THE HOST INFECTIVITY
SPECTRUM OF SARCOCYSTIS NEURONA
AND SARCOCYSTIS FALCATULA

By

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To the horses

and

To those who have touched
and shaped my life
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# TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................................................................................. iii

LIST OF TABLES ........................................................................................................................ x

LIST OF FIGURES ...................................................................................................................... xii

ABSTRACT .................................................................................................................................. xiii

CHAPTERS

1 INTRODUCTION ......................................................................................................................... 1

2 ARE SARCOCYSTIS NEURONA AND S. FALCATULA SYNONYMOUS? A HORSE INFECTION CHALLENGE .................................................. 11

   Introduction .......................................................................................................................... 11
   Materials and Methods ........................................................................................................ 12
      Opossum Infection ........................................................................................................... 12
      Horse Infection ............................................................................................................... 13
      Necropsy of Horses ........................................................................................................ 15
      Polymerase Chain Reaction ......................................................................................... 15
      Western Blot Analysis ..................................................................................................... 16
      Viability of Sporocysts .................................................................................................... 17
   Results .................................................................................................................................. 17
      Opossum Infection ........................................................................................................... 17
      Horse Infection ............................................................................................................... 18
      Gross Pathology and Histopathology ........................................................................... 18
      Viability of Sporocysts .................................................................................................... 19
   Discussion ............................................................................................................................. 19

3 EQUINE PROTOZOAL MYELOENCEPHALITIS: NASOGASTRIC ADMINISTRATION OF SARCOCYSTIS NEURONA SPOROCYSTS CAUSES SEROCONVERSION AND DISEASE IN HORSES ................................. 27

   Introduction .......................................................................................................................... 27
   Materials and Methods ........................................................................................................ 28
      Opossum Collection and Sporocyst Recovery .............................................................. 28
      Inoculum Preparation ...................................................................................................... 29
Single-Dose Horse Challenge .................................................................30
Environmental Sentinels .....................................................................31
Multiple-Dose Horse Challenge ............................................................32
Western Blot Analysis ..........................................................................32
Gross and Histopathological Examination ..........................................33
Results .................................................................................................34
Opossum Collection and Inoculum Preparation .....................................34
Single-Dose Horse Challenge ...............................................................35
Western Blot Results ...........................................................................36
Multiple-Dose Horse Challenge ...........................................................36
CSF Analysis .......................................................................................37
Discussion ...........................................................................................38

4 CHALLENGE OF PUTATIVE INTERMEDIATE HOSTS OF S. NEURONA WITH CHARACTERIZED SPOROCYSTS ........................................50

Introduction .........................................................................................50
Materials and Methods .......................................................................52
Results .................................................................................................54
Gross Necropsy and Histopathology: Non-Psittacidae .........................54
Gross Necropsy and Histopathology: Psittacidae .................................55
Gross Necropsy and Histopathology: Mice ..........................................56
Discussion ...........................................................................................57

5 DIDELPHIS VIRGINIANA: DEFINITIVE HOST OF MULTIPLE SARCOCYSTIS SP. ..............................................................74

Introduction .........................................................................................74
Materials and Methods .......................................................................76
Opossum Collection ............................................................................76
Density Purification of Sporocysts .........................................................77
Excystation of Sporocysts ....................................................................78
Polymerase Chain Reaction .................................................................78
Restriction Endonuclease Digestion .......................................................79
Results .................................................................................................80
Opossum Collection ............................................................................80
Categorization of Isolates .....................................................................80
Discussion ...........................................................................................80

6 CONCLUSIONS ................................................................................95

APPENDICES

A STANDARDIZED NEUROLOGIC EXAMINATION FORM ..............104

B NEUROLOGIC EXAMINATION OF THE HORSE .........................105
C ONLINE RESOURCES ................................................................. 108
LITERATURE CITED ........................................................................... 109
BIOGRAPHICAL SKETCH ................................................................. 115
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1. Summary of horse experiment. Horses in the challenge group were administered sporocysts characterized as <em>Sarcocystis falcata</em> in water by nasogastric tube.</td>
<td>24</td>
</tr>
<tr>
<td>3-1. Summary of single-dose horse challenge.</td>
<td>46</td>
</tr>
<tr>
<td>3-2. Summary of multiple-dose horse challenge.</td>
<td>47</td>
</tr>
<tr>
<td>3-3. Summary of western blot tests from laboratory 1 for horses in the single-dose challenge, demonstrating consistency of test results.</td>
<td>47</td>
</tr>
<tr>
<td>3-4. Summary of western blot tests from laboratory 1 and laboratory 2 for horses in the multiple-dose challenge.</td>
<td>47</td>
</tr>
<tr>
<td>3-5. Results of western blot testing of horse #817 from laboratory 1.</td>
<td>48</td>
</tr>
<tr>
<td>4-1. Administration of sporocysts to brown-headed cowbirds, boat-tailed grackles, Bobwhite quail, European starlings and redwing blackbirds.</td>
<td>68</td>
</tr>
<tr>
<td>4-2. Administration of sporocysts to budgerigars and summary of results.</td>
<td>69</td>
</tr>
<tr>
<td>4-3. Administration of sporocysts to NIH Swiss mice.</td>
<td>69</td>
</tr>
<tr>
<td>4-4. Results of histopathological examination of budgerigars.</td>
<td>70</td>
</tr>
<tr>
<td>5-1. Number of opossums collected and shedding by month for 1997, 1998 and combined.</td>
<td>91</td>
</tr>
<tr>
<td>5-2. Number of opossums collected and shedding by county in Florida for 1997 and 1998.</td>
<td>92</td>
</tr>
<tr>
<td>5-3. Numbers of isolates by <em>Sarcocystis</em> sp. or type with descriptive statistics.</td>
<td>92</td>
</tr>
<tr>
<td>5-4. Numbers of opossums shedding sporocysts by weight (where weight available), as a percentage of all collected opossums of that weight, for 1997 and 1998.</td>
<td>92</td>
</tr>
</tbody>
</table>
5-5. Numbers of opossums shedding sporocysts by weight and gender (where weight and gender available), as a percentage of all collected opossums per category, for 1997 and 1998.................................................................93

5-6. Numbers of opossums shedding sporocysts by gender (where gender available), as a percentage of all collected opossums of that gender for 1997 and 1998. ..........93

B-1. Grades of neurologic disease.........................................................107
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1.</td>
<td>Photograph of the Virginia opossum (<em>Didelphis virginiana</em>)</td>
<td>9</td>
</tr>
<tr>
<td>1-2.</td>
<td>Schematic of the life cycle of <em>Sarcocystis neurona</em> showing current state of knowledge</td>
<td>10</td>
</tr>
<tr>
<td>2-1.</td>
<td>Schematic drawing of horse pasture, showing location of some isolation stalls</td>
<td>25</td>
</tr>
<tr>
<td>2-2.</td>
<td>Photograph of the pasture fence, showing adaptations to prevent ingress of wildlife</td>
<td>26</td>
</tr>
<tr>
<td>3-1.</td>
<td>Densitometric comparison of 17 kD protein on western blot of CSF of challenged horses compared to reference standard. Each horse was challenged on day 0.</td>
<td>49</td>
</tr>
<tr>
<td>4-1.</td>
<td>Photograph of brown-headed cowbirds (<em>Molothrus ater</em>)</td>
<td>71</td>
</tr>
<tr>
<td>4-2.</td>
<td>Photograph of a boat-tailed grackle (<em>Cassidix mexicanus</em>)</td>
<td>72</td>
</tr>
<tr>
<td>4-3.</td>
<td>Photograph of European starling (<em>Sturnus vulgaris</em>)</td>
<td>72</td>
</tr>
<tr>
<td>4-4.</td>
<td>Photograph of redwing blackbird (<em>Agelaius phoeniceus</em>)</td>
<td>72</td>
</tr>
<tr>
<td>4-5.</td>
<td>Photograph of Bobwhite quail (<em>Colinus virginianus</em>)</td>
<td>73</td>
</tr>
<tr>
<td>4-6.</td>
<td>Photograph of budgerigars (<em>Melopsittacus undulatus</em>)</td>
<td>73</td>
</tr>
<tr>
<td>5-1.</td>
<td>Poster used to inform the public and horse farm personnel about opossum collection program</td>
<td>94</td>
</tr>
</tbody>
</table>
Equine protozoal myeloencephalitis has been called the most important unsolved infectious disease of horses. Its rise to this position has taken just 35 years and has stunned the equine industries of the Americas. Seroprevalence has dramatically increased to more than 50% but disease remains less common (<1%). Several milestones have passed in investigating the disease during its emergence. The suggestion of synonymy between S. neurona and S. falcatula has been disproved but caused increased scrutiny of both parasites and development of different animal models. Our primary aim was to clarify the biology of S. neurona and, in consequence, understand its relationships with other opossum-borne Sarcocystis sp. Over 400 opossums were collected and screened for sporocyst shedding to obtain isolates for host challenge. Overall, 20% (87/419) of
opossums were infected, but prevalence was highest in the 2nd quarter and lowest in the 3rd and 4th quarters. Using published molecular markers, isolates were classified into 4 types by DNA sequence. Together with published data, at least 3 are individual species. Sixteen isolates were \textit{S. neurona}, 13 \textit{S. falcatula}, 6 Type-1085, 3 Type-2079 and 40 remain uncategorized. Type-2079 isolates were shed with highest intensity. Type-1085 isolates were almost all collected in South Florida and were also shed with high intensity. \textit{Sarcocystis neurona} isolates were frequently from urban areas and were intermediate in intensity while \textit{S. falcatula} was typically shed in low numbers. Horses and potential intermediate hosts were challenged with different isolates. All \textit{S. neurona}-challenged horses seroconverted but only high doses (6 daily doses of $5 \times 10^5$ sporocyst each) caused unequivocal neurologic disease (\textbf{Grade II or worse}). Weakness was more pronounced than ataxia. The protozoan long known as \textit{S. falcatula} has a benign sylvatic cycle, kills psittaciforms at low doses but is harmless to horses. Type-1085, previously-undescribed, behaves similarly to \textit{S. falcatula} but has distinct DNA sequences. Type-2079 caused equivocal or no disease and remains least understood. The complexity of opossum-borne \textit{Sarcocystis} biology, and the diversity of hosts that may be infected, is greater than previously suggested. This horse model of EPM will be vital for pharmaceutical evaluation and understanding pathogenesis of the natural disease, but requires further development. In our opinion, it is the greatest contribution of this work. Some potential hosts for the \textit{Sarcocystis} isolates identified have been eliminated, but now 3 distinct types have unknown intermediate hosts. The descriptive statistics should contribute to solving the natural biology and sylvatic cycles of opossum-borne \textit{Sarcocystis} sp.
CHAPTER 1
INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is a potentially debilitating disease of horses in the Americas (MacKay, 1997a). The disease has been recognized in the USA since the 1960s, but may have been recognized since the 1950s in South America (Macruz et al., 1975). Equine protozoal myeloencephalitis has now been reported in Canada, Central and South America (Clark et al., 1981; De Barros et al., 1986; Granstrom et al., 1992; MacKay et al., 1992). When first recognized, the disease was called Segmental Myelitis or Focal Myelitis because lesions were found in multiple sections of the spinal cord with clinical signs referable to the affected spinal cord segment (Rooney et al., 1970). The agent was initially thought to be Toxoplasma gondii, (Beech and Dodd, 1974; Cusick et al., 1974), but was reported to be a Sarcocystis species in 1980 (Simpson and Mayhew). In 1991, Dubey et al. successfully isolated and propagated in cell culture a protozoan parasite from the spinal cord of a neurologic horse which had been diagnosed with equine protozoal myeloencephalitis: the organism was named S. neurona.

Clinical signs of EPM are highly variable among horses. The spectrum covers chronic progressive (usually mild) disease through to devastating peracute presentations. Paradoxically, although horses with chronic disease suffer only quality-of-life problems, they have the poorest chance of recovering to normal because of the duration of the
disease. Presumably, by the time of diagnosis some lesions have progressed from sites of inflammation to sites of cell death and are irreparable. The most common presentation is a mild gait abnormality, such as dragging a limb or failing to advance the limb in normal cadence. Muscle atrophy locally is also common. The subtlety of disease and the broad range of differential diagnoses make identification of the cause problematic, and as a result, most of these cases are diagnosed after a considerable intermission. Clinical signs in horses with acute disease may include recumbency, severe ataxia, paraplegia or tetraplegia, depression or dementia, vestibular disease or self-inflicted injuries. Because such cases show dramatic contrast to the normal behavior of the animal, veterinary attention is sought quickly. Early and aggressive therapy in those cases may have equally rapid results and return the animal to normal.

The differential diagnosis of EPM ultimately includes all causes of neurologic or gait abnormalities of the horse. Differential diagnoses for specific cases will depend on the time since onset, severity of condition and systems involved (e.g. locomotion versus dysphagia). Clearly, to be convinced of the diagnosis in a disease known to vary so much in its clinical signs, additional support would be very helpful. The first specific commercial diagnostic test became available in 1993 in the form of a western blot for *S. neurona* antibody (Granstrom, 1993; Granstrom et al., 1993). Two assumptions were necessary to use the test reliably. First, infected horses with EPM would mount a humoral immune response against the organism. Second, healthy horses in the general population would be unlikely to have produced specific or cross-reactive antibodies against *S. neurona* (or *S. cruzi* in the case of the older FIAX test). The second assumption could be met by having
a short-lived immune response or rare interactions between the horse and the organism. This test was a great advance and very useful in the diagnosis of EPM for several years. Since that time it has become increasingly clear that these two assumptions are no longer valid. *Sarcocystis neurona* infection is both common and widespread in the United States, with seroprevalence of ~50% reported in surveys from Ohio, Oregon, and Pennsylvania (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997). Additionally, it appears that the humoral response is either long-lived or that horses are re-challenged sufficiently frequently to maintain specific or cross-reactive antibodies. Older horses are more likely to be seropositive than young horses (Saville et al., 1997), which is compatible with both assumptions, but it may suggest that the exposure is not dramatically higher now than it was 5 or 10 years ago.

As EPM emerged as a greater threat to the horse industries of the Americas, particularly the USA, a greater effort was mounted to understand the biology of the organism *S. neurona*. These efforts would be essential groundwork for developing the tools and pharmaceuticals necessary to control and eliminate EPM. The monetary implications of this disease are enormous. The horse industries contribute $112 billion to the US economy annually (Anonymous, 1996). It is estimated that therapy of EPM costs $4 million per year in the state of Ohio alone (http://prevmed.vetmed.ohio-state.edu/epm-home.html). The greatest losses are undoubtedly the majority of horses which are mildly affected, but lose athletic ability as a consequence. The uncertain outcome with treatment together with the expense and prolonged course, means that many horses are never treated and may be unable to continue their working life whether as pleasure horse or racehorse.
Despite the frequent occurrence of EPM, the life cycle of the causative agent still was unknown in 1994. Protozoa belonging to the genus *Sarcocystis* are obligatorily heteroxenous and typically host-specific. Isolates from horses affected with EPM were available (Dubey and Miller, 1986; Davis et al., 1991a, Davis et al., 1991b, Bowman et al., 1992, Granstrom, 1992) and provided a ready source of DNA. In 1995, Fenger et al. demonstrated 99.8% concordance between the DNA sequences of 18S rRNA genes amplified from sporocysts shed by opossums (*Didelphis virginiana*) (Figure 1.1) and *S. neurona* (isolate SN5) merozoites. This was the first piece of evidence to solve the life cycle (Figure 1.2), and made a subsequent breakthrough in the intermediate host a real possibility.

Others in my laboratory hypothesized that *S. neurona* might be a previously known species. Over 200 belong to the genus *Sarcocystis* but less than a third of those have an identified definitive host (Levine and Tadros, 1980; Levine, 1986). The intermediate host is more often known because sarcocysts are identified during routine necropsy or at other examinations. Thus it was suspected that *S. neurona* had been described in its natural intermediate, and possibly definitive, host. Two known *Sarcocystis* sp. use the opossum as definitive host: *S. falcatula*, an organism that may use either brown headed cowbirds (*Molothrus ater*) or boat-tailed grackles (*Cassidix mexicanus*) as intermediate hosts and *S. rileyi* that typically cycles between the striped skunk (*Mephitis mephitis*, definitive host) and any one of numerous species of Anatidae including the pintail (*Anas acuta*), green-winged teal (*A. carolinensis*) and blue-winged teal (*A. discors*). *Sarcocystis falcatula* is unusual in that it can experimentally infect a variety of intermediate hosts spanning 3 orders of birds, whereas most *Sarcocystis* sp. use only a single species as definitive or
intermediate host. That biological behavior made *S. falcatula* an even more attractive candidate to be the agent of EPM. The host spectrum of *S. rileyi* is not as well understood but may be almost equally promiscuous: in addition to the skunk, the opossum (*Didelphis virginiana*, Duszynski and Box, 1978), the dog (*Canis familiaris*), and domestic cat (*Felis catus*) (Golubkov, 1979) have been reported to act as definitive hosts, although the latter 2 are in some question (Levine and Tadros, 1980; Levine, 1986). *Sarcocystis falcatula* was selected for initial evaluation because of its greater host range and the presence of large numbers of opossums and brown-headed cowbirds in Florida (Bull and Farrand, 1977). In contrast, striped skunks are relatively uncommon in Florida.

Generic primers were used to amplify a 742-bp segment of the 18S rRNA gene from *S. falcatula* sarcocysts recovered from naturally infected brown-headed cowbirds. There was >99.5% homogeneity between that sequence and the published *S. neurona* (SN5 isolate) sequence (Dame et al., 1995). In fact, the *S. falcatula* sequence was identical both to that reported by Fenger et al. (1995) from sporocysts isolated from 2 opossums and to another isolate of *S. neurona* (UCD-1) (Marsh et al., 1996a). On the basis of these findings, it was suggested that the 2 species (*S. neurona* and *S. falcatula*) were synonymous (Dame et al., 1995).

Box and Duszynski (1978) have demonstrated that *S. falcatula* can complete its life cycle not only in its natural intermediate hosts, but also in other birds including English sparrows (*Passer domesticus*), canaries (*Serinus canarius*) and pigeons (*Columba livia*). In stark contrast to other birds, psittacines are highly susceptible to infection by *S. falcatula*. The common budgerigar (*Melopsittacus undulatus*, Australian grass parakeet) can develop a fatal pneumonitis after ingesting as few as 25 sporocysts per gram
bodyweight (Smith et al., 1989). Higher doses result in earlier death: 3,000 sporocysts per gram bodyweight is fatal in 8 days. Other Psittaciforms are also susceptible to the organism (Clubb and Frenkel, 1992, Hillyer et al., 1991). Overall the ability of this protozoan to infect many different hosts is unusual within the genus *Sarcocystis* (Tadros and Laarman, 1982). Efforts to infect rats, cats, a dog and a ferret (as definitive hosts) were unsuccessful (Tadros and Laarman, 1982).

On the basis of these collective findings, the next logical step was to challenge naïve horses with *S. falcatula*. This experiment is described in chapter two. After two rounds of challenge of the subject horses without seroconversion or disease, further probing of the genomes revealed that, despite virtually identical 18S rRNA, *S. neurona* and *S. falcatula* were distinct species.

Once the hypothesis of synonymy was rejected, further development of a horse model reached an impasse. *Sarcocystis neurona* sporocysts could not be generated as a single population in the laboratory because no intermediate host had been identified and therefore the sarcocyst stage of the parasite was not available for challenge of naïve opossums. The only alternative was wild-acquired opossums that were naturally-infected with *S. neurona*. The molecular tools developed to differentiate *S. neurona* from *S. falcatula* could be used to characterize isolates, and if a small percentage of *S. falcatula* were present it would be unlikely to confound results, given the results in chapter two.

An essential first step to further investigation of any host was recovery of *Sarcocystis* sp. sporocysts. Several methods have been described to recover sporocysts from the gastrointestinal epithelium of the opossum. A common step is the identification of sporocyst shedding by examining slides prepared from material floated in Sheather’s
solution. In one method (Box and Duszynski, 1978), infected gut is then stripped of fecal material, washed with saline and the mucosa scraped from the intestinal wall into a beaker. Scrapings are diluted in 10 volumes of digestive fluid (pepsin 0.65% w/v, NaCl 0.86% w/v, HCl 1%, v/v in water) and stirred for 60 minutes at 38 C. The mixture is centrifuged and washed twice in water. Sediment is resuspended in 10 volumes of 0.4% (w/v) trypsin 1-300 in Ringer’s solution, pH 7.4 - 7.8, and digested for a further 60 – 90 minutes at 38 C. The final material is stored at 4 C in several times its volume of antibiotic solution (penicillin 100 U/mL, streptomycin 100 µg/mL, mycostatin 500 U/mL in sterile water or phosphate buffered saline). In an alternative procedure, the mucosa is not subjected to digestion but rather is aggressively scraped with a microscope slide and the recovered tissue placed in an equal volume of 5.25% NaOCl (commercial bleach) for 30 minutes and stirred several times to agitate the material. The bleach causes tissue clumps to denature and aggregate as well as reduce the bacterial burden. Bleach is washed out by 2-3 cycles of centrifugation and is resuspended in Hank’s Buffered Saline Solution (HBSS; Gibco BRL, Gaithersburg, Maryland) and refrigerated as above. We selected the latter method because of convenience in handling large numbers of opossums, efficiency of sporocyst recovery appears to be similar, and because tissues may be processed more expediently.

An additional quirk was reported for the first time in 1996. Marsh and colleagues (1996b) recovered a Neospora sp. from a horse exhibiting classic clinical signs and histopathology of equine protozoal myeloencephalitis. Because the disease name does not specify an individual parasite species, no nomenclature change was necessary, but an additional parasite was implicated. In the report by Marsh et al., the horse had a low titer of S. neurona antibodies when tested, although the possibility of cross-reactive antibodies
could not be excluded. Subsequently, an additional clinical case of EPM caused by a *Neospora* sp. has been reported (Hamir et al., 1998). In this second case the horse had both *S. neurona* and *Neospora* sp. antibodies. The *S. neurona* antibodies are most likely present as a consequence of the high seroprevalence already reported.

