

Experimental infection with *Sarcocystis neurona* alters the immune response: the effect on CD4+, CD8+, B-cell, monocyte and granulocyte populations in horses

Stephanie Rochelle Lewis

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Approved:

Sharon G. Witonsky, Committee Chair

Virginia Buechner-Maxwell, Committee Member

John J. Dascanio, Committee Member

Robert M. Gogal, Jr., Committee Member

David S. Lindsay, Committee Member

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Abstract

Previous studies have demonstrated differences in CD4+, CD8+ and B-cell populations between EPM affected and normal horses. The overall goal of our project was to further define the immune deficiencies associated with *S. neurona* infection. We hypothesized that PMA/I stimulated suppression in EPM horses is due to decreased proliferation of monocytes, CD4+ and CD8+ cells. Our objectives were 1) to determine whether *S. neurona* infection causes an increase in apoptosis of a particular immune subset, and 2) to determine whether *S. neurona* causes a decrease in the number of cellular divisions (proliferation) of a particular immune cell subset.

For this study, nine *S. neurona* antibody negative, immunocompetent horses were obtained. Baseline neurologic examinations, SnSAG1 (*S. neurona* Surface Antigen 1) ELISAs on cerebrospinal fluid (CSF) and serum, and baseline immune function assays were performed. Horses were randomly divided into groups. Five horses were challenged for ten days via intravenous injection of autologous lymphocytes infected with *S. neurona*. Neurologic parameters of all horses were assessed for 70 days following infection. Immune function was based on proliferation responses to mitogens, as assessed through thymidine incorporation. Enumeration of cellular subsets, degree of apoptosis and number of cellular divisions were assessed through flow cytometry. SnSAG1 ELISA of serum and CSF samples performed post-infection confirmed infection and disease. All infected horses displayed moderate neurologic signs on clinical examination. Some significant differences in cellular activities were noted. Additionally, this is the first time the method using *S. neurona* infected lymphocytes has been reproduced successfully by different investigators.

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List of Abbreviations

7-AAD 7-amino actinomycin D
AQ albumin quotient
BBB blood brain barrier
CBC complete blood count
CNS central nervous system
ConA concanavalin A
CPM counts per minute
CSF cerebrospinal fluid
DNA deoxyribonucleic acid
EPM Equine Protozoal Myeloencephalitis
FBS fetal bovine serum
FDA Food and Drug Administration
FITC fluorescein isothiocyanate
GKO gamma interferon knock-out
I ionomycin
IC immunocompetent
IFA immunofluorescence assay
IFN γ interferon gamma
Ig immunoglobulin
IFA immune fluorescence assay
IL interleukin
IV intravenous
KO knock out
LPS lipopolysaccharide
NAHMS National Animal Health Monitoring System
NF- κ B nuclear factor- κ B
NFAT nuclear factor of activated T cells
PBMCs peripheral blood mononuclear cells
PBS phosphate buffered-saline solution
PCR polymerase chain reaction
PE phycoerythrin
PFOR pyruvate ferredoxin oxidoreductase
PHA phytohemagglutinin
PI post infection
PMA phorbol 12-myristate 13-acetate
PWM pokeweed mitogen
QH quarter horse
QHX quarter horse cross
RBC red blood cell
RNA ribonucleic acid
SAG surface antigen
s.c. subcutaneous
SCID severe combined immune deficiency
Th T helper cell
TLR Toll-like receptor
WB Western Blot
WBC white blood cell

CHAPTER 1: INTRODUCTION

Equine Protozoal Myeloencephalitis (EPM) represents the most commonly diagnosed neurologic disease of horses within the United States of America (USA) ¹⁻³. Horses are most commonly aberrant hosts, and are infected by ingestion of the sporocyst form of the causative agent *Sarcocystis neurona* (*S. neurona*), through contamination of feedstuffs. Most horses exposed to the parasite mount a protective immune response, and do not experience clinical signs. For the small percentage of horses that progress to develop EPM, clinical signs are classically those associated with asymmetric neurologic deficits including gait abnormalities, ataxia, weakness and focal muscle atrophy ³⁻⁵.

A reliable diagnosis of EPM based on blood testing alone is problematic due to the large number of horses that are seropositive to *S. neurona* yet do not have the disease. Current pre-mortem diagnostic methods are based on abnormal neurologic signs in conjunction with detection of higher than normal levels of antibodies to *S. neurona* in serum and cerebrospinal fluid as detected using the Western Blot test or other diagnostic assays.

Treatment of horses with EPM relies on the use of one or more anti-protozoal drugs, in addition to supportive and often intensive care. Treatment may be prolonged, and may be required for months to years. Although 70% of horses improve one neurologic grade with treatment, this improvement may not be adequate for the horse to return to its previous level of performance. Additionally, some treated horses still deteriorate and must then be euthanized, which represents both an emotional and financial loss to the owner. The average cost of treatment for a horse with EPM is

substantial: it may range from \$400 to \$2400/ month, dependent on treatment choices and level of neurologic deficits. Additional financial losses may be incurred due to loss of winnings, time off from performance, loss of stud revenue, and the economic value of the horse if euthanasia is the end result ^{1,2}. Annual losses in the US are estimated to be \$55.4 to \$110.8 million ¹.

No effective vaccine against the disease is currently available. In order to develop a suitable product, further knowledge about the protective immune response to *S. neurona* infection is required. It is thought that a deficiency in some aspect of the normal immune response in certain horses is responsible for their susceptibility to developing the disease. Further knowledge of the immune response to *S. neurona* infection would also help researchers to develop more sensitive and specific diagnostic assays, to improve treatment outcomes, and to formulate an effective vaccine against the disease. This would dramatically benefit both the individual horse owner and the US equine industry, and reduce the financial and emotional losses currently experienced with EPM.

The goal of the present study is to further characterise the immune response in horses experimentally challenged with merozoites of an SnSAG1 positive strain of *S. neurona*. Various aspects of the immune system will be studied in detail with the aim of elucidating the protective and pathophysiologic mechanisms of the immune response associated with the development of EPM. Overall long-term goals include determining how *S. neurona* alters host immune response in experimentally infected animals, based on changes in immune cell subsets (via flow cytometry) and immune cell function (via lymphocyte proliferation assays). Previously, investigators have determined that both

naturally and experimentally infected horses have suppressed leukocyte proliferation responses when stimulated with the combined mitogens phorbol myristate acetate and ionomycin (PMA/I). The purpose of this study was to determine whether this defect was due to an increase in apoptosis or a decrease in cellular proliferation of a specific subset of leukocytes.

CHAPTER 2: LITERATURE REVIEW OF EQUINE PROTOZOAL MYELOENCEPHALITIS

2.1 HISTORY

The name “equine protozoal myeloencephalitis (EPM)” was first used by Mayhew et al in 1976, although the disease had previously been described by others. Rooney et al first referred to a “segmental myelitis” in cases of equine neurologic disease in 1970⁶. Although the causative agent was not known, the syndrome was outlined as a case series of 52 horses, largely from Kentucky and Pennsylvania. In the decade that followed, protozoa were identified in lesions from horses suffering from ‘segmental myelitis’ by three different groups of researchers⁷⁻⁹. In these early reports, the condition was mistakenly thought to be the result of *Toxoplasma gondii* infection. It was not until 1980 that Simpson and Mayhew proved, with transmission electron microscopy of lesions containing parasites, that the parasite was in fact a *Sarcocystis* species¹⁰. Unlike the tachyzoites of *T. gondii*, the merozoites of *Sarcocystis* species do not contain rhoptries and are not within a parasitophorous vacuole in the host cell. In 1991, Dubey et al. isolated a *Sarcocystis* species from a clinical case of EPM, and named the protozoan *Sarcocystis neurona*¹¹. More recently, *Neospora hughesi* (*N. hughesi*) has also been identified as a possible, albeit much less common, causative agent of EPM^{2,12}.

Once *S. neurona* was found to cause EPM, and the definitive host identified as the opossum (*Didelphis virginiana*), it was possible to use small subunit RNA (ssRNA) gene sequence comparisons of cell-culture derived merozoites and sporocysts separated from opossum intestines to identify the phylogenetic relationship of *S. neurona* to other members of the family *Sarcocystidae*^{11,13}. Using this approach, as well as other methods, four different species of *Sarcocystis* have been shown to infect the horse. These are *S. neurona*, *S. fayeri*, *S. bertrami* and *S. equicanis*.

In some countries, such as Mongolia, sarcocysts may be isolated from the muscles of up to 93% of horses examined¹⁴. Similar studies in the United Kingdom (UK)¹⁵ and Japan¹⁶ have reported cyst recovery rates of 62% and 14% respectively. The precise species of *Sarcocystis* isolated in the above studies was not determined, but was not *S. neurona*, as horses acted as intermediate hosts. Despite the presence of multiple *Sarcocystis* species in horses from varying geographic locations, *S. neurona* is the only *Sarcocystis* species known to cause EPM.

2.2 LIFE CYCLE OF *S. NEURONA*

Sarcocystis species are members of the phylum *Microsporidia*. *Sarcocystis* species typically have a two-host life cycle; the two hosts have an obligatory predator-prey relationship, thus ensuring that the parasite is passed from intermediate to definitive host. The opossum represents the natural definitive host for the parasite⁴ and exhibits minimal clinical signs. Opossums are infected through consuming the dead tissue of the intermediate host, which contains sarcocysts of *S. neurona*. Tissue cysts formed in

muscle of the intermediate host are known as sarcocysts, and are responsible for the transmission of the protozoan when the intermediate host dies or is killed and its tissues are consumed by the carnivorous definitive host. As the opossum digests the tissues, the sarcocyst wall breaks down and releases bradyzoites, which are able to invade cells in the lamina propria of the intestine of the opossum. Only sexual stages of reproduction can occur in the opossum. Sexual reproduction consists of gametogony and sporogony, and occurs in the lamina propria of the intestine of the definitive host. Sporulated oocysts contain two sporocysts, each with four sporozoites. Oocysts are then excreted in the feces of the opossum. The wall is thin, and often *S. neurona* sporocysts break apart from the oocysts while entering the small intestine. Oocysts or sporocysts remain viable in the environment for over a year. They are then ingested on feedstuffs by herbivores, which can be intermediate or aberrant hosts for *S. neurona*. This life cycle is summarized in Figure 1.

Horses are aberrant hosts as only asexual stages, consisting of schizonts and merozoites, are commonly found within equine tissues. Considerable confusion once existed regarding the natural intermediate host, yet recent work has elucidated the complete life cycle of *S. neurona*. Several intermediate hosts have been discovered: these include the raccoon¹⁷, striped skunk, sea otter¹⁸, armadillo and Fisher^{19,20}. Cats are also able to act as intermediate hosts of the protozoan²¹. Once *S. neurona* is ingested by horses or intermediate hosts, sporocysts excyst and release sporozoites that invade the small intestinal vascular endothelium of the host. Asexual reproduction occurs to form schizonts which produce merozoites². In contrast to the epithelial infection in the definitive host, this endothelial invasion can allow systemic infection. It is believed that the merozoites are transported to the tissues of the central nervous system (CNS) for

which they have a tropism, in peripheral leukocytes^{22,23}. Once in the CNS, they continue to undergo schizogony intracellularly in neurons and microglial cells^{10,24,25}. The protozoan is found only within the tissues of the CNS after 21 days of infection, although cases with very short parasitemic phases have been noted^{26,27}. In contrast to the definitive host, both aberrant and intermediate hosts of *S. neurona* can develop neurologic signs and die. Horses are described as aberrant hosts as sarcocysts had not, until recently, been found in equine muscle tissue. Mullaney et al (2005) recently found sarcocysts in the tongue muscle of a 4month old foal infected with *S. neurona*. This filly also demonstrated schizonts in the brain and spinal cord. This is the first case which documents these findings and, although Koch's postulates were not fulfilled, it raises the question as to the horse's ability to act as a natural intermediate host of *S. neurona*²⁸.

2.3 EPIDEMIOLOGY

There are many factors that influence the epidemiology of EPM. Factors include geographic restrictions on intermediate and definitive host ranges, variants in climate and season that may affect the parasite, and equine-specific factors such as age, breed, housing environment and immune status. The influence of each factor will be discussed in this section.

2.3.1 Geographic range of host species

Many studies have compared geographic range of host species and the presence of EPM affected horses. Cases of EPM have predominantly been described within the horse populations of North, South and Central America. This correlates with the presence of both the definitive host as well as large numbers of intermediate and aberrant host species. The opossums from these areas are *D. marsupialis*, *D. albiventris*

and *D. virginiana*. To date, only *D. virginiana* and *D. albiventris* have been shown to be true definitive hosts for *S. neurona*. Thus the distribution of EPM cases correlates with the geographic range of the North and South American opossums. Relatively few cases of EPM have been reported from countries outside these ranges. Sporadic cases have been reported in Europe, Southern Africa and Asia, yet in each case the affected horse had at some point been exported from the United States of America (USA) ²⁹⁻³⁴.

2.3.2 Other mammals in the life cycle

Although prevalence of opossums and wild intermediate host species can affect the prevalence of EPM, they are not the only animals involved in the life cycle of *S. neurona*. Cats were first identified as a possible intermediate host of the parasite by Dubey et al ²¹. Further studies fully elucidated the cat's role as a natural host of *S. neurona*, and this will be summarized briefly ³⁵⁻³⁹.

Gillis et al., in 2003, demonstrated sarcocysts in 10% of cats sampled, but only 5% had antibodies specifically to *S. neurona*. In addition to *S. neurona*, cats were infected with *S. felis*. When cats and horses were in the same environment, the number of cats in a population with circulating antibodies to *S. neurona* specifically increased dramatically ³⁹. Stanek et al (2003) demonstrated that 40% of cats living on horse farms were positive if a horse had been previously diagnosed with EPM. By contrast, only 10% of feral cats with unspecified access to horses were seropositive. Both groups suggested that cats might have a role in transmitting the parasite to horses, and therefore that cats can act as a risk factor in the development of EPM. This potential negative influence of cats within a horse housing environment must be weighed against the positive effect that cats may have through chasing opossums away from food and predation upon

various host species ⁴⁰.

2.3.3 Seroprevalence may be influenced by climatic factors

There have been several published studies assessing seroprevalence across the USA. The Western Blot test, which was developed in 1990, was the main method used to detect antibodies in these studies ⁴¹⁻⁴³. These studies show that approximately 50% of horses in the continental US have been exposed to the protozoan, based on seroconversion. This figure was 10-15% higher in the eastern states where the opossum is more prevalent ⁴¹, and also varied with geographical differences within an individual state.

A study by Blythe et al (1997) examining the seroprevalence of antibodies to *S. neurona* in Oregon discovered an overall seroprevalence of 45% in the state as a whole. There was considerable variation within the state; some regions were as high as 65% (the coastal west) whereas the drier, hotter east was as low as 22% ⁴². These regional differences may be a reflection of conditions that the definitive host or the protozoan prefers. For example, several studies have shown that wooded areas on the property are a risk factor in developing EPM. These areas may provide cover for the opossums, or reflect conditions that are more favorable for the protozoan itself. Regarding the influence of opossums, some studies have shown that limiting opossum's access to feed decreases the chances of them shedding sporocysts in feedstuffs, and thus decreases the risk of EPM. It follows then that higher densities of opossums could increase the risk of the disease ^{43,44}.

With regard to climate and protozoal factors, *Sarcocystis* species survive best in

moderate temperatures and 33% humidity ^{45,46} and thus sporocysts may survive in the environment longer in areas more similar to these ideal conditions than in arid areas with extremes of temperature. In a study by Morley et al in 2008, areas with highest mean annual temperature had the lowest odds of disease. Those with moderate temperatures throughout the year had the highest odds of disease ⁴⁴. This apparent preference for moderate temperatures was reflected in the season of the year in which EPM cases present. Compared to winter, spring and summer represent a 3 x greater risk, and fall a six times greater risk of developing EPM ⁴⁷.

2.3.4 Breed of horse

Factors affecting horses, as well as traits affecting other animals within the protozoan's life cycle, must be considered. These include factors affecting the individual horse's risk of developing EPM, when compared to other equines. Breed of horse is one such factor. Certain studies report a breed predisposition towards the development of EPM, with Thoroughbreds, Standardbreds and Warmblood breeds being more at risk. However, it is these breeds that are commonly used for high level competition, showing and racing: all of which have been demonstrated as additional risk factors ⁴⁴. Use of horse may thus confound breed-associated risk.

Breed not only affects the likely use of the horse, but also reflects the horse's genetic background. Genetics may influence immune response and thus susceptibility to EPM. For instance, Arabian horses have a higher prevalence of severe combined immunodeficiency (SCID) foals due to a mutated gene that is inherited in an autosomal recessive manner. Certain familial lines of Swedish Halfbred horses show increased susceptibility to developing sarcoids due to the equine leukocyte antigen (ELA) ELA-

haplotype A3W13 appearing more frequently within this breed ⁴⁸. Icelandic horses show increased hypersensitivity responses to the bites of the *Culicoides* species biting fly due to increased IgE specific binding patterns within certain members of the breed ⁴⁹. Additionally, in mice, certain strains of mice show increased susceptibility to *Toxoplasma gondii* due to genetic defects ⁵⁰. At this time, there are no known genetic factors associated with increased risk for EPM, but as certain breeds seem to be more predisposed, further investigation is warranted.

2.3.5 Age of horse

In addition to the above-mentioned factors, the horse's age may also impact prevalence of disease. In an owner-response study by Morley et al ⁴⁴, age was the risk factor most strongly associated with disease amongst individual horses. Horses between the ages of 6 and 18 months of age were 4 times more likely to develop EPM than those between 5 and 20 years of age. Horses 18 months to 5 years were twice as likely to develop the disease as those 5-20 years old. The authors theorized that this reflected the younger horses' underdeveloped immune responses, or was a virtue of the fact that horses under high competitive stressors such as those in training, racing or showing disciplines were often younger than 5 years of age. In other studies, older horses were at lower risk than those <5 years of age for developing the disease ⁵¹, although seroprevalence increased with the age of the horse, presumably due to increased exposure to the parasite with time ⁵².

Despite all the risk factors outlined above, only 14 new cases/ 10,000 horses are diagnosed with EPM per year in the USA. This suggests that there are many factors responsible for the development of clinical disease. Many horses challenged with the

parasite mount an effective immune response and are able to clear the protozoal infection without succumbing to neurologic disease ⁴⁴.

2.3.6 Immune status

There are many factors that can influence the horse's ability to respond to immune stimuli. Certain careers such as racing and high-level performance induce stress, and this has been demonstrated to affect immune capabilities. Many have postulated that horses that develop EPM may have an immune deficiency. Based on this hypothesis, some investigators have assessed components of the innate and acquired responses in horses that develop EPM. Others have studied the influence of stress on the development of clinical signs of the disease. Generally, chronic stress can suppress the normal immune response and may predispose an animal to developing many different diseases. Stress increases circulating catecholamines through upregulating sympathetic nervous system stimulation. Catecholamines, such as epinephrine and norepinephrine, act as neurotransmitters, and are released from the presynaptic membrane in response to an action potential ⁵³. They allow continuation of the nerve impulse, but also exert a neuroendocrine effect on various body systems. As lymphocytes and monocytes, and potentially other immune cell subsets, possess receptors for these hormones, these cells are affected by changes in catecholamine expression ⁵⁴.

Catecholamines modulate a range of immune cell activities, including cell proliferation, cytokine and antibody production, lytic activity, receptor expression, and cellular migration/chemotaxis ^{55,56}. Changes in epinephrine levels reflect direct lymphocyte migration from bone marrow, the extremities, and the thymus to other

areas of the body⁵⁷. Laboratory stress placed upon human subjects can suppress natural killer (NK) cell activity, suppress T-lymphocyte proliferation when stimulated with various mitogens, and lower both the percentage of CD4+ T helper cells and the ratio of CD4+ to CD8+ T lymphocytes in circulating blood⁵⁸. The exact mechanisms of this immunosuppression are not clear.

Stress is presumed to affect the horse in similar ways to the effect seen in other species; this includes suppression of the immune response. Horses in high stress environments, such as young horses in race or show training or other competition, are more at risk of developing EPM^{44,47}. This fact was utilized by Saville et al. in 2001 in the development of an experimental model of EPM. Long-distance transport stress was performed just prior to infection, and EPM was successfully induced in these horses⁵⁹.

2.4 EXPERIMENTAL ANIMAL MODELS.

There have been many hurdles to overcome in developing an experimental model of EPM in the horse. Initial attempts to experimentally reproduce EPM were unsuccessful, and limited knowledge of the parasite's natural life cycle hampered the development of the most suitable route(s) of infection. Therefore extensive work was performed on developing a reproducible model for the study of the disease in a controlled environment: to that extent, many different methods have been employed. The use of murine models has been widespread. Mice are generally less expensive to obtain, maintain and house, and they are more readily available than horses. Mice are also available in numerous immunodeficient strains, and have less genetic variation than horses, which enables more extensive studies of the immune response to *S. neurona* to be performed. Murine specific reagents are also widely available for immune

assays.

2.4.1 Models in mice

Murine models became significant in studying EPM once the potential role of the immune system in the disease pathogenesis was revealed. Marsh et al ⁶⁰ were the first to develop a mouse model in 1997. They established an *S. neurona* mouse model using an approach that was similar to that used in models for the study of *Toxoplasma gondii* and *Neospora caninum*. They found that some *S. neurona* infected mice developed encephalitis, and the immune status of the mice affected disease susceptibility. C57BL/6 nude mice, without normally functioning T cells, developed encephalitis, yet immunocompetent C57BL/6 mice or ICR severe combined immunodeficient (SCID) mice, that lacked B and T cells, did not.

Since this initial research was published, many different models have utilized various strains of mice. SCID mouse strains cannot clear the parasite, but do not develop neurologic disease. Nude mice, which have no T lymphocytes, developed clinical signs when challenged with appropriate doses of *S. neurona* merozoites or sporocysts. As lymphocytes were determined to play a key role in the pathogenesis of EPM, other mice strains have also been used to further delineate the immune response to *S. neurona* infection. Dubey and Lindsay developed a model using interferon gamma knockout mice (GKO) in 1998, which was refined in 2001 ^{26,61}. Interferon gamma mediated responses by both CD4+ and CD8+ cells had previously been demonstrated to be critical in the protection from *T. gondii* infection ⁶², with CD8+ cells being most important. It was therefore postulated that this would be the same for infection with *S. neurona*. Although depleting IFN γ compromises both innate and adaptive immune responses, some

components of the immune system are unaffected, and both T and B lymphocytes are present. GKO mice can mount an immune response, but it is delayed or reduced when compared to normal. *S. neurona* infected GKO mice develop clinical disease.

Both merozoites and sporocysts can be infectious when given orally to GKO mice²⁶. These mice exhibited dose dependent clinical signs of encephalitis, including blepharospasm, mild to severe ataxia, circling, head tilt, and paralysis⁶³. The increased susceptibility of GKO mice to *S. neurona*- induced abnormalities, when compared with normal mice or SCID mice, leads us to conclude that IFN γ , and the cell subsets that it governs, are crucial in protecting mice from developing clinical disease⁶⁴⁻⁶⁹. IFN γ is the most important cytokine in modulating the cell-mediated (CD4+ Th₁/ CD8+ Tc₁) arm of the immune response. A major function of IFN γ is to activate macrophages to phagocytose intracellular pathogens⁷⁰. It is secreted by a variety of cells, including Th₁ CD4+ cells, CD8+ Tc₁ cells, NK cells, macrophages, dendritic cells, and brain cells such as astrocytes and microglia. These cell subsets are all important in generating a protective immune response to *S. neurona* infection: however, CD4+ and CD8+ cells have been demonstrated to play the most critical roles.

Witonsky et al. demonstrated that, in immunocompetent mice, a significant increase in total CD8+ splenocytes and CD8+ peripheral blood lymphocytes occurs following infection with *S. neurona*. Endothelialitis and meningoencephalitis developed in CD8+ knockout mice following challenge with the protozoan, highlighting the importance of this cellular subset in protection against the disease. *S. neurona* infected CD8+ KO mice developed disease whereas infected CD4+ KO mice did not, suggesting that CD8+ cells had a more important role in preventing disease⁶⁶⁻⁶⁹. In addition to the adaptive immune response, innate cells and mediators also contributed to the response

to infection with *S. neurona*. Nitric oxide, a multifunctional molecule that has roles in vasodilation, immune mediation and neurotransmission, has also been studied in relation to *S. neurona* infection. Increased concentrations of NO have been found in association with infections of various parasitic protozoa, and studies by Rosypal et al. examining inducible and endothelial nitric oxide synthase gene knockout mice demonstrated that these strains were resistant to infection ⁷¹. Sellon also demonstrated that SCID mice did not develop disease unless treated with IFN γ antibodies. As these mice lacked lymphocytes, this suggested that there were other IFN γ -producing cells that helped to prevent disease. As treatment with NK antibodies did not cause disease, this suggested that IFN γ dendritic cells may have been critical in preventing encephalitis in *S. neurona* infected SCID mice ⁶⁵.

In summary, both CD4+ and CD8+ cellular subsets, plus IFN γ from multiple sources, are critical for a successful protective immune response against *S. neurona* infection in mice. Innate cells, NK cells and possibly dendritic cells also have roles in protection from disease. The question remains as to whether the same protective immune response is critical in horses.

2.4.2 Models in horses

For many years, a reliable model of inducing disease in horses did not exist; therefore, using other species for models was a necessity. As mentioned above, both the life cycle of the parasite and the horse's role in this cycle were not known until recently, thus hampering the development of a suitable model. A variety of experimental conditions were utilized, yet it was often not possible to reliably induce clinical signs of EPM, even in horses considered infected based on titer and exposure. This may be in

part due to the fact that only a fraction of exposed horses develop clinical signs of EPM: simply infecting immunocompetent horses with *S. neurona* may not be sufficient to reproduce clinical disease in all horses, as the immune system of the immunocompetent horse may limit disease.

Fenger et al reported the first equine model successful in inducing clinical signs in the horse in 1997⁴. Horses were fed sporocysts from a variety of different protozoa: only *S. neurona* induced signs of EPM. The *S. neurona* infected horses developed antibodies to the parasite in both serum and CSF, but Koch's postulates could not be fulfilled, as the protozoan could not be identified on histopathologic analysis of tissue sections. Saville et al., Sofaly et al. and Ellison et al. have generated several different models for successfully reproducing disease under experimental settings. Sofaly and Saville have developed methods by which horses are inoculated with oral sporocysts via nasogastric intubation, whereas Ellison uses intravenous infection techniques^{27,72-74}.

In Sofaly's model, published in 2002, weanling horses were inoculated on a single occasion with a variety of doses of sporocysts via nasogastric intubation⁷². Mild to moderate neurologic signs were induced in most of the challenged horses, with the most severe signs seen in those given the highest dose of sporocysts. However, not all challenged horses developed disease, or became seropositive in either serum or CSF. Seroconversion occurred in the majority of infected horses, and there was a dose and time dependent response to seroconversion. Histologic lesions consistent with *S. neurona* infection were demonstrated in 20% of infected horses, yet parasites themselves could not be identified. Inoculation of tissues from the infected horses into interferon gamma knock-out (GKO) mice did not produce disease, indicating that no

parasites were present in the tissues. Control horses did not develop clinical signs of the disease: this was in contrast to previous studies utilizing oral inoculation methods ^{59,75}. In 2005, Rossano et al used oral inoculation to induce parasitemia in immunocompetent horses: however, none of these horses developed clinical signs of the disease ⁷⁶.

As Sofaly's model was capable of reproducing disease in a moderate percentage of horses, efforts were made to improve this model both with respect to the number of horses demonstrating clinical signs as well as the ability to isolate the organism, and fulfill Koch's postulates. In order to improve the model, immunosuppressive events were incorporated. Transportation, as a means of inducing stress, led to more severe signs ⁷². As an extension to this work, Saville et al. ⁷⁴ studied the effect of two periods of shipping (representing a period of stress) on horses infected via oral sporocyst ingestion. Sporocysts of known strain and infectivity were used, and administered orally: horses were inoculated after a short transportation, and split into groups. One group was not shipped a second time, whereas the other groups were subjected to another transport period. Various intervals between inoculation and a second transport were then examined. All horses seroconverted before the end of the study period. Horses that were not subjected to a second transport exhibited more severe clinical signs than any of those that were transported a second time. This finding was in contrast to those from previous studies. The protozoan was not detectable on examination of the tissues at post mortem. The second transport stress period therefore did not create more severe disease, or enable isolation of *S. neurona* from the horses.

These studies indicate that most naturally infected horses, when challenged with *S. neurona*, can routinely mount a successful immune response and prevent serious and

long-lasting clinical disease from occurring. Stress alone may be insufficient to precipitate EPM, or may be less important than other factors, such as immunodeficiency, in mounting an appropriate response. It is likely that multiple factors contribute to susceptibility to EPM.

Besides Sofaly's and Saville's designs ^{72,74}, another successful model for experimentally inducing and studying EPM was designed by Ellison ^{27,77}. Ellison's model of experimental infection utilizes autologous cells, infected *in vitro* with merozoites, to inoculate naïve horses. Lindsay et al demonstrated that the merozoites enter equine leukocytes; most commonly they are found within mononuclear cells ⁷⁸. The infected cells migrate through the blood and traverse the blood brain barrier, allowing for infection of the CNS. *Sarcocystis neurona*, isolated from the spinal cord of an infected horse, was cultured using bovine turbinate cells, and then used to infect lymphocytes separated from peripheral blood from naïve horses. Lymphocytes provided an intracellular location and a hematogenous method of distribution for the parasite in the host, and are thought to be necessary in order for the parasite to cross the blood-brain barrier into the CNS, where they are largely sequestered from normal immune defense mechanisms.

For Ellison's model, each horse was inoculated intravenously with approximately 6000 *S. neurona*-infected autologous leukocytes on a daily basis for 14 days. Merozoites were demonstrated in circulating lymphocytes and a few monocytes following infection, and remained viable when infected lymphocytes were incubated for 24 hrs in Roswell Park Memorial Institute cell culture medium (RPMI). Horses in this study that were infected in this manner developed cranial nerve deficits, ataxia and encephalitis. Early

signs of neurologic disease coincided with an increase in *S. neurona*-specific IgG in the CSF. Histopathologic lesions were demonstrated, and were consistent with those occurring in natural cases of EPM. *S. neurona* was recovered from cultures of the spinal cord in four horses. In order to optimize the dose and onset and severity of signs, a variety of different doses of merozoites were studied. It was determined that either 1,000 or 10,000 organisms administered daily for at least 7 days was necessary to induce marked changes in the CNS, and allow recovery of the organism *in vitro* ²⁷.

In order to further elucidate the pathology associated with infection, Long et al. developed another model imitating natural infection via the gastrointestinal tract. To determine whether lymphocytes alone were responsible for transporting the parasite to the CNS, or whether other leukocytes played a role, Long et al. ²² studied the infection of SCID foals with sporocysts. They were able to recover *S. neurona* organisms from the blood of a 5-month old Arabian foal with severe combined immunodeficiency (SCID) three weeks after it was challenged with orally administered sporocysts. This foal, although undergoing parasitemia, did not develop neurologic signs, and the parasite could only be isolated from visceral tissues. Sellon et al. ²³ examined Arabian horses with and without SCID that were given oral sporocysts: those with SCID developed severe parasitemia and persistent infection of visceral tissues including the lung, liver and spleen, which they were unable to clear. The CNS remained unaffected however, and the horses did not develop clinical disease. By contrast, immunocompetent Arabians in the same experiment were quickly able to control the parasitemia induced by challenge with the parasite, but the organism could be recovered from the CNS, and these animals displayed neurologic clinical signs.

This study suggested that the parasitemia caused by *S. neurona* infection was controlled by specific, adaptive immune responses that are lacking in SCID foals. SCID animals lack functional B and T lymphocytes. It was postulated that *S. neurona* gained access to the CNS via its intracellular location within lymphocytes, and can cross the blood-brain barrier into this immunoprivileged site. This would explain why only immunocompetent animals, and not SCID animals, developed clinical signs of EPM and displayed evidence of the parasite in tissues of the CNS. The exact deficiency that led to disease in some immunocompetent animals remains to be elucidated. A study by Lindsay et al. (2000) using free merozoites inoculated directly into the CNS caused seroconversion but failed to reproduce clinical disease, suggesting that an intracellular location allows parasitic survival and potentiates disease ⁷⁹.

In order to further define potential abnormalities in the immune system, responses in naturally infected horses have also been extensively studied. Tornquist ⁸⁰ and Spencer ⁸¹ demonstrated that horses with EPM, either naturally or experimentally induced, have suppressed antigen specific immune responses to merozoites and SnSAG1 respectively. Additionally, Tornquist et al. were able to demonstrate a decreased percentage of CD4+ cells in EPM horses compared with normal equines. Spencer demonstrated that naturally and experimentally infected horses had decreased IFN γ and increased IL-4 gene expression compared to normal horses. As an extension of these studies, and in order to better define the existing immune deficiencies, Yang et al. ⁸² examined leukocytes from naturally infected horses with EPM, and compared immune cell profiles with control horses. Naturally infected horses had significantly higher percentages of CD4+ lymphocytes versus normal horses, and had less viable CD8+ lymphocytes compared to normal horses, although the latter was not statistically

significant. No significant differences in number of B cells, or in the viability or apoptosis of CD4+ cells, CD8+ cells, B cells, monocytes or neutrophils between EPM and normal horses, was observed. Leukocyte function was also assessed, and a significant difference in phorbol myristate acetate/ionomycin (PMA/I) stimulated proliferation was detected. Proliferation responses in EPM horses were significantly lower than in normal horses, after blood samples had been stored for one day. Yang et al. postulated that this decreased proliferation response may be due to increased apoptosis induced by PMA/I, interference in cell signaling pathways due to *S. neurona* infection and/or decreased proliferation responses, or changes in leukocyte subsets.

A subsequent study to determine the mechanisms of suppression, and whether the differences existed in experimentally infected horses, was performed by Witonsky et al.⁸³. Immune responses of horses infected through the intravenous model described above were examined. Infected horses had significantly decreased proliferation responses as measured by thymidine incorporation into dividing lymphocytes, when cells were incubated with the non-specific mitogen PMA/I, as soon as 2 days post infection. Infected horses had decreased percentages of viable B cells at certain time points following infection. At Day 28 post-infection, there was a significant decrease in the percentage of viable CD8 cells, and an increase in percentage of neutrophils present in infected versus control horses. No significant changes in CD4 subsets were noted. Antigen-specific proliferation responses to merozoites or SnSAG1 antigen were also not observed with the conditions used.

The mechanism behind many of these findings has not yet been elucidated, and our study aims to further examine immune suppression in response to *S. neurona*

infection, and the cellular subsets responsible.

2.5 CLINICAL SIGNS

EPM predominantly affects the equine nervous system, therefore clinical signs are largely those associated with neurologic deficits. Clinical signs may be associated with the immune response to infection, or be a direct result of neuronal damage by *S. neurona* in the CNS.

In 2003, Ellison reported the early clinical signs seen in horses experimentally infected with *S. neurona* via autologous lymphocytes⁷⁷. The first signs noted in these horses were reduced aggression within the group, a reluctance to come in for food, and a slower eating time than before infection. When eating, the infected horses drooled considerably more than previously noted, and had decreased tongue tone resulting in an increase in the amount of dropped food. Mild and often transient eyelid and lip paresis was observed in some horses. These cranial nerve signs appeared to improve as the disease progressed, and ataxia developed. Ataxia, weakness and recumbency/unusual stances were noted in most of the horses, and these signs progressively worsened throughout the experimental period. The presence of lameness, weakness and ataxia was noted later in the course of the disease than the behavioral and cranial nerve changes mentioned above.

The most commonly reported signs of EPM include ataxia, weakness and muscle atrophy. These may be symmetrical, but are more often asymmetric, and can be focal or multifocal due to the organism's capacity to infect multiple areas of the CNS concurrently. Initial signs may be mistaken for mild lameness of a musculoskeletal

origin: lameness that is unilateral, involves an element of incoordination and is not exacerbated by flexion may be the result of EPM. Lethargy, seizures, exercise intolerance, difficulties executing complex movements and further cranial nerve signs such as headshaking, dysphagia, and facial paralysis may also be reported ².

Clinical signs of the disease may be gradual in onset or may progress rapidly to recumbency and other severe neurologic deficits. Some horses plateau for an indeterminate time, although some may still progressively worsen from this static stage. On physical examination, vital signs are usually normal and most horses are bright and alert, although some horses appear thin and mildly depressed ¹. A full neurologic examination should be performed. This often reveals asymmetric weakness, ataxia, and spasticity in one or more limbs, and some horses have areas of hypalgesia. Horses with lesions localized to several different areas within the CNS, yet with relatively normal neurologic function of those areas anatomically between affected sites, should also be considered suspect for EPM.

Other differential diagnoses for a neurologic horse include Equine Herpes Myeloencephalopathy (EHM), West Nile Virus, Cervical Vertebral Myelopathy/Instability (CVM/CVI), Eastern and Western Equine Encephalitides, Rabies and Equine Motor Neuron Disease, although this list is by no means exhaustive. A full diagnostic work up of the case will help to rule out these other causes of equine neurologic disease.

2.6 DIAGNOSIS

2.6.1 Neurologic Evaluation

The neurologic evaluation of the horse is a complex examination that, when performed correctly, may reward the clinician with vital information to localize the neurologic deficits and to determine which additional diagnostics are required.

A full history is important: the age, breed, sex and primary use of the horse may make certain differentials more or less likely. The clinician will attempt to determine the duration of clinical signs, first deficits noted and owner's perception of their progression. The level of performance that the horse is currently in, and was performing at over the past year, is also important.

The horse is observed whilst at rest in its usual environment, and then a thorough physical examination, including assessment of temperature, heart rate and respiration rate, is performed. The veterinarian will assess cranial nerve responses, position of limbs and head/neck, and degree of muscling to determine whether this is symmetrical and adequate for the specific training undergone by the horse. Tests of the horse's ability to swallow, prehend food and to see and hear adequately will be performed as part of the assessment of cranial nerves. Strength tests for assessing paresis of each of the four limbs should be performed, and each side of the horse compared. This involves pulling the horse's weight to one side or the other, and assessing its ability to resist this pressure whilst standing and whilst walking. An assessment of the panniculus (skin twitch) reflex is made at this time. Tail tone and anal tone may also be evaluated.

Conscious proprioceptive deficits, as an assessment of ataxia, are evaluated by

positioning the horse's legs in abnormal locations, and observing the horse's response. A normal horse will resist this unusual placement, or quickly return the leg to a more normal position. Neurologic horses may have less perception on the exact location of their extremities, and so may leave the leg in a crossed-over or wide-based position.

Gait assessment is then performed to further assess paresis (weakness) and ataxia (incoordination). The horse is first observed walking on a flat surface and interference between limbs, the degree of lameness, and asymmetric findings are recorded. The horse is turned in large and small circles, and observed for limb interference, pivoting, circumduction of a leg, toe-dragging, or imbalance. Occasional horses will appear dizzy after circling when suffering from cerebral neurologic disease. A "tail-pull" performed whilst walking assesses the horse's ability to resist pulling from one side on their hindquarters- a normal horse without weakness or ataxia is able to resist this pull and continue to walk with all four feet in a straight line. The horse may be walked up and down an incline, backed up and asked to trot in order to further evaluate its neurologic status. Urination and defecation, along with the ability to eat normally, should also be assessed if they have not been observed already through the course of the examination^{84,85}.

Following completion of the neurologic exam and localization of the deficits, differential diagnoses are prioritized. Venous blood and other samples may then be obtained, to allow further diagnostics to elucidate the disease responsible for neurologic signs.

2.6.2 Cytology and Cerebrospinal fluid indices (CSF indices)

The collection of cerebrospinal fluid from either the atlanto-occipital or the

lumbosacral space is a key part of an equine clinician's diagnostic evaluation of a horse that presents with neurologic disease. Information can be gained from the color, consistency, protein content, level of hemorrhage and white blood cell content of the CSF. For this to be performed adequately, a portion of the sample is best submitted for a cytologic evaluation. This will enable a full examination of the cells present in order to ascertain both the quality of the sampling technique, and the likely causative agent. Excessive numbers of erythrocytes may be suggestive of trauma or vascular damage, but they may be iatrogenic, as a result of poor sampling technique. The presence of more than 8 RBC/ μ L⁸⁶ can cause a false positive result on a Western Blot in a seropositive horse whose neurologic symptoms are not the results of EPM. Elevated protein levels may be a result of contamination, but may also be the result of a leaky blood-CSF barrier, as is the case in many neurologic diseases including EPM. White blood cell numbers and differentials are important to distinguish between EPM and other diseases, although the changes induced by EPM are neither pathognomonic nor consistent¹. EPM affected horses may have an increased leukocyte count, largely due to an increase in mononuclear cells, if changes are present.

CSF samples can be analyzed for presence of *S. neurona* through a variety of diagnostics (detailed below), and CSF indices may also be calculated to determine levels of intrathecal antibody production. In a healthy and non-EPM affected animal, *S. neurona* specific IgG found in the CSF is derived from the serum or from B-lymphocytes primed in the periphery^{87,88}. The blood-brain barrier (BBB) is intact in the healthy animal, and therefore limited amounts of IgG are able to cross. In diseased animals where the CNS and BBB are affected, IgG may also be derived from the parenchymal tissues of the CNS (i.e. "intrathecal antibody production"). The IgG index was developed

to aid in the differentiation of intrathecal antibody production from serum-derived antibodies to the same organism. It is calculated by dividing the amount of IgG found in the CSF by the total serum IgG, and is also affected by the albumin quotient (AQ), as this reflects damage to the BBB. Values greater than 0.27-0.30 are thought to represent intrathecal antibody production ^{87,88}.

The albumin quotient (AQ) is the ratio of CSF albumin to serum albumin, and when elevated indicates an increased permeability of the blood-brain barrier, or blood contamination. Indices must be interpreted with caution as there are other diseases that could increase blood-brain barrier permeability and allow increased *S. neurona* antibody to be present in the CSF (due to leakage of antibody into the CSF in a seropositive animal), although EPM may not be the cause of clinical signs. Furr also demonstrated that serum antibodies or B-cells producing antibodies can cross into the CSF, using ovalbumin vaccination of healthy animals. These animals can thus have antibodies in the CSF but be neurologically normal ^{87,88}.

In addition to the IgG index, other indices such as the Goldman-Witmer Coefficient (C-value) and the antibody index (AI) have been described. Both of these techniques have been favored over the IgG index as they use antigen-specific antibody titers, rather than total IgG. These techniques have become standard in human neuro-immunology literature.

The C-value was developed based on the principle that the ratio of an antigen-specific antibody to total IgG in the CSF was equal to the ratio of that antibody to total antibodies in the serum. Intrathecal production of an antigen-specific antibody resulted

in a value greater than 1, whereas passive movement of antibodies across the blood-brain barrier (BBB) gave a value of less than or equal to 1. In work by Furr et al. (2002) on the C-value in normal horses, values up to 1.7 were seen. This suggested that values >1.7 were indicative of intrathecal antibody production. Further work by Furr in 2007 illustrated that normal horses had values around or equal to 1, whereas horses challenged with intrathecal administration of ovalbumin (or presumably another antigen) had a mean value of 7.48, representing a marked increase ⁸⁸.

