BAYLISASCARIS PROCYONIS INFECTION DYNAMICS AND TRANSMISSION AMONG WILDLIFE, DOMESTIC ANIMAL, AND HUMAN HOSTS

by

SARAH GRACE SAPP

(Under the Direction of Michael J. Yabsley)

ABSTRACT

The raccoon roundworm, *Baylisascaris procyonis,* is a common intestinal nematode of raccoons (*Procyon lotor*) and occasionally domestic dogs. Infection with larval stages following ingestion of infectious eggs in feces of these definitive hosts is capable of causing fatal neural larva migrans in a broad variety of paratenic host species. Approximately 50 human cases are recognized and incidence may be increasing. However, many knowledge gaps exist in understanding the epidemiology and transmission of *B. procyonis*. The goal of this dissertation was to employ an interdisciplinary, One Health approach to investigating *B. procyonis* in human, wildlife, and domestic hosts, including 1.) risk of occupational exposure among wildlife rehabilitators, 2.) infection dynamics and survival among rodent hosts, 3.) developing serologic tests for diagnostic purposes, and 4.) the role of dogs as *B. procyonis* hosts. Among wildlife rehabilitators, 7% (24/327) had antibodies to *Baylisascaris* suggesting prior subclinical infection. Significant risk factors included region, *B. procyonis* prevalence in raccoons, and consistency of hand hygiene after contact with raccoons/their feces. A questionnaire on knowledge, attitudes and practices revealed that correct knowledge and attitudes depend on factors such as

educational background and experience. Reported use of personal protective equipment and infection control by raccoon rehabilitators depended on similar factors. Detection of *Baylisascaris* in non-definitive hosts remains challenging, and serology is the only ante-mortem diagnostic tool available. A recombinant ES-antigen based ELISA was developed to investigate serologic responses among experimentally infected rodents, but it was not successfully adapted to human testing. Studies on tolerance and survival among *Peromyscus* spp. (deer mice) demonstrated species-level differences in infection dynamics which may influence parasite transmission and maintenance. Finally, studies on patent *B. procyonis* in dogs revealed aspects of epidemiology and infection biology. From a national reference laboratory database, *Baylisascaris* eggs were detected in 0.005% (504/9,487,672) of dogs. Experimental infections in dogs and raccoons revealed lower host competence in the domestic host versus the natural raccoon host. Only 2/12 dogs became infected compared to 12/12 raccoons, with longer prepatent periods and lower egg outputs among dogs. Collectively, these studies answer important questions on the transmission and "expanded lifecycle" of a high-consequence zoonosis.

INDEX WORDS: *Baylisascaris procyonis,* Ascaridoidea, Zoonoses, Raccoons, Wildlife rehabilitation, *Peromyscus,* Larva migrans, One Health

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DEDICATION

Life is (a) highway.... 316.

Using the conservative estimate of 100 round trips from Athens to the Centers for Disease Control in Atlanta during my time here, and 75% of those trips were via the 316-85 drag, which is 72 miles one-way, that means I've driven 10,800 miles of the 316 route during my graduate student career. Basically, I could have driven from Miami to Anchorage and back and then some. That's a whole lot of one less than scenic highway.

I gripe about the commute enough, but the reality is that, without the generosity and guidance of the many individuals in the Parasitic Diseases Branch Biology & Diagnostics team, none of this would have been possible. The near entirety of labor of two chapters were done in the lab of Sukwan Handali, and I am deeply indebted to him for his peaceful wisdom and immense patience. I'm also grateful for the two worm freaks like me, Richard Bradbury and Henry Bishop, always up for discussing wacky cases and giving me a (figurative) taste of morphology. These individuals have made me a better parasitologist overall.

On the other end of the 316, I owe it all to my advisor Michael Yabsley, the most gifted and dedicated mentor anyone could hope for. Brave enough to take in a student from outside his department, only to be bitten by endless nonsense throughout. And deal with the fact that he had three students of the same name (Three Sarah Era). I can only hope I've been a productive enough parasite disciple to make it all worth it.

Other miscellaneous acknowledgments include the many laboratory creatures who unwillingly gave their lives for this dissertation, my family for putting up with my anxiety as much as possible and providing me an Atlanta home to reduce my miles on 316, my grandparents for making sure I was soul food fed and loved, Heaven's Edge Farm for the equine therapy to keep me sane, J. Mitchell who knows more about my parasite than I do by now after listening to so many talk rehearsals, V. Vance for the support and motivation, and Der Wurmwagen for getting me there and back safely. That's a lot to cheers to. - Sapp

This dissertation is dedicated to the late Texas, loyal friend and opinionated scholar.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Baylisascaris procyonis (raccoon roundworm) is a large ascarid of raccoons capable of causing disease in a broad variety of hosts. The definitive host range encompasses raccoons, occasionally domestic dogs, and potentially other members of Procyonidae (Kazacos, 2016; Overstreet, 1970; Sapp et al., 2017). Non-definitive hosts, or those in which larval infection occurs, include over 150 avian and mammalian species including humans. The extent to which these non-definitive hosts experience larva migrans associated disease is highly variable, but many hosts develop severe to fatal neural larva migrans (NLM) and ocular larva migrans (OLM) following ingestion of infectious eggs.

First recognized as a zoonotic agent in the 1980s, approximately 50 cases of *Baylisascaris*-associated NLM and OLM have been confirmed. Approximately 25% were fatal cases, and only two patients have fully recovered with no lasting damage (Sircar et al., 2016). Treatment can be difficult and expensive, involving extended hospitalization with high doses of anthelmintics to kill migrating larvae and corticosteroids to reduce inflammation. Albendazole is the preferred anthelmintic due to its safety profile and tissue penetrance; ivermectin is unacceptable as it does not pass the blood-brain barrier (BBB) effectively, and may be toxic in patients with disrupted BBB (Cunningham et al., 1994). Unfortunately, the average cost of albendazole in the United

States is over $100 per typical daily dose, and treatment of baylisascariasis in humans requires a much higher dose and longer course than used to treat intestinal parasites (Manz et al., 2014). Therefore, the economic burden of a clinical baylisascariasis case, or even a potential exposure that is followed by prophylactic albendazole, is very high. Treatment must be initiated rapidly, because once neurologic signs are evident, irreversible damage has likely occurred (Kazacos 2016).

