Prevalence and Identity of Tissue Cyst Forming Apicomplexan Parasites in the Muscles of Raptors

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Abstract

There is little information on the distribution and diversity of Apicomplexan protozoal infections in the tissues of raptors in the United States. Protozoan encephalitis caused by *Sarcocystis* species and *Toxoplasma gondii* is being increasingly reported in raptors from various locations in the United States. To better determine the exposure of raptors to these Apicomplexan parasites, we examined breast and heart muscle tissue of raptors from the Carolina Raptor Center for the presence of *Sarcocystis* species, *T. gondii* and *Neospora caninum* via histology, Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) using *Dra*I and *Hinf*I enzymes (*Sarocystis* only). Of 187 available H&E stained tissue sections, 33 contained sarcocysts. Nineteen of these slides had a matching DNA sample to compare via PCR. Nine of these 19 were positive for *Sarcocystis* via ITS PCR. Using ITS PCR, we detected *Sarcocystis* DNA in 24 of 114 birds (21.1%). Further molecular differentiation using JNB primers showed that 9 of the 24 birds were positive for either *S. neurona* or *S. falcatula.* RFLP analysis of these 9 indicated that 4 were *S. falcatula* samples, and 3 were *S. falcatula* Arg samples that cut with both enzymes. Our *Sarcocystis* positive samples were also tested for *S. calchasi, S. columbae* and *Sarcocystis* sp. Ex. *A. nisus* using PCR primers designed for these species. These species are emerging in Europe and have already shown an expansion of their distribution. Two samples (14567 and 15203) suggestive of *Sarcocystis* sp. Ex. *A. nisus* were identified, as well as one

sample (14567), which suggested the presence of *S. columbae.* None of these samples were confirmed by sequencing the amplicons and the other 22 samples were all negative for these parasites. Recent reports have demonstrated DNA of *S. falcatula* in the brain and muscles of great horned owls (*Bubo virginianus*), golden eagles (*Aquila chrysaetos*), and bald eagles (*Haliaeetus leucocephalus*) with encephalitis in rehabilitation centers in Indiana, Minnesota, and Virginia using PCR. DNA of *S. calchasi* has been found in CNS tissue of several species of birds suffering encephalitis in an aviary in California. Hawks (*Accipiter* species) are believed to be the source of infection. The prevalence of *T. gondii* was 18.4% (21 of 114) in these birds by PCR, but none were positive by histopathology. *N. caninum* prevalence in raptors has been poorly discussed in the literature. This parasite uses canids as the definitive host in its life cycle, and is considered to have a much more restricted host range than *T. gondii*. Thirty-five of 114 birds (30.7%) were found to be PCR positive for *N. caninum*, but no tissue cysts of *N. caninum* were observed in histological sections. Co-infection of 2 or all 3 species was detected in 16 of 114 birds (14%). This study demonstrates that there may be a higher prevalence of *S. falcatula* in raptors than was previously known, including more, as yet unknown, species of *Sarcocystis* capable of infecting raptors as intermediate hosts. Our PCR prevalence for *T. gondii* is similar to the serological prevalence for this parasite in raptors. The high PCR prevalence of *N. caninum* needs to be confirmed by sequencing the amplicons and the use of additional PCR primers. Information from the present study may help to inform zoos, aviaries and wildlife rehabilitation centers about parasite host diversity and reinforce the importance of preventative measures, such as making sure opossums (*S. falcatula* and *S. falcatula*-like), feral cats (*T. gondii*), and wild raptors (*S. calchasi*) do not have access to facilities. Insect control should also be emphasized

because of their ability to serve as phoretic hosts and carry oocysts/sporocysts into zoos, aviaries, and rehabilitation center.

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Table of Contents

Acknowledgements v

List of Tables viii

List of Figures ix

Literature Review 1

1.1 *Sarcocystis* in Raptors 1

1.1a Life Cycle of *Sarcocystis* species 1

1.1b *Frenkelia* 3

1.1c Diagnosis of *Sarcocystis* 3

1.1d Molecular Identification of *Sarcocystis falcatula* 4

1.1e Treatment of Disease Caused by *Sarcocystis* 4

1.1f Opossums as a Definitive Host for Avian Infective Sporocysts 5

1.1g *Sarcocystis falcatula* infection 5

1.1h S*arcocystis* infections of the Central Nervous System 6

1.1i *Sarcocystis falcatula*-like and *S. lindsayi* Species 7

1.1j *Sarcocystis calchasi, Sarcocystis columbae* and *Sarcocystis* sp. ex *Accipiter nisus* 8

1.2 *Toxoplasma gondii* in raptors 10

1.2a Life Cycle of *Toxoplasma gondii* 10

1.2b *Toxoplasma gondii* Infection in Raptors 11

1.2c Clinical Signs of *Toxoplasma gondii* in Raptors 11

1.2d Diagnosis of Disease Caused by *Toxoplasma gondii* 12

1.2e Prevention of *Toxoplasma gondii* 12

1.3 *Neospora caninum* in raptors 12

1.3a Life Cycle of *Neospora caninum* 12

1.3b Clinical Signs of *Neospora caninum* in birds 13

1.3c Diagnosis of Disease Cause by *Neospora caninum* in birds 13

1.3d *Neospora caninum* in Raptors 13

Proposal 15

Materials and Methods 16

3.1 Raptor Samples 16

3.2 Hematoxylin and Eosin Slide Preparation 16

3.3 Primers 16

3.4 DNA Isolation 18

3.5 *Sarcocystis* species PCR 20

3.6 Restriction Fragment Length Polymorphism 20

3.7 Sequencing of *Sarcocystis* spp. 22

3.8 *Toxoplasma gondii* PCR 23

3.9 *Neospora caninum* PCR 23

3.10 Positive Apicomplexan Parasite DNA Samples 24

Results 25

4.1 General Procedures and Raptor information 25

4.1 *Sarcocystis* species 27

4.2 *Toxoplasma gondii* 33

4.3 *Neospora caninum* 35

4.4 Co-infected Birds 37

Discussion **39**
Conclusions **43**
References **44**

List of Tables

Table 1. Primers used for the detection of *Sarcocystis* species, *Toxoplasma gondii* and *Neospora caninum*. *17*

Table 2. Summary of RFLP results of several *Sarcocystis* species using *DraI* and *HinfI* enzymes with JNB 33/JNB 54 primers. *22*

Table 3. Life history and diet information for raptors found positive for one or more parasitic species. *26*

Table 4. ITS positive bird samples.. ..... *32*

Table 5. Samples positive for *Toxoplasma gondii.* *34*

Table 6. Samples positive for *Neospora caninum.* *35*

Table 7. Samples positive for multiple parasites. *37*

List of Figures

Figure 1. Life Cycle of *Sarcocystis falcatula*. 2

Figure 2. Bioline protocol for DNA isolation from animal tissue. 19

Figure 3. Flow chart of tests performed on raptor samples. 25

Figure 4. RFLP gel analysis using *DraI* and *HinfI* on *S. falcatula, S. neurona*, and 16328B. 28

Figure 5. Sporocysts from the intestines of a Red-tailed Hawk. 29

Figure 6. Heavy *Sarcocystis* infection at 4x power. 29

Figure 7. Sarcocyst from an ITS positive only raptor. 30

Figure 8. Sarcocyst from a female red-shouldered hawk (14218) positive for the *S. falcatula* Arg species with measurements. 30

Figure 9. Sarcocyst from an *S. falcatula* Arg positive sample. 31

Figure 10. Primers to detect *S. calchasi* (SCa), *S. columbae* (SCo), and *Sarcocystis* sp. *ex A. nisus* (SNi) with positive controls. 32

Literature Review

1.1 *Sarcocystis* in Raptors

1.1a Life Cycle of *Sarcocystis* species

Sarcocystis species are in the phylum Apicomplexa and have a life cycle requiring 2 hosts (Figure 1). The definitive host is the animal, typically a carnivore or omnivore, in which sexual reproduction occurs followed by endogenous sporogony. The intermediate host, usually an herbivore or omnivore, is where asexual reproduction occurs (Lindsay et al., 1995). The definitive host sheds sporulated oocysts in the feces, which each contain 2 sporocysts. Within each sporocyst are 4 sporozoites (Lindsay et al., 1995). Sporocysts typically rupture out of their thin-walled oocysts while migrating from the lamina propria of the intestinal tract as they are shed in the feces of the definitive host (Atkinson et al., 2008). Intestinal infection in the definitive host is usually asymptomatic. When they reach the external environment, these sporocysts are fully infectious for the intermediate host. Inside the intermediate host, sporozoites excyst from the sporocyst and leave the small intestine to undergo a first generation of merogony (=schizogony) and produce first-generation merozoites in the endothelial cells of arteries. A second round of merogony occurs in additional endothelial cells of capillaries and the merozoites eventually produce sarcocysts in the striated and cardiac muscle (rarely CNS) of the intermediate host. Once they penetrate the muscle cell, merozoites become metrocytes, and divide by endodyogeny to produce bradyzoites. Sarcocysts can develop in any striated muscle, with some species showing a preference for specific muscle groups (Luznar et al., 2001). Once ingested by an appropriate definitive host, bradyzoites are digested out of sarcocysts and penetrate the lamina propria of the intestinal tract. Bradyzoites develop directly into sexual stages, macrogamonts and microgamonts, and sporulation occurs in the lamina propria.

As of 2008, 12 species of *Sarcocystis* were reported to use birds as a definitive host, 22 species used birds as intermediate hosts, and 2 species used birds as both definitive and intermediate hosts (Atkinson et al., 2008). Several additional species have been described since then. Species of *Sarcocystis* that infect birds are widely distributed and are present on all continents except Antarctica (Atkinson et al., 2008). Raptors and their prey, which include passerines, rodents, deer and other animals, act as intermediate hosts for species of *Sarcocystis*. Insects such as flies and cockroaches can act as phoretic vectors of sporocysts and serve as a source of infection for intermediate hosts. These phoretic hosts may contribute to the outbreaks of *Sarcocystis* species in zoos, aviaries, and rehabilitation centers. They may also lead to the possibility of biosecurity failures with fomites increasing transmission throughout different enclosures within a facility.

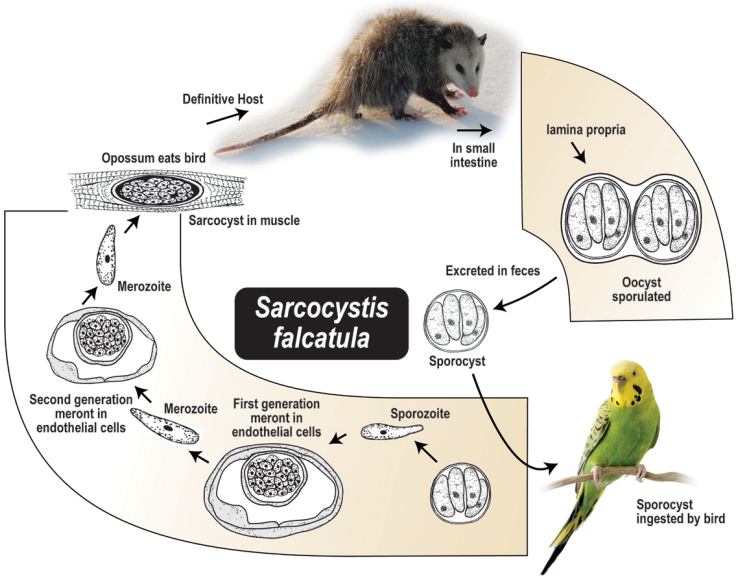

Figure 1. Life Cycle of *Sarcocystis falcatula.* Image courtesy of Dr. David S. Lindsay.