The AI was also developed to detect CSF-derived specific antibodies in the CSF. It has been found to increase significantly in a number of inflammatory diseases in people, where normal values were less than one. In normal horses, AI values up to 0.76 were recorded ⁸⁹. After intrathecal immunization in the Furr 2007 study, affected horses had an AI value of 5.9, which represented a significant increase. Furr (2007) also examined the AI and C-value of the horses immunized with ovalbumin with respect to herpesvirus antibodies; the purpose of this was to demonstrate the increase in each value due to inflammation-associated damage to the BBB, which was not associated with intrathecal production of specific antibodies. Of the two values, the AI increased only mildly in the herpesvirus horses, suggesting that this was the more sensitive of the two indices in its ability to detect active secretion of antigen-specific IgG.

These indices may provide additional, valuable information when analyzing the CSF of horse with suspect EPM.

2.6.3 Immunoblot assay (Western Blot)

The first antemortem diagnostic method to be developed for EPM testing was the

Western blot, developed by Granstrom et al. in 1991. Prior to this, there had been no definitive test for the disease in a live horse. The Western blot is an immunoblot assay that identifies *S. neurona* specific antibodies in the test fluid (serum or CSF), and separates proteins based on their molecular weights. The test uses culture-derived merozoites as antigens, and was originally developed using antisera from horses with clinical EPM and polyclonal rabbit antisera against a variety of *Sarcocystis* species⁴¹. By comparing the reactivity of these different species to the protein profile of *S. neurona*, eight proteins specific to infection with *S. neurona* were isolated. These became the basis of interpretation of the test in cases of EPM. The specific proteins used for diagnostic testing have molecular weights of 7, 13 and 14.5 kDa. Additional immunodominant bands at 16 and 30 kDa are nonspecific. The unmodified Western blot test has been shown to have a sensitivity and specificity of 89%¹.

To perform the test, *S. neurona* antigens are separated via gel electrophoresis, and then transferred to a membrane, where they are incubated with the serum or CSF from the horse to be tested. Alkaline phosphatase is used to develop the immunoblot. Rossano et al. (2000) suggested a modification of the test to boost sensitivity and specificity to close to 100%. This modification eliminates cross-reaction with *S. cruzi* (a species seen in cattle and dogs) by incubating the immunoblot with sera from cattle that have been exposed to *S. cruzi* before the addition of equine samples. This modification improves the accuracy of the test, as *S. cruzi* binds non-specific bands thus increasing the specificity of the results⁹⁰.

A positive Western Blot result will indicate the presence of antigen-specific antibodies, but does not give a quantitative result. If serum is used for the WB test, it

also does not differentiate between clinical cases of EPM and those horses that have been exposed to the protozoan, mounted an immune response, and have not or will not develop clinical disease. In some areas of the US, >65% of horses may be seropositive for *S. neurona*, yet only 1% of these develop disease: if Western blots were performed on only serum from all these horses, the majority of positive results would likely represent false positives for disease, and hence the test must be used judiciously. It is usually recommended to test only those horses demonstrating clinical neurologic disease, and the test should not be used as a screening test of clinically normal horses.

Approximately 1 in 10 horses with neurologic abnormalities due to another disease will test positive for *S. neurona* antibodies in the CSF on a Western Blot¹. This is due to the presence of blood-derived antibody within the CSF. Various neurologic diseases may result in compromise to the blood-brain barrier, causing antibodies from the bloodstream to leak into the CSF and cause a false positive test. This occurs with as few as 8 red blood cells per microliter, and may also be due to poor collection technique, due to iatrogenic hemorrhage into the CSF sample^{1,86}.

2.6.4 Immunofluorescence Assay (IFAT)

Indirect fluorescent antibody tests (IFATs) have been used for the diagnosis of protozoal diseases in many species. In horses this has included the diagnosis of infection with *Neospora spp*, *Babesia spp* and *Trypanosoma spp* species. Concern initially about the potential for cross-reactivity with other equine *Sarcocystis* species precluded the use of the IFAT for the diagnosis of EPM, although work by Duarte et al in 2003 appeared to show that the accuracy of the test was better than that of the Western Blot

91.

To perform the IFAT, merozoites of the UCD-1 strain of the protozoan were used as the test antigen: this is a SnSAG1 positive strain. Approximately 20,000 merozoites in 10 μ L were added to each well in a 12-well slide, and allowed to air dry. They were then fixed in formalin, washed twice with PBS then frozen at -70°C for later use. Fluorescein-labeled (FITC) antibodies directed against horse specific IgG were added to the wells. Serial dilutions with the test animal serum were begun at 1:5, and continued until an endpoint titer was reached. This was defined as the last serum dilution demonstrating distinct fluorescence ⁹¹.

2.6.5 Serum Agglutination Test (SAT)

A serum agglutination test was developed by Lindsay and Dubey in 2001 using experimentally infected animals. The SAT detects antibodies to *S. neurona* in serum or CSF. It does not require species-specific reagents to do so ⁹². Merozoites of the SN6 strain of *S. neurona* were collected from cell culture and used as antigen. Beta-2-mercaptoethanol was added to the antigen suspension to destroy IgM antibodies, in order to prevent nonspecific agglutination reactions. Serial dilutions of 25 μ L of serum or CSF from the test animal were then added to 75 μ L of the antigen solution; this was mixed well and incubated overnight. Positive and negative control mice were used to examine and typify the varying agglutination reactions.

The test demonstrated a sensitivity of 100% and a specificity of 90% in mice, which represented an improvement in these markers of reliability when compared with the widely utilized Western blot. The SAT has demonstrated some cross-reactivity in rabbit specimens, however. There have not been sufficient numbers of horses studied to

publish sensitivity/specificity values in this species ⁹².

2.6.6 Polymerase Chain Reaction (PCR)

Fenger et al. developed a suitable PCR test for use in cases of EPM in 1994. It was initially marketed for use on samples from the CSF of horses with neurologic disease, with the main advantage over conventional techniques being the ability to diagnose positive cases early in the course of infection ⁹³. Miller et al. (1999) reported cases that test positive for *S. neurona* DNA, but are negative for antibodies to the protozoan, when diagnostics are performed on CSF ⁸⁶. The sensitivity and specificity are not optimal for the diagnosis of EPM cases, and are less than originally reported. One of the reasons for this may be that intact merozoites are rarely found in the CSF, and free DNA is destroyed rapidly by enzymatic action ⁹⁴. *S. neurona* is a largely intracellular organism. A positive PCR result can be considered an accurate and specific diagnosis; however there are many limitations in its usefulness and therefore PCR is considered a useful adjunct diagnostic.

2.6.7 Enzyme-linked immunosorbent assay (ELISA) to *Sarcocystis neurona*

The above diagnostic methods, with the exception of PCR, require the use of culture-derived merozoites in order to perform the analyses. ELISA methods have been developed that negate this need for expensive propagation of *S. neurona* in tissue culture, relying instead on recombinant proteins that have been isolated, based on their similarity to families of antigens characterized in other protozoal species. ELISA methods also allow a quantitative result to be generated.

2.6.7a Surface Antigen 1 (SnSAG1)

Merozoites of *S. neurona* express genes for at least five different surface antigens; these have been described as SnSAGs. These proteins are abundant and relatively immunodominant, and were identified due to their similarity to similar proteins expressed by other protozoa, such as *T. gondii* and *Neospora spp*^{95,96}. Recently, the production of a large database of expressed sequence tags (ESTs) for *S. neurona* has provided significant information regarding the genetic composition of the organism⁹⁵.

Initial work by Ellison et al. in 2002 identified a dominant protein at the 29 kDa band on *S. neurona* positive immunoblot tests. The gene encoding this protein was cloned, sequenced and expressed as a recombinant protein, SnSAG1. This was then used to develop an ELISA that demonstrated the ability to positively identify horses with experimental EPM infection⁹⁶. To perform the ELISA, 96-well plates were coated with recombinant protein by adding 50 µL rSnSAG1 to each well, and incubated overnight at 4°C. The plates were then washed with phosphate buffered saline with Tween (PBST), and blocked using 1% bovine serum albumin in PBST, and incubated at room temperature for 30 mins. The plates were then washed three times in PBST, and either 50 µL of serum or 50 µL of CSF was added. This was diluted in dilution buffer. The plates were then incubated for two hrs at room temperature, and washed a further three times with PBST. 50 µL of secondary antibody (antihorse IgG-alkaline phosphatase) was added to each well, diluted to 1:3000 in PBST. Incubation of one hour followed, then three washes with PBST. Para-nitrophenol phosphate (a freshly-made substrate) was prepared in carbonate buffer, and 100 µL added to each well. After 45 mins of incubation at room temperature, the plates were read using a microplate reader equipped with a 405nm filter. All test horses in the initial study became seropositive on

the ELISA. The antibody levels were significant by 4 weeks post infection: a serum titer of 100 or greater correlated with clinical signs of EPM, and clearly differentiated between uninfected and acutely infected horses ⁹⁶.

The SnSAG1 ELISA has since been made commercially available, and has the advantage over many other diagnostic methods in that it generates a quantitative result which may be used to assess treatment efficacy and disease progression. Recent work by Crowds et al has demonstrated the presence of SnSAG1 negative strains, however. Therefore, a negative SnSAG1 result does not exclude EPM as the causative agent, and work continues in developing commercially available ELISA tests for SnSAG1 negative strains. Crowds demonstrated that the surface antigen SnSAG5 is mutually exclusive to SnSAG1 ⁹⁷, although a diagnostic test for the presence of SnSAG5 is not currently available to the practitioner.

2.6.7b Other surface Antigens (SnSAG2 etc)

One of the proposed problems with the SnSAG1 ELISA for the diagnosis of EPM is that certain strains of *S. neurona* may not display the SnSAG1 protein. The prototypic SnSAG1 has been shown to be absent in cultured *S. neurona* isolates from two different EPM horses ^{98,99}. Other surface antigens are being investigated that, it is hoped, are shared by all strains of the protozoan. These have been designated as SnSAG2, SnSAG3 and SnSAG4. A study by Hoane et al. in 2005 compared the recombinant forms of each protein, plus that of SnSAG1, with Western blot analysis of *S. neurona* merozoites to determine the reliability and accuracy of these assays ¹⁰⁰.

Based on these evaluations, the rSnSAG2 ELISA demonstrated the highest

accuracy, with a sensitivity of 95.5% and specificity of 92.9% at a cutoff positive predictive (PP) value of 20%, when compared with the Western Blot. Recombinant rSnSAG1, by contrast, had only 68.2% sensitivity and 71.4% specificity at the optimum PP of 10%. Serum antibody titers were detected against rSnSAG4 in 96.2% of EPM horses, against rSnSAG2 and rSnSAG3 in 92.3%, and against rSnSAG1 in 69.2% of EPM horses ¹⁰⁰.

SnSAG2 may cross react with *S. fayeri* at dilutions of 1:250 or less, but this is not thought to adversely affect interpretation of the test for EPM, as *S. fayeri* does not cause clinical disease in the horse. Other assays did not cross-react with *S. fayeri*. Unfortunately, ELISAs for surface antigens to proteins other than SnSAG1 are not widely or commercially available at this time ¹⁰⁰.

SnSAG5 is the most recently discovered surface antigen, and was found to be the dominant surface protein in 6 out of 13 parasite isolates. None of the SnSAG1 positive strains expressed SnSAG5 ⁹⁷. SnSAG5 is similar in amino acid sequence to SnSAG1 and was therefore mistakenly labeled as SnSAG1 in certain previous work⁹⁷.

This abundance of different surface antigens may affect the ability to generate immunity to EPM through vaccination: vaccination with one strain only will not provide immunity against subsequent challenge with a dissimilar strain, unless there is cross-reactivity between the strains ⁹⁷. A polyvalent vaccine may therefore prove more efficacious.

2.7 PATHOLOGY

Considered the absolute gold standard in the diagnosis of EPM for many years, the pathology associated with the disease is usually confined to the CNS ⁷⁻⁹. Lesions may be multifocal, focal or diffuse. Most lesions are found within the spinal cord (especially lumbar and cervical regions) ⁶, although the brainstem is the area of the brain most commonly affected. Many EPM-affected horses show no gross abnormalities on necropsy ^{1,101}. Acute lesions mainly consist of randomly distributed foci of hemorrhages, whereas chronic lesions show areas of pale to dark tan discoloration and foci of malacia. Hemorrhage may be evident in the gray or white matter, and may be diffuse or punctate. On cross section, the edematous tissue may bulge.

Lesions can be further classified by means of histology. In peracute cases of EPM, lesions contain pronounced perivascular cuffing of lymphocytes, small areas of hemorrhage, and minimal tissue destruction and phagocytosis. Horses most commonly examined are acute to subacute cases, with extensive perivascular cuffing and infiltration with a largely mononuclear cell population, although occasional eosinophils may be noted. Inflammatory responses to the disease are highly variable; infiltrates may consist of lymphocytes, neutrophils, eosinophils, multinucleate giant cells and Gitter cells ¹. Hemosiderin within macrophages surrounding affected blood vessels may be seen. Granulocytes may also be observed. Chronic lesions are less common, perhaps due to partial treatment success or disease progression. They are characterized by extensive tissue destruction, including astrocytosis and gliosis, with loss of neuronal structure seen. Phagocytes are usually present that ingest myelin in the white matter ¹⁰². Actual protozoa are rarely visualized, particularly if the horse has received treatment prior to euthanasia. When seen, protozoa are most commonly found in leukocytes and microglia,

yet are also found in neuronal cell bodies and endothelial cells. Individual merozoites may be seen extracellularly, and are typically 4.2 to 4.5 μm long, and 1.2 to 2.0 μm wide.

2.8 TREATMENT

Many different treatments have been employed for EPM, although it is only recently that specific, anti-protozoal treatments have been approved by the FDA for EPM-specific use in the horse. Anti-protozoal drugs provide the mainstay of modern therapeutic methods, and are used in conjunction with anti-inflammatories and immunomodulators. Treatment costs may be expensive: average cost per horse was estimated at \$790 in a nationwide study in 1998, although this has likely increased significantly since that time ³.

2.8.1 Sulphadiazine and pyrimethamine

Folate-inhibiting drugs have long been recommended as a suitable treatment for EPM. The drugs sulphadiazine (a sulfa antibiotic) and pyrimethamine (an anti-protozoal drug) are used most efficaciously in combination. These drugs block successive steps in protozoal folate synthesis, and have a synergistic effect when used together as each individual drug targets a different step in the metabolic process. Neither drug alone is considered an effective treatment ⁵, although pyrimethamine has been shown to have some antiprotozoal activity when used as the sole treatment for *S. neurona in vitro* ¹⁰³. Both drugs act to inhibit the action of dihydrofolate reductase (DHFR), and prevent the synthesis of purine nucleotides. Pyrimethamine selectively inhibits parasitic DHFR. *Apicomplexa* have a DHFR/thymidilate synthase enzyme that performs the same function. Sulphadiazine inhibits synthesis of dihydrofolic acid by competing with para-aminobenzoic acid.

A commonly recommended dosage regimen is 1 mg/kg pyrimethamine with 20 mg/kg sulphadiazine administered as an oral suspension once daily for at least six months. Dietary folate can interfere with the action of the drugs, therefore hay should not be fed for 2 hrs before or after treatment.

One main advantage of the sulphadiazine/pyrimethamine combination as the treatment choice for EPM is that an oral suspension can be relatively inexpensive. The modality does however require a long treatment period to be efficacious (at least 12 to 24 weeks), and there have been concerns raised about the minimal drug concentrations achieved within the structures of the CNS, where most of the protozoa are found ¹⁰³. Only 70% of treated animals responded to the drug combination, and horses were often left with residual neurologic deficits, with occasional recrudescence cases seen ¹⁰⁴. Long term treatment may decrease the horse's ability to metabolize folic acid, causing anemia, thrombocytopenia, leukopenia and bone marrow suppression ¹⁰⁵. For this reason, it is recommended that regular CBCs be performed, and that folic acid supplementation be instigated if adverse effects are seen.

2.8.2 Ponazuril

In July 2001, the FDA approved the drug ponazuril for use in the treatment of horses with EPM. Marketed as Marquis Antiprotozoal Oral Paste™, the drug is an anticoccidial compound with dose dependent activity against *S. neurona* ^a. Ponazuril is a metabolite of another coccidial drug known as toltrazuril. This triazinone derivative was used in pigs and lambs, and has been demonstrated to be efficacious against a

^a Marquis Antiprotozoal Paste, Bayer Animal Health, Shawnee Mission, KS USA

broad range of parasitic protozoal species. Its use in *Isospora suis* infection in piglets, *T. gondii* infection and infection with *Eimeria* species has been proven ¹⁰⁶. Toltrazuril has been shown to act upon the apicoplast (a chloroplast-like organelle that is involved in sending messages to the nucleus) and the respiratory chain of various avian coccidial species, and also to affect nuclear division of schizonts ¹⁰⁶. Toltrazuril is not available in the USA, although in other countries the liquid form is marketed under the name Baycox^{™b}. Studies examining toltrazuril and its metabolites toltrazuril sulfone and toltrazuril sulfoxide, demonstrated adequate and therapeutic levels of the drug and its primary metabolites in the serum and CSF. When examined after 10 days, toltrazuril sulfone was the primary metabolite found in both the serum and CSF ¹⁰⁷.

Toltrazuril sulfone is also known as ponazuril, and this has been shown to be effective against *S. neurona* in vitro. It is thought to have effects on the apicoplast ¹⁰⁸. This is an organelle not found in mammalian species but possessed by many protozoa, and important in these species for the synthesis of amino acids, transport of electrons and energy metabolism. A concentration of the drug at 1 µg/mL inhibited the growth of *S. neurona* by 98.6%, although this was reduced to 94% when a concentration of 0.1 µg/mL was used instead ¹⁰⁸. Ponazuril, as a weak acid with high lipid solubility, easily crosses the blood brain barrier, and is believed to enter the CSF via passive diffusion. The approved dosage for the treatment of EPM is 5 mg/kg once daily for 28 days, with a further 28 days of dosing at 10 mg/kg if no improvement is seen. Many practitioners will utilize the drug at 5 mg/kg for a month, and then reduce it to 1 mg/kg once daily for the consecutive month. This treatment regimen has been efficacious, and is more cost effective ¹⁰⁹.

^b Baycox, Bayer International, Newbury, Berkshire UK

Most horses tolerate ponazuril very well, and limited side effects have been seen. In a study where horses were given 10x the recommended dose for 10 days, all horses tolerated treatment, although some weight loss and mild colic signs were seen. Intermittent treatment with a higher dose (20 mg/kg every 7 days for 12 weeks) has been shown to prevent immunoconversion (generation of antibodies in a naïve horse, as based on Western Blot) when treatment is started very early in the course of infection. This may have preventative effects, and will be discussed later.

2.8.3 Nitazoxanide

Marketed as Navigator™ paste, nitazoxanide (NTZ) was approved as a treatment for EPM by the FDA in 2003^c. Nitazoxanide is used for treating *Cryptosporidium parvum* and *Giardia lamblia* in human Acquired Immunodeficiency Syndrome (AIDS) patients, and for the treatment of other protozoal infections, particularly those in immunosuppressed individuals. Having shown promise in human and other animal species, nitazoxanide interferes with pyruvate ferredoxin oxidoreductase. This enzyme is necessary for the electron transfer reaction involved in anerobic energy metabolism¹¹⁰. Limited studies have been performed regarding the drug's usefulness in cases of EPM. A study by the manufacturer reported an 81% success rate, with 78% of horses previously treated with other drugs, and then receiving nitazoxanide, appearing cured of the disease. The recommended treatment dose is 11.36 mg/lb administered orally once a day for 5 days, followed by 22.72 mg/lb orally SID for a further 23 days¹¹⁰.

The concern with the use of Navigator™ is related to its potential toxicity. Trials of

^c Navigator, IDEXX Laboratories, Westbrook, ME USA

the drug at 1.2, 2.5 and 4.9 times the recommended dose revealed the potential of the drug to cause severe erosion and ulceration of the colon. All eight horses in the highest dose range developed adverse reactions, with a fatality rate of 63%. Horses in the 2.5 times recommended dose group also suffered fatal colitis, and those in the 1.2 times recommended dose group suffered from loose feces, depression and lethargy. NTZ has very recently been taken off the commercial market, due to reduced sales to veterinarians.

In mild cases of EPM, additional therapy may not be required. However in moderate to severe cases, additional treatments to support neurologic function, reduce inflammation and boost the immune response are often utilized. Flunixin meglumine, a nonsteroidal anti-inflammatory, is commonly used at 1.1 mg/kg intravenously BID for the first week of treatment, with a reduction to once daily dosing after the first week. This may help to prevent the worsening in neurologic grade seen in some cases after treatment kills *S. neurona* and triggers an exaggerated inflammatory response. Antioxidants in the form of vitamin E may be given throughout the treatment period in an attempt to prevent oxidative damage and support neurologic function. This is usually given at a dose of 20-200 IU/kg orally once a day, although no research has been performed that supports its use ^{2,109}.

2.8.4 Levamisole and other immune stimulants

Originally used as an anthelmintic, levamisole was discovered to have the ability to boost the function of T lymphocytes, especially in immunocompromised animals. This improvement in cell-mediated immunity is advantageous when dealing with mainly intracellular organisms, such as protozoa. Evidence of levamisole's effectiveness

includes the use of the drug, again in human medicine, to combat cerebral toxoplasmosis in AIDS patients. *T. gondii* is found in a wide range of mammals, including humans, and in birds. The cat is the definitive host and passes the protozoa through its feces. In humans, infection is largely asymptomatic. However, immunocompromised patients are especially susceptible to this particular parasite, and infection may result in clinical signs, as is the case with cerebral toxoplasmosis in AIDS patients. The human disease is treated in a similar manner to EPM in horses, with a combination of sulfadiazine and pyrimethamine. Adding levamisole to the treatment regimen in humans has increased the survival rate for a year by 50% ¹¹¹. Levamisole has been used with similar results in treatments for coccidiosis of pigs, and leishmaniasis and cancer in humans ¹¹². Treatment with levamisole is in addition to other antiprotozoal drugs, and is usually administered at 1 mg/kg once daily as an oral suspension ¹¹³.

Other immunostimulants such as *Mycobacterium spp.* cell wall extract, *Propionibacterium acnes*, and transfer factor have been used in the treatment of EPM, and many clinicians believe their usage to be beneficial ^{2,109}.

2.9 PREVENTION

Prevention of clinical signs of EPM is the ultimate goal. It is possible that the prophylactic administration of antiprotozoal drugs may be preventative, but is not a cost-effective method for most horse owners. In mice, infection with *S. neurona* may be prevented by daily administration of the anti-protozoal diclazuril ¹¹⁴. However, this method does not allow the normal and potentially protective immune response to develop. If horse owners began prophylactic treatment and later discontinued its administration, this may predispose horses to infection at a later date ¹⁰⁹.

An intermittent dosing regimen of ponazuril has been studied, and this may be useful in providing a form of metaphylaxis. This regimen allows schizogony to take place, and normal immune responses to be generated, but prevents the protozoan from invading the CNS and therefore prevents the neural damage and resultant clinical signs. In a study in 2007 by MacKay et al., horses receiving weekly ponazuril at the above dosage failed to demonstrate immunoconversion in the CSF when challenged with orally administered sporocysts ¹¹⁵. However, untreated horses also failed to develop clinical signs of neurologic disease, therefore it is difficult to assess the validity of this treatment method.

A vaccine developed from lysates from *S. neurona* merozoites was available for several years, but has since been discontinued due to failure to demonstrate efficacy. Work by Ellison is currently ongoing to attempt to develop a more useful and efficacious vaccine by the use of recombinant SnSAG1 subunit antigen ¹¹⁶. Recombinant subunit vaccine technology can involve inserting a specific gene(s) from the disease-producing organism into another similar, but not disease-producing organism, which then expresses the protein(s) without presenting a risk to the horse. A subunit vaccine could then generate an immune response without introducing the entire disease-producing pathogen, thus limiting the chance to develop full-blown disease. The development of a successful vaccine for EPM is difficult due to the intracellular nature of the protozoan. Killed vaccines are less successful at stimulating protective cell-mediated immunity than they are at increasing humoral response capabilities, and the ability of certain protozoa to change surface antigens whilst in the host in an attempt to evade the host's immune response makes the development of an efficacious vaccine more challenging.

This ability has not been documented in *S. neurona*, but related protozoa such as *Trypanosoma brucei* can evade the host's immune system by changing its surface antigens during development ⁷⁰.

Management techniques may play a key role in the prevention of infection with *S. neurona*: the majority of these involve the reduction of horse and opossum interaction. Attempts to protect feedstuffs from contamination with opossum feces (such as the use of covered storage containers) may be partially effective, as may measures to reduce opossum populations (such as the use of traps or animal deterrents e.g. free ranging dogs). In areas with high opossum populations, where horses are allowed access to pasture, it may be impossible to completely avoid opossum contact with horses.

2.10 FUTURE GOALS

There has been considerable research performed on EPM in the last 25 years, and many advances in our understanding of the disease have been achieved. However, many questions remain unanswered at this point, and should form the basis of future studies. Future research is expected to focus upon gaining a more thorough understanding of the pathophysiology of disease, developing an efficacious vaccine, and developing more sensitive diagnostic tests that could easily distinguish between infected and exposed horses.

In trying to develop a protective vaccine, research has focused on identifying an immunodominant protective antigen. The surface antigen SnSAG1 is expressed by the majority of *S. neurona* merozoites isolated from EPM horses, and recent work by Ellison et al. ¹¹⁶ has focused on the use of this protein in various vaccine trials. Further

investigation of this, and other surface antigens shared between the majority of *S. neurona* strains is required.

Regarding the pathophysiology of EPM, it is not yet clear why certain horses that are exposed to *S. neurona* develop clinical signs of EPM, whereas the majority do not exhibit disease. This is a key area to focus on in the future. It is thought that susceptible horses are in some way immunodeficient, and a better understanding of the exact deficiency responsible is key to the development of more efficacious diagnostic tests, treatments and possible preventative measures. Spencer et al. (2004) documented a reduced expression of IFN γ in EPM horses, and a link to the cell-mediated immune system. Further studies indicated that this immunosuppression was at least in part due to reduced Th1 gene expression, and increased Th2 cytokine gene expression⁸¹. A decrease in mitogen stimulated lymphocyte proliferation has been noted when lymphocytes from EPM horses are exposed to PMA/I^{73,82,83,101}. An aim of future studies, such as this one, is to elucidate the precise cellular mechanism responsible for this suppressed response.

CHAPTER 3: OVERALL HYPOTHESIS AND OBJECTIVES

3.1 OVERALL GOAL OF STUDY

The overall goal of our study was to determine the mechanisms by which PMA/I stimulated proliferation is suppressed in horses with experimentally induced EPM.

3.2 HYPOTHESIS

Our working hypothesis for this project was that PMA/I stimulated suppression in

EPM horses was due to decreased proliferation of monocytes, CD4+ and CD8+ cells, due to *S. neurona* infection of these host cell types.

3.3 OBJECTIVES

The objectives for the study were as follows:

3.3.1 Objective One: To determine whether PMA/I stimulated suppression was associated with enhanced apoptosis of CD4+, CD8+ and monocyte populations

Decreased cell numbers following mitogen stimulation can be associated with either enhanced apoptosis or a decrease in the initial level of proliferation. Apoptosis in response to PMA/I stimulation were assessed in this project by assessing cell viability and incorporation of an apoptosis marker (7-amino actinomycin D) via flow cytometry, in order to validate the significance of detectable changes.

3.3.2 Objective Two: To determine whether PMA/I stimulated suppression was associated with a decrease in proliferation of CD4+, CD8+ and monocyte populations

A decrease in cell proliferation when stimulated by mitogens could cause a decrease in final cell numbers, without necessarily causing enhanced apoptosis. The presence of infected cells, a decrease in cell signaling and the presence of other suppressive molecules (for example inflammatory mediators such as lipoxin A4) could all result in an inhibition of proliferation. The level of cellular proliferation was assessed in this experiment through incorporation of carboxyfluorescein succinimidyl ester (CFSE) and flow cytometry.

3.3.3 Objective Three: To determine whether PMA/I stimulated suppression was associated with *S. neurona* egression from infected cells and cellular damage

Calcium ionophores (i.e. ionomycin) stimulate egression from infected cells. If parasitemia is present, ionomycin may cause *S. neurona* extravasation from cells, causing death of the cell and inflammation that would then inhibit cellular proliferation. In order to assess this effect, cells were stimulated with PMA and/or ionomycin, and samples collected to detect egression by electron microscopy. This objective will be performed at a later date on samples saved from this study, due to pressures of time.

3.3.4 Objective Four: To determine whether PMA/I stimulated suppression was associated with inhibition of NF- κ B mediated signaling pathways and cytokine production

Other protozoan species such as *T. gondii* can suppress host immune responses by inhibiting NF- κ B signaling and subsequent cytokine expression. Cytokine production and NF- κ B expression can be assessed through real-time PCR. Samples from this experiment were saved for additional analysis at a later date, due to pressures of time.

CHAPTER 4: MATERIALS AND METHODS

4.1 HORSES AND EXAMINATION PROCEDURES

Nine horses were obtained from Florida for use in the study. They were Quarter Horse and Quarter Horse crosses, and ranged in age from 18 months to 11 years old. There were eight mares and one gelding. Historically, none of the horses had exhibited neurologic signs. The horses had been vaccinated against equine influenza and tetanus, as well as diseases that could cause neurologic signs including West Nile Virus, Eastern

and Western Equine Encephalitis and Equine Herpes Virus. Horses were vaccinated for rabies upon arrival at VMRCVM. Horse breed, age and sex are summarized in table form in Appendix A.

Physical examinations were performed on all the horses the day of arrival at Virginia-Maryland Regional College of Veterinary Medicine. The horse's body condition was scored in accordance to the Henneke scaling system, which utilizes grades of 1 (emaciated) to 9 (obese). Ideal body condition score (BCS) is between 5 and 6¹¹⁷. One horse (Horse 9) had a BCS of 2, five horses had scores from 3-5 and three horses had a BCS of 6. Several horses were infested with lice, and had dermatitis and associated areas of alopecia. All horses were treated with oral ivermectin.

When assessing conformation and other baseline factors that could affect the results of our study, certain horses had a variety of deficits. Horse 4 was unwilling to circle in a counterclockwise direction when compared with the contralateral side, and this resulted in both pivoting and circumduction abnormalities when circled in this direction. This was not determined to be due to a neurologic cause: the horse had reduced flexibility in the neck and back when bent in this direction, although an etiology was not determined. Horse 4's level of flexibility did not vary dramatically during the experiment and data collection period. Several of the horses were very nervous and had not been handled frequently in the past: Horse 7 was particularly wary of humans. Horse 9 had an asymmetric pelvis in accordance with a previously sustained pelvic fracture, and as a result had a baseline degree of lameness in the hindend. No other physical abnormalities were noted.

4.2 NEUROLOGIC EXAMINATIONS

Neurologic evaluations were performed on all of the horses to establish baseline neurologic scores. A system based on that used by Ellison et al ⁷⁷ was utilized: this is provided in Appendix B. This scoring system had been used in several USDA approved EPM studies. Briefly, a score was given ranging from zero (normal) to 3 (severely affected) for 22 different parameters, and a total out of a possible 97 was recorded. Parameters that were assessed ranged from cranial nerve function to ataxia, weakness and deficiencies in normal movement.

Each horse was assigned a student handler whose task it was to work with the individual horses and attempt to socialize them effectively for handling by humans, including examinations and sample collections. The handlers exercised the horses in a round-pen, both on and off the lunge line, and both walked and trotted them in-hand.

4.3 EXPERIMENTAL DESIGN

The horses were given a two-week period of acclimatization before initial immune function and serology were assessed. As stated, baseline physical and neurologic exams were performed during this two week period. During this time they were handled in the manner described above, and had 24-hour access to pasture. They were fed grass hay and pelleted 'sweet feed' in accordance to normal energy requirements for weight gain. The horses were not unduly disturbed or stressed in any other manner, except for brief periods of handling/ socialization as described. Appendix D contains a flow chart detailing the steps in this experiment, and summarizes the experimental design.

4.3.1 Day -5: Sample collection five days before beginning infection of the horses

After the two-week acclimatization period, blood was collected from the jugular vein of each horse and submitted to *Pathogenes* (Pathogenes Inc, Fairfield, FL) for serum SnSAG1 ELISA. This day was designated “Day -5”. The horses were then anesthetized by routine field anesthesia, using xylazine at 1.1 mg/kg IV and butorphanol at 0.01-0.025mg/kg IV to provoke sedation, followed by intravenous administration of ketamine at 2.2 mg/kg to induce anesthesia. The region behind the poll was clipped and scrubbed aseptically with chlorhexidine and alcohol, and CSF was collected from the atlanto-occipital space. A 3.5”, 18G spinal needle was used for this procedure, and directed towards the atlanto-occipital space on midline of the sagittal plane. CSF was allowed to flow freely from the hub of the needle until any discoloration from blood contamination was no longer visualized. 5 ml of clear CSF was then collected using a sterile syringe, and submitted to *Pathogenes* for SnSAG1 ELISA and to the laboratory at VMRCVM for routine cytology ¹¹³.

Horses were assigned a number from 1 to 9, and split randomly into two groups by a person other than the main investigators, who remained blinded throughout the data collection period as to each horse’s infection status. Five horses were designated to receive the parasite in infected host leukocytes intravenously, and constituted the infected group; the remaining four were the control horses.

4.3.2 Day -1: sample collection one day before beginning infection of the horses

On the day before infection was due to begin, blood was collected from the left jugular vein of all horses into lithium heparinized blood collection tubes. The blood from horses that would constitute the infected group was infected with 6000 live

merozoites, and incubated overnight. Blood from the control horses was incubated overnight in similar conditions, without infection. This is further described below.

4.3.3 Blood collection occurred on selected days

Blood was collected from the horses on day -5, day -1, days 0-10, and then at the following time points: day 14, day 21, day 28, day 35, day 42, day 56 and day 70.

4.3.4 Proof of infection

Once all data were collected (after Day 73), the horses were anesthetized in the same manner as before, and additional CSF was collected from the AO space, in the manner described. A sample of CSF, coupled with a serum sample from each horse, was submitted for SnSAG1 ELISA in order to confirm infection. These values were compared with baseline values for each horse.

4.3.5 Treatment of infected horses post day 73, and eventual fate of animals in the experiment

On receipt of the SnSAG1 ELISA results, horses in the infected group were started on a course of trimethoprim/sulphadiazine liquid at a dose of 1 mg/kg pyrimethamine with 20 mg/kg sulphadiazine. It was administered as an oral suspension once daily for at least 12 weeks, until the horses were clinically normal and the SnSAG1 ELISA had returned to within normal range. All the horses were adopted out to suitable homes.

4.4 BLOOD COLLECTION TECHNIQUE

Blood samples were obtained from each animal via aseptic jugular venipuncture, and collected into lithium heparinized vacuum tubes. Samples were obtained from all

horses at day -5, for assessment of baseline parameters and establishment of flow cytometry protocols. One 10ml jugular blood sample per horse was obtained on day -1. For the horses to be infected, this blood was infected with merozoites and incubated to allow inoculation of horses the following day. Blood from control horses was incubated overnight but no infection was performed. The infection protocol that is described below was repeated daily for 10 days. In addition, blood was also obtained for immune function studies, on days 0, 2, 7, 14, 21, 28, 35, 42, 56 and 70. Three 10ml lithium-heparin tubes per horse were required for experimental analysis.

4.5 INFECTION PROTOCOL

Merozoites utilized in the experiment were obtained as per Ellison et al ¹¹⁶. Briefly, the SnSAG1 strain was isolated from CNS tissue from a horse previously diagnosed with EPM, and merozoites maintained in continuous culture. The merozoites were cultured in complete media (RPMI with L-glutamine, hepes buffer 25 mM, 2% heat inactivated FBS, 50 IU/ml of penicillin/streptomycin solution, 1% sodium pyruvate solution, Mediatech, Herndon, VA) The merozoites were used to establish experimental infection within the designated five horses.

Beginning on day -1, the blood drawn from each of the infected horses was inoculated in the lithium heparinized tube with 6000 *S. neurona* (SnSAG1 positive) culture-derived merozoites (harvested daily for infection), vented using a needle, and placed in an incubator with 5% CO₂ atmosphere, and at a temperature of 37°C, overnight. The inoculation was performed by Dr. Sharon Witonsky, who was blinded as to which blood sample came from which horse, and remained blinded for the duration of the experiment. Samples from control horses were not inoculated, but were vented

and incubated with those from infected horses for the same duration of time.

The following day, blood from each incubated tube was injected into the jugular vein of the horse from which it was drawn the previous day. This was performed by an assisting DVM (Dr. John Dascanio) to allow both primary investigators (Drs. Lewis and Witonsky) to remain blinded as to the infected horses' identities.

4.6 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)

Prior to enriching samples, direct blood smears were made from each horse at each time point to assess differentials. These slides were air-dried, fixed and stained with modified Wright stain (Wright's Giemsa). PBMCs were then isolated by density gradient centrifugation in the method described by Witonsky et al ¹¹⁸. Whole blood samples at room temperature were diluted with phosphate buffered saline (PBS, Mediatech, Herndon, VA) at the ratio of 1:2. Room-temperature Lymphoprep (Lymphoprep 1.077, Greiner, NJ) was placed (5mls/tube) in a number of 15ml centrifuge tubes, and twice the volume of the diluted blood was layered on top. Keeping the reagents at room temperature maximized the purity of lymphocyte samples, as cooler reagents have, in the past, led to more neutrophil contamination ⁸². Samples were centrifuged at 1500 rpm (350 x g) for 30 min at 23°C without the brake. The buffy coat was then collected into a centrifuge tube using a glass pipette, and diluted with at least an equal volume of PBS, taking care not to disturb the erythrocyte layer below to minimize contamination. Cold PBS was added at a 1:1 dilution, and the cells were centrifuged at 1300 rpm (250 x g) for 10 min at 4 °C, this time utilizing the brake. The supernatant was removed: the pelleted samples were resuspended in 5ml PBS, and washed twice (spinning at 1300 rpm (250 x g) for 5 min at 4 °C).

4.7 COULTER CELL COUNTER

Cell counts were determined with the Coulter Cell Counter (Multisizer 4 Coulter Counter, Beckman Coulter, Inc., Fullerton, CA). Once the final wash and centrifugation had been performed as above, a small volume (10 μ L) of the resuspended sample was added to 10ml cold PBS in a Coulter analysis tube. A second Coulter tube containing 10ml of cold PBS was used as a control, and to calibrate the machine. Cell counts were determined, with the desired cell population at 5 to 10 μ m diameter. Based on cell counts determined in this manner, samples were resuspended to a final concentration of 5×10^6 / ml in complete RPMI 1640 (Cellgro™ RPMI 1640 1x, Thomas Scientific, Herndon, VA) with L-glutamine, Hepes Buffer 25 mM, 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), penicillin (50 IU/ml), and streptomycin (50 IU/ml) (Sigma Chemical Co, St Louis, MO). The cells were kept on ice.

4.8 CYTOSPINS

For cytologic analysis, 125 μ l of the 2×10^6 /ml diluted enriched lymphocyte samples were mixed with 100 μ l PBS and loaded into a centrifugation chamber. Each chamber was placed into a cytocentrifuge (Cyto-Tek centrifuge, Sakura Finetechnical Co, Tokyo, Japan), and spun at 500 rpm (30 x g) for 5 mins. The resultant slides were air-dried, and then fixed and stained with modified Wright stain (Wright's Giemsa). A sample of total slides was used to determine neutrophil contamination by determining differential counts. Samples with evidence of potential neutrophil contamination on Coulter outputs (those with a second peak at larger cell size) were selected for differential counts. Differential counts were also performed on slides from a similar number of horses on the same time point that did not have a double peak on Coulter outputs. To perform a differential, 100 cells were enumerated and described as the

percentages of lymphocytes, neutrophils, monocytes, basophils, or eosinophils ¹¹⁸.

4.9 PRIMARY ANTIBODY STAINING

Cells (5×10^5 cells per sample or $100\mu\text{l}$ of the $5 \times 10^6/\text{ml}$ dilution) were plated in a 96 well round bottom plate (Corning Glass Works, Corning NY). Cold PBS($100\mu\text{l}/\text{well}$) was added, and the plate was centrifuged at 1300 rpm ($250 \times g$) for 5 min at 4°C .

Supernatant was discarded, and $50\mu\text{l}$ total volume of the following diluted monoclonal antibodies (1:100 in PBS) was added to individual wells of each sample: CD4+ (mouse anti-equine CD4+ antibody, 1 mg/ml, cell line HB61A IgG₁, VMRD, Pullman, WA), CD8+ (mouse anti-equine CD8+ antibody, 1 mg/ml, cell line HT14A, IgG₁ VMRD, Pullman, WA), B-cell (mouse anti-equine CD5+ antibody, 1 mg/ml, cell line B29A, IgG_{2a}, VMRD, Pullman, WA), and CD172a antibody (mouse anti-equine IgG₁, 1 mg/ml, DH59B, VMRD, Pullman, WA), which stains for monocytes and granulocytes. Cells were incubated with the primary antibody for 20 mins at 4°C . Cells were then washed with $175\mu\text{l}$ of cold PBS, and centrifuged at 1300 rpm ($250 \times g$) for 5 mins at 4°C . Supernatants were again discarded, and $50\mu\text{l}$ of the secondary antibody was added to the wells. The secondary antibody added to the B-cell was PE rat anti-mouse IgG_{2a} (for conjugation to B-cell antibody) (Pharmingen, San Diego, CA) at a dilution of 1:100. FITC rat anti-mouse IgG₁ was used for conjugation to CD4, CD8, and DH59b antibodies (Pharmingen, San Diego, CA), at a 1:100 dilution. The unstained cells, to be run as controls, received $50\mu\text{l}$ cold PBS. The plate was incubated for an additional 20 mins at 4°C . Following this time period, the plate was washed and centrifuged in the same manner as before, and the supernatant again discarded. The cells were then resuspended in $200\mu\text{l}$ of cold PBS for flow cytometric analysis. 7-AAD, if required in each step, was added at this time (see protocol). Flow cytometry was performed using the EPICS XL Flow cytometer (Coulter,

Hialeah, FL). Samples were run until a minimum of 5000 (with an optimal count of 10,000) cells were captured (dependent on subset) as described below.

4.10 7-AMINO ACTINOMYCIN D (7-AAD) AND CARBOXYFLUORESCCEIN SUCCINIMIDYL ESTER (CFSE) STAINING

Both stains are fluorescent DNA binding agents widely used for flow cytometry, yet they are used to analyze different cellular events. 7-AAD is used to assess viability and apoptosis of cells. This dye binds between cytosine and guanine bases in the dinucleotide strands of cellular DNA, causing the cell to fluoresce, and enabling detection by flow cytometry¹¹⁹. 7-AAD cannot bind to intact cells; the degree of binding depends on the extent of cell damage and permeability of the cell membrane. If the cell membrane is damaged, the dye can penetrate and is intercalated with the DNA, as described above. The dye can be used in combination with FITC or PE since it creates emission in the far red spectrum, and can easily be separated from the emission of FITC and PE fluorochromes. Staining intensities vary between early apoptotic and late apoptotic/necrotic populations, therefore flow cytometry can be utilized to distinguish between viable, early apoptotic, and late apoptotic/necrotic cell populations¹¹⁹.