It is likely that other human cases have been misdiagnosed or have gone unreported in light of diagnostic challenges and lack of a complete understanding of the clinical disease spectrum. Although a very rare zoonosis, the potential consequences of *B. procyonis* exposure are severe, which warrants in-depth investigation of transmission and epidemiology to reduce the risk of exposure. Thus, it is important to identify which groups are at risk of exposure. Wildlife rehabilitators possibly represent a group that is particularly at a high risk of zoonotic spillover, given their frequent and prolonged contact with diseased and stressed wildlife and the potential contamination of the work environment (Jijon et al., 2007; Steele et al., 2005). Knowledge levels and use of appropriate protective measures are variable among this community, potentially leading to elevated risk (López et al., 2011; Saito and Shreve, 2005). Therefore, this community is a potentially interesting outlet for the investigation of *B. procyonis* exposure in adults and modifiable risk factors.

Baylisascariasis is also a threat to exotic animals in zoos and other captive settings. Numerous fatalities of captive wildlife due to *Baylisascaris* spp. have been reported, and most of these were likely *B. procyonis* infections due to the ubiquity of raccoons and severity of disease (Kazacos, 2016). Free-ranging raccoons are attracted to feed and bedding that are stored for

captive animals, and can easily contaminate these items with *B. procyonis* eggs if not properly secured. In some cases, raccoons were also kept on the premises, or animals were housed in enclosures that were previously used for raccoons (Fitzgerald et al., 1991). Often, trapping of free-ranging raccoons and environmental sampling reveals contamination, which is strong evidence for *B. procyonis* as the causative species versus other *Baylisascaris* spp. (Ball et al., 1998; Desprez et al., 2017). Fatal NLM case reports in captive exotics encompass a remarkable array of taxa, including small and large rodents, lagomorphs, primates, parrots, perching birds, ratites, raptors, marsupials, and some carnivores (Agnew et al., 1994; Ball et al., 1998; Campbell et al., 1997; Kazacos et al., 1991; Larson and Greve, 1983; Roth et al., 1982; Sato et al., 2005, 2002; Wolf et al., 2007). A comprehensive list of case reports in wildlife can be found in Kazacos (2016).

Due to the potentially grave and life-altering consequences of infection, the broad non-definitive host range, the potential involvement of domestic dogs as definitive hosts, and the increasing presence of raccoons in populated areas, *B. procyonis* has emerged as a concern for public, domestic animal, and wildlife health. A One Health approach that integrates multiple disciplines is beneficial to investigations on the epidemiology and biology of this versatile parasite, in order to fill in knowledge gaps and reduce risk for exposure.

LITERATURE REVIEW

Morphology and Phylogenetics

Adult *B. procyonis* nematodes have a typical ascarid appearance (three well-developed lips, cylindrical, cream-colored body tapering at either end) and are much larger than *Toxocara* spp. Females can reach up to 30 cm in length; males typically do not exceed 11 cm. Cervical alae are present, but inconspicuous and only apparent in transverse section. Eggs are moderately ellipsoid, usually amber in color, with a thick shell and granular proteinaceous coat. The size can be variable but typically measure 68-80 x 55-61 μm. Larvae measure ~200 um when hatched from eggs and grow up to ~1800 um after ~10 days of migration within non-definitive host tissues, and have a tapering filariform esophagus (approximately 15% of the body length), granular intestinal cells, and a tapered, digitiform tail. Migrating larvae reach a maximal width of 60-75 μm mid-body, and can be identified in cross-section by lateral alae, and two roughly triangular excretory columns positioned on either side of the intestine (Bowman, 1987).

The genus *Baylisascaris* was proposed and described in 1969 by J.F.A. Sprent, in order to unite similar members of *Toxascaris* and *Ascaris.* Historically, *B. procyonis* was named *Ascaris columnaris,* which was also used to describe the skunk ascarid *B. columnaris* but Stefanski and Zarnowski (1951) designated the raccoon ascarid as its own species, *Ascaris procyonis.* This parasite was later reassigned to the novel genus *Baylisascaris* based on the presence of an area rugosa (roughened perianal patch, in males only) and an alternative arrangement of postcloacal papillae (Sprent, 1968; Stefanski and Zarnowski, 1951).

Phylogenetic studies incorporating molecular targets and morphologic characteristics nearly uniformly place *Baylisascaris* within the subfamily Ascaridinae (including *Ascaris, Parascaris,* and *Toxascaris*), with well-supported divergence from sister genera *Ascaris* and *Parascaris* (Franssen et al., 2013; Nadler et al., 2007; Nadler and Hudspeth, 2000). Studies on the molecular phylogenetics of *Baylisascaris* species suggest *B. procyonis* is most closely related to *B. columnaris* (of skunks), forming a clade that diverges from another clade incorporating *B. transfuga* (of bears), *B. schroederi* (of Giant pandas), and *B. ailuri* (of red pandas) (Franssen et al., 2013; Tokiwa et al., 2014; Tranbenkova and Spiridonov, 2017). These molecular studies support the morphologic differences observed among these species. *B. procyonis* and *B. columnaris* are nearly identical in morphology, lacking the characteristics (prominent cervical alae; large, stout midbody with a lateral groove) common to members of the other clade (Berry, 1985; Franssen et al., 2013; Sapp et al., 2017; Sprent, 1968).

Life Cycle

The life cycle of *B. procyonis* may be either monoxenous or heteroxenous depending on exposure route (Figure 1.1). Adult ascarids reside in the small intestine of the definitive host, feeding on ingesta. Mated females pass eggs that are shed in the feces, and are remarkably fecund. Egg burdens are generally correlated with infection intensity; in juvenile raccoons this averages around 26,000 EPG, but burdens of over 200,000 EPG have been documented (Kazacos, 2016; Weinstein, 2016). Prepatent periods for raccoons vary based on route of infection (eggs vs. larvae). For eggs, patency typically occurs within 50-75 days post inoculation. Patency has been documented as early as 32 days post inoculation in raccoons fed larvae in tissues (Kazacos, 1983).

