. B and T cells are able to form memory cells, which "remember" a pathogen from primary exposure in order to mount a faster, stronger and more targeted response upon secondary exposure (Immunisation Advisory Centre, 2012). An effective immune response is usually comprised of a combination of both B and T cells.

3.2 The Central Nervous System and Cerebrospinal Fluid

The CNS is no different from other tissues in requiring protection from invading organisms. Although many of the cell types mediating immune responses are the same in

the CNS as in peripheral tissues, the unique nature of the CNS mandates careful modulation of these elicited responses (Furr and Reed, 2015; Streilein, 1993).

Inflammation is used in physiological systems to maintain tissue homeostasis and as a defense against pathogens. It is used to initiate death of damaged cells, removal of dead cells, to promote healing of damaged areas, and to produce cytokines and chemokines for the direction and coordination of immune responses (Carson et al., 2006). This becomes tricky in the central nervous system, which is encased in a solid outer layer, and therefore cannot tolerate the swelling caused by cellular recruitment to damaged areas. Additionally, removal of key neurons, even if damaged, can lead to the cognitive and/or motor deficits (Carson et al., 2006). While inflammation caused by an immune response is desirable in peripheral tissues, it can cause irreparable damage to the CNS. The CNS is therefore delegated an "immunoprivileged" site.

Immunoprivileged sites are sites in the body that could suffer detrimental consequences as a result of the immunogenic inflammatory response, and include the fetoplacental unit, the central nervous system, the ovary and testes, and the eye (Streilein, 1993). While initially thought to have resulted from passive processes, immune privilege has more recently been shown to be the result of active processes derived from immunoregulatory and immunosuppressive mechanisms that govern the type of immunity elicited and expressed at the site of invasion (Carson et al., 2006; Streilein, 1993). This immune regulation is believed to be a physiological adaptation that provides control of infection in a privileged site, without the potential deleterious consequences of immunogenic inflammation (Streilein, 1993).

The CNS is coated and protected by cerebrospinal fluid, and is separated both functionally and anatomically from the rest of the body by the blood-brain barrier. The blood-brain barrier is composed of brain microvascular endothelial cells, astrocytes, and pericytes. Tight junctions between endothelial cells are a distinctive feature of this barrier, which prevent many molecules from passing through. By controlling the passage of molecules, the barrier is able to control the microenvironment in the central nervous system, and protect it from pathogens and toxins found in the blood (Carson et al., 2006; Kim, 2008).

The microenvironment is also controlled by the immune privileged site itself, which is able to produce soluble factors, thought to be cytokines. When antigens are found in the central nervous system, for example, they leave this immune privileged site in conjunction with the cytokines, and the cytokines present determine the nature of the immune response. The anti-inflammatory cytokine, transforming growth factor beta (TGF-β) seems to be particularly important in this regard (Murphy, 2011).

In the healthy mammal, lymphocytes circulate through the central nervous system as part of normal surveillance and protection. These lymphocytes are derived entirely from serum, and occur in the CSF in direct proportion those in found in serum, although in far lower quantities (Furr, 2002). In diseases of the central nervous system, antibodies are

derived from intrathecal tissues, as well as from serum. This intrathecal antibody production is a key feature of CNS infection, and results in a higher concentration of antigen-specific antibodies found in the CSF than would usually occur through passive transfer across the BBB (Furr and Reed, 2015). Therefore, differentiating between antibodies produced by the serum, and those produced intrathecally can be crucial in diagnosing neurological disease. Because CSF composition is largely dependent on a fully functional BBB, it can be used effectively as a diagnostic tool for diseases of the central nervous system (Furr, 2002).

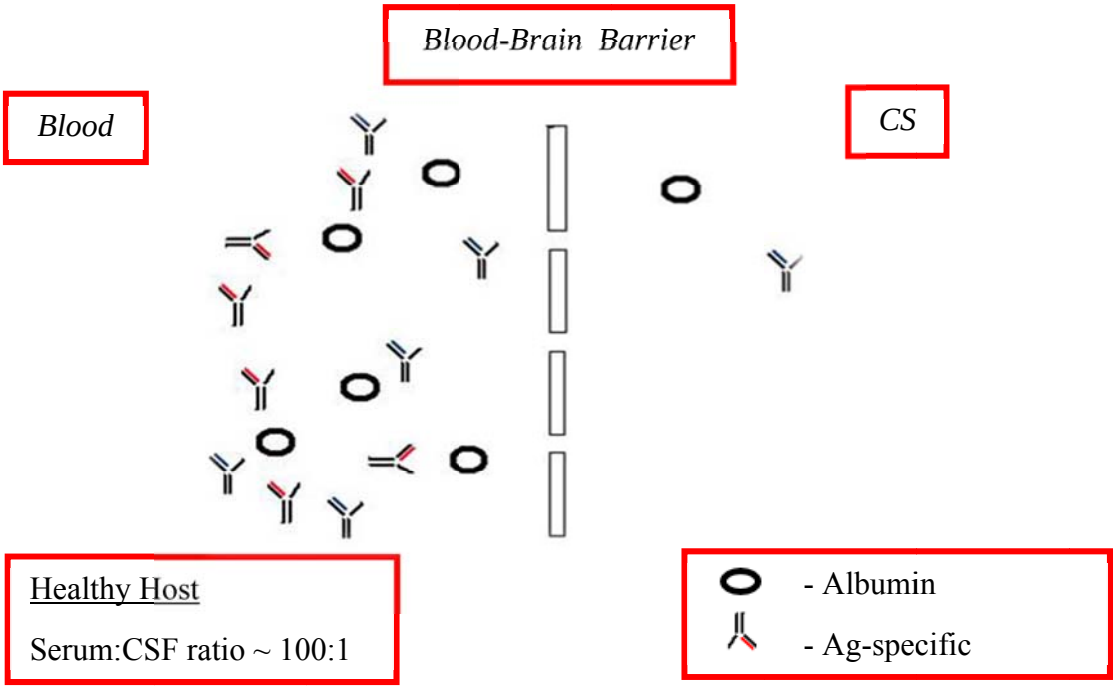

Figure 3.2.1 Schematic of proteins found in the serum and cerebrospinal fluid (CSF) of a healthy animal, showing normal passive transfer of proteins across the blood-brain barrier. Proteins in the CSF are proportional to those in serum, with a ratio of serum:CSF proteins of ~100:1.

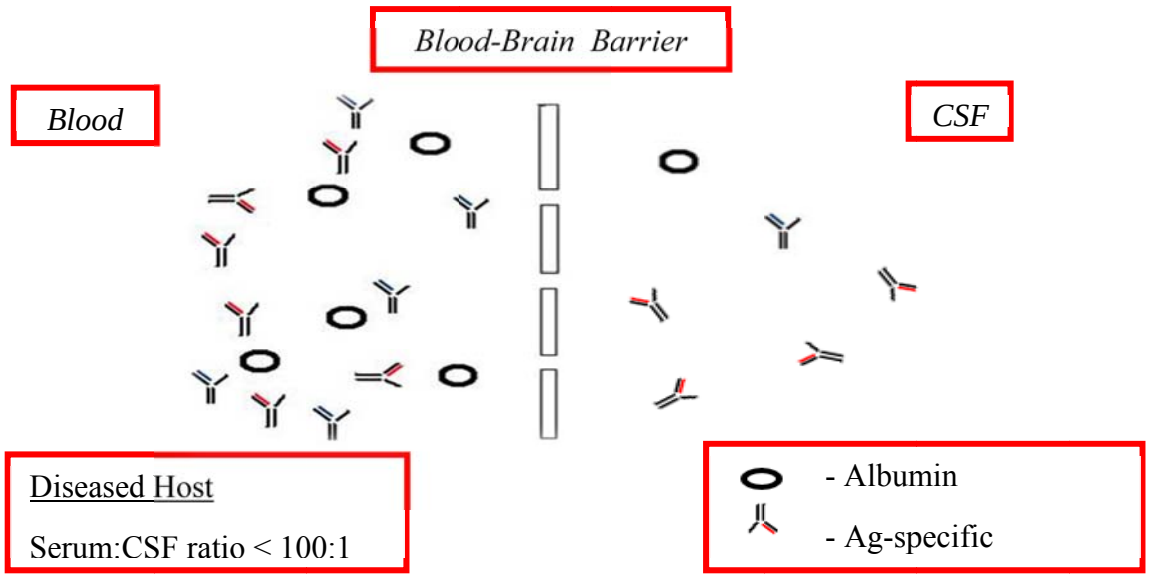

Figure 3.2.2 Schematic of proteins found in the serum and cerebrospinal fluid (CSF) of an animal showing infection of the central nervous system and intrathecal antibody production. Proteins in the CSF are disproportional to those in serum, with a ratio of serum:CSF proteins of < 100:1.

In healthy mammals, B and T lymphocytes occur in the CSF in very low numbers, with T lymphocytes occurring in a greater proportion in the CSF than in the blood, relative to B lymphocytes (Furr et al., 2001). The majority of B cells found in the CNS are memory B cells, which do not require lymphoid organs, but T cells, for differentiation into antibody secreting cells (Meinl et al., 2006). Once these B lymphocytes have had physical contact with their cognate antigen, they are retained in the CNS.

The key regulators of immunosuppression in the CNS are cytokines that suppress T cell mediated processes. When T cells respond to these particular cytokines, the result is either inactivation or apoptosis. While several cytokines have been observed in mammals to have immunosuppressive properties, only transforming growth factor-β (TGF-β) has been examined in the horse (Furr and Reed, 2015).

3.3 Components of the Adaptive Immune System

Antibodies circulating in the body bind to a foreign particle or pathogen, and either neutralize the pathogen, or mark it for action by other components of the immune system. The coating of foreign particles is known as opsonization, and signals phagocytic cells, such as macrophages and neutrophils, that a particle should be engulfed (Murphy, 2011). The binding of antibodies to a pathogen alone is not always enough to prevent infection.

Immune responses are often categorized into two broad spectrums – a type 1, or cell-mediated, response to intracellular pathogens, and a type 2, or humoral, response to extracellular pathogens. However, as research into the field of immunology continues to expand knowledge of the immune system, it is becoming evident that there are no clear

delineations between responses, and that more than two responses are at play in tackling pathogenic threats. It has also become clear that there is a complex pattern of cross-regulation throughout the development of CD4 T cell subsets, described below (Murphy, 2011).

Detailed below are key components of the immune response, with particular emphasis on those pertaining to the control and/or elimination of intracellular pathogens. It must be kept in mind that the plasticity of leukocytes is well documented (Muranski and Restifo, 2013), and cells may have more than one function, functional overlap, or may switch between functions. Similarly, the cytokines that are produced may vary depending on the stimulus signal to the expressing cells, and cytokines themselves may be pleiotropic. This list is not comprehensive.

T cells

Thymus-derived cells, or T cells, are lymphocytes in the adaptive immune response with different effector functions. T cells express the T cell receptor (TCR) on their cell surface, which is unable to bind antigen directly, and instead relies on presentation by antigen presenting cells (APC's), such as dendritic cells and macrophages. Effector differentiation depends on the location in the body (different locations will have different APC's), the nature of the pathogen, and the cytokine environment.

Major Histocompatibility Complex (MHC)

MHC molecules are expressed on the surface of cells, and responsible for the presentation of antigen for recognition by T cells. These molecules are highly polymorphic and vary between individual animals. There are two classes of MHC: Class I and Class II. MHC Class I molecules bind to short peptides, 8-10 amino acids in length, which come from intracellular pathogens. Class II molecules bind longer peptides, which are derived from extracellular pathogens that have been taken up by a phagocytic cell. The polymorphism of MHC molecules allows the immune system to respond to a huge diversity of pathogens (Murphy, 2011).

CD8 T cells

CD8 T cells, or cytotoxic T cells, express glycoprotein CD8 on the cell surface. These cells bind to MHC Class I molecules and induce cell death. Because of their destructive nature, CD8 T cell activation can result in tissue damage. Therefore, a strong signal is required for CD8 T cell activation to ensure that this response is not triggered unnecessarily. These cells are activated by dendritic cells or CD4 T cells. Like all T cells, proliferation is regulated by IL-2.

CD8 T cells are crucial in controlling intracellular pathogenic infections – not only do they destroy infected cells, but they secrete IFN-γ, which increases intracellular pathogen processing and MHC Class I expression (leading to a positive feedback look for increased CD8 T cell activation), and activates macrophages, which are important for the clearance of dead cells and debris.

CD4 T cells

CD4 T helper (T_H) cells are so-called due to the expression of the glycoprotein CD4 on the cell surface. These cells are a key component of the adaptive immune response, ensuring normal functioning of all parts of the immune system by modulating CD8 T cell and B cell responses, and through interaction with the innate arm of the immune system. Although some plasticity can occur between them, T_H cells are largely polarized to have specific effector functions, which are determined by the site of antigenic interaction, pathogen type, and the presence or absence of environmental signaling molecules (Muranski and Restifo, 2013). T_H effector cells are characterized by the expression of a master regulator, and the cytokines they produce (Murphy, 2011), and bind to antigen presented on MHC Class II molecules.

Early investigations into the field of immunology classified immune responses as predominantly either Type 1 or Type 2 (T_H1 or T_H2). However, upon the discovery of CD4 T cells expressing FoxP3, the addition of regulatory T cells was added to the list of polarized T cell responses. More recently, T_H17 cells have also been characterized (Muranski and Restifo, 2013). Other described T cells include T follicular helper, T_H9, and T_H22 cells, but these go beyond the scope of this paper, and will not be discussed.

Type I (T_H1) Immune Response

T_H1 cells are linked to the cell-mediated arm of adaptive immunity, and their response is induced by intracellular pathogens, such as viruses or intracellular bacteria and protozoa (Murphy, 2011). The main role of T_H1 cells is the activation of macrophages and CD8 T cells. However, they can also help stimulate B cells in the production of the opsonizing or neutralizing antibody subisotypes (Lopez et al., 2003; Murphy, 2011).