1.1b *Frenkelia*

Frenkelia species (=M organisms) were once classified in the family Sarcocystidae, along with *Sarcocystis* species. Raptors are the only known definitive hosts for this genus (Lindsay et al., 1987). In North America, red-tailed hawks (*Buteo jamaicensis*) are proven to be the definitive host for *F. microti* (Upton and McKnown, 1992). It uses rodents as intermediate hosts and birds of prey as definitive hosts (Mugridge et al., 1999). The tissue cysts of *Frenkelia* are lobate and are located exclusively in the CNS (Lindsay et al., 1992). *Frenkelia*-like sporocysts were found in 8 of 16 red-tailed hawks examined in Alabama (Lindsay and Blagburn, 1989). Upton and McKnown (1992) found by bioassay that sporocysts of *F. microti* were present in the feces of 1 of 6 hawks (*Buteo* spp.) in Kansas that were positive for sporocysts in the feces. Some true *Sarcocystis* species will invade neurological tissue including the peripheral and central nerves and make sarcocysts, but prefer skeletal and cardiac muscle. Both *Frenkelia* and *Sarcocystis* share antigens and have similar cyst wall structure (Votypka et al., 1998). Current literature suggests synonymizing these two genera and that classification is used herein (Mugridge et al., 1999; Votypka et al., 1998).

1.1c Diagnosis of *Sarcocystis*

Diagnosis of infection in the definitive host depends on the demonstration of characteristic sporocysts in the feces. Sporocyst structure is not definitive for each species, but can be used for identification of the genus. Some species of *Sarcocystis* are grossly visible in the muscles of the intermediate host. The structure of sarcocysts and the sarcocyst wall were used to identify *Sarcocystis* to the species level, but this is becoming less common as more species are discovered. Ultrastructural features of the sarcocysts were often used to name *Sarcocystis* species in the past, but molecular sequencing data based on the Internal transcribed Spacer 1 (ITS-1)

gene is becoming more commonly utilized. Acid-pepsin digestion can be used to demonstrate *Sarcocystis* bradyzoites in muscle tissue. This method has the advantage of being able to examine several grams of tissue rather than the small amount available using histology or PCR.

1.1d Molecular Identification of *Sarcocystis falcatula*

Molecular testing is necessary to distinguish between species of *Sarcocystis* in the muscles of birds because of the similar morphology of the sarcocysts and lack of life cycle knowledge. Primers Yabsley 18S9L (5'-GGATAACCTGGTAATTCTATG-3') and Yabsley 18S1H (5'-GGCAAATGCTTTCGCAGTAG-3') use the Internal Transcribed Spacer 1 (ITS-1) region to identify *Sarcocystis* species (Li et al., 2002). The JNB primer set (JNB 33 5'-CGAACAGAGATGAGGAAAAT-3'; JNB 54 5'-GTTGTGGTGTTGCGTGAGTC-3') amplifies an 1100 bp ITS region, which contains useful restriction sites for differentiation (Tanhauser et al., 1999). The *Hinf*I restriction site is unique to *S. falcatula* creating 745 and 355 bp fragments. The *Dra*I restriction site is unique to *S. neurona* and creates 884 and 216 bp fragments (Tanhauser et al., 1999). Other restriction sites appear along fragments amplified by different JNB primers.

1.1e Treatment of Disease Caused by *Sarcocystis*

Most coccidia that occur in the GI tract are treatable with sulfonamides. For raptors, the drug is inserted into a piece of meat or a gelatin capsule to avoid regurgitation (Tully et al., 2009). Pyrimethamine is a drug used in protozoal infections and is administered orally. It is a folic acid antagonist and acts synergistically with sulfonamides. These drugs are also active against *Plasmodium* and *T. gondii* (Coles and Coles, 2007). Supportive care can be administered to patients in conjunction with anti-protozoal drugs (Aguilar et al., 1991; Dubey et al., 1991; Wunschmann et al., 2010; Wunschmann et al., 2009). Because only sexual stages are present in

the intestines and cause no clinical signs, treatment of intestinal infection is not needed or justified.

1.1f Opossums as a Definitive Host for Avian Infective Sporocysts

Opossums (*Didelphis* species) have been shown to be definitive hosts for 5 or more species of *Sarcocystis* including *S. falcatula, S. falcatula*-like*, S. lindsayi*, *S. neurona,* and *S. speeri* (Box and Duszynski, 1978; Box and Smith, 1982; Dubey and Lindsay, 1998, 1999; Dubey et al., 2001b; Fenger et al., 1997; Tanhauser et al., 1999). Budgerigars are commonly used in bioassays to identify *S. falcatula, S. falcatula*-like, and *S. lindsayi* parasites in opossums while interferon-gamma gene knockout mice are used to identify *S. neurona, S. speeri,* and *S. neurona*-like species in opossums. Until 1995, *S. falcatula* was the only *Sarcocystis* species thought to use birds as intermediate hosts and opossums as a definitive host, but at present 2 additional avian pathogenic species (*S. lindsayi* and *S. falcatula*-like) have been identified to use *Didelphis* spp as a definitive host. The *S. falcatula*-like parasite is distinct from *S. falcatula* because it contains recognition sites for both the *Dra*I and *Hinf*I restriction enzymes, not just *Hinf*I, in a PCR RFLP analysis. The third species, *S. lindsayi* cuts at the *Dra*I restriction site using JNB 33/54 primers, like *S. neurona* does (Dubey et al., 2001b). This species has also been found in an opossum species from Brazil (*Didelphis aurita*) (da Silva Stabenow et al., 2012). All 3 bird infective *Sarcocystis* species excreted by opossum are highly pathogenic for budgerigars.

1.1g *Sarcocystis falcatula* infection

S. falcatula is recognized as a highly pathogenic species and has caused outbreaks of fatal disease in zoos and aviaries (Ecco et al., 2008; Suedmeyer et al., 2001). Larger birds seem better equipped to handle infections while smaller birds succumb to the infection. During schizogony of *S. falcatula*, inflammatory cells infiltrate the damaged tissues and result in blockage of blood

vessels, edema, and pulmonary congestion (Smith et al., 1987b). Most meronts develop in the lungs, with a smaller portion occurring in the kidney. Merogony can begin as early as 12 hours after infection of the intestinal lamina propria by sporozoites. By day 2, meronts can be found in the lungs and liver. The first sarcocysts develop in the cardiac muscles by day 7, though these cysts degenerate within 30 to 40 days post infection. Sarcocysts develop in the skeletal muscle, including pectoral and leg muscle, by day 8. Those in the pectoral muscles usually degenerate, but don't always do so. Most avian deaths caused by *S. falcatula* are attributed to pneumonitis with some cases having evident inflammation of the liver, muscles, kidney and brain. Captive psittacines can develop acute sarcocystosis with pulmonary edema and hemorrhage. Parrots develop an enlarged spleen and liver as well as marked inflammation of other internal organs (Atkinson et al., 2008).

Sporocysts of *S. falcatula* can also infect canaries, cowbirds, budgerigars, grackles, house sparrows, zebra finches and rock pigeons (Box and Duszynski, 1978). Domestic chickens and helmeted guineafowl do not develop disease after ingestion of *S. falcatula* sporocysts (Atkinson et al., 2008; Box and Smith, 1982).

1.1h S*arcocystis* infections of the Central Nervous System

Sarcocystis associated encephalitis was observed in an immature northern goshawk (*Accipiter gentilis atricapillus*) from a falconer in Minnesota (Aguilar et al., 1991) and a golden eagle (*Aquila chrysaetos*) in southwest Virginia (Dubey et al., 1991), but the species was not identified in either report. DNA of *S. falcatula* has been identified in the brain and muscles of great horned owls (*Bubo virginianus*), golden eagles (*Aquila chrysaetos*) and bald eagles (*Haliaeetus leucocephalus*) in raptor rehabilitation centers in Indiana, Minnesota and Virginia using PCR (Olson et al., 2007; Wunschmann et al., 2010; Wunschmann et al., 2009).

Neural sarcocystosis can simulate toxoplasmosis and has been found in the northern gannet (*Morus bassanus*), northern goshawk, wild turkeys, the Eurasian capercaillie from Finland, and straw necked ibis, (Aguilar et al., 1991; Atkinson et al., 2008; Dubey et al., 1991; Dubey et al., 1998; Olson et al., 2007; Spalding et al., 2002; Teglas et al., 1998). Many of these cases have not been clearly identified to a particular *Sarcocystis* species. Of these, the bald eagle from the Olson et al. (2007) study was determined to have *S. neurona* by immunohistochemistry. Additional confirmation is needed because of the close relationship between *S. falcatula* and *S. neurona*. It is unlikely that the wild turkeys or Eurasian capercaillie would be positive for *S. falcatula*, since this species does not infect gallinaceous birds. It was not noted whether or not these birds were exposed to opossums.

1.1i *Sarcocystis falcatula*-like and *S. lindsayi* Species

S. falcatula was recently identified for the first time in Argentina (Dubey et al., 1999). Budgerigars succumbed to *Sarcocystis* infection after inoculation with sporocysts from a naturally infected South American opossum (*Didelphis albiventris*). Recent reports have found *S. falcatula*-like parasites in opossums from South America that cut with both *Dra*I and *Hinf*I enzymes (Dubey et al., 2000a; Dubey et al., 2001a; Dubey et al., 2000b). In 2000, Dubey et al. used 2 *S. falcatula* isolates from *D. albiventris* of Argentina and infected CV-1 cells in culture (Dubey et al., 2000a). PCR was performed and products were amplified using JNB 33/JNB 54. Unfortunately, products were only digested with *Hinf*I. Dubey et al. performed PCR on isolates SF-1 and SF-2 using JNB33/JNB 54 and restriction digest with both enzymes. Both isolates, as well as bird 152 showed cuts at both restriction sites, which is unlike *S. falcatula* isolates from North America (Dubey et al., 2000a). *S. falcatula*-like isolates from *D. marsupialis* and *D. albiventris* from São Paolo, Brazil were used to infect budgerigars in a 2001 study (Dubey et al.,

2001a). Molecular characterization was performed on several birds, including bird 62 from the first Dubey et al. study, and bird 200, which was infected using sporocysts from *D. marsupialis* (Dubey et al., 2001a; Dubey et al., 2000b). Both samples showed cuts at restriction sites for *Dra*I and *Hinf*I and sequencing did not distinguish the *D. marsupialis* isolate from the *S. falcatula*-like SF-1 and SF-2 isolates (Dubey et al., 2001a). Both species of South American opossum are widespread in South and Central America, but clinical pulmonary sarcocystosis in avian species had not been reported in the region. This lack of observation of parasitic infection may be due to birds being asymptomatic. A report by Spalding et al. (2002) discussed a case of fatal *Sarcocystis*–associated encephalitis in a northern gannet from Florida. This *Sarcocystis* species had both restriction sites for *Dra*I and *Hinf*I enzymes using JNB33/JNB54 primers. DNA was isolated from the brain and compared to isolates 1085 and 1086 from opossums (*Didelphis virginiana*) in a previous study (Spalding et al., 2002; Tanhauser et al., 1999). These results indicate that this *S. falcatula*-like parasite is present in opossums and birds from both North and South America.

Sarcocystis lindsayi was obtained from the lungs and muscles of budgerigars (*Melopsittacus undulatus*) fed sporocysts from a naturally infected South American opossum, *Didelphis albiventris* (Dubey et al., 2001). Molecular studies demonstrated that *S. lindsayi* was genetically distinct from other *Sarcocystis* species that also cycle between opossums and avian species. Sporocysts of *S. lindsayi* have been demonstrated in the feces of *Didelphis aurita* from Brazil (da Silva Stabenow et al., 2012).