Eggs are passed unembryonated, containing a zygote. Over a period of ~14 days, the zygote develops into an infectious larva. Debate exists as to which stage is represented *in ovo* (L2 vs L3). Morphologic studies of larvae hatched from eggs and molts that occur *in vitro* can be difficult, and subtle differences between developmental stages may be subject to variation by interpreter (Bowman, 1987; Boyce et al., 1988a; Sprent et al., 1973). Like most ascarid eggs, *B. procyonis* eggs are remarkably hardy; neither freezing to -20 C for extended periods of time, freeze-thaw cycles, nor exposure to typical disinfecting agents (e.g. ethanol, bleach, chlorine) render them unviable (Shafir et al. 2011; Sapp unpublished data). Viability only begins to decline after exposure to temperatures exceeding 57 degrees C (Shafir et al., 2011). This durability allows eggs to overwinter and likely retain infectivity for years in the environment.

When infectious eggs are ingested by non-definitive hosts, the larvae penetrate the wall of the small intestine following hatching, and enter circulation. Liver-lung migration takes place early in infection, followed by somatic migration to various sites. The distribution of larvae following liver-lung migration is host-dependent and may be related to the host's ability to contain migrating larvae within inflammatory processes. Both naturally and experimentally infected squirrels had larval granulomas primarily in the anterior carcass (thoracic cavity, lung, heart, intercostal muscle), compared to rabbits and rats which had abundant granulomas in the intestinal wall, mesentery, and liver (Tiner, 1953). Migration of larvae through tissues (larva migrans; LM) is capable of producing severe disease in many hosts (discussed in the next section).

The behavioral changes in the infected non-definitive host with NLM render it susceptible to predation by raccoons, or scavenging after death. Encapsulated larvae are released in the small intestine of the raccoon host; however, it is not known if these larvae undergo tracheal migration (as with *Ascaris* and *Toxocara* spp.) or complete their development to adulthood solely in the intestinal wall and lumen (as with *Toxascaris leonina*) (Matoff and Wassileff, 1958; Schacher, 1957; Sprent, 1958). It is also possible that both routes occur under different conditions; in cats inoculated with *Toxocara cati* larvae, tracheal migration does not always occur (Sprent, 1956).

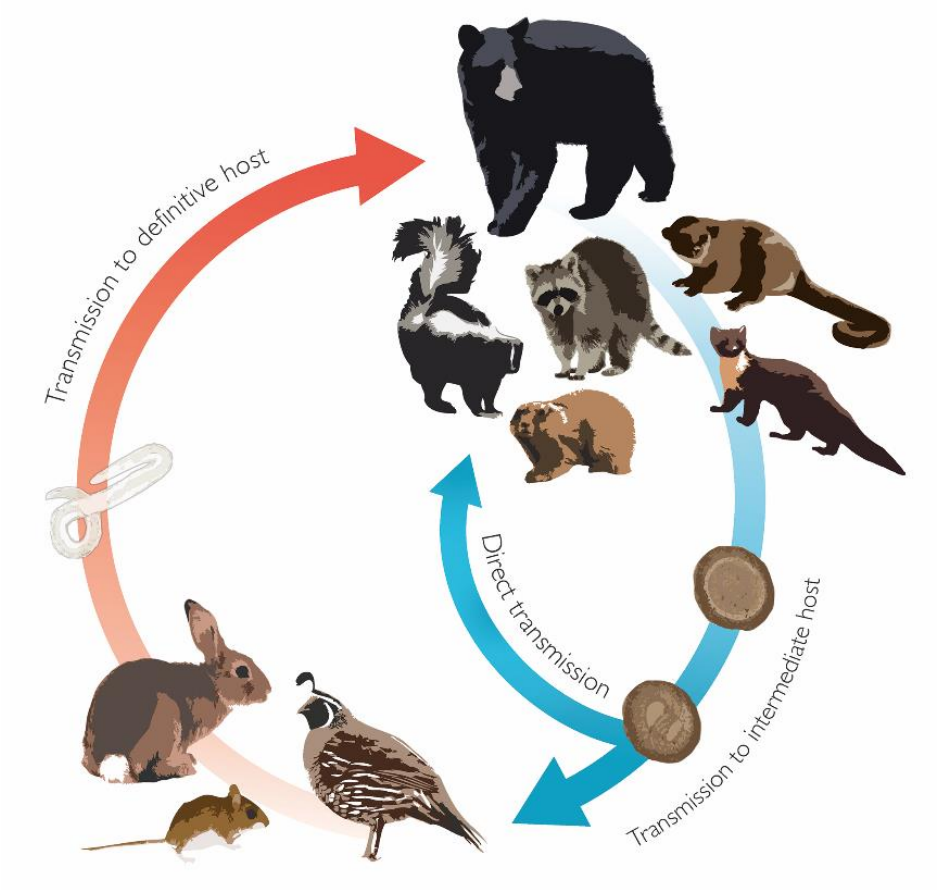

Figure 1.1. Generalized life cycle scheme for *Baylisascaris* spp. (from Sapp et al. 2017)

Larva Migrans and Disease

The pathology that accompanies larva migrans is also host-dependent. For example, *Peromyscus leucopus* and *Mus musculus* differ in the size and extent of inflammatory reactions, with *Mus musculus* producing large, very conspicuous granulomas compared to the less pronounced lesions of the other species (Sheppard and Kazacos, 1997). However, some general features are shared among the broad number of hosts, including petechial hemorrhagic in lungs following larval egress, and peripheral eosinophilia. Histopathology on granulomas typically shows infiltration by eosinophils and macrophages, fibrosis, and perivascular cuffing around the larva, with malacia in tracks following larval migration (Campbell et al., 1997; Coates et al., 1995; Rowley et al., 2000). Some examples of gross lesions and disease presentation are presented in Figure 1.2.