The defining transcription factor for T_H1 cells is T-box 21, or T-bet. Their differentiation is stimulated by IL-12 and IFN-γ, and they are characterized by the cytokines they produce, which are the proinflammatory cytokines IL-12 and IFN-γ, and T cell regulating cytokine IL-2.

T_H1 cells operate in part on a positive feedback cycle. They continue to respond to signals during an infection, and produce IL-12, a cytokine required for an effective T_H1 immune response (Muranski and Restifo, 2013; Murphy, 2011). Because of their proinflammatory nature, T_H1 cells are viewed as the main perpetrators of autoimmunity (Muranski and Restifo, 2013).

Type 2 (T_H2) Immune Response

T_H2 effector cells are linked to humoral immune responses, and are involved in the stimulation of B cells to produce antibodies (Muranski and Restifo, 2013; Murphy, 2011). The humoral arm of adaptive immunity is responsible for responding to extracellular pathogens and parasites, and is also implicated in the development of atopy and allergic reactions (Muranski and Restifo, 2013).

The defining transcription factor for T_H2 cells is GATA3, and they are stimulated to differentiate by the cytokine IL-4. T_H2 cells are characterized by the cytokines they produce, IL-4, IL-5, and IL-13, and to a lesser extent, IL-10 (Muranski and Restifo, 2013).

T_H2 cells are produced in balance with T_H1 cells. The inducing cytokines of T_H1 cells (IL-12 and IFN-γ), and the T_H1 transcription factor (T-bet) inhibit T_H2 differentiation. Conversely, IL-4 and transcription factor GATA3 stimulate T_H2 differentiation but inhibit T_H1 polarization (Muranski and Restifo, 2013).

T_H17 Effector Cells

The master regulator of T_H17 cells is retinoic acid–related orphan receptor γ thymus (RorγT), and they are stimulated to differentiate by transforming growth factor beta (TGF-β) and interleukin-6 (IL-6), and the absence of IL-4 and IL-5 (Muranski and Restifo, 2013). IL-6 is the key to T_H17 differentiation, as without it, regulatory T cells will be induced instead of T_H17 cells. T_H17 cells display site-dependent plasticity, and can work in conjunction with T_H1 cells to mount a functionally similar immune response. They are thought to augment T_H1 responses to some intracellular pathogens, such as Mycobacteria and *Francisella tularensis* (Muranski and Restifo, 2013). As with T_H1 cells, T_H17 cells have been implicated in the development of autoimmune disease (Muranski and Restifo, 2013). However, T_H17 cells have higher self-renewal capacity, less senescence, and less phenotypic stability (i.e. more plasticity) than T_H1 cells (Muranski and Restifo, 2013). T_H17 cells are characterized by production of the cytokine interleukin-17 (IL-17). IL-17 stimulates local tissues to produce an additional proinflammatory cytokine, interleukin-8 (IL-8). IL-17 and IL-8 can both often be found in a T_H17 mediated immune response. Cytokine IL-23 is not required for T_H17 polarization, but stabilizes and expands pathogenic T_H17 cells once a T_H17 response has been established (Murphy, 2011). Cytokines IFN-γ, IL-27, and IL-12 block differentiation of T_H17 cells (Muranski and Restifo, 2013).

In the absence of IL-6, naïve T cells will differentiate into regulatory T cells (Butler et al., 2013; Murphy, 2011).

Regulatory T cells (Tregs)

When a naïve T cell encounters its peptide:MHC ligand in the presence of TGF-β alone (i.e. in the absence of IL-6), it differentiates into a regulatory T cell (Murphy, 2011). Regulatory T cells are a type of effector CD4 T cell involved in downregulating immune reactions. They inhibit responses by other types of T cells, and assist in preventing autoimmunity. Treg cells are particularly important in maintaining immune tolerance and limiting potentially pathogenic immune reactions (Butler et al., 2013; Murphy, 2011). There are various subsets of Tregs, but the two most well-categorized are the natural regulatory T cells (nTregs), which are produced in the thymus, and activation-induced regulatory T cells (iTregs), which differentiate from naïve CD4 T cells in specific cytokine environments in the periphery (Butler et al., 2013; Murphy, 2011).

The master regulator of regulator T cells is forkhead box P3 **(**Foxp3), and their differentiation is stimulated by transforming growth factor beta **(**TGF-β). Tregs are characterized by the cytokines they produce, which are the anti-inflammatory IL-10 and TGF-β.

Tregs have a known role in modulating inflammation and limiting immunopathology, and are thought to act in maintaining a balance between immune protection and pathology. This could be critical in managing diseases affecting the central nervous system, such as toxoplasmosis, malaria (Butler et al., 2013), and, potentially, EPM.

Failure of natural Tregs has been implicated in autoimmunity (Murphy, 2011).

Table 3.3.1 Summary of four main effector T cell types, inducing cytokines, transcription factors, characteristic cytokines produced, and T cell function (Murphy, 2011).

Effector T Cell	**T_H1**	**T_H17**	**T_H2**	**Tregs**
Inducing cytokines	IL-12, IFN-γ	IL-6, TGF-β	IL-4	TGF-β
Transcription Factor	Tbet	RORγT	GATA3	FoxP3
Characteristic cytokines	IL-12, IFN-γ, IL-2	IL-17, IL-6	IL-4, IL-5	TGF-β, IL-10
Function	Activate macrophages	Enhance neutrophil response	Activates cellular and antibody response to parasites	T cell suppressor

B Cells

B cells are lymphocytes in the adaptive immune response, and are responsible for antibody production. B cells express the B cell receptor (BCR) on their cell surface, which binds its cognate antigen directly. B cells can also serve as APC's to T cells. They are activated by components of the innate immune system.

Antibodies

Antibodies are proteins produced by effector B cells (plasma cells) which bind to extracellular pathogens, marking them for neutralization, destruction, or opsonization. These Y-shaped proteins have two arms that are capable of binding antigen (Fab region) in a highly specific manner, and a region that binds to receptors on other cells (Fc region). Antibodies vary by isotype and subisotype, which governs their effector function. Mammals have five isotypes: IgA, IgD, IgE, IgG, and IgM. Antibody subisotypes vary between species.

B Regulatory Cells

B cells produce antigen-specific antibodies in response to infection. They can also negatively regulate immune responses with a subset of B cells called regulatory, or B10, cells. B10 cells are named after to their ability to express the regulatory cytokine IL-10 (Butler et al., 2013; Kalampokis et al., 2013; Yoshizaki et al., 2012). Through IL-10 production, B10 cells are able to function as critical regulators of T_H1-driven inflammation, T cell dependent autoimmune disease, and innate and antigen-specific adaptive immune responses (Butler et al., 2013; Yoshizaki et al., 2012). They are also shown to expand in number and modulate immunity to protozoan infections, such as human babesiosis (Kalampokis et al., 2013) and the known immunomodulatory parasite schistosomiasis (Tang et al., 2013). Although no research has been conducted into the expansion of B10 cells during Plasmodium or Toxoplasma infections, given the critical immunoregulation reported on T_H1 cells by IL-10 for these two Apicomplexan infections, a functional B10 cell role would be unsurprising (Butler et al., 2013). Additionally, given the similarity between *S. neurona* and *T. gondii*, it would not be improbable to also expect a functional role of B10 in *S. neurona* infections.

Interferon-γ (IFN-γ)

IFN-γ is a cytokine of the interferon structural family produced by effector CD4 T_H1 cells, CD8 T cells, and Natural Killer cells. It is the key cytokine for the activation of mononuclear cells (macrophages), increases expression of MHC molecules, and suppresses T_H2 responses (Murphy, 2011; Olias et al., 2013). IFN-γ has been found to be significant in host resistance to protozoan diseases, such as toxoplasmic encephalitis and cryptosporidiosis (Sellon et al., 2004). Overexpression of IFN-γ can lead to autoimmunity (Murphy, 2011).

When microbes are able to resist the effects of activated macrophages (activated by IFN-γ), or if there is insufficient IFN-γ, and therefore insufficient macrophage activation, chronic infection can with inflammation can develop in the form of granulomas, which are damaging to tissues (Murphy, 2011)

Interleukin-12 (IL-12)

IL-12 is most commonly produced by cells of the innate arm of the immune system, and is widely recognized as a critical component in influencing adaptive cell-mediated immunity by promoting the differentiation and maintenance of CD4 T cells into mature T_H1 effector cells (Denkers, 2003; Kastelein et al., 2007). IL-12 also activates natural killer and CD8 T cells to produce IFN-γ, which further stimulates the differentiation of T_H1 cells. Numerous studies have illustrated the importance of IL-12 in developing protection against intracellular pathogens, such as *T. gondii* (Denkers, 2003; Kastelein et al., 2007). As a component of a T_H1 polarized response, IL-12 has also been implicated in the development of inflammatory autoimmunity (Kastelein et al., 2007).

Transforming Growth Factor- β (TGF-β)

The primary role of TGF-β in the immune system is to suppress effector T cells and promote the differentiation and maintenance of Tregs. By supporting the subsistence of Tregs, TGF-β assists in mediating inflammation and maintaining immune tolerance through regulation of T_H1 cells and cytotoxic CD 8 cells. Because of this immunomodulatory role, it has been linked to regulating immunopathogenesis in both Plasmodium and Toxoplasma infections, and it would not be unreasonable to assume its role in *S. neurona* infections. The presence of TGF-β is correlated with increased IL-10 production, and the improved outcome of both severe malarial anemia and cerebral anemia, through the suppression of effector T cell activation (Butler et al., 2013; Fitzgerald et al., 2007).

As previously mentioned, when in the presence of IL-6, TGF-β can also trigger differentiation of the inflammatory T_H17 response, which has the opposite effect of suppressing effector T cells, which would happen in the absence of IL-6. TGF-β functions in a complex manner to both promote and suppress T cell activity and inflammation (Butler et al., 2013).

TGF-β is produced by CD4 T_H2 cells and regulatory cells in conjunction with IL-10, and inhibits macrophage activation (Murphy, 2011). In the horse, TGF-β blocks secretion of IFN-γ, and suppresses MHC class II expression (Furr and Reed, 2015). The T_H2 response is therefore critical in modulating the potential overexpression of a T_H1 response, the occurrence of which could result in tissue damage.

The importance of TGF-β has been demonstrated in controlling pathology due to overactive proinflammatory responses, such as in the case of cerebral malaria (CM). Development of CM is associated with T_H1 cells, cytotoxic CD8 cells, and cytokine storms involving various proinflammatory cytokines. TGF-β suppresses the effector cells triggering this immune response, and thereby reduces to severity of CM (Butler et al., 2013).

Conversely, lack of TGF-β can also cause pathology. In mice studies conducted on Toxoplasma infection of the CNS, it was found that neuronal cells directly respond to IL-6 driven inflammation by expressing regulatory cytokines TGF-β and IL-27 (Butler et al., 2013). When neuronal cells were blocked from detecting IL-6, they were no longer able to express either TGF-β of IL-27, and the mice died as a result of encephalitis associated with an uncontrolled T_H1 and T_H17 cell proliferation in the CNS (Butler et al., 2013). Under expression of TGF-β has also been linked to autoimmunity (Murphy, 2011).

Interleukin-10 (IL-10)

IL-10 is secreted by regulatory T cells, and acts on effector T cells to inhibit proliferation. It also acts on dendritic cells to inhibit secretion of IL-12, which in turn inhibits T cell

activation. IL-10 exhibits important immune regulatory functions and suppresses T cell-mediated inflammation (Fitzgerald et al., 2007; Murphy, 2011).

Interleukin-6 (IL-6)

Interleukin-6 is a proinflammatory cytokine that, in conjunction with TGF-β, stimulates the differentiation of naïve T cells into T_H17 cells. IL-6 is generated by cells of the innate immune system, and is regulated by the presence or absence of pathogens. In the absence of pathogens, production of IL-6 is low, which favors the differentiation of immunosuppressive regulatory T cells, thereby preventing unwanted or overactive immune responses (Murphy, 2011).

Interleukin-17 (IL-17)

IL-17 is a proinflammatory cytokine, most commonly secreted by T_H17 cells, but can also be secreted by other cells, including neutrophils. Its effects are mediated though the IL-17 receptor, which is expressed on a wide number of cells in the immune system (both lymphocytes and neutrophils), as well as other tissue types. IL-17 is a pleiotropic cytokine, linking inflammatory effects of the adaptive and innate immune system (Muranski and Restifo, 2013).

Interleukin-27 (IL-27)

The main sources of IL-27 are thought to be the antigen presenting dendritic cells and macrophages, acting during the initial stage of T cell activation (Kimura 2016). Although initially considered a proinflammatory cytokine indicative of a T_H1 response, IL-27 has subsequently been shown to have a predominantly regulatory role in limiting immunopathology. This is particularly notable in the parasitic diseases caused by *T. gondii*, *T. cruzi*, and Plasmodium infections, where IL-27 has a profoundly suppressive effect on IL-2 production, thereby inhibiting CD4 cell proliferation. IL-27 suppresses the development of the inflammatory T_H17 cells, and stimulates the production of regulatory cytokine IL-10 (Fitzgerald et al., 2007; Kimura et al., 2016). In mice lacking a functional IL-27 receptor, excessive inflammation occurs during infection and autoimmunity.

3.4 Polarization of Immune Responses

It is clear that there are many factors that play into an immune response to infection. The age old adage of "too much of a good thing is a bad thing" is certainly clear in immunology, and it is the balance between all such components that is critical to remember.