Despite intense investigation the literature on EPM was confusing. Few data were unequivocal and further investigation was hampered by lack of clear direction. We felt strongly that a better understanding of the biology of *S. neurona* was critical to further progress. The purpose of the studies reported herein, therefore, are threefold: firstly, to create a model of EPM in the horse; secondly, to examine selected species as possible intermediate hosts, and; thirdly, during collection of materials for the first and second objectives to record descriptive data on infected opossums. It is a prerequisite of pharmaceutical trials and vaccine development that a characterized method is available that can reliably induce EPM in horses. Discovery of the intermediate host would permit both easier access to sporocysts for the horse model and development of strategies to control the sylvatic cycle of *S. neurona*. Understanding the risk factors for infecting opossums will permit some preventative measures until the intermediate hosts are identified.
Figure 1-1. Photograph of the Virginia opossum (*Didelphis virginiana*).
Figure 1-2. Schematic of the life cycle of *Sarcocystis neurona* showing current state of knowledge.

Opossum

*flesh-eater*

sarcocyst

in muscle

Intermediate host

*plant-eater*

? months

Horse

sporocysts

in feces

? 10 - 13 d
CHAPTER 2

ARE SARCOCYSTIS NEURONA AND S. FALCATULA SYNONYMOUS? A HORSE INFECTION CHALLENGE

Introduction

Equine protozoal myeloencephalitis (EPM) is a potentially life-threatening neurologic disease of horses which appears to be increasing in incidence (MacKay, 1997a). The disease has been recognized throughout the USA, as well as in Canada and Central and South America (De Barros et al., 1986; Granstrom et al., 1992; MacKay et al., 1992). Since the 1960s when the disease was first recognized, and throughout its further description, much debate has surrounded the clinical diagnosis of the disease and the identification of its etiologic agent. The agent initially was thought to be Toxoplasma gondii, (Cusick et al., 1974), but subsequently was identified as a Sarcocystis sp. (Simpson and Mayhew, 1980). Dubey et al. (1991) successfully isolated and propagated in cell culture a protozoan parasite from the spinal cord of a horse diagnosed with EPM and named it S. neurona. A western blot for S. neurona antibody was developed and made commercially available (Granstrom, 1993). Since then, it has become increasingly clear that S. neurona infection is common and widespread in the United States, with seroprevalence of ~50% reported in surveys from Ohio, Oregon, and Pennsylvania (Bentz
et al., 1997; Blythe et al., 1997; Saville et al., 1997). Despite the frequent occurrence of EPM, the life cycle of the causative agent still is incompletely understood.

Because there was 99.8% concordance between the DNA sequences of 18S rRNA genes amplified from opossum sporocysts and *S. neurona* (isolate SN5) merozoites, Fenger et al. (1995) suggested that the opossum (*D. virginiana*) was the likely definitive host for *S. neurona*. Work from our laboratory showed that there was >99.5% homogeneity between a 742-bp segment of the 18S rRNA gene from *S. falcatula*, an organism known to parasitize opossums (Box et al., 1984), and *S. neurona* (SN5 isolate) (Dame et al., 1995). The *S. falcatula* sequence was identical both to that reported by Fenger et al. (1995) for sporocysts isolated from 2 opossums and to another isolate of *S. neurona* (UCD-1) (Marsh et al., 1996a). On the basis of these findings, it was suggested that the 2 species (*S. neurona* and *S. falcatula*) were synonymous (Dame et al., 1995). The objective of this study was to extend these findings by attempting to induce EPM in horses challenged with an authenticated population of *S. falcatula* sporocysts.

**Materials and Methods**

**Opossum Infection**

Brown-headed cowbirds (*Molothrus ater*) were trapped and killed. The external surfaces of thigh and breast muscles were examined for the presence of sarcocysts. Brown-headed cowbird muscles containing sarcocysts were refrigerated at 4 C and were fed to opossums within 4 hr of collection. Muscle sections were fixed in 10% neutral-buffered formalin for later histologic examination. Sarcocysts were identified as *S. falcatula* based upon sarcocyst wall morphology and host species (Box et al., 1984).
Seven hand-raised opossums (5– to 6-mo-old) were purchased and housed in individual cages. Opossums were not shedding sporocysts according to results of fecal flotation with Sheathers' sugar. Muscles from infected brown-headed cowbirds were fed to opossums (1 bird/opossum). Two of the opossums were not fed brown-headed cowbird muscle and were kept as controls. Feces were collected and examined daily. Once sporocysts were detected, feces were collected and examined twice weekly. Opossums were killed (Beuthanasia-D Special, Schering Plough Animal Health, Kenilworth, New Jersey) at 42–73 days post-feeding and their gastrointestinal tracts harvested. Sporocysts were recovered and processed as follows: small intestinal mucosal scrapings were collected and placed in an equal volume of 5.25% NaOCl (commercial bleach) on ice for 30 min, and were stirred every 10 min. Tissue aggregates were removed by passage of the suspension through a gauze mesh, and then sporocysts were washed in deionized water by 3 cycles of centrifugation at 800 $g$ for 10 min each. The sporocysts were resuspended in phosphate-buffered saline solution containing 100 U/mL penicillin G and 100 $\mu$g/mL streptomycin and stored at 4 C.

**Horse Infection**

Ten horses were identified which were negative for both $S. \text{neurona}$ antibody (as detected by western blot analyses) and Sarcocystis sp. DNA in blood (as detected by PCR amplification using specific primers; Dame et al., 1995). A group of seronegative horses for this study was identified on Prince Edward Island, Canada, which is outside the known geographical range of the opossum (Gardner, 1982). Horses were transported to Florida, vaccinated against eastern and western equine encephalomyelitides and tetanus, dewormed
(Eqvalan; Merck AgVet, Rahway, New Jersey) and kept isolated for 14 days prior to beginning the experiment. To minimize exposure of the horses to naturally-shed sporocysts of *S. neurona* or *S. falcatula*, horses were fed only a complete pelleted ration (Purina Horse Chow 100; Purina Mills, Inc., St Louis, Missouri). The pelleting process requires heating beyond 65 C, a temperature which destroys *S. gigantea* sporocysts (McKenna and Charleston, 1992). Horses were kept in a 4,600 m² pasture that was modified to prevent opossum access (Figure 2-1). Specifically, the pasture was surrounded by a 1.2 m high, 5 cm by 10 cm welded-wire-mesh fence, that was buried to 15 cm below ground-surface, and had a pulsed-electric wire on the outer upper edge (Figure 2-2). No tree limb or other structure overhung the fences or enclosure.

Horses were assigned randomly (5/group) into challenge and control groups. Challenged horses were relocated to isolation stalls and approximately $10^6$ sporocysts in 1 L of water were administered by nasogastric tube (Table 2-1). Horses were kept in stalls for 7 days after challenge to ensure that pasture contamination did not occur due to passage of unexcysted sporocysts in feces. During the 3-mo study period, blood was drawn at least twice weekly and cerebrospinal fluid (CSF) samples were collected every 14 days. Blood for PCR was drawn every 2-3 days up to 30 days post-challenge. Complete neurologic examinations were performed every 14 days on each horse and recorded on videotape. Atlanto-occipital cisternal puncture was performed under short-term injectable anesthesia with xylazine (1.1 mg/kg bodyweight; Xylaject, Phoenix Pharmaceuticals, St Joseph, Missouri) and ketamine (2.2 mg/kg bodyweight; Ketaject, Fort Dodge Labs Inc, Fort Dodge, Iowa) to acquire CSF samples. Horses were examined daily and heart and respiratory rates and rectal temperature were recorded.
At the conclusion of the initial observation period (12 wk), the 4 challenged horses
that remained seronegative were reinoculated with $10^6$ sporocysts and followed for an
additional 8 weeks.

**Necropsy of Horses**

The horse that developed disease was subjected to a full necropsy after 7 days of
daily intravenous dexamethasone (0.1 mg/kg) administration. Gross examination of
organs included central nervous system, lung, heart, liver, kidneys, spleen, gastrointestinal
tract and mesenteric lymph node and musculature. Representative sections were placed in
10% neutral-buffered formalin for fixation. Sections from the central nervous system were
taken as follows: 2 sections each from cervical, thoracic and lumbar spinal cord; the
occipital lobe, diencephalon at thalamus, mesencephalon at rostral colliculus,
metencephalon at pons at cerebellar peduncles, caudal medulla, and cerebellum. After
fixation, histological sections were cut at 6 µm intervals, stained with hemotoxylin and
eosin and examined under light microscopy by a specialty pathologist. Sections with
suspect abnormalities were subjected to immunohistochemical staining (laboratory of Dr.
Brad Barr, University of California, Davis) to identify *S. neurona* or *S. falcatula*.

**Polymerase Chain Reaction**

Ribonucleic acid was extracted from 0.25 mL of whole blood using the Triazol
reagent kit according to manufacturer’s instructions (BRL GIBCO, Life Sciences,
Gaithersburg, Maryland). RNA was resuspended in 50 µl H$_2$O, and RT-PCR was
performed on 5 µl aliquots using the Access RT-PCR System (Promega, Madison,
Wisconsin) employing primers JD351 (5'-CAGCCAGTCCGCCCTTTGT-3') and JD352 (5'-CATGCTGCAGTATTCAAGGCAAC-3'). These primers were designed to specifically amplify a 160-bp segment of the 18S rRNA from *S. neurona* and *S. falcata*. RT-PCR was performed in a final volume of 50 µl of AMV/Tfl reaction buffer containing 1 µM primers, 0.2 mM dNTPs, 1 mM MgSO₄, 5 units AMV reverse transcriptase and 5 units Tfl DNA polymerase. After an initial 45-min incubation at 48 C, the PCR mixtures were subjected to 40 of the following thermocycles: 93 C for 2 min, 60 C for 30 seconds, then 68 C for 1 min. After the last cycle, the reaction mixture was kept at 68 C for 7 min and then cooled to 4 C and held. A positive control consisting of RNA extracted from uninfected horse blood containing 10³ *S. neurona* (isolate UCD-1) merozoites/mL was included in each reaction series. A negative control was included which contained all components of the PCR mixture except template. The possibility of contamination by extraneous amplicons was minimized by performing reaction setup, sample extraction, and amplification/analysis in 3 separate laboratories. Reaction tubes were closed in the room in which the reaction mixtures were prepared and only opened after amplification. PCR products were analyzed by electrophoresis using a 1% agarose gel (NuSieve 3:1, FMC, Rockport, Maine).

**Western Blot Analysis**

Serum and CSF samples were submitted to a commercial diagnostic laboratory (Equine Biodiagnostics Inc., Lexington, Kentucky) and analyzed as previously described (Granstrom et al., 1993). Test results are reported by the laboratory as either negative, suspect-positive or positive. It is our observation that samples classified as “suspect-
positive” frequently are negative when retested; therefore, we report as positive only those samples so classified by the diagnostic laboratory. All other samples are considered *S. neurona*-antibody negative.

**Viability of Sporocysts**

The pool of sporocysts used for challenge of experimental horses was examined for excystation. Aliquots of 10,000 sporocysts each were pelleted by centrifugation and resuspended in undiluted equine bile (pH 7.0) containing 2% w/v trypsin (Sigma T-8642, Sigma Chemical Company, St. Louis, Missouri). Sporocysts were then incubated for 4 hr at 37 C for maximal excystation. Counts were performed on a hemocytometer at 200 x magnification by evaluating 5 fields of 100 sporocysts each and counting the sporocyst wall fragments. To test whether sporocysts from the same lot given to horses were biologically active, 500 sporocysts were administered orally to each of 5 brown-headed cowbirds. Birds were maintained in a 2 m by 2 m by 2 m wire enclosure and fed crushed corn. After 90 days, cowbirds were killed and examined in an identical manner to the naturally-infected cowbirds described previously.

**Results**

**Opossum Infection**

Beginning 8-10 days after infected muscles were fed, sporocysts were found in the feces of all challenged opossums. In contrast, the unchallenged opossums remained negative. Opossums shed sporocysts until they were killed (range 42-73 days after challenge). Maximal shedding occurred on day 72 (range 26-112) post-feeding.
Horse Infection

Control horses remained negative for *S. neurona* antibody and *Sarcocystis* sp. DNA in both blood and CSF. One of the experimental horses (#56) had *S. neurona* antibody detected on western blot. When additional samples were analyzed, it was discovered that horse 56 had seroconverted prior to the administration of any sporocysts, i.e., was naturally exposed and infected before challenge. *Sarcocystis neurona* antibody was frequently, but not invariably, detected in serum and CSF from that horse for the remainder of the project. In addition, beginning on day 28, horse 56 showed progressive clinical signs typical of EPM, including moderate to severe ataxia and weakness of the limbs, generalized muscular atrophy, and obvious weight loss. The remaining *S. falcatula*-challenged horses did not develop clinical signs or have *S. neurona* antibody or DNA detected in blood or CSF. After the second challenge of these horses with *S. falcatula* sporocysts, there still was no clinical or other evidence of infection or disease. All positive controls for the PCR process yielded appropriately-sized DNA fragments, whereas no negative control amplified this band on any occasion.

The nucleated cell count in CSF remained within reference range (≤8 nucleated cells/µl) at all examination times. Repeated CSF collection did not appear to induce an inflammatory response in the CNS.

Gross Pathology and Histopathology

Only horse 56 was killed because it was apparent that, at most, only equivocal evidence of neurologic disease was present in other horses. The only abnormal finding on gross examination was thin condition. Histopathological examination of lung, kidney,
skeletal muscle, myocardium, tongue, esophagus, pancreas, stomach, colon, duodenum, ileum and cecum revealed no lesions. There was moderate diffuse degeneration of the liver parenchyma. Rarely, kidney tubule cells contained mineralized material and scattered glomeruli had thickened basement membranes. Mesenteric lymph nodes showed severe depletion of cells throughout. A similar, less severe, pattern was present in the spleen. All brain sections were normal except some vacuoles contained axonal debris in white matter of the medulla. In the spinal cord, lesions were present at all levels (cervical, thoracic and lumbar) but were most pronounced in the mid-cervical area (C₃ and C₄). Changes primarily comprised vacuolar degeneration of white matter (predominantly lateral and ventral) and degenerating nerve cell bodies in gray matter, with occasional mild hemorrhages in the lumbar spinal cord. Immunohistochemical staining (for *S. neurona* and for *S. falcatula*) was negative on all sections examined.

**Viability of Sporocysts**

All brown-headed cowbirds (N = 5) given sporocysts from the same pool used for horse challenge had sarcocysts present when examined 90 days after infection. Each experimentally-infected bird had a large number of sarcocysts in all skeletal muscles, particularly the breast and thigh muscles. Excystation of sporocysts was consistently 30-40%.

**Discussion**

We believe this experiment casts considerable doubt over the previous hypothesis that *S. neurona* and *S. falcatula* are synonymous ([Dame et al., 1995](#)). Challenge of horses with $10^6$ *S. falcatula* sporocysts resulted in neither seroconversion against *S. neurona* nor
clinical signs of EPM. The results from the second challenge of 4 horses from the same experimental group were likewise negative. Control experiments demonstrated that the sporocysts were viable and infectious.

The hypothesis of synonymy was based on finding virtual sequence identity of the 18S rRNA gene from *S. falcatula* bradyzoites and *S. neurona* merozoites. Typically, divergence of the 18S rRNA gene is slow and closely related species might have identical 18S rRNA gene sequences (*Hillis and Dixon, 1991*). Subsequent analyses in our laboratory, however, have identified several DNA sequence differences elsewhere in the genome which discriminate between isolates of *S. falcatula* and *S. neurona* (*Tanhauser et al., 1999*). Further, the sarcocysts and sporocysts used in the various challenge experiments in this report have been examined by these additional criteria and verified to be solely *S. falcatula* (*Tanhauser et al., 1999*).

The failure of any control horse to seroconvert against *S. neurona* indicates that when substantive measures are taken to block exposure of horses to wildlife and sporocysts, infection of horses with *S. neurona* can be prevented. Although 1 experimental horse did develop clinical EPM, the presence of *S. neurona* antibody on the day of challenge with *S. falcatula* sporocysts indicates strongly that this was a natural infection acquired prior to introduction to the protected enclosure. *Sarcocystis* sp. rRNA was not amplified by RT-PCR from this horse's blood, which suggests either that the agent may already have completed its blood-borne phase, or that the parasitemia was below the limit of detection of our RT-PCR test. In contrast, it was consistently possible to amplify RNA from $10^3$ *S. neurona* merozoites (UCD-1) grown in cell culture added to normal horse blood. Horizontal transmission between horses would not be expected because
Sarcocystis species are obligatorily heteroxenous (Dubey et al., 1989). Histopathological findings on horse 56 were compatible with EPM, although no parasite was seen microscopically. Immunohistochemical staining of spinal cord did not identify any S. neurona or S. falcatula antigen. Together these findings are still most compatible with a natural occurrence of EPM.

Seroprevalence studies conducted in Ohio, Pennsylvania, and Oregon suggest that the prevalence of S. neurona infection may be approximately 50% throughout much of the USA (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997). We found that all 29 horses kept in college pastures close to our own were seropositive, suggesting that prevalence may approach 100% in the area surrounding our experimental enclosure. A further testament to the protective efficacy of our enclosure was the finding that, after the second challenge experiment was complete, when experimental horses were transferred to another pasture, all seroconverted within 30 days. The fact that horses remained seronegative despite unfettered exposure to birds and insects from outside the enclosure additionally indicates that these species did not act efficiently as transport hosts for S. neurona sporocysts under these conditions.

Our results are somewhat in conflict with those reported by Fenger et al. (1997a). In the previous study, pooled sporocysts obtained from 10 wild opossums behaved like infective S. neurona in that they induced both seroconversion to S. neurona and neurologic signs in most subject horses. The same inoculum also was S. falcatula-like in that it killed budgerigars (Melopsitticus undulatus) and caused sarcocyst development in sparrows (Passer domesticus). These results can be interpreted to mean either that the 2 species (S. neurona and S. falcatula) are the same, as previously suggested, (Dame et al.,
or that they are different and both were present in the pooled inoculum used for horse and bird challenges. On the basis of the study we report here, we believe that the latter interpretation is correct, and that there is compelling evidence that *S. falcatula* sporocysts are harmless to horses, whereas *S. neurona* sporocysts cause the disease we recognize clinically as EPM. Clearly, both species use the opossum as definitive host, but then complete their life cycles in different intermediate hosts. It is possible that other as yet unidentified *Sarcocystis* sp. also use the opossum as a definitive host, and thus epidemiological work aimed at defining risk factors cannot merely identify sporocysts in opossum feces, but will need to characterize the species with DNA-based or other tests. The opossum has been shown to support transient infection with *S. rileyi* although the striped skunk (*Mephitis mephitis*) is the recognized definitive host in the wild (Duszynski and Box, 1978; Levine, 1986).

Currently, there is no evidence to indicate whether EPM is more likely to result from single or multiple episodes of ingestion of infective sporocysts. We chose our challenge dose to be larger than the single LD$_{50}$ oral challenge doses for *S. cruzi* (200,000 sporocysts; Fayer and Dubey, 1986) and *S. tenella* (100,000 sporocysts; Leek et al., 1977) previously given to cattle and sheep, respectively. The dose of $10^6$ sporocysts also was considerably greater than the total number of sporocysts shed in a single day by any opossum during our experimental *S. falcatula* experiment. Fenger et al., (1997a) reported that seroconversion and neurologic signs were induced when large numbers of opossum-shed sporocysts (1.2-4 x $10^7$) were given to foals. Seroconversion occurred after doses of $10^6$ and $2 x 10^6$ sporocysts. There was a trend toward earlier appearance of antibody and disease with larger doses, although these results are not strictly comparable to our
experiment as previously discussed. We believe strongly that a single dose of $10^6$
sporocysts should be sufficient to cause infection and perhaps even disease in a species
that naturally can function as host.

The results of this work are important. We present strong biological evidence that
*S. neurona* and *S. falcatoria* are not synonymous. The data suggest that *S. falcatoria* does
not infect horses. This difference reopens the fundamental question of which vertebrate
species act as intermediate host for *S. neurona*. This is practically important because
pharmacological studies and vaccine trials will require highly-characterized sporocysts
produced in the laboratory under controlled conditions.
Table 2-1. Summary of horse experiment. Horses in the challenge group were administered sporocysts characterized as *Sarcocystis falcatoria* in water by nasogastric tube.

<table>
<thead>
<tr>
<th>Horse ID#</th>
<th>Breed</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Group</th>
<th>Sporocysts $(x \times 10^6)$</th>
<th>Neurologic disease</th>
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<tr>
<td>50</td>
<td>Std</td>
<td>5</td>
<td>g</td>
<td>Control</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>51</td>
<td>Std</td>
<td>3</td>
<td>f</td>
<td>Control</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>52</td>
<td>Std</td>
<td>3</td>
<td>f</td>
<td>Challenged</td>
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<td>No</td>
</tr>
<tr>
<td>53</td>
<td>Std</td>
<td>6</td>
<td>f</td>
<td>Control</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>54</td>
<td>Std</td>
<td>3</td>
<td>f</td>
<td>Challenged</td>
<td>1.0</td>
<td>No</td>
</tr>
<tr>
<td>55</td>
<td>Std</td>
<td>4</td>
<td>g</td>
<td>Challenged</td>
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<td>No</td>
</tr>
<tr>
<td>56</td>
<td>Std</td>
<td>4</td>
<td>f</td>
<td>Challenged</td>
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<td>Yes</td>
</tr>
<tr>
<td>57</td>
<td>Std</td>
<td>3</td>
<td>g</td>
<td>Challenged</td>
<td>1.0</td>
<td>No</td>
</tr>
<tr>
<td>58</td>
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<td>3</td>
<td>f</td>
<td>Control</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>59</td>
<td>TB</td>
<td>3</td>
<td>g</td>
<td>Control</td>
<td>0</td>
<td>No</td>
</tr>
</tbody>
</table>

Std = Standardbred, TB = thoroughbred; f = filly, g = gelding.
Figure 2-1. Schematic drawing of horse pasture, showing location of some isolation stalls.
Figure 2-2. Photograph of the pasture fence, showing adaptations to prevent ingress of wildlife.
CHAPTER 3

EQUINE PROTOZOAAL MYELOENCEPHALITIS: NASOGASTRIC ADMINISTRATION OF SARCOCYSTIS NEURONA SPOROCYSTS CAUSES SEROCONVERSION AND DISEASE IN HORSES

Introduction

It has previously been demonstrated that the administration of characterized *S. falcatula* sporocysts to horses does not induce seroconversion against *S. neurona* nor does it induce disease (Cutler et al., 1999). In a similar horse-challenge experiment using an uncharacterized mixture of sporocysts from wild-caught opossums, disease and seroconversion occurred in challenged horses and disease also occurred in challenged budgerigars (Fenger et al., 1997a). This apparent paradox is resolved if a mixture of *Sarcocystis* sp. sporocysts was present in the inoculum for the latter experiment. Ideally, a new horse challenge is indicated utilizing biologically-purified *S. neurona* sporocysts to fulfill Koch’s postulates. The intermediate host of *S. neurona* is not known, however, and therefore it is not possible to generate a single population of *S. neurona* sporocysts by infecting naïve laboratory animals. The closest alternative is to collect wild opossums, screen for infection, and then characterize sporocyst isolates using molecular markers. This technique does not exclude the presence of other species at low concentrations. However, the risk of confounding the results by accidentally including *S. falcatula* is
minimal in light of the negative data obtained previously when horses were challenged with *S. falcatula* sporocysts.