Neural larva migrans (NLM) typically produces severe or fatal disease in non-definitive hosts. *B. procyonis* L3 grow as they migrate, from ~200 μm after hatching to a maximum length of ~1,800 μm, which may be achieved as early as 10 dpi in laboratory mice and *Peromyscus* spp. (Kazacos 2016; Sapp unpublished data) (Tiner, 1953). It is believed that the severity of *B. procyonis* associated NLM compared to that of *Toxocara* NLM is greater due to the growth and much greater terminal size as *T. canis* typically only reaches 150-200 μm in length during migration (Sprent, 1955). In laboratory mice, the ratio of larvae in the brain to carcass in mice infected with equal numbers of eggs is markedly greater for *T. canis* (31.85) than *B. columnaris* (0.04), which shares similar migratory and morphologic characteristics with *B. procyonis* (Tiner, 1953; Sprent, 1955). However, neurologic disease is observed far less often in *T. canis* infected hosts than with *Baylisascaris* spp. infected hosts, suggesting that larval size is the major factor contributing to the

severity of NLM (Sprent, 1955, 1952). Visceral larva migrans (VLM) may produce an array of usually non-specific signs depending on which organs are affected, although a VLM syndrome for *B. procyonis* is poorly defined as most research emphasis is placed on the neurological manifestations of disease.

Behaviors associated with cerebral baylisascariasis include a wide variety of dysfunction. Among mammalian hosts, limb and face tremors, torticollis, partial to full paresis, ataxia, continuous circling, rolling along the lateral axis, inability to right, stupor, and seizures are commonly observed (Figure 1.2). Preceding these signs, more non-specific somatic manifestations may become apparent including respiratory distress, anorexia, reduced activity, hunched posture, abdominal tenderness, and a ruffled coat, although in many cases the onset of neurologic disease is rapid and these signs may not be observed. If larvae migrate out of the brain or are otherwise contained in a host immune reaction, neurologic signs may improve, although the mechanical damage from migrating larvae typically results in permanent sequellae (Kazacos, 2016).

Invasion of the eye is referred to as ocular lava migrans (OLM) and *Baylisascaris* spp. are increasingly recognized as a causative agent of disseminated unilateral subacute neuroretinitis (DUSN). Inflammatory responses including retinitis, choroiditis, and vitritis are typically observed early, and cases that are left untreated may result in permanent, necrotic damage to the optic nerve and retina (Shafir et al., 2006). Granulomas containing *Toxocara* or *Baylisascaris* spp. larvae may superficially resemble retinoblastoma (Cortez et al., 2010). The incidence of *Baylisascaris*-associated OLM may be underestimated, as these cases are not generally reported and species identification is typically not attempted in ocular parasitosis cases (Shafir et al., 2006).

The majority of reported ocular cases occur without concomitant neurological disease. Some human patients with confirmed *Baylisascaris* OLM do not show evidence of seroconversion, suggesting that perhaps these were from very low-level infections in which a single larva happened to migrate to the eye. Squirrel monkeys (*Samiri scurius*) and macaques (*Macaca fascicularis*) inoculated with relatively large doses of 5,000-20,000 *B. procyonis* eggs developed OLM along with severe CNS disease, further suggesting that cases in which OLM and NLM occur together result from high-level exposure (Kazacos et al., 1984). In some *Baylisascaris* NLM cases, visual signs were observed with neurologic disease, but it is difficult to ascertain whether this is true OLM or due to damage to the visual cortex and optical nerve.

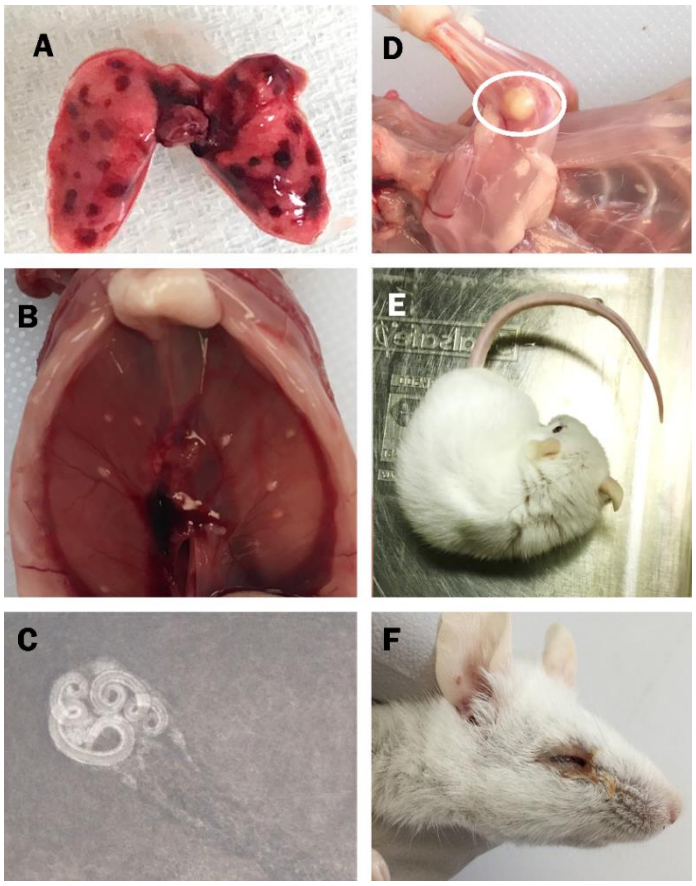

Figure 1.2. Gross pathologic lesions associated with *Baylisascaris procyonis* larva migrans in experimentally infected rodents. A. Petechial hemorrhage in lungs early in infection (5 days); B. Granulomatous lesions on diaphragm of infected laboratory mouse; C. Two larvae in a "squash" prep of the brain of an experimentally infected *Peromyscus leucopus*, showing host reaction and tissue damage following migratory path of larvae; D. Purulent abscess possibly associated with a larval granuloma; E. Abnormal torticollic posture typical of rodents with late stage neural larva migrans; F. Ocular discharge and swelling of eyelids, possibly associated with uveitis and other inflammatory pathology following ocular larva migrans. All photos by S.G.H. Sapp during experimental trials.

Distribution and Ecology

In general, *B. procyonis* appears to be ubiquitous in distribution in southern Canada and throughout the United States (Figure 1.3). Geographic expansion or new recognition has occurred in recent years into areas where the species was previously very rare or absent, such as areas of the Southeast and Southwest (Blizzard et al., 2010a, 2010b; Hernandez et al., 2013; Roug et al., 2016). Prevalence remains highest in areas of the upper Midwest, Northeast, and the West coast, which may be as high as >90% of juvenile raccoons at any given time (Evans, 2001; Jacobson et al., 1982; Kazacos, 2016; Snyder and Fitzgerald, 1987; Weinstein, 2016). The parasite has also been reported in raccoons in Costa Rica so it presumably occurs throughout Mexico and Central America (Baldi et al., 2016).