The correct functioning of the immune response is a product of millions of years of evolution in the face of infections by a diverse array of pathogens (Dent, 2002). The polarization of the immune response that is mounted is largely determined by the cytokine environment at the time of introduction of a pathogen. Cytokines creating this environment may originate from infected or activated somatic cells, or from cells of the immune system that have been recruited to the site of infection. Antigen presenting cells,

such as the so-called "professional" antigen presenting dendritic cells, present antigen to T cells and deliver the necessary cytokines and co-stimulatory molecules necessary for T cell activation (Dent, 2002). Dendritic cells are highly diverse, and can be of either myeloid or lymphoid origin, with each sub-type producing differing cytokine profiles, and thereby directing immune response polarization (Dent, 2002).

In addition to the cytokine environment, the immune response may be stimulated in either direction by the invading pathogen itself – for example, the lipopolysaccharide-containing cell wall of gram-negative bacteria directs a T_H1 immune response. The pathogen may impose an immune bias to promote its own survival and replication in the host (Dent, 2002). Therefore, co-infections by other pathogens can also affect the cytokine environment.

3.5 Imbalance of Immune Responses

There is no single component of the immune system that can in itself resolve pathogenic invasion. Rather it is the balance of the various components that lead to either successful eradication or tolerance. This section will focus on what happens when the balance gets disrupted, drawing from known examples in the literature.

The primary goal of the immune response is to efficiently and swiftly eradicate the pathogen without damaging the host. For intracellular microbial infections, such as *T. gondii* and *S. neurona*, the default response in immunocompetent hosts is type I immunity (T_H1 polarized response). In a healthy host, the T_H1 response will swiftly clear the pathogen, and naturally switch from the production of type I to type II cytokines over time, in order to prevent an overly polarized immune response and re-establish homeostasis (Spellberg and Edwards, 2001).

If the type I response is unable to fully clear the intracellular infection, continued antigenic stimulation may occur, leading to chronic type I responses and immunopathological consequences (most often tissue destruction) (Oldenhove et al., 2009; Romagnani, 1996; Spellberg and Edwards, 2001). To prevent this from occurring, the immune system has regulatory components, such as regulatory T cells. However, in some cases, such as pathogen-driven diseases and autoimmune disorders, T_H1 cells are able to avoid immune regulation (Oldenhove et al., 2009).

Under-regulation of Th1 and Th17 responses

T_H1 cells and T_H17 cells both produce an inflammatory response to pathogens (Kastelein et al., 2007). As mentioned, IL-12 stimulates the differentiation of T_H1 cells, which in turn produce the pro-inflammatory cytokine, IFN-γ. This pathway is predominantly cytotoxic, and is important for the direct killing of microbes or infected cells (Kastelein et al., 2007). IL-23 stimulates the differentiation of T_H17 cells, which in turn produce the pro-inflammatory cytokine IL-17. IL-17 stimulates local tissues to produce IL-8, another proinflammatory cytokine, and induces the key signs of infection – swelling, heat, and

pain, thus setting up an environment conducive to heightened immune responses (Kastelein et al., 2007).

The innate immune system can provoke an immunopathological response without the aid of the adaptive immune response. IL-12 and IL-23 are key modulators of this innate immunity, with IL-23 directing local inflammation, and IL-12 directing systemic responses. In immune privileged organs, such as the central nervous system, inflammation requires infiltration of T_H17 cells. The initial inflammatory response is likely mediated by T_H17 cells, and once this initial danger signal has been processed, the effector arm of the adaptive immune system is stimulated to promote and influx of activated T_H1 and CD8 T cells (Kastelein et al., 2007).

Dysregulated production of pro-inflammatory cytokine IL-17 can result in pathological chronic immune-mediated tissue destruction (Kastelein et al., 2007). Because the consequence of this IL-23/IL-17 pathway can be immunopathology and/or autoimmune inflammation, the evolutionary pressures to conserve such a pathway must be strong. In the healthy body, IL-17 is expressed at low levels and likely contributes to the overall health of the host and resistance to damage from invading pathogens. After an initial heightened inflammatory response by T_H17 cells in response to danger, when CD4 and CD8 T cells are recruited to the area, a T_H1 or a T_H2 response will likely become the more prominent pathway. This dominance is thought to play a role in ensuring control of the inflammatory response so that it does not lead to the breakdown of tissue-specific immune tolerance responsible for autoimmune pathology (Kastelein et al., 2007).

Leprosy

The development of clinical disease from *Mycobacterium leprae* infection is a result of a T_H1/T_H2 imbalance (Murphy, 2011). Patients who mount a T_H1 immune response to infection will control infection and develop the much milder, self-limiting form of disease, tuberculoid leprosy. However, if a patient mounts a T_H2 response to infection, the body will not be able to clear infection, and the result will be the much more severe lepromatous leprosy. This is a systemic infection resulting in gross lesions all over the body. In patients with lepromatous leprosy, CD8 T cells also play a role in suppressing T_H1 responses, and producing the cytokines with suppressive capabilities, IL-10 and TGF-β (Murphy, 2011)

Leishmania

The protozoan parasites *Leishmania* are also known regulators if the IFN-γ response. They inhibit the upregulation of IFN-γ, and the production of IL-12 by macrophages, which would be pivotal in mounting a successful Th1 immune response (Sibley, 2011).

In mice experimentally infected with the protozoan parasite Leishmania, mice that mounted a T_H1 response to the parasite were immune to disease, whereas mice that mounted a T_H2 response could not be protected from infection (Spellberg and Edwards, 2001).

Malaria

As described earlier, IL-27 has a profoundly suppressive effect on the production IL-2, which inhibits a T cell response. IL-27 also suppresses development of inflammatory Th17 cells, and stimulates the production of regulatory IL-10, making it a critical suppressor of immune responses. As with all pathogenic infections, there must be a balance between pathogen eradication and host damage. Malaria, one of the most important infectious diseases in the world, is caused by the species of protozoan parasite, Plasmodium. Malaria has been the focus of much research, not least when it comes to the immune system. Protection against malaria infection is largely regulated by CD4 T cells. When in the blood-stage form of infection, Plasmodium parasites can exacerbate the immune response, resulting in severe pathology. Malaria infection can have an immunosuppressive effect, which, while blocking the severe immunopathology just mentioned, can also block the protective immune response to the parasite itself, and immune responses by other pathogenic agents and vaccines (Kimura et al., 2016). In mouse models of malaria infection, a subpopulation of Plasmodium-specific CD4 T cells was discovered that produced IL-27 in response to receptor stimulation. By producing IL-27, IL-2 production was inhibited, and T cell responses were diminished. This indicates IL-27 as an immune modulator of responses to Plasmodium infection (Kimura et al., 2016).

It would not be unreasonable to propose similar T cell suppression in the immune response to *S. neurona*.

Toxoplasma gondii

Toxoplasma gondii is a widespread human pathogen, and has been widely studied. Resistance to *T. gondii* involves both innate immunity and the development of T cell responses required to control parasite survival, replication, and persistence (Butler et al., 2013). The immune response to *T. gondii*, as with most intracellular pathogens, revolves around the production of IL-12, which is the main physiological inducer of IFN-γ and the differentiation of T_H1 cells (Sacks and Sher, 2002). A T_H1 response is necessary to keep parasite populations in check, and *T. gondii* parasites have evolved mechanisms for controlling IL-12 production. The parasites are not only able to suppress IL-12 expression directly, but can induce expression of the down-regulatory cytokines IL-10 and TGF-β, and by modulating pro-and anti-inflammatory cytokines can ensure survival (Denkers, 2003; Sacks and Sher, 2002). However, unregulated IL-12, and thereby IFN-γ, responses from the host can also cause immunopathogenesis to the host, and must be controlled. Induction of cytokines IL-10 and IFN-γ as part of the same immune response is common with infection by intracellular parasites (Gazzinelli et al., 1996). IL-10 production is found to be critical to this regulation. Research conducted in mice has shown that IL-10 deficient mice may have lower parasite burdens, due to elevated IL-12 levels, but die from cytokine-associated tissue damage (Butler et al., 2013; Denkers, 2003; Gazzinelli et al., 1996; Sacks and Sher, 2002). When T cells are appropriately activated, they will express inhibitory receptors on their cell surface, which keeps the

immune response in balance and is critical for controlling immunopathogenesis (Butler et al., 2013). Sustained high levels of IFN-γ, and other proinflammatory cytokines are responsible for fever and can initiate cell death and/or damage healthy tissue (Butler et al., 2013).

Research conducted in *Sarcocystis Calchasi*

S. calchasi heteroxenous parasite not unlike *S. neurona*, and cycles between *Accipiter* hawks and domestic pigeons (*Columba livia* f. *domestica*). As with *S. neurona*, *S. calchasi* reproduces sexually in the definitive host (accipiter hawk), and is shed in sporocyst form, where it is taken up by the domestic pigeon. When infecting domestic pigeons, *S. calchasi* causes the emerging central-nervous disease pigeon protozoal encephalitis (PPE). PPE causes (often fatal) brain lesions and skeletal muscle sarcocysts (Olias et al., 2013).

PPE is biphasic disease in the domestic pigeon, with the first phase resulting in diarrhea and apathy, and the second phase causing severe neurological signs and cerebral lesions, akin to *S. neurona*. In research conducted on the immune response to *S. calchasi*, it was found that infectious dose of *S. calchasi* sporocysts was not correlated to the strength and composition of the host immune response. Additionally, it was found that in the first phase of the disease, the important T_H1 cytokines IL-12, IL-18, IFN-γ, and IL-15 were significantly down-modulated. This absence of strong cellular immune response in the first stage, and ability to modulate the IL-12/IL-18/IFN-γ pathway, may be a sign of parasite evasion, as is seen with other protozoan parasites, such as *T. gondii* (Olias et al., 2013; Sacks and Sher, 2002). Also, as with *T. gondii*, this temporary immune suppression seen in the first phase of the disease, may allow the parasite to invade cells undetected, and replicate (Olias et al., 2013).

However, in the neurological second phase of PPE, IFN-γ was significantly up-modulated. As previously mentioned, IFN-γ is a key cytokine in the activation of mononuclear cells, such as lymphocytes and monocytes (Olias et al., 2013). This corresponds well with the prominence of T cells detected in the pigeon's immune system, MHC-II signaling in the pigeon's brain, and the granulomatous nature of the lesions. The cerebral lesions are a result of the host's immune response, triggered by cytokines and parasite antigen. Indeed, despite the low parasite load in infected pigeons, there appears to be a strong T_H1-biased T cell driven immune response, suggesting that a T cell mediated delayed-type hypersensitivity reaction may be what causes the cerebral lesions and subsequent neurological signs (Olias et al., 2013).

It is reasonable to assume *S. neurona* is capable of acting in a similar manner to *S. calcashi*, and that it is in fact the host's overly polarized T_H1 immune response to parasite infection that causes lesions of the central nervous system.

3.6 Type IV Hypersensitivity (Delayed Hypersensitivity) and Autoimmunity

Type IV hypersensitivity reactions, or delayed type hypersensitivity reactions, are mediated by antigen-specific effector T cells. When T_H1 cells recognize their cognate antigen on MHC-II antigen presenting cells, they release cytokines, including IFN-γ, which further activates T cells, and TNF-α, causing local tissue destruction (Murphy, 2011). In a healthy animal, this is a desirable response to eliminate intracellular pathogens or damaged cells. However, in the case of delayed hypersensitivity, the immune response is undesirable, and causes excessive inflammation through the activation of macrophages, which release inflammatory cytokines also capable of causing inflammatory lesions (Murphy, 2011).

Genetic differences in MHC II complexes are known to play a role in autoimmunity (Murphy, 2011). Research conducted in the intracellular parasite *Sarcocystis cruzi* has shown a correlation between increased MHC II expression on vascular endothelial cells and destructive immune responses, such as delayed hypersensitivity reactions (Dubey et al., 2016). It is thought that endothelial cells assist in eliciting an overactive immune response by processing and presenting antigens on MHC II complexes, resulting in the sensitization of T cells (Dubey et al., 2016).

Autoimmunity due to IL-23/IL-17 pathway

IL-17 is implicated in several autoimmune diseases. In psoriasis, production of IL-17 by resident CD4 T cells encourages the recruitment of other inflammatory cells, such as neutrophils, which results in psoriatic lesions (Kastelein et al., 2007; Muranski and Restifo, 2013).

In the disease, Experimental Autoimmune Encephalomyelitis, the mouse model of human Multiple Sclerosis, T_H17 cells revealed their capacity to induce disease when stimulated by IL-23 rather than IL-17. Other human autoimmune diseases were also susceptible to IL23-induction of T_H17 cells, including inflammatory bowel disease, rheumatoid arthritis, uveitis, and allergies (Muranski and Restifo, 2013). Tissue damage caused by T_H17 cells can either be the result of direct recognition of an antigen-specific target, and/or the recruitment of other inflammatory immune components, such as neutrophils and macrophages (Muranski and Restifo, 2013). It would appear that when stimulated by IL-17, T_H17 cells develop into their characteristic phenotype, yet when stimulated by IL-23, pathogenic T_H17 cells usually evolve, displaying T_H1-like inflammatory characteristics (Muranski and Restifo, 2013).

The effects of environmental factors, such as toxins, drugs, and co-infection, cannot be overlooked in the development of autoimmune disease, although at present, these are poorly understood (Murphy, 2011).

3.7 Research in SCID animal models

Animals with severe combined immunodeficiency syndrome (SCID) make useful research tools when studying the immune system. While SCID mice are the traditional

research tool, Arabian SCID foals can be used in studying equine diseases. Arabian horses with SCID lack T and B cells due to a DNA mutation, and have been used experimentally in determining the role of the adaptive immune response in disease development of equine infectious anemia and equine piroplasmosis (Sellon et al., 2004).