1.1j *Sarcocystis calchasi, Sarcocystis columbae* and *Sarcocystis* sp. ex *Accipiter nisus*

There are three *Sarcocystis* species that are emerging parasites in birds from Europe; *S. calchasi, S. columbae* and *Sarcocystis* sp. ex *A. nisus*. *S. calchasi* is a novel species, first

described in Berlin, Germany that cycles between the intermediate host, the domestic pigeon (*Columba livia* f. *domestica*), and the definitive host, the northern goshawk (*Accipiter gentilis*) (Olias et al., 2010a; Olias et al., 2009b). This parasite will cause severe CNS disease in the intermediate host, but is non-pathogenic in the definitive host. A recent study has expanded the host range to include cockatiels (*Nymphicus hollandicus*), which were experimentally infected with *S. calchasi* after oral inoculation with sporocysts (Olias et al., 2014). Within 7 to 12 days post infection (dpi), pigeons infected with at least 8 x 10^4 sporocysts died, with lower doses causing neurological symptoms 51 to 64 dpi. Olias et al. (2010) showed that there was no infectivity of sarcocyst positive skeletal muscle tissue that was orally administered to mice (*Mus musculus domesticus*), rats (*Rattus norvegicus* f. *domestica*), ferrets (*Mustela putorius furo*), dogs (*Canis familaris*, beagle) and gyr-saker (*Falco rusticolis* x *Falco cherrug*) hybrid falcons (Olias et al., 2009a). Sporocysts were not infective to chickens (*Gallus gallus* f. *domestica*). Morphologically distinct sarcocysts were described for *S. calchasi* that differed from *S. columbae*.

Psittacines are also susceptible to *S. calchasi* infection and an outbreak has been reported in an enclosed zoological aviary (Rimoldi et al., 2013). Five psittacine birds in an enclosed zoological exhibit (2 princess parrots and 3 cockatoos of 2 different species) developed severe CNS signs over a 2–3-month period and died or were euthanized (Rimoldi et al., 2013). Histologically, all birds had a lymphoplasmacytic and histiocytic encephalitis and sarcocysts were present in muscles. PCR and sequencing identified *S. calchasi* as the etiologic agent. This report and a report of CNS disease in a domestic pigeon caused by *S. calchasi* demonstrate that this highly pathogenic species is present in intermediate hosts in the United States (Wunschmann

et al., 2011). Additional studies are needed to determine which raptor is the definitive host in North America.

More work is needed to determine the prevalence of this parasite, its distribution in definitive or intermediate hosts as well as using molecular techniques to characterize and differentiate species. The clinical signs associated with disease are also very similar to those associated with salmonellosis and paramyxovirosis (Olias et al., 2009b; Olias et al., 2010b). *S. columbae* and *Sarcocystis* sp. ex *A. nisus* are less characterized. *S. columbae* uses the wood pigeon (*C. palumbus*) as its intermediate host and has an unknown definitive host. It was first described in Northern Germany. It is speculated to also use the northern goshawk as it's definitive host (Olias et al., 2010c). *Sarcocystis* sp. ex *A. nisus* may use northern goshawks and European sparrowhawks as definitive hosts.

1.2 *Toxoplasma gondii* in raptors

1.2a Life Cycle of *Toxoplasma gondii*

Toxoplasma gondii is a protozoan parasite with a worldwide distribution. It is in the phylum Apicomplexa, family Sarcocystidae. It uses felids as the definitive host and is potentially infective to all warm-blooded animals, making all species of birds potentially susceptible. This parasite is also zoonotic, which is of particular concern in pregnant women and immunocompromised individuals. Transmission occurs from ingestion of tissue cysts in an infected carcass or through ingestion of oocysts in contaminated food or water. There is only a single species in this genus, but genetic differences occur between isolates. They are classified by biological characteristics as mouse virulent or avirulent and distributed into lineages: Type I,

II, and III (Atkinson et al., 2008). Type I is the most virulent in mice but the effects demonstrated in this host may not reflect pathogenicity in other hosts.

With such a wide range of intermediate hosts, it is hard to control the spread of this parasite. Oocysts shed by the felid definitive host are environmentally resistant. They are shed unsporulated, and sporulate in the environment for 1-5 days. An oocyst contains 2 sporocysts, each of which contain 4 sporozoites. Although about 1% of infected cats may shed oocysts at any given time, each may shed 100,000,000 during its lifetime. Congenital infection may also occur in cats. Oocysts can also be found in paratenic hosts. They can be mechanically spread by flies, cockroaches, dung beetles and earthworms (Atkinson et al., 2008).

1.2b *Toxoplasma gondii* Infection in Raptors

A previous report showed a 26.7% (n=27) prevalence in 101 raptors in Alabama based on parasite isolation from heart and skeletal muscle (Lindsay et al., 1993). In raptors, *T. gondii* may or may not be pathogenic. Pathogenicity is dependent upon the asexual reproduction in the host tissue causing focal tissue necrosis resulting in anorexia, weight loss, diarrhea, respiratory signs and CNS signs (Coles and Coles, 2007).

1.2c Clinical Signs of *Toxoplasma gondii* in Raptors

Toxoplasma gondii encephalitis in a bald eagle and hepatitis in a barred owl have been reported (Mikaelian et al., 1997; Szabo et al., 2004). Clinical signs are nonspecific and cannot be used to make a definitive diagnosis. They include: anorexia, depression, dull ruffled feathers, diarrhea, dyspnea, with some cases showing ocular or CNS involvement (Atkinson et al., 2008). In ocular cases, patient's eyes are dull, remain closed or shrunken into the head and sight may be lost. In many cases, birds are still alert and feeding.

1.2d Diagnosis of Disease Caused by *Toxoplasma gondii*

Histology, serology, immunohistochemistry and molecular methods can be used to diagnose *T. gondii (*Atkinson et al., 2008; Coles and Coles, 2007*)*. Presence of antibodies to this parasite is only indicative of exposure, and does not provide information on whether or not there is a current infection (Atkinson et al., 2008). The modified agglutination test has, so far, proven to be the most specific and sensitive. It is also simple, reliable, does not require specific reagents and works well with plasma (Atkinson et al., 2008).

1.2e Prevention of *Toxoplasma gondii*

Preventative measures can be taken to reduce *T. gondii* exposure to raptors in captivity. Aviaries should be made cat proof, if possible, and proper food storage for the birds should be practiced. For meat eating birds (and humans), meat should be frozen for 1-2 days at -20˚C prior to feeding if cooking is impractical (Atkinson et al., 2008; El-Nawawi et al., 2008). Cooking meat at 60-100˚C for 10 minutes is sufficient to kill tissue cysts (El-Nawawi et al., 2008).

1.3 *Neospora caninum* in raptors

1.3a Life Cycle of *Neospora caninum*

N. caninum is an Apicomplexan parasite of family Sarcocystidae with a worldwide distribution, which is closely related to *T. gondii* (Darwich et al., 2012). Like *T. gondii* and *Sarcocystis* species, *N. caninum* is capable of forming tissue cysts. It uses a canine definitive host and is a major cause of reproductive disorders and abortions in cattle (Bartels et al., 1999; Dubey, 2003; Dubey et al., 2007; Gondim, 2006). This parasite was first recognized in dogs in Norway (Bjerkas et al., 1984). Domestic dogs can act as the intermediate or definitive host of this coccidian parasite with the most severe cases occurring in puppies that were congenitally

infected (Basso et al., 2001; Dubey, 2003; Dubey et al., 2002; Lindsay et al., 1999; Lindsay et al., 2001; McAllister et al., 1998). *N. caninum* uses deer as a natural intermediate host, along with other ruminants, which may contribute to the idea that carrion is a significant source of infection, especially in carnivorous birds (Gondim, 2006; Gondim et al., 2004; Vianna et al., 2005). An early study with raptors found that they were not definitive hosts for *N. caninum* and that no disease occurred after inoculation with infected mouse carcasses (Baker et al., 1995).

1.3b Clinical Signs of *Neospora caninum* in birds

Birds appear to be resistant to clinical infection with *N. caninum*. Naturally occurring clinical disease has not been reported. Few studies have demonstrated infections in birds using PCR or serology, but disease has not been reported.

1.3c Diagnosis of Disease Cause by *Neospora caninum* in birds

There is no consensus on how best to diagnose *N. caninum* infection in birds. Examination of serum using experimental ELISAs, indirect fluorescent antibody tests (IFAT) and the *Neospora* agglutination test (NAT) can be used to detect antibodies. PCR can also be used for detection, but the sensitivity may vary. Histpathology and immunohistochemistry can be used if tissue cysts are present.

1.3d *Neospora caninum* in Raptors

There is little information on the prevalence of *N. caninum* in birds, including raptors, but it has been suggested that the rate of neosporosis in cattle increases with birds present (Bartels et al., 1999). Quails (*Coturnix coturnix japonica*) and zebra finches (*Poephila guttata*) have shown resistance to the disease while chickens (*Gallus domesticus*), domestic pigeons (*Columbia livia*) and sparrows may be susceptible (Costa et al., 2008; de Oliveira et al., 2013; Gondim et al.,

2010; McGuire et al., 1999). Raptors are resistant to oral infection (Baker et al., 1995). Some rodents have also been implicated as an intermediate host in the life cycle of *N. caninum* since they are a food source for carnivorous birds (Huang et al., 2004; Jenkins et al., 2007). It has been suggested that birds may be an intermediate host in this cycle, since foxes have been found seropositive, and they will frequently feed on birds (McGuire et al., 1999).

Proposal

Reports of *Sarcocystis*-associated encephalitis in raptors due to *S. falcatula* and *S. falcatula*-like parasites have been emerging in the literature from the United States. Additional species (particularly *S. calchasi*) have been emerging as a cause of encephalitis in birds from Europe. We propose that there are several species of *Sarcocystis* that are capable infecting raptors, but that *S. falcatula* will be the most prevalent species identified in North America. Little is known about the occurrence of *Toxoplasma gondii* and *Neospora caninum* infections in raptors. In this study, we examined the prevalence of these parasites, along with *Sarcocystis* species in raptors from the Carolina Raptor Center.

Materials and Methods

3.1 Raptor Samples

The samples used in this study were obtained from patients at the Carolina Raptor Center in Huntersville, North Carolina. The birds either died or were humanely euthanized due to poor prognosis. A tissue sample was obtained from the breast and heart of each bird. The DNA was tested by PCR and further characterized by RFLP, if applicable, at CMMID. Hematoxylin and eosin (H&E) slides were prepared as described below. Muscle was kept at -20˚C until used for DNA isolation. Isolated DNA samples were stored in a freezer at -20˚C until examined.

3.2 Hematoxylin and Eosin Slide Preparation

Portions were fixed in 10% neutral buffered formalin solution for histological examination and other portions were frozen at -20˚C for DNA isolation. Tissues were embedded in paraffin and 6 µm sections were cut and stained with H&E stain and examined to determine the presence of sarcocysts. DNA was extracted from the frozen muscle at either the Department of Natural Sciences and Mathematics, College of Science, Technology, Engineering and Mathematics, Johnson C. Smith University (JCSU), Charlotte, North Carolina or from frozen muscle samples sent to the Center for Molecular Medicine and Infectious Disease (CMMID), Virginia Tech, Blacksburg, Virginia.

3.3 Primers

Isolated DNA was examined by PCR using 7 different primers (Table 1). Five were designed to detect and identify *Sarcocystis* species and one each was used to detect *Toxoplasma gondii* or *Neospora caninum*.