Raccoon translocations for the fur and pet trade have introduced *B. procyonis* to several continental European and East Asian countries, including Denmark, Norway, Germany, Poland, Czech Republic, Slovakia, Netherlands, Spain, China, and Japan (Al-Sabi et al., 2015; Bauer, 2011; Davidson et al., 2013; Jimenez et al., 2015; Miyashita, 1993; Popiołek et al., 2011; Xie et al., 2014). The popularity of pet raccoons is what drove the import of raccoons into Japan in the 1970s. Outbreaks in captive non-definitive hosts have occurred in Japan; however, no cases in humans have been reported (Miyashita, 1993; Sato et al., 2005, 2002). Extensive control measures including trapping and euthanasia of free-ranging raccoons appear to have been effective in minimizing or eliminating *B. procyonis* from feral raccoon populations in Japan according to an extensive survey of 1,688 individuals (Matoba et al., 2006).

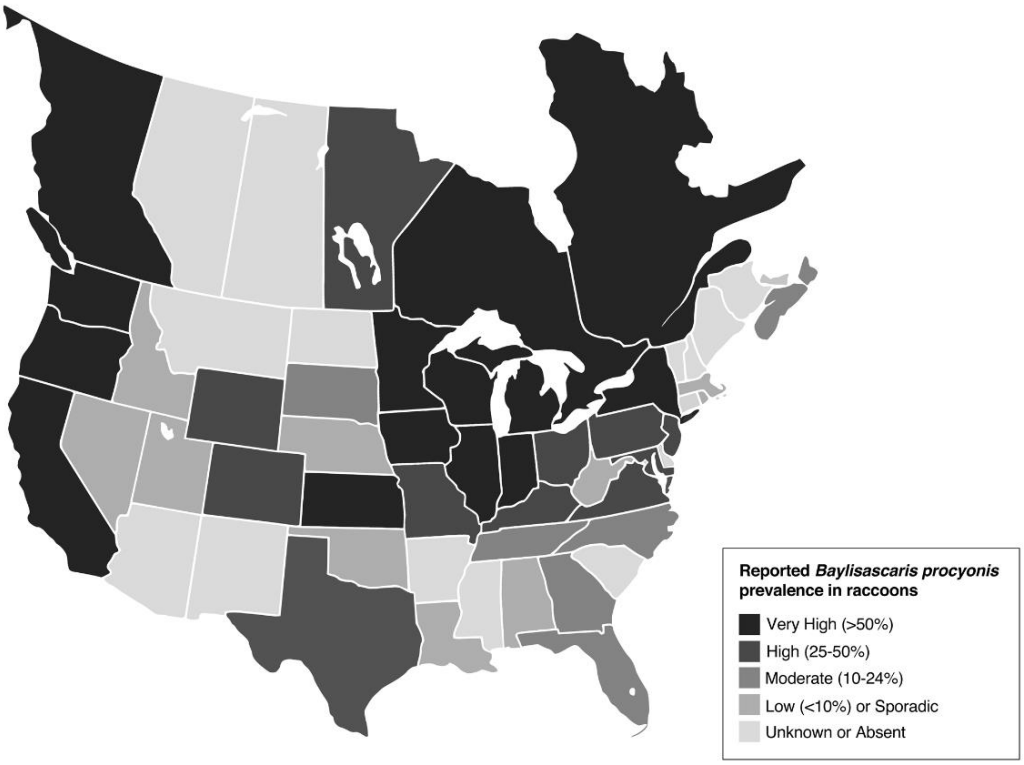

Figure 1.3. Distribution and general prevalence estimation of *Baylisascaris procyonis* in raccoons in the United States and Canada based on published reports.

The ecological and demographic factors influencing the prevalence of *B. procyonis* in raccoons and non-definitive hosts are complex and generalization is difficult. The environmental hardiness of eggs allows the infective stages to persist in diverse environments. Temperature does not appear to be an important factor, although denser soil types may influence the ability of eggs to persist in the topsoil (where they are accessible to hosts) instead of being swept to more basal subsoil horizons (Kresta et al., 2010). The impacts of habitat fragmentation and urbanization on

B. procyonis prevalence are not clear, with studies finding conflicting results (Kellner et al., 2012; Page et al., 2008). The differences in the distinction between rural and urban sites will differ across region and this may confound observed differences.

For demographic factors, it is generally observed that juvenile raccoons are more frequently and intensely infected than older animals although in some studies this relationship is not always found (Chavez et al., 2012; Jardine et al., 2014; Page et al., 2009; Pipas et al., 2014; Weinstein, 2016). In experimental studies, young raccoons are susceptible to infection via eggs, after which infection efficiency drops off presumably due to acquired immunity. However, adult raccoons are susceptible to infection via ingestion of non-definitive hosts containing larvae (Kazacos, 1983). This may explain the age intensity pattern observed in the wild, as raccoons are not obligate predators and generally rely on plant, arthropod, and anthropogenic food sources with occasional opportunistic predation of small mammals (Page et al., 2008; Rulison et al., 2012). Furthermore, seasonal shifts in the proportion of small mammals in raccoon diets could ultimately influence the prevalence of *B. procyonis* in adult raccoons (Rulison et al., 2012). Prevalence also appears to increase with increased contact rates and congregation of infected individuals (Gompper and Wright, 2005). Home range sizes and social behavior vary widely across the raccoon's range, which further interact with environmental factors in the maintenance and transmission of *B. procyonis* (Gehrt et al., 2008; Hauver, 2003).

The composition of non-definitive host species in an ecosystem likely has a role in maintenance of *B. procyonis* in raccoons as well. The apparent resistance of older raccoons to infection by eggs implies that the consumption of an infected non-definitive host is required for infection maintenance in adult raccoons. In habitats where raccoons rely more on small mammals

and birds as food sources as opposed to plant material or anthropogenic waste, prevalence in adult raccoons is higher. Rodent species that employ a foraging-caching feeding strategy (e.g. foraging undigested plant material from feces and storing) are likely to be exposed to *B. procyonis* eggs in latrines during feeding (Logiudice, 2001; Page et al., 2001). Therefore, these species at a high risk of exposure are likely to be important in the transmission of *B. procyonis* larvae to adult raccoons. Woodrats (*Neotoma* spp.) and white-footed mice (*P. leucopus*) are common across the range of *B. procyonis* and are implicated as potentially important non-definitive host species (Logiudice, 2001; Page, 2013; Page et al., 2012). *B. procyonis* larvae are relatively frequently found in *P. leucopus* in areas of high *B. procyonis* endemicity (Beasley et al., 2013; Tiner, 1954). Non-definitive host species capable of harboring large numbers of larvae, such as *Rattus rattus*, may also provide an important reservoir of infection for the maintenance of *B. procyonis* in adult raccoons (Weinstein, 2017).