Studies conducted in SCID mice infected with *Sarcocystis neurona* found SCID mice to be resistant to encephalitis, while immunocompetent mice were susceptible (Marsh et al., 1997).

SCID foals were successfully infected with the *S. neurona* parasite, and subsequently, parasites were found in the host blood and tissue. However, whether the mode of infection was oral or intravenous, *S. neurona* was undetectable in tissues of the central nervous tissue of these foals, and none of them developed clinical signs of EPM (Dubey et al., 2015; Sellon et al., 2004). This is in contrast to immunocompetent animals, which are able to successfully control parasitemia and clear infection from visceral tissues, but in whom parasites are often found in the central nervous system, and clinical signs of neurological disease are common (Sellon et al., 2004).

This research in both SCID mice and horses demonstrates the role of the adaptive immune system in the development of *S. neurona* infection and disease. The adaptive immune response is important in controlling parasite replication, as shown by the immunocompetent animals' ability to limit parasitemia and visceral tissue invasion, but may not be as important in controlling neuroinvasion, as shown by the presence of parasites in the CNS of immunocompetent animals but not SCID animals (Dubey et al., 2015; Sellon et al., 2004).

While the adaptive immune response may not be responsible for controlling neuroinvasion, should neuroinvasion occur, the adaptive immune response could well be implicated in the development of CNS lesions and clinical disease.

3.8 The equine immune system

Immunoglobulin isotypes are defined by amino acid differences in the constant region of the heavy chain. Different isotypes, and subisotypes, have different biological functions, including complement fixation and the binding of phagocytic cells (Sheoran et al., 2000).

The isotype immunoglobulin G (IgG) is the predominant antibody class in equine serum, and is a critical component of immune response to both intracellular equine pathogens, such as equine herpesvirus, *Rhodococcus equi*, and *Theileria equi,* and extracellular pathogens, such as nematodes (Keggan et al., 2013). In the horse, IgG has four subisotypes – IgGa, IgGb, IgGc, and IgG(T).

In adult horse serum, IgGb is the predominant isotype. Consisting of over 60% of total isotype concentration, it is significantly higher than all other subisotypes. Subisotypes IgGa and IgG(T) occurred at significantly higher concentrations to IgGc, which occurs at the less than 1% of total isotype concentration, as demonstrated in Figure 3.9.1 (Sheoran et al., 2000).

Table 3.8.1 Original and updated classification of equine IgG subisotypes (Wagner, 2006).

Original Subisotype Classification	Updated Subisotype Classification
IgGa	IgG1
IgGb	IgG4, IgG7
IgGc	IgG2
IgG(T)	IgG3, IgG5

IgGa and IgGb have been demonstrated to be the most important equine antibody subisotypes in eliciting a protective response against intracellular pathogens, and are therefore indicative of a type I, cell-mediated immune response. Isotypes IgGc and IgG(T) are thought to be indicative of a type II, humoral immune response (Keggan et al., 2013; Lewis et al., 2008).

IgGc is detected in very low serum concentrations of the clinically normal horse, and in horses infected with *Streptococcus equi* and equine influenza virus, and is not able to bind complement. It is, therefore, considered to be the least important equine IgG subclass in protective humoral immune responses (Sheoran et al., 2000).

As a consequence of equine genome sequencing, equine subisotypes have been reclassified according to their position on the chromosome. Research has shown seven genes for IgG constant heavy chains, which correspond to the original nomenclature as described in Figure 3.2.2.

Table 3.8.2 Data showing equine serum IgG and subisotype concentrations (Sheoran et al., 2000).

Antibody	Quantity (mg/ml)
IgG	27.2
IgGa	3.4
IgGb	19.6
IgGc	0.2
IgG(T)	4.0

Due to the limitation of materials available for use in equine research, monoclonal antibodies were used in this research with the original designations of IgGa, b, and (T), and such nomenclature shall be used throughout this document.

3.9 Antibody subisotypes as proxies for T_H1 vs T_H2 Immune Responses

Immunoglobulin subisotype profiles are useful as immunological markers in indicating the overall type of immune response (T_H1 or T_H2) being mounted by the host animal. This is evaluated particularly noting by the differences between levels of IgGa, IgGb, and

IgG(T). In horses infected with the Apicomplexan *Babesia equi* (*Theileria equi*), successful eradication of parasitemia occurred with a prevalence of subisotypes IgGa and IgGb (Cunha et al., 2006). This was indicative of a T_H1 response, dependent on the production of interferon gamma (IFN-γ) by T cells (Cunha et al., 2006).

In horses infected with equine influenza virus, protective immunity generated by natural infection is associated with IgGa and IgGb subisotype responses. When an IgG(T) response is elicited, as by vaccination, clinical signs and viral shedding occur (Nelson et al., 1998). This demonstrates well the important of having the correct subisotype response to infection.

Knowledge of the protective immune response required to eliminate or control pathogenic infection provides a basis for rational vaccine design, where the end goal is for optimal stimulation of functionally effective antibodies (Sheoran et al., 2000). By measuring which antibody subisotype classes are present in their respective concentrations, an idea of which immune response is being mounted can be formulated.

Chapter 4: Analysis of humoral immune responses in horses with equine protozoal myeloencephalitis.

4.1 Introduction

Equine protozoal myeloencephalitis (EPM) is one of the most important neurological diseases of horses in the Americas (Dubey et al., 2015), and is most commonly caused by the protozoan parasite *Sarcocystis neurona*. In general, Sarcocystis species convey low pathogenicity, and infections are well tolerated (Dubey et al., 2016). While the seroprevalence of *S. neurona* in horses is high, EPM occurs in less than 1% of the infected horses (NAHMS, 2001). Clinical signs of EPM can vary, and antemortem diagnosis has proven challenging. Although EPM is usually a progressively debilitating disease with a gradual onset, this varies widely from horse to horse. The disease may stabilize in some horses, remaining static for periods of time, but can have a rapid onset with focal or multifocal signs of neurologic disease in others (Dubey et al., 2015). Early clinical signs of disease can include stumbling, and are often confused with hind end lameness (Dubey et al., 2015). The presence of serum antibodies against *S. neurona* is an indicator of infection but not necessarily disease. It is, therefore, apparent that neurologic disease is not the outcome of every *S. neurona* infection. Upon necropsy, parasites can be numerous and easily found in the central nervous system (CNS) in some EPM cases. However, other cases may present with severe lesions despite parasites that are sparse or non-detectable. This implies that pathogenesis is, at least in part, immune-based (Dubey et al., 2015; Furr and Reed, 2015).

The correct functioning of the immune response is a product of millions of years of evolution in the face of infections by a diverse array of pathogens (Dent, 2002). There is no single component of the immune system that can alone resolve pathogenic invasion – rather it is the balance of the various components that lead to either successful eradication or tolerance. The primary goal of the immune response is to efficiently and swiftly eradicate the pathogen without damaging the host. Polarization of an immune response means that cells become specialized to perform specific functions in response to the particular type of pathogen encountered. For intracellular microbial infections, such as *Toxoplasma gondii* and *S. neurona*, the default response in immunocompetent hosts is type I immunity (T_H1 polarized response). In a healthy host, the T_H1 response will swiftly clear the pathogen, and naturally switch from the production of type I to type II cytokines over time in order to prevent an overly polarized immune response and re-establish homeostasis (Spellberg and Edwards, 2001). If the T_HI response is unable to fully clear the intracellular infection, continued antigenic stimulation may occur, leading to chronic T_H1 responses and immunopathological consequences (most often tissue destruction) (Oldenhove et al., 2009; Romagnani, 1996; Spellberg and Edwards, 2001).

Aberrant immune responses to pathogenic organisms are well documented. The development of clinical disease from *Mycobacterium leprae* infection is a result of a T_H1/T_H2 imbalance (Murphy, 2011). If a T_H1 immune response is mounted, infection will be controlled and the patient will develop the much milder, self-limiting form of disease,

tuberculoid leprosy. However, a T_H2 response will not clear infection, resulting in the much more severe lepromatous leprosy. Similarly, in mice experimentally infected with the protozoan parasite *Leishmania*, a T_H1 response provides immunity to disease, whereas a T_H2 response does not protect the mice from infection (Spellberg and Edwards, 2001). In horses infected with equine influenza virus, protective immunity generated by natural infection is associated with IgGa and IgGb subisotype responses. When an IgG(T) response is elicited, as by vaccination, clinical signs and viral shedding occur (Nelson et al., 1998).

The variety and amplitude of antibodies produced during an infection are largely influenced by this T_H1 or T_H2 bias, and bias in the "wrong" direction could lead to disease. As such, an in-depth examination of antibody responses may reveal aberrations in the immune status of horses that are actively infected with *S. neurona* and afflicted with neurological disease. The isotype immunoglobulin G (IgG) is the predominant antibody class in equine serum, and is a critical component of immune response to both intracellular and extracellular equine pathogens (Keggan et al., 2013). In the horse, four IgG subisotypes have been described – IgGa, IgGb, IgGc, and IgG(T). IgGa and IgGb have been demonstrated to be the equine antibody subisotypes associated with the protective response against intracellular pathogens, and are therefore indicative of a type I, cell-mediated immune response. Isotypes IgGc and IgG(T) are thought to be indicative of a type II, humoral immune response (Keggan et al., 2013; Lewis et al., 2008).

As mentioned above, infection of horses by *S. neurona* is fairly common, but the annual incidence of EPM is quite low (less than 1%). We hypothesize that EPM occurs due to an aberrant immune response, which will be discernable in the IgG subisotypes that recognize *S. neurona* in EPM positive horses versus infected but clinically healthy horses.

4.2 Materials and Methods

Serum and CSF

Serum was collected from 116 clinically healthy horses on the University of Kentucky Research Farm. CSF from 30 EPM-negative (infected normal) horses was provided by the Equine Diagnostic Solutions (EDS). Ninety-three serum samples and 91 CSF samples from horses diagnosed with clinical EPM (infected diseased) were also provided by EDS. The cut-off for diseased horses was determined at a CSF:serum antibody ratio of 1:25 (Reed et al., 2013). To ensure a positive serum antibody titer for infected normal horses, the cut-off for a percent positivity was determined to be 10%, as previously demonstrated (Yeargan et al., 2015).

Enzyme-Linked Immunosorbent Assays (ELISAs)

The *S. neurona* trivalent recombinant protein rSnSAG2/4/3 was produced and used as antigen in the ELISAs. The *S. neurona* positive control serum was from a clinically affected horse that had EPM confirmed by postmortem examination. The negative control

serum was from a pre-infection foal used in a prior infection experiment. All samples were tested in duplicate wells.

Unknown Samples

Corning high-binding, 96-well plates were coated with antigen and incubated overnight at 4°C. Antigen was diluted at 1:800 with PBS, as per Bradford Assay results to determine the correct concentration for antigen use.

After overnight incubation, the antigen was removed from the wells and plates were rinsed three times with PBS–0.05% Tween 20 (PBST). Wells were blocked by incubating for 1 hour at room temperature (RT) with 200 µl PBS containing 1.0% Tween 20, 5% NGS, and 0.01 g/ml nonfat dry milk. After one wash, 50 µl of primary sera or CSF were added to the wells containing antigen. Sera and CSF were diluted with antibody diluent (1:10 mix of block solution with PBST) in a two-fold dilution series described in Table 4.2.1.

Table 4.2.1 Serum and CSF dilutions used against rSnSAG2/4/3 antigen to test antibody concentrations in infected normal and infected diseased horses.

Subisotype	**Serum Dilution**	**CSF Dilution**	**Subisotype**	**Serum Dilution**	**CSF Dilution**
Total IgG	1:400	1:12.5	**IgGb**	1:25	1:6.25
	1:800	1:25		1:50	1:2512.5
	1:1,600	1:50		1:100	1:25
IgGa	1:100	1:6.25	**IgG(T)**	1:12.5	1:6.25
	1:200	1:12.5		1:25	1:12.5
	1:400	1:25		1:50	1:25

These dilutions were previously determined empirically to most often show an optical density (OD) within the typical working range, between 0.5 and 2.5 OD. The plate was incubated for 1 h at 37 °C. After five washes with PBST, 75 µl of monoclonal antibodies IgGa, IgGb, and IgG(T) (Lunn et al., 1995) diluted with antibody diluent were added to the appropriate wells at dilutions described in Table 4.2.3.

Table 4.2.2 Monoclonal antibody dilutions for IgG subisotypes.

Subisotype	**Concentration**
IgGa	1:16
IgGb	1:128
IgG(T)	1:16

The dilutions of these monoclonal antibodies were previously determined empirically to give data within the best working range of OD.

The plate was incubated for 1 h at 37 °C. After five washes with PBST, 75 µl of HRP-conjugated goat anti-horse IgG diluted to 1:5,000 with antibody diluent was added to the standard curve wells, and HRP-conjugated goat anti-mouse IgG diluted to 1:10,000 with antibody diluent was added to the unknown sample wells. The plate was again incubated for 1 hour at 37 °C. After washing the wells five times, 75 µl of the chromagenic substrate TMB (3,3′,5,5′,-tetramethylbenzidine; Pierce, Thermo Fisher Scientific, Inc.) was added to each well, and color was allowed to develop for 10 min at RT in the dark. The reaction was stopped by the addition of 75 µl of 1 M sulfuric acid, and the OD was measured at 450 nm in an E_{max} microplate reader (Molecular Devices).

Standard Curves

Standard curves were generated using a pooled serum sample from 21 healthy horses of mixed gender, age, and breed (kindly provided by Dr. Alex Estellar-Vico, University of Kentucky, Lexington, KY, USA). Pooled serum was also diluted in PBS, and the wells were coated to create standard curves in a two-fold dilution series, as shown in Table 4.2.3.

Table 4.2.3 Serum dilutions used to create standard curves for total IgG and IgG subisotypes a, b, and (T).