Table 1. Primers used for the detection of *Sarcocystis* species, *Toxoplasma gondii* and *Neospora caninum.*

Primers	Description	Sequence	Amplicon Size/ Target Site (TS)
ITS 18S 9L	*Sarcocystis* species	5'- GGATAACCTGGTAATTCTATG-3'	800 bp
ITS 18S 1H		5'-GGCAAATGCTTTCGCAGTAG-3' (Li et al., 2002)	
JNB 33	*S. falcatula* or *S. neurona*	5'-CGAACAGAGATGAGGAAAAT-3'	1100 bp
JNB 54		5'- GTTGTGGTGTTGCGTGAGTC-3' (Tanhauser et al., 1999)	
SCa1	*S. calchasi*	5'-CTCCTTGCTCGAGAATGAACATGAG-3'	(TS) 276-300
SNCa3		5'-TCCAGAGAAGATCCCCTGGCTAC-3 (Olias et al., 2011)	(TS) 389-411
SCo1	*S. columbae*	5'-TTCCTCGCTTGAGGATGAATAAG-3'	(TS) 281-303
SNCo3		5'-TCCAGGGATGATTTCATGGTTACAC-3' (Olias et al., 2011)	(TS) 385-409
SNi1	*Sarcocystis* sp. Ex. *A. nisus*.	5'-CGCGCAGAAAGATGAACAACGA-3'	(TS) 294-315
SNNi3		5'-GATCTCCCTGGTACACCATCCTA-3' (Olias et al., 2011)	(TS) 394-417
NP21+	*Neospora caninum*	5'-CTCGCCAGTCAACCTACG-3'	328 bp
NP6+		5'-CCCAGTGCGTCCAATCCTGTAAC-3' (Liddell et al., 1999; Yamage et al., 1996)	
TOX4	*Toxoplasma gondii*	5'-CGCTGCAGACACAGTGCATCTGGATT-3'	529 bp
TOX5		5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3' (Homan et al., 2000)	

3.4 DNA Isolation

DNA from samples were initially isolated at JCSU using a commercial kit (Quiagen DNA Mini Kit, Valencia, CA) by students of Dr. Alexa C. Rosypal. The DNA was extracted using protocol as per manufacturer's instructions. Briefly, 25 mg of tissue was cut into small pieces using a sterile razor blade. The sample was mixed with 180µl of lysis buffer and 20µl of Proteinase K in a 1.5 ml microcentrifuge tube. The tube was incubated at 56˚C for 1 hour or until the sample was completely lysed. Next, 200 µl of Buffer AL was added to the tube, vortexed and incubated at 70˚C for 10 minutes. After incubation, 200 µl ethanol was added to precipitate the DNA. The sample was then applied to the minispin column placed inside a collection tube, then centrifuged at 8,000 rpm to bind the DNA to the column. The column was then placed in a clean collection tube and 500 µl of wash buffer was added. The sample was centrifuged at 8,000 rpm for 1 minute. A second application of wash buffer was added to the column and spun for 3 minutes at 14,000 rpm. The column was then placed in a clean collection tube and 200 µl elution buffer was added. The sample was centrifuged a final time at 8,000 rpm to elute the DNA from the spin column. The isolated DNA was stored at -20˚C in a freezer.

The Bioline Isolate Genomic DNA mini kit was used on samples of raptor muscles that were not processed at JCSU but shipped frozen to CMMID. Figure 2 shows the protocol for DNA isolation from animal tissue, provided by Bioline. DNA was isolated from 40 mg of tissue, to which 400 µl of lysis buffer D, 25 µl of Proteinase K and 4 µl of 100mg/ml RNase Inhibitor were added. The sample was incubated at 50˚C for 1-3 hours, with intermittent vortexing, until sample was lysed. The tube was spun at 12,000 rpm for 30 seconds and the supernatant was transferred to another 1.5 ml tube. 400 µl binding buffer was added and the tube was vortexed. The sample was transferred to a spin column placed in a collection tube and spun down at 12,000

rpm for 2 minutes. The collection tube was discarded and the spin column was placed in a clean collection tube with 700 µl wash buffer, and spun at 12,000 rpm for 1 minute. The wash buffer step was repeated. To remove traces of ethanol, the tube was spun at max speed (14,800 rpm/21,100 x g) for 2 minutes and the collection tube was discarded. The spin column was then placed in a 1.5 ml elution tube with 200 µl of elution buffer applied directly to the spin column. We let the tube sit at room temperature for 2 minutes. The tube was then spun at 8,000 rpm for 1 minute to elute the DNA. All centrifugations were performed in a Legend Micro 21R Cetrifuge, rotor #75003424. The concentration of DNA was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and the sample was stored at -20˚C in the freezer.

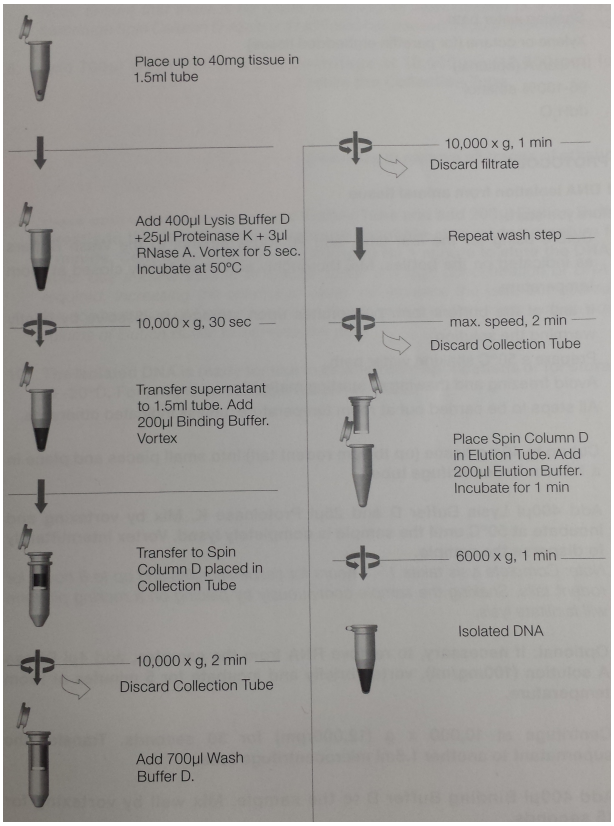

Figure 2. Bioline protocol for DNA isolation from animal tissue. (Bioline ISOLATE DNA Kits product manual)

3.5 *Sarcocystis* species PCR

Test DNA was in a 25 µl reaction consisting of 12.5 µl of GoTaq Green, 10.5 µl of nuclease free H_2O, 0.5 µl of forward primer, 0.5 µl of reverse primer and 1 µl of sample template. Some reactions were modified to 6.5 µl of water and 5 µl of sample template to produce more visible bands. The samples were tested with 2 or more primer sets. The first set was based on the Internal Transcribed Spacer (ITS) region. This primer set is used to determine the presence of all *Sarcocystis* species. The second set of primers, JNB 33/JNB 54, were designed to detect *S. falcatula* and/or *S. neurona* following RFLP (see below).

Based on emerging reports of fatal, neurological *Sarcocystis* infections caused by *S. calchasi* in pigeons and the involvement of raptors in its life cycle, we chose to test the samples found positive for ITS for *S. calchasi, S. columbae* and *Sarcocystis* sp. *ex A. nisus* (Table 1) using primer sets SCa1/SNCa3 (*S. calchasi*), SCo1/SNCo3 (*S. columbae*), and SNi1/SNNi3 (*Sarcocystis* sp. from *Accipiter nisus*) respectively (Olias et al., 2011).

The PCR protocol was performed on an Eppendorf Mastercycler (Hamburg, Germany) under the following conditions: 94˚C, 10 min; (94˚C, 45 sec; 52˚C, 1 min; 72˚C, 1 min) x 35 cycles; 72˚C, 10 min; 4˚C holding temperature. PCR products were run on a 1% agarose gel that consisted of either 50 ml TBE, 0.5 g room temperature setting agarose and 7 µl ethidium bromide (or 150 ml TBE, 1.5 g room temperature setting agarose and 21 µl ethidium bromide) at 90V for 90 minutes.

3.6 Restriction Fragment Length Polymorphism

The QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) was used to extract PCR products from 1% agarose gels. Instructions in the commercial kit were followed with a few modifications. The gel was excised using either a gel punch or blade, on a UV transilluminator

and added to a collection tube. Once the gel slice was added, the tube was weighed again and 3 volumes of QG buffer was added per 1 volume of gel. The tube was incubated at 50˚C for 10 minutes, or until the gel slice dissolved, inverting the tube every 2-3 minutes. One gel volume of isopropanol was then added to the sample and mixed. The sample was pipetted into a QIAquick column and centrifuged to bind the DNA. Once the entire sample had been spun through the column, 500µl of Buffer QG was added to the column and was spun to dissolve any residual agarose. A second wash step with 750 µl buffer PE was conducted. We allowed the buffer PE to sit in the column for 2-5 minutes before centrifugation. After discarding the flow-through, the column was spun again to ensure residual ethanol was removed. DNA was eluted into a 1.5 ml microcentrifuge tube using 30-50 µl of buffer EB or nuclease free H_2O if the DNA was to be sent for sequencing. When using nuclease free H_2O, we heated the water on a heating block ensuring it is no more than 70˚C upon addition to the spin column. Buffer EB or nuclease free H_2O was allowed to sit on the column for up to 4 minutes before centrifugation to produce a higher yield. The eluted DNA was passed through the same spin column a second time after it stood one minute on the column.

For RFLP, the initial amplification step was performed in a 50 µl reaction (25 µl GoTaq Green, 21 µl nuclease free H_2O, 1 µl forward primer, 1 µl reverse primer and 2 µl sample template or 25 µl GoTaq Green, 13 µl nuclease free H_2O, 1 µl forward primer, 1 µl reverse primer and 10 µl sample template) and run on a 1% agarose gel. The PCR protocol for the thermalcycler is the same as listed above. Two wells were loaded with 20 µl of each sample to increase the DNA yield from the extraction kit on a 1% gel. We used a QIAquick Gel Extraction Kit with minor modifications such as running the elution through the spin column twice in the last step and using warmed nuclease free H_2O for the elution step. The DNA concentration

(ng/µl) of the extracted samples was quantified using a NanoDrop ND-1000 Spectrophotometer for calculations necessary to perform the digest. We used the Promega Restriction Enzyme Digest Protocol (Promega, Madison, WI) to result in a 20 µl reaction. After the incubation step, the digested products were run on 2% agarose gel at 90V for 100 minutes. We used enzymes *Dra*I and *Hinf*I as they cut *S. neurona* and *S. falcatula* respectively (Tanhauser et al., 1999). *S. falcatula* Arg is present when both *Dra*I and *Hinf*I cut the DNA fragment (Figure 3) (Dubey et al., 2001a; Dubey et al., 2000b). A summary of RFLP results for different species of *Sarcocystis* using the aforementioned materials is shown in Table 2.

Table 2. Summary of RFLP results of several *Sarcocystis* species using *DraI* and *HinfI* enzymes with JNB 33/JNB 54 primers.