Once a highly characterized method for the experimental induction of EPM exists in horses, further progress can be made in understanding the pathogenesis of the natural disease. Refinements in challenge dose and administration protocol would permit better characterization of the risks of developing EPM and identify horse populations placed at greatest risk by athletic, or other, activity. Ultimately, repeatable and dependable induction of EPM in horses is an essential prerequisite to further progress in understanding and eliminating this disease.

**Materials and Methods**

**Opossum Collection and Sporocyst Recovery**

Opossums were acquired either as roadkill or were trapped and euthanized. Gender and weight were recorded and a unique identification number was assigned. Some samples were provided to my laboratory as intestinal tracts only, (without other information). Necropsies were performed as soon as possible after collection. The gastrointestinal tract was isolated and removed intact through an incision of the midline ventral abdominal wall. The mesentery was stripped off and the intestinal contents milked into a clean, labeled container. A section of distal ileum was incised, flattened and the mucosal surface exposed. Excess ingesta were washed off the mucosa with deionized water and then mucosa was collected by scraping with a microscope slide at a 45 degree angle. A 2-g sample of feces and a 1-mL sample of intestinal-mucosal scrapings were taken from the original samples and each was homogenized in 10 mL deionized water.
Samples were passed through cheesecloth into labeled 15 mL tubes and were centrifuged for 10 minutes at 800 g. Pellets were re-suspended in saturated Sheather’s sugar solution and transferred onto coverslips by centrifugation for 10 minutes at 800 g. Coverslips were scanned at 100 x magnification to determine if sporocysts were present. Sporocysts were frequently noted to have a pink hue if examined within 12 hours of removing the intestinal tract. When sporocysts were identified, the remaining gastrointestinal tract mucosa was recovered. Mucosal scrapings were mixed in to an equal volume of 5.25% NaOCl (commercial bleach) on ice for 30 minutes and stirred every 10 minutes. Tissue aggregates were removed by pouring the sporocyst suspension through a gauze mesh. Bleach was removed by 2-3 washes in deionized water. The sporocysts were collected by centrifugation at 800 g for 10 minutes between washes and finally stored at 4°C in phosphate-buffered saline (PBS) with 10,000 U/mL, 100 µg/mL streptomycin and 50 µg/mL gentamicin to prevent bacterial overgrowth.

**Inoculum Preparation**

An aliquot of 5,000-10,000 sporocysts from each isolate was pelleted at 800 g and re-suspended in equine bile (pH 7.0) with 2% w/v purified trypsin (Sigma T-8642; Sigma Chemical Company, St Louis, Missouri) followed by incubation at 37°C for up to 4 hours. Excystation was confirmed by examination of a slide at 100 x total magnification. Estimated percentage excystation was made by examining 10 fields and identifying excysted sporocyst cases and motile sporozoites. DNA from the sporocysts was extracted using a non-ionic detergent (NP-40; Sigma Chemical Company, St. Louis, Missouri) method and PCR was performed as previously described (Tanhauser et al., 1999) using 6
different primer-pairs, in separate reactions, for each sporocyst isolate. According to published protocol, sporocysts were characterized as either neurona-like, falcatula-like, dissimilar to both or, rarely, a mixture (Tanhauser et al., 1999). An aliquot of sporocysts from any isolate to be used for horse challenge was re-examined to confirm concentration and ability of the sporocysts to excyst prior to use. All doses for each horse were prepared in advance and each vial was labeled and re-counted prior to beginning the experiment. All counts were performed twice.

**Single-Dose Horse Challenge**

Five horses were identified which had no serum *S. neurona* antibodies (as detected by western blot analyses). Horses were vaccinated against eastern and western equine encephalitis and tetanus, dewormed (Eqvalan; Merck AgVet, Rahway, New Jersey) and kept in an isolation pasture for a minimum of 21 days before beginning the experiment to permit acclimation. To ensure minimal chance of accidental exposure of the horses to naturally-shed sporocysts of *S. neurona* or *S. falcatula*, only feed which had been heat-treated beyond 65 C, a process which appears to destroy sporocysts (McKenna and Charleston, 1992), was fed. Heat-extrusion of pelleted feeds (Purina Horse Chow 100 and Purina Pure Pride 300; Purina Feed Mills, St Louis, Missouri) occurs at temperatures in excess of 140 C and is followed by air-drying and immediate bagging, eliminating any chance of recontamination. No hay was fed. The isolation pasture has previously been described (Cutler et al., 1999) and used for previous horse challenge experiments. As in earlier experiments, no structures were permitted to overhang the enclosure as possible access. An area of the pasture was sub-divided to allow separation of horses into smaller
groups for handling and feeding. Two horses were randomly selected for challenge and
were housed in individual isolation stalls. On day 2, \(5 \times 10^5\) sporocysts of the
characterized \(S.\) neurona isolate pool were administered by nasogastric tube in 10 mL
PBS followed by 1 L deionized water. Challenged horses were kept in the isolation facility
for 8 days thereafter to ensure pasture contamination did not occur by passage of
sporocysts in equine feces. During the 4-month study period, whole blood for serum
preparation was drawn at least three times weekly and CSF samples were collected and
neurologic examinations performed every 14 days on each horse and recorded on
videotape. Atlanto-occipital cisternal puncture was performed under short-term injectable
anesthesia with xylazine (Xylaject; Phoenix Pharmaceuticals, St. Joseph, Missouri; 1.1
mg/kg bodyweight) and ketamine (Ketaject; Fort Dodge Laboratories Inc.; Fort Dodge,
Iowa; 2.2 mg/kg bodyweight) to acquire the CSF samples. Evaluation of CSF was
performed within 1 hour of collection for WBC concentration and cytospin slides were
prepared for differential cell counts. Horses were examined daily to monitor for presence
of intercurrent disease and for evidence of neurologic clinical signs.

**Environmental Sentinels**

Contemporary environmental sentinels were necessary to reflect any breach of
biosecurity in the isolation pasture. Sentinels were handled identically to challenged-
horses except they never left the isolation pasture. One young horse (#801) was separated
from the others at feed time and was permitted a typical quantity (approx. 2%
bodyweight) of hay in addition to the pelleted diet for a 4-week period prior to beginning
the experiments. This protocol deviation was necessary for the dietary management of
this horse and, in consequence, allowed potential evaluation of the risk of feeding hay on the serostatus of this individual. After 4 weeks, horse #801 was handled and fed identically to the others, although it was still separated at feed times. No hay was fed to any horse at any other time. The “hay-challenge” horse was euthanized and necropsied at the end of the first horse challenge. The 2 environmental sentinels were maintained in the pasture until the end of the second horse challenge. One of the challenge horses for the multiple-dose experiment was introduced into the pasture before the end of the experiment and served as an additional environmental sentinel during its quarantine period.

**Multiple-Dose Horse Challenge**

Two additional horses were acquired, acclimated and handled as previously described. After the quarantine period, both horses were assigned as subjects and were transferred to an isolation stall in a closed building. On days 2 – 7, horses received $5 \times 10^5$ sporocysts daily in 10 mL PBS followed by 1 L deionized water by nasogastric tube. The inoculum consisted of a single opossum isolate (#2009). On days 9-11 horses were given 500 g psyllium mucilloid (approximately 1 g/kg) in 4 L water by nasogastric tube to assist in flushing unattached sporocysts out of the gastrointestinal tract before return to the isolation pasture. This experiment began 6 weeks after the completion of the single-dose experiment, lasted 50 days, and used the same 2 environmental sentinel horses.

**Western Blot Analysis**

Serum was prepared by centrifugation of clotted blood at 800 g for 10 minutes. Serum and CSF were aliquoted, and those aliquots not submitted for analysis immediately were frozen at -80 C for archival purposes. All submitted samples were coded non-
sequentially and accession sheets did not include specific details on the animal sampled. At least one portion of every sample was submitted to the commercial diagnostic laboratory Equine Biodiagnostics Inc., Lexington, Kentucky and analyzed as described (Granstrom, 1993). In the multiple-dose challenge, portions of some samples were also submitted to the commercial diagnostic laboratory Neogen Inc., Lexington, Kentucky and were analyzed according to their standard protocol. All results not designated “positive” by the laboratory are considered “negative” for purposes of comparison. One laboratory (Neogen Inc., Lexington, Kentucky) reported a densitometric comparison of the 17 kD protein band in each CSF sample to the same band in a series of standards, expressed as a unit-less number between 0 and 100.

**Gross and Histopathological Examination**

Challenged horses and the “hay-challenged” horse were euthanized using an overdose of sodium pentobarbitone intravenously (Beuthanasia-D Special; Schering Plough Animal Health, Kenilworth, New Jersey). Gross examination of organs included lung, heart, liver, kidneys, spleen, pancreas, mesenteric lymph node, multiple sites within the gastrointestinal tract and multiple sites (epaxial, appendicular and axial) of skeletal muscle. Representative sections of each were placed in 10% neutral-buffered formalin for fixation. The spinal cord was recovered cleanly in 3 parts—namely, cervical, thoracic and lumbosacral sections. Each piece of spinal cord, with dura intact, was placed into a sterile disposable jar of 1000 mL normal saline containing penicillin 100 U/mL and streptomycin 100 µg/mL, transferred to a steriley-draped laminar flow hood and was sectioned at 5-mm intervals. Particular attention was paid to segments of spinal cord considered suspect
on neurologic exam of the living animal. Areas appearing discolored were bisected and one half removed and placed aseptically into a petri dish containing culture media (RPMI-1640; Gibco-BRL, Gaithersburg, Maryland) while the remaining spinal cord was fixed in 10% neutral-buffered formalin. Sections from the brain were taken as follows: the occipital lobe, diencephalon at thalamus, mesencephalon at rostral colliculus, metencephalon at pons at cerebellar peduncles, caudal medulla, and cerebellum. After fixation, histological sections of all tissues were made at 6-µm intervals and stained with hematoxylin and eosin and were examined under light microscopy by a specialty pathologist. Sections were also sent to the laboratory of Dr. Brad Barr, University of California, Davis for immunohistochemical staining to identify *S. neurona* or *S. falcatula* merozoites.

**Results**

**Opossum Collection and Inoculum Preparation**

One hundred seventy three road-kill opossums were collected in 1997 and 66 were collected in 1998 in time to characterize before horse challenge. Forty-five isolates were obtained from these animals, of which 14 were identified as being *S. neurona* alone on at least 4 tests and were considered for use as inocula. Some isolates appeared to be neurona-like with some molecular tools, but falcatula-like with others: these isolates were not used. For the single-dose challenge, the inoculum consisted of 4 isolates in a 3:1:1:1 ratio (isolate #1112, #1013, #1067 and #1071). Additional aliquots of the inoculum were reserved for further host challenge. A mix of sporocysts had the potential added advantage of controlling for varied infectivity among isolates. In the multiple-dose
challenge a single high count isolate (#2009) was used to provide the large stocks required without consuming too many isolates. Isolate #2009 also contained a small percentage of sporocysts identified as *S. falcata*. However, in light of previous negative data from horses challenged with biologically-purified *S. falcata* it was considered unlikely that low level contamination by this parasite would confound the results of this trial (Cutler et al., 1999).

**Single-Dose Horse Challenge**

No sentinel horse ever produced *S. neurona* antibodies. Both challenged horses produced serum antibodies (day 19 and day 26) and CSF antibodies (day 40) [Table 3-1]. Challenged horses showed subtle neurologic signs from day 26 until euthanasia but these deficits never progressed to be unequivocal. The hay-challenge horse (#801) showed variable subtle clinical neurologic signs that were somewhat ephemeral, but predominantly hindlimb and never exceeded grade I/V (Appendix B). Neither of the sentinel horses showed neurologic signs (subtle or otherwise). The hay-challenge horse produced antibodies in serum (day 30) and CSF (weak positive on day 30, strong positive on day 60). All antibodies persisted to the time of euthanasia without apparent diminution. No therapy was necessary during the experiment for intercurrent disease. All horses showed clinical signs of conjunctivitis and tracheitis, believed to be caused by a *Streptococcus* sp., which resolved without therapy. No gross abnormality was noted at necropsy except that a large amount of sand was present in the gastrointestinal tract of each horse.
Western Blot Results

Results are available only from laboratory 1 (EBI) for the single-dose challenge. Table 3-3 summarizes the results giving number of consecutive negative and then positive tests and the number of consecutive days in each category. Data are presented for both challenged horses, both environmental sentinels, the hay-challenge horse and a horse that was in its quarantine period prior to being challenged in the subsequent multiple-dose trial. In total, there was only a single discordant result in one of the challenged horses (#817). Before the multiple-dose horse challenge began, all 4 remaining horses seroconverted while still in the pasture. Results from laboratory 2 are available from the time of seroconversion onwards. The summarized data are presented in Table 3-4. The results for horse #817 from laboratory 2 are consistent. The full results for horse #817 from laboratory 1 are presented in Table 3-5 because they are discordant. Some samples were resubmitted to the same diagnostic laboratory in 1999 for comparative purposes. These results are also presented in Table 3-5.

With the exception of horse #817 results from laboratory 1, the consistency of within-horse results from either laboratory are excellent, with only a single negative result being reported in any horse after first seroconversion. The agreement between laboratories for horse #816 is complete.

Multiple-Dose Horse Challenge

Both challenged horses became unequivocally neurologic by day 13 (Table 3-2). No definitive signs of cranial nerve dysfunction were observed. The predominant clinical sign in each horse was weakness and ataxia of the pelvic limbs. Horse #817 was grade 2
or worse in pelvic limbs from day 13 until euthanized, and at least grade 1 in both thoracic limbs. Horse #816 showed similar limb deficits. Diminution of the cervicofacial and panniculus responses was noted in horse #816 on 4 of 6 examinations. Muscle atrophy was a minor component of the presentation in both horses. Weight loss was not a prominent feature of the disease in these horses. Neither of the environmental sentinel horses developed evidence of clinical neurologic disease at any time during the trial.

The sequential densitometric comparisons of 17 kD protein to reference standard protein for both challenged horses are shown in Figure 3-1.

At necropsy no gross abnormality was noted except that a large amount of sand was present in the gastrointestinal tract. Both horses had mild myocarditis, mild lymphoid hyperplasia in lymph nodes, and moderate lymphoplasmacytic to eosinophilic enterocolitis. Histopathological lesions within the CNS consisted of neuroaxonal degeneration with spongiosis, gliosis and occasional spheroids. Lymphoid cuffing was occasionally present around vessels, but was mild or minimal. In horse #816, the lesions were most severe in the cervical spinal cord at C6 and C7, consisting of neuroaxonal degeneration. Acute severe meningeal hemorrhage was present multifocally throughout the cervical region. In horse #817, lesions were similar but milder and most pronounced in the cervical region. No meningeal hemorrhage was present.

**CSF Analysis**

Concentration of nucleated cells in the CSF was normal at all time-points (<8 cells / µL). The highest cell count recorded was 5, in horse #799 on day 12 post challenge. Minor contamination of CSF with blood occurred on three occasions: the RBC counts
were 137, 700 and 770 / µL. On each occasion, RBC concentration returned to normal before the next CSF aspiration.

**Discussion**

This paper describes the first successful induction of equine protozoal myeloencephalitis using molecularly-characterized *S. neurona*. Challenged horses either seroconverted (single-dose study) or developed disease (multiple-dose study), whereas contemporaneous environmental sentinels did not. Previous publications on this subject (Dame et al., 1995; Fenger et al., 1997a; Cutler et al., 1999) clearly underline the great importance of correctly and fully identifying the *Sarcocystis* species being used. Otherwise, induction of EPM underscores the fact that opossums shed the infectious agent, but does nothing to confirm the agent’s identity.

Data reported elsewhere (chapter four) is relevant to the single-dose horse challenge. Five species of birds were challenged in separate groups with 3 of the 4 *S. neurona* isolates that constituted the inoculum for the single-dose challenge. The fact that no bird developed sarcocysts when administered characterized *S. neurona* sporocysts further supports the biological difference between *S. neurona* and *S. falcatula*, as has been previously suggested (Tanhauser et al., 1999). Bird species challenged included the brown-headed cowbird (*Molothrus ater*) and the boat-tailed grackle (*Cassidix mexicanus*) which are known intermediate hosts of *S. falcatula*. In a previous bird challenge using *S. falcatula* sporocysts, sarcocysts were very numerous and easily recognized in all muscles at 90 days post-feeding (Dame et al., 1995).

It is unfortunate that it is still not possible to replicate the entire life cycle of *S. neurona* in the laboratory in order to produce a single population of sporocysts:
nonetheless, these results do clearly demonstrate that opossum-shed *S. neurona* alone (as identified by 6 molecular tools) is harmless to birds, but can induce EPM in horses. Eventually, the ability to generate sporocysts in the laboratory will remove a large burden of collection and identification of isolates and will be a necessary component of fulfilling Koch’s postulates.

First evidence of seroconversion at days 19 and 26 is in agreement with previously published data (Fenger et al., 1997a) where seroconversion occurred as early as 19 days after challenge. Fenger reported development of clinical neurologic signs at day 28 (n=2) or day 42 (n=2) whereas in this study definitive (grade II/IV or worse) were noted earlier, by day 13. It is extremely difficult to design a truly objective neurologic examination for horses: therefore, we report only those signs that were repeatedly identified by a blinded examiner, using standard forms (Appendix A) and a standardized examination methodology (Appendix B). Clinical neurologic signs were somewhat progressive, and in one horse (#816) showed some improvement before euthanasia (at day 50).

Clinical experience suggests that development of EPM is dependent on multiple factors, and therefore it should not be surprising that neurologic signs are variable among horses, or that signs might develop after variable periods or different ingested doses. The predominance of pelvic limb disease in the challenged horses reported here reflects the predominant presentation of naturally-occurring EPM cases. There is still no good pathophysiological explanation for the development of devastating peracute disease in animals known to be clinically normal only hours before (T. Cutler and R. MacKay, unpublished observations). This must remain a concern for those investigating EPM because it indicates either that the horse challenge protocol is incomplete (causing only
mild disease), or that the organism is adapting, changing the disease manifestation and that peracute disease is becoming even rarer. Although adaptation of the parasite is unlikely to occur over such a short period (at most 40 years), there is substantial evidence that most horses are quite capable of withstanding *S. neurona* infection without developing EPM.

Necropsy findings are moderate or mild in all challenged horses. No protozoa were identified in any sections examined. The meningeal hemorrhage in some horses may be related to the collection of a maximal quantity of CSF immediately post-mortem and did not occur in one horse where only 60 mL of CSF was recovered. Clinical impression was that both horses #816 and #817 were improving prior to euthanasia, and this may explain the end-stage neural disease that we identified (neuroaxonal degeneration and gliosis). Even when sampling CSF twice monthly it appears that at most only a mild inflammatory response occurs and does not induce a pleocytosis in CSF. In future work, it will be important to sample frequently so as to identify more accurately the date of first appearance of antibodies in the CSF and to increase the attempts to isolate parasite from the CSF. When RBCs contaminated the CSF aspirate there was no evidence of residual contamination on the next aspiration from that horse (14 days later).

In this study either 1 or 6 doses of 5 x 10⁵ sporocysts were administered to each horse. This dose was selected on the basis of extrapolations from our previous work and a previous experiment where daily output of sporocysts was determined in opossums fed *S. falcatula*-infected cowbirds (R. Porter & E. Greiner, personal communication). We were also guided by the LD₅₀ oral challenge doses reported for cattle (2 x 10⁵ sporocysts of *S. cruzi*, Fayer and Dubey, 1986) and sheep (10⁵ sporocysts of *S. tenella*, Leek et al., 1977) and seroconversion of pony foals in another study with a dose less than or equal to
1 x 10⁶ sporocysts (Fenger et al., 1997a). No evidence exists to infer whether a single or continuous exposure is more effective in inducing development of the natural disease although the definitive host for many Sarcocystis sp. sheds sporocysts for a short period of weeks to months rather than lifelong (Dubey et al., 1989). We suggest that disease induction and seroconversion validate the dose selected and that the eventual model of EPM will use a challenge similar to the multiple-dose protocol described herein. In large animals acting as typical intermediate hosts an additional round of merogony probably occurs before sarcocyst formation (Dubey et al., 1989). If a similar pathogenesis is involved in causing EPM in horses, which are atypical intermediate hosts of S. neurona, then ingested dose may only influence the rate of development and/or the severity of disease. Although we have no data to predict whether merogony in the horse has a finite or unlimited number of iterations, the latter appears more likely. The fact that some cases of EPM have developed up to 2 years after export from EPM-endemic areas may lend support to such an extended process (I. G. Mayhew, personal communication). After challenge, horses were held in the isolation facility and received psyllium mucilloid orally to prevent shedding of viable, unexcysted sporocysts in the horse pasture. No data are available to validate either the duration or effect of the psyllium loading. However, lambs orally-challenged with very high sporocyst doses (3.5 x 10⁶) were still shedding viable sporocysts in feces 7 days post-dosing (Munday, 1985). An even longer “washout” period may therefore be indicated in horses, although the lambs were not fed psyllium to aid in flushing unattached sporocysts out of the gut lumen.