7-AAD staining was utilized in our study in order to address Objective One, and was performed as follows: once cells had been incubated with both primary and secondary antibodies, washed and resuspended, 1 μ g of 7-AAD (Molecular Probes, Eugene, OR) in 200 μ l PBS, was added to each sample in plates for Objective One. Plates were incubated for a maximum of 30 mins on ice in the dark. Flow cytometry was then performed: five or ten thousand cells gated events per sample were collected by the flow cytometer (EPICS XLflow cytometer, Coulter, Hialeah, FL). Based on the intensity of

staining, cells were classified by their subset as 7AAD_{dull} (live cells), 7AAD_{moderate} (early apoptosis), and 7AAD_{bright} (late apoptosis) ¹²⁰. In order to study the effect of PMA/I on apoptosis (to accomplish Objective 1), additional cells were plated in three separate 96 well round bottom plates, as is shown in Figure 2. PMA/I was added (at a concentration of 20 ng/ml PMA plus 10 pg/ml ionomycin) to one set of samples, and plates were incubated for 24, 48 and 72 hrs in a controlled environment (37°C, 5% CO₂). Non-stimulated samples were also included on each 96 well plate, to act as controls. Flow cytometric analysis was performed on each of these plates, as above ⁸³.

CFSE is a marker used to determine the number of divisions by each cell in a population. CFSE passively diffuses into cells and remains colorless (nonfluorescent) until acetate groups on the molecule are cleaved by intracellular esterases. This cleavage results in formation of carboxyfluorescein succinimidyl ester, a highly reactive and highly fluorescent product. The succinimidyl ester group reacts with intracellular amines, forming conjugates that are well retained within the cell and generate high levels of fluorescence. The protein-dye adducts that form within labeled cells may be fixed with aldehyde fixatives, allowing unconjugated reagents to be washed away. Daughter cells from the labeled population inherit the fluorescent conjugate, as this is retained in a cell throughout division and meiosis, although the level of fluorescence exhibited by each generation from the parent is proportionally reduced. This enables the study of cellular proliferation and division, which is the purpose of Objective 2 in our study ^{83,121}.

For Objective 2, cells were labeled with CFSE, then plated and stimulated appropriately and incubated for 72 hrs, after which cells were stained with antibodies

for subset differentiation. Briefly, aliquots of cells were resuspended in 5 μ M of CFSE (Molecular Probes, Eugene, OR) for 10 mins at 37°C. Cells were then washed, plated and stimulated with or without PMA/I for 72 hrs in a controlled environment (37°C, 5% CO₂). Following incubation, primary and secondary antibodies were added as previously described. Flow cytometry was then performed: five or ten thousand cells gated events per sample were collected by the flow cytometer (EPICS XLflow cytometer, Coulter, Hialeah, FL). Based on the intensity of staining, the numbers of divisions each cell had undergone was determined as follows: cells undergoing no divisions, one, two, three and greater than three divisions¹²¹. Both PMA/I containing and control media containing wells were included for each stain, as shown in Appendix C.

4.11 LIVE MEROZOITE PREPARATION

For the assessment of antigen specific proliferation and apoptosis, merozoites were used for stimulating cells in some assays for the experiment. They were utilized in lymphocyte proliferation assays, as well as 7AAD and CFSE-stained samples for flow cytometry from Day 21 onwards.

Live *S. neurona* merozoites of the SN-37R strain⁷² were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Rockville, MD, USA). The *S. neurona* merozoites were then harvested from CV-1 cells by removing the complete media (RPMI with L-glutamine, hepes buffer 25 mM, 2% heat inactivated FBS, 50 IU/ml of penicillin/streptomycin solution, 1% sodium pyruvate solution, Mediatech, Herndon, VA), including the merozoites. The suspension was filtered through a 3 μ M filter and spun at 1,500 rpm (350 x g) for 10 mins at room temperature, and resuspended in the

fresh complete media. The merozoites were enumerated with a hemocytometer, then resuspended at a concentration of 1×10^5 /ml with complete media containing 10% heat inactivated FBS¹²². Merozoites (100 μ l/well) were then incubated with cells as indicated.

4.12 LYMPHOCYTE PROLIFERATION ASSAYS

These assays were used to determine whether differences in lymphocyte proliferation existed between EPM and control horses, as well as to determine which particular subsets of lymphocytes (T cells and/or B cells) were affected. Non-antigen specific differences (mitogen stimulated) and parasite (antigen) specific differences were both measured. Non-antigen specific differences in proliferation were assessed using a variety of mitogens to stimulate different lymphocyte subpopulations. T lymphocytes (T cells) were selectively stimulated through the use of Concanavalin A (ConA), and B lymphocytes (B cells) were stimulated preferentially by pokeweed mitogen (PWM). Phorbol Myristate Acetate (PMA) and Ionomycin (I) in combination (PMA/I) were used to stimulate all leukocytes. The effect of these mitogens (PMA and I) was also studied individually¹¹⁸. Antigen-specific blastogenesis was assessed by using live merozoites added to the plate as an antigen.

Plates containing 100 μ l aliquots of each horse's enriched lymphocytes (2×10^6 /ml) in triplicate wells were cultured together with 100 μ l of the appropriate mitogen or live merozoites (1×10^5 /ml)⁸³. Final concentrations of mitogens in the wells were 5 μ g/ml ConA (Sigma Chemical Co, St Louis, MO), 1 μ g/ml PWM (Sigma Chemical Co, St Louis, MO), 20 ng/ml PMA and 10 pg/ml ionomycin (Sigma Chemical Co, St Louis, MO). Spontaneous proliferation was assessed by culturing a triplicate of aliquot-containing

wells with only enriched media added.

Cells were incubated for 48 hrs at 37°C in humidified 5% CO₂. The plates were then pulsed with 1 µCi³H-thymidine. Plates were harvested 18-24 hrs later using a Filtermake Harvester (Packard Bioscience, Billerica, MA). Stimulation indices were calculated for each mitogen by dividing the mean counts per minute (CPM) of wells with mitogen by the mean CPM of the unstimulated cells in media, which represented spontaneous proliferation. The effect of mitogens was also assessed through calculating the delta values: the change in proliferation was assessed by subtracting average spontaneous proliferation from the average mitogen-stimulated count.

In previous studies, more significant differences in PMA/I-induced suppression in EPM horses was seen with samples that were stored overnight at 4°C¹⁰¹. Therefore, at Day 21, it was decided to prepare additional samples at each experimental time point and incubate them overnight in the refrigerator at 4°C. Following overnight incubation, blood samples were warmed to room temperature. Blood samples were processed and lymphocytes collected as previously described. Cells were plated and stimulated with ConA, PWM and PMA/I as previously described. Cells were also cultured and proliferation determined in a manner identical to non-overnight blood samples.

CHAPTER 5: STATISTICS AND DATA ANALYSIS

Horses 1, 2, 4 and 8 were control horses, and did not receive daily doses of parasite. Horse 2 received 1 dose of *S. neurona* in error, and could no longer be considered a control. Data were still collected from this animal, but they were not used

in the statistical analysis of the control horses. Horses 3, 5, 6, 7 and 9 received ten doses of the protozoan in autologous lymphocytes via intravenous injection and constituted the infected group.

The purpose of our study was to determine how infection altered immune subsets including specific populations as well as function. More specifically, we wanted to determine whether PMA/I stimulated suppression in EPM horses was due to decreased proliferation of monocytes, CD4+ and CD8+ cells, due to *S. neurona* infection of these host cell types. Results from infected horses were compared with those from the control group. Time was also a factor in our experiment: both as the number of days since infection, and also as a result of differing incubation times with various mitogens. Our statistical analysis needed to account for the 'treatment effects' of both infection status and of time, and for the wide degree of variability within the horse population.

In considering how to analyze specific sets of data, the majority of results involved either proliferation data or results from flow cytometry. For the proliferation data, stimulation indices were calculated for the results of lymphocyte proliferation assays, and statistical analysis performed on these calculated values. Stimulation indices were obtained by dividing the average of the three wells stimulated with a specific mitogen, by the average of wells in triplicate that contained no mitogen (media only) that represented spontaneous proliferation. Deltas were also calculated by subtracting the average spontaneous proliferation from the average mitogen-stimulated result. Analysis of both stimulation indices and deltas were compared, and stimulation indices were chosen as they demonstrated a more significant difference.

Besides proliferation data, we also collected data from flow cytometric analysis. Flow cytometry data were tabulated and enumerated using the FlowJo software package (Tree Star Inc, Ashland, OR). Unstained samples (background) were used to establish the correct location of gates for each sample at each time point in order to distinguish between positively stained samples and background (unstained, non-specific staining) and autofluorescence. Care was taken to ensure these gates represented the best fit for data on individual days, and for the cellular subset as a whole. Examples of this gating are demonstrated in Figures 2, 3 and 4. With longer incubation periods, another separate cell population became apparent. This was described as the dying cell population based on a small cellular size and increased apoptosis within the group, and the presence of multiple different cell types in this population. Dying cells were analyzed in addition to the “live lymphocyte”, “monocyte” and “neutrophil” gates, for each cellular subset.

Standard residual plots were used to assess model adequacy, and logarithmic values were also analyzed for many data sets in order that the variance between individual data points could be standardized. Lines of best fit were examined, and data points for logarithmic values lay closer to these lines.

There were more than two treatment effects that we wished to assess in this study, therefore the majority of simple statistical tests did not apply or would have created results that did not adequately reflect the data. Infection status and time were both factors in our experiment, along with analysis of the specific variable (for instance cellular viability) being studied. A statistical test that could assess more than two factors simultaneously was therefore required. Analysis of variance was chosen as the most

suitable test. Analysis of variance was conducted using the GLM procedure of the SAS system (version 8.2, SAS Institute Inc, Cary, NC). This procedure was utilized for most of the individual analyses performed, including results of flow cytometry, neurologic scoring over time, and lymphocyte proliferation assays. As there were dramatically fewer time points available for CD8+ cells in late apoptosis (i.e. not all time points contained cells in late apoptosis), a Wilcoxon Exact two-ended test was performed on CD8+ cells in late apoptosis.

Results were expressed as an adjusted P value, and were referenced against the mean value for each group \pm standard deviation. The actual numbers of horses used to generate each individual result may be different on a small number of occasions, due to limited data collection of some samples, or reduced cellular survival in these individuals.

CHAPTER 6: RESULTS

6.1 INFECTION STATUS

All horses had complete blood count profiles within normal reference intervals, prior to enrollment in the study. After the acclimatization period, serum and CSF samples were submitted for SnSAG1 ELISA on Day -5. All horses had serum titers of <2 except for horse 6 which had a titer of 4. A titer ≥ 32 is considered significant in cases with accompanying neurologic signs, and in experimentally induced cases with neurologic signs that have received 6000 organisms/day for 14 days.

After all immune function data had been collected for the experiment, additional

serum and CSF SnSAG1 results were obtained at day 73. All experimentally infected horses demonstrated a rise in serum titer from pre-experiment values (Table 1). All infected horses had a serum titer of ≥ 32 : this is considered positive. Two of the infected horses (horses 3 and 7) had serum titers of 80. Several infected horses also had elevated CSF titers. Horses 3, 5 and 7 were considered positive, as their CSF titer was ≥ 1 . However, infected horses were not the only animals in the study with increased SnSAG1 ELISA results: some of the control horses also demonstrated a rise in serum or CSF titer. Horses 1 and 8 had positive serum titers of 32. Horse 4 had a positive CSF titer of 2. These results are summarized in Table 1.

Cytologic analysis performed on the 1stCSF sample obtained via atlanto-occipital puncture was within normal ranges for white blood cell (WBC), total protein, turbidity and color (Table 2). Several of the horses had increased red blood cell (RBC) levels, presumably due to iatrogenic contamination. This did not adversely affect the SnSAG1 ELISA on the initial samples: all horses had a titer of < 2 prior to the experiment. These results are summarized in Table 2.

6.2 NEUROLOGIC SCORING

All five horses infected with the protozoan *S. neurona* exhibited neurologic signs at some point during the experiment. Horses were scored out of a possible 97 in accordance with the scoring system included as Appendix B. An increase in neurologic score was seen in all infected horses over the course of the study (Figure 5), although the horses varied as to when their neurologic deficits were most significant. Logarithmic values were used in the statistical analysis in an attempt to standardize variance, and as these were closer to a line of best fit. A significant difference ($p < 0.05$)

between the control and infected population's mean overall neurologic score was present at Week 10. A trend towards a difference ($0.05 < p < 0.1$) was seen at Weeks 1 and 4. The results are represented graphically in Figure 5.

All horses received baseline neurologic evaluations several weeks before the experiment began. They were evaluated again after the acclimatization period. The overall mean score for the five horses that would receive the parasite did not change during the acclimatization period, although the control horse group did experience a small rise in overall score. This was largely due to Horse 1's increased overall score from a baseline score of 2 to 5 at Week -1. Horse 1's decreased aggression was attributed to decreased fear of humans; however this attitude change resulted in an increased neurologic score. Decreased aggression or depression is a common early clinical sign of EPM^{77,116}. Thus changes in attitude can be associated with disease and warrant monitoring on neurologic examination.

Neurologic signs were first noted on Day 5 of infection. Horse 5 began to eat more slowly, and with increased effort, than before infection. Subsequently, Horse 3 demonstrated a slowness to finish grain, and Horse 5 became ataxic by Day 7 of infection. Horses 2 and 9 were slow to eat on Day 8 of infection. By Day 10 of infection, all challenged horses were demonstrating neurologic signs. These were graded as moderately severe in most cases, and it was decided to stop infecting the horses at Day 10 instead of continuing until Day 14 as had been originally planned, as a suitable level of neurologic deficits had been achieved for this stage of infection. It was known that the horses would become progressively worse from this time point, and it was therefore anticipated that they would continue to develop into Grade II signs (the standard level

of ataxia induced with Ellison's model).

Neurologic scores for horses 3, 5 and 9 peaked within the first two weeks of the experiment. Horses 6 and 7 peaked slightly later, between 6 and 8 weeks into the study. All infected and control horses except horse 8, had a higher score in week 8.

Gait abnormalities associated with ataxia are the dominant clinical signs observed in naturally occurring cases of EPM. When scores for parameters assessing ataxia were analyzed by treatment group, without inclusion of other parameters, the infected horses appeared more ataxic than the control horses at weeks 1, 3 and 10. This is shown in Figure 6.

Although ataxia is one of the most common neurologic signs in naturally and experimentally occurring cases of EPM, it is not the only clinical symptom exhibited. Cranial nerve signs have also been reported in both experimentally induced and naturally occurring EPM, and they were one of the first signs noted by Ellison when the model was developed⁷⁷. Figure 7 illustrates cranial nerve signs as well as ataxia of infected horses. The infected horses exhibited a peak in cranial nerve signs prior to the peak in ataxia, which was more pronounced towards the end of the study period.

6.3 LYMPHOCYTE SEPARATION

6.3.1 Differential counts from peripheral blood smears

Direct blood smears were examined, and differential counts performed. A direct blood smear was counted from each horse at the beginning of the experiment, and the average differential count was 62% neutrophils, 31% lymphocytes, 3% monocytes, 5%

eosinophils and 0% basophils. These differential counts were within normal ranges for adult horses ¹²³.

6.3.2 Cytology of enriched lymphocyte populations

Differential counts of cytopsin slides of enriched lymphocyte populations were examined to assess lymphocyte purity and neutrophil contamination. Differential counts were performed on 28 different cytopsin slides, from a variety of horses and time points throughout the experiment. Samples were not chosen if they exhibited a double peak on the Coulter data: other samples were picked at random. Cytospins were made on every sample following lymphocyte isolation. An average differential count of 6% neutrophils, 87.5% lymphocytes and 6.5% monocytes was obtained.

6.3.3 Coulter Counter analysis for enumeration of cells and neutrophil contamination

Coulter cell counter outputs of cellular size and distribution were examined for each horse at each time point. On Coulter analysis, a peak in cellular size was seen between 5 and 10 μm diameter. This represented the lymphocytes. A small number of the samples had a second peak of a larger diameter cell population but containing fewer cells. Only 13 samples demonstrated this double peak, representing an increased number of neutrophils. Differential counts performed on cytopsin with the Coulter samples displaying a double peak demonstrated a minimal increase in neutrophil numbers. The average percentage of neutrophils in the double peak samples was 6.2%, compared with an average of 5.8% in samples without this double peak. Results of this analysis are shown in Appendix D.

6.4 PROLIFERATION RESPONSES TO MITOGENS

When non-log transformed stimulation indices for PMA/I were compared between control and infected horses there were no significant differences at any time points studied. Due to the variance in the samples associated with thymidine, and the unequal variance between horses, logarithmic values were also compared (Figure 8). A significant difference was seen at Day 35. At this time point, the control horses had a significantly higher stimulation index when leukocytes were stimulated with PMA/I than that of the infected group. This is shown in Figure 8. In general, based on the average log transformed stimulation indices, infected horses had a higher average stimulation index (not significant) in the first half of the study period, whereas control horses had a higher index in the second 35 days of the experiment. This is also shown in Figure 8.

Stimulation indices were chosen in this experiment to analyze results of proliferation assays. Deltas, the change in proliferation when spontaneous proliferation was subtracted from mitogen-stimulated results, were also calculated. The deltas did not demonstrate a significant difference between treatment groups. This was therefore not submitted to the statistician. Delta values are shown in Appendix E.

Once methods of analysis were selected, additional examination of the data was performed to determine whether a specific immune cell subset was affected by *S. neurona* infection. Analysis of ConA results, which reflect T-lymphocyte function, and PWM, which reflects B-cell function, revealed no apparent significant differences between infected and control horses. Therefore, the results were not submitted to the statistician for mathematical analysis. A very small difference between treatment

groups was seen at Day 35 for both mitogens, which was very likely not significant. A graphical representation of these results is shown in Appendices F and G.

Antigen-specific stimulation with merozoites did not reveal any significant differences in proliferation responses between infected and control horses. Infected horses had a non-significant increase in proliferation at Days 14 and 35 relative to controls. At later time points, (Days 42, 56 and 70), infected horses had a non-statistically significant decrease in merozoite stimulation responses when compared to controls. Results are shown in Appendix H.

For samples that were incubated overnight before plating with mitogens, a significant difference was seen at Day 70 when samples were stimulated with PMA/I. The infected horses had a lower stimulation index than the controls at this time point. In general, infected horses had a higher stimulation index than control horses at most time points. Figure 9 demonstrates these results.

6.5 TOTAL CELLULAR SUBSET PERCENTAGES ON FLOW CYTOMETRY

The **total percentages of positively staining cells for each subset** within the main (“live”) gate were examined. Percentages of CD4+ and CD8+ cells within the live lymphocyte gate were compared across treatment groups. Percentages of B-cells within the lymphocyte gate, and percentages of monocytes and neutrophils within the respective gates for these cell types were also evaluated. Differences between infected and non-infected horses were present. Table 3 demonstrates the significant results ($p < 0.05$) and trends ($0.05 \leq p < 0.1$) in percentages of each subset between treatment groups, at each specific time point (i.e. baseline, 24 hrs incubation, 48 hrs incubation, 72

hrs incubation) for each treatment day. The percentage of certain cellular subsets present varied with infection status. These results are summarized below, and sorted via cellular populations. Stimulated results refer to those cells incubated with PMA/I, and unstimulated refer to results obtained at baseline (after no incubation, and not exposed to a mitogen).

6.5.1 CD4+ Total Percentage (Stimulated and Unstimulated)

At all incubation periods studied, the control horses usually had a higher total percentage of CD4+ cells than the infected animals. Due to variation between individual horses, the difference was not always significant. The effect was most pronounced after 24 hrs of incubation with PMA/I, although it was also present in unstimulated samples after no incubation period (Figure 10 and Appendices J to L).

Significant differences were seen after 24 hrs of incubation with PMA/I. At Day 70, the control horses had a significantly higher total percentage of CD4+.

At all time points measured except Day 7, the control horses had a higher average percentage (but not always a significantly higher percentage) of CD4+ cells. The aberrant result on Day 7 was present because of a large standard deviation of the control horses on this day. This was predominantly due to Horse 1's low total percentage CD4 on this day compared to this horse's other results at different time points, and those of the other horses in the control group. These results are shown in Figure 10.

6.5.2 CD8+ Total Percentage (Stimulated and Unstimulated)

At most time points studied, the control group had a higher total percentage of

CD8+ cells than the infected horses. This difference was most pronounced later in the experiment (at Day 70) and after longer incubation periods. A significant difference was not always seen due to high amounts of variation between horses. Results are shown in Appendices M to P.

Significant results were seen at certain time points. At Day 14, after 72 hrs of incubation, the infected group had a higher percentage of CD8+ cells. At Day 42, after no incubation, the infected group had a higher percentage of CD8+ cells. At Day 70, after 72 hrs of incubation, a trend towards a higher percentage of CD8+ in the control group was seen.

6.5.3 Neutrophil Total Percentage (Stimulated and Unstimulated)

At most time points studied, the infected horses had a higher percentage of neutrophils than the control group. There was not a significant difference in total percentages of neutrophils between the treatment groups at every time point studied.

Significant results were seen at the following days: at Days 0 and 14, with no incubation, the infected group had a significantly higher percentage of neutrophils. At Day 70, after 24 hrs incubation with PMA/I, the infected group had a significantly higher percentage of neutrophils (Appendices Q - S). There was a significantly lower total percentage of neutrophils in both infected and control populations at Day 0 and Day 14 (no incubation), when compared with total percentages from other days studied (Figure 11).

6.5.4 Total Monocytes (Stimulated and Unstimulated)

In general, the infected group had a slightly higher total percentage of monocytes than the control group at most time points studied, although no significant differences were seen. The effect became more marked after longer incubation with PMA/I (Appendices U - W). Significant differences were not seen. A trend towards a significant difference was noted after 48 hrs incubation on Days 28 and 35.

6.5.5 Total B cells (Stimulated and Unstimulated)

At most time points, after short incubation periods, the infected horses had a higher total percentage of B cells than control horses. After longer incubation times the control horses had a higher total percentage of B cells. These differences were not always significant. Significant differences were seen at the following days: at Day 21, after 72 hrs incubation, the control group had a significantly higher percentage of B cells. At Day 28, after 24 hrs incubation, the infected group had a significantly higher percentage of B cells. At Day 28, after 72 hrs incubation, a trend towards a significantly higher percentage of B cells in the control group was seen (Appendices X-AA).

6.6 RESULTS FROM OBJECTIVE ONE: FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS

All samples were run as described in the materials and methods section of this thesis. Examples of data from the flow cytometer are shown in Figures 2 and 3. Results for baseline flow cytometry (no incubation) and those for 24, 48 and 72 hrs of incubation with PMA/I are shown. Samples that were stimulated with PMA/I had less defined populations than unstimulated samples. Cells were more widely distributed, with more variety in forward and side scatter than in the unstimulated samples.

At longer periods of incubation, a greater proportion of cells of all cell types were found in the “dying” gate, as these cells underwent apoptosis and necrosis and therefore lost cell volume. These cells were evaluated separately from the “live lymphocyte,” “monocytes” and “neutrophils” gates. Both the effects of infection and stimulation with PMA/I were evaluated with respect to apoptosis.

6.6.1 The effect of *S. neurona* infection on apoptosis

The effect of infection status on cell viability was assessed through examining baseline (no incubation) samples that had not been stimulated with PMA/I. **An overview of significant data is presented in Table 4. Data are shown as percentages of viable, early and late apoptotic cells.**

6.6.1a CD4+ Lymphocytes

Infection with *S. neurona* did not alter live CD4+ lymphocyte viability or levels of apoptosis between treatment groups. No significant results were seen.

In the dying cell CD4+ population, some effect of infection status was seen. In general, there were lower percentages of cells in early apoptosis in infected horses than in control, although this result was not consistently seen at all time points. At some time points, a decrease in early apoptosis was countered by an increased percentage of cells in late apoptosis in the infected group, whereas at others, an increase in viable cells was seen. Significant results were seen at the following days: at Day 0, the infected horses had a significantly higher percentage of cells in late apoptosis. At Days 2, 7 and 14, the infected horses had a significantly lower percentage of cells in early apoptosis. At Day 21, the infected horses had a significantly higher percentage of viable cells and a

significantly lower percentage of cells in early apoptosis. At Day 42, there was a trend towards a decreased percentage of viable cells in the infected horses. At Day 70, there was a trend towards an increased percentage of cells in early apoptosis in the infected group, and a significantly higher percentage of cells in late apoptosis in infected horses.

6.6.1b CD8+ Lymphocytes

Infection had little effect on the viability or level of apoptosis of live CD8+ populations, although several significant results were seen: at Day 14, the infected horses had a significantly higher percentage of viable cells. At Day 28, the infected horses had a significantly higher percentage of cells in early apoptosis. At Day 42, there was a trend towards an increased percentage of cells in early apoptosis in the infected group.

By contrast, there was a higher correlation between apoptosis in dying CD8+ lymphocytes and *S. neurona* infection. In general, the percentage of CD8+ cells undergoing apoptosis was higher in infected horses, with significantly higher percentages of cells in late apoptosis observed at several time points. The following significant results were seen: at Day 0, there was a trend towards an increase in early apoptotic cells in the infected group, and a significantly higher percentage of cells in late apoptosis in infected horses. At Day 2, there was a trend towards a decrease in the percentage of cells in late apoptosis in the infected horses. At Day 7, the infected horses had a significantly lower percentage of cells in late apoptosis. At Day 35, there was a trend towards an increase in early apoptotic cells in the infected group. At Day 42, the infected horses had a significantly higher percentage of cells in late apoptosis. At Day 56, the infected horses had a significantly lower percentage of cells in late apoptosis. At

Day 70, the infected horses had a significantly lower percentage of cells in late apoptosis.

6.6.1c Neutrophils

Infection resulted in a limited effect on the percentages of viable and apoptotic neutrophils. On the few time points when significant results were achieved, infection usually resulted in a decrease in early apoptosis. The following significant results were obtained: at Day 2, there was a trend towards a decrease in the percentage of neutrophils in early apoptosis in the infected horses. At Day 21, the infected horses had a significantly higher percentage of neutrophils in late apoptosis. At Day 35, the infected horses had a significantly lower percentage of neutrophils in early apoptosis. At Day 42, the infected horses had a significantly lower percentage of neutrophils in early apoptosis.

6.6.1d Monocytes

A consistent effect of infection on the apoptosis of monocytes was not seen. The few significant results seen were as follows: at Day 14, the infected horses had a significantly lower percentage of viable monocytes. At Day 21, the infected horses had a significantly higher percentage of monocytes in early apoptosis. At Day 35, the infected horses had a significantly lower percentage of monocytes in early apoptosis.

6.6.1e B-cells

Infection with *S. neurona* did not cause a consistent effect on live B-cell populations. The following significant results were obtained: at Day 14, there was a trend towards a decreased percentage of B-cells in early apoptosis in the infected

horses. At Day 21, the infected horses had a significantly higher percentage of B-cells in early apoptosis.

Infection had a limited effect on B-cells within the dying population, but caused a slight increase in apoptosis. The following significant results were seen: at Day 56, there was a trend towards an increased percentage of B-cells in late apoptosis in the infected horses. At Day 70, there was a trend towards an increased percentage of B-cells in early apoptosis and a significantly higher percentage of B-cells in late apoptosis in the infected horses.

6.6.2 The effect of incubation with PMA/I on apoptosis in infected and control horses

A decrease in cellular proliferation in infected horses had been seen in previous experiments, when cells were stimulated with the combined mitogens PMA and I. As the purpose of this experiment was to assess the reason for this suppression, cells were stimulated in culture for 24, 48 and 72 hrs with PMA/I and then analyzed via flow cytometry in order to assess cell viability and levels of apoptosis. Samples were run using the same protocols as described for unstimulated cells above. The gates were placed using unstimulated cells as a control, and can be seen in Figure 3. Gates needed to be wider in stimulated samples due to the greater variety in cellular size, as was explained previously. These results are summarized in Table 5.

6.6.2a CD4+ Lymphocytes

Incubation with PMA/I did not affect apoptosis in the live CD4+ population, except at Day 7. At Day 7, after 24 hrs incubation, the infected horses had a significantly higher percentage of cells in late apoptosis, and a trend towards an increased percentage of

cells in early apoptosis. Also at Day 7, after 72 hrs incubation, the infected horses had a significantly higher percentage of viable cells.

Incubation with PMA/I had minimal effects on apoptosis in the dying CD4+ population. At Day 7, after 24 hrs incubation, the infected horses had a significantly lower percentage of cells in late apoptosis. At Day 14, after 24 hrs of incubation, the infected horses had a significantly lower percentage of cells in late apoptosis.

6.6.2b CD8+ Lymphocytes

Incubation with PMA/I had a mixed but minimal effect on apoptosis of CD8+ live lymphocytes. At day 7, after 48 hrs incubation, there was a significantly higher percentage of viable cells in the infected group. At Day 42, after 48 hrs incubation, there was a trend towards an increased percentage of cells in early apoptosis in the infected group. At Day 70, after 48 hrs incubation, there was a trend towards an increased percentage of viable cells in the infected group. After 72 hrs incubation, a trend towards a decrease in early apoptotic cells in the infected group was seen.

At Day 70, after 72 hrs incubation, there was a trend towards a decreased percentage of viable cells in the infected group in the dying CD8+ population. This was the only significant result or trend seen within the dying CD8+ lymphocytes when stimulated with PMA/I, therefore it appeared that the effect of incubation with the combined mitogens was minimal.

6.6.2c Neutrophils

The effect of incubation with PMA/I on neutrophils was minimal. At Day 14, after

72 hrs incubation, there was a trend towards a decreased percentage of neutrophils in early apoptosis in the infected horses. At Day 35, after 24 hrs incubation, there was a significantly lower percentage of viable neutrophils in the infected group. Also on this day, after 48 hrs incubation, there was a trend towards a lower percentage of neutrophils in late apoptosis in the infected group. At Day 42, after 48 hrs incubation, there was a significantly lower percentage of viable neutrophils in the infected group.

6.6.2d Monocytes

The most significant effect of incubation with PMA/I was seen on the monocyte population. Early in the experiment, a decreased percentage of monocytes in late apoptosis was seen in infected horses after short periods of incubation. Later in the experiment this trend was reversed, with a decreased percentage of viable cells and an increase in late apoptosis seen in the infected horses.

The following significant results were obtained: at Day 21, after 48 hrs incubation, there was a significantly lower percentage of monocytes in late apoptosis in the infected group. At Day 28, after 24 hrs incubation, there was a significantly lower percentage of monocytes in late apoptosis in the infected group. At Day 35, after 24 hrs incubation, there was a significantly lower percentage of viable monocytes in the infected group. After 48 hrs, there was a significantly higher percentage of monocytes in early apoptosis in the infected group. After 72 hrs incubation, there was a trend towards a higher percentage of monocytes undergoing late apoptosis in the infected group. At Day 42, after 48 hrs incubation, there was a trend towards a lower percentage of viable monocytes in the infected group. At Day 56, after 24 hrs incubation, there was a trend towards a lower percentage of viable monocytes in the infected group. At Day 70, after

48 hrs and 72 hrs incubation, there was a trend towards a higher percentage of monocytes undergoing late apoptosis in the infected group.

6.6.2e B-cells

Incubation with PMA/I had mixed effects on live B-lymphocytes. Early in the experiment, an increase in late apoptosis was seen in the infected horses. An increase in viability of live B-lymphocytes in the infected group was seen after short incubation periods during the middle of the experiment, than towards the end of the study period, an increase in levels of apoptosis was seen. The following results were statistically significant: at Day 0, after 24 hrs incubation, the infected horses had a significantly higher percentage of B-cells in late apoptosis. At Day 2, after 48 hrs incubation, the infected horses had a significantly higher percentage of B-cells in late apoptosis. At Day 14, after 48 hrs incubation, there was a trend towards a decreased percentage of viable B cells in the infected group. At Day 21, after 24 hrs incubation, there was a significantly higher percentage of viable B-cells in the infected group. After 72 hrs incubation, there was a significantly higher percentage of B-cells in early apoptosis in the infected horses. At Day 28, after 24 hrs incubation, there was a significantly higher percentage of viable cells in the infected group. At Day 35, after 48 hrs incubation, there was a significantly higher percentage of B-cells in late apoptosis in the infected group. At Day 70, after 24 hrs incubation, there was a significantly higher percentage of B-cells in early apoptosis in the infected group.

Incubation with PMA/I had less of an effect on dying B-lymphocytes, with a small number of significant results seen. At Day 21, after 24 hrs incubation, there was a trend towards an increased percentage of dying cells in early apoptosis in the infected horse

population. At Day 35, after 72 hrs incubation, there was trend towards a lower percentage of viable cells in the infected group. At Day 70, after 24 hrs incubation, there was a significantly lower percentage of cells in late apoptosis in the infected group.

6.6.3 The effect of antigen-specific stimulation on apoptosis (Table 6)

6.6.3a CD4+ Lymphocytes

Effects of merozoite stimulation were only seen after 24 hrs incubation in the live CD4+ group. Merozoites induced an increase in early and late apoptosis in the infected horses: the following results were significant. At day 35, after 24 hrs incubation, the infected horses had significantly more cells in late apoptosis. At day 70, after 24 hrs incubation, the infected horses had significantly more cells in early and late apoptosis.

Merozoites induced a difference in apoptosis in the dying CD4+ population only at Day 70. At day 70, after 24 hrs incubation, the infected horses had a trend towards a higher percentage of viable cells. Also after 24 hrs incubation, there was a significantly lower percentage of cells in early apoptosis and late apoptosis in the infected horses. After 72 hrs incubation, there was a trend towards a lower percentage of viable cells in the infected horses, and a trend towards an increased percentage of early apoptotic cells in the infected group.

6.6.3b CD8+ Lymphocytes

Merozoites induced a significantly higher percentage of apoptosis in the infected group on certain time points towards the end of the experiment. The following results were significant: at Day 42, after 24 hrs incubation, the infected horses had a significantly higher percentage of cells in early apoptosis. At Day 70, after 72 hrs

incubation, the infected horses had a significantly higher percentage of cells in late apoptosis. Merozoites caused no significant effects on the dying CD8+ lymphocyte population.

6.6.3c Neutrophils (merozoites)

Merozoites induced a higher percentage of apoptosis in the infected group on certain time points towards the end of the experiment. The following results were significant: at Day 28, after 24 hrs incubation, the infected horses had a significantly lower percentage of neutrophils in early apoptosis. At Day 56, after 24 hrs incubation, there was a trend towards an increased percentage of viable neutrophils in the control horses. At Day 70, the control horses had a significantly higher percentage of viable neutrophils, and the infected horses had a significantly higher percentage of neutrophils in early apoptosis. There was a trend towards a higher percentage of cells in late apoptosis in the infected horses. After 72 hrs incubation there was a trend towards an increased percentage of cells in early apoptosis in the control horses.

6.6.3d Monocytes (merozoites)

Merozoites had less effect on the monocyte population. The following results were significant: at Day 56, after 24 hrs incubation, there was a trend towards an increased percentage of viable monocytes in the control group. At Day 70, after 24 hrs incubation, there were significantly higher percentages of monocytes in late apoptosis in the infected horses.

6.6.3e B-cell (merozoites)

Merozoites had mixed effects on the B cell population. In the live B-cells,

merozoites caused a decrease in apoptosis in the infected horses on certain time points. The following results were significant: at Day 35, after 72 hrs incubation, the infected horses had a significantly higher percentage of viable B cells. At the same time point, the control horses had a trend towards a higher percentage of cells in early apoptosis, and a significantly higher percentage of B cells in late apoptosis.

In the dying B cell population, the effect of merozoite incubation was mixed. The following significant results were seen: at Day 35, after 72 hrs incubation, the infected horses had a trend towards a higher percentage of viable B cells. At Day 56, after 72 hrs incubation, the infected horses had a trend towards a higher percentage of viable B cells. At Day 70, after 24 hrs incubation, the infected horses had significantly higher percentages of B cells in early apoptosis. At Day 70, after 72 hrs incubation, the infected horses had a trend towards a decreased percentage of viable B cells and a significantly higher percentage of B cells in early apoptosis.

6.7 RESULTS FROM OBJECTIVE TWO: FLOW CYTOMETRIC ANALYSIS OF CELLULAR DIVISIONS

All samples were run as described in the materials and methods section of this thesis. Examples of outputs from the flow cytometer are shown in Figure 4. Results for flow cytometry after 72 hrs of incubation with PMA/I and with merozoites are shown. Samples that were stimulated with mitogens have less defined populations than unstimulated samples. Cells were more widely distributed, with more variety in forward and side scatter than in the unstimulated samples.

At longer periods of incubation, a greater proportion of cells of all cell types were

found in the “dying” gate, as these cells underwent apoptosis and necrosis and therefore lost cell volume. In order to discern the effects of infection between immune cell subsets, these cells were evaluated separately from the “live lymphocyte,” “monocyte” and “neutrophil” gates.

Both the effects of infection and stimulation with mitogens were evaluated with respect to cellular divisions. In general, stimulated samples underwent more cellular divisions than unstimulated samples. This is also demonstrated in Figure 4.

6.7.1 The effect of PMA/I on cellular divisions/ proliferation based on CFSE staining in infected and non-infected horses

6.7.1a CD4+ Lymphocytes

When stimulated with PMA/I, a greater number of cellular divisions occurred in infected horses than in control animals. This was true for both the live and dying CD4+ populations. These results are shown in Table 7. The following results were significant in the live population: at Day 2, there was a significantly higher percentage of lymphocytes not undergoing division in the control group. At Day 21, there were significantly more lymphocytes having undergone three cellular divisions in the infected group. At Day 56, there was a trend towards a higher percentage of cells that had undergone three divisions in the infected group.

When considering the dying CD4+ population, a greater number of divisions were again seen in infected horses. At Day 2, there was a trend towards an increased percentage of CD4+ cells that had undergone two or three divisions in the infected group. At Day 7, there was a significantly higher percentage of CD4+ cells having undergone three divisions in the infected group. At Day 21, there was a trend towards

an increased percentage of CD4+ cells that had undergone more than three divisions in the infected group. At Day 56, there was a significantly higher percentage of cells in the control group that had undergone two cellular divisions, and a significantly higher percentage of cells in the infected horses that had undergone three or more divisions.

6.7.1b CD8+ Lymphocytes

When stimulated with PMA/I, a greater number of cellular divisions occurred in infected horses than in control animals. This was true for both the live and dying CD8+ populations. These results are shown in Table 7. In the live CD8+ population, the following results were significant: at Day 2, there was a significantly higher percentage of CD8+ cells that underwent two divisions in the infected group, and a trend towards a higher percentage of cells in the control group that had undergone no divisions. At Day 21, there was a trend towards a higher percentage of cells in the infected group that had undergone three or more divisions. At Day 28, there was a significantly higher percentage of CD8+ cells that underwent two divisions and a trend towards a higher percentage that underwent three divisions, in the infected group. At Day 56, not enough cells survived in PMA/I to perform a reliable analysis.

In considering the dying CD8+ population, a higher percentage of cells underwent two or more cell divisions in the infected group, with control horses having significantly fewer numbers of cellular divisions. The following results were significant: at Day 2, there was a significantly higher percentage of CD8+ cells that underwent three divisions in the infected group. At Day 21, there was a significantly higher percentage of CD8+ cells that underwent more than three divisions in the infected group. At Day 28, there was a significantly higher percentage of CD8+ cells that underwent one division in the

control group, and a trend towards a higher percentage of cells in the infected group that had undergone two divisions. At Day 56, not enough cells survived in PMA/I to perform a reliable analysis.

It was our aim to assess the effect of infection status and that of incubation with various mitogens on neutrophils, monocytes and B-lymphocytes. These cellular subsets did not survive well enough in CFSE plates for us to perform reliable analysis: resultant cell numbers were very small, and made assessment of the number of cell divisions impossible.

6.7.2 Effect of stimulation with merozoites on number of cellular divisions in infected and non-infected horses

6.7.2a CD4+ Lymphocytes

When stimulated with merozoites, trends were the opposite to those seen with PMA/I stimulation. In the live CD4+ population, merozoites caused more cellular divisions in control horses, and a higher percentage of cells undergoing no divisions in the infected animals. No significant results or trends were seen in the dying population.

When considering live CD4+ lymphocytes, the following results were significant: at Day 28, there were significantly more cells in the control group that had undergone three cellular divisions. At Day 42, there was a trend towards an increased number of cells in the infected population that had not undergone any divisions (Table 8).

6.7.2b CD8+ Lymphocytes

When stimulated with merozoites, trends were the opposite to those seen with PMA/I stimulation. In the live CD8+ population, merozoites caused more cellular

divisions in control horses, and we observed a higher percentage of cells undergoing no divisions in the infected animals. Fewer significant results were seen in the dying population.

In the live CD8+ population, the following results were significant: at Day 28, there was a trend towards a higher percentage of cells in the control group that had undergone three divisions. At Day 56, there was a trend towards a higher percentage of cells in the control group that had undergone two divisions.

In the dying CD8+ cells, at Day 70, there was a trend towards a higher percentage of cells in the control group that had undergone two divisions.

CHAPTER 7: DISCUSSION

In this section I will present the explanations of and for our results and possible reasons why certain expected patterns were not seen. The results will be interpreted, with reference to current literature, and a view to future study.

7.1 NUMBER OF HORSES

Small numbers of horses were used in this study, which was a disadvantage in demonstrating significant results. Individual variation between animals is large. Therefore, within a normal population of horses, there will be considerable variation in adaption to a new environment, responses to external stressors, gaits exhibited, normal range of motion and of course immune function and response to infectious challenge. Nine horses were used as this was the maximum number of animals that budget allowed.

One control horse (Horse 2) received a dose of parasite in error on day 4 of the experiment. This mistake was realized, and the horse was treated with a single dose of intravenous flunixin meglumine at 1.1 mg/kg, and temperature monitored twice a day. This horse did not suffer any adverse effects to the administration of another horse's blood, yet the horse was excluded from all of the data analysis. It was decided that this horse no longer represented a naïve animal, and therefore could not be included as a control subject. Horse 6 was the infected horse that had been administered the sample intended for Horse 2; this horse also did not suffer any adverse effects, and was also treated with flunixin meglumine at the same dosage. A replacement dose of the parasite was given to this horse, and it remained part of the infected group for all data analyses. The error in dosing was not believed to have influenced the infected animal, yet it did decrease the number of control animals and thus negatively impact the experiment.

7.2 SnSAG1 ELISA METHODS

Changes in SnSAG1 ELISA on both serum and CSF samples were used in this experiment to document exposure and thus infection. A rise in titer was seen in all infected horses, yet also in some control animals. Cytologic evaluations were performed on CSF samples obtained from the atlanto-occipital space before the experiment. Red blood cells and white blood cells were enumerated, and CSF color, turbidity and protein content were assessed. All parameters were within normal limits for equine reference values. The total RBC count allowed us to visualize those samples excessively contaminated due to iatrogenic hemorrhage: on baseline samples, CSF from Horse 1 contained 1575 RBC/ μ L. Horses 2, 4 and 6 also contained more blood than the 8 RBC/ μ L specified by Miller to adversely affect the outcome of certain diagnostic tests for

EPM, such as the Western Blot ⁸⁶. None of the horses had a pre-experiment SnSAG1 titer on CSF that represented even a weak positive: therefore, this contamination did not affect interpretation of these results. Cytology was not performed on CSF obtained after the experiment for financial reasons. A total red blood cell (RBC) count would have been useful, in order to prove that elevations in CSF SnSAG1 titer were not due to iatrogenic hemorrhage when sampling. Seropositive horses may generate a false positive result on CSF analysis due to the presence of blood components in the sample, and/or associated antibody. Many of the horses in the study had an increased serum titer at the post-experiment sample, therefore if blood was present within the CSF sample submitted it could have falsely elevated results. However, our technique in performing the atlanto-occipital puncture had improved, and less frank hemorrhage was noted. The initial 2ml of CSF withdrawn from the spinal needle was discarded in order to minimize blood contamination of the sample. If a red-tinge was noted to the fluid withdrawn following this, CSF was discarded until it ran clear. One horse required the withdrawal of 3.5ml before a clear sample was obtained.