Species	**Host**	**JNB33/JNB54**	***DraI***	***HinfI***
S. falcatula	Bird	Pos 1100	No	Pos 745/355
S. neurona	Mammal	Pos	Pos 884/216	No
S. lindsayi	Bird	Pos	Pos 884/216	No
S. falcatula Arg	Bird	Pos	Pos 884/216	Pos 745/355
S. speeri	Mammal	Neg		

3.7 Sequencing of *Sarcocystis* spp.

DNA fragments to be sequenced were submitted to the Virginia Bioinformatics Institute (VBI) at Virginia Tech in Blacksburg, VA. The reaction mixture used contained a 45 µl aliquot of platinum mix, 1 µl forward primer, 1 µl reverse primer, and 5 µl template. When used with

ITS primers, the thermalcycler protocol was slightly modified from the one previously used to lower the extension temperature (94˚C, 10 min, [94˚C, 45 sec; 52˚C, 1 min; 68˚C, 1 min] x 35 cycles, 72˚C, 10 min, and 4˚C holding temperature). The PCR products were run on a 1% agarose gel with 2 wells for each sample. The wells were loaded with 20 µl sample and 4 µl loading dye. Gel fragments were excised and a QIAquick Gel Extraction kit was used to extract DNA. The resulting DNA was then analyzed for concentration using a Nanodrop spectrophotometer. If more than 10 ng/µl was present, the sample was deemed suitable for submission. VBI received 30 µl of sample with water added as necessary and 10 µl of primer (5 µl stock primer, 5 µl nuclease free H_2O).

3.8 *Toxoplasma gondii* PCR

DNA previously isolated from raptors and stored at -20˚C was used to conduct PCR. The DNA was in a 25 µl reaction consisting of 12.5 µl of GoTaq Green, 10.5 µl of nuclease free H_2O, 0.5 µl of forward primer, 0.5 µl of reverse primer and 1 µl of sample template. Some reactions were modified to 6.5 µl of water and 5 µl of sample template to produce brighter results. *Toxoplasma gondii* was identified with TOX4/TOX5 primers (Table 1). The PCR protocol was performed on Eppendorf Mastercycler with a protocol similar to that described in section 3.4. The hot start at 94˚C was 7 minutes long, the denaturation step was 1 minute long, the annealing step was changed to 61˚C for 1 minute, and all other steps remained constant. PCR products were run on a 1% agarose gel (150 ml) at 90V for 90 minutes.

3.9 *Neospora caninum* PCR

DNA previously isolated from raptors and stored in the freezer will be used to conduct PCR. The DNA was in a 25 µl reaction consisting of 12.5 µl of GoTaq Green, 10.5 µl of nuclease free H_2O, 0.5 µl of forward primer, 0.5 µl of reverse primer and 1 µl of sample

template. Some reactions were modified to 6.5 µl of water and 5 µl of sample template to produce brighter results. *Neospora caninum* was identified with NP6+/NP21+ primers (Table 1). The PCR protocol performed on an Eppendorf Mastercycler with a protocol similar to that described in section 3.4. The denaturation step was only 30 seconds, the annealing temperature was 55˚C, and all other steps remained constant. PCR products were run on a 1% agarose gel (150 ml) at 90V for 90 minutes.

3.10 Positive Apicomplexan Parasite DNA Samples

The Apicomplexan parasites *S. falcatula, S. neurona, S. lindsayi, T. gondii,* and *N. caninum* are routinely maintained in mammalian cell cultures in the Zoonotic Parasitic Diseases Laboratory at CMMID. Samples of DNA isolated from these Apicomplexan parasites are kept frozen at -20˚C and were used as positive controls in appropriate PCR reactions in this study.

Results

4.1 General Procedures and Raptor information

The following flow chart breaks down the general steps taken with each sample.

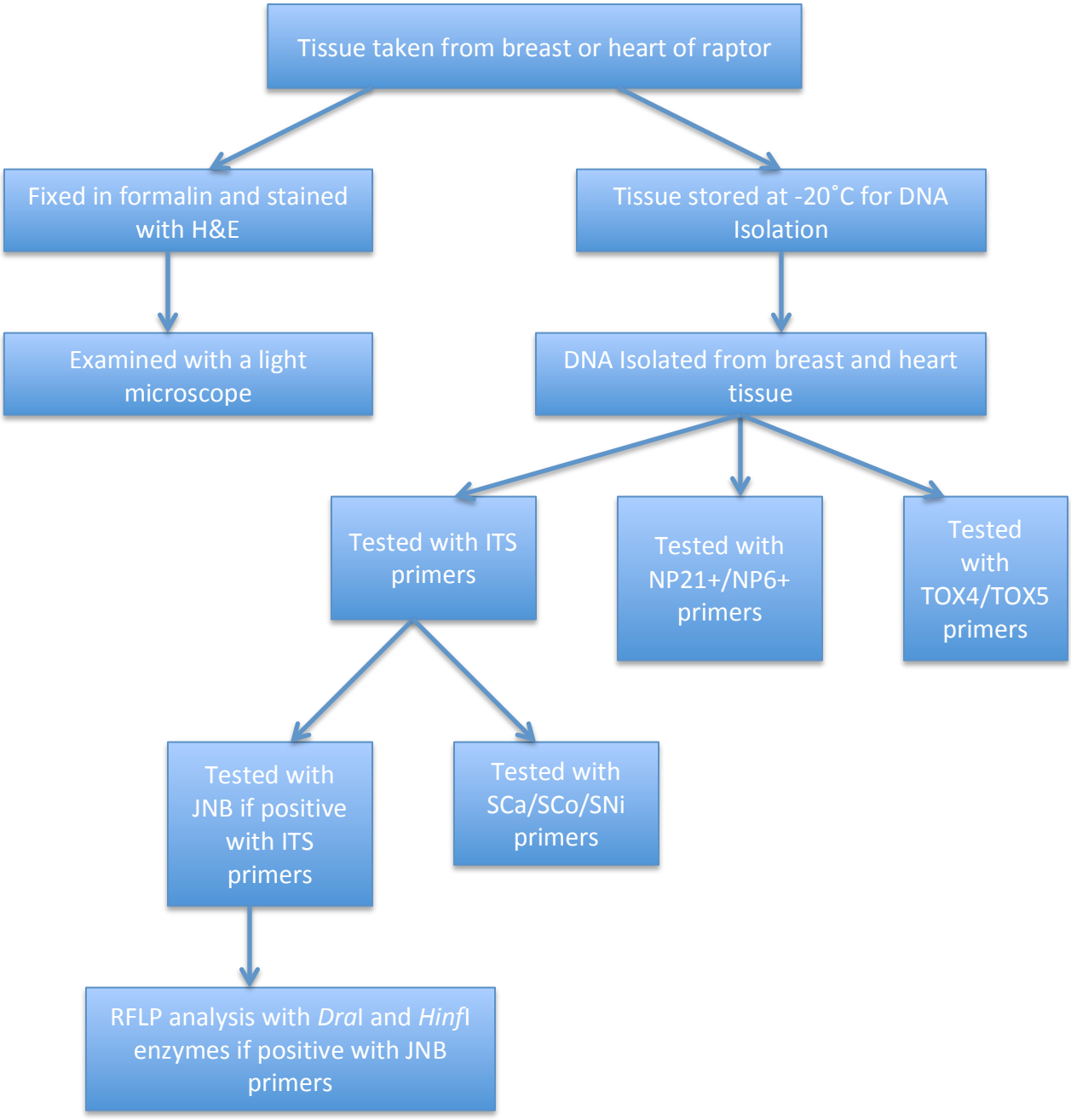

Figure 3. Flow chart of tests performed on raptor samples.

In order to better understand the organization of the following tables, as well as the background and life history of some of the raptors in this study, Table 3 includes information on raptors that we found positive for one or more parasites via PCR.

Table 3. Life history and diet information for raptors found positive for one or more parasitic species. Information provided by the Sibley Field Guide to Birds of Eastern North America (2003) and www.allaboutbirds.org managed by the Cornell Lab of Ornithology.

Species	**Scientific Name**	**Diet**	**Other facts**
-Diurnal Raptors-			
Black Vulture	*Coragyps atratus*	Carrion	Pairs or small groups. Often mixes with Turkey Vultures
Turkey Vulture	*Cathartes aura*	Carrion	Solitary or small groups.
Osprey	*Pandion haliaetus*	Fish (~99%); also seen feeding on birds, snakes, voles, squirrels, muskrats and salamanders	Solitary
Mississippi Kite	*Ictinia mississippiensis*	Cicadas, dragonflies, other insects. Pluck from mid air or off of leaves	Uncommon. Small groups or larger flocks during migration.
Cooper's Hawk	*Accipiter cooperii*	Birds (medium sized) and mammals	Uncommon. Solitary.
Red-shouldered Hawk	*Buteo lineatus*	Small mammals, lizards, snakes, amphibians, crayfish and occasionally birds	Uncommon. Solitary.
Broad-winged Hawk	*Buteo platypterus*	Small mammals, amphibians, insects	Smallest buteo hawk. Uncommon. Usually solitary but migrate in large flocks or congregate in certain locations.
Red-tailed Hawk	*Buteo jamaicensis*	Mammals, birds, snakes, carrion	Uncommon, but widespread.

Merlin	*Falco columbarius*	Small birds, insects, bats, small mammals	Uncommon in open habitats. Solitary but pair up occasionally.
-Owls-			
Eastern Screech Owl	*Otus asio*	Small birds and mammals, invertebrates	Uncommon, but widespread. Usually solitary. No larger than a pint glass.
Great Horned Owl	*Bubo virginianus*	Mammals and birds including raptors (Barred Owls and house cats included in diet)	Uncommon, but widespread. Usually solitary.
Barred Owl	*Strix varia*	Small mammals, small birds, reptiles, amphibians, invertebrates	Common to uncommon (depending on the region). Usually solitary.

4.1 *Sarcocystis* species

The results of ITS positive birds are listed in Table 4. Unfortunately, species, gender and location data was not available for all birds. 24 of 114 (21.1%) birds were positive for a *Sarcocystis* species via PCR with ITS primers. We then tested the 24 birds with JNB primers to determine the presence of either *S. neurona* or *S. falcatula.* Of the 24 birds, 9 were positive (34.6%). Restriction digest using the enzymes *Dra*I and *Hinf*I was then required to discern which species was present in the bird. Four birds were determined to have *S. falcatula* (*Hinf*I cut) and 3 to have *S. falcatula* Arg (*Dra*I and *Hinf*I cut). Figure 4 shows a gel of the RFLP results for *S. falcatula, S. neurona* and sample 16328B, which was positive for *S. falcatula* Arg.