There is considerable concern amongst equine clinicians about the interpretation of western blot results of serum or CSF. The data presented in Tables 3-3 and 3-4 should
provide further assurance that general agreement within and between the laboratories included in this study is excellent. When a horse is identified as seropositive the test result is highly repeatable. The value of a negative western blot on serum is particularly underscored. The data presented in Table 3-5 are difficult to interpret. The laboratory results may simply be erroneous. However, when the results reported on the resubmitted samples are considered, laboratory error seems an unsatisfactory explanation. Alternatively, horse #817 may have responded to the natural point-challenge with a poor humoral response which peaked and then dissipated quickly. When the horse was experimentally challenged an anamnestic response ensued together with disease. The repeated samples are compatible with this result. The 17 kD data from laboratory 2 also corroborates this second possibility, and elevated from 5 at challenge to 75 on day 20 post challenge. In contrast, the results for horse #816 were already elevated before challenge, possibly reflecting disease due to natural exposure and not due to challenge. Considerably more data will be necessary to determine the full value of the 17 kD protein densitometry reading. Nonetheless, it remained at or below 5 in the environmental sentinel horses and, therefore, in this limited instance was capable of differentiating the horses who were naturally exposed and then experimentally challenged from the horses that were naturally exposed alone.

It has become popular to discount results of serum western blot tests simply because seroprevalence is so high. The implication is that the test is providing unusable results. An alternative, and perhaps more likely, explanation is that seroprevalence is high but disease incidence is low because innate and humoral immunity usually prevent disease (EPM) from occurring after infection with *S. neurona*. 
The hay-challenged horse (#801) seroconverted and developed CSF antibodies at 30 and 60 days respectively. The longer time to first appearance of antibodies may be related to a lower challenge dose ingested with the hay, or that the horse was adjusting from a low level of nutrition and was partially immunocompromised as a result, thus delaying the humoral response. In either case, although this is a single animal, the only factor different between this individual and the others in the pasture was its diet. No other horse was permitted access to the hay during the 4 weeks that it was fed to horse #801. All horses had access to the holding pen area when hay was not being fed. The most likely explanation is that exposure to *S. neurona* occurred consequent to ingesting contaminated hay. An alternative explanation is that this horse alone had an additional point exposure to contaminant *S. neurona* sporocysts. However, its poor nutritional state would suggest that this horse was less likely, rather than more, to seroconvert after a group exposure.

The isolation pasture where horses were kept was successful in protecting the environmental sentinels during the first experiment. All horses in the pasture experienced a point exposure, presumably to *S. neurona*, and seroconverted after the conclusion of the single-dose horse challenge and prior to the multiple-dose horse challenge. This demonstrated that these horses were susceptible and were truly protected during the first challenge. When horses were transported from the isolation paddock to the necropsy facility, a truck and trailer were driven through the pasture and may have acted as a mechanical vector. Extensive measures were taken to prevent wildlife access to the isolation paddock because it was known that opossums were present around the facility. No opossum was ever seen inside the enclosure, and so it is assumed that such was not the source of contamination. Alternative sources of sporocyst contamination include the city.
water supply and contaminated food given in error by technical personnel or visitors to the compound. We have frequently observed turkey vultures (*Cathartes auna*) and other prey birds preferentially scavenging opossum intestines during our roadkill collections. Potentially, although perhaps less likely, prey birds may act as mechanical vectors as a result. Because feed pellets are scattered on the paddock ground while the horses feed, small birds are frequently present eating the lost food and are an additional potential source.

EPM has apparently increased in incidence recently (MacKay, 1997a), and seroprevalence has been documented to be increasing (Granstrom, 1993; Saville et al., 1997). It is important to distinguish seroconversion, which represents exposure to the organism and probably infection, and the disease EPM which additionally involves invasion of the central and/or peripheral nervous system by the parasite, asexual replication at those sites, a resultant inflammatory response and the manifestation of neurologic disease. An increased seroprevalence could be a result of increases in the population or distribution of either the intermediate or definitive host, or both. Surveys performed by the State of Kentucky recorded a decrease in the number of opossums killed by trappers and furriers from 70,000 in 1982 to 2,000 in 1994 (Cramer, 1997). Removing this pressure on the population could therefore permit an increase in feral opossum numbers and result in further alterations in population dynamics. Roadkill surveys of opossums are not available from that state, but the raccoon highway mortality index increased by 386% between 1986 and 1991. It is possible that without trapper pressure that opossum populations have dramatically risen (at the same time that seroprevalence has), and that in turn has forced opossums to disperse (and change their distribution). The
intermediate host remains unknown and precludes speculation on its role in the emergence of EPM.

It is clear from the combined results of this and previous studies (Fenger et al., 1997a) that *D. virginiana* is the definitive host of both *S. neurona* and *S. falcultula*, and probably of other *Sarcocystis* sp. (Tanhauser et al., 1999). The identification of opossum-shed sporocysts categorized as non-neurona/non-falcatula indicates that a considerable amount of work remains to be completed to understand the role of the opossum in dispersing *Sarcocystis* sporocysts into the environment. Of ongoing concern is the failure of any investigator to identify the true intermediate host(s) of *S. neurona*. This remains a priority area if we are to be able to identify and endorse logical wildlife control and preventative measures with confidence.

This is the first report of EPM induced in horses using characterized *S. neurona* sporocysts. It seems unlikely that natural disease only occurs after ingestion of such enormous numbers of sporocysts, and it is probable that additional uncontrolled factors reduce the ID$_{50}$ in horses that spontaneously develop EPM. The observed dose increment necessary to cause disease in addition to antibody induction may reflect clinical experience with prevalence of *S. neurona* antibodies compared to prevalence of EPM. The clinical implications of each possibility (i.e. seroconversion alone or with disease) should be considered when interpreting western blot results. Published serosurveys indicate that approximately 50% of the horses in the USA have antibodies (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997), perhaps now an underestimate (T. Cutler and R. MacKay, unpublished observations), whereas it has been suggested that only 1-2% of horses actually have EPM (R. MacKay, personal communication). Thus the state of knowledge
is that opossums have an unusual position as host of both *S. neurona* and *S. falcatula*.

Secondly, the western blot test is capable of differentiating exposed and truly infected horses from unexposed horses, and is highly repeatable. *Sarcocystis neurona* causes the often devastating disease we know as EPM while *S. falcatula* causes disease in birds but not horses. The horse model described herein remains to be validated and refined with a larger group before it is used for more advanced work.

Table 3-1. Summary of single-dose horse challenge.

<table>
<thead>
<tr>
<th>Horse ID#</th>
<th>Number of days in quarantine</th>
<th>Group</th>
<th># 5 x 10⁵ sporocyst doses</th>
<th>1st day Serum WB+ve</th>
<th>1st day CSF WB+ve</th>
<th>1st day Neurol. signs</th>
<th>Isolate ID#</th>
</tr>
</thead>
<tbody>
<tr>
<td>797</td>
<td>50</td>
<td>Challenge I</td>
<td>1</td>
<td>19</td>
<td>40</td>
<td>40</td>
<td>mix*</td>
</tr>
<tr>
<td>799</td>
<td>30</td>
<td>Challenge I</td>
<td>1</td>
<td>26</td>
<td>40</td>
<td>40</td>
<td>mix*</td>
</tr>
<tr>
<td>798</td>
<td>114</td>
<td>Sentinel I &amp; II</td>
<td>0</td>
<td>WB-ve</td>
<td>WB-ve</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>815</td>
<td>42</td>
<td>Sentinel I &amp; II</td>
<td>0</td>
<td>WB-ve</td>
<td>WB-ve</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

Challenge I = single-dose challenged horses; Sentinel I & II = environmental sentinel horses for both studies; WB+ve = western blot results positive; WB-ve = western blot result negative throughout *The inoculum mix for horses in challenge group I comprised 50% isolate #1112 and 16.67% each of isolates #1013, #1067 and #1071.
Table 3-2. Summary of multiple-dose horse challenge.

<table>
<thead>
<tr>
<th>Horse ID#</th>
<th>Number of days in quarantine</th>
<th>Group</th>
<th># 5 x 10^5 sporocyst doses</th>
<th>1st day Neurol. signs</th>
<th>Isolate ID#</th>
</tr>
</thead>
<tbody>
<tr>
<td>816</td>
<td>115</td>
<td>Challenge II</td>
<td>6</td>
<td>8</td>
<td>2009</td>
</tr>
<tr>
<td>817</td>
<td>21</td>
<td>Challenge II</td>
<td>6</td>
<td>13</td>
<td>2009</td>
</tr>
<tr>
<td>798</td>
<td>114</td>
<td>Sentinel I &amp; II</td>
<td>0</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>815</td>
<td>42</td>
<td>Sentinel I &amp; II</td>
<td>0</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

Challenge II = multiple-dose challenged horses; Sentinel I & II = environmental sentinel horses for both studies; WB+ve = western blot results positive; WB-ve = western blot results negative.

Table 3-3. Summary of western blot tests from laboratory 1 for horses in the single-dose challenge, demonstrating consistency of test results.

<table>
<thead>
<tr>
<th>Horse ID#</th>
<th>Group</th>
<th>Chall. 797</th>
<th>Chall. 799</th>
<th>Sentinel 798</th>
<th>Sentinel 815</th>
<th>Hay 801</th>
<th>Quarantine 816</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum WB-ve consecutive tests duration (d)</td>
<td>6</td>
<td>50</td>
<td>3</td>
<td>12</td>
<td>19</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>WB+ve consecutive tests duration (d)</td>
<td>5</td>
<td>61</td>
<td>6 *1</td>
<td>75</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>CSF WB-ve consecutive tests duration (d)</td>
<td>5</td>
<td>61</td>
<td>5</td>
<td>46</td>
<td>9</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>WB+ve consecutive tests duration (d)</td>
<td>4</td>
<td>49</td>
<td>4</td>
<td>49</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Serum WB-ve= western blot result negative, WB+ve= western blot result positive, Chall.= challenged horse; Hay= hay challenged horse; Quarantine= horse entering Sentinel status; *1= 1 result discordant (negative).

Table 3-4. Summary of western blot tests from laboratory 1 and laboratory 2 for horses in the multiple-dose challenge.

<table>
<thead>
<tr>
<th>Horse and Laboratory</th>
<th>Sample</th>
<th># tests WB-ve</th>
<th>Duration (days)</th>
<th># tests WB+ve</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#816 Lab 1</td>
<td>Serum</td>
<td>8</td>
<td>75</td>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>4</td>
<td>49</td>
<td>5</td>
<td>56</td>
</tr>
<tr>
<td>#816 Lab 2</td>
<td>Serum</td>
<td>n/a</td>
<td>n/a</td>
<td>9</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>n/a</td>
<td>n/a</td>
<td>5</td>
<td>56</td>
</tr>
<tr>
<td>#817 Lab 1</td>
<td>Serum</td>
<td>3</td>
<td>10</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>0</td>
<td>0</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>#817 Lab 2</td>
<td>Serum</td>
<td>n/a</td>
<td>n/a</td>
<td>9</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>n/a</td>
<td>n/a</td>
<td>6</td>
<td>68</td>
</tr>
</tbody>
</table>

WB-ve= western blot result negative, WB+ve= western blot result positive, *= results discordant, see Table 3-5.
Table 3-5. Results of western blot testing of horse #817 from laboratory 1.

<table>
<thead>
<tr>
<th>Day #</th>
<th>Sample</th>
<th>Date</th>
<th>1998 Western Blot result</th>
<th>1999 Western Blot result</th>
</tr>
</thead>
<tbody>
<tr>
<td>-39</td>
<td>Serum</td>
<td>18-May</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>-31</td>
<td>Serum</td>
<td>26-May</td>
<td>Negative</td>
<td>&lt;&gt; Positive</td>
</tr>
<tr>
<td>-29</td>
<td>Serum</td>
<td>28-May</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>-24</td>
<td>Serum</td>
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<td>46</td>
<td>CSF</td>
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Day # is from first dose; * laboratory reports albumin concentration elevated in CSF compared to expected range. Weak Positive is considered Negative for comparisons. “=” same result reported when tested in 1998 and 1999; “<>” results discordant.
Figure 3-1. Densitometric comparison of 17 kD protein on western blot of CSF of challenged horses compared to reference standard. Each horse was challenged on day 0.
CHAPTER 4

CHALLENGE OF PUTATIVE INTERMEDIATE HOSTS OF *S. NEURONA* WITH CHARACTERIZED SPOROCYSTS

**Introduction**

There is an urgent need to be able to generate *S. neurona* sporocysts under controlled circumstances in the laboratory. Firstly, only by taking naïve opossums and feeding them characterized *S. neurona* sarcocysts would it be possible to assure that the resulting sporocysts are a single population. Minor contamination with *S. falcatula*, for instance, would complicate challenge of budgerigars because as few as 25 sporocysts / gram bodyweight can cause death (Smith et al., 1989). Secondly, collecting wild opossums and screening them for infection is costly, time consuming, unreliable, and an unpredictable method of acquiring sufficient sporocysts for challenge. The intermediate host of *S. neurona* needs to be identified before control of the sylvatic life cycle of the parasite will be feasible. Therefore, the primary aim of this experiment was to find a vertebrate host that would allow completion of the life cycle, and a secondary aim was to find the true natural intermediate host or hosts for *S. neurona*.

Equine protozoal myeloencephalitis is most common in the eastern USA but occurs across the country. It is likely that the intermediate host(s) have a similar distribution. The North American opossum may be found across the continental USA and
therefore is unlikely to limit parasite spread (Gardner, 1982). Additionally, EPM occurs in Central and South America (De Barros et al., 1986; Granstrom et al., 1992; MacKay et al., 1992) and while Didelphis virginiana does not, the two other members of the genus (D. marsupialis and D. albiventris) are found in those regions (Gardner, 1982).

*Sarcocystis falcatula* can infect at least 3 orders of birds as intermediate host. It is quite possible, therefore, that more than one intermediate host exists for *S. neurona*, because the parasites are closely related genetically (Tanhauser et al., 1999). We considered as intermediate host candidates birds which are common in the southeastern US and which, by virtue of body size and population density, would likely be included in a free-ranging opossum’s diet.

Laboratory animals were selected in an attempt to fulfill the primary aim of completing the life cycle. Psittacines are known to be very susceptible to infection by *S. falcatula* (Smith et al., 1989). Even low dose infection can be sufficient to induce a fatal pneumonitis. If the behavior of *S. neurona* in budgerigars (*Melopsittacus undulatus*) was different (i.e. it did not cause fatal disease) it would provide a useful technique to corroborate the results obtained from molecular tools and DNA sequencing. If *S. neurona* could infect budgerigars and sarcocysts were produced then the primary aim would be fulfilled. Immunologically intact NIH Swiss mice (*Mus musculus*) were also selected for challenge as an alternative possibility to complete the *S. neurona* life cycle. Although *S. falcatula* cannot infect mice (differentiating it from *Toxoplasma gondii* [Dubey, 1999]), the ubiquitous use of the mouse in laboratory medicine made it a priority animal to screen for *S. neurona* challenge.
Materials and Methods

Ten brown-headed cowbirds (*Molothrus ater*), 10 boat-tailed grackles (*Cassidix mexicanus*), 10 European starlings (*Sturnus vulgaris*) and 10 redwing-blackbirds (*Agelatus phoeniceus*) were obtained from the United States Department of Agriculture, Gainesville, Florida (courtesy of Dr. Mike Avery). There were five males and five females in each group. Ten female Bobwhite quail (*Colinus virginianus*) were donated by a local breeder. Birds were randomly assigned to 5 groups of 2. Three *S. neurona* isolates and 2 type-2079 isolates were selected for administration to birds. Sporocyst isolates processed as previously described (*chapter three*) were diluted in deionized water to a concentration of 5000 sporocysts /mL. Each bird was administered 100 µL containing 500 sporocysts by dribbling the suspension in their beaks (*Table 4-1*). Any material lost by dripping was noted. Quail were housed in a closed room at Animal Resources and were fed commercial bird food. All other birds were maintained in covered-outdoor aviaries, separated by species, and were fed crushed corn. All birds were euthanized by decapitation at 88 days post-challenge except for the cowbirds, which were euthanized at 74 days post-challenge. All muscle surfaces were examined for evidence of sarcocysts with and without a dissecting microscope. Representative sections of muscles, brain, liver, lung, spleen, heart (myocardium) and kidney were taken and fixed in formalin for histopathological examination.

Fifteen budgerigars were purchased from a local pet store. Fifteen sporocyst isolates were diluted to a working concentration of 10,000 sporocysts /mL deionized water (*Table 4-2*). Birds were housed individually in a single room. Cages were arranged in 5 rows of 3 and had solid floors to minimize cross-contamination with feces. Birds
were challenged in the same room in which they were housed. Birds were first challenged with 250 sporocysts each (for isolates, see Table 4-2) in 250 µL deionized water by dribbling the inoculum into the beak. Clinical signs were monitored for evidence of respiratory distress, feather fluffing, tachypnea or grossly abnormal behavior. Birds were re-challenged at day 27 because no evidence of disease had been noted. For the second challenge, each bird was given 1000 sporocysts in 250 µL deionized water by dribbling the inoculum in to the beak. No loss of inoculum out of the beak was noted for any bird. During the experiment, birds were re-housed twice because of circumstances beyond my control. Birds that survived to day 90 were euthanized by decapitation. Necropsy was performed as soon as possible after death in each case. All muscle surfaces were examined for presence of sarcocysts with and without a dissecting microscope. Representative sections of muscles, brain, liver, lung, spleen, heart (myocardium) and kidney were taken and fixed in formalin for histopathological examination.

Eighteen NIH Swiss mice (24 to 26 g) were purchased from a commercial laboratory animal producer and assigned four per box in a single room. All mice were female. Mice were identified by indelible markings on the tail, which were re-applied as necessary to ensure readability. Mice were assigned randomly into 9 groups (Table 4-3). All isolates were administered in 200 µL deionized water. Five groups received 500 sporocysts of a single isolate per mouse, the 6th group received 100 sporocysts per mouse of each of the same isolates (total 500 sporocysts per mouse), the 7th and 8th groups received 500 sporocysts of a single isolate each and the 9th group received water only. Mice were maintained according to Animal Resources protocol, fed a commercial laboratory animal feed, and checked daily for evidence of overt clinical disease or behavior
change. At 88 days, mice were euthanized by exposure to CO₂ for 15 minutes. Mice were transferred to our laboratory, and were examined for presence of sarcocysts by gross and dissecting microscope examination of all muscle surfaces and on cut section. Representative sections were taken of appendicular and trunk muscles. Sections of internal organs were also sampled for histopathological examination as follows: brain, liver, lung, spleen, heart (myocardium), kidney and tongue.

**Results**

All birds belonging to families Icteridae, Sturnidae and Phasianinae survived the experiment until euthanized at day 74 (cowbirds) or day 88 (all others). Some birds belonging to Psittacidae (budgerigars) succumbed before conclusion of the experiment (Table 4-2). All mice survived until they were euthanized at day 88.

**Gross Necropsy and Histopathology: Non-Psittacidae**

No bird had grossly visible sarcocysts on the surface or cut-section of any of the muscles examined. No stages of *Sarcocystis* sp. or compatible lesions were identified in any bird. Seven of 10 cowbirds had some degree of pulmonary mineralization with or without granulomatous inflammation. Lesions were centered around major airways. Two cowbirds had few, and one had many, intravascular microfilariae. One starling had an extensive granulomatous hepatitis in multiple sections examined. Four other starlings had small scattered inflammatory foci of macrophages and occasional heterophils in liver sections. Individual hepatocellular necrosis was associated with some foci.

Two redwing blackbirds had pulmonary mineralization and granulomatous inflammation around major airways. One bird (RWB#8) had microfilariae within the lung
Three birds had mild multifocal acute hepatic necrosis. There were no abnormal findings in either the grackles or the quail.

**Gross Necropsy and Histopathology: Psittacidae**

In all cases cell necrosis was non-suppurative unless specifically indicated. Results for all birds are summarized in Table 4-4.

Five budgerigars died spontaneously and one was euthanized because of tachypnea and feather fluffing. Not all tissues were available for all birds. Five of the six birds were examined by gross and histopathological methods. One bird (BUD-1, isolate #0000) was too autolyzed to be included in the study. In a second bird (BUD-5, #1112) only the brain was suitable for examination and it was normal. Three birds had been challenged with *S. falcatula* (BUD-1, 3 & 13) and one each with *S. neurona* (BUD-10), type-1085 (BUD-5) and an *S. falcatula* / type-1085 mixture (BUD-14). Three birds had merozoites present in at least one organ (BUD-3, 10 & 13), and one other had what appeared to be merozoites present (BUD-14). One of the *S. falcatula*-challenged birds had skeletal and cardiac muscle sarcocysts (BUD-3), while two others (*S. falcatula*-challenged (BUD-13) and *S. neurona*-challenged (BUD-10)) had skeletal muscle sarcocysts alone. Budgerigar 14 (#1092) had markedly distended liver sinusoids filled with infiltrates of degenerating leukocytes. Moderate autolysis was present throughout which impaired definitive identification of merozoites. Although the major blood vessels and airways of the lung were infiltrated with inflammatory cells and associated necrotic cells, merozoites were not seen. Budgerigar 3 (#1035) had moderate to severe sarcocystosis throughout the skeletal muscle section. Mild inflammatory foci were associated with individual myofiber loss but
not with sarcocysts. Cardiac muscle contained large sarcocysts but also free merozoites associated with infiltrated leukocytes. Hepatic sinusoids were distended with leukocytes but merozoites were not definitively identified. The spleen was similarly affected.

The 9 remaining birds were euthanized on day 90. Two of these birds had merozoites present in at least three organs (BUD-11 & 15). One bird had been challenged with the original type-1085 isolate (#1085) while the other bird received an isolate of unknown species (#1093). Budgerigar #15 (#1093) had a non-suppurative mild encephalitis with merozoites present and with some necrotic cells. Merozoites were also present intraleesionally in sections of skeletal muscle and were associated with severe diffuse myositis. The bird also had an acute pneumonitis with merozoites present intraleesionally, moderate diffuse necrotizing hepatitis and a subacute diffuse severe splenitis with merozoites present.