Diagnosis

The "gold standard" for diagnosis in definitive hosts is recovery of adult nematodes in the gastrointestinal tract, usually post-mortem, although worms may pass and be identified after anthelmintic treatment. Fecal flotation to recover eggs from feces is specific and also commonly employed, although this is not without challenges and may result in an underestimation of true prevalence (Page et al., 2005). Eggs may not be shed constantly or the animal may be in the pre-patent period, resulting in false negatives. New coproantigen-based ELISAs, which detect antigens shed by adult stage ascarids, may aid in diagnosis prior to patency but have not been validated for detection of *Baylisascaris* spp. (Elsemore et al., 2017).

Diagnosis in non-definitive hosts is less straightforward because larvae are sequestered within tissues. The primary post-mortem methods for detection of *B. procyonis* larvae include microscopic examination of brain tissue that has been flattened between glass plates ("brain squash"), or by artificial digestion or Baerman examination of tissues to recover migrating larvae (Kazacos, 2016). After recovery, larvae can be identified morphologically, or preferably, molecularly. These methods are sensitive but are only appropriate for use on animals. Biopsy on impacted organs is only useful if the tissue sample happens to contain a migrating larva, which can be identified morphologically to species in a histologic section. The probability of successfully capturing a migrating larva in a host as large as a human is extremely low, so biopsy is not typically utilized.

Serology for detection of antibodies to *Baylisascaris* is the mainstay of clinical diagnosis in people. Prior to the development of appropriate serology, diagnosis was primarily based on clinical presentation (e.g. high peripheral and cerebrospinal eosinophilia, neurologic complications) and epidemiologic characteristics (i.e. known raccoon contact). In a number of fatal cases, larvae were identified during autopsy. Occasionally, provisional western blots on crude *B. procyonis* were used, but these have a high degree of cross-reactivity to *Toxocara* anti-sera. However, the existence of a few excretory-secretory (ES) antigens unique to *Baylisascaris* spp. allow the serologic differentiation of baylisascariasis from toxocariasis (Boyce et al., 1988b; Dangoudoubiyam and Kazacos, 2009). This is particularly important, as exposure to *Toxocara* spp. is highly prevalent in most populations; in the United States, an estimated 14% of people have antibodies to *Toxocara* spp. (Won et al., 2008). Currently, a Western blot based on a recombinant ES antigen unique to *Baylisascaris* [*B. procyonis* repeat antigen-1 (rBpRAG-1)] is used for detection

of anti-*Baylisascaris* IgG in serum and CSF. This assay has a sensitivity of 88% and a specificity of 98%, with little to no cross-reactivity to toxocariasis specimens. Since validation was performed on a very limited set of sera or CSF from confirmed baylisascariasis cases (n=16), some of which may have been taken early during infection (prior to seroconversion), sensitivity may be underestimated (Rascoe et al., 2013). Similarly, negative control sera were a set of samples from 'normal' individuals and while they are expected to be negative, their true exposure history is unknown.

Radiologic imaging of the brain typically reveals a great degree of atrophy and white matter loss, although this is not necessarily informative as many other pediatric neurologic diseases can cause similar changes. Considerable variability can likely occur in human cases as well, depending on larval migration, host response, and chemotherapy (e.g. use of corticosteroids) (Rowley et al., 2000). Therefore, radiologic findings are useful in diagnosis only when paired with epidemiologic factors and serologic results (presence of antibody and peripheral or CNS eosinophilia).

Epidemiology of human cases

Historically, most human baylisascariasis cases have occurred in very young children or older developmentally disabled persons. Pica or geophagia is considered the most major risk factor for exposure to *B. procyonis* eggs and were known to occur in several reported cases (Cunningham et al., 1994; Kazacos et al., 2002; Sorvillo et al., 2002). Given the high numbers of eggs that an infected raccoon may shed in feces, direct ingestion of infected feces or oral contact with contaminated objects may represent a massive inoculum and lead to very severe NLM with

large numbers of larvae in the brain. Some patients had a noted history of raccoon contact prior to the onset of disease, and nearly all documented cases originated in areas where *B. procyonis* is highly prevalent (Sircar et al. 2016). As raccoon population density seems to be positively associated with *B. procyonis* prevalence, the density of raccoons should be considered a risk factor. In the neighborhood of one patient, raccoons occurred at an incredibly high density (30 animals/0.25 acres) (Sorvillo et al. 2002).

Two clinical cases in previously healthy, cognitively normal adults have been identified in recent years, likely through exposure via more indirect means (e.g. contact with contaminated soil or objects). The relatives of one adult patient reported that he seldom washed his hands prior to eating, after working outdoors on construction sites where the soil was likely contaminated (Sircar et al., 2016). Another adult patient had a pet raccoon, and developed eosinophilic meningitis with seroconversion. After a three week course of albendazole the patient's clinical signs improved but despite the treatment and removal of the pet raccoon, clinical signs returned 16 months later. The pet raccoon was never tested for *B. procyonis*, although it remains probable that this was the original source of exposure. Limited evidence for subclinical or asymptomatic infection exists, most notably, the finding of a single *Baylisascaris* spp. larva in the brain of an elderly patient who died of Alzheimer's disease (Hung et al., 2012).