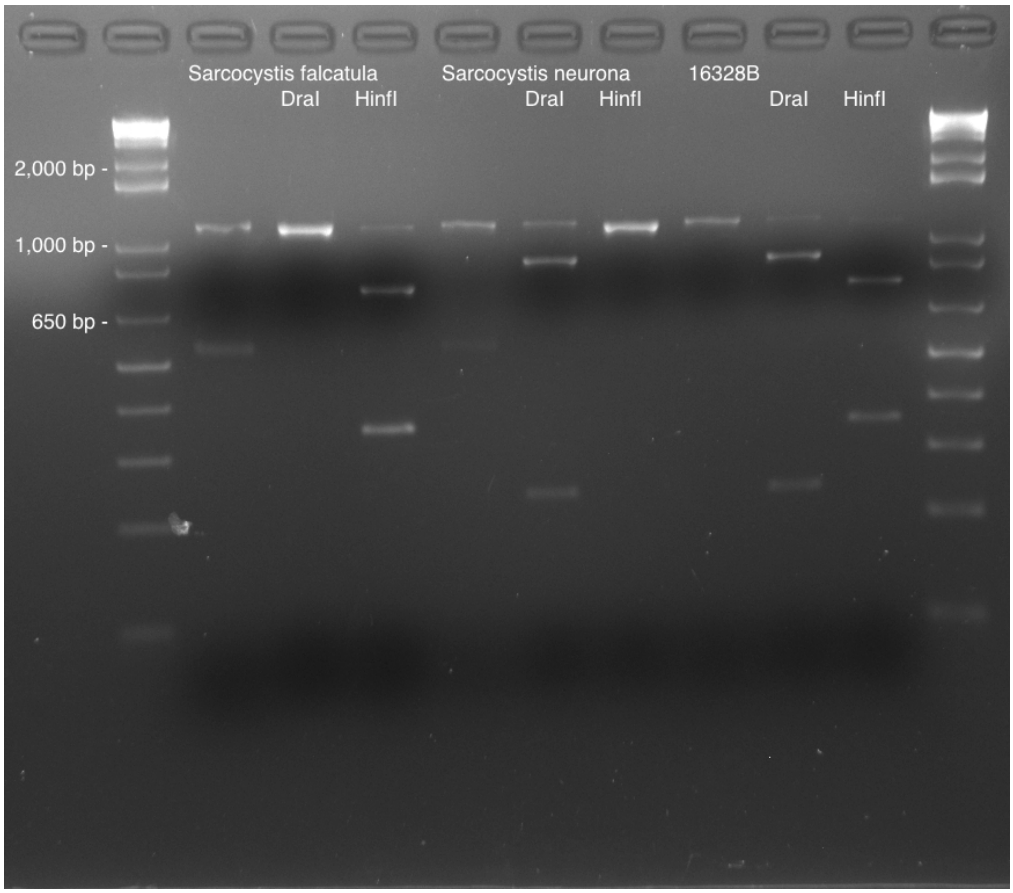

Figure 4. RFLP gel analysis using *Dra*I and *Hinf*I on *S. falcatula, S. neurona*, and 16328B. The first lane of each group is the undigested PCR product. *S. falcatula* cuts with the *HinfI* enzyme. *S. neurona* cuts with the *DraI* enzyme. Sample 16328B, a red-shouldered hawk, cuts with both enzymes.

For histology, we examined 187 hematoxylin and eosin stained slides. Of these slides, 33 were positive for sarcocysts or another parasite. Twenty-two of the 30 slides had an associated frozen DNA sample. Thirteen slides corresponded to birds that tested positive for *Sarcocystis* via PCR with ITS primers. Figure 5 shows sporocysts and Figures 6, 7, 8 and 9 show sarcocysts for *Sarcocystis* species.

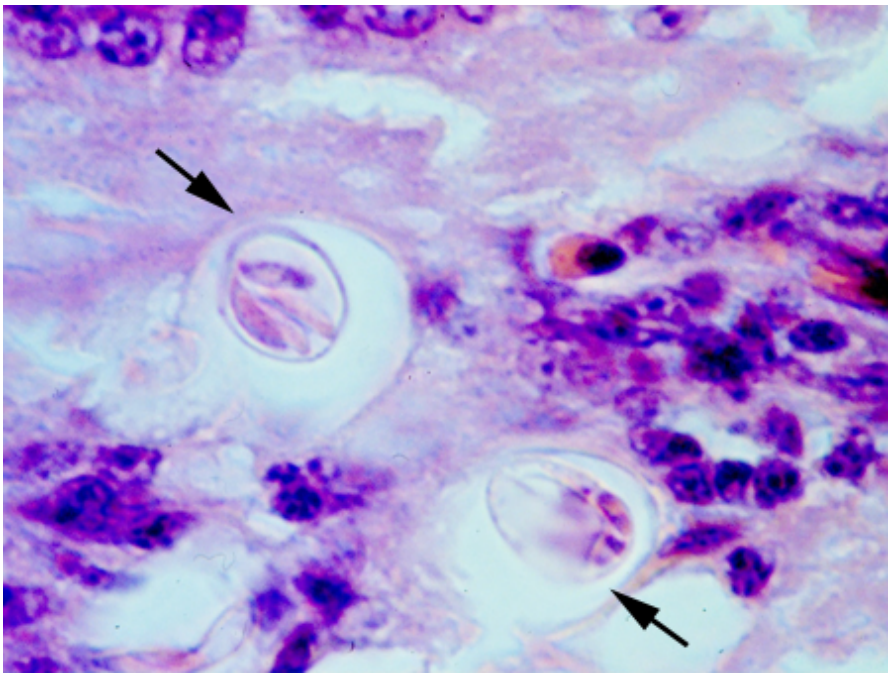

Figure 5. Sporocysts from the intestines of a Red-tailed Hawk.

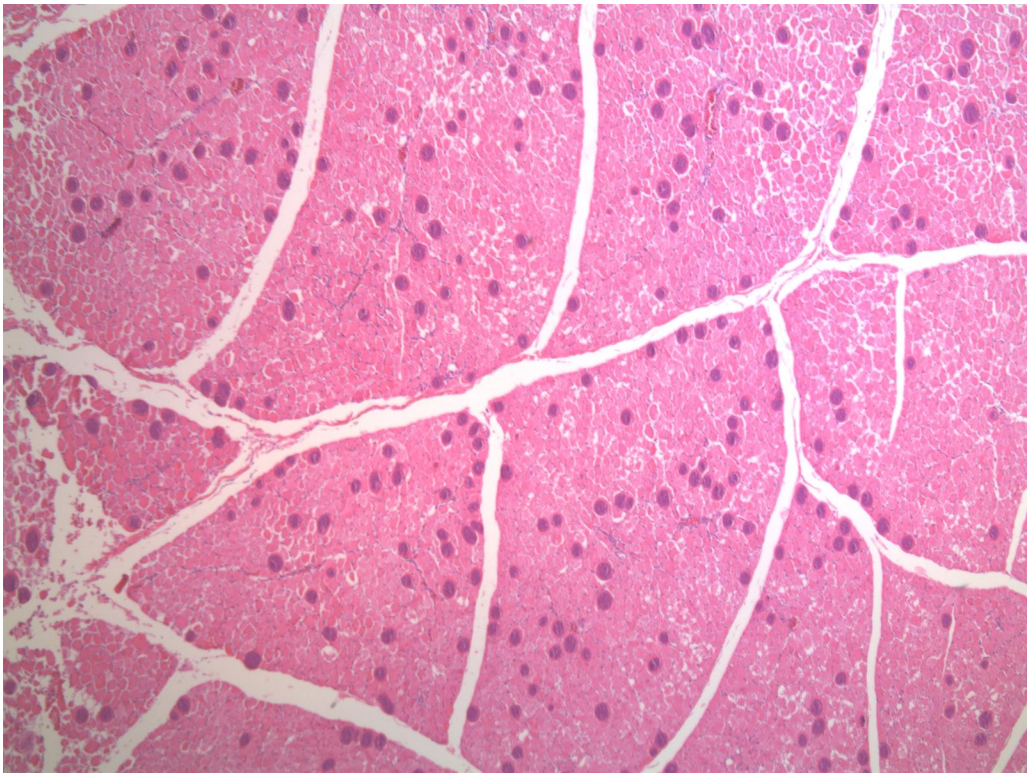

Figure 6. Heavy *Sarcocystis* infection at 4x power.

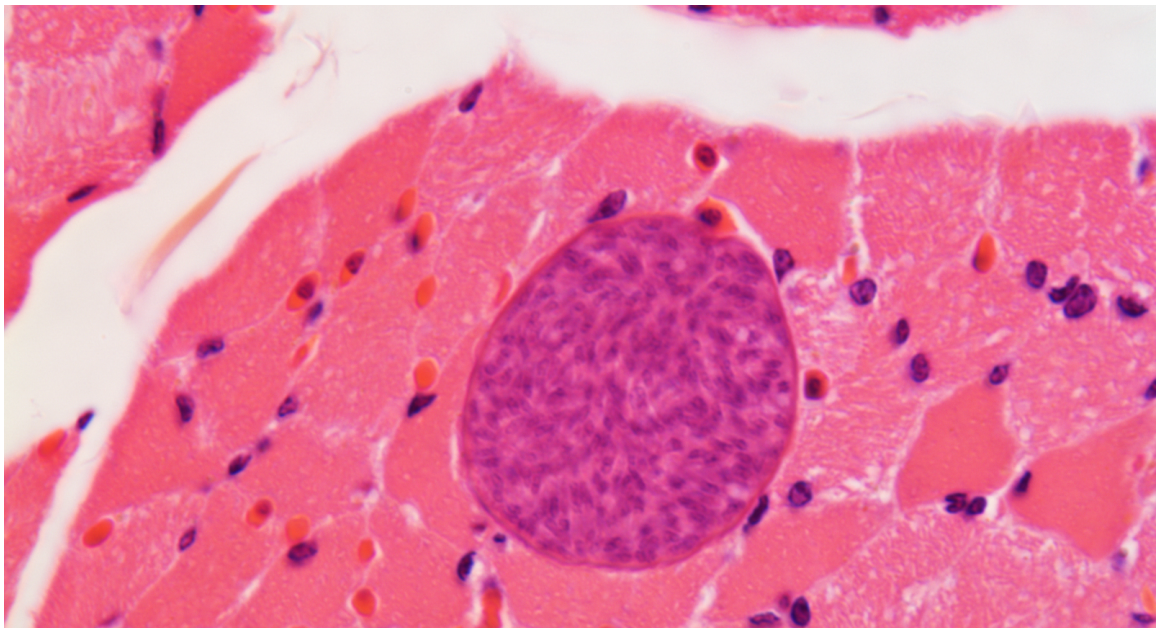

Figure 7. Sarcocyst from an ITS positive only raptor. This raptor was infected with an identified species of *Sarcocystis*.

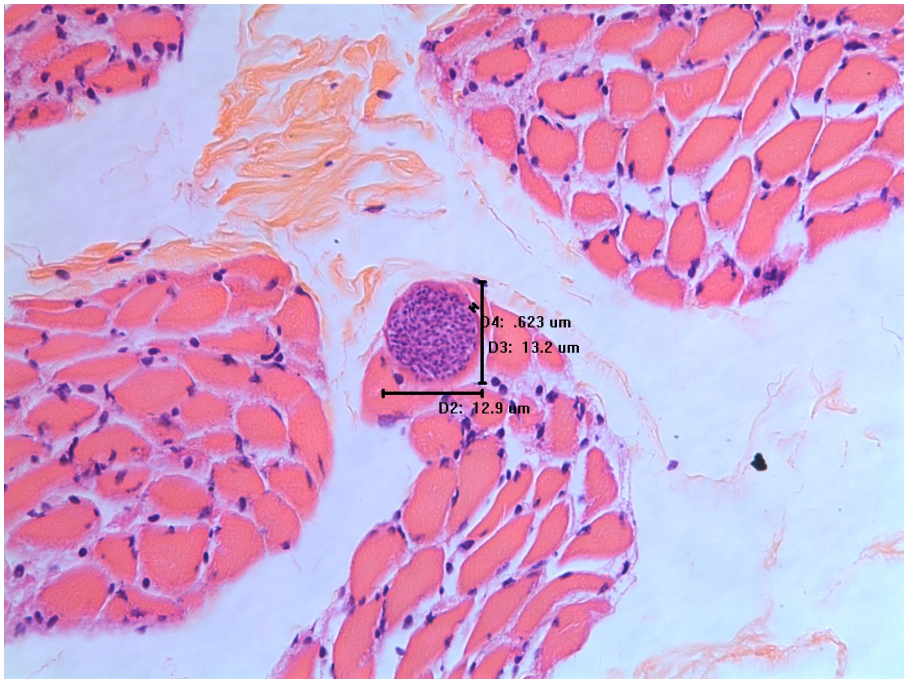

Figure 8. Sarcocyst from a female red-shouldered hawk (14218) positive for the *S. falcatula* Arg species with measurements.