When merozoites were present they were most common in liver, lung and spleen. When sarcocysts were present they were not associated with inflammation. When merozoites were present they were always associated with inflammatory cells, predominantly lymphocytes and plasma cells and frequently with necrotic cells in the vicinity. Other organs showed mild, unrelated changes that were not considered significant.

**Gross Necropsy and Histopathology: Mice**

No grossly visible sarcocyst was found on the surface or cut-section of any of the muscles examined. No stage of *Sarcocystis* sp. or compatible lesion was identified in any mouse. Two mice had mild multifocal hepatitis without organisms present.
Discussion

The data presented exclude brown-headed cowbirds, grackles, redwing blackbirds, starlings, quail, and mice as natural or alternative intermediate hosts for *S. neurona*. In addition, budgerigars appear unlikely to be able to complete the life cycle, although a single bird administered an *S. neurona* isolate did die. None of the hosts challenged were able to complete the life cycle of type-2079 isolates. All budgerigars challenged with *S. falcatula* developed pneumonitis and died. Some isolates of type-1085 also caused pneumonitis and death in budgerigars. The host infectivity spectrum of *S. falcatula* and type-1085 therefore appear to overlap to include psittacines, but we can only exclude some possible intermediate hosts of *S. neurona* and type-2079. Non-psittacines were not challenged with type-1085 in this trial, and therefore the degree of overlap of host infectivity between type-1085 and *S. falcatula* remains unknown.

All budgerigars that were challenged with *S. falcatula* sporocysts died or were euthanized. One bird that was challenged with *S. neurona* (isolate #1071) also died: it appears unlikely, although not impossible, that this bird developed pneumonitis due to *S. neurona* infection. No other *S. neurona*-challenged bird became diseased or even had subtle histopathological changes. It is possible that isolate #1071 included *S. falcatula* at a concentration below the threshold detectable by our DNA-based classification tools. Isolate #1071 has been retyped and again appeared to be a single population of *S. neurona*. All birds were identified with leg bands and each bird received the same isolate when re-challenged so inadvertent cross-contamination is considered very unlikely. It is possible that the budgerigar accidentally ingested some *S. falcatula*, although we took measures to prevent this. Clubb and Frenkel (1992) reported an acute fatal illness in Old
World psittacines held in an outdoor breeding collection in Florida which was reproduced when cockroaches that had ingested opossum feces were fed to cockatoos and induced an identical fatal illness. In those birds merozoites (presumably *S. falcatula*) were sufficiently numerous to completely occlude pulmonary capillaries. Budgerigars were moved twice during the experiment because of practicalities within Animal Resources, and this may be an additional source of cross-contamination if birds and bird cages were not rematched appropriately.

*Sarcocystis* type-1085 has not been reported previously, other than the defining molecular work from my laboratory (*Tanhauser et al., 1999*). Therefore, we had no basis on which to predict its behavior in birds. Biologically, it appears that type-1085 behaves more like *S. falcatula* than like *S. neurona* in that it can infect, and kill, budgerigars. Coincidentally, limited genomic-DNA sequence comparison shows that type-1085 is more similar to *S. falcatula* than to *S. neurona* (*Tanhauser et al., 1999*). It has been recognized for some time that *S. falcatula* is unusual as a *Sarcocystis* sp. in that it appears to be able to use multiple different intermediate hosts (*Tadros and Laarman, 1982*). Box and Duszynski (1978) have suggested that *S. falcatula* may be more than one species. When originally described, Stiles (1893) reported *S. falcatula* and *Balbiana falcatula* as different species parasitizing the cowbird, although they are now regarded as synonyms (*Levine, 1986*). Box et al. (1984) have also demonstrated that *S. falcatula*, as described, may infect passeriform, psittaciform and columbiform birds. In the latter experiment, infected passeriforms were fed to opossums, the sporocysts generated were fed to columbiforms through two cycles or to psittaciforms through one cycle causing infection in each. Thus, even if Box’s original suggestion is correct, *S. falcatula* as a single organism may infect...
multiple species of bird. Our data are compatible with two similar sarcocystid parasites, although they do not address whether type-1085 can infect brown-headed cowbirds or grackles. In either case, further investigation of type-1085 will be required to characterize its relationship to \textit{S. falcatus}.

Our original hypothesis that budgerigars could act as a biological filter and differentiate between \textit{S. falcatus} and \textit{S. neurona} is strongly supported. With the exception of the budgerigar described above, all \textit{S. falcatus} challenged budgerigars died whereas no type-2079 or \textit{S. neurona}-challenged birds died. Type-1085 appears to yield intermediate results, and will require additional investigation. In addition, the exquisite sensitivity of the budgerigar to \textit{S. falcatus}-challenge may make it valuable in determining whether any given feral opossum isolate has a small component of \textit{S. falcatus} or possibly type-1085 in it. With the administration of $2 \times 10^4$ or more sporocysts to a bird, a threshold detection level of 5\% or less may be attainable. The test would take 10-14 days and in some instances may be almost as rapid as DNA-based techniques given the frequent difficulty in excystation and also the common PCR inhibition thought to be due to other components of opossum feces. The budgerigar-challenge system may even be more sensitive than PCR for identifying small \textit{S. falcatus} components in individual isolates.

Our results are in agreement with Marsh et al. (1997b) who reported fatal pneumonitis in budgerigars after the administration of culture-derived \textit{S. falcatus} merozoites but no disease or pathologic lesion after the administration of \textit{S. neurona} merozoites. The data are complementary because Marsh et al. used merozoites for challenge, whereas we had administered sporocysts. Bypassing the excystation stage did not alter the ability of \textit{Sarcocystis} sp. to cause disease in budgerigars.
The challenge protocol for the budgerigars was unusual in that there were two time points when sporocysts were administered. The presence of sarcocysts in some of the birds that died (range 20-29 days after second challenge, 47 – 56 days after first challenge) is compatible with development from either the first or second challenge (Smith et al., 1989). Sarcocysts may be present in cardiac muscle as early as day 7, but in skeletal muscle rarely occur before day 28 (Smith et al., 1989). It is unclear whether the fatal extent of the pneumonitis was due to the first or second administration because *S. falcatula*-challenged birds succumbed soon after the second challenge (Smith et al., 1987a). Smith et al. (1989) reported death after 39 days with a dose of 25 sporocysts per gram bodyweight. At a dose of 60 sporocysts per gram, death occurred on approximately day 28. The initial dose that we administered was between 10 – 15 sporocysts per gram while the second dose was 40 – 60 sporocysts per gram. The survival of all birds to 27 days after low dose challenge agrees with Smith et al. (1989). It is quite possible that some *S. falcatula*-challenged birds may have developed disease and died if we had waited longer before re-challenging them. However, our primary interest was in *S. neurona* and therefore we chose to re-challenge the birds with a half-order of magnitude increase in dose. The fact that some birds died 10 – 13 days after the higher dose challenge appeared to be temporally related to that second challenge, but is compatible with either.

It is worthy of note that some budgerigars had moderate to severe infiltrates in association with intralesional merozoites on some sections whereas other sections of the same organ appeared normal or had non-specific cellular infiltrates. In light of this finding, it is possible that we did not identify all birds or organs that were infected. Because of the small size of the birds, autolysis had a rapid onset and this was thought to partially obscure
definitive identification of merozoites on some sections. In Table 4-4, where appropriately-sized and stained 1 – 2 µm-wide bodies were seen, but could not be definitively identified, the symbol “?” designates the possible presence of merozoites.

The budgerigar (BUD-15) that was challenged with an unknown *Sarcocystis* species is of particular interest because myositis was severe with many intralesional merozoites present. No sarcocysts were present, however. It is possible that sarcocysts were rare and were present on adjacent sections, but it appears more likely that in this instance the parasite was not able to reach sarcocyst formation. The severe inflammation is not compatible with sarcocyst formation but rather with merogony. The observed pathology may represent infection of an atypical host for this *Sarcocystis* species. No other bird had intralesional merozoites in skeletal muscle. Budgerigar #3 had some free merozoites but sarcocysts were numerous. Further classification of isolate #1093 and challenge of additional birds may provide evidence to explain this unusual finding.

Clinical signs were useful in identifying budgerigars that were developing pneumonitis. Most birds developed feather fluffing and some degree of tachypnea. It would have been useful to have sequentially measured body weight but this is difficult to perform accurately in live budgerigars. A number of budgerigars exhibited significant loss of pectoral muscle at necropsy, although this was obscured by feathering during life. It is likely that development of this group of clinical signs is more useful as an end-point than death because it would permit intervention and euthanasia and also better quality tissue recovery.

The development of sarcocysts in budgerigars is not novel, but is apparently uncommon and was first reported in 1982 (*Box and Smith*). Presumably, most birds do
not survive long enough to develop skeletal muscle sarcocysts (present in an immature form as early as 28 days post infection) and either cardiac muscle was not examined or sarcocysts were not present there. Smith et al. (1989) have stated that cardiac muscle sarcocysts rarely mature, and are absent in budgerigars killed many months after challenge. Regression of myocardial sarcocysts was not observed in our trial because no *S. falcatula*-challenged birds were alive after 40 days.

The presence of merozoites in the liver, lung and spleen of most *S. falcatula*-challenged budgerigars is in agreement with previously published data (Smith et al., 1987a, Smith et al., 1987b). Merozoites were most abundant in pulmonary vasculature, but also were numerous in the liver sinusoid. This is also in agreement with previous reports.

The lack of sarcocyst development in the birds challenged with other *Sarcocystis* sp. was disappointing but not surprising. *Sarcocystis neurona* may be a more typical *Sarcocystis* sp. and have a narrow intermediate host range. The range of aberrant hosts that have developed disease after infection with *S. neurona* or *S. neurona*-like organisms stands in contrast to this, however (Dubey et al., 1987; Dubey et al., 1990; Dubey and Hedstrom, 1993; Dubey et al., 1994; Klumpp et al., 1994; Dubey et al., 1996; Dubey et al., 1998;). If *S. neurona* were capable of infecting brown-headed cowbirds and grackles, we might have expected to identify a mixed infection in laboratory opossums fed naturally-infected cowbirds in previous experiments (Dame et al., 1995). The presence of only incidental findings in all birds examined by histopathology provides strong evidence that future investigations into possible hosts should be directed towards other bird species, other mammals, or even amphibians.
We did not attempt to identify sarcocysts in any species of bird by digesting muscle, although a pepsin and trypsin digestion method was described by Box and Smith (1982). It may be more sensitive than gross examination alone but that method failed to identify the rare sarcocysts in pigeons (*Columba livia*) which subsequently infected naïve opossums. The early conclusion that pigeons did not complete the life cycle of *S. falcataula* was revised when it was demonstrated that pigeons do in fact form limited numbers of sarcocysts when challenged (Box et al., 1984).

The location of the pulmonary lesions identified in the brown-headed cowbirds and redwing blackbirds (centered on major airways) suggests that birds aspirated a portion of the inoculum. The inoculum was administered by dripping into the buccal cavity to avoid the possible trauma of passing an oro-esophogeal tube in wild birds. Birds were held firmly during the challenge, but were permitted limited control of their heads. No evidence of respiratory or other distress was noted during challenge or within the 12 hours before returning the birds to the aviaries.

The microfilariae seen in redwing blackbird #8 possibly could have been merozoites rather than fragments of microfilariae; however, this is considered unlikely because so few were present, neither its group mate RWB#7 nor other challenged birds had merozoites identified, and the birds were wild-caught adults with natural parasite burdens. Many of the cowbirds also had intravascular microfilariae definitively identified and had been housed in adjacent aviaries.

The hepatitis described in 3 blackbirds was mild and was considered unlikely to be clinically significant. Additionally, no organisms were identified in any of the foci. However, we did not attempt to recover viable organisms by tissue culture of
homogenized organs. This could have been a useful corroboration of the histopathology results, or would have been a more sensitive test if organisms were isolated.

In retrospect, and in light of the results of the other experiments, it is not surprising that no mice challenged here developed sarcocysts. Marsh et al. (1997a) had demonstrated that *S. neurona* administration to nude mice resulted in development of encephalitis. The suggestion that the immune status was a critical factor in determining the susceptibility of those mice to *S. neurona* is supported by our results. In another experiment (Dubey and Lindsay, 1998), nude and gamma interferon-knockout mice were fed *S. neurona* sporocysts and became lethargic and developed encephalitis. *Sarcocystis neurona* was grown in tissue culture from a liver / spleen / brain homogenate taken from a nude mouse on day 11. Protozoa were identified in neural tissue as early as day 21. In contrast, *S. falcatula* was harmless to both species of mouse when given as either merozoite or sporocyst. Finally, Dubey fed sporocysts from two wild-caught (Dubey et al., 1998) opossums to budgerigars, nude mice, and gamma-interferon knockout mice. No mouse fed sporocysts of *S. falcatula* developed infection. Mice challenged with *S. neurona* developed encephalitis, and merozoites were confirmed to be *S. neurona* by immunohistochemistry. A third *Sarcocystis* sp. (possibly the species we designate type-2079, [S. Tanhauser, personal communication]) described in that paper was also administered to mice. Disease was similar to that induced by *S. neurona* but schizonts and merozoites, which were predominantly found in the liver, did not react with *S. neurona* antibodies. Additionally, sarcocysts were found in leg muscles of 2 mice killed on days 50 and 54.
The presence of hepatitis in two mice in our study may be coincidence or may represent subtle disease due to *Sarcocystis* sp. infection in partially-resistant mice. One of two mice challenged with a type-2079 isolate (#1058) had a mild necrotizing, focal, suppurative hepatitis infiltrated with degenerate neutrophils. No organisms were identified with Giemsa stain. Its group mate was normal. The second mouse was 1 of 2 challenged with *S. neurona* isolate #1112 and developed necrosuppurative hepatitis with multiple small foci of acute hepatocellular necrosis. Giemsa staining of slides did not reveal organisms. The second group mate was normal. Although disease cannot be completely excluded, the NIH Swiss mice challenged here offer less potential for understanding the life cycle of opossum-shed *Sarcocystis* sp. than do the nude or gamma-interferon knockout mice used by others.

Box and Smith noted in 1982 that pigeons, which are Columbiforms, were intermediate in their susceptibility to *S. falcatula* infection. Merogony was present but the authors felt pigeons were resistant to muscle meronts. Although in that instance sarcocysts were later identified (Box et al., 1984), the salutary lesson is that infection in partially-susceptible prey (or intermediate hosts) is likely to be subtle and all means to detect it should be employed. We are confident that we have not erroneously excluded the true intermediate host of *S. neurona*, but it is possible that merogony did occur at low levels in some of these birds and was not identified.

Future challenge of mice should be attempted in animals that have been selectively bred to be specifically immunodeficient. The identification of some birds and mice with mild nonspecific hepatitis is of concern. This may represent unrelated disease, but it is also possible that we missed evidence of merozoites. Future efforts in these areas would
be well improved with the addition of a tissue culture protocol to attempt recovery of any viable organisms present. Dubey has demonstrated the potential value of this corroborative evidence (Dubey et al., 1998).

Members of the genus *Sarcocystis* (Lankester, 1882) undergo asexual proliferation by processes of merogony and external budding and replication by endodyogeny. Sarcocysts are typically present in striated muscle and merozoites are elongate. The sarcocyst form of *S. neurona* has yet to be described, although it clearly must exist. The only known host is the horse which is considered aberrant because sarcocysts of *S. neurona* have not been identified in horses and because the organism has a neurotropism in horses. Affected horses, therefore, present no risk to group mates, nevertheless, outbreaks of disease have been reported (Fenger et al., 1997b). In contrast with other *Sarcocystis* sp. the tendency for *S. neurona* (or *S. neurona*-like organisms) to infect atypical hosts and cause neurologic disease has been reported on numerous occasions. Encephalitis due to *S. neurona* (or *S. neurona*-like organisms) has been reported in a colony of mink (Dubey and Hedstrom, 1993), a striped skunk (Dubey et al., 1996), a rhesus monkey (Klumpp et al., 1994), a cat (Dubey et al., 1994), a steer (Dubey et al., 1987), harbor seals (Lapointe et al., 1998) and caused fatal disease in a raccoon (Dubey et al., 1990). This is a very broad range of hosts by any comparison. The fact that aquatic mammals can be affected is interesting. Hepatitis was recently reported in polar bears caused by a *Sarcocystis* sp., but the merozoites did not stain with *S. neurona* antibodies (Garner et al., 1997). It is quite possible that some of the *S. neurona*-like organisms are not *S. neurona* at all, but that epitopes have been conserved among some *Sarcocystis* sp. The strong neurotropism of *S. neurona* and the host spectrum are very uncharacteristic of
Sarcocystis sp. in general, and mean some question remains about the final classification of S. neurona. The encephalitis experimentally induced in laboratory mice by two different Sarcocystis sp. (Dubey et al., 1998) is the logical extension of this literature and capitalizes on the commonality of immunosuppression in most of the aberrant hosts affected. The mouse model opens a new line of investigation which should prove very productive.

In summary, the results presented suggest that unlike S. falcatula, neither S. neurona nor type-2079 can use the brown-headed cowbird or grackles as intermediate hosts. In addition no quail, starling or redwing blackbird developed convincing evidence of disease after challenge with either S. neurona or type-2079. Type-1085 isolates can behave similarly to S. falcatula and induce fatal pneumonitis in budgerigars. Merozoites can be found in multiple organs, and the pathology is similar to that seen with S. falcatula infection. However, not all type-1085 organisms caused disease, and no bird developed sarcocysts. The experimental information provided is incomplete and further evaluation is certainly warranted. Immunocompetent mice are not a suitable intermediate host substitute for S. neurona, S. falcatula, type-1085 or type-2079 on the basis of the limited replicate pairs described here. The information presented is in agreement with previously published molecular evidence that the opossum can be infected with at least 4 different types of Sarcocystis sp. Furthermore, these data suggest that the 4 different classifications are different species on the basis of their individual ability to infect the different hosts challenged and reported here. Immunodeficient laboratory animals may provide the next major advance in understanding the life cycle of these opossum-borne Sarcocystis sp., and show considerable promise as an alternative model to study S. neurona.
Table 4-1. Administration of sporocysts to brown-headed cowbirds, boat-tailed grackles, Bobwhite quail, European starlings and redwing blackbirds.

<table>
<thead>
<tr>
<th>Bird ID#</th>
<th>Bird ID#</th>
<th>Bird ID#</th>
<th>Bird ID#</th>
<th>Number of sporocysts</th>
<th>Isolate ID#</th>
<th>Species Identity</th>
<th>Test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHCB-1</td>
<td>BTG-1</td>
<td>BWQ-7</td>
<td>ES-1</td>
<td>500</td>
<td>1013</td>
<td>neurona</td>
<td>10 n</td>
</tr>
<tr>
<td>BHCB-2</td>
<td>BTG-2</td>
<td>BWQ-8</td>
<td>ES-2</td>
<td>500</td>
<td>1013</td>
<td>neurona</td>
<td>10 n</td>
</tr>
<tr>
<td>BHCB-3</td>
<td>BTG-3</td>
<td>BWQ-5</td>
<td>ES-3</td>
<td>500</td>
<td>1062</td>
<td>type-2079</td>
<td>sequenced</td>
</tr>
<tr>
<td>BHCB-4</td>
<td>BTG-4</td>
<td>BWQ-6</td>
<td>ES-4</td>
<td>500</td>
<td>1062</td>
<td>type-2079</td>
<td>sequenced</td>
</tr>
<tr>
<td>BHCB-5</td>
<td>BTG-5</td>
<td>BWQ-1</td>
<td>ES-5</td>
<td>500</td>
<td>1062</td>
<td>neurona</td>
<td>10 n</td>
</tr>
<tr>
<td>BHCB-6</td>
<td>BTG-6</td>
<td>BWQ-2</td>
<td>ES-6</td>
<td>500</td>
<td>1062</td>
<td>neurona</td>
<td>10 n</td>
</tr>
<tr>
<td>BHCB-7</td>
<td>BTG-7</td>
<td>BWQ-9</td>
<td>ES-9*</td>
<td>500</td>
<td>1071</td>
<td>neurona</td>
<td>10 n</td>
</tr>
<tr>
<td>BHCB-8</td>
<td>BTG-8</td>
<td>n/a</td>
<td>ES-10</td>
<td>500</td>
<td>1071</td>
<td>neurona</td>
<td>10 n</td>
</tr>
<tr>
<td>BHCB-9</td>
<td>BTG-9</td>
<td>BWQ-3</td>
<td>ES-7</td>
<td>500</td>
<td>1058</td>
<td>type-2079</td>
<td>sequenced</td>
</tr>
<tr>
<td>BHCB-10</td>
<td>BTG-10</td>
<td>BWQ-4</td>
<td>ES-8</td>
<td>500</td>
<td>1058</td>
<td>type-2079</td>
<td>sequenced</td>
</tr>
</tbody>
</table>

BHCB = brown-headed cowbird, BTG = boat-tailed grackle, BWQ = Bob White quail, ES = European starling, RWB = redwing blackbird. Bird ES-9* died shortly after challenge due to a severe mite infestation. Bird BWQ-10 died prior to challenge and is not shown in the table. Brown-headed cowbirds were euthanized on day 74 while all other birds were euthanized on day 88. Test results are number of marker positions indicating the *Sarcocystis* sp. in the isolate is falcata-like (f) or neurona-like (n).
Table 4-2. Administration of sporocysts to budgerigars and summary of results.