Improvements in diagnostics, particularly the development of the *Baylisascaris*-specific Western blot, and awareness by physicians have enhanced early detection and therefore clinical outcomes. Since 2009, only one case of *Baylisascaris* NLM was fatal. However, of confirmed survivors, 88% (22/25) were left with permanent neurologic sequellae, with or without eventual improvement. Generally these persistent deficits are severe (e.g. loss of cognitive function,

paralysis, blindness, seizures, brain atrophy, etc.), although occasionally less severe sequellae have been reported (e.g. mild vision impairment, weakness, sensory disturbances) (Kazacos, 2016; Sircar et al., 2016). Ocular larva migrans may also result in vision deficits ranging from mild to severe. The prophylactic use of albendazole in individuals with a high suspicion of exposure (20-50 mg/kg for 10-20 days) mitigates the risk of complications by halting the migration of larvae to the CNS and allowing more time for further diagnostic testing (Sircar et al. 2016).

It is important to note that determination of the specific species involved is difficult to impossible, as L3 larvae of different *Baylisascaris* spp. are morphologically indistinguishable and are serologically cross-reactive (Boyce et al., 1988b; Sapp et al., 2017). *B. procyonis* is typically assumed the species implicated in human cases, due to its ubiquity and pathogenicity, and in many instances is supported by epidemiological evidence (e.g. pet raccoon, recovery of *B. procyonis* eggs around the home) (Sircar et al., 2016). However, this does not rule out the possibility that other *Baylisascaris* spp. are capable of zoonotic transmission. *B. columnaris* and *B. melis* in particular can cause considerable pathology in experimentally infected hosts, although not to the extent of *B. procyonis* (Sprent, 1955). *B. potosis* produces larva migrans associated lesions in the intestines, liver, and brain experimentally infected primates (*Saimiri scurius*), although overt clinical signs were not observed (Tokiwa et al., 2015).

Domestic dogs as hosts

Despite very serious potential public health consequences, little is known about canine *B. procyonis* infections. Both patent intestinal infections and larva migrans have been documented in dogs. In the limited number of reports of larva migrans induced by natural and experimental

infections, infection resulted in rapid neurologic degeneration (Table 1.1). Experimentally infected dogs given very high numbers of eggs (>100,000) developed clinical signs of VLM within a few days of inoculation, including abdominal tenderness and lethargy. Neurologic disease occurred not long after, and both dogs were dead by 19 days post inoculation (Snyder, 1983). Only three instances of naturally-acquired *Baylisascaris* larva migrans in dogs have been documented, which were also fulminant, fatal infections (Table 1.1). Given the diagnostic challenges and minimal awareness, it is possible that *B. procyonis* is an under-recognized causative agent of severe neurological disease in dogs. It may be that these published reports are the result of very high dose levels, similar to what is hypothesized with human NLM cases; exposure to a smaller number of eggs may result in subclinical or milder disease. Due to its similarity in presentation to rabies, including baylisascariasis as a differential diagnosis in dogs with CNS signs is important.

Patent intestinal infections in dogs are a particularly concerning public health issue, as human contact with infected dog feces is more likely than infected raccoon feces. Furthermore, dogs defecate indiscriminately unlike raccoons which typically use defined latrine areas (Page et al., 1998), which may contribute to contamination of a broader area. Estimation of prevalence of patent canine infections is difficult. *Toxocara canis* eggs superficially resemble *B. procyonis*, and although these can be distinguished based on size, technicians may only diagnose "ascarids" and not determine species or low numbers of *B. procyonis* eggs may be missed when large numbers of *T. canis* eggs are present. It is unknown how many practicing veterinarians or veterinary technicians may also be unaware of *B. procyonis* in dogs.

Coprophagy is a confounding factor in fecal examinations of dogs in particular (Nijsse et al., 2014). It may lead to spurious infections/pseudoparasitism as eggs found in consumed feces simply pass through the gastrointestinal tract, but these results may be interpreted as true infections. While this does not necessarily mean a dog is not truly infected, it suggests a spurious infection. Spurious "infection" with *Baylisascaris procyonis* in dogs still remains a public health hazard, perhaps even more so than a true patent infection, as dogs are depositing eggs (which may be larvated and infectious) into domestic environments. Dogs engaging in coprophagy are also at a greater risk of eventually acquiring a true infection through ingestion of eggs. Fortunately, intestinal *B. procyonis* infections are easily treated with anthelmintics that have activity against nematodes (e.g. ivermectin, milbemycin, fenbendazole), although heavy burdens may require multiple doses to be completely eliminated (Bauer and Gey, 1995; Bowman et al., 2005).

Table 1.1. Summary of published instances of *Baylisascaris* infections in domestic dogs.

Number of dogs	Age	Natural/ Experimental	Route	Signs/Outcome	Diagnosis	Source
1	10 mos.	Natural	Likely egg†	Encephalomyelitis; Acute onset ataxia, deterioration, euthanized w/in 48 hr.	Histopathology	Thomas, 1988
2	5-6 mos.	Natural	Unk.	Patent intestinal infection in one dog which passed 3 adult; 2 adult females passed by other dog which did not reach patentcy.	Fecal flotation, recovery of worms after treatment	Greve and O'Brien, 1989
2	NS	Natural	Unk.	Patent intestinal infection; 7 male and 19 females from both dogs recovered.	Fecal flotation, necropsy	Averbeck et al., 1995
1	12 wks.	Natural	Likely egg†	Progressive weakness, ataxia, imbalance deteriorating to severe CNS disease within 24 hours; euthanized.	Histopathology	Rudmann et al., 1996
1	NS	Natural	Unk.	Vomiting, anorexia, eosinophilia (peripheral and CSF), sublumbar lymphadenopathy, progressive neurologic deterioration, euthanized w/in 24 hr.	MRI and histopathlogy	Windsor et al., 2009
7	NS	Experimental	Larvae*	Patent intestinal infection in 4/7 dogs after 42-80 d.	Fecal flotation, necropsy	Miyashita, 1993
7	NS	Experimental	Eggs	Unthriftiness and abdominal tenderness followed by severe CNS signs within 10 days in 2 dogs fed 100,000 and 200,000 eggs, dead at 14 days. Ataxia and incoordination in one dog fed 10,000 eggs by day 19.	Necropsy	Snyder, 1983
4	10 mos.	Experimental	Larvae*	Patent intestinal infection in 3/4 dogs	Fecal flotation; recovery of worms after treatment followed by necropsy	Bowman et al., 2005

* larvae in tissues of infected laboratory mice; † Infected raccoons were kept in proximity to dogs suggesting egg ingestion as the likely route of exposure; NS = not specified

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CHAPTER 2

BAYLISASCARIS PROCYONIS ROUNDWORM SEROPREVALENCE AMONG WILDLIFE REHABILITATORS, UNITED STATES AND CANADA, 2012–2015[1]

[1] Sapp, S.G.H., Rascoe, L.N., Wilkins, P.P., Handali, S., Gray, E.B., Eberhard, M., Woodhall, D.M., Montgomery, S.P., Bailey, K.L., Lankau, E.W. and Yabsley, M.J. 2016. *Emerging Infectious Diseases*, 22(12): 2128-2131. Open access. Reprinted here with permission of publisher.