CSF titers must be interpreted in conjunction with serum titers, as horses with high numbers of circulating antibodies in the blood, and/or damage to the BBB, may experience cross-over of antibodies into the CSF that are not generated in this region. Horses with SnSAG1 CSF titers ≥ 1 likely represent intrathecal production of antibodies, and thus active infection. Horses with high serum titers, but low CSF values (such as the two control horses mentioned in the following section), may represent horses whose immune system has been presented with sufficient time to quell the *S. neurona* infection prior to entry into the CNS, or may be those horses in early stages of disease. Blood contamination of CSF at sampling may be particularly significant in cases with a high

serum titer, as it may falsely elevate CSF values. Horses with low titers in both serum and CSF titers are less likely to have been infected. In future experiments, it would be ideal to perform RBC and cytologic analysis on the post-experiment CSF samples as well, to conclusively rule out hemorrhagic contamination of CSF, and to prove that results were accurate. SnSAG1 titers on CSF pre and post-experiment must be interpreted with caution as the lowest possible value for the test changed during our study period. Prior to the experiment, and given that all of the horses had a low serum titer, the first dilution performed was 1:2. Therefore the lowest possible (negative) result of the test was read out as <2. During the time in which we performed our experiment, and thus applicable to post-experiment CSF SnSAG1 results, the testing laboratory had changed the protocol so that the first dilution performed was a 1:1 dilution, and the lowest possible (negative) value was thus <1. The reduced sensitivity in our pre-experiment samples is unlikely to have resulted in a false negative result, as all the horses had negative serum titers at this time.

7.3 NATURAL EXPOSURE

Based on serum titers, it appeared that all of the control horses were naturally exposed to the parasite during the experiment. Two of the control horses (Horses 1 and 8) experienced a significant rise in serum SnSAG1 titer during the study period. Both horses had baseline titers of <2, and recorded post-experimental SnSAG1 titers of 32. These horses never received contact with the experimental source of the protozoan. The intravenous model of infection used in this study did not create a source of infective *S. neurona* in the feces or saliva of experimentally infected horses, and therefore infected horses do not pose a risk to control animals (Ellison, unpublished data). Several studies have reported likely cross-contamination of control horses in experimental settings^{59,75},

yet these have all been seen with oral inoculation methods, and not through intravenous challenge.

The rise in serum SnSAG1 titer seen in these two control horses likely represents a natural, environmental exposure. It is possible that the experimentally infected horses were also exposed to environmental sources of the pathogen, and thus received additional challenge of unknown dose and strain at an unknown time point within the experiment. This may, in addition to experimental infection, have resulted in the change in SnSAG1 results and neurologic signs seen. Horses were permanently housed in a large pasture with access to shelter, and were managed as a large single group. They were fed grain, which was stored in sealed bins and represented minimal risk of contamination with opossum feces, and hay that was fed from the ground and stored in a barn elsewhere on the property. It is possible that the hay or pasture could have been contaminated with excreted sporocysts, and was a source of environmental challenge to all horses. However, no opossums were seen within the barn, and hay was not visibly contaminated. It is possible that opossums, as nocturnal mammals, may have been present at night, in either barns or fields. Our study was conducted in southwest Virginia, in an area with a relatively high density of wild opossums, and although every effort was taken to minimize potential exposure, horses could potentially have come into contact with these wild opossums at any point during the study. Sporocysts excreted by opossums onto the grass within the pasture could have been ingested by the horses, although no opossum feces was noted in the fields containing or surrounding our study horses at any time. It is difficult to state with certainty that this proposed environmental exposure occurred and even harder to attempt to qualify at what point in the study exposure could have happened. If finances had allowed, and for

future experiments, it would be recommended to repeat SnSAG1 titers on serum more frequently within the study to be able to more accurately determine when natural exposure occurred in the control horses.

It would have been interesting to correlate the titers with the neurologic signs, although this may not have yielded significant findings, for a number of reasons. Horse 1's total neurologic score varied considerably. This was in part due to the perceived change in attitude, which resulted in a change in score, as this horse became more trusting towards humans. However, the attitude change could have been due to environmental exposure to the protozoan, and subsequent natural clearance of infection. If this behaviour change was associated with infection, one would have expected the horse to become aggressive upon resolution of the infection, which did not occur. As the horse's improved attitude persisted, this suggested that behaviour changes were due to socialization. Horse 8 remained largely normal throughout the study, and was the most consistent of all the horses in neurologic scoring: this horse had a maximum total score of 5 on Week 9, and often represented the horse with the lowest score at each evaluation. Horse 4 was the only control horse whose serum titer did not change, and therefore represents the most reliable control animal, based on serum SnSAG1 titers alone. This horse had considerable musculoskeletal abnormalities prior to the experiment, however, which varied in severity on a daily basis. This meant that the total score for this horse was often relatively high (sometimes higher than that of some of the infected horses) and varied with time. This horse also recorded a high post-experiment SnSAG1 CSF titer, representing intrathecal production of antibodies and active infection. A CSF titer of 1 or 2 is considered significant¹¹⁶. Thus it is possible that the variation in the horse's signs were also associated with natural exposure in addition

to the baseline musculoskeletal issues. Most of the infected horses, and Horse 4, mounted CSF titers high enough to be considered positive and likely indicative of intrathecal antibody production in those horses with low serum values. It is therefore misleading to present Horse 4 as the most valid of the control horses in all data analysis methods. None of the control group horses represented perfect control animals due to this presumed environmental exposure, and this may have negatively impacted the results of the study, and decreased the observed differences between treatment groups. The experimentally infected horses may also have been challenged through natural sources of the protozoan, and some of the results we saw could have been attributable to this additional infective dose. It is impossible to tell if, and when, natural exposure occurred in the experimentally infected horses. Most horses that are naturally exposed to the protozoan do not develop clinical disease, therefore exposure in our control group is not equivalent to clinical EPM. Although less ideal, we can still compare the effects of experimental infection with those of non-experimental infection in these horses. Natural exposure can still potentially confound the results.

7.4 AGE

The wide range of ages of horses in our study may have affected the results. The horses in our study group ranged in age between 1.5 and 11 years of age. Previous studies have often focused on young horses from 1-3yrs old. The immune response varies with age in all animal species; young animals may respond very differently to antigenic stimulus than aged individuals^{124,125}. The affect of age on the immune system is wide-ranging: innate, cell-mediated and humoral immune responses all decline with advancing age in a phenomenon known as immunosenescence. Macrophages express lower levels of receptors, such as the toll-like receptor family (TLRs) and thus cytokine

levels may be reduced ⁷⁰. Changes in the levels and types of cytokines expressed may alter the type of immune response that an animal can mount in response to challenge (for example bias towards a Th2/Tc2 response if less IL-12 and more IL-4 is present). Aged humans and older dogs may have decreased numbers of CD4+ lymphocytes and increased CD8+ lymphocytes: indeed the ratio of CD4+ to CD8+ cells has been shown to be inverted in human subjects that are very old ^{70,126}. Lymphocytes from older animals lose their ability to progress through the cell cycle, and thus early events in the T cell response to antigens such as a rise in intracellular calcium may be impaired. This response is not normalized through additional exposure to high levels of IL-2. Somatic mutation within the variable region of immunoglobulins also ceases, so antibody affinity and adaptability may be compromised. Older horses have reduced antibody responses to influenza vaccination, and also demonstrate reduced lymphocyte responses to mitogens ^{70,124}. The majority of our horses were between 2 and 5yrs old: unfortunately it was not possible to obtain age matched horses that were also matched for state of origin, antigenic naivety to *S. neurona*, breed and size. The horses were randomly allocated to groups; therefore mean age between treatment groups was not identical. The average age for the control group was 3.7 years, whereas the mean infected age was 5.1 years. This difference of 1.4 years is unlikely to result in a vast difference in immune system maturation or immune response capabilities. Our older age range of horses may have made them more resistant to the development of infection, displaying a more mature immune response, and therefore influencing our results.

7.5 BREED

Breed of horse influences genetic makeup, and therefore capabilities of immune response: different breeds may respond differently to certain antigenic stimuli. Horses

obtained for use in our study were all Quarter Horse or Quarter Horse Cross breed horses. Some other studies⁸² used mainly Thoroughbreds in the study population. Breed has been shown to be a significant factor in the development of EPM in some studies, yet this is thought to represent an association of certain breeds with high-stress environments, such as racing or competition. Breed can also affect the immune system. There are many breed-related susceptibilities or associations with certain diseases, such as insect hypersensitivity, sarcoid development, and SCID, as mentioned earlier. It therefore may be difficult to make direct comparisons of efficacy of immune response between studies, when different breeds (and ages) of horse are used. Within this study, however, most of the horses were Quarter Horse or Quarter Horse crosses, and therefore the effect of breed on the results is likely to be minimal.

7.6 NEUROLOGIC DISEASE DEVELOPMENT AND EXPERIMENTAL MODEL

We were successful in inducing clinical neurologic disease in previously normal horses through inoculation of horses with autologous venous blood infected with live merozoites. This model was developed by Ellison, and our study constitutes the first time that this model has been effectively reproduced at a separate institution.

The horses in our study demonstrated neurologic signs earlier than reported in Ellison's original experimental model. This may be an effect of natural exposure in addition to experimental infection. However, personal communication with Ellison revealed that her experimentally infected horses developed cranial nerve signs and behavioral changes in a manner similar to horses in this study. In previous studies⁷⁷, Ellison noted that all challenged horses demonstrated neurologic signs (ataxia) by sixty days post-challenge, when the parasite was given intravenously for 14 days. However,

Ellison also reported that horses in both challenged groups in her 2009 study¹¹⁶ experienced neurologic signs as little as 6 days after challenge; the cranial nerve signs spontaneously resolved in some animals, although ataxia still developed later. These signs were attributed to the presence of the organism in the CNS, and to the inflammatory response to *S. neurona*.

It is possible that a similar effect was seen in our study, and might explain why some infected horses were improving neurologically prior to initiating treatment. Early signs seen in our horses were largely those affecting cranial nerves: this is shown in Figure 7 and is in accordance with signs seen by Ellison^{27,77}. Ataxia peaked later in the experiment, as is demonstrated in Figure 7. This is also in accordance with Ellison's work^{27,77,116}. In Ellison's 2009 study, ataxia was seen late in the study period, and was observed at 60 to 125 days post intravenous challenge. A rising ataxia score was seen in the infected horses towards the end of our study, although the level of neurologic signs expressed by the horses had a biphasic pattern. Peaks were seen early in the experiment (largely due to cranial nerve signs, as discussed) and at the last weeks of the experiment (mainly due to ataxia). This biphasic nature of neurologic signs has not been extensively reported in natural cases. It is possible that signs are exhibited in a biphasic pattern in field cases of EPM, but that owners miss the more subtle cranial nerve signs and only call a veterinarian when the later signs are noted, as ataxia and movement deficits may be more noticeable to the owner and may be more pronounced, especially in a horse that is ridden. It is also possible that the biphasic nature of neurologic signs in this experiment (shown in Figure 7) is as a result of the occurrence of both natural and experimental exposure to the protozoan at varying time points. This is unlikely however, as disease in the experimentally infected horses progressed in a similar

manner to that demonstrated in experimentally infected horses in Ellison's previous experiments, which were not exposed to natural sources of the protozoan. Thus, this suggests that the progression of signs is associated with experimental infection alone. The most significant difference between infected and control horses' neurologic scores was displayed at week 10 (the last week of the experiment). If we had been able to continue the experiment for a longer period post-challenge, the infected horses may have become more ataxic and more neurologic overall and greater significant differences may have become evident. Unfortunately, financial constraints made continuing the experiment an impossibility.

In order to accurately and quantitatively assess the onset and development of neurologic deficits associated with EPM, it was necessary to monitor and record a wide range of parameters in addition to those relating to ataxia. Cranial nerve signs, paresis, lameness, muscle atrophy, hypermetria and spasticity have all been reported in both naturally occurring and experimentally induced cases of EPM, and these signs were all seen in this study. The wide range of parameters evaluated, and the need to grade severity, resulted in a high maximal possible score on the neurologic grading system used. In comparison with total possible score, the maximum score achieved by any horse in this study was fairly low. As such a high total score was possible, the effect of ataxia (the most common sign associated with EPM) did not contribute significantly to the total score, although may represent a clinically significant increase in neurologic deficits.

In an attempt to create a scoring system that was very sensitive to the development of neurologic signs, our system was affected by signs (i.e. change in

attitude) that were non-neurologic in origin, but that could occur due to a neurologic basis. Changes in attitude for reasons other than the development of neurologic disease, (i.e. improvement in socialization) could not be distinguished on the scoring system. For example, Horse 1 experienced an attitude change due to decreased fear of humans, yet this caused an elevation in this horse's score during the acclimatization period. Another example of this involves lameness. Various horses were seen to suffer from musculoskeletal lameness at certain points before and during the study. This was recorded in a separate observation, but may have falsely elevated the score for these horses on certain weekly observations. Ambient conditions (i.e. excessive mud, ice or snow) also affected the horses' abilities to move normally and thus impacted scoring differently, dependent on conditions. Environmental conditions affect all horses; thus they may be the cause of daily and/or weekly variation in scores and may affect standard error, yet should apply to all horses equally. The distribution of scores within the population is more likely to be affected by individual horse factors such as lameness.

Another factor that influenced the outcome of the overall neurologic status of the infected animals was the number of days of infection (total infective dose). As our horses developed behavior changes and cranial nerve signs more rapidly than those reported by Ellison, and as we also knew from previous studies that the horses would become progressively worse, we stopped infecting the horses after 10 doses of the parasite. The horses were expected to (and later did) develop Grade II ataxia. We were concerned, had we continued infecting the horses for the full 14 days as planned, that the horses would become more severely affected than Grade II. Therefore we stopped infecting the horses after 10 days instead of continuing to 14 days. As a decreased total dose of *S. neurona* was used in this study, we may not have caused the same levels of

clinical signs, SnSAG1 titer elevation, and changes in immune function, as that in previous similar studies¹¹⁶. An additional consideration was that if we had continued to infect horses, and they got progressively worse, this would likely lessen overall treatment success. We hoped to adopt the horses out to suitable homes following treatment at the end of the study.

In summary, we successfully reproduced neurologic disease in our infected group of horses. Analysis was complicated by external factors (i.e. weather and musculoskeletal disease) that influenced scoring, and by probable environmental exposure within the control group. If the experiment were repeated, and a larger budget were available, more horses should be used, and inside housing, feeding, food storage and exercise facilities utilized in order to minimize the above effects. Younger horses without pre-existing musculoskeletal deficits should be chosen for inclusion in future studies.

7.7 CELL SEPARATION

Peripheral blood mononuclear cells (PBMCs) were isolated through density gradient centrifugation. A sample with a high purity of lymphocytes was desired. Previous experiments have documented that increased numbers of neutrophils can negatively impact the ability of lymphocytes to proliferate⁸². Differential counts were performed on cytopins to assess the level of neutrophil contamination (see Appendix D). Examination of 28 slides from a random selection of horses and time points generated an average differential count of 6% neutrophils, 87.5% lymphocytes and 6.5% monocytes. This represented a high level of purity for lymphocytes in these samples. A very slight increase in neutrophil count in samples exhibiting a second peak

in Coulter Counter outputs was seen: the average neutrophil percentage for double peak samples was 6.2%. The effect of the increased neutrophil percentage was believed to be insignificant.

7.8 PROLIFERATION

A significant difference in response to PMA/I between infected horses and those in the control group was not seen, except in the logarithmic values at Day 35 (Figure 8). This was in contrast to previous studies^{73,82,83}, when infected horses demonstrated a markedly suppressed lymphocyte proliferation response when stimulated with PMA/I. This could have been due to environmental exposure (that was presumed to have occurred in this experiment). A pattern towards a decreased stimulation index in the infected horses towards the end of our experiment was seen, although this difference was only significant at Day 35. The decreased responsiveness to PMA/I in infected horses may still represent a biologically significant difference, however. Also, it should be noted that when assessing the deltas for PMA/I response compared to the stimulation indices, the deltas for both groups of horses decreased over time. The thymidine used in this study was newly purchased, and other tissue culture conditions were functioning normally. Therefore, it is possible that this decrease in the deltas was an effect of natural exposure and/or experimental infection. Both treatment groups were affected by natural sources of the pathogen, hence this may be why we did not see a significant treatment difference.

PMA/I is a pan-leukocyte stimulating mitogen, and therefore stimulates not only T lymphocytes and B lymphocytes, but also monocytes, dendritic cells and natural killer cells. It may stimulate different subsets of lymphocytes than other mitogens⁸². The fact

that a significant difference in ConA or PWM stimulated proliferation was not seen in our study suggests that the ability of T and B lymphocytes to proliferate in response to mitogens was unaffected. It is therefore possible that the suppressed response to PMA/I observed in infected horses was due to a decreased responsiveness in a non-lymphocyte cellular subset, such as mononuclear or dendritic cell lines.

Mononuclear cells are those most commonly found to contain merozoites, and have a key role in mounting an effective cell mediated immune response through their ability to phagocytose infected cells. Dendritic cells (DCs) are highly effective antigen presenting cells, and also secrete differing cytokines in response to the detection of antigens, in order to generate a bias towards a humoral (Th2/Tc2), cell-mediated (Th1/Tc1) or T-regulatory immune response ⁷⁰. The exact cytokines secreted by DCs, and thus the type of T-cell response that ensues, depends on the characteristics of the antigen detected and its location within the host. A defect in either monocyte or dendritic cellular subsets could therefore be detrimental to the host, and result in a decreased capability to detect and destroy intracellular pathogens. This is further discussed later in this section.

PMA/I suppressed responses in all cellular subsets may be a direct effect of *S. neurona* on PMA/I induced signaling: inflammation from infected cells could alter PMA/I induced signaling, or this could be a direct effect of infection. To determine if such an effect is present, future studies could test the activity or concentration of tyrosine kinases and signaling molecules, i.e. Raf, MAPK/ERK kinase (MEK), extracellular signal-regulated protein kinase, protein kinase C isoenzymes, and MAPK in infected cells ¹⁰¹. Alternatively, ionomycin has been shown to cause *S. neurona*

egression from the host cells, albeit at a much higher concentration than was used in this experiment ¹⁰¹. If the PBMCs incubated with PMA/I from infected horses contained *S. neurona* merozoite infected cells, merozoites could be released causing inflammation that would inhibit cell proliferation and/or merozoites could infect other PBMCs. This could possibly result in the lower proliferation response to PMA/I in EPM horses compared to normal horses.

There were many factors that may explain why our results did not display a significant decrease in proliferation responses in the infected horses on other time points studied, and why the difference in PMA/I responses was not as marked as had been seen in previous studies. Some general factors included age, breed and dose of parasite received, and were discussed above, whereas others were more specific to lymphocyte proliferation responses and therefore will be mentioned subsequently.

7.8.1 Individual horse variability

One of the infected horses (Horse 3) demonstrated a high spontaneous proliferation on many of the time points studied. This contributed to the high degree of variability within the results, and decreased the likelihood that results would be significant. At baseline, this horse's spontaneous proliferation was at least twice as high as most of the infected and control horses, with an average count of 23017 CPM. The highest average spontaneous proliferation in the other horses was 11897 CPM for Horse 2; this horse was excluded from the analysis for reasons detailed above. Horse 3 had the highest spontaneous proliferation on Day 7 (twice as high as the other horses), Day 14 (almost three times as high as most of the other horses), Day 21 (almost three times as high as the other horses), Day 28 (over twice as high as any other horse), Day

35 (over twice as high as any other horse) and Day 70 (twice as high as other horses). Mitogen stimulation induced a proliferative response that was similar to that of the other horses, and not proportionally increased when compared to Horse 3's spontaneous proliferation. Therefore, stimulation indices were affected.

Horse 3 was the oldest horse in our study, at 11 years old. It may be that this horse's immune response was different than the other horses studied due to age-related changes. Noticeable differences in this horse's leukocyte numbers of cell subset percentages were not seen, however. A high spontaneous lymphocyte proliferation is sometimes seen in animals suffering from neoplastic conditions (e.g. lymphoma) as may be induced by leukemic retrovirus infection, such as Bovine Leukemia Virus and human T-cell leukemia virus ^{127,128}. A complete blood count, cytologic examination of a blood smear by a board-certified clinical pathologist, and an assessment of immunoglobulins by radioimmunoassay were all within normal limits; there was no evidence of lymphoma or leukemoid disease. The horse remained otherwise healthy throughout and following the study period.

One of the other infected horses (Horse 5) demonstrated a low spontaneous proliferation on many of the time points studied. This also increased the variability within the results, as this meant that the stimulation index for this horse was usually considerably higher than that of the other horses. At baseline, this horse had the joint lowest spontaneous proliferation, at 9017 CPM. At Day 2, horse 5's spontaneous proliferation was half that of the majority of the other horses, but her PMA/I stimulated average was equal to those of the other horses, creating a high stimulation index. On day 7, Horse 5 again had the lowest spontaneous proliferation. On day 21, this horse's

spontaneous proliferation was approximately half that of most of the other horses. On day 28, it was significantly lower than any other horse. On days 35 and 42, Horse 5 had the lowest spontaneous proliferation. On day 56, it was again at least half the value of most of the other horses. Horse 5 was 7 years old, and thus not outside the ranges of ages of the other horses in the study. We did not identify a reason for her low spontaneous proliferation.

The fact that the infected group contained both Horse 3 and Horse 5 may have affected the averages for this group, as the horses ‘cancelled each other out’: one had a low index and one had a higher index at most of the time points. Although this is not a linear relationship, it may have reduced the impact of each of these horse’s individual results on the overall average for the infected group. For example, without Horse 5 the overall average index for infected horses may be lower. It also illustrated the variability in the capabilities of immune responses between individual animals.

7.8.2 Stimulation index calculation

There are several different ways of comparing responses to mitogens. Previous experiments using mitogen-stimulated lymphocyte proliferation assays have used either stimulation indices, as in this study, or calculated the change in proliferation (“deltas”) by subtracting the average spontaneous proliferation from the mitogen-stimulated CPM. Both methods were used in our initial analysis of the data, although neither yielded a large number of significant results. Stimulation indices were chosen as they remained more consistent throughout the course of the study. The ‘deltas’ tended towards a decreasing value as the study progressed, for both the infected and control populations (see Appendix E). This may be due to a level of tolerance developing in both

treatment populations, or be due to natural exposure within both treatment groups resulting in decreased PMA/I responses, as demonstrated in previous studies^{82,83}.

7.8.3 Individual well variability

There were also factors that influence the results from an individual well for an assay (i.e. mixing inaccuracies of reagents and mitogens, and equipment malfunction in aspirating or washing samples) that may have applied to our study. To limit the effect of individual well variability on overall result, samples were plated in triplicate so that each horse could act as its own internal control at each time point. Average counts for the three wells were then calculated, and it was on these values that statistical analysis was performed. Stimulation indices were calculated for PMA/I by dividing the average of the three wells stimulated with this mitogen, by the average of wells in triplicate that contained no mitogen (media only) that represented spontaneous proliferation. At several time points, a small number of samples in triplicate yielded widely differing results. On one occasion this was noticed to be the result of the cell harvester, as several of the vacuum assisted harvester tips appeared to be malfunctioning, and the corresponding sample was not aspirated completely from the plate as required. On other occasions a reason for these very low individual well counts was not immediately evident. These spurious results were highlighted, and an average of the two remaining wells was calculated if one well represented a greater than three-fold difference to the other two wells. A separate statistical analysis of these results was not performed as the difference between treatment groups, once the spurious results had been discounted, was less on each occasion than when they remained within the data set. It was therefore believed that these abnormal results were not the reason why we did not achieve significant values in this part of the study.

On day 70, most of the horses recorded a low spontaneous proliferation, and lower than average mitogen-stimulated CPM. The incubator where the plates were housed was noted to have run out of CO₂ (due to a leaky valve) when it came time to harvest the samples, which may explain this generalized suppressed proliferation response on this time point. It did not appear to dramatically alter the stimulation indices, however, as both spontaneous and mitogen-stimulated responses were suppressed.

7.8.4 Overnight incubation of samples

At Day 21 of the experiment, additional blood was pulled from the horses and incubated overnight, when it was then processed and plated for lymphocyte proliferation assays in the same manner as described. This step was added as it had been noted in previous experiments to exacerbate the difference in PMA/I proliferation responses^{82,101}. In our study, a significant difference was seen at Day 70 when samples were stimulated with PMA/I. The infected horses had a lower stimulation index than the controls at Day 70, although in general, infected horses had a higher stimulation index than control horses at most time points (Figure 9). The reason for the significantly lower stimulation index at Day 70 is not known, although it is interesting that this correlates with a significant difference in neurologic scores between infected and control populations. Infected horses also had a lower stimulation index than controls on Day 70 on non-overnight lymphocyte proliferation assay results, although this was not statistically significant (Figure 8). Reasons why this significantly decreased response to PMA/I in infected horses was seen are likely the same as those detailed earlier, when discussing the proliferation results as a whole (Section 7.8). The reason why this difference was only significant at Day 70 may be due to the fact that there was the

greatest difference in clinical neurologic deficits between treatment groups at this time point (and therefore a greater difference in immune responses might be expected), might be due to exhaustion of proliferative capabilities of a particular affected cellular subset in infected horses, might be due to the incubator losing CO₂ gas, or might be due to chance. It would have been interesting to see whether, had we continued the experiment for longer, this difference would have remained. However, as discussed previously, financial constraints made this unfeasible.

7.8.5 Antigen-specific mitogen responses

No significant differences between treatment groups were seen in wells stimulated with merozoites. This represented antigen-specific stimulus. Infected horses have already been exposed to *S. neurona* prior to inclusion of merozoites in the assay plate, and it was hypothesized that this priming (pre-sensitization) of the immune capability to react to this specific stimulus might generate a higher stimulation index for the infected horses. This was not the case. However, a potential suppressive effect of merozoites on proliferation responses has also been suggested.

It appears from SnSAG1 titers that many if not all of the control horses were exposed to environmental sources of the parasite, and therefore would also have been 'primed' to respond to this antigen. A difference between treatment groups, given this fact, was therefore less likely.

7.8.6 Other mitogen responses

There did not appear to be a large difference between infected and control horses' responses to the other mitogens studied. This data was reviewed and, due to the

large amount of variability and small possibility of statistical significance, the results were not submitted for statistical analysis. Graphs of both ConA and PWM stimulation indices over time are included in Appendices F and G, and horses in both groups experienced similar fluctuations in stimulation index over the course of the experiment. Since there was no significant difference in ConA and PWM responses between infected and non-infected horses, the proliferative capacity of T cells and B cells seemed to be intact, and these mitogen-stimulated proliferative abilities were not affected by *S. neurona* infection. ConA stimulates T lymphocytes specifically, and PWM stimulates B lymphocytes. The fact that no differences were seen between treatment groups in response to the addition of either of these mitogens suggested that T and B lymphocyte proliferative capabilities are not affected by *S. neurona* infection. PMA/I is a pan-leukocyte stimulating mitogen, and therefore stimulates other cell types such as mononuclear and dendritic cells, as mentioned earlier, in addition to lymphocytes. No difference was seen in ConA and PWM responses in this experiment, which suggested that a suppression in PMA/I responses may have been more likely to be the result of a non-lymphocyte subset. It is possible that it is a defect in the proliferative capacity of dendritic cell or mononuclear populations that leads to a decreased PMA/I stimulated proliferation in EPM horses, and therefore suggests that further study of these cellular subsets and their response to *S. neurona* infection is warranted.

7.9 FLOW CYTOMETRY

In order to enumerate and classify cellular subsets, as well as study various cell cycle events such as apoptosis and division into daughter cells, flow cytometry was performed. To assess how these immune responses changed with time, several incubation times were examined. Additionally, cells were stimulated (with PMA/I or

merozoites) to determine which cells were involved in the mechanisms of PMA/I suppression, and to determine if merozoites stimulated or suppressed select populations. Considerable data were obtained, and there were both significant and non-significant differences. The limited number of significant results may have been due to a number of reasons: some of these are presented below, and along with those mentioned earlier in this discussion section, may indicate why greater overall significance over more time points was not demonstrated.

7.9.1 Suitability of gates within the established protocols

The initial gates on populations of live lymphocytes, monocytes and granulocytes all contained distinct groups of cells that, on analysis, represented the populations in question. The creation of another gate to include a large population of smaller (less forward scatter) cells was necessary during the experiment, particularly for samples incubated for lengthy time periods. This new group represented dying cells. Lymphocytes cultured without growth factors lose cell volume as a consequence of nutrient deprivation ¹²⁹ and hence are represented closer to the y-axis on flow cytometric representation of forward scatter against side scatter. It was necessary to include these cells in our analysis as this population likely contained a mixture of different cellular subsets, including some of those that we were attempting to enumerate. Results from this additional group are included in the results section, and referred to under the category “dying cells”.

Gates for stimulated and non-stimulated samples varied slightly in their exact position and in their shape. Stimulated samples contained cells with a wider range of forward scatter than unstimulated samples. Stimulation with mitogens caused an

increase in cellular proliferation (divisions) and generated higher numbers of immature cells. Young cells in blast form are larger in diameter than mature cells, and therefore a wider range in cell age will generate more variety in forward scatter.

7.9.2 Total cellular subset percentages

Total percentages of each cellular subset were compared between infected and non-infected horses. Some differences were found, although significant differences did not exist at every time point.

A higher total percentage of CD4+ and CD8+ lymphocytes was seen in the control group. There were many possible reasons why this result might have been seen. Infected horses may have had decreased percentages of T lymphocytes due to a direct effect of infection of the host or the cell with the parasite. This may have resulted in a defect in cellular expansion, or be due to normal immune cell turnover in detecting and fighting an infection. It may also have been the result of another disease, or parasitic infection, although all horses remained clinically normal and were suitably dewormed throughout the experiment. An imbalance in the percentages of T lymphocytes between infected and non-infected animals has previously been noted^{80,81,101} and reflected a suppressed cell-mediated (Th1/Tc1) immune response. A bias towards a Th2/Tc2 type of response might therefore be observed. Protozoa have developed various means by which to avoid host immune responses: these means may decrease the amounts of T lymphocytes present, and/or alter the efficacy of the host immune system to respond to stimulus. *Trypanosomes* induce T cell anergy, and produce substances that downregulate the cell mediated immune response¹³⁰. *Leishmania* parasites suppress IL-12 production, and thus block a protective Th1 response, and also inhibit normal

cellular events such as apoptosis¹³⁰. These different infectious agents can alter the normal percentages of cell subsets within infected animals. Suppression of IL-12 decreases the Th1/Tc1 response, and indirectly causes the release of IL-4 from CD4+ and CD8+ cells. This cytokine further downregulates the Th1/Tc1 responses and causes a switch to Th2/Tc2 responses. Levels of IFN γ and TNF α are suppressed due to the blockage of Th1 responses. A decreased Th1/Tc1 response could also generate a switch to a T-regulatory response through the release of IL-10. This kind of response would not be protective against intracellular pathogens, and would not produce the same cytokines as the expected Th1 class of responses⁷⁰.

In this study, the infected horses had a relative decreased percentage of T lymphocytes compared to the controls. This may reflect a true lymphopenia, or could be due to an increase in the percentage of another cell type, such as neutrophils. Initial examination of CBC values did not show an overall difference in number of WBCs in any of the horses; however CBCs were performed prior to infection. Lymphocyte numbers were still within normal reference ranges. If I were to repeat the experiment, or for future experiments, additional CBCs at weekly intervals would be helpful to assess cell numbers as well as cellular percentages. It would be possible to back-calculate from flow cytometric data to achieve these numbers of each of the different subsets. Differential counts performed on a sample of blood smears did not demonstrate a difference in WBC numbers between infected and non-infected horses.

Total cell subset percentages were measured through evaluation of the live cell population for each subset. Infected horses may have more T lymphocytes within the dying cell gate, which would not have been analyzed as part of the total percentage

analysis. This methodology could have created the appearance of decreased lymphocytes without an overall difference in numbers present. For Objective One, the purpose was to determine whether *S. neurona* infection altered the degree of apoptosis across cell subsets. Our data and analysis did not reveal a significant increase in apoptotic CD4+ or CD8+ cells in the infected horses.

At most time points studied, the infected horses had a higher total percentage of neutrophils than the control animals. It is possible that the increased percentage of neutrophils within infected horses represented a relative or actual neutrophilia in these animals. It is known that an increase in circulating neutrophils is seen as a response to inflammatory stimulus, such as would occur with protozoal infection ¹³¹. In this event, more neutrophils are released into the peripheral blood system, and therefore the overall percentage of WBCs that are neutrophils is increased. This effect could have been seen in our infected animals. Comparison of cellular percentages with CBC or WBC numbers on a weekly basis would have been useful, had finances allowed.

When considering total B-cell numbers, the infected horses had a higher percentage after shorter incubation periods, yet a lower total percentage after longer incubation. An increase in B-cell numbers in the infected horses likely reflected increased antigenic stimulus. Infection with *S. neurona* could have triggered the clonal proliferation of B-lymphocytes responsible for the production of antibodies specific to the protozoan, and thus may have resulted in a greater percentage of B cells in the infected population. This was the most probable explanation for the rise in percentage of B cells seen after short incubation periods.

The decreased percentage of B cells seen after longer incubation periods likely

reflected the fact that these cells did not survive as well in culture as lymphocytes. Infected horse B-cells may have been more sensitive to apoptosis than cells from control horses. After long incubation periods, a decrease in B cell (and neutrophil/monocyte) percentages was seen in samples when assessing total numbers, and in samples for Objectives 1 and 2. Separation and purification of the blood samples was optimized for lymphocyte isolation, and conditions for primary cultures such as B cells were not ideal for the survival of these cellular subsets. If we were to repeat the experiment, increasing the number of B-cells stained or running additional samples optimized for B-cell survival would be necessary.

7.9.3 Apoptosis (Objective One)

There were comparisons at individual time points that produced significant results at various stages of the experiment. There were also patterns in the degree of apoptosis of various subsets with infection status, over the course of the experiment. Increased apoptosis was a potential reason why EPM horses could have exhibited suppressed cell numbers following mitogen stimulation. In previous experiments that used lymphocyte proliferation assays, a PMA/I stimulated suppression had been demonstrated, and we aimed to further investigate this suppressed response in this study. We were not able to demonstrate suppression in PMA/I stimulated lymphocyte assays, as measured by thymidine incorporation, except on Day 35. Therefore it was difficult to make/state a final conclusion on whether increased apoptosis was the reason why infected horses can have a decreased response to mitogens. However, some patterns and differences were seen when apoptosis was examined via flow cytometry. The 7-AAD assay for apoptosis may be more sensitive to this particular treatment effect than the PMA/I proliferation assays, as these did not show a difference. This lack of

difference may be due to natural exposure, although control animals did not demonstrate clinical neurologic disease.

Infection with *S. neurona* did not appear to affect the levels of apoptosis within the “live lymphocyte” gate for CD4+ or CD8+. More of an effect was seen in these cells within the “dying” population. This was not surprising, given that this “dying” gate was created in order to capture cells of shrinking size as they underwent apoptosis or necrosis. Within the dying cell population, infection caused an increase in apoptosis in both CD4+ and CD8+ lymphocytes. In CD4+ cells, an increase was mainly seen in early apoptosis. In CD8+ cells there was a significant increase in cells in late apoptosis on a number of time points. This could have been due to a direct effect of the protozoan on programmed cellular events, may reflect cellular death due to intracellular parasitism, or be due to a dysregulation in normal cellular signaling pathways that could cause increased apoptosis. The effect may also have been the normal immune response to infection with *S. neurona*.

In assessing the degree of apoptosis, cells were classified as either viable, early apoptotic or late apoptotic, out of a total 100%. Therefore, if a significant difference in one classification was present, for example a significant increase in early apoptotic cells, a decrease in either viable cells or late apoptotic cells should also have been present. As variance between horses and immune responses was considerable, the differences in the other two classifications were not always significant.

The effect of the combined mitogen PMA/I on apoptosis in infected and control horses was also studied. The majority of significant results were seen on evaluation of

the monocyte population. Early in the experiment, decreased percentages of monocytes in late apoptosis were seen in infected horses after short periods of incubation. Later in the experimental period this trend was reversed, with a decreased percentage of viable cells and an increase in late apoptosis seen in the infected horses. In lymphocyte proliferation assays, a mitogen specific for monocytes was not used. In addition, the percentage of monocytes in the total population of cells was small compared to lymphocytes. Therefore, it is possible that infection with *S. neurona* caused a difference in monocyte proliferation responses, and that the effect seen in the apoptosis flow results reflected this difference. Previous studies have suggested that PMA/I may not stimulate equine monocytes similarly to other cell populations¹⁰¹. Thus, in future studies, an assay specifically designed to assess monocytes would be ideal. This will be discussed later in more detail.

Monocytes are circulating precursors to macrophages, and hence are important cells in the phagocytosis and destruction of material that is foreign to the host. Parasitemia will therefore trigger phagocytosis of the protozoan by macrophages and similar cells. Protozoa have evolved mechanisms by which the organism attempts to block host attempts at phagocytosis: *Leishmania* parasites produce substances that inhibit the antigen-presenting capabilities of macrophages, and also that reduce apoptosis of infected macrophages, once the protozoan has been phagocytosed¹³⁰. If *S. neurona* has similar capabilities, it may be that apoptosis of mononuclear cells was inhibited early in the experiment, when the horses would have been parasitemic. As the experiment progressed, this inhibition may have waned, and other cells could have stimulated monocytes through other pathways and cytokines, thus resulting in an overall increase in apoptosis towards the end of the experiment.

Monocytes are also the cell type most commonly found to contain merozoites⁷⁸. Parasites may inhibit apoptosis early in infectivity. However, a key function of monocytes is to destroy the foreign (*S. neurona*) organisms; thus they may be capable of becoming activated at a later stage, and killing the protozoal organisms as well as undergoing apoptosis themselves. A parasite-induced stimulation of another immune subset could also stimulate feedback mechanisms, leading to monocyte apoptosis later in the experiment.

Apoptosis in response to stimulation with merozoites (antigen-specific mitogen) was also evaluated. In general, an increase in apoptosis in infected horses was seen when cells were incubated in media containing merozoites. This effect was seen in both CD4+ and CD8+ lymphocytes. Prior exposure to *S. neurona* may prime the immune response, so that when rechallenged through antigen-specific mitogen stimulation, cells more quickly become apoptotic. This may be a host animal defence mechanism in order to destroy the parasite, in addition to the infected cell. Lymphocytes infected with merozoites may be more quickly identified and targeted for destruction, or an increase in apoptosis of CD4+ and CD8+ cells may occur as a “survival mechanism” by the host in an attempt to avoid parasitism. Certain protozoan parasites (such as *Blastocystis hominis*) have been demonstrated to undergo programmed cell death in a manner similar to cells from vertebrates¹³². Other protozoa can induce apoptosis in host cells as a means of exiting the cell, or to avoid the inflammatory trigger of cellular necrosis and hence limit detection¹³³. Studies have demonstrated that *Trypanosoma cruzi* induces apoptosis in T and B lymphocytes in order to evade the host immune system¹³⁴. It is not known whether *S. neurona* is capable of inducing apoptosis in order for merozoites to

be released from an intracellular location in host cells, or in an attempt to evade detection.

7.9.4 Cellular divisions (Objective Two)

CFSE was effectively used to demonstrate the number of cellular divisions that occurred in CD4+ and CD8+ lymphocyte populations. B lymphocytes, granulocytes and monocytes did not survive or proliferate well enough in culture to perform analysis (some of these populations contained less than 50 cells on flow cytometric outputs). In comparing the ability of B and T cells to survive in culture for the experimental assays, we noticed that B-cells survived poorly compared to T-cells. Possible explanations for these findings are that 1) the half lives of neutrophils and monocytes are shorter than T-cells, thus the cells do not survive as well in culture 2) T-cells can respond to the mitogens used without additional antigen presentation by antigen presenting cells, thus they would be able to respond more readily to mitogens 3) B-cells, monocytes and neutrophils are less responsive than T-cells to the mitogens used. In the T lymphocyte populations, cells dividing once, twice, three and more times were noted, as well as those that did not divide. A difference that was significant at every time point was not seen, although some individual data points demonstrated a significant difference, and a tendency towards a relationship between infection status and number of divisions was shown.

When assessing whether infection status had an effect on these lymphocyte subsets' proliferative ability in response to PMA/I, it was noted that, in general, cells from infected horses underwent more cellular divisions than those in the control group, which were shown at several time points to have significantly more CD4+ and CD8+

cells that did not divide. Both CD4+ and CD8+ lymphocytes divided more times in the infected horses when cells were incubated with PMA/I.

Although this difference was not statistically significant at every point in the experiment, many individual time points follow this pattern, as can be seen in Table 7. The reasons why infected horses' T-lymphocytes proliferated more than non-infected horses' were not clear: there were several potential explanations. It is possible that infection caused a relative or actual leukocytosis in the challenged horses, and that these animals had more lymphocytes initially, prior to mitogen stimulation. Complete blood counts would have been useful in assessing whether this was indeed the case, yet were not performed for financial reasons. Differential counts performed on blood smears and cytopins, and did not demonstrate a difference in infected versus non-infected horses. Coulter counter data also did not demonstrate a difference in total counts. Another possible explanation is that infection with *S. neurona* may have stimulated cells in some way, and allowed them to respond quicker and more vigorously to mitogen stimulation. A third explanation is that merozoite infection of the cells caused a change in intracellular signaling and itself prompted cellular divisions. T-lymphocytes have been shown to undergo proliferation in response to the cytokines IL-4, IL-7 and IL-15, with IL-7 playing a key role. Increased amounts of IL-7 and IL-15 have been linked to increased NK cell activity in protozoal infections, and may thus also have an effect on lymphocyte proliferation¹³⁵. Merozoites are most commonly found in monocytes⁷⁸, although they can be found in all equine leukocytes. If the merozoites themselves caused an increase in cellular proliferation, it may have been the case that, were we able to examine cellular divisions in the monocyte subgroup, a more significant difference would have been demonstrated. Unfortunately, due to poor cellular survival,

analysis of these samples was not possible.

Granulocytes, monocytes and B cells were present in the sample in lesser numbers than T-lymphocytes, and therefore a failure of cells to survive of a similar magnitude across all subsets studied resulted in fewer of these cells at time of flow cytometry. These cells were more fragile at extended primary culture. B lymphocytes removed from their normal microenvironment undergo death by neglect that can be blocked by inducers of cell growth and antiapoptotic molecules. It may have been the case that conditions within the plate allowed for B cell death in a ratio higher to that of T lymphocytes ¹³⁶.

Although nutrients are provided in cell culture, their entry and use can be restricted by the lack of transporters and enzymes regulated by growth factors, which may not be adequately provided ¹³⁷. Thus, the cells may not survive well in long-term culture. Mature circulating polymorphonuclear cells have the shortest half-life among leukocytes and undergo rapid programmed cell death *in vitro* ¹³⁸ therefore it is not surprising that these subsets survived less well to 72 hrs in our plates.