Subisotype	**Pooled Serum Dilution**	**Subisotype**	**Pooled Serum Dilution**
Total IgG	1:640,000	**IgGb**	1:160,000
	1:1,280,000		1:320,000
	1:2,560,000		1:640,000
	1:5,120,000		1:1,280,000
	1:10,240,000		1:2,560,000
IgGa	1:160,000	**IgG(T)**	1:320,000
	1:320,000		1:640,000
	1:640,000		1:1,280,000
	1:1,280,000		1:2,560,000
	1:2,560,000		1:5,120,000

Wells were coated with 75 µl pooled serum and incubated overnight at 4°C. Standard curves were generated based on previously determined serum concentrations, shown in Table 4.1.4. Blocking, washing, and antibody detection was conducted the same as above.

Table 4.2.4 Mean (± SD) serum concentrations (mg/ml) of immunoglobulin isotypes and 27 adult mixed-breed horses (Sheoran et al., 2000)

Immunoglobulin	Serum Concentration (mg/mL)
IgGa	3.4 ± 2.0
IgGb	19.6 ± 6.5
IgG(T)	4.0 ± 2.5

Calculations

The standard curve was plotted in excel. A polynomial trend line with an R^2 value of 0.95 or higher was added, and the curve equation exhibited on the graph. The equation was then used to plot the data of unknown serum and CSF samples, with the 'y' variable as antibody amount (ng), and the 'x' variable as the OD reading given by the microplate reader. From these readings, the antibody concentration was calculated using the following equation:

Antibody concentration (mg/mL) = Antibody amount (ng) * dilution factor / µl serum used in well * 0.001 ***(Equation 1)***

The average of three possible positive values for each sample were used. If one or more value was negative, these values were excluded from the average. If all three values were negative, the sample was recorded as showing missing data.

Statistical Analysis

Data was analyzed as a nested design using PROC GLIMMIX in SAS 9.4 (Carny, NC). Individual horse was used as replicates and were nested within their respective treatment groups (i.e. either infected normal or infected diseased). Mean comparisons between treatment groups was done using least significant difference. Linear regressions were conducted between (Y-variables) and (X-variables) using PROC REG. Significance of all analyses were made at a $P < 0.05$ level.

4.3 Results

Since ELISA's are semi-quantitative, standard curves were set up to more precisely measure the amount of *S. neurona*-specific total IgG and IgG subisotypes a, b, and (T) in equine serum samples. As shown in Figures 4.3.1 and 4.3.2, total amounts of antigen-specific IgG, IgGa, IgGb, and IgG(T) were determined for the infected diseased and infected normal groups of horses. No difference in amounts of antigen-specific IgG, IgGa, and IgGb were observed between the infected diseased and infected normal group. However, a significant difference ($p < 0.05$) was observed between the two groups for IgG(T), with the infected normal group having higher concentrations of IgG(T) than the infected diseased group. These results suggest a lower T_H2 response in infected diseased horses.

Next, ratios between the IgG subisotypes were determined for the infected normal and infected diseased groups, with the aim of showing a bias in immune response. As shown in figure 4.3.2, there were no significant differences in the ratios of IgGa to IgGb, IgGb to IgG(T), or combined IgGa and IgGb to IgG(T) between groups. However, there was a significant difference in the ratio of IgGa to IgG(T), with infected diseased horses having a higher ratio than infected normal horses. This finding is consistent with infected diseased horses having a lower concentration of IgG(T) than infected normal horses, and suggest a bias towards a T_H1 and/or away from a T_H2 response in the infected diseased horses.

Because the central nervous system is separated both functionally and anatomically from the rest of the body, the IgG subisotypes in both serum and CSF of infected diseased horses were analyzed to determine whether responses were compartmentalized and showed a difference between the intrathecal immune response and the systemic immune response to *S. neurona* (Figure 4.3.4). The CSF data from one horse were excluded from the analysis as an outlier (horse 90). Significant differences were found in the ratios of IgGa to IgGb and IgGa to IgG(T) ($p < 0.05$) between serum and CSF, with these ratios being higher in CSF. Results from this analysis show that there is a significant difference between intrathecal and systemic immune responses, and suggest a bias toward a T_H1 response in the central nervous system.

CSF data was analyzed for the infected normal group of horses but antibody concentrations were undetectable and therefore not useful for further analysis.

Since differences in IgG subisotypes were observed between infected diseased and infected normal horses, we examined the possibility of developing a simple serum test for identifying EPM diseased horses. For IgG(T) concentrations, a 0.05 ng/ml cut-off yielded specificity of 57% and sensitivity of 18% for EPM diagnosis (Figure 4.3.5). Increasing the cut-off to 0.15ng/ml provided 80% specificity but only 10% sensitivity These results indicate that an IgG(T) serum test will have little diagnostic value for EPM.

The ratio of IgGa to IgG(T) was higher in infected diseased horses, so its diagnostic potential was also evaluated (Figure 4.3.6). With a ratio of 100:1 IgGa to IgG(T) as the cut-off for determining EPM, specificity was 67% but sensitivity was only 2%. With a ratio cut-off of 25:1 IgGa to IgG(T), specificity was 56% and sensitivity was 16%. Based on these results, a ratio of IgGa to IgG(T) serum test will have little diagnostic value for EPM.

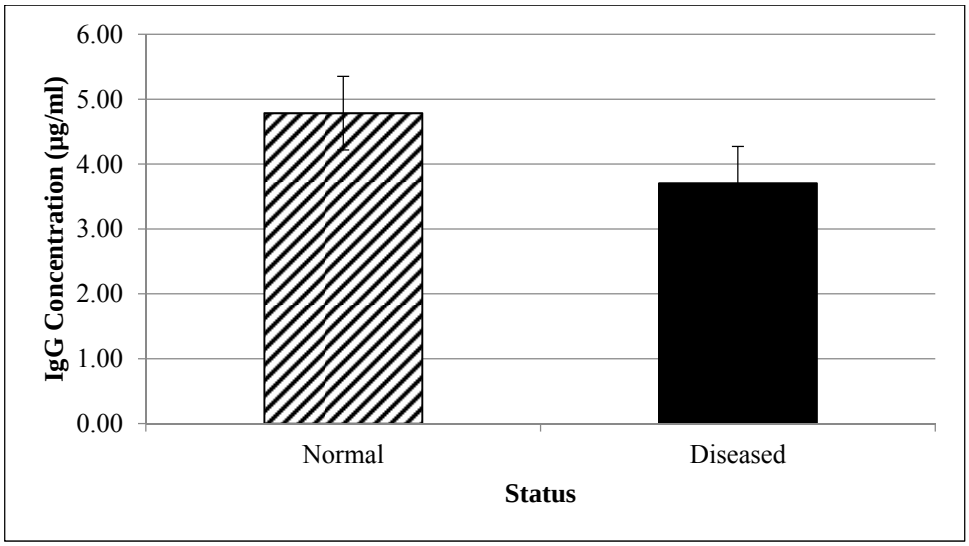

Figure 4.3.1 Difference in total immunoglobulin G (IgG) (µg/ml) in the serum of infected normal and infected diseased horses. IgG concentration in infected normal horses was 0.005 µg/ml (SE ± 0.001 µg/ml), and IgG concentration in infected diseased horses was 0.004 µg/ml (SE ± 0.001 µg/ml), with no significant difference between concentrations detected ($p = 0.2069$).

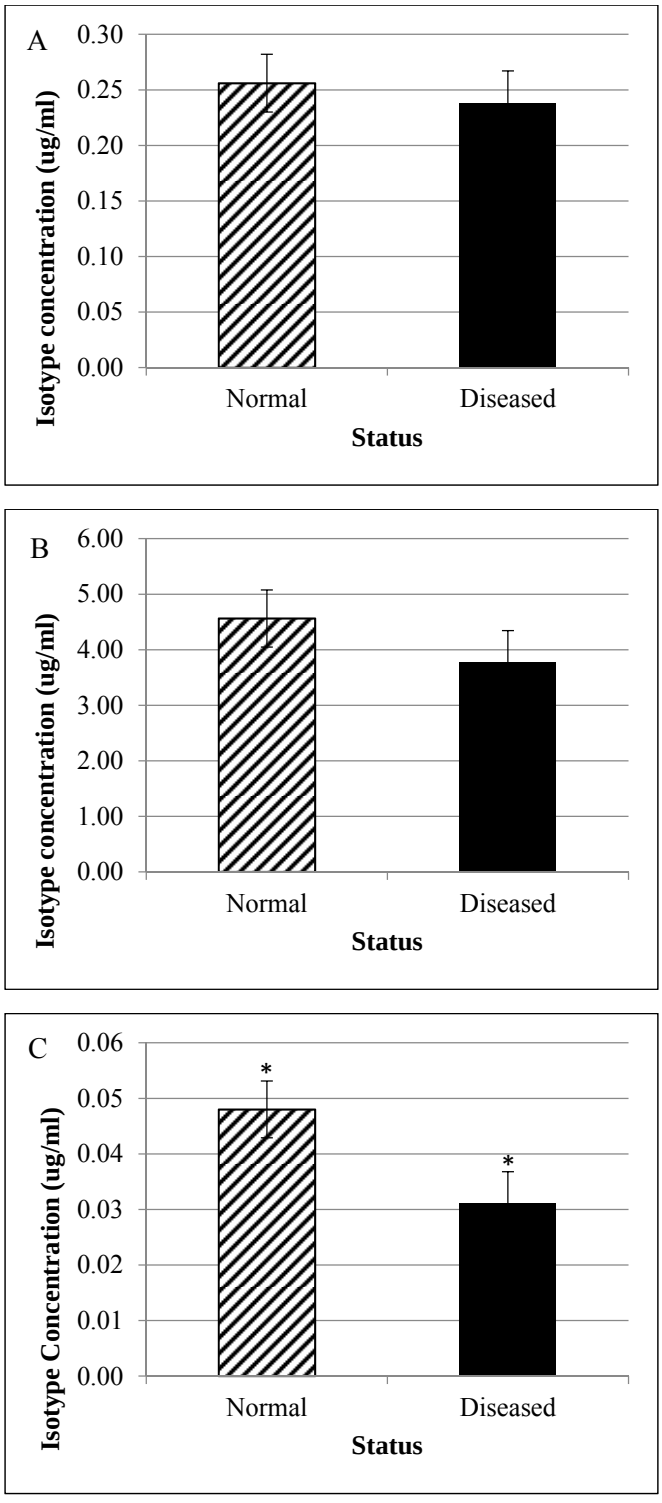

Figure 4.3.2 Difference in immunoglobulin G (IgG) subisotype concentrations (ng/ml) in the serum of infected normal and infected diseased horses. A) IgGa concentration in healthy horses was 0.256 ng/ml (SE ± 0.026 ng/ml), and IgGa concentration in diseased horses was 0.238 ng/ml (SE ± 0.029 ng/ml), with no significance between concentrations detected ($p = 0.6544$). B) IgGb concentration in healthy horses was 4.562 ng/ml (SE ± 0.514 ng/ml), and IgGb concentration in diseased horses was 3.773 ng/ml (SE ± 0.572 ng/ml), with no significance between concentrations detected ($p = 0.3065$). C) IgG(T) concentration in healthy horses was 0.048 ng/ml (SE ± 0.005 ng/ml), and IgG(T) concentration in diseased horses was 0.031 ng/ml (SE ± 0.006 ng/ml), with a significant difference between concentrations detected ($p = 0.0359$).