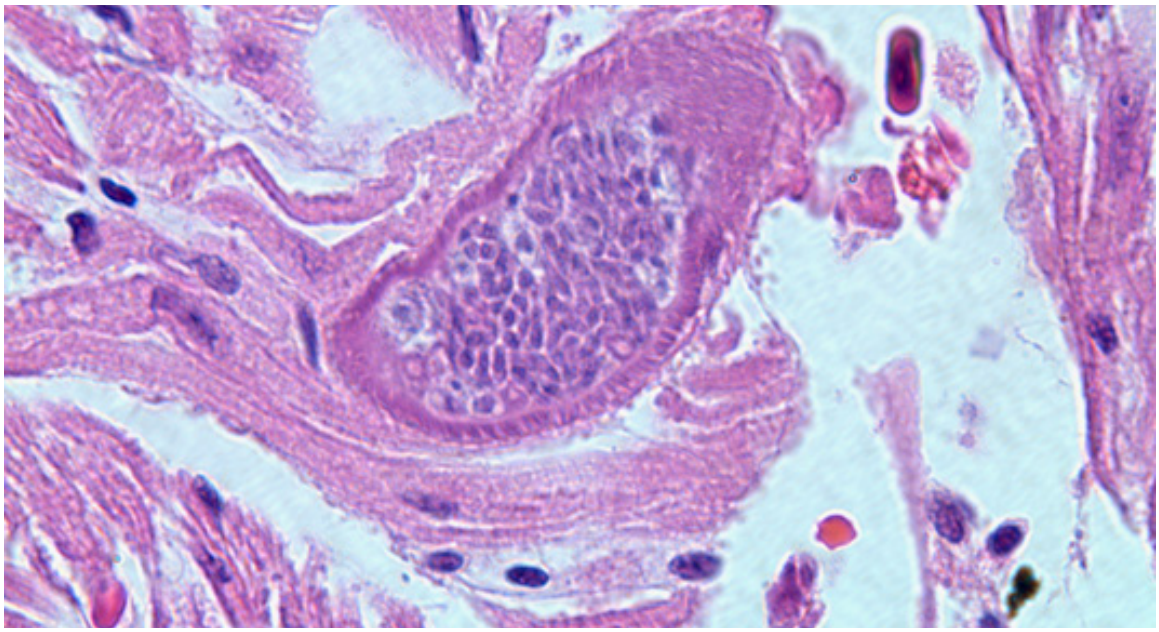

Figure 9. Sarcocyst from an *S. falcatula* Arg positive sample.

Amongst the 24 samples tested for *S. calchasi* (SCa) we found no positives. 14567 had a light band present for the SCo primers. With *Sarcocystis* sp. *ex A. nisus* (SNi), sample 14567, a Great Horned Owl, showed a bright band for the breast muscle and a slightly lighter band for the heart muscle. Sample 15203, a Red-tailed Hawk, had a heart sample positive with the SNi primers. We found, however, that the SNi primers reacted with the positive controls for *S. falcatula*, *S. neurona* and *S. lindsayi* (Fig 10). We were unable to run them against an SNi positive control due to lack of a positive DNA control sample.

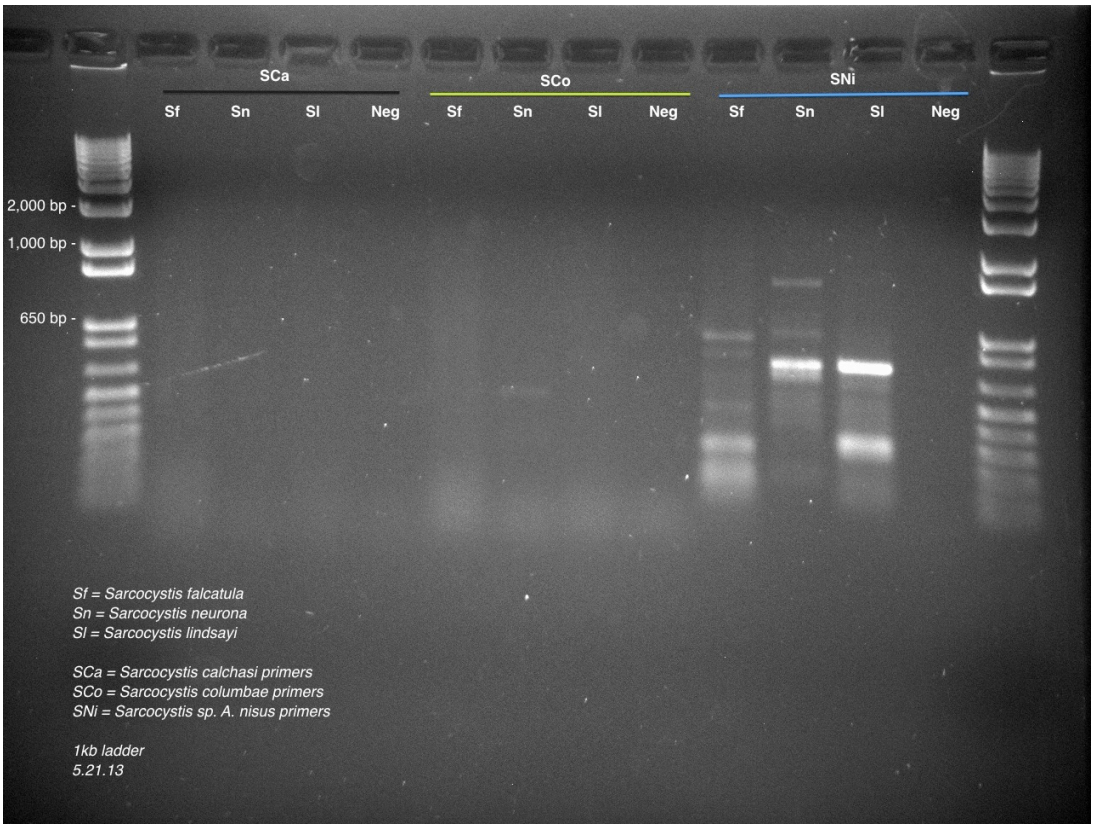

Figure 10. Primers to detect *S. calchasi* (SCa)*, S. columbae* (SCo), and *Sarcocystis sp. ex A. nisus* (SNi) with positive controls.

Table 4. ITS positive bird samples. Birds infected with *Sarcocystis* and *T. gondii* (*), *T. gondii* and *N. caninum* (▲), *Sarcocystis* and *N. caninum* (✧), and all three parasites (❧). ITS = *Sarcocystis* species present. B = breast. H = heart. JNB = *S. falcatula* or *S. neurona* present.

Bird Number /Sex		**Species**	**ITS**	**JNB**	**RFLP results**
14512□	M	Black Vulture	B- H+	Neg	n/a
15021□	M	Turkey Vulture	B+ H-	Neg	n/a
15588□	n/a	Osprey	B+ H-	Neg	n/a
14499	F	Mississippi Kite	B+ H-	B+	*S. falcatula*

14166	M	Cooper's Hawk	B+ H+	Neg	n/a
14224	F	Cooper's Hawk	B+ H-	Neg	n/a
14574	M	Cooper's Hawk	B- H+	Neg	n/a
14218	F	Red-shouldered Hawk	B+ H-	B+	*S. falcatula* Arg
14232*	M	Red-shouldered Hawk	B+ H-	Neg	n/a
14596	M	Red-shouldered Hawk	B+ H-	Neg	n/a
15772	n/a	Red-shouldered Hawk	B+ H+	B+	(too little DNA)
16328	M	Red-shouldered Hawk	B+ H+	B+ H+	*S. falcatula* Arg
14288	M	Red-tailed Hawk	B+ H-	B+	*S. falcatula* Arg
14521	M	Red-tailed Hawk	B+ H-	Neg	n/a
15203	M	Red-tailed Hawk	B- H+	Neg	n/a
15247	M	Red-tailed Hawk	B+ H-	B+	*S. falcatula*
15107□	n/a	Merlin	B+ H+	H+	(too little DNA)
14567	n/a	Great Horned Owl	B+ H+	Neg	n/a
	*SNi positive breast and heart. Possible SCo positive breast.				
14283	F	Barred Owl	B+ H-	Neg	n/a
14319	M	Barred Owl	B+ H-	B+	*S. falcatula*
14863□	F	Barred Owl	B- H+	Neg	n/a
15781	n/a	Barred Owl	B+ H-	Neg	n/a
16129	n/a	n/a	B+ H-	Neg	n/a
16921	n/a	n/a	B+ H+	B+ H+	*S. falcatula*

4.2 *Toxoplasma gondii*

The results of TOX 4/TOX 5 positive birds are listed in Table 5. Species, gender and location data was not available for all birds. 21 birds of 114 (18.4%) tested positive for *Toxoplasma gondii.*

Table 5. Samples positive for *Toxoplasma gondii.* Birds infected with *Sarcocystis* and *T. gondii* (*), *T. gondii* and *N. caninum* (▲), *Sarcocystis* and *N. caninum* (□), and all three parasites (□). TOX4/TOX5 primers detect *T. gondii*. B = breast. H = heart.

Bird Number	**Sex**	**Species**	**TOX4/TOX5**
14254▲	M	Turkey Vulture	B+ H-
15021□	M	Turkey Vulture	B+ H-
14666▲	M	Cooper's Hawk	B+ H-
14256▲	M	Red-shouldered Hawk	B+ H-
14294	F	Red-shouldered Hawk	B+ H+
14477	M	Red-shouldered Hawk	B- H+
14490	M	Red-shouldered Hawk	B+ H-
14491	M	Red-shouldered Hawk	B+ H-
14503▲	M	Red-shouldered Hawk	B- H+
14510▲	M	Red-tailed Hawk	B- H+
14560	M	Red-tailed Hawk	B- H+
14626▲	F	Red-tailed Hawk	B+ H-
14566▲	M	Eastern Screech Owl	B- H+
14247▲	M	Great Horned Owl	B+ H+

14248	F	Great Horned Owl	B- H+
14277	F	Barred Owl	B+ H-
14548	F	Barred Owl	B- H+
14251	n/a	n/a	B+ H+
14506▲	n/a	n/a	B- H+
14641	n/a	n/a	B+ H+
14684	n/a	n/a	B- H+

4.3 *Neospora caninum*

The results of NP21+/NP6+ positive birds are listed in Table 6. Species, gender and location data was not available for all birds. 35 birds of 114 (30.7%) tested positive for *N. caninum*.

Table 6. Samples positive for *Neospora caninum.* Birds infected with *Sarcocystis* and *T. gondii* (*), *T. gondii* and *N. caninum* (▲), *Sarcocystis* and *N. caninum* (□), and all three parasites (□). NP6+/NP21+ primers detect *N. caninum*. B = breast. H = heart.