In considering cellular divisions in response to merozoite (antigen-specific) stimulation, a decrease in numbers of divisions was seen in infected horses. This could be due to a direct suppression of cellular division by the parasite. Certain intracellular parasites are transmitted to daughter cells when the host cell divides through close association to microtubules ¹³⁹, yet others are not and therefore seek to inhibit host cell division in order to maximize parasite survival. It is not known whether *S. neurona* merozoites are capable of suppressing host cell division and proliferation. Antigen-

specific responses depend on the ability of antigen presenting cells, such as B lymphocytes and dendritic cells, to recognize foreign material within the host environment, and to process and present antigen on their surface in a manner that allows for T cell activation and proliferation. A defect in the ability to present or process antigen could be responsible for the decreased responsiveness seen in infected horses when cells were incubated with merozoites ⁷⁰. Normal ConA and PWM responses were seen in this experiment, which suggested that the proliferative capacity of T and B lymphocytes remained intact. The defect thus is possibly within the mononuclear or dendritic cell populations, thus limiting these cells' ability to present antigen to T-cells. To assess whether the suppression was due to decreased ability to present antigen, the response to other specific antigens could also be studied ⁷⁰.

When an infection occurs, foreign antigens within the host are detected by cells of the innate immune system (largely neutrophils and mononuclear cells such as macrophages) and phagocytosed. This process is enhanced when antibodies are present to act as opsonins. Upon phagocytosis of the foreign material, cells of the innate immune system destroy pathogenic material in two different manners: respiratory burst and the generation of lytic enzymes. A failure of either of these methods may promote pathogenic survival. Some protozoal species, such as *Brucella abortus* and *Listeria monocytogenes*, have been demonstrated to possess the ability to interfere with phagosomal maturation and the fusion of the phagosome with lysosomal enzymes ⁷⁰. It is not known whether *Sarcocystis spp* also possess this ability.

The innate immune system is not the only arm of the immune system to respond to antigens. The acquired immune system is stimulated to respond through the

secretion of varying cytokines by antigen presenting cells ⁷⁰. Antigen presenting cells recognize pathogen-associated molecular patterns (PAMPs) on foreign material through the activation of toll-like receptors (TLRs) on the cell surface and within the cytoplasm of sentinel cells. Once TLRs bind to PAMPs, nuclear factor $\kappa\beta$ increases and activates IL-1, IL-6 and TNF α . These cytokines stimulate macrophage activation and further enhance phagocytosis. Dendritic cells are also activated by PAMP recognition- pattern recognition causes DCs to retain antigen, and present it to T cells. Mature DCs are the only antigen-presenting cell that can stimulate naïve T cells to proliferate: the type of immune response triggered depends on the conditions under which DCs are stimulated. Some microbes, particularly intracellular pathogens, cause DCs to upregulate the secretion of costimulatory markers, to differentiate into DC1 cells, and secrete IL-12 (and IL-18), which leads to a Th1/Tc1 response, and the secretion of IFN γ and TNF α by CD4+ and CD8+ cells. B-cells possessing CD80 act as co-stimulatory factors. Extracellular pathogens are recognized by different TLRs, and stimulate DC2s to release IL-4, and to provide costimulation through CD86. This promotes B-cell proliferation and immunoglobulin secretion. A lack of IL-12 secretion can cause a switch to Th2 types of responses ⁷⁰. Natural killer cells are also activated by IL-12, and attack infected cells. A failure in any part of this cascade may decrease the ability of certain animals to respond appropriately to intracellular infections, and thus create a decrease in antigen-specific responses.

The normal protective response to ingestion of *S. neurona* is multifaceted, and depends on protective mechanisms of the gastrointestinal tract, innate and acquired immune system. The gastrointestinal tract in a normal animal has features that decrease the ability of protozoa to gain systemic access to the host organism. The low pH of a

normal equine stomach, coupled with associated protease enzymes in the stomach and saliva, may cause destruction of the parasite before systemic infection can occur. The intact mucosal lining of the normal gastrointestinal tract is impermeable to pathogens due to enterocyte tight junctions, and contains specialized epithelial cells known as Paneth cells that secrete enzymes to prevent pathogenic colonization. The gut also contains specialized lymphoid tissue in the form of Gut Associated Lymphoid Tissue (GALT) that contains all components necessary to initiate an appropriate immune response ⁷⁰. Specialized APCs in the intestinal wall include M cells and DCs: M cells rapidly present antigen to intraepithelial lymphocytes or pass the antigen to interstitial fluid, allowing a systemic immune response to be generated. Some pathogens use M cells to gain access to the systemic circulation of the host : it is possible that this may be the method by which *S. neurona* enters lymphocytes. The innate immune system is stimulated on infection of the host to phagocytose the foreign material, and further activation of macrophages occurs following the release of IL-1, IL-6 and TNF α , as described earlier. *S. neurona* is a largely intracellular pathogen; therefore a successful protective immune response relies on the generation of Th1/Tc1 types of responses, and a downregulation of Th2/Tc2 stimulation. This is achieved through the release of IL-12 from APCs. CD4+ (Th1) and CD8+ (Tc1) cells then proliferate, and release IFN γ and TNF α . IFN γ is particularly important, and causes downregulation of Th2/Tc2 cell types. It also promotes macrophage activation, and allows them to carry out antibody dependent cell-mediated cytotoxicity, allowing antigen specific phagocytosis to occur ⁷⁰. A defect in any part of this highly specialized and interlinked system of events could lead to the changes in aspects of the immune response seen in our study, and ultimately make horses suffering from such a defect more susceptible to EPM.

These experiments have raised some interesting questions as to the precise immune response of cellular subsets to infection with *S. neurona*. Results demonstrated here may be investigated further by additional studies, and will be discussed in the following section.

CHAPTER 8: FUTURE DIRECTIONS

Future studies should involve further investigation into possible mechanisms associated with differences in the proliferation response to PMA/I in EPM affected horses, and should aim to elucidate reasons for the differences in cellular subset percentages and in the incidence of cellular events that were observed in this study.

A consistently observable difference in PMA/I stimulated proliferation, based on thymidine incorporation, between infected and non-infected horses was not seen in this study, yet had been observed in previous experiments. Reasons for this have been discussed. Further studies to investigate this effect should consist of greater numbers of age-matched horses than were used here. Possible differences between infected and non-infected animals include changes in lymphocyte subpopulations and altered signaling in the PMA/I pathway associated with *S. neurona* infection. An alteration in the signaling pathway for PMA/I could be the most important reason for the decreased proliferation response to PMA/I in EPM horses. Future studies can measure the activity or concentration of tyrosine kinases, other signaling molecules (i.e. Raf) and/or intracellular protein kinase C isoenzymes in the PMA signaling pathway of *S. neurona* infected equine lymphocytes¹⁴⁰. The lack of statistically significant differences in thymidine incorporation results, compared with the presence of significant differences

in apoptosis and cellular proliferation as assessed through CFSE, is interesting. This discrepancy may be due to the large amount of variability involved in using thymidine, thus reducing the likelihood of achieving significance in these results. A difference in response to PMA/I was seen on most time points studied, yet this was only significant at Day 35. This difference may have represented a biologically, yet not statistically, significant distribution and therefore should not be ignored. PMA/I stimulates dendritic cells and mononuclear cells, as well as lymphocytes ⁸³. It is therefore possible that EPM develops in animals with a defect in either dendritic cells or mononuclear populations. This should be further elucidated. Dendritic cells are important antigen presenting cells, and a defect in this population may explain the decrease in antigen-specific proliferation seen in infected animals and discussed later in this section. DCs differentiate into DC1s and DC2s, dependent on the nature of stimulus received. DC1s trigger a Th1 response through the secretion of IL-12, and DC2s bias the organism towards a Th2 response through IL-4 release ⁷⁰. Future studies could assess relative abundance and functionality of dendritic cell subsets. The use of Flt3-ligand to dramatically expand mature lymphoid and myeloid-related DC subsets has been recently reported. Flow cytometry can then be used to sort these cells into their different subsets, and intracellular cytokine staining used to assess the secretion of various cytokines ¹⁴¹. These subset percentages and cytokine abundances should be compared between infected and control horses.

Changes in cellular subset percentages were seen between infected and control horses in this study. In general, infected horses had a decreased percentage of positively staining CD4+ and CD8+ T-lymphocytes, and a greater percentage of neutrophils than control animals. It would be helpful to look at the reasons behind these observed effects.

Performing flow cytometric analysis in conjunction with regular CBCs would aid an investigator in ascertaining whether these effects reflected a true change in cellular subset numbers.

When apoptosis was examined, PMA/I stimulated a decrease in the level of monocyte apoptosis at the beginning of the experiment, and an increased level of apoptosis in monocytes towards Day 70. Merozoites are most commonly found within monocytes, and it is possible that they exert a direct effect over cell signaling pathways and the stimulus to undergo apoptosis⁷⁸. Future experiments should be designed to elucidate whether this is indeed the case. Other methods of assessing apoptosis exist, and may prove more sensitive than using 7-AAD alone. These include the use of propidium iodide, measuring the levels of DNA fragmentation via terminal deoxynucleotidyl transferase mediated UTP nick end-labeling (TUNEL), or utilizing stains for caspases inherent to the process of apoptosis. Many of these methods can be used in conjunction with flow cytometry to quantify eventual results¹⁴².

In assessing cellular divisions, this experiment demonstrated an increase in cellular divisions in T- lymphocytes from infected horses when stimulated with PMA/I. Potential reasons for this difference include a direct stimulatory effect of *S. neurona* on cellular proliferation, or an effect on intracellular signaling. Future experiments might focus on measuring cytokines that signal cells to divide, such as growth factors, Src kinase, and the proteins Ras and p53. Levels of these signals for cellular division could then be correlated with infection status. Certain cytokines such as IL-4, IL-7 and IL-15 have also been demonstrated to cause T-cell clonal proliferation: infection with *S. neurona* may upregulate the expression of these cytokines⁷⁰. Future studies measuring

expression and activation of such intracellular signaling molecules should be performed. Methods of measuring cytokine production by T-cells include the use of flow cytometry, using real-time PCR to identify cytokine mRNA and immunohistochemical intracellular cytokine staining methods¹⁴⁰. Additional experiments could be designed to utilize 5-bromo-2-deoxyuridine (BrdU) and cell surface marker antibodies to detect which subpopulations proliferate in response to stimulation with PMA/I. As discussed, it may be that *Sarcocystis spp* are able to bias the type of adaptive immune response demonstrated by a host animal: in normal protective immunity to intracellular pathogens, a Th1/Tc1 response is necessary versus a Th2/Tc2 response. EPM animals may demonstrate a bias towards the Th2/Tc2 immune response, which may be inherent or a direct effect of infection with *S. neurona*. Future studies measuring levels of cytokines, in particular IL-12 and IFN γ (secreted and upregulated in a Th1/Tc1 response) and IL-4 (secreted in a Th2/Tc2 response) may be helpful in assessing the type of immune response generated by infected and control animals⁷⁰. Intracellular cytokine staining plus flow cytometry would be one method of assessing levels and types of cytokines present¹⁴⁰. If I were to repeat the study, an effort to increase the total amount of positively staining cells would need to be made, in order to ensure that enough monocytes, granulocytes and B-lymphocytes survived for analysis. A higher concentration or volume of cells could be selected for initial plating, or conditions optimized for survival of cells other than T-lymphocytes. Our separation protocol was designed with the aim of achieving a sample that had a high purity for lymphocytes. Cell culture media were also chosen for optimal lymphocyte survival. A future experiment that emphasized monocyte and granulocyte populations would therefore use different protocols. Performing flow cytometry at 48 hrs as opposed to 72 hrs may increase cellular survival in these subsets. A decrease in the number of cellular

divisions observed would be expected if results were harvested after a shorter incubation period.

A decrease in the number of cellular divisions when cells were stimulated with merozoites was seen in this experiment. This could reflect a direct suppressive effect of *S. neurona* on cellular proliferative capabilities, or the animals developing tolerance to the parasite. Intracellular signaling in response to infection with the protozoan should be investigated as detailed previously¹⁴⁰. As the responses examined here were antigen specific, incubation with merozoites for a longer time period could be performed.

If using SnSAG1 ELISA on serum and CSF to assess infection in the future, it would be advisable to repeat these diagnostic tests on a weekly basis for the duration of the experiment, in order to correlate cellular events with infection of peripheral blood and structures of the CNS.

At present, there is not an effective vaccine available to prevent horses from developing clinical EPM. The ideal vaccine would generate protective immunity to the parasite, yet allow the clinician to distinguish between vaccinated and infected animals on diagnostic testing. As *S. neurona* is an intracellular pathogen, a vaccine should be designed to bias the immune response to a Th1/Tc1 response, and maximize cell-mediated immunity. This could be achieved in traditional vaccines through careful adjuvant selection. It appears from previous work that modified live vaccines are more efficacious in generating effective cell-mediated immunity, and that irradiated merozoites are a better choice than heat-killed organisms in this respect. A polyvalent vaccine should be utilized, as it has been shown that *S. neurona* exists in a number of different strains, and that strains that are SnSAG1 negative exist. A vaccine with SnSAG1

and SnSAG5 strains is likely necessary, as these surface antigens appear to be mutually exclusive¹⁰⁰. Work is ongoing in various laboratories in order to develop such a vaccine, and has recently focused on recombinant techniques.

In summary, with the use of the reproducible equine model of EPM, this study provides the groundwork for further investigation into the protective and pathophysiologic immune responses to *S. neurona* infection in horses. Once the mechanisms are elucidated, the overall pathophysiology of disease will be better understood. Additional studies will focus on addressing potential mechanisms of immunosuppression, and elucidating the reasons behind the differing incidence of cellular events such as apoptosis and cellular division in infected and non-infected horses. Hopefully, through united efforts, we will ultimately be more successful in diagnosing, treating and preventing this devastating disease.

CHAPTER 9: SUMMARY

These studies investigated how *S. neurona* altered host immune responses in experimentally infected horses. Leukocyte function and proliferative ability were assessed through mitogen stimulation and thymidine incorporation. A decrease in proliferation following PMA/I stimulation was seen in infected horses at Day 35.

When cellular subsets were examined via flow cytometry, a consistent but non-significant decrease in total percentages of CD4+ and CD8+ T-lymphocytes was seen in infected horses. This may reflect a direct effect of the parasite on lymphocyte survival and function. The infected horses had an increased total percentage of neutrophils,

which likely represents increased circulating numbers of neutrophils as part of the normal response to inflammatory stimulus and infection. Variations in total B cell numbers were also seen: antigenic stimulation likely created an increase in clonal proliferation of B cells in infected horses, yet due to poor B cell survival in tissue culture, these cells did not survive after long incubation periods.

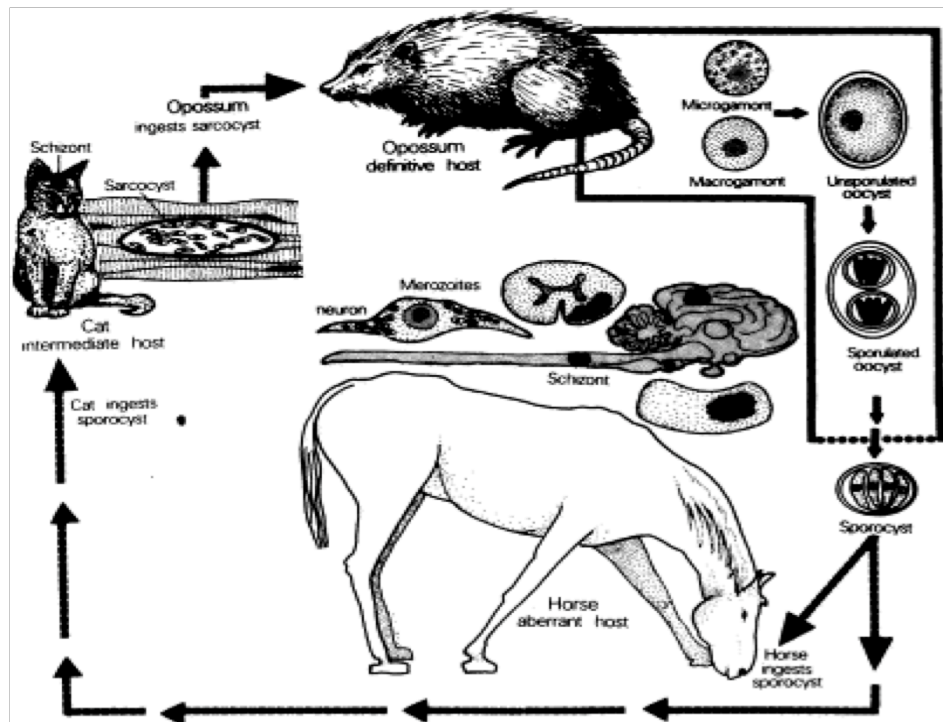
To address Objective 1, cellular viability and apoptosis was studied using flow cytometry. An increase in apoptosis of dying CD4+ and CD8+ T-lymphocytes was seen in infected horses at certain time points. This may be a normal immune response to infection, or may be due to a parasite-induced dysregulation in normal cellular signaling pathways. Intracellular parasitism could also target cells for programmed cell death. Culture of cells with PMA/I was shown to affect the percentage of monocytes undergoing apoptosis. Early in the experiment, infected horses had a decreased percentage of monocytes undergoing apoptosis. Later in this experiment, this was reversed, and infected horses had an increased percentage of monocytes in stages of apoptosis. Monocytes are the cell type most commonly parasitized by merozoites ⁷⁸ and therefore monocytes may be preferentially targeted by the protozoan in an attempt to evade normal host immune responses. A decrease in early apoptosis potentially reflects a parasite-induced inhibition of monocyte apoptosis during parasitemia. The later increase in apoptosis may be a result of waning inhibition and thus a compensatory increase in apoptosis, or be due to other factors. Monocytes were not examined cytologically or microscopically to look for merozoites in this experiment, although this could be a target for future work.

To address Objective 2, the numbers of cellular divisions were assessed using

CFSE as detected by flow cytometry. Infected horses underwent more divisions within their T-lymphocyte populations, when stimulated with PMA/I. This may be due to a leukocytosis following infection, or be due to *S. neurona* infection priming the cells, leading cells to divide more readily following stimulation with mitogens. When antigen-specific responses were assessed through the use of merozoites as mitogens, the infected horses underwent fewer cellular divisions in T-lymphocyte populations. This may be due to a suppressive effect of *S. neurona* on proliferative ability, changes in the type of immune response generated, or may be due to an exhaustion of the horse's ability to respond to merozoite stimulation.

Further studies are still needed before the immunopathogenesis of EPM is fully elucidated. Results from this study raise interesting questions, and provide the basis for future studies of the effect of *S. neurona* on the equine immune response.

FIGURE 1: LIFE CYCLE OF *S. NEURONA*, DEMONSTRATING THE ROLE OF THE OPOSSUM AS A DEFINITIVE HOST AND THE HORSE AS AN ABERRANT HOST SPECIES



From: Dubey et al 2001 ¹

Figure 1: Life cycle of *S. neurona*, demonstrating the role of the opossum as a definitive host and the horse as an aberrant host species. *S. neurona*, the causative agent for EPM, is ingested by the horse in sporocyst form. It undergoes asexual replication within the horse and localizes to the CNS causing clinical signs. The opossum is the definitive host, and excretes the sporocyst in its feces, which may then contaminate the horse’s foodstuffs and allow infection to occur.

Fig 2A

FIGURE 2: FLOW CYTOMETRY RESULTS FROM OBJECTIVE ONE (APOPTOSIS): UNSTIMULATED

Fig 2B

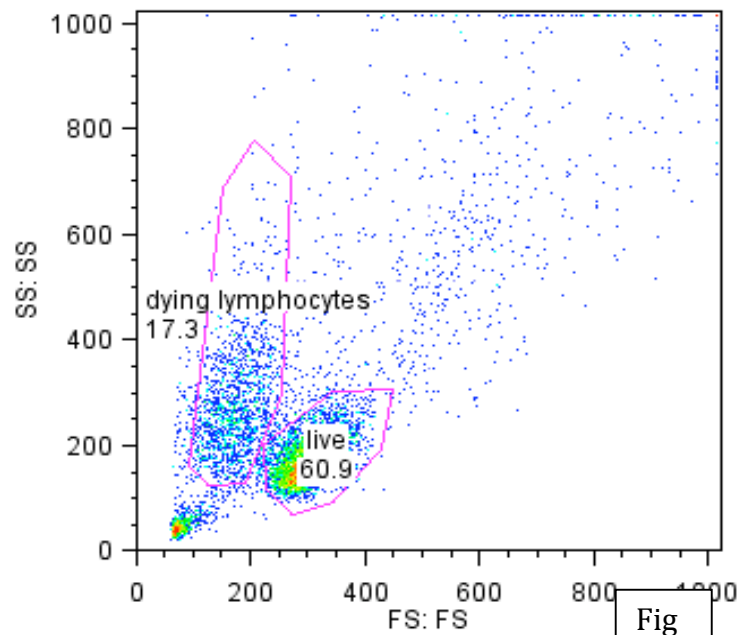
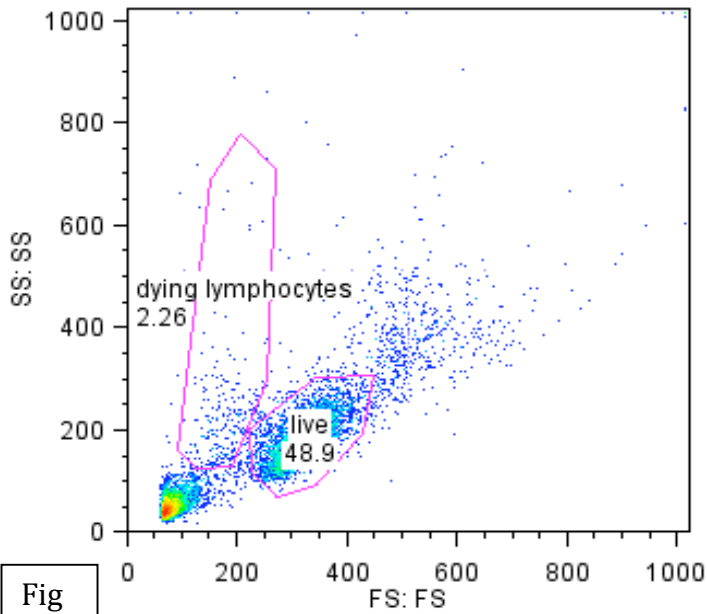


Fig 2C

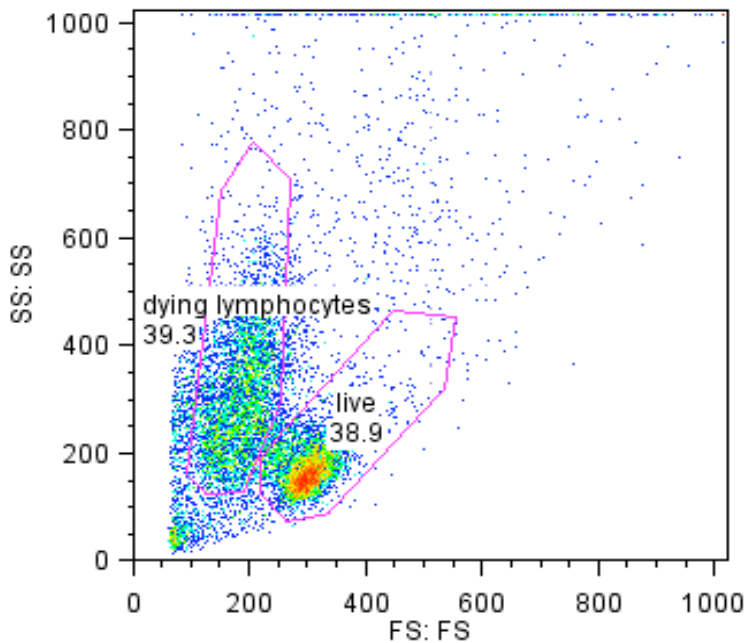


Fig 2D

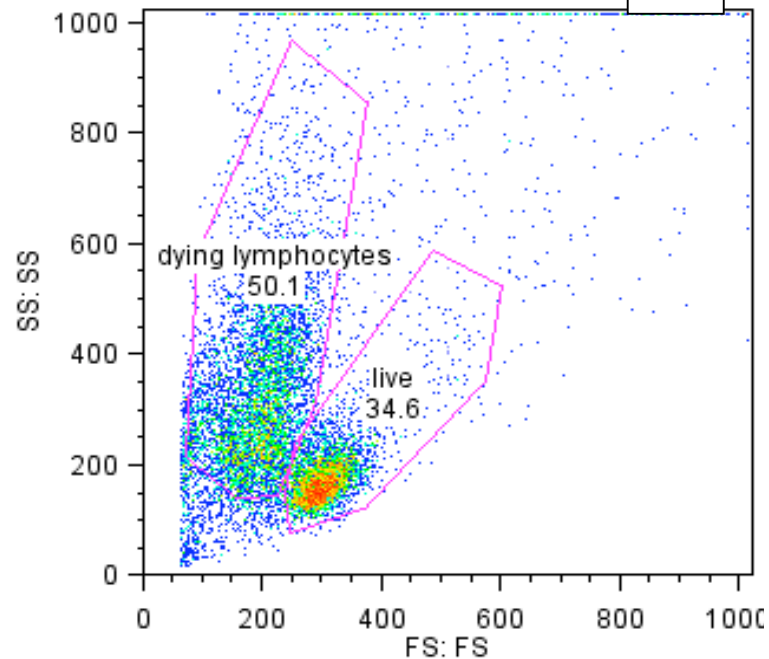


Figure 2: Flow cytometry results from Objective 1 (apoptosis): unstimulated samples. Figure 2 demonstrates that, at longer incubation times, with no mitogen stimulation, cells are more widely dispersed as they have greater variation in size and granularity (forward and side scatter). At longer incubation times, there is a greater percentage of cells within the dying gate (labeled “dying lymphocytes”) than in the live gate (labeled “live”), and than in non-incubated samples.

Cells were enriched for lymphocytes, plated and stained with primary and secondary antibodies. Plates were incubated with no mitogens for varying periods, and flow cytometry was performed. Gates were established based on size and granularity of cellular subsets. Figure 2A shows data after no incubation. Figure 2B shows data after 24 hrs of incubation. Figure 2C shows data after 48 hrs incubation. Figure 2D shows data after 72 hrs incubation. FS= forward scatter. SS = side scatter. Live 38.9 = 38.9% of lymphocytes staining with primary antibody are within the “live” gate.

Fig 3A

**FIGURE 3: FLOW CYTOMETRY RESULTS FROM OBJECTIVE ONE (APOPTOSIS):
STIMULATED CELLS**

Fig 3B

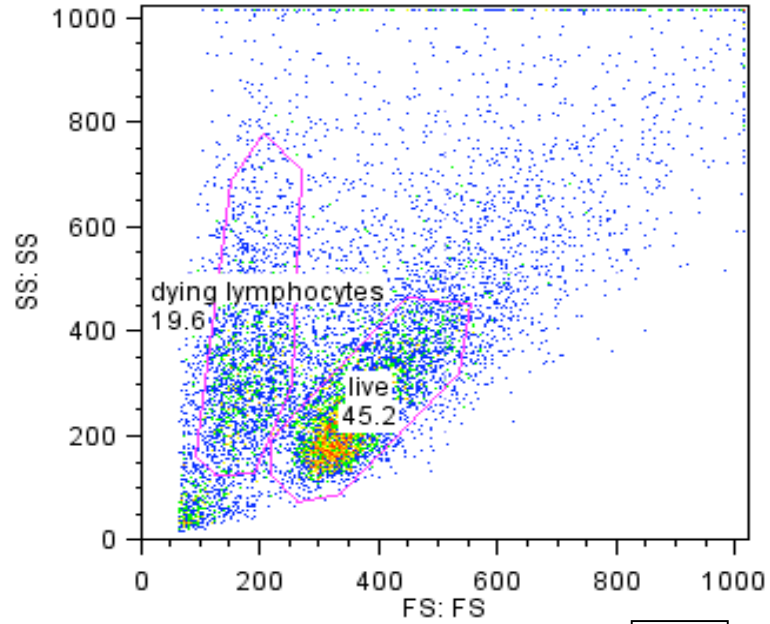
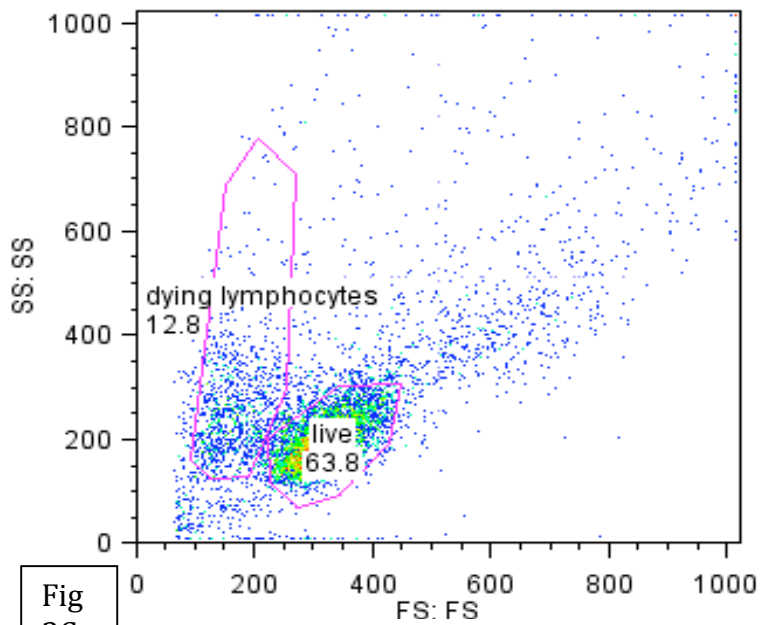


Fig 3C

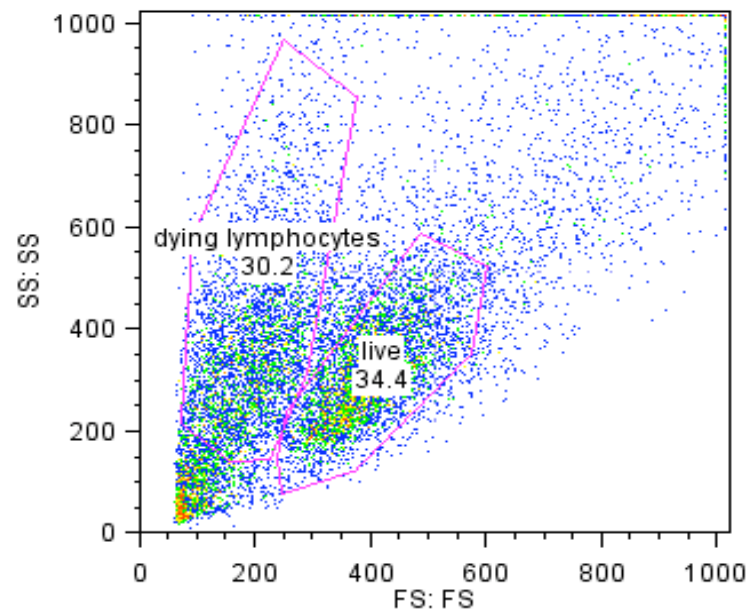


Fig 3D

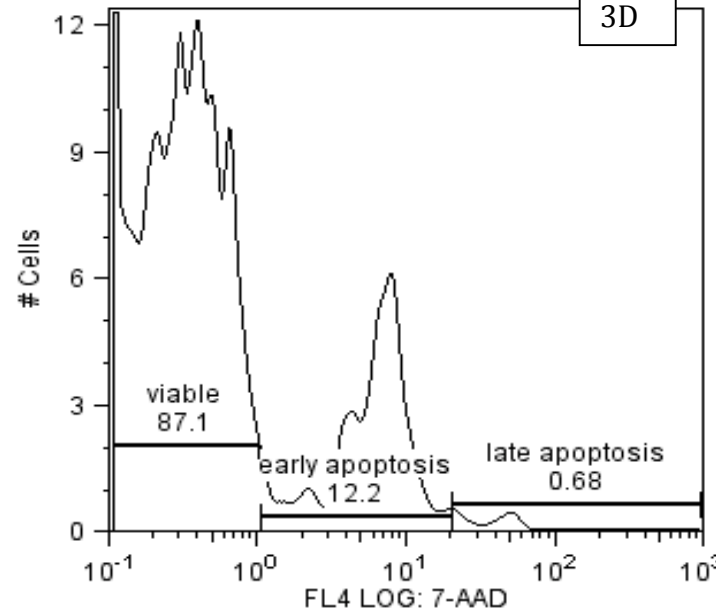


Figure 3: Flow cytometry results from Objective One (apoptosis): stimulated cells. Stimulated cells have more variety in cell size (FS) than unstimulated cells shown in Figure 2. At longer incubation times, there is a greater percentage of cells within the dying gate (labeled "dying lymphocytes") than in the live gate (labeled "live"). Cells were enriched for lymphocytes, plated and stained with primary and secondary antibodies. Plates were incubated with PMA/I for varying periods, and flow cytometry was performed. Gates were established based on size and granularity of cellular subsets. Figure 3A shows data after 24 hrs incubation with PMA/I. Figure 3B shows data after 48 hrs of incubation with PMA/I. Figure 3C shows data after 72 hrs incubation with PMA/I. FS= forward scatter. SS = side scatter. Live 34.4 = 34.4% of lymphocytes staining with primary antibody are within the "live" gate. Figure 3D demonstrates flow cytometric analysis of cell viability. Gating on each population (for example "live lymphocyte" population, and for cells positive for staining with primary antibody), gates for viability and stage of apoptosis were established. Individual peaks can be seen on Fig 3D for viable, early and late apoptotic cells, based on the level of 7-amino actinomycin D incorporation

Fig 4A

FIGURE 4: FLOW CYTOMETRY RESULTS FROM OBJECTIVE TWO (CELLULAR DIVISIONS): STIMULATED AND UNSTIMULATED CELLS

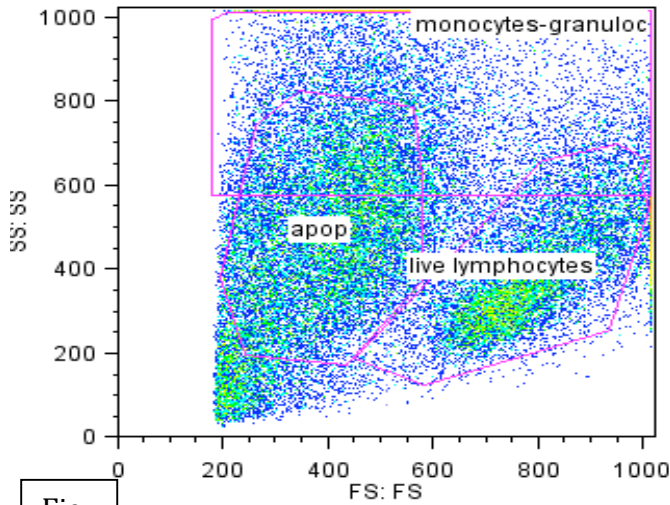


Fig 4C

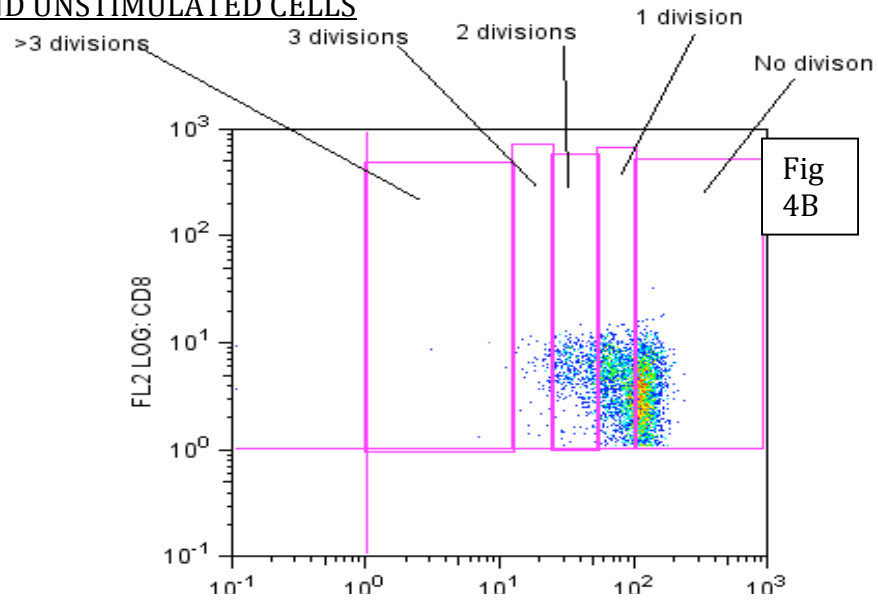
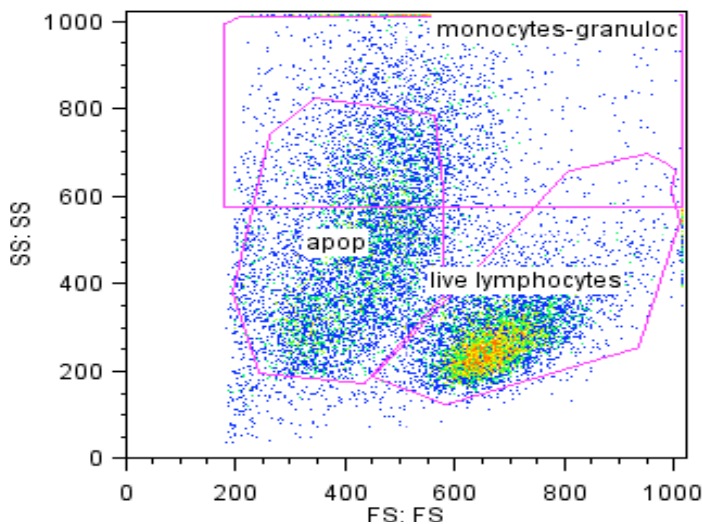


Fig 4B

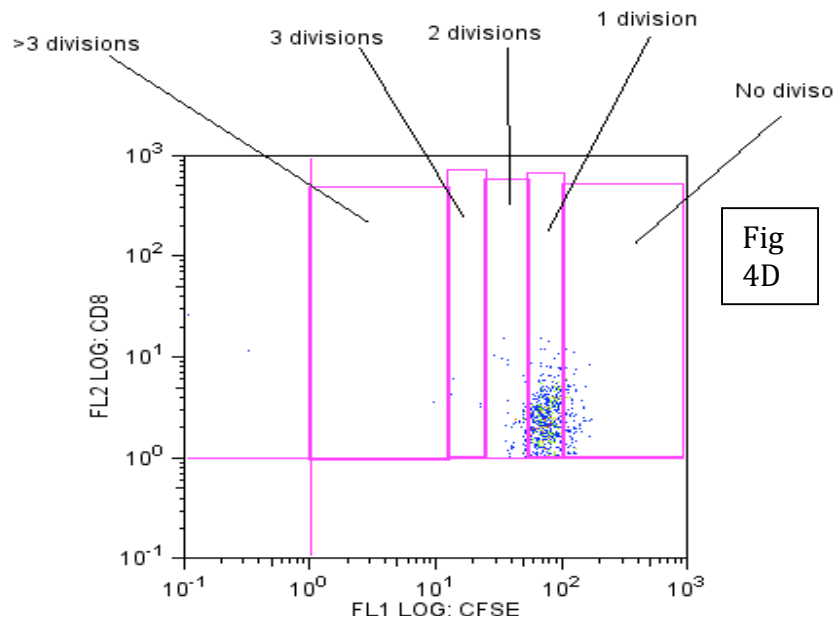


Fig 4D

Figure 4: Flow cytometry results from Objective Two (cellular divisions): stimulated and unstimulated cells. Cells stimulated with PMA/I (Fig 4A) have more variety in cell size (FS) than unstimulated cells (Fig 4C) Samples enriched for lymphocytes were stained with primary and secondary antibodies, and flow cytometry was performed. Gates for each cellular subset were determined based on size and granularity, prior to the experiment. FS = forward scatter. SS= side scatter. When cells are stimulated with PMA/I (Fig 4B), they undergo more cellular divisions than when unstimulated (Fig 4D) Cells were incubated with carboxyfluorescein succinimidyl ester (CFSE), which fluoresces when cells divide. It is inherited as cells undergo division, with progressive dilution as cells proliferate. The amount of CFSE incorporated in each cell is shown on the x-axis. Gates were determined using unstained samples prior to the experiment, and numbers of cellular divisions assessed. Less CFSE is incorporated in cells that have undergone more divisions.

FIGURE 5: LOG-TRANSFORMED NEUROLOGIC SCORES (ALL PARAMETERS) OVER TIME

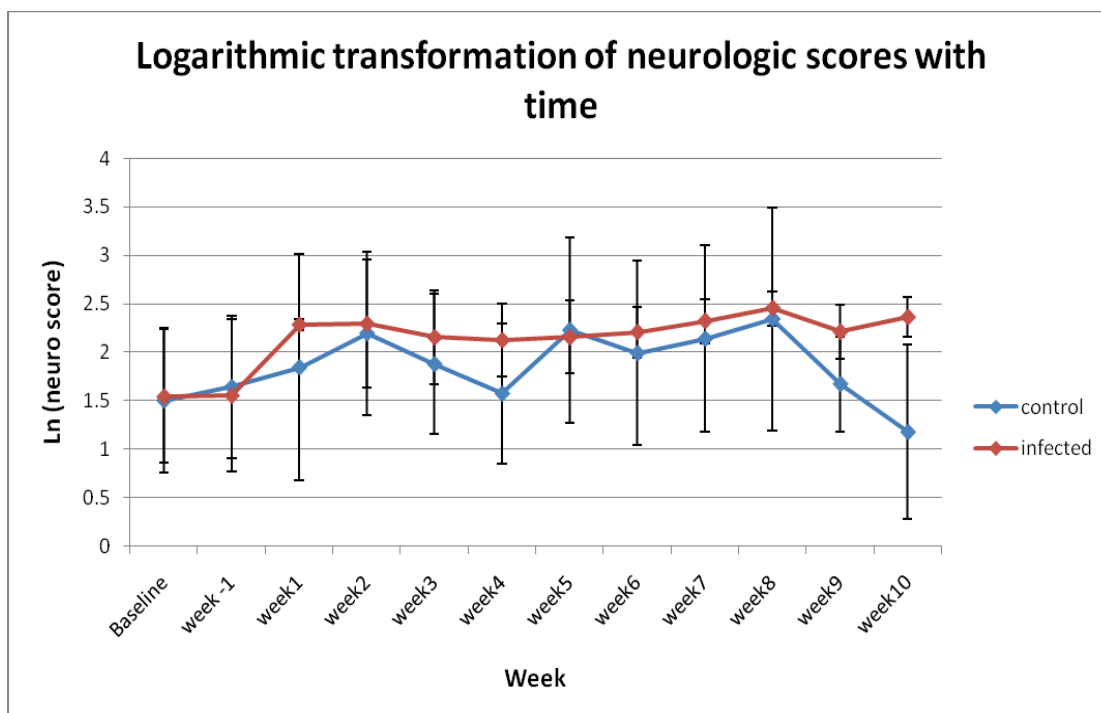


Figure 5: Log-transformed neurologic scores (all parameters) over time. All horses were scored biweekly for a multitude of different parameters in order to detect neurologic signs. Each parameter was assessed a score from 0 (normal) to 3 (very neurologic) creating a possible total of 97. The average neurologic score was log-transformed and plotted against experimental time to equalize variance between samples. Baseline = pre-acclimatization score. Week -1 = score following acclimatization, but prior to infection. Week 1 = first week of infection with *S. neurona*. Error bars represent one standard deviation about the mean.