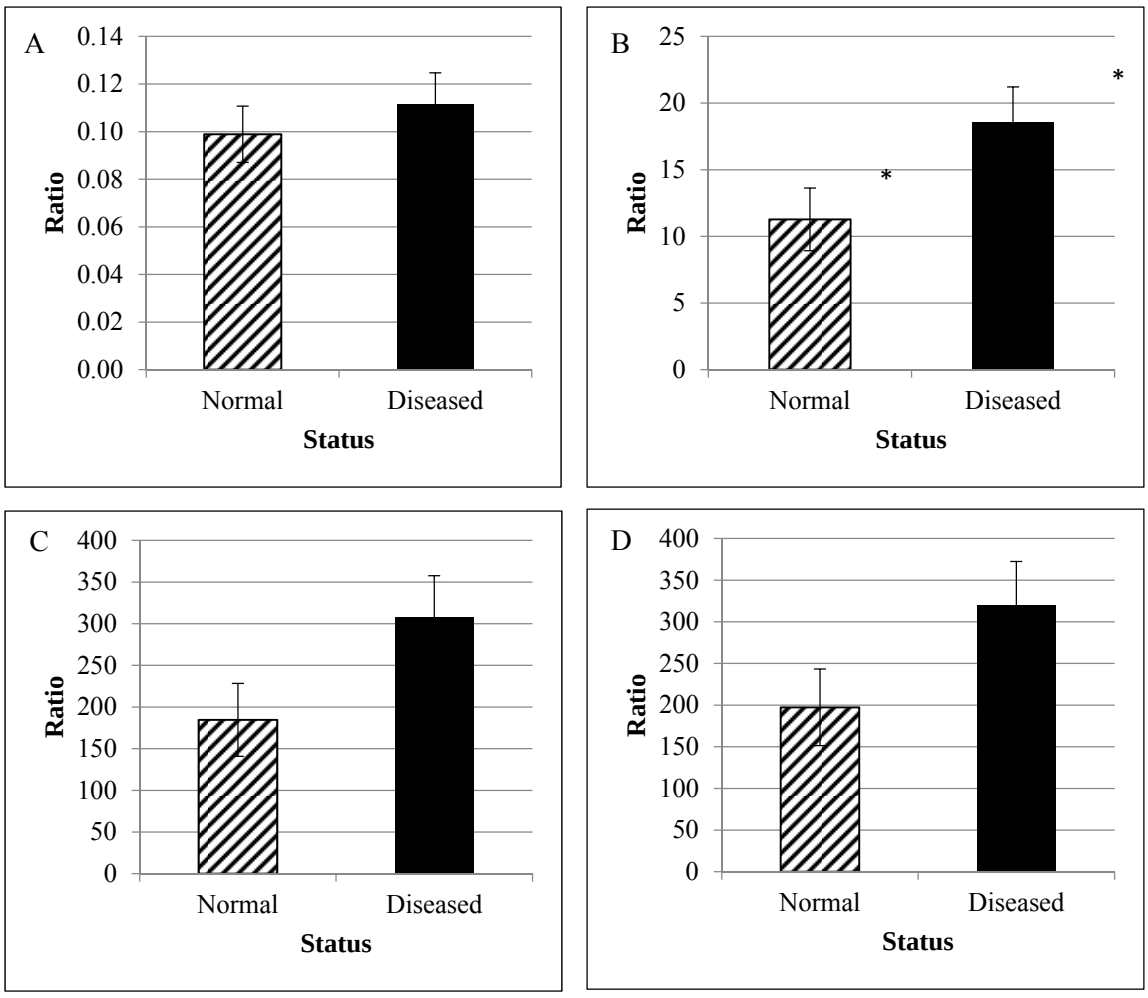

Figure 4.3.3 Difference between immunoglobulin G (IgG) subisotype concentration ratios in the serum of infected normal and infected diseased horses. A) The ratio of IgGa to IgGb in infected normal horse serum was 0.099 (SE ± 0.012), and the ratio of IgGa to IgGb in diseased horse serum was 0.111 (SE ± 0.013), with no significance between ratios detected ($p = 0.4705$). B) The ratio of IgGa to IgG(T) in infected normal horse serum was 11.275 (SE ± 2.357), and the ratio of IgGa to IgG(T) in diseased horse serum was 18.543 (SE ± 2.667), with a significant difference between ratios detected ($p = 0.0425$). C) The ratio of IgGb to IgG(T) in infected normal horse serum was 184.580 (SE ± 43.783), and the ratio of IgGb to IgG(T) in diseased horse serum was 301.850 (SE ± 49.741), with the difference between ratios approaching significance ($p = 0.0783$). D) The ratio of IgGa plus IgGb to IgG(T) in infected normal horse serum was 197.350 (SE ± 46.019), and the ratio of IgGa plus IgGb to IgG(T) in diseased horse serum was 320.390 (SE ± 52.045), with the difference between ratios approaching significance ($p = 0.0781$).

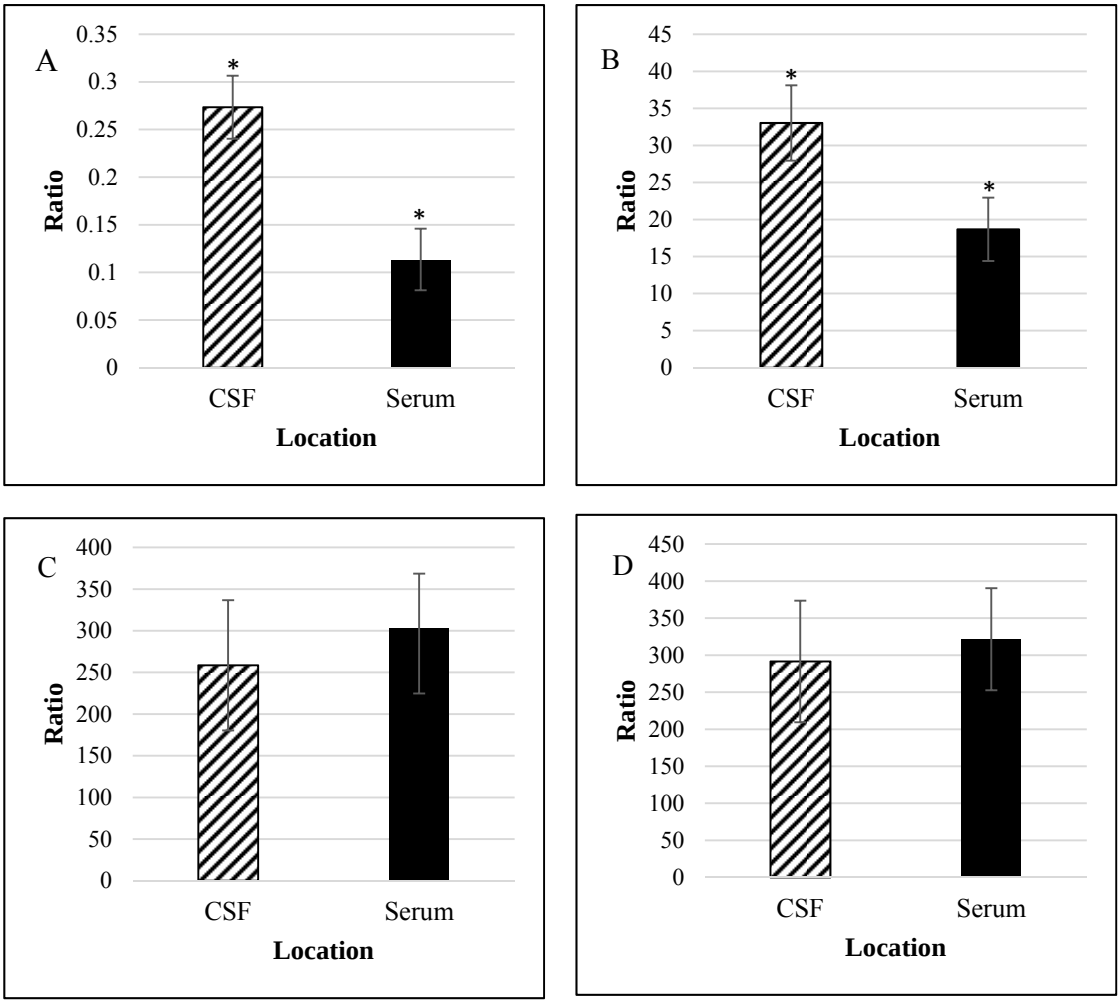

Figure 4.3.4 Difference between immunoglobulin G (IgG) subisotype concentration ratios in the serum and cerebrospinal fluid (CSF) of infected diseased horses. A) The ratio of IgGa to IgGb in CSF was 0.274 (SE ± 0.033), and the ratio of IgGa to IgGb in serum was 0.114 (SE ± 0.032), with a significant difference between ratios detected ($p = 0.0007$). B) The ratio of IgGa to IgG(T) in CSF was 33.033 (SE ± 5.084), and the ratio of IgGa to IgG(T) in serum was 18.669 (SE ± 4.272), with a significant difference between ratios detected ($p = 0.032$). C) The ratio of IgGb to IgG(T) in CSF was 258.57 (SE ± 78.180), and the ratio of IgGb to IgG(T) in serum was 302.88 (SE ± 65.685), with no significant difference between ratios ($p = 0.665$). D) The ratio of IgGa plus IgGb to IgG(T) in CSF was 291.600 (SE ± 82.085), and the ratio of IgGa plus IgGb to IgG(T) in serum was 321.54 (SE ± 68.965), with no significant difference between ratios ($p = 0.780$).

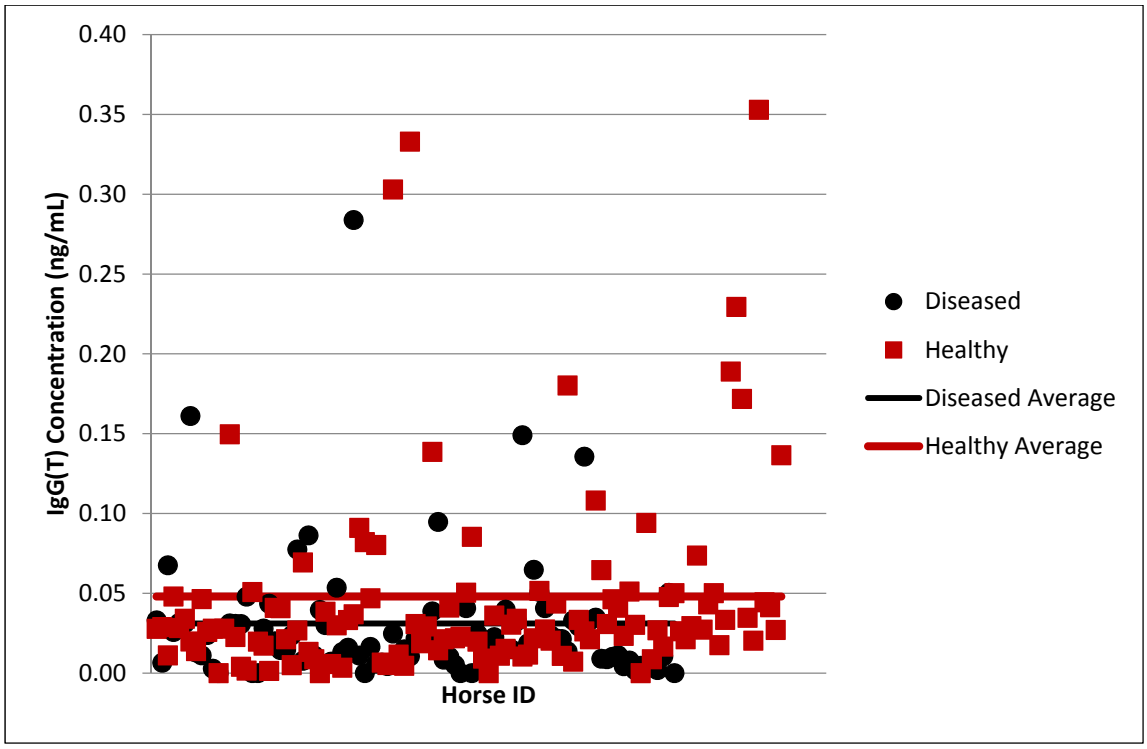

Figure 4.3.5 Serum IgG(T) concentrations in infected diseased and infected normal horses to determine the diagnostic potential of IgG(T) for a serum-based diagnostic test.

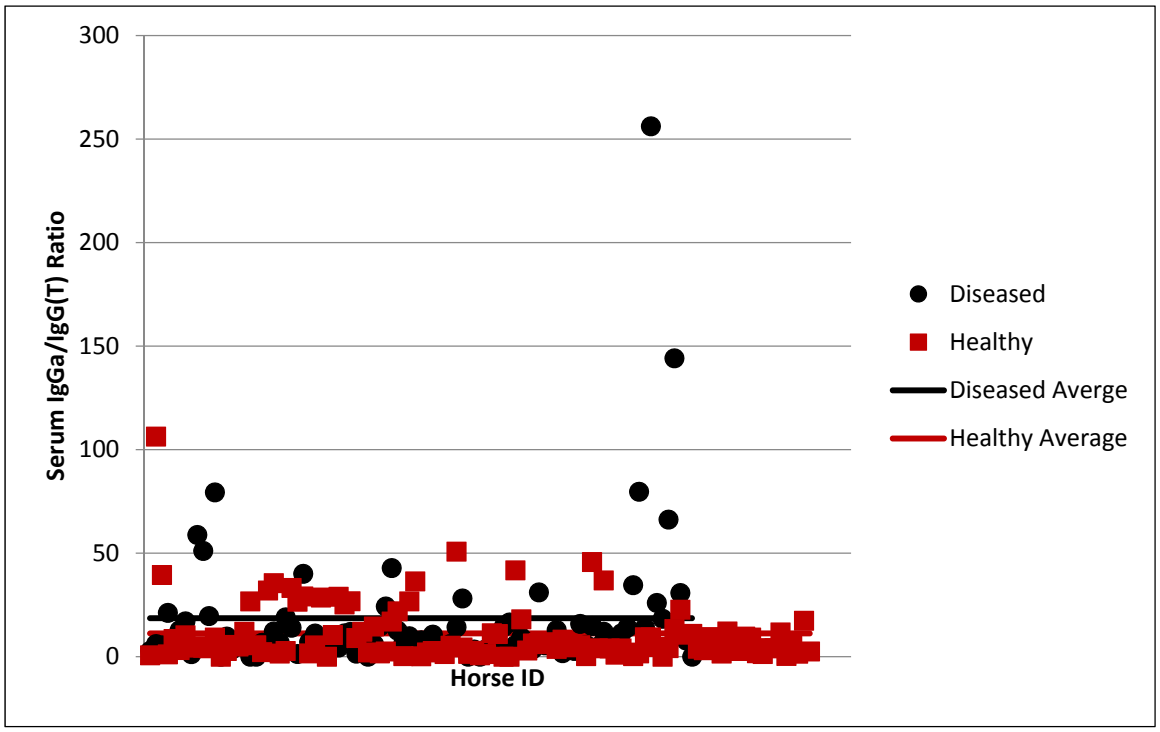

Figure 4.3.6 Serum IgGa/IgG(T) ratios in infected diseased and infected normal horses to determine the diagnostic potential of a serum-based test using IgGa/IgG(T) ratio.

4.4 Discussion

Why most horses infected with *S. neurona* remain clinically unaffected while a small proportion develop EPM has remains a major barrier in the understanding and control of the disease. The purpose of this study was to assess the immune response in horses seropositive for *S. neurona*, and compare those that develop clinical disease with those that were infected but remained normal. The rSnSAG2/4/3 ELISA was used and modified to detect amounts of IgG and subisotypes in serum from these two groups of horses. Collectively, our results suggest that infected diseased horses have an immune response biased towards a T_H1 response relative to horses that have been infected but remained clinically unaffected. However, it is currently unclear whether it is the difference in immune responses that is causing the development of clinical disease, or whether the chronic CNS infection by *S. neurona* is leading to a change in the immune response.