<table>
<thead>
<tr>
<th>Bird ID#</th>
<th>Group</th>
<th>Number of sporocysts</th>
<th>ID# of isolate</th>
<th>Species identity</th>
<th>Test results</th>
<th>Day # died</th>
<th>Manner of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUD-1</td>
<td>1</td>
<td>1000</td>
<td>0000</td>
<td>falcatus</td>
<td>10 f</td>
<td>13</td>
<td>died</td>
</tr>
<tr>
<td>BUD-2</td>
<td>2</td>
<td>1000</td>
<td>1051</td>
<td>neurona</td>
<td>10 n</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-3</td>
<td>3</td>
<td>1000</td>
<td>1035</td>
<td>falcatus</td>
<td>10 f</td>
<td>28</td>
<td>euthanized early</td>
</tr>
<tr>
<td>BUD-4</td>
<td>4</td>
<td>1000</td>
<td>1129</td>
<td>unknown</td>
<td>--</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-5</td>
<td>5</td>
<td>1000</td>
<td>1114</td>
<td>type-1085</td>
<td>3 n, 1 f</td>
<td>20</td>
<td>died</td>
</tr>
<tr>
<td>BUD-6</td>
<td>6</td>
<td>1000</td>
<td>1013</td>
<td>neurona</td>
<td>10 n</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-7</td>
<td>7</td>
<td>1000</td>
<td>1112</td>
<td>neurona</td>
<td>10 n</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-8</td>
<td>8</td>
<td>1000</td>
<td>1058</td>
<td>type-2079</td>
<td>sequenced</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-9</td>
<td>9</td>
<td>1000</td>
<td>1062</td>
<td>type-2079</td>
<td>sequenced</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-10</td>
<td>10</td>
<td>1000</td>
<td>1071</td>
<td>neurona</td>
<td>10 n</td>
<td>29</td>
<td>died</td>
</tr>
<tr>
<td>BUD-11</td>
<td>11</td>
<td>1000</td>
<td>1085</td>
<td>type-1085</td>
<td>8 n, 2 f</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-12</td>
<td>12</td>
<td>1000</td>
<td>1086</td>
<td>type-1085</td>
<td>8 n, 2 f</td>
<td>90</td>
<td>experiment ended</td>
</tr>
<tr>
<td>BUD-13</td>
<td>13</td>
<td>1000</td>
<td>1089</td>
<td>falcatus</td>
<td>10 f</td>
<td>20</td>
<td>died</td>
</tr>
<tr>
<td>BUD-14</td>
<td>14</td>
<td>1000</td>
<td>1092</td>
<td>falcatus &amp; type-1085</td>
<td>mixture</td>
<td>6</td>
<td>died</td>
</tr>
<tr>
<td>BUD-15</td>
<td>15</td>
<td>1000</td>
<td>1093</td>
<td>unknown</td>
<td>---</td>
<td>90</td>
<td>experiment ended</td>
</tr>
</tbody>
</table>

Day # killed is from date of second challenge (with 1000 sporocysts), which was 27 days after initial challenge with 250 sporocysts per bird. Test results are numbers of marker positions indicating the *Sarcocystis* sp. in the isolate is falcatus-like (f) or neurona-like (n).

Table 4-3. Administration of sporocysts to NIH Swiss mice.

<table>
<thead>
<tr>
<th>Mouse ID#</th>
<th>Group</th>
<th>Number of sporocysts</th>
<th>Isolate ID#</th>
<th>Species identity</th>
<th>Test results</th>
<th>Notes on administration</th>
<th>Day # killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 bar dot 1 bar</td>
<td>1</td>
<td>500</td>
<td>1051</td>
<td>neuron</td>
<td>10 n</td>
<td>OK</td>
<td>88</td>
</tr>
<tr>
<td>2 bar dot 2 bar</td>
<td>2</td>
<td>500</td>
<td>1051</td>
<td>neuron</td>
<td>10 n</td>
<td>OK</td>
<td>88</td>
</tr>
<tr>
<td>3 bar dot 3 bar</td>
<td>3</td>
<td>400-500</td>
<td>1013</td>
<td>neuron</td>
<td>10 n</td>
<td>175 uL (not 200 uL)</td>
<td>88</td>
</tr>
<tr>
<td>4 bar dot 4 bar</td>
<td>4</td>
<td>500</td>
<td>1085</td>
<td>neuron</td>
<td>10 n</td>
<td>OK</td>
<td>88</td>
</tr>
<tr>
<td>5 bar dot 5 bar</td>
<td>5</td>
<td>500</td>
<td>1071</td>
<td>neuron</td>
<td>10 n</td>
<td>OK</td>
<td>88</td>
</tr>
<tr>
<td>6 bar dot 6 bar</td>
<td>6</td>
<td>500</td>
<td>Mix*</td>
<td>neuron</td>
<td>10 n</td>
<td>OK</td>
<td>88</td>
</tr>
<tr>
<td>7 bar dot 7 bar</td>
<td>7</td>
<td>500</td>
<td>1112</td>
<td>neuron</td>
<td>10 n</td>
<td>OK</td>
<td>88</td>
</tr>
<tr>
<td>8 bar dot 8 bar</td>
<td>8</td>
<td>500</td>
<td>0000</td>
<td>falcatus</td>
<td>10 f</td>
<td>OK</td>
<td>88</td>
</tr>
<tr>
<td>black tail Control</td>
<td>--</td>
<td>500</td>
<td>0000</td>
<td>[water]</td>
<td>[water]</td>
<td>n/a</td>
<td>88</td>
</tr>
<tr>
<td>black tail Control</td>
<td>--</td>
<td>500</td>
<td>0000</td>
<td>[water]</td>
<td>[water]</td>
<td>n/a</td>
<td>88</td>
</tr>
</tbody>
</table>

mix* is a composite of the following isolates #1051, #1058, #1013, #1085 and #1071. n/a= not applicable. Test results are number of marker positions indicating the *Sarcocystis* sp. in the isolate is falcatus-like (f) or neurona-like (n).
## Table 4-4. Results of histopathological examination of budgerigars.

<table>
<thead>
<tr>
<th>Bird ID#</th>
<th>ID# of isolate</th>
<th>Species identity</th>
<th>Notes</th>
<th>Sc</th>
<th>Merozoites</th>
<th>Brain</th>
<th>Skeletal muscle</th>
<th>Liver</th>
<th>Kidney</th>
<th>Lung</th>
<th>Heart</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUD-1</td>
<td>0000 falcataula</td>
<td>died</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>BUD-2</td>
<td>1051 neuronana</td>
<td>--</td>
<td>--</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>Ureter with subepithelial infiltrate lymphs</td>
<td>NSL</td>
<td>NSL</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>BUD-3</td>
<td>1035 falcataula</td>
<td>killed</td>
<td>H Sk</td>
<td>H Lu ?Lv</td>
<td>NSL</td>
<td>mild myositis</td>
<td>severe diffuse hepatitis</td>
<td>--</td>
<td>lymph. infiltrate</td>
<td>mild myocarditis</td>
<td>mononuc infiltrate</td>
<td></td>
</tr>
<tr>
<td>BUD-4</td>
<td>1129 unknown</td>
<td>--</td>
<td>--</td>
<td>NSL</td>
<td>NSL</td>
<td>--</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>BUD-5</td>
<td>1114 type-1085</td>
<td>died</td>
<td>--</td>
<td>NSL</td>
<td>--</td>
<td>autolysis</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-6</td>
<td>1013 neuronana</td>
<td>--</td>
<td>NSL</td>
<td>--</td>
<td>lipidosis</td>
<td>NP</td>
<td>congested, diffuse acute hemorrhage</td>
<td>NSL</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-7</td>
<td>1112 neuronana</td>
<td>--</td>
<td>--</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>BUD-8</td>
<td>1058 type-2079</td>
<td>--</td>
<td>NSL</td>
<td>--</td>
<td>NSL</td>
<td>NSL</td>
<td>interstitial nephritis</td>
<td>NSL</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-9</td>
<td>1062 type-2079</td>
<td>--</td>
<td>NSL</td>
<td>--</td>
<td>--</td>
<td>chronic moderate periportal hepatitis and fibrosis</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-10</td>
<td>1071 neuronana</td>
<td>died</td>
<td>Sk Lu ?Sp</td>
<td>?Lv</td>
<td>some necrotic cells</td>
<td>mild myositis</td>
<td>focal hepatic necrosis*</td>
<td>--</td>
<td>necrosis</td>
<td>mononuc. infiltrate</td>
<td>diffuse infiltrate</td>
<td></td>
</tr>
<tr>
<td>BUD-11</td>
<td>1085 type-1085</td>
<td>--</td>
<td>C Lu Lv ?Sp</td>
<td>vacuolar degeneration</td>
<td>NP</td>
<td>severe hepatitis, diffuse</td>
<td>moderate interstitial nephritis; mild acute tubular necrosis</td>
<td>mononuc. infiltrate</td>
<td>moderate myocarditis, multifocal, with mononuc. infiltrate</td>
<td>splenitis with infiltrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-12</td>
<td>1086 type-1085</td>
<td>--</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>NSL</td>
<td>multifocal lymphocytic infiltrates</td>
<td>NSL</td>
<td>NSL</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Code</td>
<td>Species</td>
<td>Died</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------------</td>
<td>-------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-13</td>
<td>1089</td>
<td>falcata</td>
<td>died</td>
<td>Sk</td>
<td>Lu</td>
<td>vacuolar degeneration</td>
<td>mild myositis</td>
<td>severe diffuse -- hepatitis, subacute</td>
<td>interstitial pneumonia</td>
<td>severe acute diffuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-14</td>
<td>1092</td>
<td>falcata &amp; type-1085</td>
<td>--</td>
<td>?Lv</td>
<td>congested</td>
<td>mild autolysis</td>
<td>infiltrate in sinusoids*</td>
<td>--</td>
<td>infiltrate in airways*</td>
<td>mild autolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUD-15</td>
<td>1093</td>
<td>unknown</td>
<td>--</td>
<td>B</td>
<td>Sk</td>
<td>Li</td>
<td>encephalitis</td>
<td>severe myositis**</td>
<td>hepatitis</td>
<td>moderate**</td>
<td>moderate pneumonia, acute, multifocal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lu</td>
<td>Sp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NSL</td>
<td>severe splenitis, diffuse</td>
<td></td>
</tr>
</tbody>
</table>

*=some autolysis; **=merozoites intralesionally. H= heart, Lu= lung, Lv= liver, Sp= spleen, ?= merozoites not identified with certainty; mononuc.= mononuclear cells; lymph= lymphocyte; Sk= skeletal muscle; Sc= sarcocyst; NP= not present in block; NSL= no significant lesions.

Table 4-4. Results of histopathological examination of budgerigars, continued.

Figure 4-1. Photograph of brown-headed cowbirds (*Molothrus ater*).
Figure 4-2. Photograph of a boat-tailed grackle (*Cassidix mexicanus*).

Figure 4-3. Photograph of a European starling (*Sturnus vulgaris*).

Figure 4-4. Photograph of a redwing blackbird (*Agelaius phoeniceus*).
Figure 4-5. Photograph of Bobwhite quail (*Colinus virginianus*).

![Bobwhite quail](image)

Figure 4-6. Photograph of budgerigars (*Melopsittacus undulatus*).

![Budgerigars](image)
CHAPTER 5

DIDELPHIS VIRGINIANA: DEFINITIVE HOST OF MULTIPLE SARCOCYSTIS SP.

Introduction

The North American opossum (Didelphis virginiana) is the only identified definitive host of S. neurona (Fenger et al., 1995). The intermediate host(s) in this heteroxenous life cycle is (are) as yet unidentified. Levine (Levine and Tadros, 1980; Levine, 1986) reported 4 Sarcocystis sp. of Didelphis sp. opossums: 2 use D. virginiana as a definitive host (S. falcatula Stiles, 1893; Box et al., 1984) and S. rileyi (Stiles, 1893) and 2 use D. marsupialis as an intermediate host (S. garnhami and S. didelphidis). Sarcocystis falcatula had been extensively studied and re-described by Box and colleagues in the 1980s (Box and Duszynski, 1980; Box and Smith, 1982; Box et al., 1984). Sarcocystis neurona was named when first recovered from diseased equine spinal cord and grown in cell culture (bovine monocyte M617) by Dubey et al. (1991). All attempts to recreate the life cycle of S. neurona must incorporate collection of infected opossums as a source of sporocysts. Ultimately, the identification of an intermediate host would allow generation of sporocysts in the laboratory by experimental infection of definitive and intermediate hosts. Large scale collection of opossums would provide not only a source of sporocysts, but also would provide descriptive data of the natural population that might
lead to a better understanding of the natural spread of the organism and perhaps identify risk factors for the development of EPM in horses.

It was postulated that *S. neurona* was synonymous with *S. falcatus* (Dame et al., 1995) and, in fact, the DNA sequence of the 18s rRNA gene of *S. falcatus* was identical to that published for *S. neurona* (Fenger et al., 1994). The inability to recreate EPM in horses with *S. falcatus* sporocysts from laboratory opossums fed infected brown-headed cowbirds (*Molothrus ater*) forced reconsideration of that hypothesis (Cutler et al., 1999). Molecular tools have now been reported that demonstrate *S. neurona* and *S. falcatus* are different, and that additional *Sarcocystis* sp. are shed by the opossum (Tanhauser et al., 1999). Equine protozoal myeloencephalitis has been reproduced both using an uncharacterized sporocyst homogenate (Fenger et al., 1997a) and using characterized *S. neurona* sporocysts (chapter three).

We undertook a large scale collection of road killed and trapped opossums as a source of sporocysts for our host challenge experiments. It was suspected that the descriptive data collected during this survey might be useful in determining risk factors for infection of individual opossums. Although a number of state agencies collect data on numbers of nuisance animals killed on the highways, we are not aware of any data describing opossum-borne *Sarcocystis* sp. The results reported here describe the molecular classifications of 87 sporocyst isolates from 419 opossums collected over a 2-year period. Factors which might affect shedding pattern and intensity of infection, such as age, gender, weight, time of year and location are of specific interest and are also reported.
Materials and Methods

Opossum Collection

Opossums were collected either as roadkill or were live-trapped in the state of Florida between November 1996 and December 1998. Posters and radio advertising was used to maximize reporting of roadkilled opossums (Figure 5-1). Collections were made in a discontinuous fashion because of the seasonality of opossum movements (and hence numbers killed on roads) and also to permit examination of collected material. Overall numbers collected were in part driven by the need to accumulate *S. neurona* sporocysts for horse challenge experiments.

Gender and weight were recorded and a unique identification number was assigned. Some samples were provided to my laboratory as intestinal tracts only, (without other information). Necropsies were performed as soon as possible after collection. The gastrointestinal tract was isolated and removed intact through an incision in the midline of the ventral abdominal wall. The mesentery was stripped off and the intestinal contents milked into a clean, labeled container. A section of distal ileum was incised, flattened and the mucosal surface exposed. Excess ingesta were washed off the mucosa with deionized water and then mucosa was collected by scraping with a microscope slide held at a 45-degree angle. A 2-g sample of feces and a 1-mL sample of intestinal-mucosal scrapings were taken from the original samples and each was homogenized in 10 mL deionized water. Mucosal scrapings were passed through cheesecloth into labeled 15 mL tubes and were centrifuged for 10 minutes at 800 g. Pellets were re-suspended in saturated Sheather’s sugar solution and transferred onto coverslips by centrifugation for 10 minutes
at 800 \text{g}. Coverslips were scanned at 100 x magnification to determine if sporocysts were present. Sporocysts were frequently noted to have a pink hue if examined within 12 hours of removing the intestinal tract. When sporocysts were identified, the remaining gastrointestinal tract mucosa from that animal was recovered. Infected live opossums were killed (Beuthanasia-D Special; Schering Plough Animal Health, Kenilworth, New Jersey) and their gastrointestinal tracts recovered. Mucosal scrapings were mixed in to an equal volume of 5.25\% NaOCl (commercial bleach) on ice for 30 minutes and stirred every 10 minutes. Tissue aggregates were removed by pouring the sporocyst suspension through gauze mesh. Bleach was removed by 2-3 washes in deionized water. The sporocysts were pelleted at 800 \text{g} for 10 minutes between washes and finally stored at 4 \text{C} in phosphate-buffered saline (PBS) with 100 U/mL penicillin, 100 \mu g/mL streptomycin and 50 \mu g/mL gentamicin to prevent bacterial overgrowth. The final sporocyst count of each isolate was established by counting at 400 x magnification using a hemocytometer. Periodically, samples were recounted to determine if spontaneous excystation was occurring in the storage media.

**Density Purification of Sporocysts**

A density gradient was prepared by underlayering a 2 mL aliquot of isolate (containing 10,000 sporocysts), with 3 mL each of 60\%, 30\% and finally 20\% Percoll gradient. Sample tubes were centrifuged in a swinging bucket rotor SW41 (Beckman L870 centrifuge; Beckman Instruments, California) at 22,500 \text{g} for 30 minutes at 25 \text{C}. Most sporocysts were present at the interface between 30\% and 60\% percent Percoll. This material was collected and washed twice with Hanks’ buffered saline solution (HBSS),
and finally stored in HBSS with 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL gentamicin. Sporocysts so purified were used for DNA extraction when DNA extracts from unpurified samples failed to amplify (due to PCR inhibition).

**Excystation of Sporocysts**

Aliquots of 10,000 sporocysts each were pelleted by centrifugation and resuspended in 200 µL undiluted equine bile (pH 7.0) containing 2% w/v purified trypsin (Sigma T-8642; Sigma Chemical Company, St. Louis, Missouri). Sporocysts were then incubated at 37 C for up to 12 hours to allow maximal excystation. Excystation was confirmed by examination at 100 x magnification.

**Polymerase Chain Reaction**

Excysted sporozoites were pelleted by centrifugation for 10 minutes at 1,600 g in a microcentrifuge. DNA was extracted and then amplified using up to 6 separate pairs of RAPD primers (Tanhauser et al., 1999) as previously described. A negative control was included which contained all components of the PCR mixture except template. The possibility of contamination by extraneous amplicons was minimized by performing reaction setup, sample extraction, and amplification / analysis in three separate laboratories. Reaction tubes were closed in the room in which the reaction mixtures were prepared and only opened after amplification. PCR products were analyzed by agarose gel electrophoresis using a 1% agarose gel (NuSieve 3:1; FMC, Rockport, Maine).

Up to six molecular tools were used to characterize most isolates. Briefly, a tool consists of two specific primers used to amplify a DNA sequence and a corresponding
restriction endonuclease digestion enzyme pair capable of positively identifying a neurona-
like or falcatula-like sequence. Identifications were by positive criteria, i.e. neurona-like
isolates were cut like *S. neurona* only, and falcatula-like isolates were cut like *S. falcatula*
only. One tool amplifies a different length product from neurona-like isolates than from
falcatula-like isolates. Final categorization depends on the collective results from all tools:
certain combinations of results characterize the non-falcatula/non-neurona isolates termed
type-1085 (named after the initial isolate numbers showing those characteristics). Isolates
designated type-2079 were classified after sequencing of portions of the ITS sequence
because those isolates do not amplify with most of the molecular tools utilized. The DNA
sequences are compared with the sequences of both *S. neurona* and *S. falcatula* to ensure
that they are different.

**Restriction Endonuclease Digestion**

Amplified DNA from isolates was first run on an 1.5% agarose gel to demonstrate
that DNA was present in sufficient quantity to be clearly visible after digestion. Two
 aliquots of 10 µL of PCR-amplified product were incubated with 5 uL of one or other
restriction endonucleases in a waterbath at 37 C for 1-2 hours according to manufacturer’s
instructions (Pharmacia, Kalamazoo, Michigan). At the conclusion of the digest, another
agarose gel is prepared with the following lanes: ladder, undigested product, product
digested with first enzyme, product digested with second enzyme. In each case, the digest
enzyme pairs are selected to positively identify a neurona-like or falcatula-like sequence.
Results

Opossum Collection

Four hundred nineteen opossums were collected between November 1996 and November 1998. Eighty seven were infected (21% infection prevalence). Descriptive data are available for 81 of those isolates, 78 of which were classified as single isolates. Summary data are shown by month (Table 5-1) and by county (Table 5-2). Two skunks (M. mephitis) were also collected and were negative.

Table 5-3 shows the average, median, minimum and maximum yield for each species or type and for the total collection. Table 5-4 shows the shedding of sporocysts by weight and in Table 5-5 the data are further classified by gender. Table 5-6 shows the breakdown of shedding in opossums by gender.

Categorization of Isolates

All positive and negative controls appeared as expected, confirming that no accidental contamination of samples occurred during preparation or handling, and that reaction conditions were suitable for amplification of DNA. The breakdown of the 81 isolates by species and type examined is given in Table 5-3. Excystation of sporocysts of most isolates was poor (average 15%; range 10-40%).

Discussion

Equine protozoal myeloencephalitis is a common and often devastating disease in horses in the United States. The apparent increase in disease incidence and confusion about the life cycle of the causative organism required further investigation of the
definitive host and dissemination of infective sporocysts. In a review of all *Sarcocystis* sp. known at the time, Levine (1986) listed just one (*S. falcatula*) that utilized the opossum as a natural definitive host. In addition, experimental evidence was available that the opossum could become infected with, and shed, *S. rileyi* (Levine, 1986). It had been speculated that *S. falcatula* might not be a single species, but rather be several very similar parasites that infected different birds (Box and Duszynski, 1978). Early horse-challenge experiments suggested that sporocysts from more than one *Sarcocystis* species was shed by opossums (Fenger et al., 1997a; Cutler et al., 1999). Further investigation using molecular techniques confirmed this. Consequently, our laboratory has recently reported a series of molecular tools which differentiate among *S. neurona*, *S. falcatula* and other *Sarcocystis* sp. parasites of the opossum (Tanhauser et al., 1999). Those tools were used to characterize sporocysts from naturally infected opossums and permit the description of sporocysts in this paper. These data again call in to question whether *S. falcatula*, as defined, is a single organism or not. The isolate that we have designated type-1085 can at least infect and kill budgerigars (chapter four), and may well behave similarly to *S. falcatula*.

It is now clear that opossums can shed sporocysts of at least 4 distinguishable DNA types. These include at least 2 species (*S. neurona* and *S. falcatula*). It remains to be demonstrated that the other classifications we have used actually represent independent species. It is possible that 1 or more of them do not even belong to the genus *Sarcocystis*. The identification of possible additional *Sarcocystis* sp. is not surprising on the basis of the ability of opossum-derived sporocysts to infect different hosts as described elsewhere in this thesis. Indeed, some of these species may already be known in their intermediate
hosts. A number of traditional parasitology questions involving life cycle are therefore raised.

Some of the isolates collected have been administered to horses: *S. neurona* sporocysts induced the disease EPM (chapter three) whereas *S. falcatula* did not (Cutler et al., 1999).