FIGURE 6: DIFFERENCES IN ATAXIA SCORE BETWEEN EXPERIMENTALLY INFECTED AND CONTROL HORSES

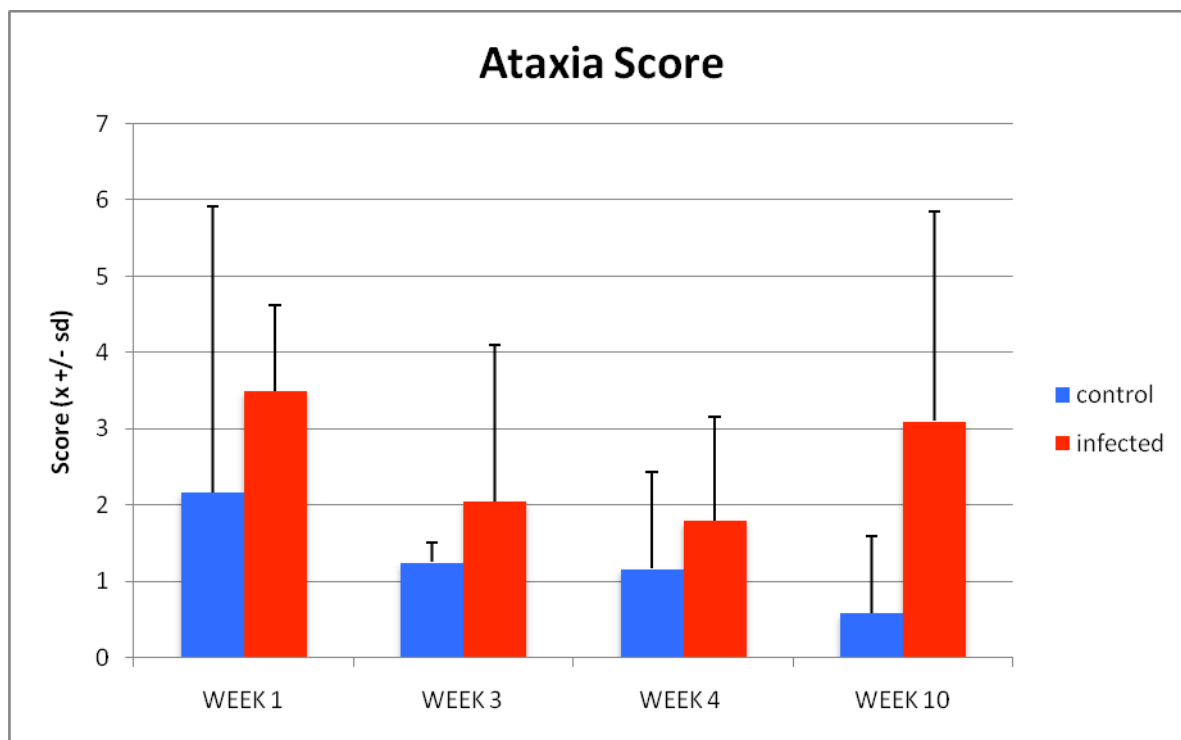


Figure 6: Differences in ataxia score between control and experimentally infected horses. Mean neurologic score attributable to ataxia was plotted for both infected and control populations. Weeks where infected horses had an increased ataxia score with respect to the control horses are shown. Horses were scored biweekly on a variety of neurologic parameters, including those attributable to ataxia. Ataxia included conscious proprioceptive deficits and movement ataxia. Week 1 = first week of the experiment (infection with *S. neurona*). Error bars represent standard deviation about the mean.

FIGURE 7: DIFFERENCES IN ATAXIA AND CRANIAL NERVE SCORES IN EXPERIMENTALLY INFECTED HORSES

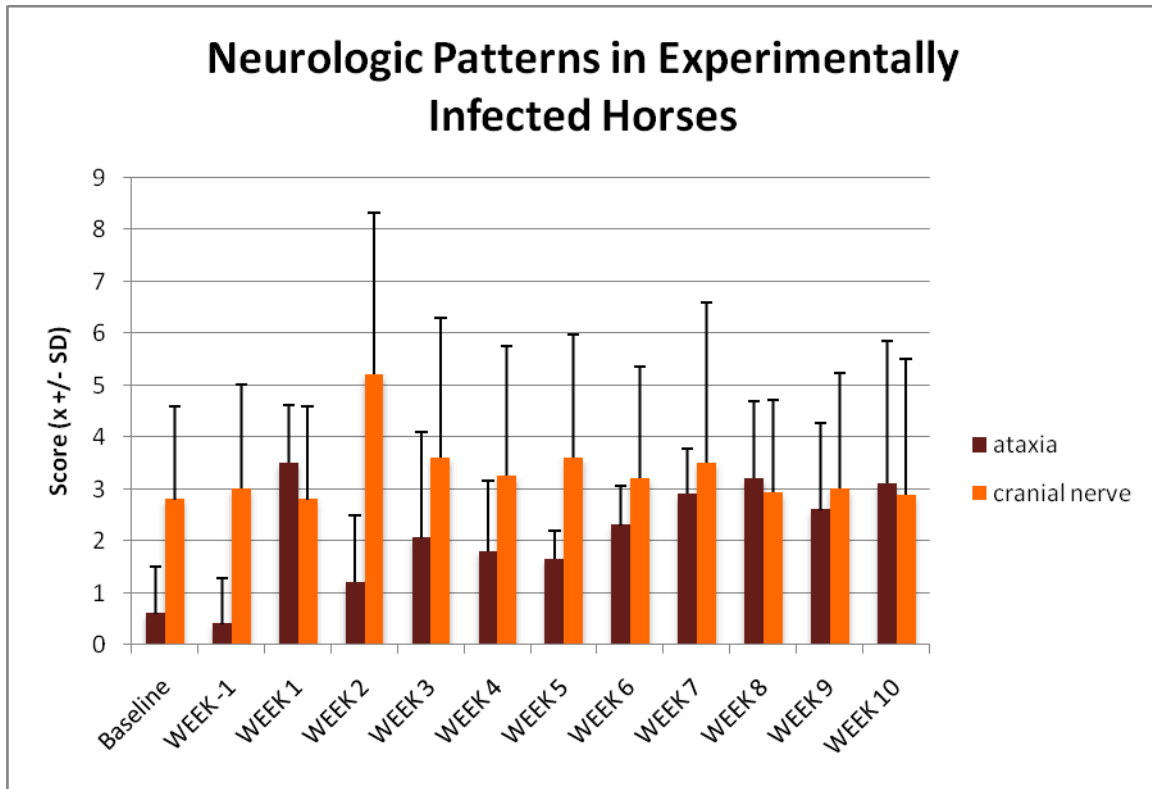


Figure 7: Differences in ataxia and cranial nerve scores in experimentally infected horses. Mean neurologic score attributable to ataxia (maroon bars) and cranial nerve signs (orange bars) are shown, for horses that were experimentally infected with *S. neurona*. Cranial nerve signs were most marked in the initial stages of the experiment, and ataxia increased towards the end of the experimental period. Horses were evaluated biweekly, and scored with respect to many parameters, out of a possible 97 total. Ataxia and cranial nerve signs were demonstrated by all infected horses at some point in the experiment, and contributed to the horses' overall neurologic scores. These results were isolated from the total score, and are shown above, for experimentally infected horses only. Baseline = score prior to acclimatization. Week -1 = score following acclimatization, but prior to experimental infection. Week 1 = first week of experimental infection. Error bars represent one standard deviation about the mean.

FIGURE 8: DIFFERENCES IN LOG-TRANSFORMED STIMULATION INDEX FOR INCUBATION WITH PMA/I BETWEEN EXPERIMENTALLY INFECTED AND CONTROL HORSES

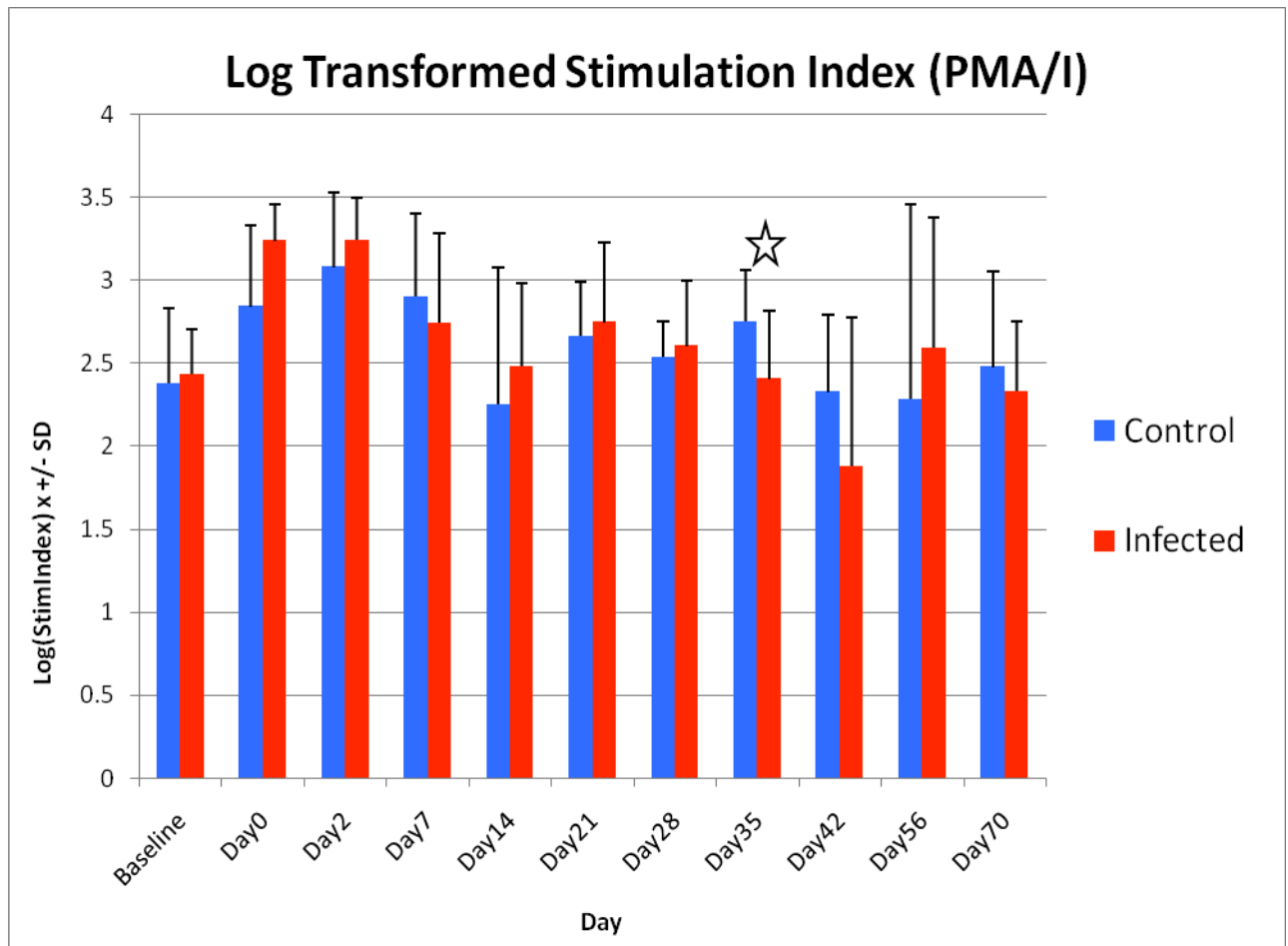


Figure 8: Differences in log-transformed stimulation indices for incubation with PMA/I between experimentally infected and control horses. Control horses had a significantly higher stimulation index for PMA/I responses on Day 35, with a pattern demonstrating higher indices in the control group towards the end of the experiment. Lymphocyte proliferation assays were performed with a variety of mitogens, including the pan-leukocyte stimulant, PMA/I. Cells were incubated with mitogens for 48 hrs, and then radioactive thymidine was added to the plate. A cell harvester was used to assess levels of proliferation via thymidine uptake 18-24 hrs later. Stimulation indices were calculated by dividing the average counts per minute for cells stimulated with each mitogen, by the counts per minute for spontaneously proliferating cells. Average stimulation index was log-transformed and plotted against time for control (blue bar) and infected (red bars) horses. The error bars represent standard deviation about the mean. The star (☆) indicates a significant difference ($p < 0.05$). Day 0 = first day of experiment (Day 1 of infection).

FIGURE 9: DIFFERENCES IN STIMULATION INDICES FOR PMA/I IN CELLS THAT HAD BEEN INCUBATED OVERNIGHT AT ROOM TEMPERATURE

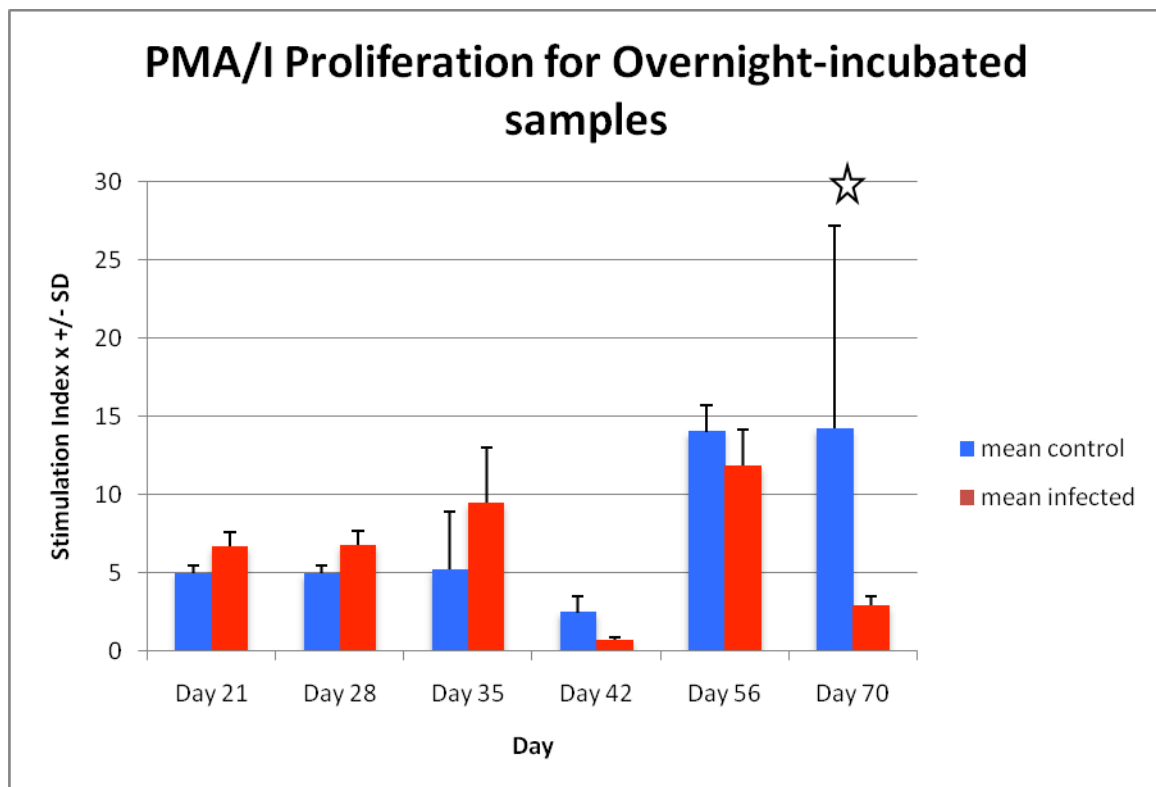


Figure 9: Differences in stimulation indices for PMA/I cells that had been incubated overnight at room temperature. Samples were drawn from horses and incubated at room temperature for 24 hrs before performing lymphocyte proliferation assays as before. Thymidine was used to determine levels of proliferation. Stimulation indices were calculated by dividing the average counts per minute for cells stimulated with each mitogen, by the counts per minute for spontaneously proliferating cells. A significantly decreased proliferation response was seen in infected horses at Day 70, as indicated by the star (☆). The error bars represent standard deviation about the mean.

FIGURE 10: DIFFERENCES IN TOTAL PERCENTAGE OF CD4+ CELLS AFTER 24 HRS INCUBATION WITH PMA/I BETWEEN INFECTED AND CONTROL HORSES

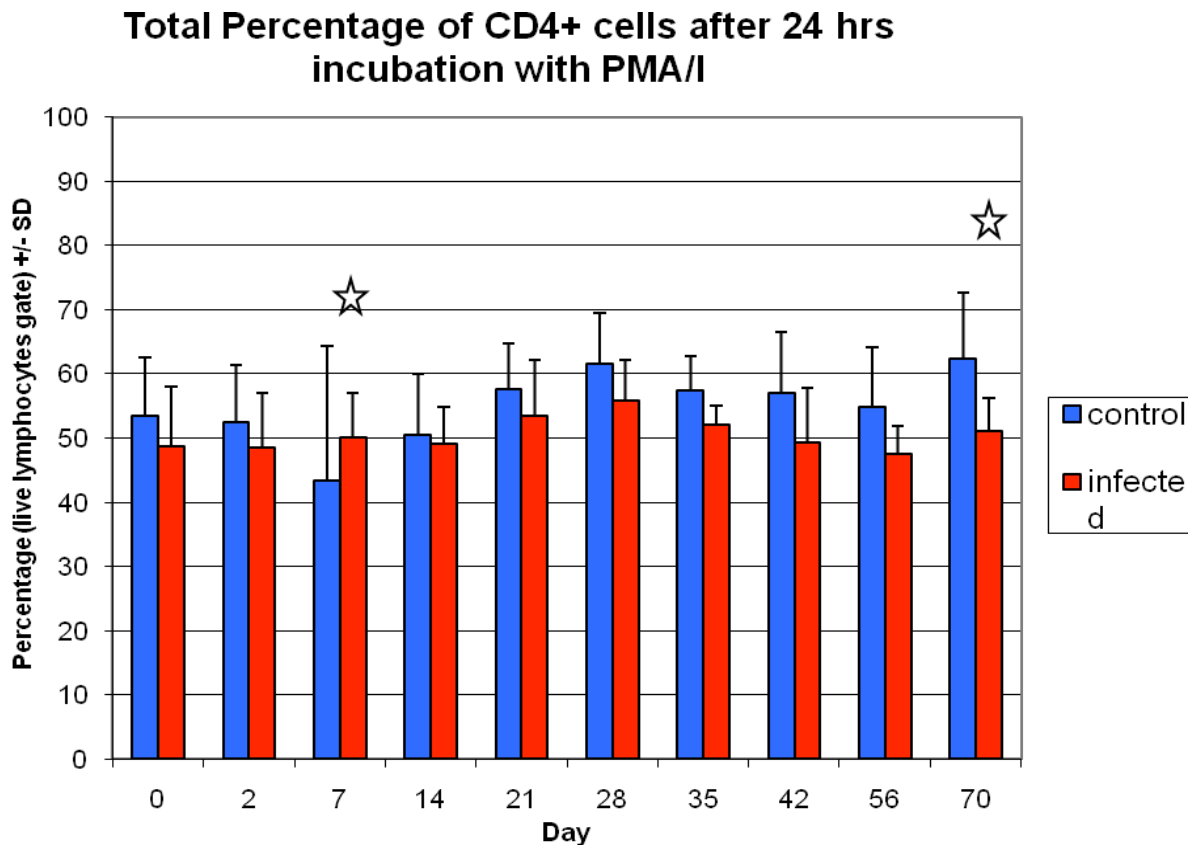


Figure 10: Differences in total percentage of CD4+ cells after 24 hours incubation with PMA/I between infected and control horses. Average percentage of total CD4+ staining cells in the “live” lymphocyte gate was plotted for infected and control horse populations, after 24hours incubation with PMA/I. Significant ($p<0.05$) results were seen at Days 7 and 70, and are indicated by a star (☆). Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

FIGURE 11: DIFFERENCES IN TOTAL PERCENTAGE OF NEUTROPHILS AFTER NO INCUBATION WITH PMA/I BETWEEN INFECTED AND CONTROL HORSES

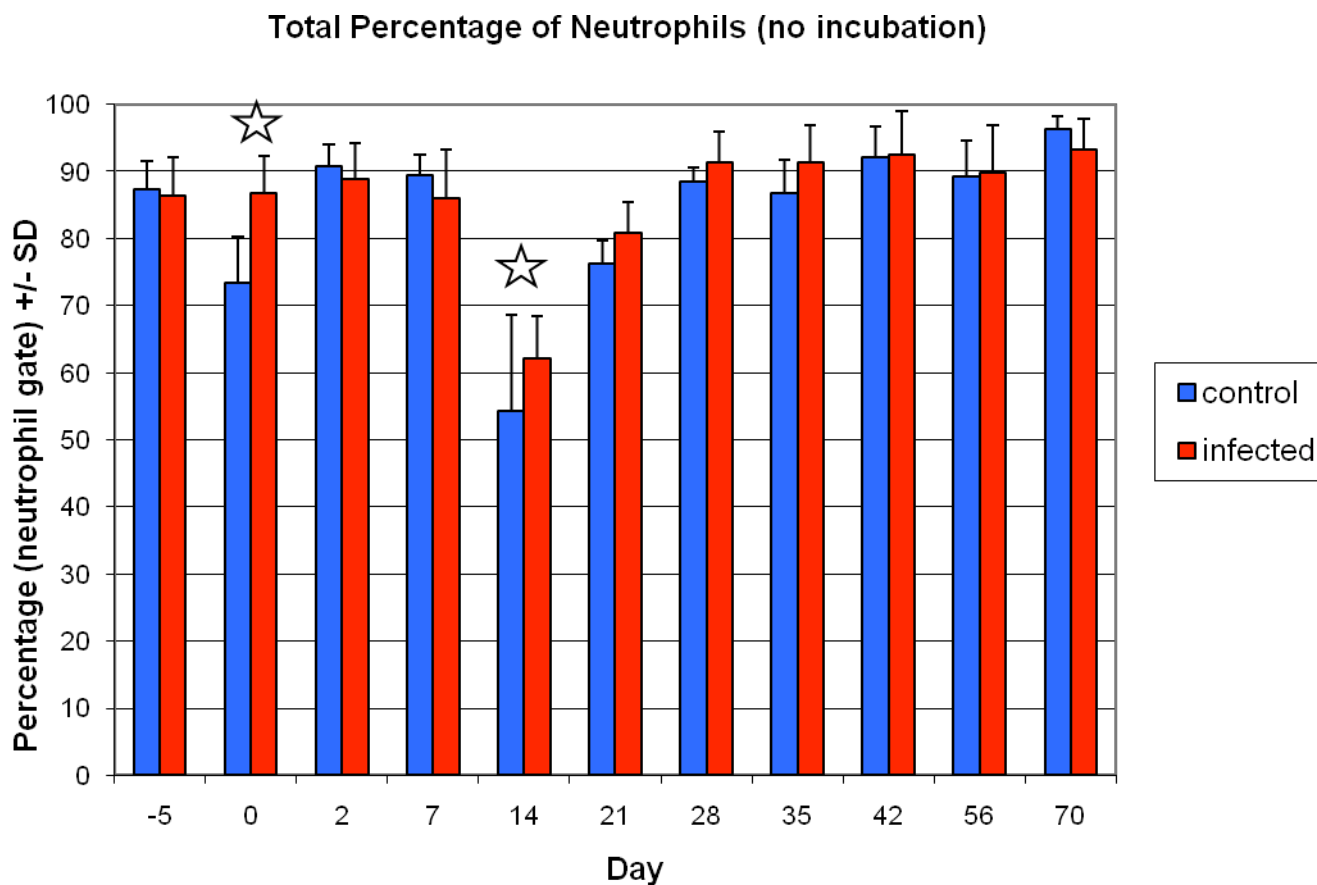


Figure 11: Differences in total percentage of neutrophils after no incubation with PMA/I between infected and control horses. Average percentage of total positively staining cells in the “granulocyte” gate was plotted for infected and control horse populations, at baseline (no incubation). Significant ($p < 0.05$) results were seen at Days 7 and 70, and are indicated by a star (☆). Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

TABLE 1: SnSAG1SERUM AND CSF TITERS FOR ALL HORSES, BEFORE AND AFTER EXPERIMENTAL INFECTION

Horse	Initial Serum SnSAG1 titer	Initial CSF SnSAG1 titer	Post-experiment Serum SnSAG1 titer	Post-experiment CSF SnSAG1 titer
1	<2	<2	32	<1
2	<2	<2	16	1
3	<2	<2	80	2
4	<2	<2	<2	2
5	<2	<2	32	2
6	4	<2	32	<1
7	<2	<2	80	1
8	<2	<2	32	<1
9	<2	<2	32	<1

Table 1: SnSAG1 serum and CSF titers for all horses, before and after experimental infection. Horses were anesthetized following the acclimatization period, and atlanto-occipital CSF taps and jugular blood draws were performed. Horses 3, 5, 6, 7 and 9 were then infected through an intravenous method of inoculation for 10 days of infection. After the conclusion of the experiment (after Day 73) the horses were reanesthetized and samples obtained. This table summarizes the results of pre-experiment and post-experiment SAG1 ELISA results for all the horses. Both serum and CSF results are included. A titer ≥ 32 is considered positive for serum. A titer ≥ 1 is considered positive for CSF. Infected horses are highlighted (Horses 3, 5, 6, 7 and 9) in red. Control horses (Horses 1, 4 and 8) are highlighted in blue. Horse 2 (grey) received a dose of the parasite in error, and was excluded from our statistical analysis

TABLE 2: CSF CYTOLOGY DATA FOR ALL HORSES FROM INITIAL CSF TAP ON DAY -5

Horse	Color	Transparency	WBC/uL	RBC/uL	Glucose (mg/dL)	Protein (mg/dL)	SAG1 ELISA titer
1	Colorless	Clear	1	1575	49.1	62.4	<2
2	Colorless	Clear	2	158	50.2	59.1	<2
3	Colorless	Clear	0	1	49.3	45.1	<2
4	Colorless	Clear	1	52	50.9	28.2	<2
5	Colorless	Clear	1	0	48.6	73.8	<2
6	Colorless	Clear	2	11	51.3	42.0	<2
7	Colorless	Clear	1	0	50.8	33.4	<2
8	Colorless	Clear	0	0	52.2	37.1	<2
9	Colorless	Clear	0	0	56.5	55.7	<2

Table 2: CSF cytology data for all horses from initial CSF tap on Day -5. Results of cytologic analysis of the pre-experiment CSF sample are shown. This sample was taken prior to infection of the horses, yet after the acclimatization period. Horses were anesthetized with xylazine and ketamine, and atlanto-occipital CSF taps were performed. Each sample was submitted for cytologic analysis.

TABLE 3: SIGNIFICANT DIFFERENCES IN TOTAL PERCENTAGE OF POSITIVELY STAINING CELLS FOR EACH CELLULAR SUBSET

	Day										
	-5	0	2	7	14	21	28	35	42	56	70
CD4+				24h I							24h C
CD8+					72hI				0h I		(72h C)
Neutrophils		0hI			0h I						24h I
Monocytes							(48h I)	(48hI)			
B cell						72h C	24h I				

Table 3: Significant differences in total percentage of positively staining cells for each cellular subset. This table shows the time points at which significant differences ($p < 0.05$) in total cellular subset percentages were seen between infected and non-infected horses. The treatment group with the higher percentage of cells of each subset is shown, alongside the period of incubation with PMA/I at which it was significant. Trends ($0.05 \leq p < 0.1$) are shown in parentheses. I = infected horses. C = control horses. Oh = no incubation with PMA/I. 24h = 24 hours incubation with PMA/I

**TABLE 4: EFFECT OF INFECTION ON APOPTOSIS IN UNSTIMULATED SAMPLES:
SIGNIFICANT RESULTS WITH RESPECT TO INFECTED HORSES**

		Day 0	Day 2	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56	Day 70
CD4+	Live										
	Dying	↑L	↓E	↓E	↓E	↑V ↓E			(↓V)		(↑E) ↑L
CD8+	Live				↑V		↑E		(↑E)		
	dying	(↑E) ↑L	(↑L)	↑L				(↑E)	↑L	↑L	↓L
Neutrophils			(↓E)	Few cells		↑L		↓E	↓E		
Monocytes				Few cells	↓V	↑E		↓E			
B Cell	Live			Few cells	(↓E)	↑E					
	Dying			Few cells						(↑L)	(↑E) ↑L

Table 4: Effect of infection on apoptosis in unstimulated samples: significant results with respect to infected horses. The table shows the time points at which significant differences ($p < 0.05$) or trends ($0.05 \leq p < 0.1$) in apoptosis were seen between infected and non-infected horses, after no incubation. The arrow dictates whether infected horses had increased percentages (↑) or decreased percentages (↓) of viable/apoptotic cells when compared to control horses. Trends are shown in parentheses. V = viable cells. E = cells in early apoptosis. L = cells in late apoptosis

TABLE 5: SIGNIFICANT RESULTS FROM THE EFFECT OF STIMULATION WITH PMA/I ON APOPTOSIS WITH RESPECT TO INFECTED HORSES

		Day									
		0	2	7	14	21	28	35	42	56	70
CD4+	Live			(24h ↑E) 24h ↑L 72h ↑V							
	Dying			24h ↓L	24h ↓L						
CD8+	Live			48h ↑V					(48h ↑E)		(48h ↑V) (72h ↓E)
	Dying										(72h ↓V)
Neutrophils				Few cells	(72h ↓E)			24h ↓V 48h ↓L			
Monocytes				Few cells		48h ↓L	24h ↓L	24h ↓V 48h ↑E (72h ↑L)	(48h ↓V)	(24h ↓V)	(48h ↑L) (72h ↑L)
B Cell	Live	24h ↑L	48h ↑L	Few cells	(48h ↓V)	24h ↑V 72h ↑E	24h ↑V	48h ↑L			24h ↑E
	Dying			Few cells		(24h ↑E)		(72h ↓V)			24h ↓L

Table 5: Significant results from the effect of stimulation with PMA/I on apoptosis with respect to infected horses. Cells were incubated with PMA/I for varying periods, and the levels of apoptotic cells were compared between treatment groups. The table shows the time points at which significant differences ($p < 0.05$) or trends ($0.05 \leq p < 0.1$) in apoptosis were seen between infected and non-infected horses, after incubation with PMA/I. The arrow dictates whether infected horses had increased percentages (↑) or decreased percentages (↓) of viable/apoptotic cells when compared to control horses. Trends are shown in parentheses. V = viable cells. E = cells in early apoptosis. L = cells in late apoptosis. 24h = incubation with PMA/I for 24 hours

TABLE 6: SIGNIFICANT RESULTS FROM THE EFFECT OF STIMULATION WITH MEROZOITES ON APOPTOSIS WITH RESPECT TO INFECTED HORSES

Cell	Subset	Day 28	Day 35	Day 42	Day 56	Day 70
CD4+	Live		↑ L 24h			↑ E 24h ↑ L 24h
	Dying					(↑ V 24h) (↓ V 72h) ↓ E 24h (↑ E 72h) ↓ L 24h
CD8+	Live			↑ E 24h		↑ L 72h
	Dying					
Neutrophils		↓ E 24h			(↓ V 24h)	↓ V 24h ↑ E 24h (↓ E 72h) (↑ L 24h)
Monocytes					(↓ V 24h)	↑ L 24h
B cells	Live		↑ V 72h (↓ E 72h) ↓ L 72h			
	Dying		(↑ V 72h)		(↑ V 72h)	(↓ V 72h) ↑ E 24h ↑ E 72h

Table 6: Significant results from the effect of stimulation with merozoites on apoptosis with respect to infected horses: significant results. Cells were incubated with merozoites for varying periods, and the levels of apoptotic cells were compared between treatment groups. The table shows the time points at which significant differences ($p < 0.05$) or trends ($0.05 \leq p < 0.1$) in apoptosis were seen between infected and non-infected horses, after incubation with PMA/I. The arrow dictates whether infected horses had increased percentages (↑) or decreased percentages (↓) of viable/apoptotic cells when compared to control horses. Trends are shown in parentheses. V = viable cells. E = cells in early apoptosis. L = cells in late apoptosis. 24h = incubation with merozoites for 24

**TABLE 7: SIGNIFICANT DIFFERENCES IN PMA/I STIMULATED CELLULAR DIVISIONS
(USING CFSE AS A MARKER)**

	CD4+					CD8+				
	No division	1 division	2 divisions	3 divisions	>3 divisions	No division	1 division	2 divisions	3 divisions	>3 divisions
Day 2	Live control		(Dying infected)	(Dying Infected)		(Live control)		Live infected	Dying infected	
Day 7				Dying infected						
Day 21				Live infected	(D infected)				(Live infected)	Dying infected (Live infected)
Day 28							Dying control	(Dying infected) Live infected	(Live infected)	
Day 56			Dying control	Dying infected (Live infected)	Dying infected					

Table 7: significant differences in PMA/I stimulated cellular divisions (using CFSE as a marker). Cells were incubated with merozoites for varying time periods, and numbers of cellular divisions were assessed through CFSE incorporation, using flow cytometry. The table shows the time points at which there was a significant difference ($p < 0.05$) or a trend ($0.05 \leq p < 0.1$) in PMA/I stimulated cellular divisions, between infected and control horses. The treatment group with the higher percentage of cells undergoing that number of divisions is listed. Live = live lymphocyte population. Dying = dying population. Trends are listed in parentheses.

**TABLE 8: SIGNIFICANT DIFFERENCES IN MEROZOITE-INDUCED CELLULAR DIVISIONS
(CFSE)**

	CD4+					CD8+				
	No division	1 division	2 divisions	3 divisions	>3 divisions	No division	1 division	2 divisions	3 divisions	>3 divisions
Day 28				Live control					(Live control)	
Day 42	(Live infected)									
Day 56								(Live control)		
Day 70								(Dying control)		

Table 8: Significant differences in merozoite-induced cellular divisions (CFSE). Cells were incubated with merozoites for varying time periods, and numbers of cellular divisions were assessed through CFSE incorporation, using flow cytometry. The table shows the time points at which there was a significant difference in PMA/I stimulated cellular divisions, between infected and control horses. The treatment group with the higher percentage of cells undergoing that number of divisions is listed. Trends are shown in parentheses. Live = live lymphocyte population. Dying = dying population.

APPENDIX A: INDIVIDUAL HORSE INFORMATION INCLUDING ASSIGNED NUMBER, TREATMENT STATUS AND SIGNALMENT

Horse Number	Group	Age at onset of study in years	Breed	Sex
1	Control	2	QH	Female
2	One Dose	4	QHX	Female
3	Infected	11	QHX	Female
4	Control	5	QHX	Female
5	Infected	7	QHX	Female
6	Infected	4	QH	Female
7	Infected	1.5	QHX	Gelding
8	Control	4	QH	Female
9	Infected	2	QHX	Female

Appendix A: Individual horse information including number, treatment status and signalment. Neurologically normal, and SnSAG1 negative, horses were obtained and split randomly into two groups: the infected group contained horses 3, 5, 6, 7 and 9, and the control group contained horses 1, 4 and 8. Horse 2 received a dose of the parasite in error in the infection process, and was therefore excluded from our results. The group, age in years, breed and sex of horses is shown above. QH= quarter horse. QHX = quarter horse cross.

APPENDIX B: NEUROLOGIC SCORING SHEET FOR OBSERVATIONS OF CLINICAL PARAMETERS (TOTAL POSSIBLE = 97)

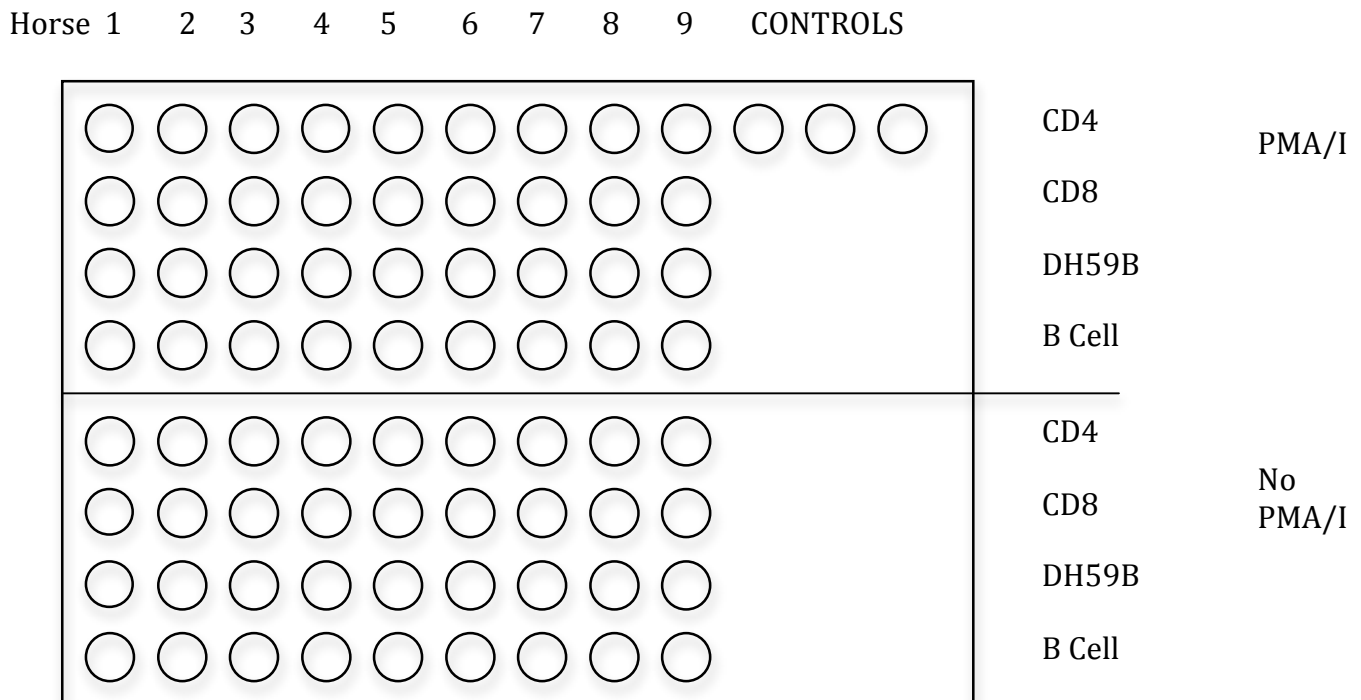
Category	Action	Description	Number	Description 2
Eating	Drops Feed	< ¼ lb	1	
		≥ ¼ lb ≤ ½ lb	2	
		> ½ lb	3	
	Tongue Tone Decreased	Normal mastication	1	
		Abnormal mastication	2	
		Paresis	3	
	Abnormal Feed Prehension	Normal	0	
		Unable to masticate	1	
		Bites Food	2	
Head	Drooling	Does not drool	0	
		When eating	1	
		Continuously	2	
	Lip Paresis	Normal	0	
		Perceptible when eating	1	
		Perceptible Continuously	2	
		Lip Hangs	3	
	Facial Nerve Paresis	Normal	0	
		Perceptible	1	
		Moderate	2	
		Severe	3	
	Eyelid paresis	Normal	0	
		Ventrally away from eye	1	
		Over ¼ of eye	2	
		With corneal lesion	3	
	Attitude	Normal	0	
		Depressed	1	
		Aggressive	2	
		Somnolent	3	
Muscle	Muscle Atrophy	Normal	0	
		Just perceptible	1	
		Immediately Noticeable	2	
		Severe Atrophy	3	
Rear End	Cauda Equina	Normal	0	
		Holds Tail Rigid	1	
		Dribbling Urine	2	
		Rectum Paretic	3	
Movement	Weakness	Normal	0	Left Fore (LF), Right Fore (RF), Left Hind (LH), Right Hind (RH)
		While Walking	1	
		Markedly reduced strength	2	
		Can pull horse over easily/ recumbent	3	
	Lameness	None seen	0	
		Just noticed at walk	1	
		Head bob or hip drop at	2	

		walk Reluctant to use limb	3	
Conscious Proprioception	Crossing Over Limbs	Normal	0	LF, RF, LH, RH
		Slightly slow to place limb back to normal position	1	
		No resistance to abnormal placement of limb	2	
		Horse unaware of limb position and unable to correctly place limb	3	
	Abduction of Hind Limbs	Normal	0	LH, RH
		Slightly slow to place limb back to normal position	1	
		No resistance to abnormal placement of limb	2	
		Horse unaware of limb position and unable to correctly place limb	3	
	Tripping	None seen	0	
		Sometimes	1	
		Often	2	
		Often and Falls to knees	3	
Limbs	Paresis	Normal	0	
		Detectable at normal gaits but exacerbated by manipulative actions	1	
		Obvious at normal gaits or postures	2	
		Very prominent at normal gait	3	
	Circling	Normal	0	Counterclockwise, clockwise
		Occasional abnormal limb placement	1	
		Does not move normally in circle	2	
		Refuses to circle/ falls	3	
	Pivoting	No pivoting seen	0	Counterclockwise, clockwise
		Pivots occasionally on inside hind limb	1	
		Pivots frequently	2	
		Will not pick up hind leg-pivots continuously	3	
	Toe Dragging	No toe dragging seen	0	Left, Right
		Occasionally drags a hind	1	

		limb Frequently drags hind limb Constantly drags hind limbs	2 3	
Backing Up	Hypermetria	Normal movement Slightly hypermetric in one limb Moderately hypermetric in one limb/ slight hypermetria in both hind limbs Very obvious hypermetria	0 1 2 3	
	Inconsistent Placement	Normal placement Slightly wide based on placement of hind limbs Wide based on hind limb placement Places limbs very abnormally (touching, very wide), almost falls	0 1 2 3	
Tail Pull	At the walk	Normal Slight lack of resistance to tail pull Easily pull horse off track Horse almost falls when tail pulled	0 1 2 3	Left, Right
Maximum Total Possible			97	

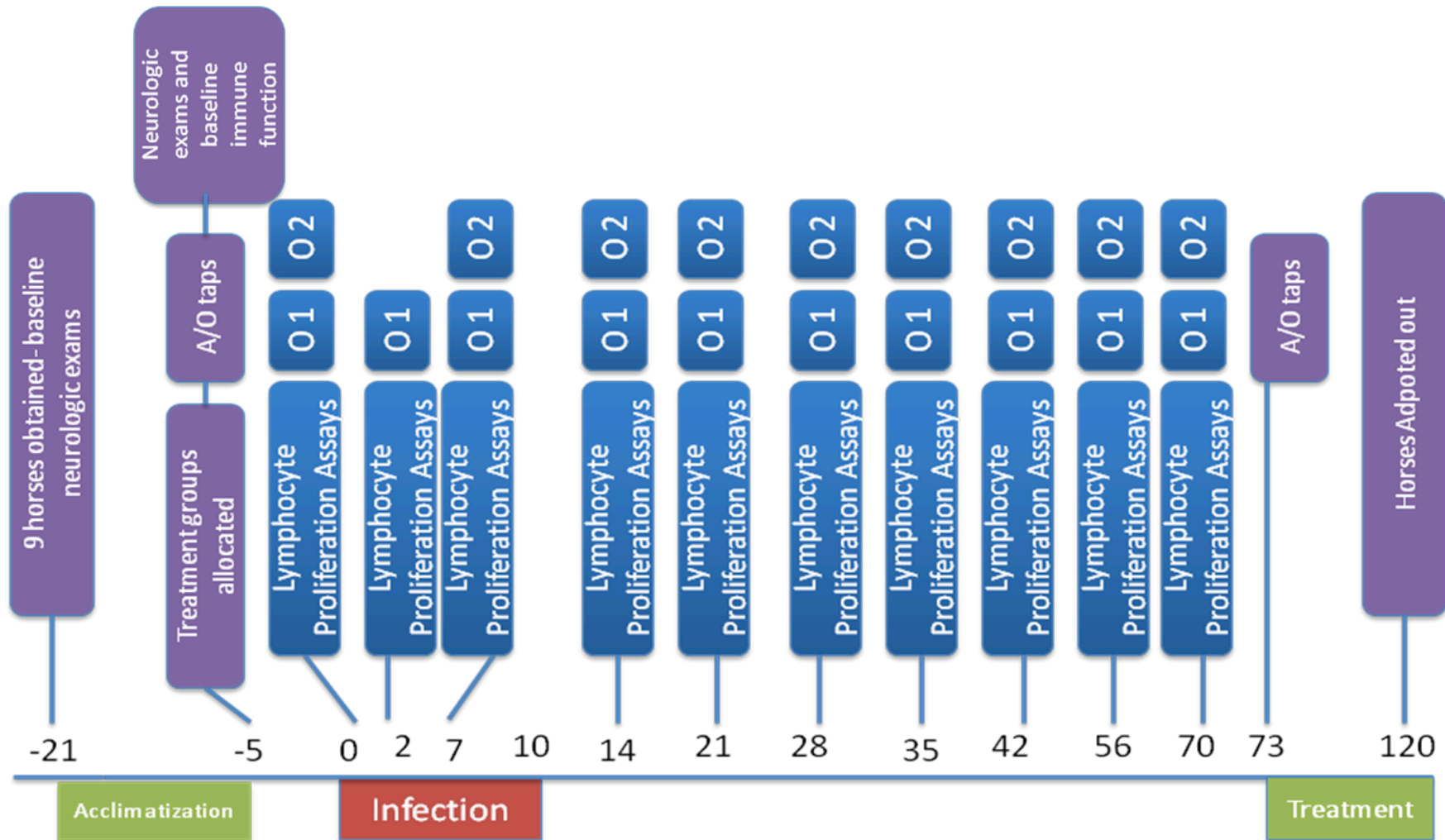
Appendix B: Neurologic scoring sheet for observations of clinical parameters (total possible 97). Horses were examined biweekly and scored based on a variety of different parameters, listed above. For most categories, a score of zero represented a normal response to evaluation of this parameter, and a score of three was the maximum neurologic score possible. The scoring system was based on that used in previous experiments by investigators using the intravenous method of infection.