It is well established that an imbalance in immune responses can result in clinical disease. Development of clinical disease from the intracellular pathogen *Mycobacterium leprae* infection is a result of a T_H1/T_H2 imbalance (Murphy, 2011). Protozoan parasites *Leishmania* spp. and *T. gondii* are also known immunomodulators, with a T_H2 response to Leishmania resulting in uncontrolled infection, and an overly polarized T_H1 response to *T. gondii* resulting in immunopathogenesis (Butler et al., 2013; Spellberg and Edwards, 2001).

A disease known as Pigeon Protozoal Encephalitis (PPE) caused by *Sarcocystis calchasi* has recently been documented in the domestic pigeon (*Columba livia* f. *domestica*). PPE is a biphasic disease, with the second phase causing severe neurological signs and cerebral lesions, similar to EPM (Olias et al., 2013). The cerebral lesions are a result of the host's immune response, triggered by cytokines and possibly parasite antigen. Despite the low parasite load in infected pigeons, there appears to be a strong T_H1-biased, T cell driven immune response, suggesting that a T cell-mediated delayed-type hypersensitivity, or type IV hypersensitivity, reaction may be what causes the cerebral lesions and subsequent neurological signs (Olias et al., 2013). Delayed type hypersensitivity reactions are mediated by antigen-specific effector T cells. When T_H1 cells recognize their cognate antigen on MHC-II antigen presenting cells, they release cytokines, including IFN-γ, which further activates T cells, and TNF-α, causing local tissue destruction (Murphy, 2011). In a healthy host, this is a desirable response to eliminate intracellular pathogens or damaged cells. However, in the case of delayed-type hypersensitivity, the immune response causes excessive inflammation and inflammatory lesions (Murphy, 2011). It is reasonable to hypothesize that *S. neurona* infection in EPM horses is similar to *S. calcashi* infection of pigeons, and that it is the host's overly polarized T_H1 immune response to parasite infection that causes lesions of the central nervous system.

The findings of this study are consistent with research conducted in severe combined immunodeficient (SCID) foals infected with *S. neurona*. In SCID foals, *S. neurona* was

undetectable in tissues of the central nervous tissue, and none of them developed clinical signs of EPM (Dubey et al., 2015; Sellon et al., 2004). Immunocompetent animals infected with *S. neurona* were successfully able control parasitemia and clear infection from visceral tissues, but parasites were often found in the central nervous system, and clinical signs of neurological disease were common (Sellon et al., 2004). The infection study with SCID foals show importance of T_H1 response in immunopathology, which is consistent with findings for *Sarcocystis calchasi* infection and PPE (Olias et al., 2013).

The results from this study are in contrast to prior studies examining cell-mediated immune responses in EPM-positive and EPM-negative horses (Spencer et al., 2005; Spencer et al., 2004). The 2004 study looked at lymphocyte responses in vitro, and found that IFN-γ was reduced in horses with clinical EPM, indicative of a reduced T_H1 response in diseased horses. The 2005 study also found an increase in IL-4 production, indicative of a higher T_H2 response, contrary to what we observed. The results from the current study are also in contrast to previous work looking at horses experimentally infected with *S. neurona* (Lewis et al., 2014; Witonsky et al., 2008). These studies found decreased cell-mediated immune (T_H1) responses as the result of experimental infection, although the 2014 study did note an inability to distinguish a unique and consistent change in equine immune response. The differences between the present study's results and these previous studies could be explained by the difference in research methods: the results of the present study were observatory from live, infected animals, whereas the results of previous studies were from in vitro stimulation of lymphocytes and examination of experimental infection in horses.

Inappropriate cross-regulation

Although the immune response has traditionally been characterized as either T_H1 or T_H2, the response is far more complex, involving other important T cell subsets, such as T_H17 and regulatory T cells (Tregs) (Murphy, 2011).

T_H17 cells display site-dependent plasticity, and can work in conjunction with T_H1 cells to mount a functionally similar immune response. They are thought to augment T_H1 responses to some intracellular pathogens, such as Mycobacteria and *Francisella tularensis* (Muranski and Restifo, 2013). In immune privileged organs, such as the central nervous system, inflammation requires infiltration of T_H17 cells. The initial inflammatory response is likely mediated by T_H17 cells, and once this initial danger signal has been processed, the effector arm of the adaptive immune system is stimulated to promote and influx of activated T_H1 and CD8 T cells (Kastelein et al., 2007). T_H17 cells are characterized by production of the cytokine interleukin 17 (IL-17). IL-17 stimulates local tissues to produce an additional proinflammatory cytokine, interleukin-8 (IL-8). IL-17 and IL-8 can both often be found in a T_H17 mediated immune response that induces the key signs of infection – swelling, heat, and pain, thus setting up an environment conducive to further heightened immune responses (Kastelein et al., 2007). Dysregulated production of pro-inflammatory cytokine IL-17 can result in pathological chronic immune-mediated tissue destruction (Kastelein et al., 2007), and IL-8 was seen to be

upregulated in *S. calchasi* infection (Olias et al., 2013). It is, therefore, possible, that the dysregulation of T_H17 cells plays a role in EPM.

Tregs have a known role in modulating inflammation and limiting immunopathology, and are thought to act in maintaining a balance between immune protection and pathology. In some cases, such as pathogen-driven diseases and autoimmune disorders, T_H1 cells avoid immune regulation (Oldenhove et al., 2009). Therefore, Tregs could be critical in managing diseases affecting the central nervous system, such as toxoplasmosis, malaria (Butler et al., 2013), and, potentially, EPM.

Coinfection

Protozoal parasites are not the only pathogens known to modulate immune responses. Concurrent infections by both pathogenic and non-pathogenic organisms are ubiquitous in nature, and in horses infected with *S. neurona*, it is possible that this microenvironment is a result of coinfection with another organism (Ezenwa and Jolles, 2011). For example, helminths are known immunomodulators, and tend to skew the immune response in the direction of a T_H2 type response (Dent, 2002; Ezenwa and Jolles, 2011). In contrast, viruses tend to skew the immune response in the direction of a T_H1 type response, while simultaneously subduing regulatory responses (Dent, 2002; Romagnani, 1996). Given that a lower T_H2 response and T_H1 dominant response is seen in horses that are infected with *S. neurona* and go on to develop clinical disease, the presence or absence of other infectious organisms could be part of the etiology of EPM.

Future Directions

In summary, it has now been demonstrated that the immune response to *S. neurona* may play a role in EPM pathogenesis. However, it is not currently clear what is causing the difference in immune responses between infected normal and infected diseased horses. Whether disease is a result of the difference in immune response or whether the difference in immune response is the result of disease still needs to be further elucidated. Additionally, further research is required into the factors driving the difference in immune response, and what makes certain horses more susceptible to the development of disease. Cytokine profiles in infected normal and infected diseased horses could be compared, particularly when examining the potential of T_H17 and Tregs cells in causing immunopathogenesis. Investigation into coinfections by other organisms may also lead to better understanding of why the difference in immune responses occurs.

4.5 Acknowledgements

We thank Dr. A. Estellar-Vico and Dr. A. Adams from the Department of Veterinary Science, University of Kentucky for providing reagents required for this study; Equine Diagnostic Solutions, LLC, for providing serum and CSF samples; and Dr. B. Goff from Plant and Soils Science, University of Kentucky for his assistance with statistical analysis. This research was supported by funds from the Amerman Family Equine Research Endowment.

Conflicts of Interest: D.K. Howe is the inventor listed on patents covering the assays used in this study.

Bibliography

Arias, M., Yeargan, M., Francisco, I., Dangoudoubiyam, S., Becerra, P., Francisco, R., Sanchez-Andrade, R., Paz-Silva, A., Howe, D.K., 2012. Exposure to Sarcocystis spp. in horses from Spain determined by Western blot analysis using Sarcocystis neurona merozoites as heterologous antigen. Veterinary parasitology 185, 301-304.

Barbosa, L., Johnson, C.K., Lambourn, D.M., Gibson, A.K., Haman, K.H., Huggins, J.L., Sweeny, A.R., Sundar, N., Raverty, S.A., Grigg, M.E., 2015. A novel Sarcocystis neurona genotype XIII is associated with severe encephalitis in an unexpectedly broad range of marine mammals from the northeastern Pacific Ocean. International journal for parasitology 45, 595-603.

Berdoy, M., Webster, J.P., Macdonald, D.W., 2000. Fatal attraction in rats infected with Toxoplasma gondii. Proceedings. Biological sciences 267, 1591-1594.

Blader, I.J., Coleman, B.I., Chen, C.T., Gubbels, M.J., 2015. Lytic Cycle of Toxoplasma gondii: 15 Years Later. Annual review of microbiology 69, 463-485.

Boy, M.G., Galligan, D.T., Divers, T.J., 1990. Protozoal encephalomyelitis in horses: 82 cases (1972-1986). Journal of the American Veterinary Medical Association 196, 632-634.

Butler, N.S., Harris, T.H., Blader, I.J., 2013. Regulation of immunopathogenesis during Plasmodium and Toxoplasma infections: more parallels than distinctions? Trends in parasitology 29, 593-602.

Carson, M.J., Doose, J.M., Melchior, B., Schmid, C.D., Ploix, C.C., 2006. CNS immune privilege: hiding in plain sight. Immunological reviews 213, 48-65.

Cunha, C.W., McGuire, T.C., Kappmeyer, L.S., Hines, S.A., Lopez, A.M., Dellagostin, O.A., Knowles, D.P., 2006. Development of specific immunoglobulin Ga (IgGa) and IgGb antibodies correlates with control of parasitemia in Babesia equi Infection. Clinical and vaccine immunology : CVI 13, 297-300.

Cutler, T.J., MacKay, R.J., Ginn, P.E., Gillis, K., Tanhauser, S.M., LeRay, E.V., Dame, J.B., Greiner, E.C., 2001. Immunoconversion against Sarcocystis neurona in normal and dexamethasone-treated horses challenged with S. neurona sporocysts. Veterinary parasitology 95, 197-210.

Dangoudoubiyam, S., Oliveira, J.B., Viquez, C., Gomez-Garcia, A., Gonzalez, O., Romero, J.J., Kwok, O.C., Dubey, J.P., Howe, D.K., 2011. Detection of antibodies against Sarcocystis neurona, Neospora spp., and Toxoplasma gondii in horses from Costa Rica. The Journal of parasitology 97, 522-524.

Denkers, E.Y., 2003. From cells to signaling cascades: manipulation of innate immunity by Toxoplasma gondii. FEMS immunology and medical microbiology 39, 193-203.

Dent, L.A., 2002. For better or worse: common determinants influencing health and disease in parasitic infections, asthma and reproductive biology. Journal of reproductive immunology 57, 255-272.

Dubey, J.P., 2001. Migration and development of Sarcocystis neurona in tissues of interferon gamma knockout mice fed sporocysts from a naturally infected opossum. Veterinary parasitology 95, 341-351.

Dubey, J.P., Calero-Bernal, R., Rosenthal, B.M., Speer, C.A., Fayer, R., 2016. Sarcocystosis of Animals and Humans, Second Edition Edition. CRC Press, Boca Raton, FL.

Dubey, J.P., Fayer, R., Seesee, F.M., 1978. Sarcocystis in feces of coyotes from Montana: prevalence and experimental transmission to sheep and cattle. Journal of the American Veterinary Medical Association 173, 1167-1170.

Dubey, J.P., Howe, D.K., Furr, M., Saville, W.J., Marsh, A.E., Reed, S.M., Grigg, M.E., 2015. An update on Sarcocystis neurona infections in animals and equine protozoal myeloencephalitis (EPM). Veterinary parasitology 209, 1-42.

Dubey, J.P., Lindsay, D.S., Saville, W.J., Reed, S.M., Granstrom, D.E., Speer, C.A., 2001. A review of Sarcocystis neurona and equine protozoal myeloencephalitis (EPM). Veterinary parasitology 95, 89-131.

Ezenwa, V.O., Jolles, A.E., 2011. From host immunity to pathogen invasion: the effects of helminth coinfection on the dynamics of microparasites. Integrative and comparative biology 51, 540-551.

Fenger, C.K., Granstrom, D.E., Langemeier, J.L., Stamper, S., Donahue, J.M., Patterson, J.S., Gajadhar, A.A., Marteniuk, J.V., Xiaomin, Z., Dubey, J.P., 1995. Identification of opossums (Didelphis virginiana) as the putative definitive host of Sarcocystis neurona. The Journal of parasitology 81, 916-919.

Fitzgerald, D.C., Zhang, G.X., El-Behi, M., Fonseca-Kelly, Z., Li, H., Yu, S., Saris, C.J., Gran, B., Ciric, B., Rostami, A., 2007. Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. Nature immunology 8, 1372-1379.

Furr, M., 2002. Antigen-specific antibodies in cerebrospinal fluid after intramuscular injection of ovalbumin in horses. Journal of veterinary internal medicine 16, 588-592.

Furr, M., MacKay, R., Granstrom, D., Schott, H., 2nd, Andrews, F., 2002. Clinical diagnosis of equine protozoal myeloencephalitis (EPM). Journal of veterinary internal medicine 16, 618-621.

Furr, M., Pontzer, C., Gasper, P., 2001. Lymphocyte phenotype subsets in the cerebrospinal fluid of normal horses and horses with equine protozoal myeloencephalitis. Veterinary therapeutics : research in applied veterinary medicine 2, 317-324.

Furr, M., Reed, S.M., 2015. Equine Neurology, 2nd Edition Edition. Wiley-Blackwell, 504 p.

Gazzinelli, R.T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G., Sher, A., 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. Journal of immunology (Baltimore, Md. : 1950) 157, 798-805.