The most recent data available in the United States indicate that approximately half of US horses have antibodies against *S. neurona* (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997). Our experiences attempting to identify a large group of seronegative horses is that seroprevalence may already be higher than that (T. Cutler and R. MacKay, unpublished observations). On the basis of these data, it is somewhat surprising that the prevalence of infection among opossums reported in this paper is so low (21%). Furthermore, to date, only 3.8% (16) of the total (419) have been identified as shedding *S. neurona*. Because of difficulty extracting DNA from low concentration isolates, and possibly because of inhibitors contained in the samples, the slight majority of isolates (40) have yet to be definitively typed. Therefore, the proportion of *S. neurona* isolates may currently be underestimated. Of the other isolates classified, 13 were *S. falcatula*, 6 were type-1085 and 3 were type-2079. Six isolates were classified as mixed infections of 2 species. Three of these isolates had only a small percentage of the second species present and for data purposes in this paper are considered single isolates of the predominant species.

Prevalence of infection was highest in opossums recovered in the 2nd quarter and was similar in both years (mean, 35%, Table 5-1). This quarter also included the highest collection rates. It is possible that an interaction exists between diet and opossum
movement, or consequences of searching for a new territory, such as stress. Opossums recovered in the 1st quarter were almost as likely to be infected (mean, 21%) as in the 2nd quarter, while in the 3rd and 4th quarters far fewer opossums were infected (10% and 12% respectively). There was a strong statistical difference between prevalence in the 2nd quarter and other quarters in 1997 ($p=1 \times 10^{-6}$), between 1st and 2nd quarters compared to 3rd and 4th quarters in both years ($p=3 \times 10^{-6}$, $p=5 \times 10^{-11}$), and a strong tendency between 2nd quarter of 1998 and other quarters combined ($p=0.012$). The increased prevalence in opossums in the 2nd quarter may indicate a seasonal change in either the numbers of intermediate hosts or the proportion of intermediate hosts that are infected. The data may support a seasonal host which winters in Florida. If shedding of sporocysts lasts 4 – 6 months after infection, few opossums are likely to be still shedding in the last quarters of the year. In the 3rd and 4th quarters of 1998, we additionally began collecting opossums from Alachua County Animal Control (ACAC) and it is possible that this changed the prevalence in our collection. However, the prevalence in the 3rd quarter of 1997 was similar to 1998 (13% vs. 9%). The prevalence of *Sarcocystis* in opossums from ACAC for 3rd and 4th quarters is low (17 of 155, 11%) but not statistically different from 3rd quarter prevalence in 1997 ($p=0.77$). Nonetheless, a number of factors might affect prevalence in the ACAC opossums. All animals collected by ACAC are live-trapped and may be healthier than, or just different from, the roadkill population. Secondly, ACAC collects opossums from across Alachua County whereas we noted a higher prevalence of infection in opossums collected in cities. Thirdly, our roadkill population may be biased by the motivation of people notifying us of roadkill or live-trapped opossums. This latter reason
appears unlikely because many calls came from radio-listeners in addition to farm managers and horse owners.

Two changes in collection protocol occurred during this study and are relevant to our report. Firstly in June 1997 we significantly increased advertising and more vigorously pursued collecting roadkill opossums. Secondly, in July 1998 we began collecting opossums from Alachua County Animal Control as described above. It is not possible to determine the significance of the effects of these alterations, but we feel that the data from June 1997 through July 1998 are likely most representative because we collected a large percentage of all opossums road killed in Alachua County during that time. The fact that the other seasonal data are comparable suggests that it is appropriate to make some early conclusions.

Most opossums were collected from Alachua and Marion counties, because they were closest to our College. Data by county are presented in Table 5-2, although because of the disproportionate numbers in each group, it is hard to make many inferences. We did note that many of the infected opossums, including most of the *S. neurona* isolates, were collected in Alachua County and specifically from the city of Gainesville (population approximately 100,000) rather than from the rural areas surrounding it. Additionally, prevalence of infection in opossums collected in Gainesville was higher than other areas. Opossums have adapted well to being city dwellers (Gardner, 1982), and it may be that intermediate hosts are more commonly found, or more commonly infected, in cities. There were some associations between the type of *Sarcocystis* sp. recovered from infected opossums and area of the state the opossum was collected. Five of 6 isolates identified as type-1085 were from South Florida (Dade and Broward counties) and the 6th was from
Marion County. Four of those isolates had greater than $2 \times 10^6$ sporocysts recovered compared with only 22 of the remaining 75 isolates of all other species. It is noted that the collections systems were different for these counties, and therefore these data are likely to be biased. In 1998, the prevalence of infection per county and total dropped because of the greater numbers of opossums collected in the 4th quarter, and the low prevalence of infection during that period (Table 5-1). The overall prevalence drop from 1997 to 1998 was not statistically significant ($p=0.11$), but had a tendency to significance when Broward County data was excluded ($p=0.027$). As expected there is no statistical difference in prevalence of Broward County opossums between the years ($p=0.11$).

From the isolates already classified there is a trend between type and intensity of shedding. Type-1085 (mean $1.2 \times 10^7$) and type-2079 (mean $2.4 \times 10^7$) were shed in the highest numbers (Table 5-3). The lowest recovery was $3.4 \times 10^5$ for a type-1085 isolate and $5.8 \times 10^5$ for a type-2079 isolate. *Sarcocystis falcatula* was recovered in the lowest numbers (mean, $7 \times 10^5$), while *S. neurona* was intermediate (mean, $2.4 \times 10^6$). Some isolates of each type were recovered in very low numbers. Two possibilities may explain the data. Either the intensity of shedding was always low in that individual opossum, or it was collected near the beginning or end of infection and, therefore, of shedding. In support of the second possibility, many *Sarcocystis* sp. are known to infect definitive hosts for only a short period of time (Dubey et al., 1989). For some isolates, an additional cause was the severely decomposed state of the gastrointestinal tract, preventing proper recovery technique. Therefore, the differences in mean sporocyst recovery between *Sarcocystis* types is likely underestimated.
The lowest infection intensities of all the species tend to overlap, but this is probably an artificial confounding due to either poor recovery technique or a very early or very late infection.

In a previous experiment conducted by investigators in my group, naturally-infected brown-headed cowbirds were fed to opossums and sporocyst shedding was monitored by examining fecal samples (R. Porter and E. Greiner, personal communication). The numbers of *S. falcatula* sporocysts shed by those laboratory opossums appears to be somewhat different than the numbers shed by the opossums naturally infected with *S. falcatula*. The peak shedding in those laboratory opossums was $2 \times 10^5$ sporocysts per gram (R. Porter and E. Greiner, personal communication). With a ‘standardized’ fecal pellet weighing 30 g, up to $6 \times 10^6$ sporocysts could be deposited at once. This appears to be considerably higher than the average numbers recovered in the opossums reported here. Even though the comparison is not direct, the implication of such intense fecal shedding is that large numbers of sporocysts were present in the gut mucosa.

In multiple experimentally-induced *S. falcatula* infections in opossums, Box and Smith (1982) reported recoveries of between 1 and $6.3 \times 10^8$ sporocysts using their gut-digestion method. Data from these experimental *S. falcatula* infections appear to correlate well. Of note, in the latter study more sporocysts were recovered when the opossums were euthanized soon after infection. Even if Box’s digestion method increases yield 10-fold, the discrepancy in sporocyst recovery between naturally-infected and experimentally-infected opossums is very large. Brown-headed cowbirds experimentally challenged with 5,000 *S. falcatula* sporocysts had a very severe sarcocyst burden (Dame et al., 1995; E. Greiner, personal communication). If natural *S. falcatula*, and possibly *S. neurona*,
infections most commonly occur at low or very low intensities, the dissonance between the 50% prevalence of *S. neurona* infection in horses (as revealed serologically) and an ID$_{50}$ which approaches $5 \times 10^5$ sporocysts per horse, is further underscored.

Many of the *S. neurona* isolates have relatively lower numbers of sporocysts ($<5 \times 10^5$). This is of interest considering the difficulty we have reported in inducing clinical signs of EPM in challenged horses under controlled circumstances. Because few isolates of *S. neurona* exceeded $5 \times 10^6$ (the minimum challenge dose successful in inducing disease in challenged horses), it appears that additional factors must be involved in progression of subclinical infection to overt EPM. This bodes well for the eventual control of EPM, because if most horses are naturally resistant to disease (either because of innate, cell-mediated or humoral immunity), and large infective doses are necessary to cause disease in the remainder, a limited additional degree of protection (such as annual vaccination) may be sufficient to eliminate the disease completely.

It is likely that we have underestimated the true infection rates of wild opossums because of false negatives. However, the false-negative opossums would be shedding very low numbers of sporocysts and hence likely play little or no role in causing disease in horses. The false negative rate is hard to estimate, but is contributed to by (a) inexperienced in identifying rare sporocysts in the beginning of our survey, (b) difficulty in recovering high quality samples in gut that was decomposing, (c) the possibility of intermittent shedding in opossums, and (d) shedding of very low numbers of sporocysts (McKenna and Charleston, 1988).

Prevalence of shedding was highest amongst opossums weighing 1.5 – 3 kg (~23%), with opossums weighing 2.0 – 2.5 kg being most likely to be infected (Table 5-
4). It was rare to find infected opossums with a bodyweight less than 0.75 kg. Of 313 opossums of known weight, 2 of 34 were infected and weighed 0.75 kg or less while 8 of 70 weighed 1 kg or less and were infected. None of 12 opossums with a bodyweight below 0.45 kg were infected. Although there were few animals collected that weighed over 3.5 kg, a lower percentage (10%) were found to be shedding sporocysts. Thus, low-weight (young) opossums appear less likely to have captured or consumed infected intermediate hosts. Heavier, and perhaps older, opossums also appear less likely to be shedding sporocysts. Because few such opossums were collected, it is unclear whether or not heavier opossums genuinely have a reduced susceptibility to infection.

In the first year of collection 15 (39.5%) males, 16 (21.3%) females and 6 (17.7%) of unknown gender (only gastrointestinal tract provided) out of 178 were infected (Table 5-6). It was rationalized that a greater percentage of males might be infected because their territory is larger (0.5 acres versus 0.25 acres for female; Gardner, 1982). However, in 1998 only 16 (15.2%) males were infected compared with 27 (23.5%) females and 7 (13.7%) unknowns. This was unexpected and led us to investigate whether we had collected fewer males in the 2 – 2.5 kg category, which has amongst the highest infection prevalence. The results of this analysis are presented in Table 5-5, and show that a very low percentage (8%) of 2 – 2.5 kg males were infected in 1998 (in our collection). We do not have a good explanation for this change. The relatively small numbers in each category should prevent overly interpreting the data, but a similar trend is present for each gender. Although we have collected females weighing more than 3 kg, none were infected. It will be of interest to review collection data from future years to determine if these trends continue or if the data vary randomly in a Gaussian distribution.
The agreement between identification of sporocysts (and therefore infected opossums) by either flotation of fecal samples or intestinal mucosal scrape was very high. On those 12 occasions when a disagreement occurred, 9 times the intestinal mucosal scrape was positive while the fecal float was negative and on 3 occasions the intestinal mucosal scrape was negative while the fecal float was positive. One sample had 0.5 – 1 x 10^6 sporocysts; the others were less than 5 x 10^5. As expected, there was no correlation between discordant results and a particular species of \textit{Sarcocystis} sporocyst. It is possible that we have underestimated the correlation between intestinal mucosal and fecal flotation because live-trapped opossums were not usually euthanized unless they had a positive fecal float.

EPM in horses is a disease that has approached epidemic proportions in the 1990s. It is unclear what factor has permitted its increasing prevalence (Granstrom, 1993; Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997). Data collected by the state of Kentucky shows that numbers of opossums killed by trappers dropped from 70,000 in 1982 to 2,000 in 1994 (Cramer, 1997). Over a similar period, the raccoon highway mortality index increased 386% (Cramer, 1997). No similar data are available for opossums, but it is probable that a similar population increase has occurred. Any factor which might increase the opossum population, or cause it to be more mobile, may be important in understanding the emergence of EPM. Opossums are increasingly forced into urban areas in search of food, which may alter their diet. Our data showed higher prevalence of infection in Gainesville than in the surrounding rural areas. However, infected urban opossums seem unlikely to cause increased disease in horses which usually are located outside cities.
The motivation for this opossum survey was primarily the collection of *S. neurona* sporocysts to use in host-challenge experiments. Granstrom et al. (1995) surveyed 215 wildlife and found *S. neurona*-specific antibodies in 22/37 skunks. One additional skunk and 6/72 raccoons recognized epitopes shared with other *Sarcocystis* sp. Definitive hosts of *Sarcocystis* sp. are not infected systemically: the entire sexual phase of reproduction occurs in gut endothelium. Serum antibodies would be unlikely, therefore, in the definitive host. Other work from the Kentucky group identified the opossum as the definitive host (Fenger et al., 1995). As a result, we did not collect other species of roadkill, except 2 striped skunks (*Mephitis mephitis*) from which we were interested in obtaining an isolate of *S. rileyi*. Neither skunk was infected. Interestingly, although the opossum can act as definitive host of *S. rileyi* in an experimental setting (Levine, 1986), *S. neurona* in the striped skunk has caused encephalitis (Dubey et al., 1996). Most of the skunks examined from Kentucky, Michigan and Panama had been exposed to *S. neurona*, or at least had antibodies that cross-reacted with it. This suggests that not only are horses frequently exposed to *S. neurona*, but so also are skunks. The only practical significance to this is that *S. neurona* appears to be a promiscuous organism, which is already not a novel observation.

Other work from my laboratory has initiated the field of molecular characterization of opossum-shed *Sarcocystis* sp. (Tanhauser et al., 1999). Such work is an essential component of the understanding of this somewhat complex area. Biological and life cycle data that complements the molecular data should eventually lead to full comprehension of the interactions of these 4 parasites, the opossum, intermediate hosts and horses. To date we have demonstrated that multiple *Sarcocystis* sp. are shed by opossums, that few
opossums shed more than a single species at a time, and that even though infection can be devastating in some hosts, the organism is harmless in others. Opossums most frequently shed *Sarcocystis* sp. parasites in the 2nd quarter of the year, and most commonly middleweight (2 – 3 kg) animals are infected. There may be an association between location (rural/urban or area of the state) and the *Sarcocystis* sp. being shed.

Table 5-1. Number of opossums collected and shedding by month for 1997, 1998 and combined.

<table>
<thead>
<tr>
<th>Month</th>
<th>1997 No.</th>
<th>1997 Pos. %</th>
<th>1998 No.</th>
<th>1998 Pos. %</th>
<th>Total No.</th>
<th>Total Pos. %</th>
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<td>18</td>
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<td>0%</td>
<td>39</td>
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<td>10</td>
<td>10%</td>
<td>24</td>
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<td>8%</td>
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<td>3rd Quarter</td>
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<td>13%</td>
<td>82</td>
<td>9%</td>
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<td></td>
<td>22</td>
<td>23%</td>
<td>22</td>
<td>23%</td>
</tr>
<tr>
<td>November</td>
<td></td>
<td></td>
<td>47</td>
<td>13%</td>
<td>47</td>
<td>13%</td>
</tr>
<tr>
<td>December</td>
<td></td>
<td></td>
<td>24</td>
<td>0%</td>
<td>24</td>
<td>0%</td>
</tr>
<tr>
<td>4th Quarter</td>
<td></td>
<td></td>
<td>93</td>
<td>12%</td>
<td>93</td>
<td>12%</td>
</tr>
<tr>
<td>unknown</td>
<td>15</td>
<td>27%</td>
<td>3</td>
<td>67%</td>
<td>18</td>
<td>33%</td>
</tr>
<tr>
<td>Total</td>
<td>148</td>
<td>25%</td>
<td>271</td>
<td>19%</td>
<td>419</td>
<td>21%</td>
</tr>
</tbody>
</table>
Table 5-2. Number of opossums collected and shedding by county in Florida for 1997 and 1998.

<table>
<thead>
<tr>
<th>County</th>
<th>1997 Total</th>
<th>Positive</th>
<th>Prevalence</th>
<th>1998 Total</th>
<th>Positive</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alachua</td>
<td>63</td>
<td>17</td>
<td>27.0%</td>
<td>230</td>
<td>41</td>
<td>17.8%</td>
</tr>
<tr>
<td>Bradford</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Broward</td>
<td>29</td>
<td>11</td>
<td>37.9%</td>
<td>7</td>
<td>5</td>
<td>71.4%</td>
</tr>
<tr>
<td>Citrus</td>
<td>1</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clay</td>
<td>9</td>
<td>2</td>
<td>22.2%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dade</td>
<td>3</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>De Soto</td>
<td>14</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hernando</td>
<td>2</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Highland</td>
<td>5</td>
<td>3</td>
<td>60.0%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Levy</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>1</td>
<td>14.3%</td>
</tr>
<tr>
<td>Marion</td>
<td>12</td>
<td>4</td>
<td>33.3%</td>
<td>25</td>
<td>2</td>
<td>8.0%</td>
</tr>
<tr>
<td>Palm Beach</td>
<td>3</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sumter</td>
<td>1</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>--unspecified--</td>
<td>6</td>
<td>0</td>
<td>0.0%</td>
<td>1</td>
<td>1</td>
<td>100.0%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>148</td>
<td>37</td>
<td>25.0%</td>
<td>271</td>
<td>50</td>
<td>18.5%</td>
</tr>
<tr>
<td># counties</td>
<td>12</td>
<td>5</td>
<td>n/a</td>
<td>6</td>
<td>5</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 5-3. Numbers of isolates by *Sarcocystis* sp. or type with descriptive statistics.

<table>
<thead>
<tr>
<th></th>
<th>All opossums</th>
<th>S. neurona</th>
<th>S. falcata</th>
<th>type-1085</th>
<th>type-2079</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number isolates</td>
<td>81</td>
<td>16</td>
<td>13</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Average yield</td>
<td>3,262,037</td>
<td>2,346,200</td>
<td>720,000</td>
<td>11,993,833</td>
<td>23,887,000</td>
</tr>
<tr>
<td>Median yield</td>
<td>620,000</td>
<td>720,000</td>
<td>492,000</td>
<td>3,972,000</td>
<td>7,632,000</td>
</tr>
<tr>
<td>Minimum yield</td>
<td>6,000</td>
<td>32,000</td>
<td>6,000</td>
<td>336,000</td>
<td>583,000</td>
</tr>
<tr>
<td>Maximum yield</td>
<td>63,446,000</td>
<td>12,960,000</td>
<td>2,165,000</td>
<td>56,915,000</td>
<td>63,446,000</td>
</tr>
</tbody>
</table>

Table 5-4. Numbers of opossums shedding sporocysts by weight (where weight available), as a percentage of all collected opossums of that weight, for 1997 and 1998.

<table>
<thead>
<tr>
<th>Weight category</th>
<th>1997 # positive</th>
<th>% of wt category</th>
<th>1998 # positive</th>
<th>% of wt category</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 - 0.5 kg</td>
<td>0</td>
<td>0%</td>
<td>2</td>
<td>10%</td>
</tr>
<tr>
<td>0.5 - 1 kg</td>
<td>1</td>
<td>14%</td>
<td>5</td>
<td>14%</td>
</tr>
<tr>
<td>1 - 1.5 kg</td>
<td>2</td>
<td>33%</td>
<td>7</td>
<td>16%</td>
</tr>
<tr>
<td>1.5 - 2 kg</td>
<td>2</td>
<td>18%</td>
<td>7</td>
<td>20%</td>
</tr>
<tr>
<td>2 - 2.5 kg</td>
<td>10</td>
<td>39%</td>
<td>9</td>
<td>25%</td>
</tr>
<tr>
<td>2.5 - 3 kg</td>
<td>3</td>
<td>25%</td>
<td>8</td>
<td>19%</td>
</tr>
<tr>
<td>3 - 3.5 kg</td>
<td>2</td>
<td>67%</td>
<td>2</td>
<td>15%</td>
</tr>
<tr>
<td>3.5 - 4 kg</td>
<td>1</td>
<td>100%</td>
<td>1</td>
<td>25%</td>
</tr>
<tr>
<td>4 - 4.5 kg</td>
<td>1</td>
<td>33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>21</td>
<td>27.8%</td>
<td>42</td>
<td>17.6%</td>
</tr>
</tbody>
</table>
Table 5-5. Numbers of opossums shedding sporocysts by weight and gender (where weight and gender available), as a percentage of all collected opossums per category, for 1997 and 1998.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># pos.</td>
<td>% pos.</td>
<td># pos.</td>
<td>% pos.</td>
<td># pos.</td>
</tr>
<tr>
<td>0.1 - 0.5</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>2</td>
</tr>
<tr>
<td>0.5 - 1</td>
<td>1</td>
<td>25%</td>
<td>0</td>
<td>0%</td>
<td>1</td>
</tr>
<tr>
<td>1 - 1.5</td>
<td>1</td>
<td>100%</td>
<td>1</td>
<td>25%</td>
<td>3</td>
</tr>
<tr>
<td>1.5 - 2</td>
<td>2</td>
<td>40%</td>
<td>0</td>
<td>0%</td>
<td>3</td>
</tr>
<tr>
<td>2 - 2.5</td>
<td>4</td>
<td>80%</td>
<td>6</td>
<td>32%</td>
<td>7</td>
</tr>
<tr>
<td>2.5 - 3</td>
<td>1</td>
<td>25%</td>
<td>2</td>
<td>25%</td>
<td>4</td>
</tr>
<tr>
<td>3 - 3.5</td>
<td>1</td>
<td>33%</td>
<td>2</td>
<td>22%</td>
<td>1</td>
</tr>
<tr>
<td>3.5 - 4</td>
<td>1</td>
<td>100%</td>
<td>1</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>4 - 4.5</td>
<td></td>
<td></td>
<td>1</td>
<td>33%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>40.1%</td>
<td>9</td>
<td>22.5%</td>
<td>15</td>
</tr>
</tbody>
</table>

In 1997, no opossum of unknown gender was positive.

Table 5-6. Numbers of opossums shedding sporocysts by gender (where gender available), as a percentage of all collected opossums of that gender for 1997 and 1998.