APPENDIX C: EXAMPLE OF 96-WELL PLATES FOR FLOW CYTOMETRY (7-AAD AND CFSE) PLATED FOR INCUBATION TIME = 24, 48 OR 72 HRS



Appendix C: Example of 96-well plates for flow cytometry (7-AAD and CFSE) plated for incubation time = 24, 48 and 72 hours. Cells were enriched for lymphocytes and separated using density centrifugation methods, then washed and plated in 96-well plates. The figure above demonstrates the layout of most of the plates used in flow cytometric analyses. Columns labeled 1-9 represent individual horses, and each column contains cells from the same horse. The three final columns represented cells that were unstained to act as controls. The rows of the plate were stained with different primary antibodies, listed to the right of the plate in the diagram. DH59B is a surface antibody stain for granulocytes and monocytes. Cells in the top four rows of the plates were incubated with PMA/I, and cells in the lower four rows were incubated without PMA/I, containing media only.

APPENDIX D: FLOW CHART DEMONSTRATING EXPERIMENTAL METHOD



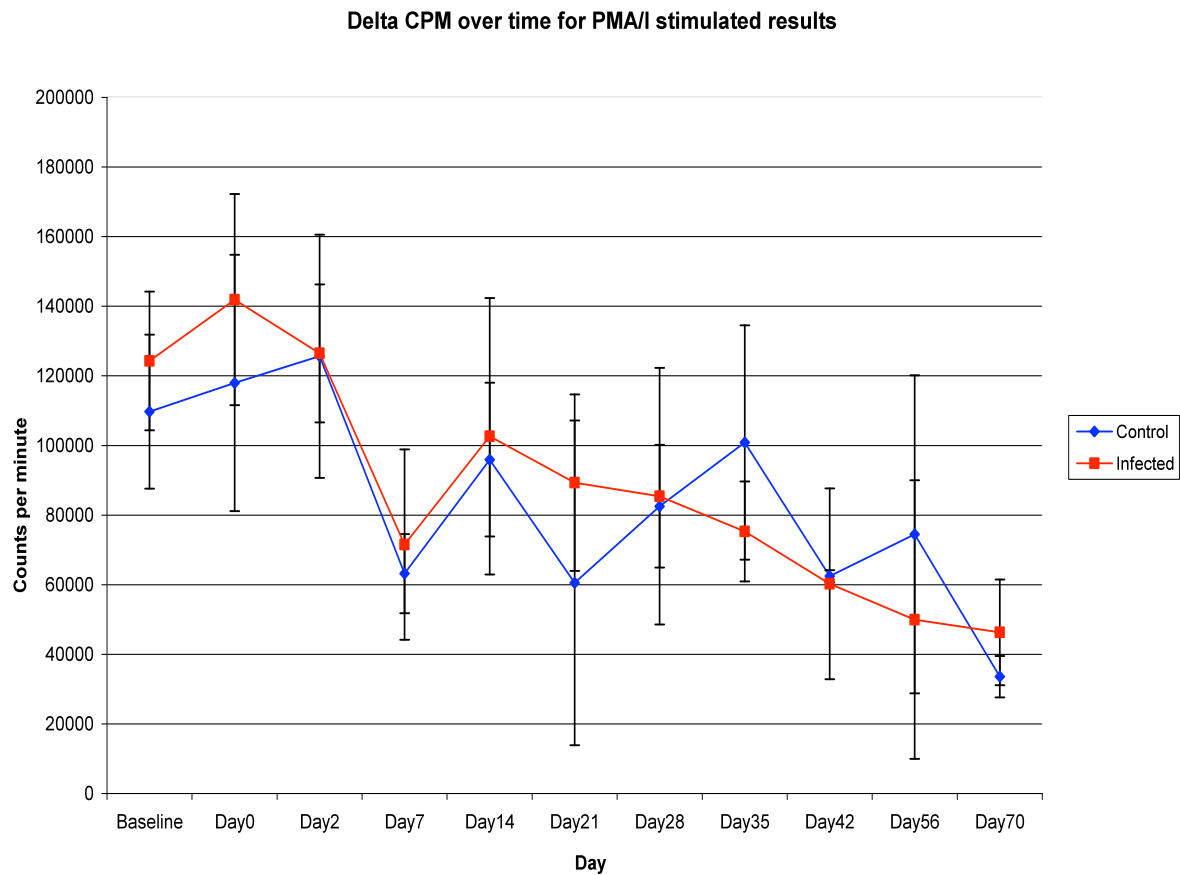
Appendix D: Flow chart demonstrating experimental method. Horses were obtained at Day -21, shown on the left of the time line, and initial neurologic exams were performed. The horses underwent an acclimatization period, and then baseline tests of immune function were performed on Day -5. Horses were anesthetized and CSF taps were performed. Infection began on Day 0 and continued for ten days. Time points at which proliferation assays and flow cytometry were performed are listed. After Day 73, CSF sampling was repeated, infected horses were treated and eventually all horses were adopted out. O1 = Objective 1. O2 = Objective 2. The number on the time line represents day post infection. 154

APPENDIX E: CYTOSPIN DIFFERENTIAL COUNTS FROM SELECTED HORSES

Double Peak					
Horse Number	Day	Neutrophil %	lymphocyte %	monocyte %	total
6	-5	2	91	7	100
6	2	2	95	3	100
5	2	6	88	6	100
5	14	3	92	5	100
3	14	1	90	9	100
8	14	3	87	10	100
4	14	3	87	10	100
2	28	13	84	3	100
5	28	9	87	4	100
4	28	12	85	3	100
6	28	4	87	9	100
9	35	15	78	7	100
6	70	8	86	6	100
	average	6.2	87.5	6.3	
No Double Peak (normal)					
Horse number	Day	neutrophil %	lymphocyte %	monocyte %	total
5	-5	3	92	5	100
9	-5	10	87	3	100
9	0	12	84	4	100
3	2	3	91	6	100
9	2	9	87	4	100
5	7	1	89	10	100
2	7	6	84	10	100
2	14	8	82	10	100
1	14	11	81	8	100
7	21	5	87	8	100
5	21	4	90	6	100
9	28	6	89	5	100
1	28	0	93	7	100
4	35	3	92	5	100
1	70	6	84	10	100
	average	5.8	87.5	6.7	
	overall average	6.0	87.5	6.5	

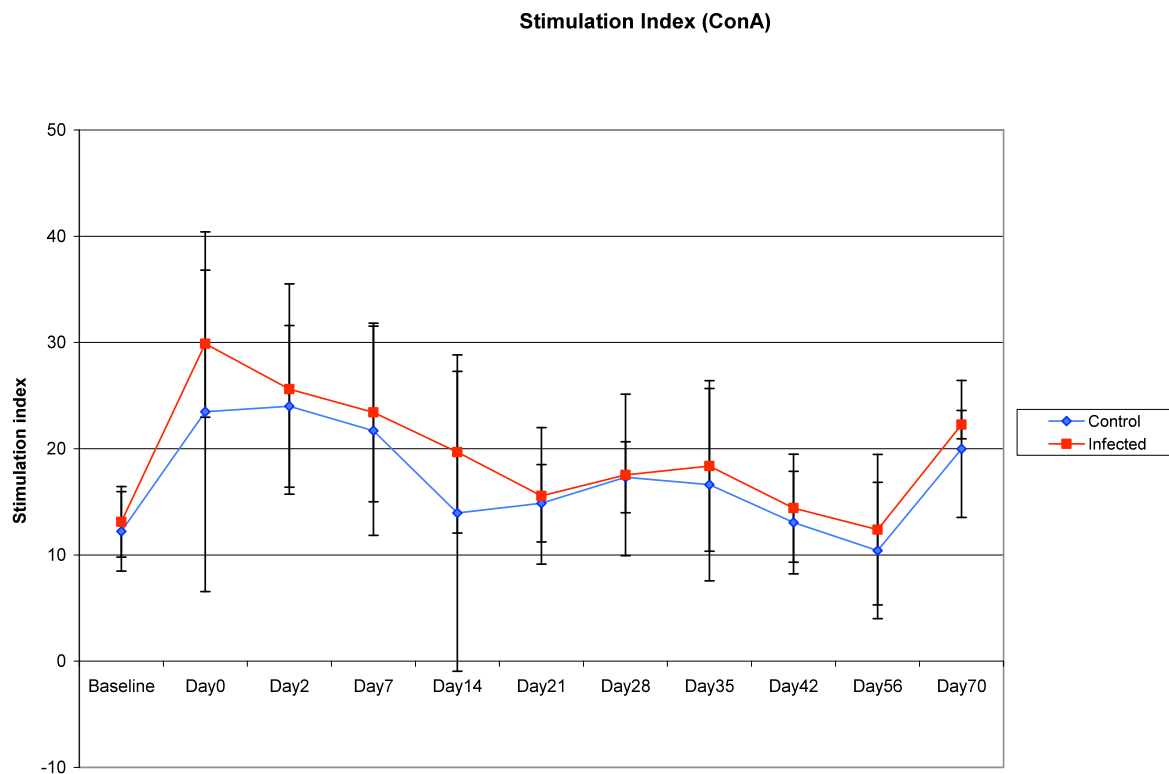
Appendix E: Cytospin differential counts from infected horses. Samples were selected for differential counts after examining Coulter data: those samples demonstrating a double peak on Coulter data are shown on the top half of the table. Samples from horses demonstrating a single peak (“normal” data distribution) on the same day were chosen at random, and differential counts were also performed on these samples. The results are shown on the bottom half of the table.

APPENDIX F: CHANGE IN PROLIFERATION INDEX (DELTAS): MITOGEN STIMULATED AVERAGE – AVERAGE SPONTANEOUS PROLIFERATION



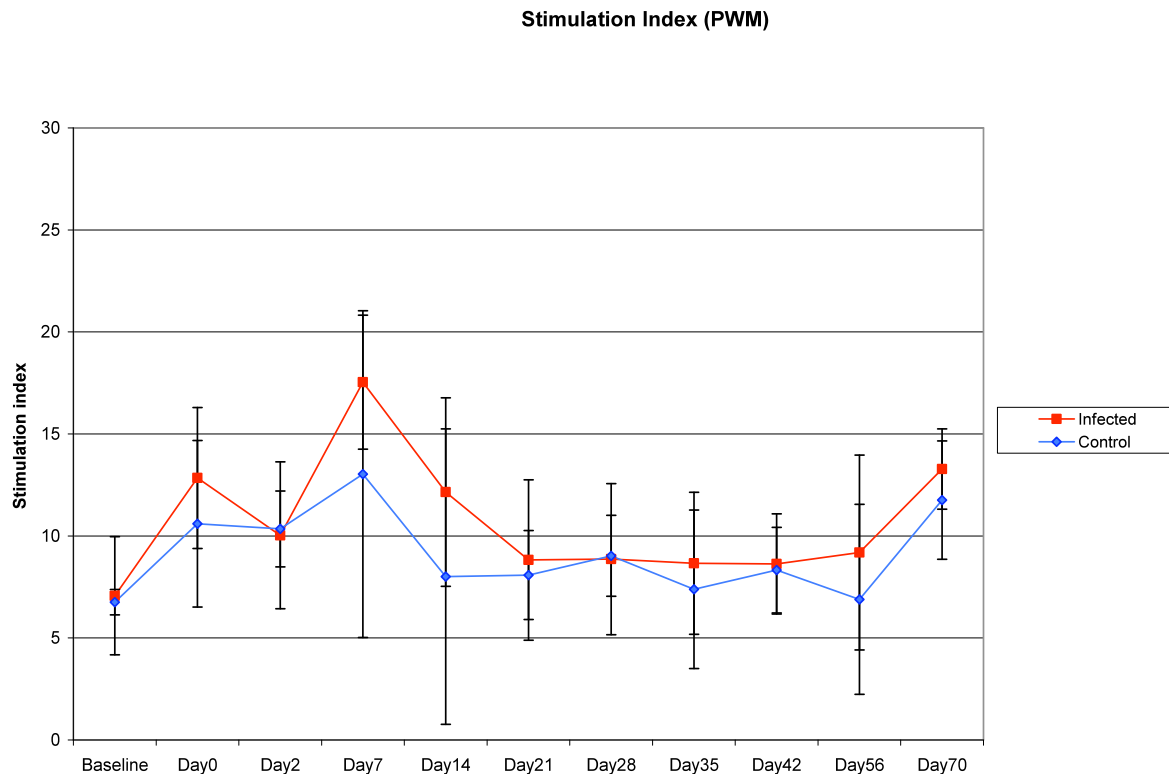
Appendix F: Change in proliferation index (deltas): mitogen stimulated average minus spontaneous proliferation. Lymphocyte proliferation assays were performed with a variety of mitogens, including the pan-leukocyte stimulant PMA/I. Cells were incubated with mitogens for 48 hours, and then radioactive thymidine was added to the plate. A cell harvester was used to assess levels of proliferation via thymidine uptake 18-24 hours later. Deltas were calculated as described above, and are plotted on the graph. Day = Days following infection on Day 0. Baseline = proliferation responses before beginning the experiment. Error bars represent one standard deviation about the mean. Statistical analysis was not performed on these results, as they were calculated to compare methodologies only and enable us to decide which method of presenting proliferation results was most appropriate.

APPENDIX G: DIFFERENCES IN STIMULATION INDICES FOR CONA OVER TIME (T LYMPHOCYTE STIMULATION) BETWEEN CONTROL AND EXPERIMENTALLY INFECTED HORSES



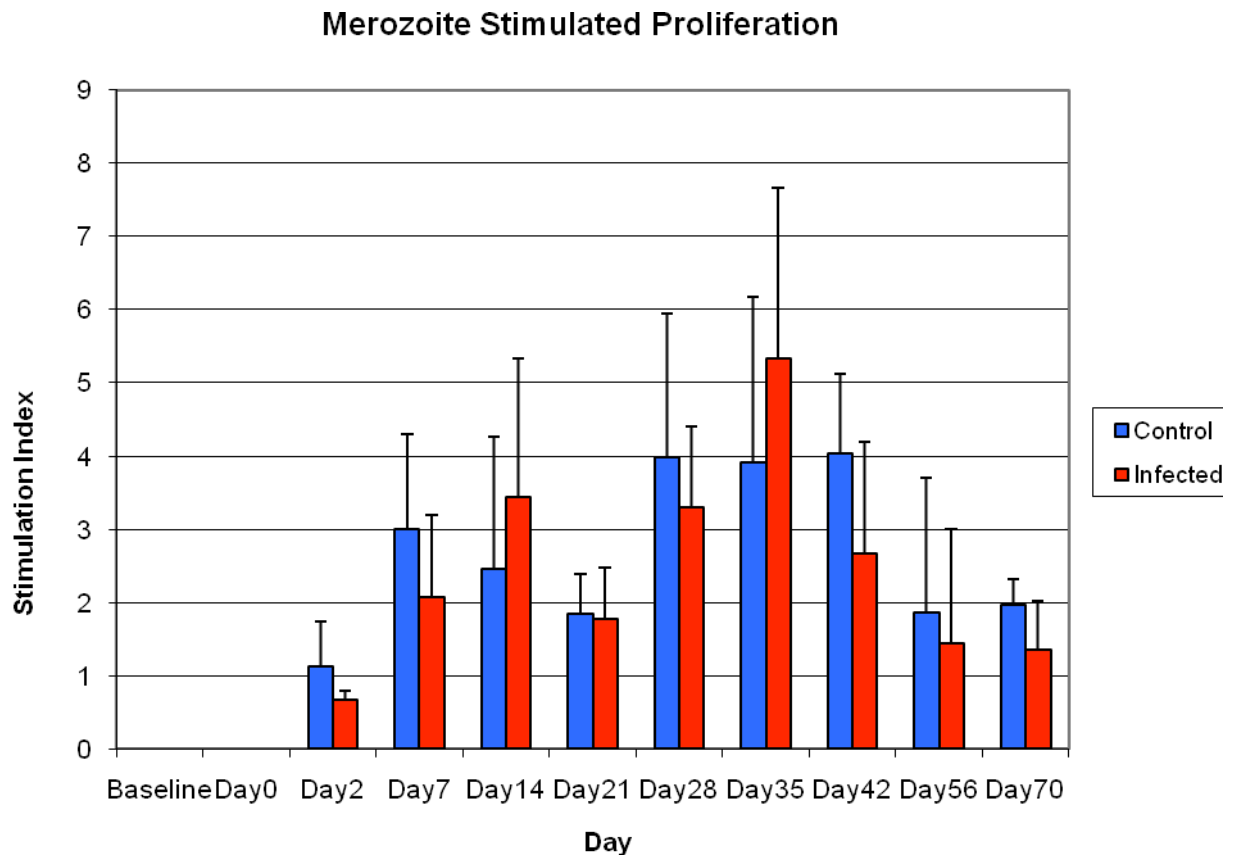
Appendix G: Differences in stimulation indices for ConA (T lymphocytes) over time, between experimentally infected and control horses. Lymphocyte proliferation assays were performed with a variety of mitogens, including the T lymphocyte stimulant, ConA. Cells were incubated with mitogens for 48 hours, and then radioactive thymidine was added to the plate. A cell harvester was used to assess levels of proliferation via thymidine uptake 18-24 hours later. Stimulation indices were calculated by dividing the average counts per minute for cells stimulated with each mitogen, by the counts per minute for spontaneously proliferating cells. Average stimulation index was plotted against time for control (blue line) and infected (red line) horses. Day = days from first day of infection (Day 0). Baseline = responses prior to beginning infection. Error bars represent one standard deviation about the mean.

APPENDIX H: DIFFERENCES IN STIMULATION INDICES OVER TIME FOR PWM (B CELL RESPONSES) BETWEEN CONTROL AND EXPERIMENTALLY INFECTED HORSES



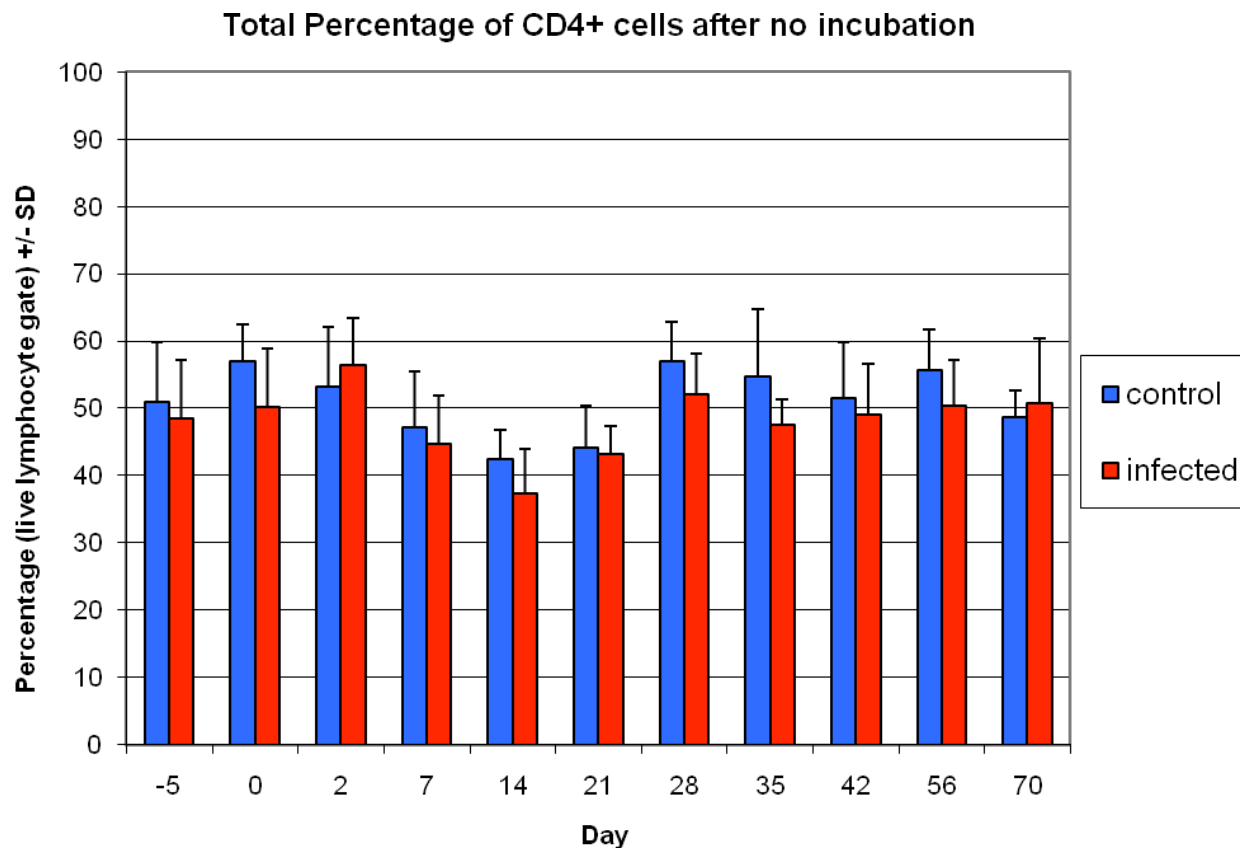
Appendix H: Differences in stimulation indices for PWM (B lymphocytes) over time, between experimentally infected and control horses. Lymphocyte proliferation assays were performed with a variety of mitogens, including the B lymphocyte stimulant, PWM. Cells were incubated with mitogens for 48 hours, and then radioactive thymidine was added to the plate. A cell harvester was used to assess levels of proliferation via thymidine uptake 18-24 hours later. Stimulation indices were calculated by dividing the average counts per minute for cells stimulated with each mitogen, by the counts per minute for spontaneously proliferating cells. Average stimulation index was plotted against time for control (blue line) and infected (red line) horses. Day = days from first day of infection (Day 0). Baseline = responses prior to beginning infection. Error bars represent one standard deviation about the mean.

APPENDIX I: STIMULATION INDEX OVER TIME FOR ANTIGEN-SPECIFIC STIMULATION (INCUBATION WITH MEROZOITES) FOR EXPERIMENTALLY INFECTED AND CONTROL HORSES



Appendix I: Stimulation index over time for antigen-specific responses (incubation with merozoites) between experimentally infected and control horses. Lymphocyte proliferation assays were performed with a variety of mitogens, including merozoites to study antigen specific responses. Cells were incubated with mitogens for 48 hours, and then radioactive thymidine was added to the plate. A cell harvester was used to assess levels of proliferation via thymidine uptake 18-24 hours later. Stimulation indices were calculated by dividing the average counts per minute for cells stimulated with each mitogen, by the counts per minute for spontaneously proliferating cells. They were then plotted against time for control (blue bar) and infected (red bars) horses. The error bars represent standard deviation about the mean. Day 0 = first day of experiment (Day 1 of infection). Merozoites were not included on the plates as mitogens until Day 2, hence data is missing from the graph at Baseline and Day 0.

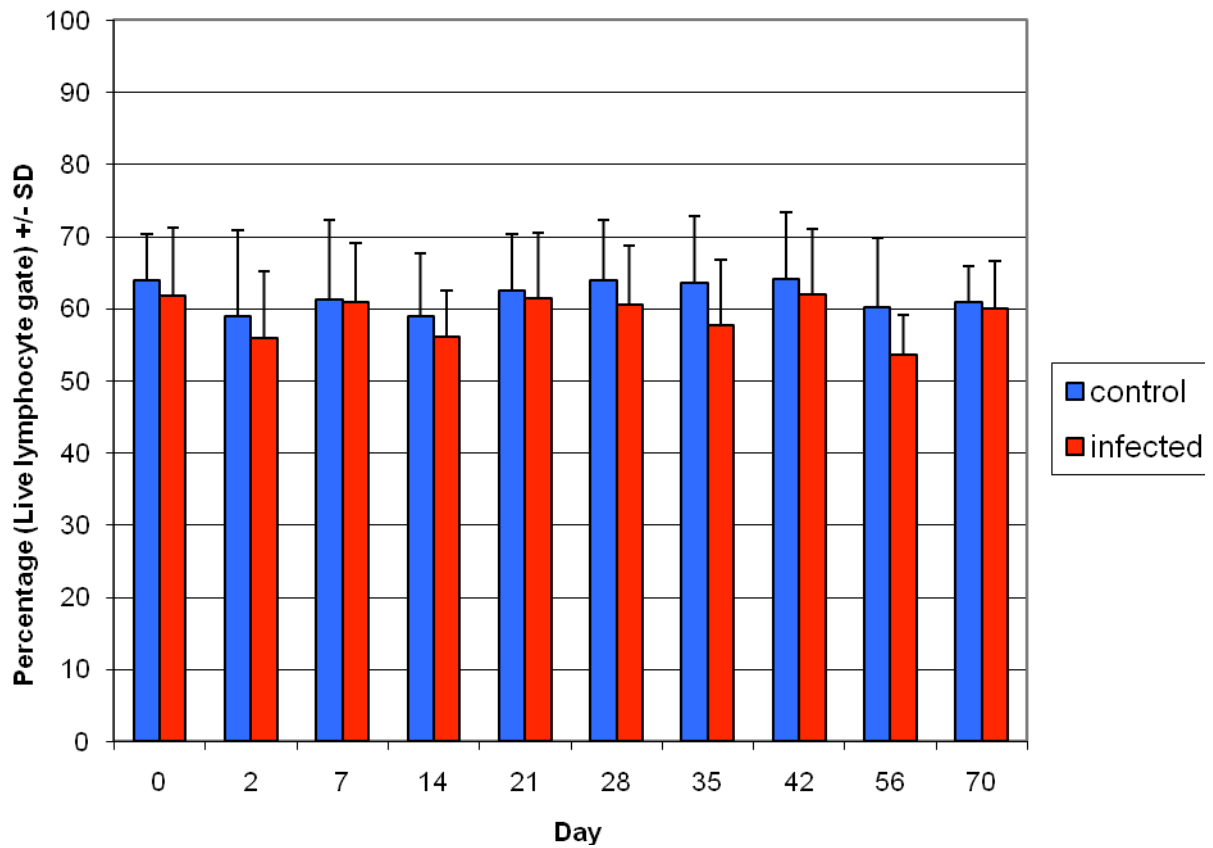
APPENDIX J: DIFFERENCES IN TOTAL PERCENTAGE OF CD4+ LYMPHOCYTES BETWEEN CONTROL AND INFECTED HORSES AT BASELINE (NO INCUBATION)



Appendix J: Differences in total percentage of CD4+ cells after no incubation with PMA/I between infected and control horses. Average percentage of CD4+ staining cells in the “live lymphocyte” gate was plotted for infected and control horse populations, at baseline (no incubation). Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

APPENDIX K: DIFFERENCES IN TOTAL PERCENTAGE OF CD4+ LYMPHOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 48 HRS INCUBATION WITH PMA/I

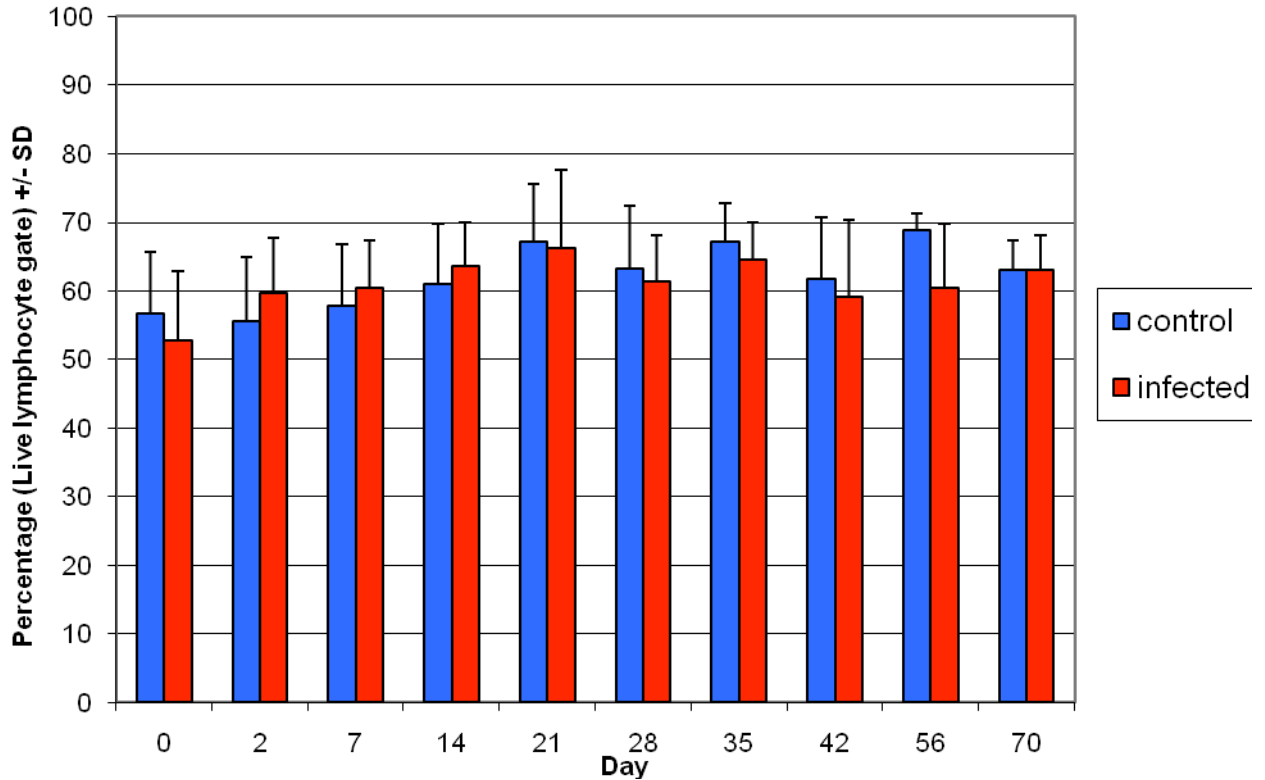
Total Percentage of CD4+ cells after 48 hrs incubation



Appendix K: Differences in total percentage of CD4+ cells after 48 hours incubation with PMA/I between infected and control horses. Average percentage of CD4+ staining cells in the “live lymphocyte” gate was plotted for infected and control horse populations, after 48 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

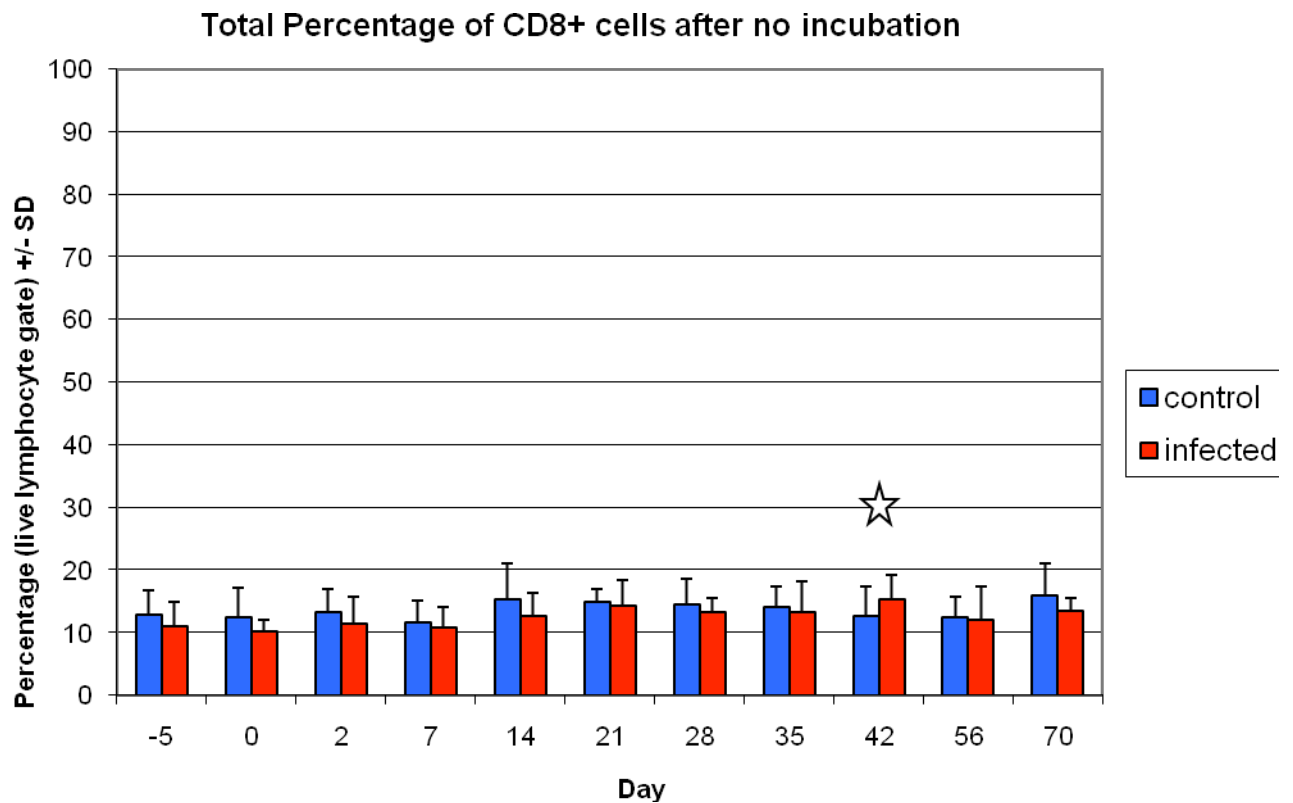
APPENDIX L: DIFFERENCES IN TOTAL PERCENTAGE OF CD4+ LYMPHOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 72 HRS INCUBATION WITH PMA/I

Total Percentage of CD4+ cells after 72 hrs incubation



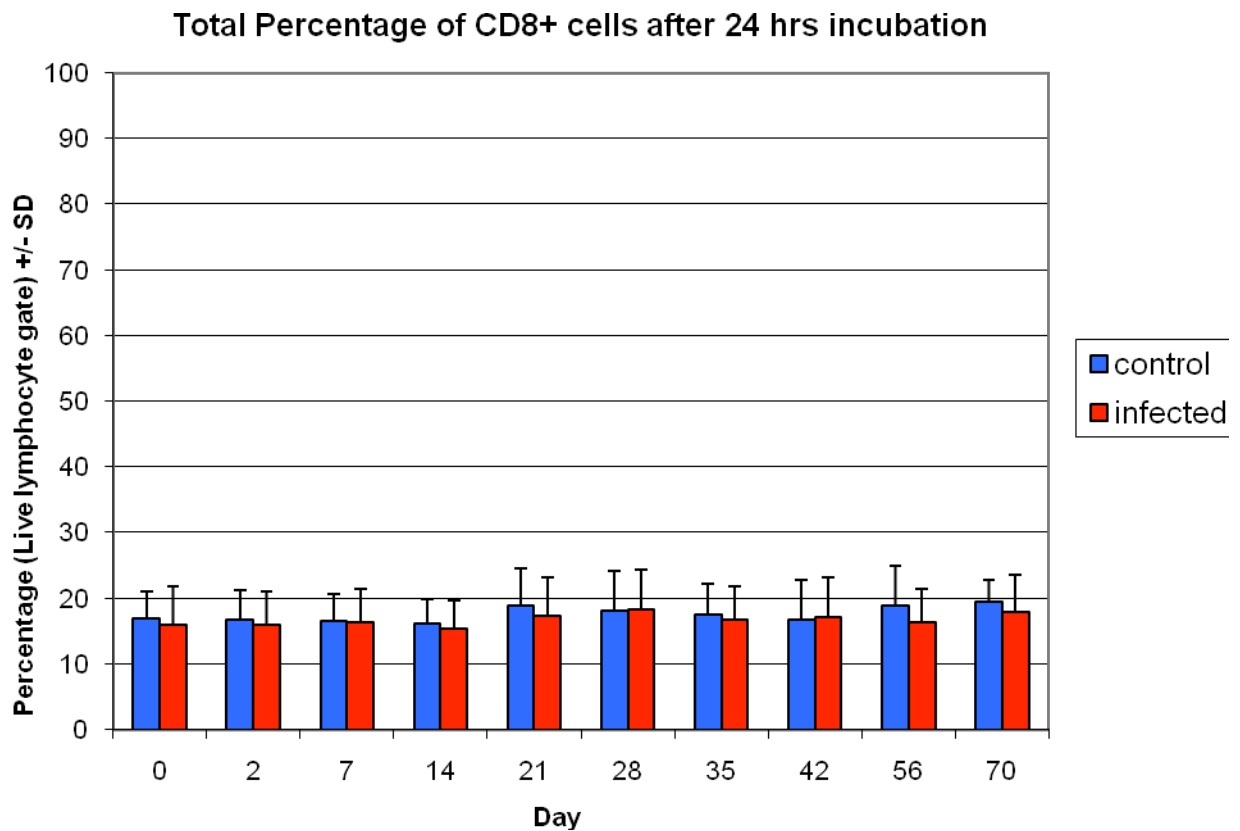
Appendix L: Differences in total percentage of CD4+ cells after 72 hours incubation with PMA/I between infected and control horses. Average percentage of CD4+ staining cells in the “live lymphocyte” gate was plotted for infected and control horse populations, after 72 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

APPENDIX M: DIFFERENCES IN TOTAL PERCENTAGE OF CD8+ LYMPHOCYTES BETWEEN CONTROL AND INFECTED HORSES AT BASELINE (NO INCUBATION)



Appendix M: Differences in total percentage of CD8+ cells after no incubation with PMA/I between infected and control horses. Average percentage of CD8+ staining cells in the “live lymphocyte” gate was plotted for infected and control horse populations, after no incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Significant results ($p < 0.05$) are indicated by a star (☆). Statistical analysis was performed on all of the results for cellular percentages, using a multivariate ANOVA test.