Hoane, J.S., Gennari, S.M., Dubey, J.P., Ribeiro, M.G., Borges, A.S., Yai, L.E., Aguiar, D.M., Cavalcante, G.T., Bonesi, G.L., Howe, D.K., 2006. Prevalence of Sarcocystis neurona and Neospora spp. infection in horses from Brazil based on presence of serum antibodies to parasite surface antigen. Veterinary parasitology 136, 155-159.

Immunisation Advisory Centre 2012. The immune system and vaccination (Online, Immunisation Advisory Centre 2013).
Kalampokis, I., Yoshizaki, A., Tedder, T.F., 2013. IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. Arthritis research & therapy 15 Suppl 1, S1.
Kastelein, R.A., Hunter, C.A., Cua, D.J., 2007. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annual review of immunology 25, 221-242.
Keggan, A., Freer, H., Rollins, A., Wagner, B., 2013. Production of seven monoclonal equine immunoglobulins isotyped by multiplex analysis. Veterinary immunology and immunopathology 153, 187-193.
Kennedy, T., Campbell, J., Selzer, V., 2001. Safety of ponazuril 15% oral paste in horses. Veterinary therapeutics : research in applied veterinary medicine 2, 223-231.
Kim, K.S., 2008. Mechanisms of microbial traversal of the blood-brain barrier. Nature reviews. Microbiology 6, 625-634.
Kimura, D., Miyakoda, M., Kimura, K., Honma, K., Hara, H., Yoshida, H., Yui, K., 2016. Interleukin-27-Producing CD4(+) T Cells Regulate Protective Immunity during Malaria Parasite Infection. Immunity 44, 672-682.
Konradt, C., Ueno, N., Christian, D.A., Delong, J.H., Pritchard, G.H., Herz, J., Bzik, D.J., Koshy, A.A., McGavern, D.B., Lodoen, M.B., Hunter, C.A., 2016. Endothelial cells are a replicative niche for entry of Toxoplasma gondii to the central nervous system. Nature microbiology 1, 16001.
Lachenmaier, S.M., Deli, M.A., Meissner, M., Liesenfeld, O., 2011. Intracellular transport of Toxoplasma gondii through the blood-brain barrier. Journal of neuroimmunology 232, 119-130.
Lewis, M.J., Wagner, B., Woof, J.M., 2008. The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Molecular immunology 45, 818-827.
Lewis, S.R., Ellison, S.P., Dascanio, J.J., Lindsay, D.S., Gogal, R.M., Jr., Werre, S.R., Surendran, N., Breen, M.E., Heid, B.M., Andrews, F.M., Buechner-Maxwell, V.A., Witonsky, S.G., 2014. Effects of Experimental Sarcocystis neurona-Induced Infection on Immunity in an Equine Model. Journal of veterinary medicine 2014, 239495.
Lopez, A.M., Hines, M.T., Palmer, G.H., Knowles, D.P., Alperin, D.C., Hines, S.A., 2003. Analysis of anamnestic immune responses in adult horses and priming in neonates induced by a DNA vaccine expressing the vapA gene of Rhodococcus equi. Vaccine 21, 3815-3825.
Lunn, D.P., Holmes, M.A., Schram, B., Duffus, W.P., 1995. Monoclonal antibodies specific for equine IgG sub-isotypes including an antibody which recognizes B lymphocytes. Veterinary immunology and immunopathology 47, 239-251.
MacKay, R.J., Granstrom, D.E., Saville, W.J., Reed, S.M., 2000. Equine protozoal myeloencephalitis. The Veterinary clinics of North America. Equine practice 16, 405-425.
Marsh, A.E., Barr, B.C., Lakritz, J., Nordhausen, R., Madigan, J.E., Conrad, P.A., 1997. Experimental infection of nude mice as a model for Sarcocystis neurona-associated encephalitis. Parasitology research 83, 706-711.

McKenna, P.B., Charleston, W.A., 1992. The survival of Sarcocystis gigantea sporocysts following exposure to various chemical and physical agents. Veterinary parasitology 45, 1-16.
Meinl, E., Krumbholz, M., Hohlfeld, R., 2006. B lineage cells in the inflammatory central nervous system environment: migration, maintenance, local antibody production, and therapeutic modulation. Annals of neurology 59, 880-892.
Miller, M.A., Conrad, P.A., Harris, M., Hatfield, B., Langlois, G., Jessup, D.A., Magargal, S.L., Packham, A.E., Toy-Choutka, S., Melli, A.C., Murray, M.A., Gulland, F.M., Grigg, M.E., 2010. A protozoal-associated epizootic impacting marine wildlife: mass-mortality of southern sea otters (Enhydra lutris nereis) due to Sarcocystis neurona infection. Veterinary parasitology 172, 183-194.
Mitchell, S.M., Zajac, A.M., Davis, W.L., Kennedy, T.J., Lindsay, D.S., 2005. The effects of ponazuril on development of apicomplexans in vitro. The Journal of eukaryotic microbiology 52, 231-235.
Morley, P.S., Traub-Dargatz, J.L., Benedict, K.M., Saville, W.J., Voelker, L.D., Wagner, B.A., 2008. Risk factors for owner-reported occurrence of equine protozoal myeloencephalitis in the US equine population. Journal of veterinary internal medicine 22, 616-629.
Muranski, P., Restifo, N.P., 2013. Essentials of Th17 cell commitment and plasticity. Blood 121, 2402-2414.
Murphy, K., 2011. Immunobiology: The Immune System (Janeway), 8th Edition Edition. Garland Science, 888 p.
NAHMS 2001. Equine Protozoal Myeloencephalitis (EPM) in the U.S., USDA:APHIS:VS, ed. (Fort Collins, CO, Centers for Epidemiology and Animal Health).
Nelson, K.M., Schram, B.R., McGregor, M.W., Sheoran, A.S., Olsen, C.W., Lunn, D.P., 1998. Local and systemic isotype-specific antibody responses to equine influenza virus infection versus conventional vaccination. Vaccine 16, 1306-1313.
Oldenhove, G., Bouladoux, N., Wohlfert, E.A., Hall, J.A., Chou, D., Dos Santos, L., O'Brien, S., Blank, R., Lamb, E., Natarajan, S., Kastenmayer, R., Hunter, C., Grigg, M.E., Belkaid, Y., 2009. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. Immunity 31, 772-786.
Olias, P., Meyer, A., Klopfleisch, R., Lierz, M., Kaspers, B., Gruber, A.D., 2013. Modulation of the host Th1 immune response in pigeon protozoal encephalitis caused by Sarcocystis calchasi. Veterinary research 44, 10.
Parham, P., 2015. The Immune System, Fourth Edition Edition. Garland Science, Taylor & Francis Group, LLC, New York, New York, USA.
Pitel, P.H., Pronost, S., Gargala, G., Anrioud, D., Toquet, M.P., Foucher, N., Collobert-Laugier, C., Fortier, G., Ballet, J.J., 2002. Detection of Sarcocystis neurona antibodies in French horses with neurological signs. International journal for parasitology 32, 481-485.
Pusterla, N., Packham, A., Mackie, S., Kass, P.H., Hunyadi, L., Conrad, P.A., 2015. Daily feeding of diclazuril top dress pellets in foals reduces seroconversion to Sarcocystis neurona. Veterinary journal (London, England : 1997) 206, 236-238.

Reed, S.M., Howe, D.K., Morrow, J.K., Graves, A., Yeargan, M.R., Johnson, A.L., MacKay, R.J., Furr, M., Saville, W.J., Williams, N.M., 2013. Accurate antemortem diagnosis of equine protozoal myeloencephalitis (EPM) based on detecting intrathecal antibodies against Sarcocystis neurona using the SnSAG2 and SnSAG4/3 ELISAs. Journal of veterinary internal medicine 27, 1193-1200.

Romagnani, S., 1996. Th1 and Th2 in human diseases. Clinical immunology and immunopathology 80, 225-235.

Rooney, J.R., Prickett, M.E., Delaney, F.M., Crowe, M.W., 1970. Focal myelitis-encephalitis in horses. The Cornell veterinarian 60, 494-501.

Sacks, D., Sher, A., 2002. Evasion of innate immunity by parasitic protozoa. Nature immunology 3, 1041-1047.

Saville, W.J., Reed, S.M., Granstrom, D.E., Hinchcliff, K.W., Kohn, C.W., Wittum, T.E., Stamper, S., 1997. Seroprevalence of antibodies to Sarcocystis neurona in horses residing in Ohio. Journal of the American Veterinary Medical Association 210, 519-524.

Saville, W.J., Sofaly, C.D., Reed, S.M., Dubey, J.P., Oglesbee, M.J., Lacombe, V.A., Keene, R.O., Gugisberg, K.M., Swensen, S.W., Shipley, R.D., Chiang, Y.W., Chu, H.J., Ng, T., 2004. An equine protozoal myeloencephalitis challenge model testing a second transport after inoculation with Sarcocystis neurona sporocysts. The Journal of parasitology 90, 1406-1410.

Saville, W.J., Stich, R.W., Reed, S.M., Njoku, C.J., Oglesbee, M.J., Wunschmann, A., Grover, D.L., Larew-Naugle, A.L., Stanek, J.F., Granstrom, D.E., Dubey, J.P., 2001. Utilization of stress in the development of an equine model for equine protozoal myeloencephalitis. Veterinary parasitology 95, 211-222.

Savini, G., Robertson, I.D., Dunsmore, J.D., 1996. Viability of the sporocysts of Sarcocystis cruzi after exposure to different temperatures and relative humidities. Veterinary parasitology 67, 153-160.

Sellon, D.C., Knowles, D.P., Greiner, E.C., Long, M.T., Hines, M.T., Hochstatter, T., Tibary, A., Dame, J.B., 2004. Infection of immunodeficient horses with Sarcocystis neurona does not result in neurologic disease. Clinical and diagnostic laboratory immunology 11, 1134-1139.

Sheoran, A.S., Timoney, J.F., Holmes, M.A., Karzenski, S.S., Crisman, M.V., 2000. Immunoglobulin isotypes in sera and nasal mucosal secretions and their neonatal transfer and distribution in horses. American journal of veterinary research 61, 1099-1105.

Sibley, L.D., 2011. Invasion and intracellular survival by protozoan parasites. Immunological reviews 240, 72-91.

Sofaly, C.D., Reed, S.M., Gordon, J.C., Dubey, J.P., Ogleebee, M.J., Njoku, C.J., Grover, D.L., Saville, W.J., 2002. Experimental induction of equine protozoan myeloencephalitis (EPM) in the horse: effect of Sarcocystis neurona sporocyst inoculation dose on the development of clinical neurologic disease. The Journal of parasitology 88, 1164-1170.

Spellberg, B., Edwards, J.E., Jr., 2001. Type 1/Type 2 immunity in infectious diseases. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 32, 76-102.

Spencer, J.A., Deinnocentes, P., Moyana, E.M., Guarino, A.J., Ellison, S.E., Bird, R.C., Blagburn, B.L., 2005. Cytokine gene expression in response to SnSAG1 in horses with equine protozoal myeloencephalitis. Clinical and diagnostic laboratory immunology 12, 644-646.

Spencer, J.A., Ellison, S.E., Guarino, A.J., Blagburn, B.L., 2004. Cell-mediated immune responses in horses with equine protozoal myeloencephalitis. The Journal of parasitology 90, 428-430.

Streilein, J.W., 1993. Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. Current opinion in immunology 5, 428-432.

Tang, H., Ming, Z., Liu, R., Xiong, T., Grevelding, C.G., Dong, H., Jiang, M., 2013. Development of adult worms and granulomatous pathology are collectively regulated by T- and B-cells in mice infected with Schistosoma japonicum. PloS one 8, e54432.

Wagner, B., 2006. Immunoglobulins and immunoglobulin genes of the horse. Developmental and comparative immunology 30, 155-164.

Witonsky, S.G., Ellison, S., Yang, J., Gogal, R.M., Lawler, H., Suzuki, Y., Sriranganathan, N., Andrews, F., Ward, D., Lindsay, D.S., 2008. Horses experimentally infected with Sarcocystis neurona develop altered immune responses in vitro. The Journal of parasitology 94, 1047-1054.

Yeargan, M., de Assis Rocha, I., Morrow, J., Graves, A., Reed, S.M., Howe, D.K., 2015. A new trivalent SnSAG surface antigen chimera for efficient detection of antibodies against Sarcocystis neurona and diagnosis of equine protozoal myeloencephalitis. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 27, 377-381.

Yoshizaki, A., Miyagaki, T., DiLillo, D.J., Matsushita, T., Horikawa, M., Kountikov, E.I., Spolski, R., Poe, J.C., Leonard, W.J., Tedder, T.F., 2012. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. Nature 491, 264-268.

VITA

- CATHERINE-JANE ANGWIN

- PLACE OF BIRTH:

  Rotterdam, The Netherlands

- EDUCATIONAL INSTITUTIONS:

  University of Kentucky, Lexington, Kentucky

  University of Wyoming, Laramie, Wyoming
  Bachelor of Science in Rangeland Ecology and Watershed Management
  (Minor Wildlife and Fisheries Biology and Management)

  Northwest College, Powell, Wyoming
  Associates of Science in Range Management
  Associates of Applied Science in Production Agriculture

- PROFESSIONAL POSITIONS:

  Graduate Research Assistant, University of Kentucky
  Undergraduate Research Assistant, University of Wyoming

- SCHOLASTIC AND PROFESSIONAL HONORS:

  Kentucky Opportunity Fellowship
  Gamma Sigma Delta
  Phi Theta Kappa

- PROFESSIONAL PUBLICATIONS:

  Meta-analysis of diet composition and potential conflict of wild horses with livestock and wild ungulates on western rangelands of North America. Scasta, J. D., J. L. Beck, and C. J. Angwin. 2016. Rangeland Ecology and Management 69:310–318.