<table>
<thead>
<tr>
<th>Gender</th>
<th>1997</th>
<th></th>
<th>1998</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Males, total</td>
<td>38</td>
<td>--</td>
<td>105</td>
<td>--</td>
</tr>
<tr>
<td>Females, total</td>
<td>75</td>
<td>--</td>
<td>115</td>
<td>--</td>
</tr>
<tr>
<td>Unspecified, total</td>
<td>34</td>
<td>--</td>
<td>51</td>
<td>--</td>
</tr>
<tr>
<td>Males, positive</td>
<td>15</td>
<td>39.5%</td>
<td>16</td>
<td>15.2%</td>
</tr>
<tr>
<td>Females, positive</td>
<td>16</td>
<td>21.3%</td>
<td>27</td>
<td>23.5%</td>
</tr>
<tr>
<td>Unspecified, positive</td>
<td>6</td>
<td>17.7%</td>
<td>7</td>
<td>13.7%</td>
</tr>
</tbody>
</table>
Figure 5-1. Poster used to inform the public and horse farm personnel about opossum collection program.

Roadkill
or not to roadkill?

- *It's not just a dead opossum*
- ... *it's the solution to a deadly problem!*

- Once more, we *desperately* need Opossums to continue our investigation of their role in a serious, often *fatal*, neurologic disease of horses (EPM)
- Use of dead Opossums reduces need for lab. animals and helps find risk areas for horses in Florida
- If you **spot one**, can help, or need more information

📞 **3 9 2 - 4 7 0 0**

- x3333 EPM/Opossum hotline and leave a message describing the location (give cross-street) when the machine answers. Thanks!
The most pertinent data described herein are the horse-challenge experiments. The failure to induce disease with characterized *S. falcatula* sporocysts in horses was an important contrast to another report describing seroconversion and EPM consequent to administration of opossum-borne *Sarcocystis* species (inadvertently ascribed to *S. falcatula*). Subsequent horse challenge with a single, and then multiple, $5 \times 10^5$ characterized *S. neurona* sporocyst doses demonstrated that seroconversion and disease could be induced in horses. Disease development with the higher total dose used in the multiple-dose horse challenge contrasts with seroconversion alone at the single dose. Thus the horse model of EPM is still a work in progress, but the data suggest that a reliable and repeatable model will be achieved in the near future. The multi-dose challenge reported here is the first induction of EPM using characterized *S. neurona* sporocysts.

The intermediate host challenge data presented represents a substantial addition to the life-cycle information available for opossum-borne *Sarcocystis* sp. Six species of birds are excluded as natural intermediate hosts of *S. neurona*, and appear unlikely to be useful as laboratory hosts. We report the first (negative) life-cycle data for the eimeriid (presumably Sarcocystid) we designated type-1085. We present data that supports the previously published notion that mice must be immunologically incompetent to develop
disease. Dubey et al. (1998) described encephalitis when nude or gamma knockout mice were challenged with *S. neurona* or *S. americana* (which we believe to be the same species that we designate type-2079). Further sporocyst challenge of these mice should provide exciting new insights into the biological differences between the 4 *Sarcocystis* sp. we have collected from opossums.

Finally, data accumulated during the collection of over 400 individual opossums that were screened for infection is presented and raises a number of interesting questions. This is the first time, to my knowledge, that such data have been available and they may form an important component of the eventual control system to eliminate EPM. Perhaps most significant are the seasonal variations in the percentage of infected opossums. Association of type-1085 isolates with opossums collected in South Florida may reflect previously unrecognized intermediate host-habitat patterns.

The experiments described also suggest possible improvements in methodology and additional fields for investigation. It would be beneficial to use both PCR and tissue culture when attempting to demonstrate the parasite in blood, CSF or organ sections. In addition, when protozoa are isolated in tissue culture, extracted DNA may be subjected to molecular tools and sequencing to potentially confirm that the specific isolate administered has been recovered.

Equine clinicians have recognized for some time that EPM in horses is temporally associated with stressful circumstances. Young racehorses beginning training programs, horses subjected to intensive preparation for public auction, mares in the peripartum period and all animals receiving corticosteroids as therapeutic or preventative medications are all too often the individuals who succumb to EPM. Undoubtedly, these situations are
confounded by other factors including close housing, transportation and feeding of commercial feeds and hay. Nonetheless, high endogenous cortisol concentrations and immunosuppression appear to be important. Therefore, the role of immunosuppressed animals as natural or alternative hosts in life-cycle models is strongly endorsed. Dubey et al. (1998) have demonstrated the value of nude and gamma-interferon knockout mice. Immunosuppressed, or preferably selectively immunocompromised, horses may allow an additional more specialized model of EPM that would complement the model we described, and permit more complete study of the variable, and sometimes aggressive, disease clinicians are forced to treat and diagnose. There may even be some value to immunosuppressing and re-challenging some of the passeriform birds described in chapter four.

One naturally occurring immunosuppression model in horses is severe combined immunodeficiency (SCID) of foals. Preliminary work at Washington State University with a SCID foal suggests that this model may complement the understanding of disease pathogenesis in horses. Protozoa were isolated from blood culture from a SCID foal challenged with the same inoculum, *S. neurona* isolate #2009, as the multiple-dose challenged horses described in chapter three. The protozoa were subjected to the molecular markers used to characterize the original opossum isolate and were again classified as *S. neurona*. Further investigation of the behavior of *S. neurona* in SCID foals will be a useful addition to the horse model.

The difficulty of handling *S. falcataula* near budgerigars may well have confounded our bird experiment. Occurrence of pneumonitis, and subsequently death, in a bird challenged with isolate #1071 (*S. neurona*) is in stark contrast to the results for 3 other *S.
neurona isolates. This experiment requires repeating to verify whether S. neurona has isolate-specific ability to kill psittacines or if S. falcatula was a contaminant. It is recommended that S. falcatula challenge of budgerigars be performed after other budgerigar experiments are concluded, or alternatively that those birds be housed in a separate enclosed room without shared personnel. Additional isolates of type-1085 should also be administered to budgerigars to better determine that species’ infectivity for psittacines. If some, but not all, type-1085 isolates can cause pneumonitis in psittacines it is possible that this is also true for S. neurona isolates. When unexpected results occur, additional emphasis should be placed on identifying the parasite present in lesions, whether by using immunohistochemistry or molecular tools on extracted DNA.

Review of the literature suggests several additional hosts that might be examined as candidate hosts for S. neurona. Firstly, English sparrows (Passer domesticus domesticus) which were shown by Box et al. (1984) to be infected by S. falcatula-shedding opossums and to be able to infect naïve opossums. We have excluded most intermediate hosts of S. falcatula as possible intermediate hosts of S. neurona but did not address the role of this common bird. Secondly, many species of duck are found throughout the United States, in contrast with the distribution of passeriform species. Most ducks are considerably larger than the birds we have investigated in this paper, but they may be consumed as carcasses or be killed by other predators and scavenged upon by opossums. Box and Duszynski (1978) administered S. falcatula sporocysts to the anseriform Anas platyrhynchos and found no evidence of disease or lesions. In a separate report, Duszynski and Box (1978) described an opossum that shed sporocysts after consuming sarcocysts from a pintail duck (Anas acuta) but no shedding in other opossums.
fed sarcocysts from either a green-winged teal (*Anas carolinensis*) or a shoveller (*Spatula chopeata*). Sarcocysts in those ducks were visible to the naked eye. It is likely that this opossum was briefly infected with *S. rileyi*, which is known to infect many species of duck, but the possibility that Anatidae may act as intermediate hosts of *S. neurona* requires resolution. Thirdly, the potential role of amphibian hosts requires investigation because review of opossum intestinal contents has demonstrated that frogs are common constituents of the opossum diet.

The collective data presented do not provide any illumination on the disparity between the prevalence of *S. neurona* infection in horses (approximately 50% of the US horse population and probably higher) and the prevalence of EPM (<1%). The ID$_{50}$ for horses is probably below $5 \times 10^5$ sporocysts administered at a single time, on the basis of seroconversion data presented in chapter three. The dose that will result in unequivocal disease (i.e., EPM) in 50% of horses appears to be at least an order of magnitude higher. The spectrum of clinical signs in our horse infection protocol do not approach the severe debilitation that infrequently develops in naturally-infected horses. Final determination of an ID$_{50}$ is likely to be complicated because there may well be an interaction between total dose and time elapsed until infection is detectable. Such an interaction may be analogous to that described for *S. falcatula* infection in budgerigars (*Smith et al., 1989*). Whatever the ID$_{50}$ is, it clearly is reached in the environment of many horses in the US, particularly in the southeast. This may be the solution to the paradox. From data presented in chapter three, we believe that *S. neurona* may be spread in horse feed and hay (and possibly water). Contamination of the food chain is likely to be at a very low concentration, particularly in commercial products. Chronic exposure to sporocysts at concentrations far
below the dose necessary to induce disease could be responsible for the observed
“background” seroprevalence. Occasional exposure of individual horses to much higher
doses, or exposure of horses which are particularly susceptible, might be superimposed as
a rare event. Therefore, we may be observing two different phenomena which are actually
unrelated. If that is the case, then current attempts to control spread of infection by
changing feed sources may be essentially flawed. Additionally, any possible protective
effect of specific antibodies on disease development is, at most, speculative at this time.
Disease induction in seroconverted horses, described in chapter three, is the only data I
know of that may address this issue.

The contribution of mechanical vectors to horse infection have been largely
ignored in the literature. One horse in chapter three was shown to seroconvert and have
CSF antibodies while in the isolation paddock. We ascribe infection in that horse to the
feeding of hay while the horse was maintained in the isolation pasture. While searching for
seronegative horses, we also observed that 16/20 thoroughbreds on the racetrack in San
Juan, PR, had seroconverted although opossums do not live in Puerto Rico. All these
horses were fed imported US hay. Clearly, much more evidence will be required in order
to properly address the risks associated with feeding hay. Nonetheless, these early
observations should not be entirely dismissed. In the collection of roadkill opossums we
have frequently noted birds feeding on carcasses. Prey birds, including turkey vultures
(Cathartes aura), appear to consume selectively the intestinal tract of the opossum. As a
result, it is conceivable that sporocysts are spread mechanically by scavengers and that
horses may subsequently be exposed and infected despite rigorous opossum control
measures. Four horses that had been kept in the isolation pasture for up to 160 days all
seroconverted within 14 days of an unexplained point exposure to *S. neurona*. It seems reasonable to speculate that this exposure occurred as a result of a mechanical vector.

After further description and investigation of the behavior of the non-falcatula/non-neurona *Sarcocystis* sp. in budgerigars, development of pneumonitis and clinical signs may be a useful additional test to complement the range of molecular markers that are currently in use in our laboratory to classify isolates. At a minimum, prescreening wild-caught opossum isolates through budgerigars would prevent confounding further *S. neurona* intermediate host investigations with isolates that kill psittacines.

A number of biological issues remain to be resolved before final control of the disease can become a reality. The intermediate host of *S. neurona* must be identified. The relationship of *S. neurona* to *S. falcatula* and other members of the genus *Sarcocystis* will be important. There may be interaction between multiple *Sarcocystis* sp. within a single opossum. Whether multiple species compete within the definitive host or whether individual opossums’ dietary preference is the major risk factor for infection with a specific species is unknown. In the first published description of a third opossum-borne *Sarcocystis* sp. Dubey et al. (1998) reported that one opossum was shedding all 3 species. This is in contrast to our results wherein only 3 of 41 classified isolates appeared to be a mixture of species. None were identified as shedding 3 species. However, our molecular tools may be less sensitive in identifying type-1085, and type-2079 can only be identified by amplifying the ITS sequence. Thus, interactions of *Sarcocystis* sp. within an individual definitive host are not understood yet, but mixed infections appear to be uncommon in our data set.
Little is known about the long-term viability of *Sarcocystis* sp. sporocysts in the environment. The only data are reported by McKenna and Charleston (1992) who examined excystation percentages of *S. gigantea* sporocysts buried in soil (in a protective vented container) and discovered that “loss of viability” occurred most quickly in the summer months, and that it was unlikely sporocysts could survive beyond 12 months. The relevance of sporocyst survival in the environment is clear. Investigation of this will be a simple task when either lower challenge doses can successfully cause disease in horses or sporocysts can be generated through laboratory-based life cycles. In an unrelated, but novel, observation Wilber et al. (1994) reported that 0/21 sporocyst isolates of *Eimeria jemezi* recovered from pocket gophers (*Thomomys talpoides*) which lived in a radon-rich area were able to sporulate compared to 16/19 isolates (84%) from gophers residing in an area with normal soil radon concentration 7 km away. Local variations in disease prevalence may be affected by many environmental factors. Saville et al. (1997) reported lower seroprevalence among horses residing in northeastern Ohio than those in southwestern Ohio and cited a greater number of days-below-freezing as a likely cofactor.

The occurrence of EPM in Central and South America suggests that either *D. marsupialis* and/or *D. albiventris* or, alternatively, an unrelated mammal can also be definitive host to *S. neurona*. The breadth of intermediate hosts which can be infected remains a matter of debate, but the wide distribution of EPM again suggests that more than one intermediate host may exist for *S. neurona*.

It would be very useful, especially from an epidemiologic standpoint, to develop a method for concentrating sporocysts to permit identification of contaminated feed and
water supplies. However, this is a task of considerable magnitude and the low concentrations that need to be detected may fall below the sensitivity of any available technologies.

The possible existence of partially susceptible hosts may allow experimental induction of sarcocysts and investigation of pathogenesis. Production of sarcocysts involves factors in addition to those that permit merozoites merely to replicate. Box and coworkers (Box et al., 1984) have classified pigeons as partially-susceptible to *S. falcatula* infection, and we cannot exclude that some of our *S. neurona*-challenged passeriform birds experienced mild or subtle infection below the sensitivity of our detection protocol. Infection of partially-susceptible hosts may lead to divergence of species under the appropriate population forces. The recent report of encephalomyelitis in harbor seals (Lapointe et al., 1998) due to an *S. neurona*-like organism is interesting because those mammals also had sarcocysts in cardiac muscle. It is unfortunate that the identity of the organism that caused the sarcocyst is unknown.

As we enter the 21st century the major focuses of research in EPM must be (a) identification of more-effective chemotherapeutic agents for affected horses, (b) development and validation of a test which can distinguish between infection alone and disease, and (c) acquiring *S. neurona* sarcocysts, either by experimental challenge or by identifying the natural intermediate host. These are significant challenges, but appear attainable on the basis of recent progress. The horse model will be most reliable when clones of, or biologically-purified, *S. neurona* sporocysts are available and consequently confounding is eliminated. Ultimately, such a model is highly desirable for chemotherapeutic and vaccine trials.
APPENDIX A

STANDARD NEUROLOGIC EXAMINATION SHEET

EPM Research Group

Neurologic examination record

<table>
<thead>
<tr>
<th>Horse ID#</th>
<th>Horse Name</th>
<th>Study#</th>
</tr>
</thead>
</table>

Exam date: __/__/199__

Video Record: YES / NO

Tape ID: ____________

Exam Series # __

Examiner: RJM / TJC / ______

Sedation / Anes. within 8 days? YES/NO

Overall impression

Behavior: __________

Mentation: __________

Head posture / co-ordination: __________

WAS STANDING EXAMINATION NORMAL? YES, then add any comments below and proceed to Gait Section

NO, then complete Standing Exam section below, proceed to Gait Section

WAS GAIT EXAMINATION NORMAL? YES, then add any comments below and initial at bottom of form

NO, then complete rest of form and initial at bottom of form

Comments: ____________________________________________________________

________________________________________________________________________

Standing examination

Cranial nerves: II III IV V VI VII VIII IX X XII

Specific comments: _______________________________________________________

Cervicofacial reflex: Normal / Abnormal

Panniculus: Normal / Abnormal

Tail / anus: Normal / Abnormal

Gait analysis

<table>
<thead>
<tr>
<th>LF</th>
<th>RF</th>
<th>LR</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARESIS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATAXIA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTHER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments:

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________

KEY CD: circumduction DR: dragging INT: interference

Exam performed by me, report completed/dictated by me: _______
Neurologic examination of the horse provides a number of difficulties even for the expert examiner. For the purposes of these studies, it was essential that examination results were repeatable. In addition, because subtle neurologic signs were likely to represent a significant proportion of the disease that we identified we elected to report only signs that were repeatedly identified. To standardize the reporting of the examination we created a specialized form derived from the form used for clinical examinations. In contrast to the standardized clinical examination, we focused mostly on gait abnormalities and devoted additional time and effort to identifying any deviations from normal. The examiner remained blinded to the challenge status of the individual horses until the end of the examination period. All examinations were recorded on videotape to permit subsequent re-examination if necessary by other blinded investigators.

Gait examination was performed first on the dirt surface of the isolation pasture in which the horses were kept. The horse was walked in a straight line by one handler while being observed by the examiner from the side and from behind. Particular attention is paid to the weight bearing of each limb and the tracking of one limb relative to both the contralateral and ipsilateral limb. Any evidence of lameness (musculoskeletal disease) is
noted at this time. The horse is then walked while the tail is held and pulled with varying
degrees of force to each side in turn. The ability of the horse to resist and continue
moving in a forward direction allow assessment of strength and to a lesser degree
conscious proprioception. The horse is circled (forward movement while turning) in
variably sized arcs as an incremental increase in test difficulty. Pivoting is performed,
where the horse turns without moving forwards, and this provides information on the
horse’s conscious proprioception and coordination skills. Particular attention is paid to
misplacement of the hoof such as standing on itself, tripping or pivoting around a single
limb without repositioning it. If neurologic disease is suspected, but the clinical signs are
equivocal, the horse may also be subjected to serpentine movements at the walk, thus
increasing the difficulty of the maneuver and permitting a greater chance for errors in
placement and coordination. The ultimate test of coordination and strength is termed the
Florida fling, and requires the handler to hold the horse by its lead shank and by its tail, the
horse and examiner forming a complete and equal circle. The examiner initiates
movement by applying gentle pressure to the shank and they turn like a carousel.
Typically the handler cannot continue in this manner for more than a couple of turns with
normal horses (greater than 150 cm in height at the withers). Weak or clumsy horses,
however, have difficulty performing this task and the handler is able to continue to dictate
the speed and position of the carousel.

All results are considered together when making a determination of normality.
Musculoskeletal disease causing lameness is excluded from the grading of the deficiency
insofar as is possible. The degree of disease is characterized for strength, coordination (or
lack thereof) and other specific gait abnormalities such as hypermetria (more joint
movement than normal). The greatest conviction results when multiple tests identify similar abnormalities. A final grading is assigned based on the individual scores given. For the purposes of our examination we use a scale from zero to five as shown in Table B-1. The reader is additionally referred to specialized texts for further information (Mayhew, 1985).

Table B-1. Grades of neurologic disease.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Characterization</th>
<th>Appearance of horse to untrained observer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>Normal horse</td>
<td>Cannot see any problem</td>
</tr>
<tr>
<td>One</td>
<td>Subtle disease- only occasional mistakes made, may need sequential examinations to be convinced</td>
<td>Cannot see any problem</td>
</tr>
<tr>
<td>Two</td>
<td>Mild disease- makes mistakes consistently, typically weak and neurologic disease is diagnosed on first examination</td>
<td>Probably appears abnormal but may easily be confused with lameness</td>
</tr>
<tr>
<td>Three</td>
<td>Moderate disease – horse makes many small errors (like standing on other feet, tripping), is weak and may even fall during complicated tasks</td>
<td>Obviously not normal, appears very clumsy and not lame</td>
</tr>
<tr>
<td>Four</td>
<td>Severe disease – horse is likely to fall over performing even simple tasks such as walking in a straight line. Horse is a risk to itself and others.</td>
<td>Horse is obviously severely affected</td>
</tr>
<tr>
<td>Five</td>
<td>Horse is recumbent and unable to rise. If thrashing around trying to rise, may represent significant risk to handlers.</td>
<td>Clearly unable to stand</td>
</tr>
</tbody>
</table>
APPENDIX C

ONLINE RESOURCES

The following web sites are maintained relatively current with major developments in equine protozoal myeloencephalitis.

http://www.vetmed.ufl.edu
http://prevmed.vetmed.ohio-state.edu/epm-home.html
http://ourworld.compuserve.com/homepages/drfenger/homepage.htm
http://www.uky.edu/agriculture/vetscience/parasitology/epmhome.htm
http://www.cvm.missouri.edu/cvm/courses/epm/index.html

These additional web sites provide further information directed primarily at public education, and as resources for horse owners.

http://www.horsechannel.com/library/veterinary/diseases/epm.asp
http://thehorse.com/0398/epm_update0398.html


Golubkov, V. I. 1979. Zarazhenie sobak i koshek sarkotsistami ot kur I utok. [Infection of the dog and cat with sarcocysts from the chicken and duck.] Veterinariya **1**: 55-56.


MacKay, R. J. 1997b. Serum antibodies to *Sarcocystis neurona*--half the horses in the United States have them! Journal of the American Veterinary Medical Association 210: 482-483.


Munday, B. L. 1985. Demonstration of viable Sarcocystis sporocysts in the faeces of a lamb dosed orally. Veterinary Parasitology 17: 355-357


The author was born in Brighton, England on September 6th, 1970 and grew up around horses in Iran and Ireland until attending boarding school at the Dundalk Grammar School, County Louth, Ireland. Immersion in veterinary medicine and animal care from an early age naturally led him into a career in veterinary medicine. In 1993, he graduated MVB with second class honors from University at the Veterinary College of Ireland in Dublin. While a senior student, an externship at the University of Florida had exposed him to the challenges and excitement of specialty medicine. Upon graduation he elected to pursue advanced qualifications, and completed an internship in 1994 at The Animal Health Trust in Newmarket, Suffolk, England, under the mentorship of Dr. Joe Mayhew. Following another transoceanic move, he entered into a residency in Large Animal Internal Medicine at the University of Florida, which he completed in 1997. He achieved Diplomate status in the American College of Veterinary Internal Medicine in 1998. His professional interests are neurologic disease of the horse, particularly the subject of this thesis ~ EPM, and ophthalmology. He is a novice skier, runner and autocrosser when time and circumstance permit. He has one cat named Simon, and is not married.