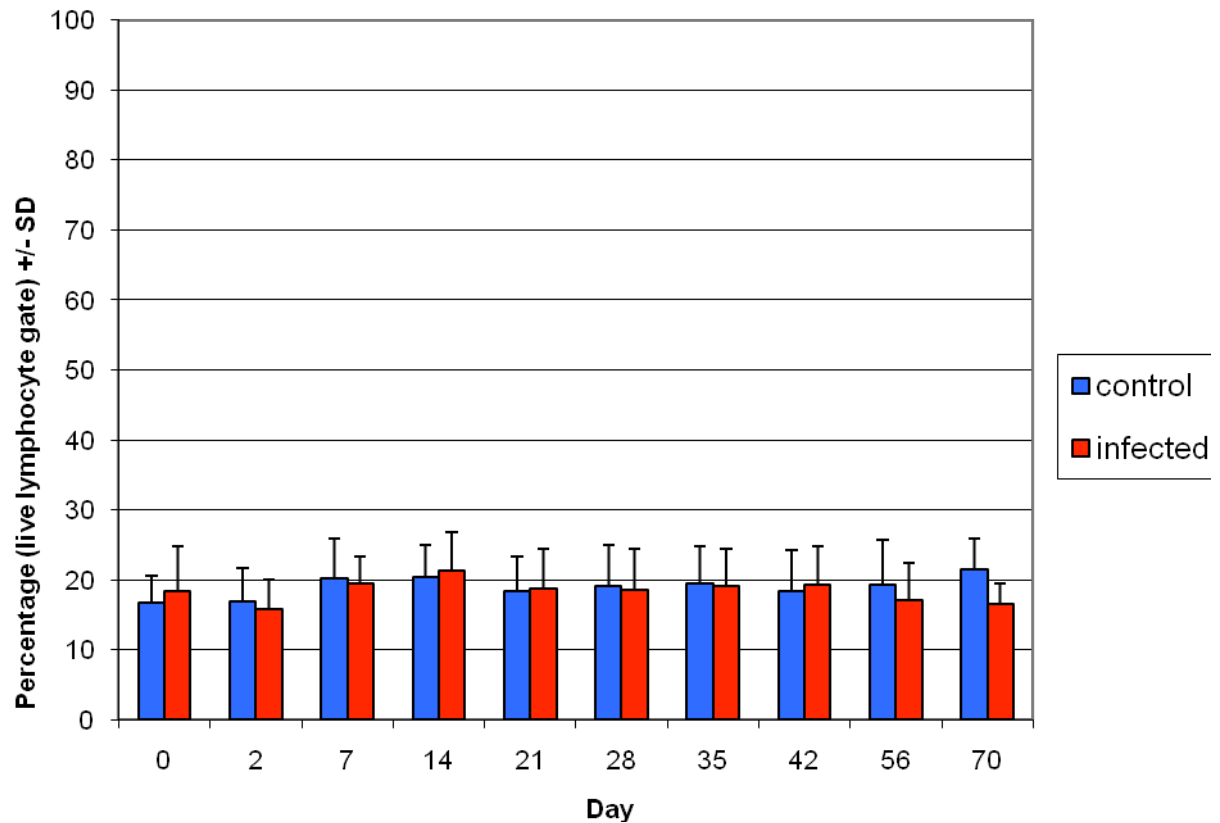
APPENDIX N: DIFFERENCES IN TOTAL PERCENTAGE OF CD8+ LYMPHOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 24 HRS INCUBATION WITH PMA/I



Appendix N: Differences in total percentage of CD8+ cells after 24 hours incubation with PMA/I between infected and control horses. Average percentage of CD8+ staining cells in the “live lymphocyte” gate was plotted for infected and control horse populations, after 24 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

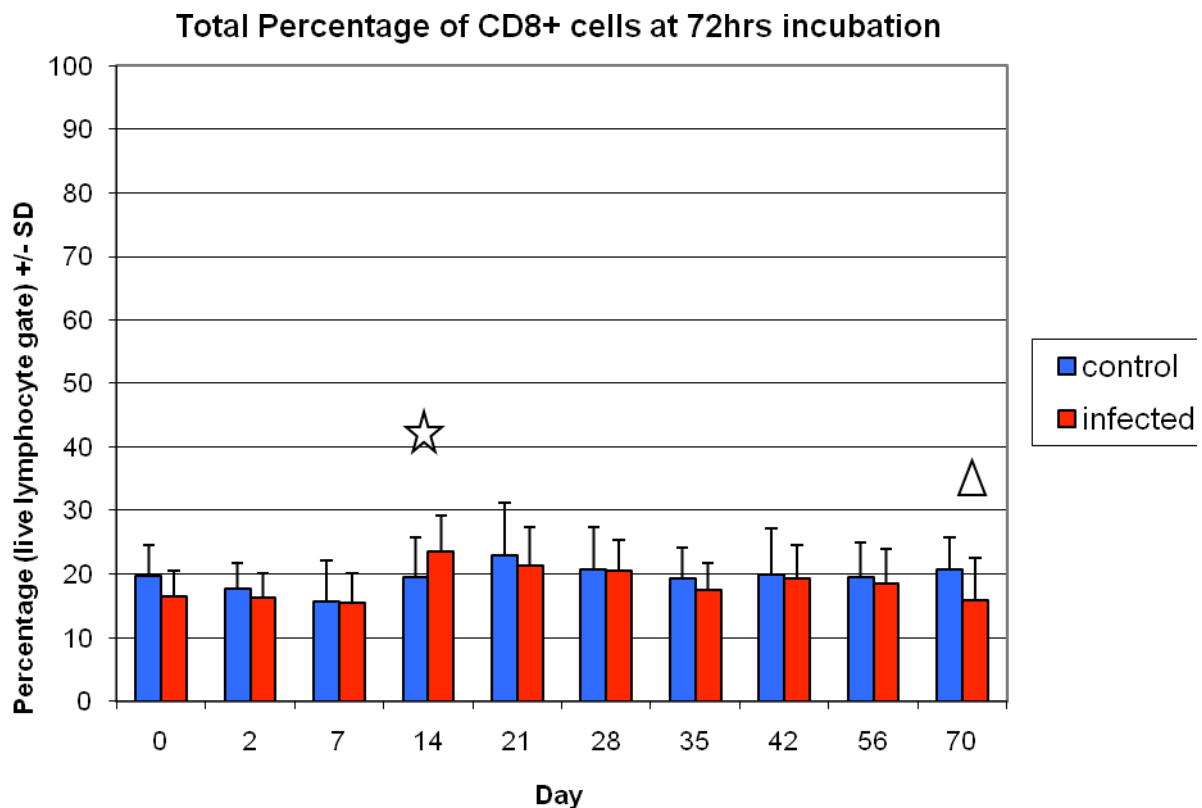
APPENDIX O: DIFFERENCES IN TOTAL PERCENTAGE OF CD8+ LYMPHOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 48 HRS INCUBATION WITH PMA/I

Total Percentage of CD8+ cells after 48 hrs incubation



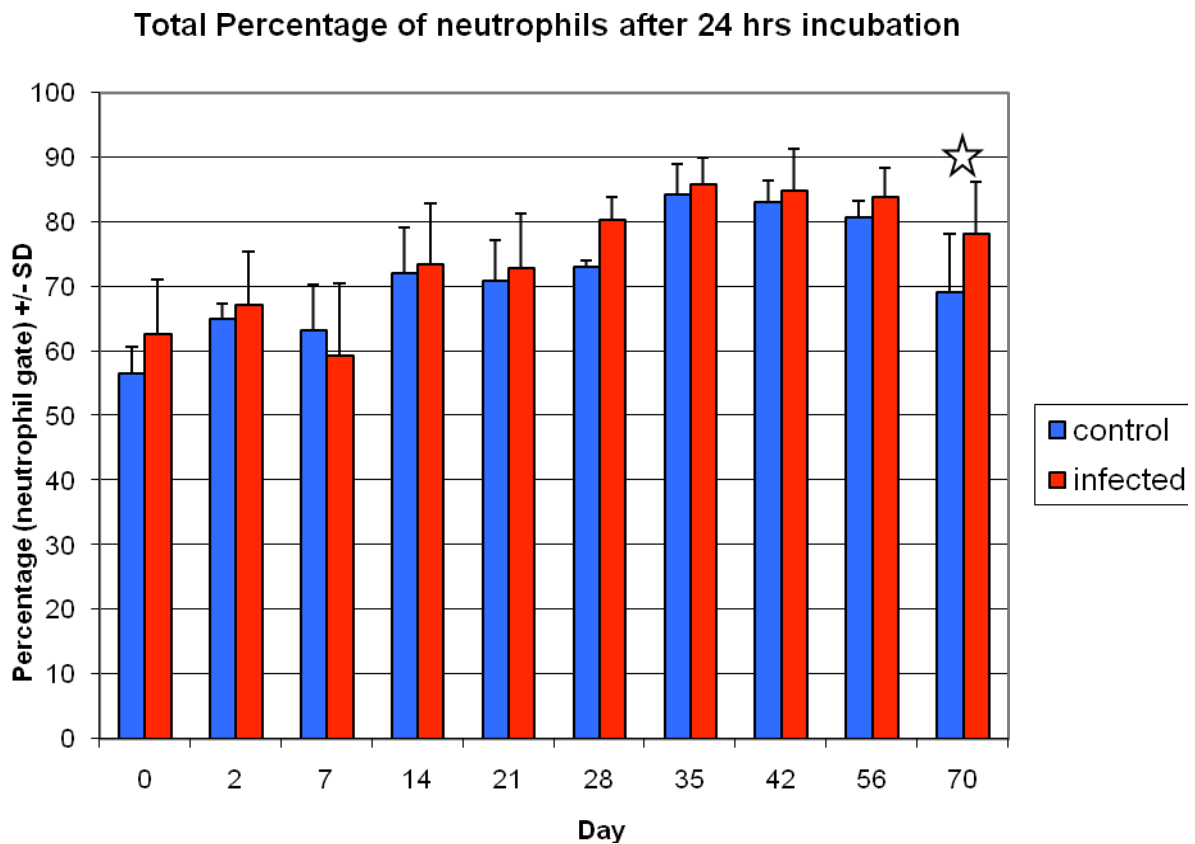
Appendix O: Differences in total percentage of CD8+ cells after 48 hours incubation with PMA/I between infected and control horses. Average percentage of CD8+ staining cells in the “live lymphocyte” gate was plotted for infected and control horse populations, after 48 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Statistical analysis was performed using a multivariate ANOVA.

APPENDIX P: DIFFERENCES IN TOTAL PERCENTAGE OF CD8+ LYMPHOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 72 HRS INCUBATION WITH PMA/I



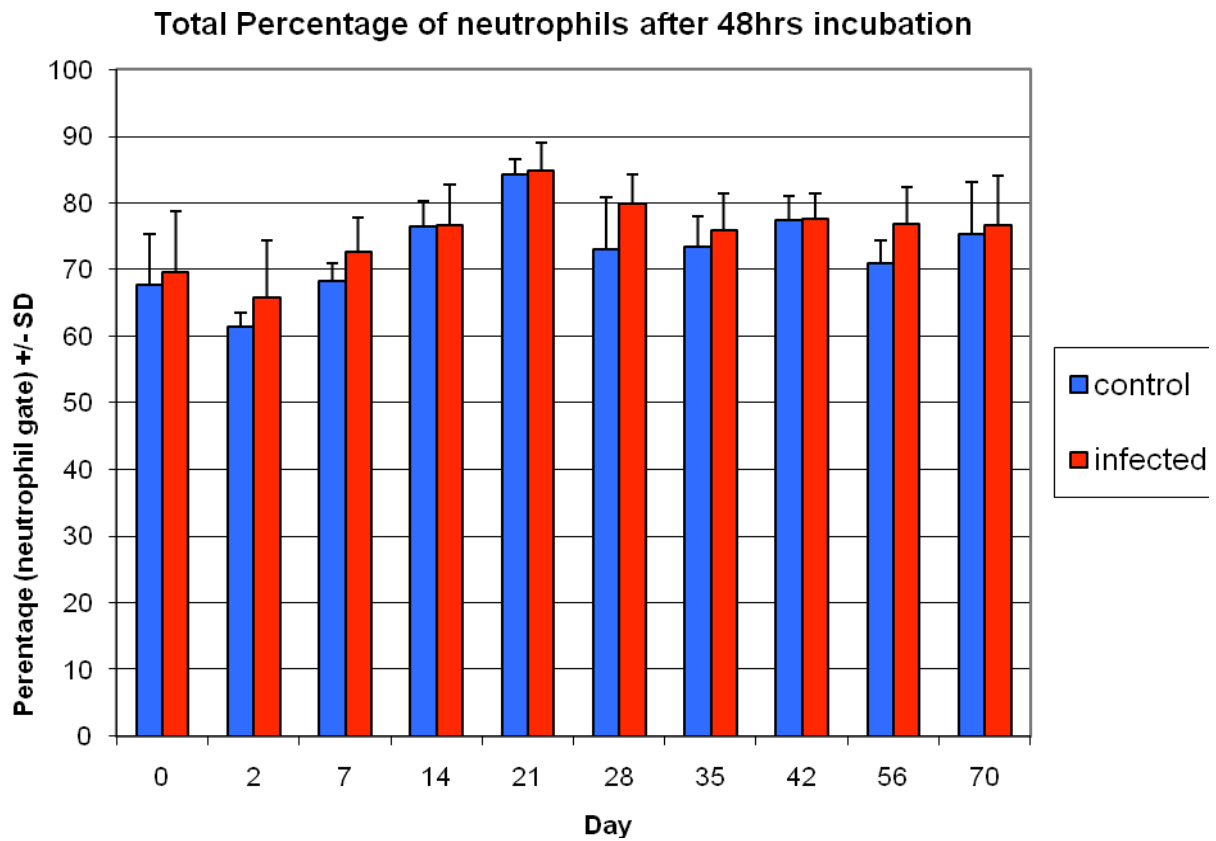
Appendix P: Differences in total percentage of CD8+ cells after 72 hours incubation with PMA/I between infected and control horses. Average percentage of CD8+ staining cells in the “live lymphocyte” gate was plotted for infected and control horse populations, after 72 hours incubation. Day (x axis) = days since infection began at Day 0. Error bars represent one standard deviation about the mean. Significant results ($p < 0.05$) are indicated by a star (☆). Results demonstrating a trend towards significance ($0.05 \leq p < 0.1$) are indicated by a triangle (△). Statistical analysis was performed using a multivariate ANOVA.

APPENDIX Q: DIFFERENCES IN TOTAL PERCENTAGE OF NEUTROPHILS BETWEEN CONTROL AND INFECTED HORSES AFTER 24 HRS INCUBATION WITH PMA/I



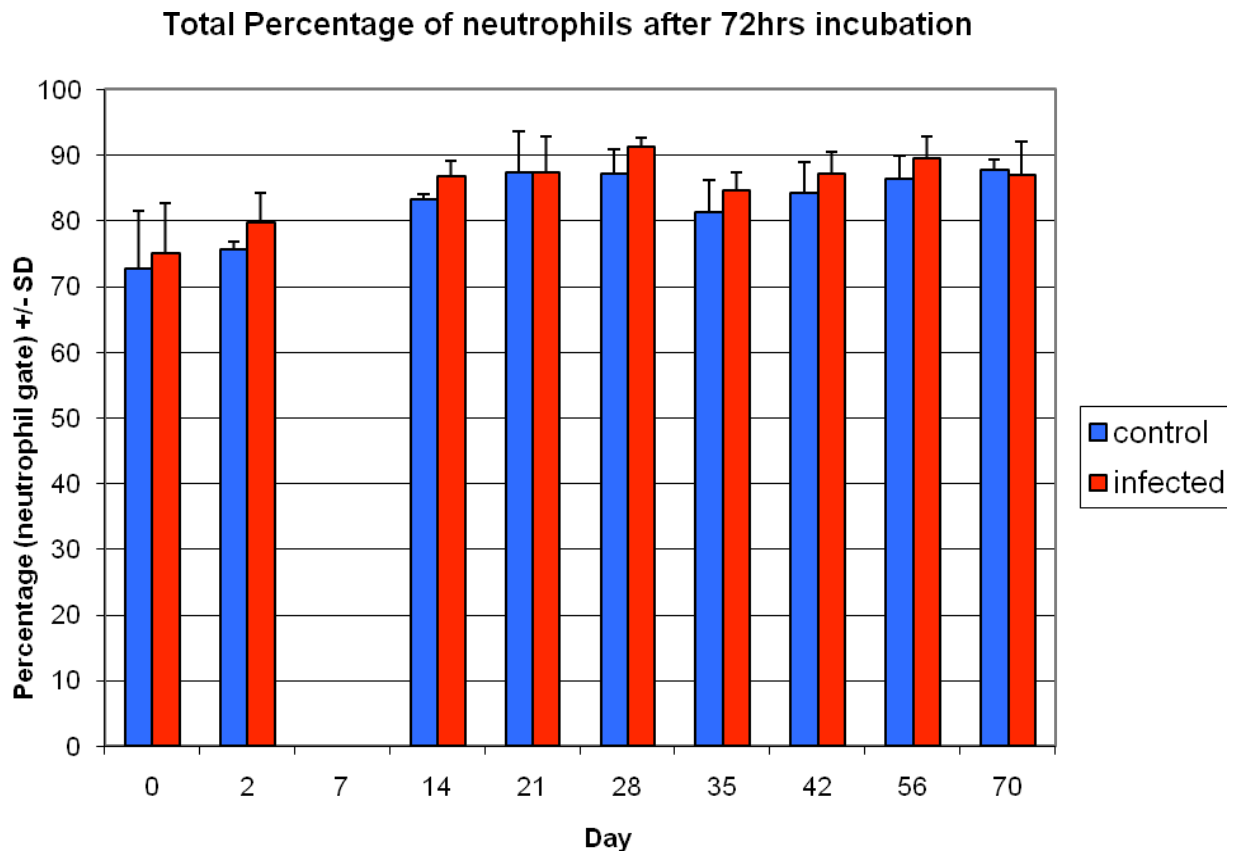
Appendix Q: Differences in total percentage of neutrophils after 24 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “granulocyte” gate was plotted for infected and control horse populations, after 24 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Significant results ($p < 0.05$) are indicated by a star (☆).

APPENDIX R: DIFFERENCES IN TOTAL PERCENTAGE OF NEUTROPHILS BETWEEN CONTROL AND INFECTED HORSES AFTER 48 HRS INCUBATION WITH PMA/I



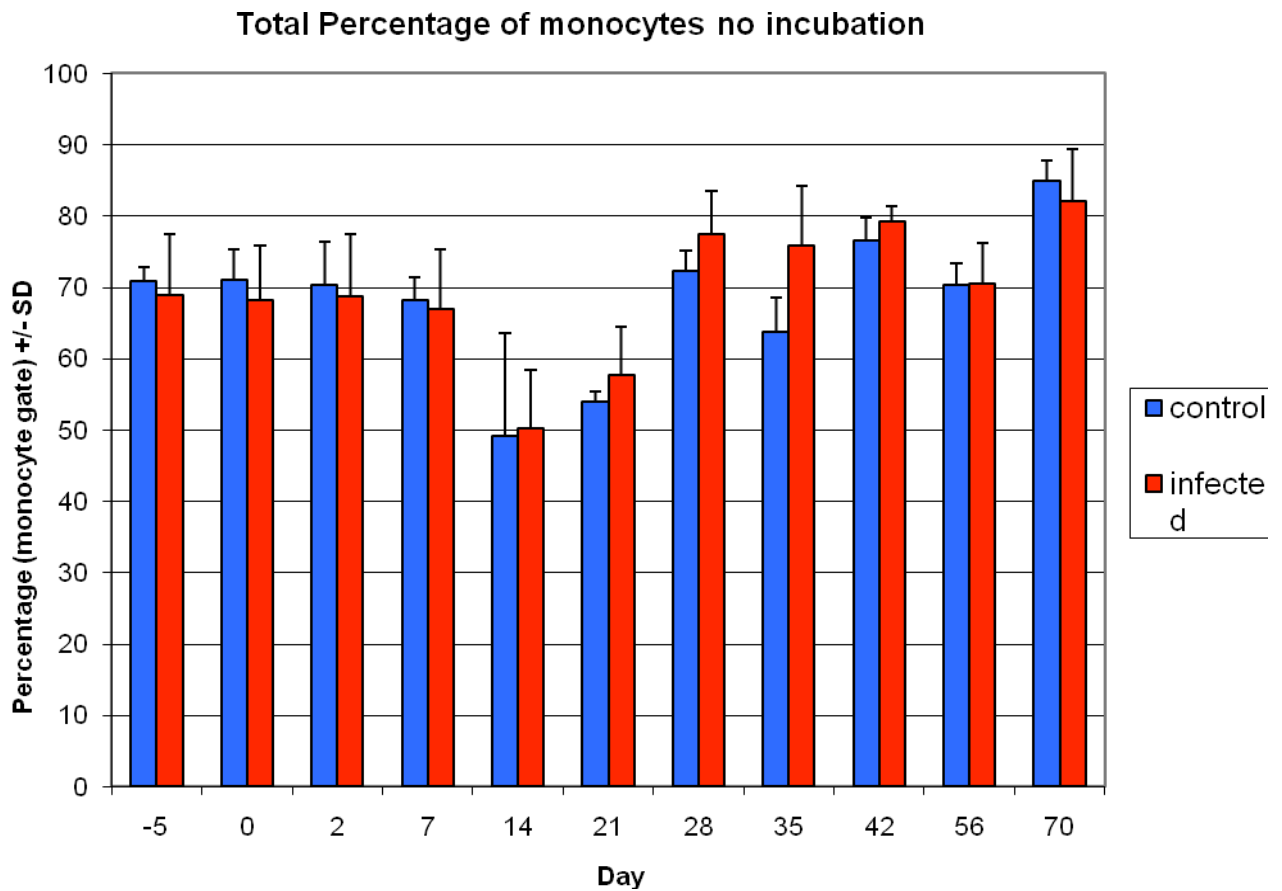
Appendix R: Differences in total percentage of neutrophils after 48 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “granulocyte” gate was plotted for infected and control horse populations, after 48 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

APPENDIX S: DIFFERENCES IN TOTAL PERCENTAGE OF NEUTROPHILS BETWEEN CONTROL AND INFECTED HORSES AFTER 72 HRS INCUBATION WITH PMA/I



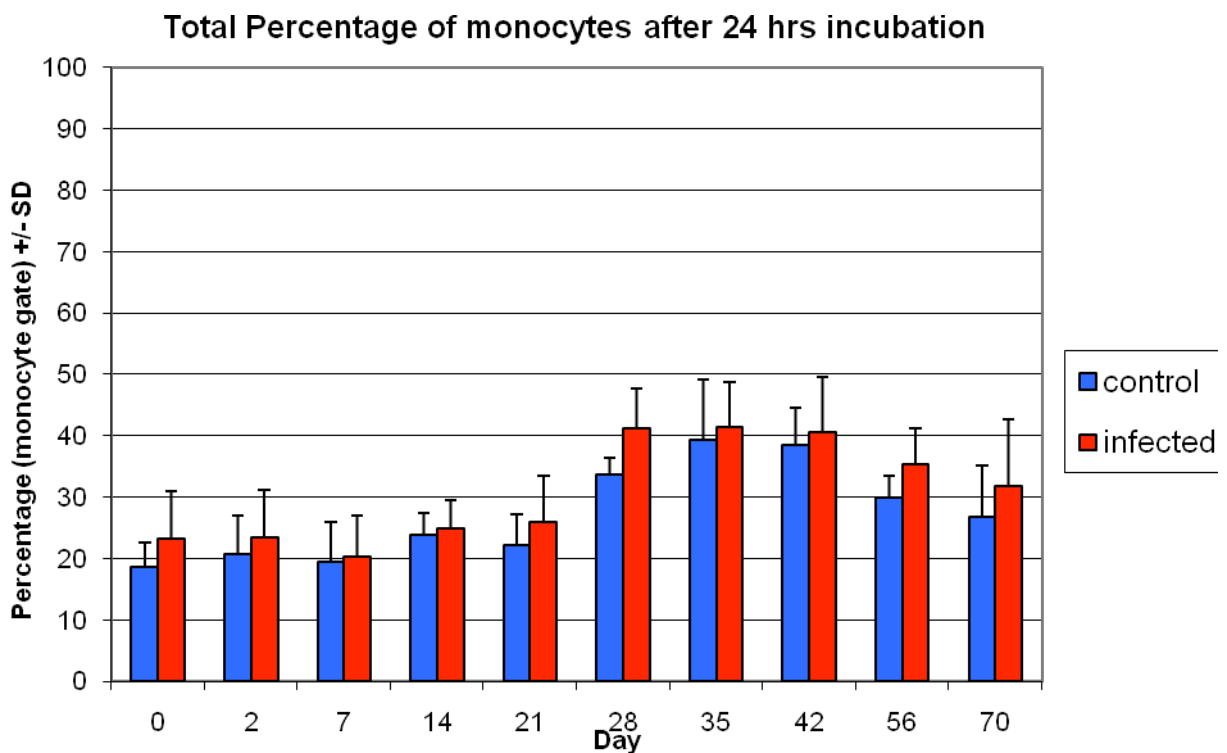
Appendix S: Differences in total percentage of neutrophils after 72 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “granulocyte” gate was plotted for infected and control horse populations, after 72 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Not enough neutrophils survived to analysis at Day 7, hence this day is blank.

APPENDIX T: DIFFERENCES IN TOTAL PERCENTAGE OF MONOCYTES BETWEEN CONTROL AND INFECTED HORSES AT BASELINE



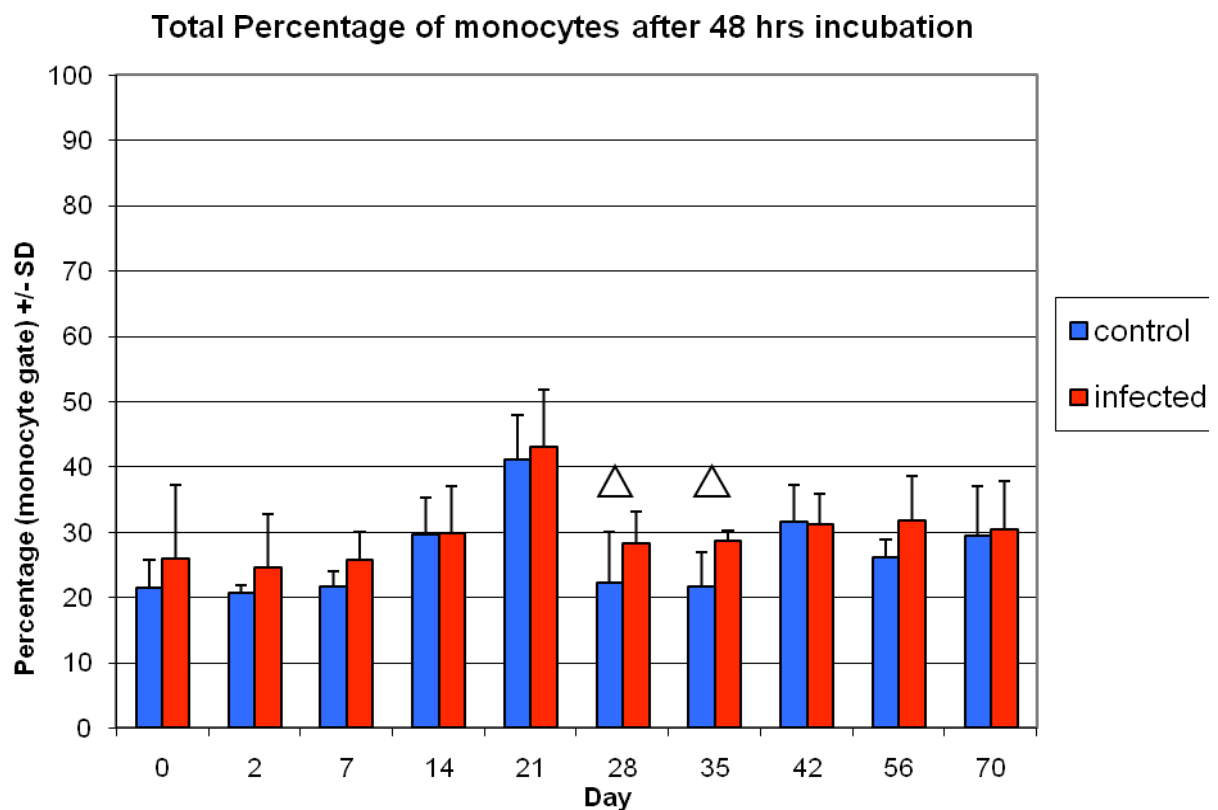
Appendix T: Differences in total percentage of monocytes after no incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “monocyte” gate was plotted for infected and control horse populations, after no incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

APPENDIX U: DIFFERENCES IN TOTAL PERCENTAGE OF MONOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 24 HRS INCUBATION WITH PMA/I



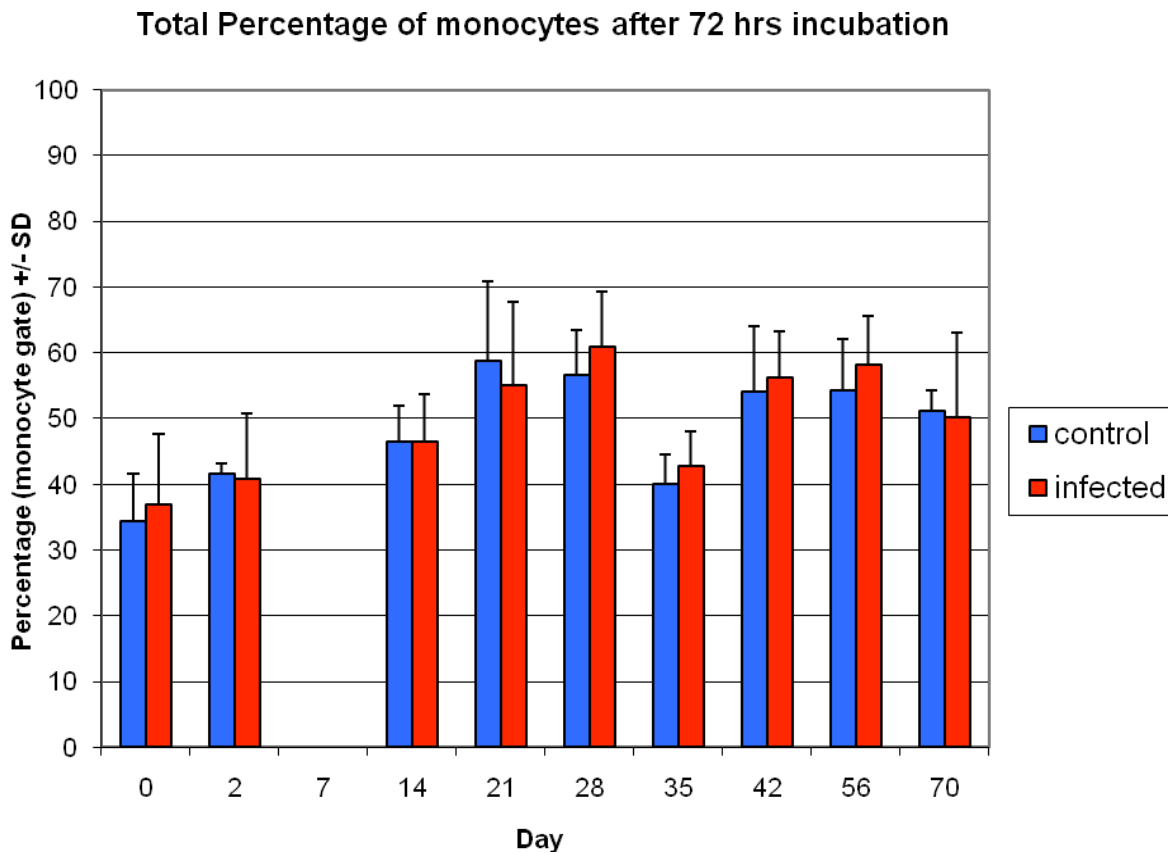
Appendix U: Differences in total percentage of monocytes after 24 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “monocyte” gate was plotted for infected and control horse populations, after 24 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

APPENDIX V: DIFFERENCES IN TOTAL PERCENTAGE OF MONOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 48 HRS INCUBATION WITH PMA/I



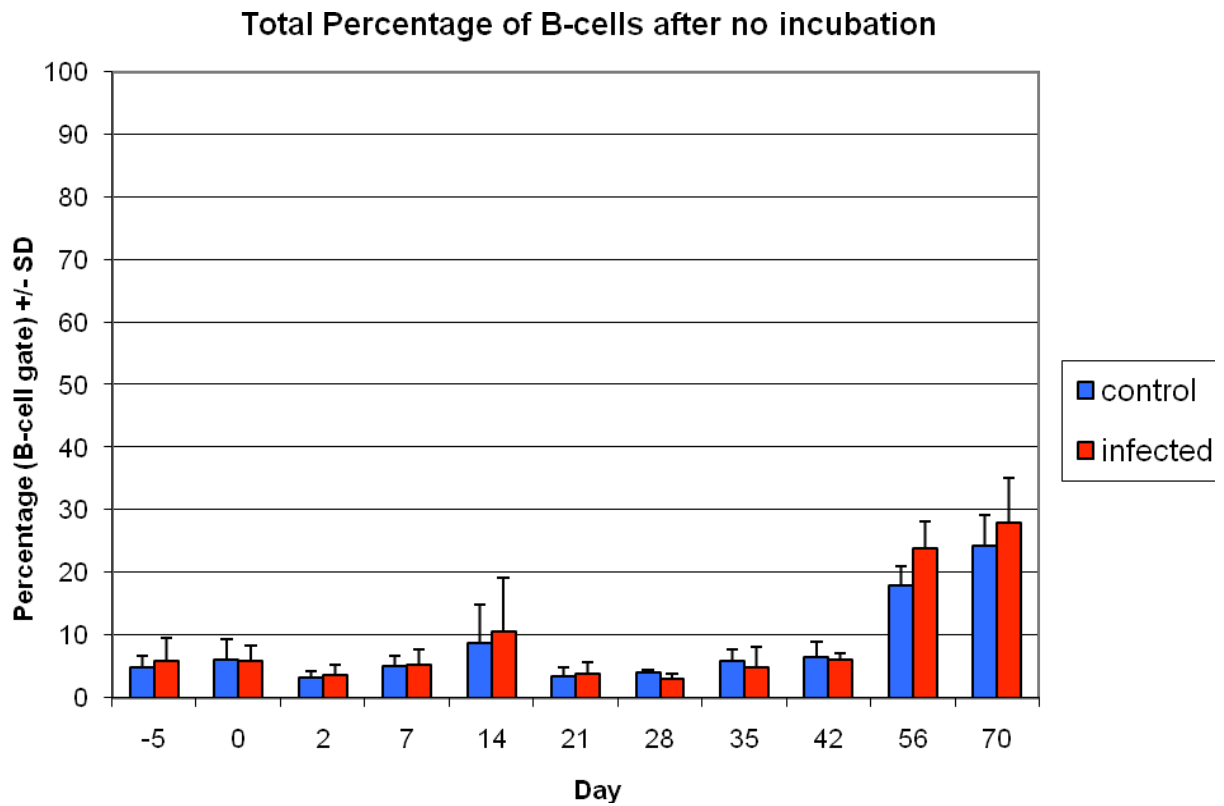
Appendix V: Differences in total percentage of monocytes after 48 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “monocyte” gate was plotted for infected and control horse populations, after 48 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Results demonstrating a trend towards significance ($0.05 \leq p < 0.1$) are indicated by a triangle (Δ). Statistical analysis was performed using a multivariate ANOVA.

APPENDIX W: DIFFERENCES IN TOTAL PERCENTAGE OF MONOCYTES BETWEEN CONTROL AND INFECTED HORSES AFTER 72 HRS INCUBATION WITH PMA/I



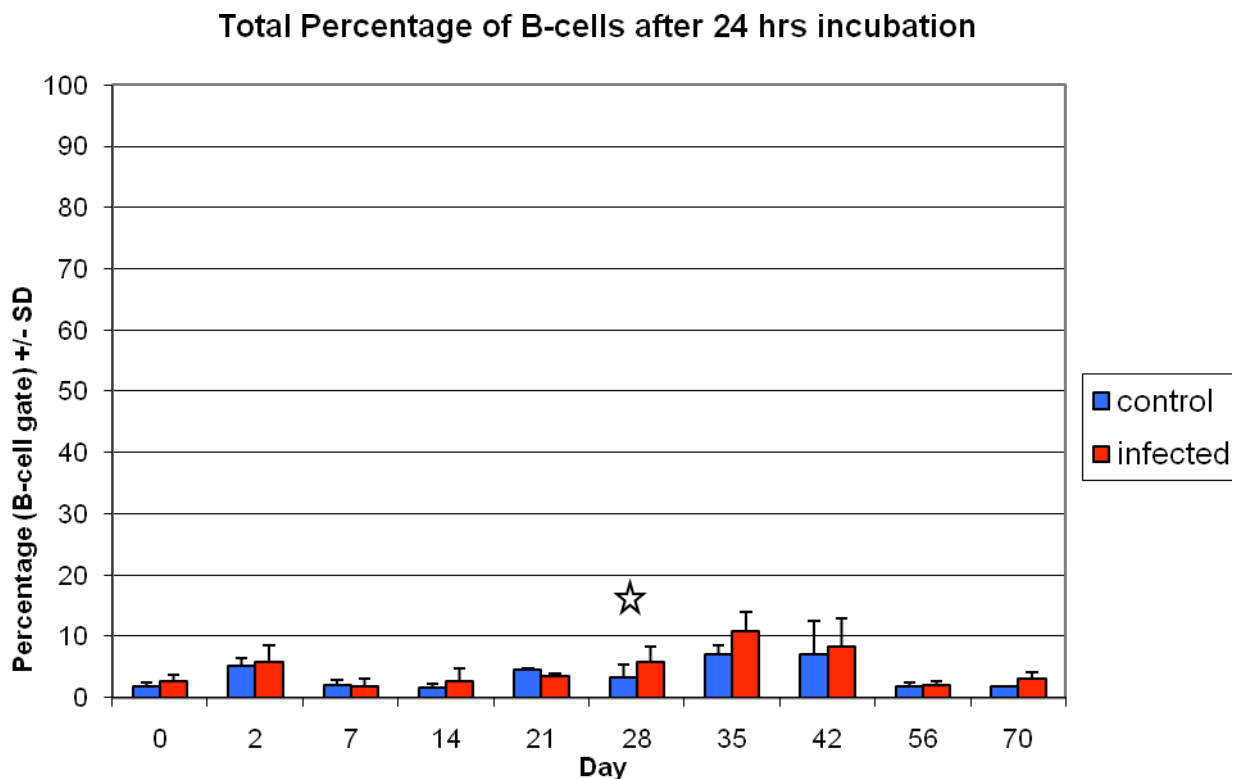
Appendix W: Differences in total percentage of monocytes after 72 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “monocyte” gate was plotted for infected and control horse populations, after 72 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Not enough monocytes survived to analysis at Day 7, hence this day is blank.

APPENDIX X: DIFFERENCES IN TOTAL PERCENTAGE OF B CELLS BETWEEN CONTROL AND INFECTED HORSES AT BASELINE (NO INCUBATION)



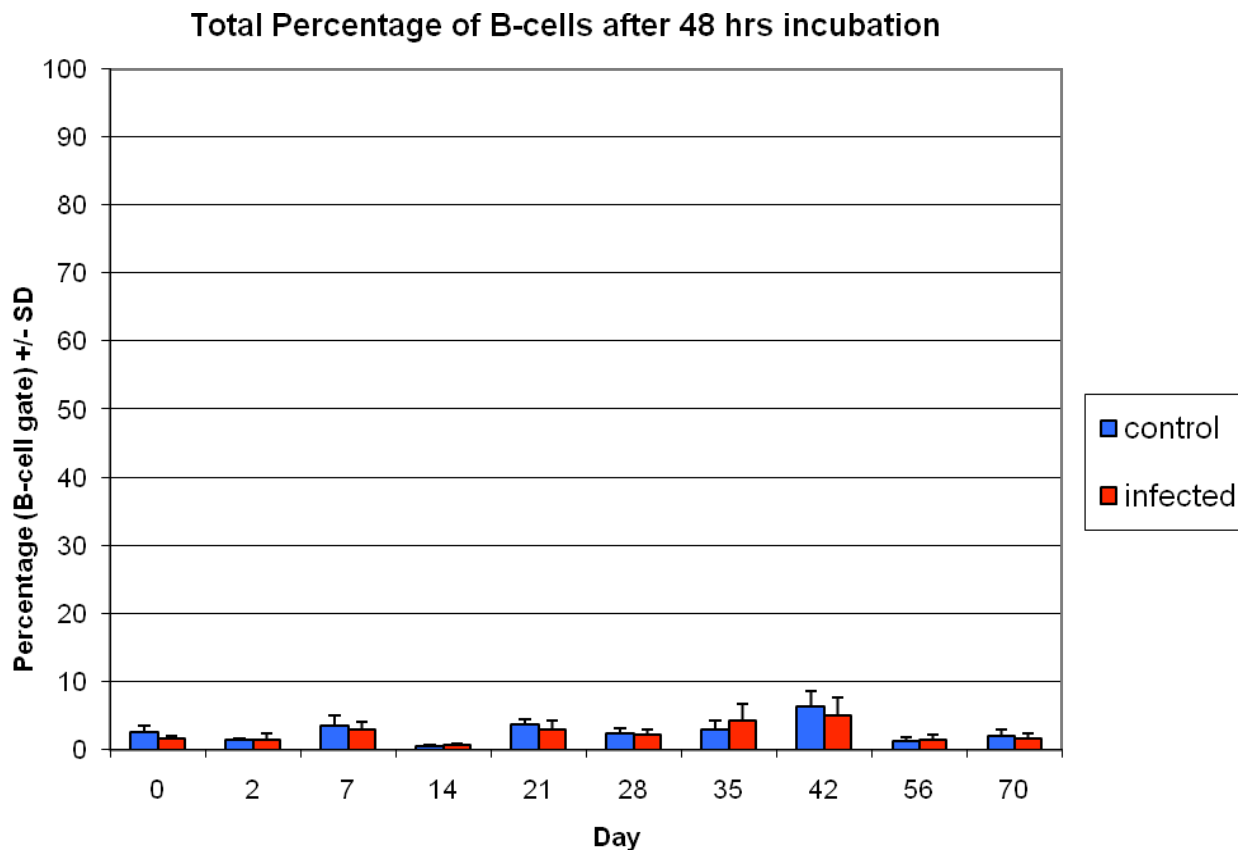
Appendix X: Differences in total percentage of B cells after no incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “B lymphocyte” gate was plotted for infected and control horse populations, after no incubation. Day (x axis) = days since infection began at Day 0. Error bars represent one standard deviation about the mean.

APPENDIX Y: DIFFERENCES IN TOTAL PERCENTAGE OF B CELLS BETWEEN CONTROL AND INFECTED HORSES AFTER 24 HRS INCUBATION WITH PMA/I



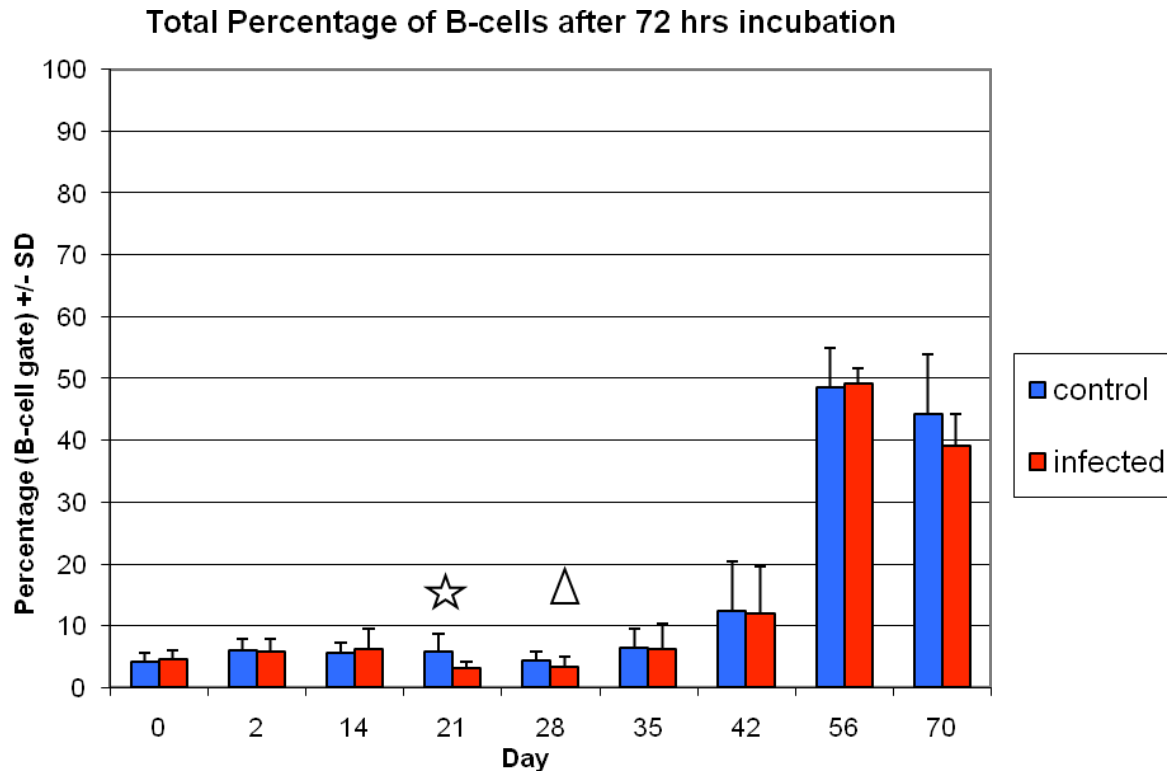
Appendix Y: Differences in total percentage of B cells after 24 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “B lymphocyte” gate was plotted for infected and control horse populations, after 24 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Significant results ($p < 0.05$) are indicated by a star (☆) and were seen at Day 28. Statistical analysis was performed using a multivariate ANOVA.

APPENDIX Z: DIFFERENCES IN TOTAL PERCENTAGE OF B CELLS BETWEEN CONTROL AND INFECTED HORSES AFTER 48 HRS INCUBATION WITH PMA/I



Appendix Z: Differences in total percentage of B cells after 48 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “B lymphocyte” gate was plotted for infected and control horse populations, after 48 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean.

APPENDIX AA: CHANGES IN TOTAL PERCENTAGE OF B CELLS BETWEEN CONTROL AND INFECTED HORSES AFTER 72 HRS INCUBATION WITH PMA/I



Appendix AA: Differences in total percentage of B cells after 72 hours incubation with PMA/I between infected and control horses. Average percentage of positively staining cells in the “B lymphocyte” gate was plotted for infected and control horse populations, after 72 hours incubation. Day (x axis) = days since infection began at Day0. Error bars represent one standard deviation about the mean. Significant results ($p < 0.05$) are indicated by a star (☆) and were seen at Day 21. Trends towards significance are indicated by a triangle (△) and were seen at Day 28. Statistical analysis was performed using a multivariate ANOVA.

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