Bird Number	**Sex**	**Species**	**NP6+/NP21+**
14512□	M	Black Vulture	B+
14254▲	M	Turkey Vulture	B- H+
15021□	M	Turkey Vulture	B+ H+
15588□	n/a	Osprey	B- H+
14511	M	Cooper's Hawk	B+ H+

14525	M	Cooper's Hawk	B- H+
14529	F	Cooper's Hawk	B- H+
14666▲	M	Cooper's Hawk	B+ H-
14232*	M	Red-shouldered Hawk	B- H+
14256▲	M	Red-shouldered Hawk	B+ H-
14488	M	Red-shouldered Hawk	B- H+
14503▲	M	Red-shouldered Hawk	B- H+
14530	M	Red-shouldered Hawk	B- H+
14531	F	Red-shouldered Hawk	B+ H+
14533	M	Red-shouldered Hawk	B+ H-
14581	F	Red-shouldered Hawk	B+ H+
14675□	M	Red-shouldered Hawk	B+ H+
14522	n/a	Broad Winged Hawk	B+ H-
14510▲	M	Red-tailed Hawk	B+ H-
14586	M	Red-tailed Hawk	B+ H-
14626▲	F	Red-tailed Hawk	B+ H+
14631	M	Red-tailed Hawk	B- H+
15107□	n/a	Merlin	B+ H+
14566▲	M	Eastern Screech Owl	B+ H+
14247▲	M	Great Horned Owl	B+ H-
14287	M	Great Horned Owl	B+ H-
15160	n/a	Great Horned Owl	B- H+
14595	F	Barred Owl	B+ H+

14648	F	Barred Owl	B- H+
14863□	F	Barred Owl	B+ H+
14506▲	n/a	n/a	B+ H+
14523	n/a	n/a	B+ H-
14671	M	n/a	B+ H-
14683	n/a	n/a	B+ H+
14972	n/a	n/a	B+ H+

4.4 Co-infected Birds

There were 15 cases of co-infection in the 114 birds (13.2%). The tables above have symbols denoting birds infected with *Sarcocystis* and *Toxoplasma* (*), *Toxoplasma* and *Neospora* (▲), *Sarcocystis* and *Neospora* (□), and all three parasites (□). Additionally, 14567, a male Red-shouldered Hawk, may contain 2 species of *Sarcocystis*, but further investigation is needed. Co-infected birds include 4 red-shouldered hawks, 1 great horned owl, 2 turkey vultures, 1 black vulture, 1 eastern screech owl, 2 red-tailed hawks, 1 cooper's hawk, 1 barred owl, 1 Merlin, 1 Osprey, and 1 bird whose species information was unavailable.

Table 7. Samples positive for multiple parasites.

Bird Number	**Sex**	**Species**	**Parasites**
14512	M	Black Vulture	*Sarcocystis, Neospora*
14254	M	Turkey Vulture	*Toxoplasma, Neospora*
15021	M	Turkey Vulture	*Sarcocystis, Toxoplasma, Neospora*
15588	n/a	Osprey	*Sarcocystis, Neospora*

14666	M	Cooper's Hawk	*Toxoplasma, Neospora*
14232	M	Red-shouldered Hawk	*Sarcocystis, Toxoplasma*
14256	M	Red-shouldered Hawk	*Toxoplasma, Neospora*
14503	M	Red-shouldered Hawk	*Toxoplasma, Neospora*
14510	M	Red-tailed Hawk	*Toxoplasma, Neospora*
14626	F	Red-tailed Hawk	*Toxoplasma, Neospora*
15107	n/a	Merlin	*Sarcocystis, Neospora*
14566	M	Eastern Screech Owl	*Toxoplasma, Neospora*
14247	M	Great Horned Owl	*Toxoplasma, Neospora*
14863	F	Barred Owl	*Sarcocystis, Neospora*
14506	n/a	n/a	*Toxoplasma, Neospora*

Discussion

Previous literature has shown an increase in the number of reported cases of *Sarcocystis*- or *S. falcatula*-associated encephalitis in raptors from North America (Olson et al., 2007; Wunschmann et al., 2010; Wunschmann et al., 2009). Encephalitis presents with various clinical signs including head tilt, deficits in the limbs, poor grasp reflex and other neurological signs (Wunschmann et al., 2010; Wunschmann et al., 2009). Encephalitis is the major cause of death in these raptors. In small passerines, schizogony in the lungs leads to obstruction of pulmonary vessels, endothelial cell hypertrophy and lysis as well as an increase in inflammatory cells and death (Smith et al., 1987a, b). Outbreaks of sarcocystosis have also been described in zoos and indoor enclosures in psittacines and columbiform (Victoria Crowned pigeons) birds (Ecco et al., 2008; Rimoldi et al., 2013; Suedmeyer et al., 2001). These outbreaks are usually associated with the presence of opossums in the facilities.

A recent disease outbreak due to *S. calchasi* was reported from an enclosed zoological aviary in California, USA (Rimoldi et al., 2013). Birds in the exhibit (2 princess parrots and 3 cockatoos) developed severe central nervous system clinical signs over a 2-3 month period resulting in death or euthanasia (Rimoldi et al., 2013). Results of necropsy examination demonstrated that all birds had a lymphoplasmacytic and histiocytic encephalitis consistent with *Sarocycstis* species and had sarcocysts in their muscles (Rimoldi et al., 2013). The source of *S. calchasi* infection was not determined, but it was speculated that Cooper's Hawks (*Acipiter cooperii*) and Sharp-shinned Hawks (*Accipiter striatus*) could be possible definitive hosts (Rimoldi et al., 2013). Additional work is needed to determine the prevalence of *S. calchasi* in raptors as potential definitive hosts and examination of other birds for their ability to serve as intermediate hosts.

The JNB PCR primers and RFLP assay were designed to differentiate *S. falcatula* from *S. neurona* form the feces of opossums (Tanhauser et al., 1999). The results of our study demonstrate that *S. falcatula* infection in raptors may be more common than previously known and that raptors are serving as natural intermediate hosts. We found 4 raptors positive for *S. falcatula* and 3 raptors positive for *S. falcatula*-like DNA. The JNB primer PCR positive samples that cut at both the *Dra*I and *Hinf*I restriction sites suggests that the *Sarcocystis falcatula*-like species discovered in Argentina, and transmitted by South American opossums (*Didelphis* spp.) is also present in North America and may be transmitted by *D. virginianus* (Dubey et al., 2000a; Dubey et al., 2001a; Dubey et al., 2000b). This is supported by the finding of a similar *S. falcatula*-like parasite in a Northern gannet from Florida and 2 opossums from a previous study (Spalding et al., 2002; Tanhauser et al., 1999). Our Figure 6 has a thick sarcocyst wall and appears similar to Figure 1 of Spalding et al. (2002). None of our JNB positive samples cut with only *Dra*I in our study and suggests that the opossum transmitted *S. lindsayi* was not in any of our raptors and that *S. neurona* may not occur in our raptors.

None of our DNA samples were amplified by PCR primers for *S. calchasi,* a cause of neurological disease in pigeons. *S. columbae* primers amplified one of our samples and two of our samples were amplified by *Sarcocystis* sp. *Ex. A. nisus* primers. None of these samples were confirmed by sequencing. These results suggest that these species are not common in raptors in North America, but additional work is needed. There is one report of *S. calchasi* in a pigeon from North America, indicating that a raptor definitive host was present. The outbreak of encephalitis associated with *S. calchasi* in California also suggests that a raptor definitive host is present in North America. It is possible that although we may not be involved in the migration patterns of

the definitive hosts, birds involved in the life cycle of these parasites as intermediate hosts may have been imported into the US.

Muscle tissues from 24 samples were positive for *Sarcocystis* species by ITS based PCR. Our other PCR studies ruled out *S. falcatula, S. falcatula*-like, *S. lindsayi, S. calchasi, S. columbae* and *Sarcocystis* sp. Ex. *A. nisus* in these samples. This indicates that other, potentially new species of *Sarcocystis* are present in these raptors.

We may have seen a lower number of samples positive for *Sarcocystis* via PCR than those seen by histology due to using only small sections of tissue from which to extract DNA. Sarcocysts were microscopic and if the infection was maintained at a lower level, our small tissue sample may have missed sarcocysts entirely. If we had homogenized entire tissue (breast and heart separately), we may have achieved better results.

Our results have caused us to question the presence of *Sarcocystis* in the tissues of an Osprey. About 99% of their diet is comprised of live fish (Sibley, 2003). The other approximately 1% may be made of bird carcasses, voles, snakes and other small animals. This leaves a very small chance of exposure to infection, because there is yet no known species of *Sarcocystis* that uses fish in its life cycle. This particular bird was also positive for *Neospora caninum* via PCR.

We found that 21 of our samples (18.4%) were positive for *T. gondii.* This is comparable to the 26.7% prevalence reported by Lindsay et al. (1993) done with a wild population of raptors. We were unable to genotype our samples to determine if they are Type I, II or III, but previous studies by Dubey et al. (2004) used this technique on black-winged lories and a Canada goose in 2004. All isolates from these birds were Type III (Dubey et al., 2004). A study done in Colorado, which examined 382 birds (29 species) was done in 2010. Sixty of these birds were raptors, and

7 of these 60 (11.7%) were positive for *T. gondii* (Dubey et al., 2010). Genotyping done the next year identified 4 birds with Type II, one with Type III, one with Type 12 and two with atypical genotypes (Dubey et al., 2011). Genetic characterization on a red-shouldered hawk in Alabama revealed that they had Type I (Yu et al., 2013). These studies may offer an idea as to the possible genotypes that may have parasitized the birds in this study.

We found that 35 of our samples (30.7%) were positive for *N. caninum.* A study done in Spain detected 1.5% prevalence (n=3) from 200 wild birds (Darwich et al., 2012). One of these 3 birds was a common buzzard (*Buteo buteo*).Out of the 200 wild birds, 105 of the birds were Griffon vultures (*Gyps fulvus*), 17 were common buzzards (*Buteo buteo*) and 3 were black kites (*Milvus migrans*). Over half of the birds in this study were raptors. In comparison, our *N. caninum* prevalence is extremely high. It is not clear how their birds were collected, but it appears no rehabilitation center was involved.

Our study is a snap shot of the complex life cycle of raptors. We can use it to gain insight into how these end-stage carnivores interact with their environment and the prey that composes the intermediate hosts of these two-host parasites. Because our samples were from patients at a rehabilitation center, and not collected at random in the wild, our results cannot be used to comment on the role of these parasites on the health of these raptors.

Overall, our study may help a variety of wildlife personnel, zoos, aviaries and those with personal pets in diagnosing the clinical signs of their birds.

Conclusions

The prevalence of *Sarcocystis* species, *Toxoplasma gondii* and *Neospora caninum* in 114 raptors from the Carolina Raptor Center were 21.1% (n=24), 18.4% (n=21) and 30.7% (n=35), respectively. Fifteen of the 114 birds (13.2%) were co-infected with 2 of the aforementioned species and 1 of the 114 birds (0.88%) was infected with all three. The prevalence rates of *Sarcocystis* and *T. gondii* found in our study are similar to the literature. The prevalence of *N. caninum* in raptors has not been examined extensively. This study may be biased in terms of prevalence rates suggesting they may be higher in an enclosed or captive facility than they are in the wild. The high rate of ITS positive only samples (*Sarcocystis* species present) suggests that there are more species of *Sarcocystis* that have yet to be characterized. From there, the use of different primers and restriction enzymes may be necessary to differentiate between species since histology alone has not proven to be useful to distinguish them.

In future studies, we may utilize different primer sets, particularly those of JNB, which cover different restriction sites on the *Sarcocystis* species genome. This may help us to elucidate subtle genetic differences between the species of *Sarcocystis* that are emerging in the literature. Sequencing of each ITS positive breast and/or heart sample would be useful in uncovering genetic differences between *Sarcocystis* species. It may also be helpful to genotype strains of *Toxoplasma gondii* from the raptor samples. This may give us some indication of the distribution and prevalence of more virulent avian strains. It is harder to infer any effects of infection on the bird without knowing severity of infection and virulence of the strain.

The results of our study can be used to aid wildlife rehabilitators, wildlife veterinarians, wildlife biologists, zoos, aviaries and owners of avian pets during assessment of clinical signs.

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