ABSTRACT

Baylisascaris procyonis roundworms can cause potentially fatal neural larva migrans in many species, including humans. However, the clinical spectrum of baylisascariasis is not completely understood. We tested 347 asymptomatic adult wildlife rehabilitators for *B. procyonis* antibodies; 24 were positive, suggesting that subclinical baylisascariasis is occurring among this population.

INTRODUCTION

Baylisascaris procyonis, a roundworm of raccoons (*Procyon lotor*) and rarely dogs, can cause fatal neural larva migrans or ocular larval migrans in numerous bird and mammal species, including humans (1). At least 54 human cases have been reported; however, cases may not have been recognized or reported, especially ocular cases, for which parasite identification is rare (1–3). Most diagnosed cases have been in children and were severe or fatal. Treatment is difficult after onset of neurologic symptoms, and neural larva migrans survivors may have permanent neurologic sequelae (1).

The clinical spectrum of baylisascariasis is not fully understood. Limited evidence suggests that subclinical disease may occur (1,2,4,5). *Baylisascaris* larvae were an incidental finding in the brain of an Alzheimer disease patient (4), and *B. procyonis* antibodies were reported in the parents of a child with baylisascariasis and in 4 of 13 adults in Germany with raccoon contact; assay specificity was not reported (2,5). The occurrence of subclinical infections with related ascarids (e.g., *Toxocara* species) is well established; up to 14% of persons in the United States are seropositive, although it is unknown how many have clinical manifestations (6).

Wildlife rehabilitators may represent a population at risk for subclinical baylisascariasis due to frequent contact with raccoons and their feces, which may contain infectious larvated *B. procyonis* eggs. We assessed the occurrence of antibodies to *B. procyonis* in a sample of wildlife rehabilitators from the United States and Canada and administered a questionnaire on rehabilitation experience and procedures.

THE STUDY

During 2012–2015, we collected serum samples from and administered questionnaires to wildlife rehabilitators (details in Technical Appendix). We tested serum samples for *B. procyonis* IgG using a recombinant *B. procyonis* repeat antigen 1 protein Western blot as described (7).

Of 347 enrolled persons (Table 1), 315 (91%) reported current involvement in rehabilitation activities. Participants had an average of 10.5 (median 7.0) years of animal rehabilitation experience. Most respondents (92%) reported having contact with raccoons at some point; 64% reported actively rehabilitating raccoons in the past year (Table 2).

Twenty-four (7%; 95% CI 4.7%–10.1%) participants tested positive for *B. procyonis* antibodies; adjusted prevalence, considering assay performance characteristics, was 5.7% (95% CI 2.2%–9.2%) (Figure) (12). Of those 24 participants, 22 (92%) were actively rehabilitating wildlife; the other 2 reported occasional wildlife contact, including contact with raccoons, through veterinary clinic activities. All but 2 seropositive persons reported raccoon contact, and 2 practiced rehabilitation in the same household. Nineteen (79%) of the 24 seropositive persons resided in a US state or Canadian province classified as having very high or high *B. procyonis* prevalence among raccoons (Table 2).

CONCLUSIONS

We detected antibodies to *B. procyonis* roundworms in 7% of wildlife rehabilitators we tested, suggesting that exposure to this zoonotic parasite may occur without clinical disease. Participants reported various degrees of raccoon contact. Although the transmission source could not be determined (i.e., from rehabilitation of raccoons or from exposure to eggs during other activities), use of gloves and handwashing was generally inconsistent among the seropositive persons in this study (S.G.H. Sapp, data not shown). *B. procyonis* is transmitted by ingestion of larvated eggs; thus, proper use of personal protective equipment (PPE), adherence to cleaning and disinfection protocols, and proper hand hygiene should minimize the risk associated with exposure to feces.

Transmission risk can also occur when handling animals whose fur has been contaminated by infective raccoon eggs, as shown for *Toxocara canis* parasites and dog fur (13). More investigations are needed regarding the occurrence of *B. procyonis* eggs on raccoon fur and transmission implications. Lapses in PPE use and hand hygiene may indicate a lack of caution or risk awareness for other pathogens.

Wildlife rehabilitators in areas with a very high prevalence of *B. procyonis* infection among raccoons may be at elevated risk for subclinical infections. Only 1 *B. procyonis*–seropositive wildlife rehabilitator resided in a state with low or sporadic prevalence (Alabama); however, that person lived in an area adjacent to a Florida county where the prevalence of *B. procyonis* infection in raccoons was 9% (M.J. Yabsley, unpub. data) (Figure). Data on *B. procyonis* prevalence in raccoons are outdated or missing for many US states and Canadian provinces. Furthermore, raccoon infections with *B. procyonis* are now being reported in areas where the parasite has

historically been absent (e.g., the southeastern United States); thus, awareness of this parasite may be limited in those areas (8). More surveillance is needed on the distribution and prevalence of *B. procyonis* infection among raccoons to assess the association with exposure risks among humans.

Rehabilitation facilities housing raccoons can easily be contaminated with *B. procyonis* because high numbers of environmentally hardy eggs are passed by infected raccoons (1). Our finding of 2 seropositive raccoon rehabilitators operating out of the same household highlights the importance of infection-control practices. Facility contamination can be prevented by treating raccoons for parasites at intake and at regular intervals thereafter and by sterilizing enclosures using heat-based methods (14). Several anthelmintic drugs can kill adult *B. procyonis*, but raccoons with high worm burdens may require retreatment (15). Raccoon enclosures and housing should be constructed with materials that are easy to clean and disinfect using heat-based methods.

We tested persons with wildlife (mostly raccoon) contact, so our results describe an exposure risk that likely does not apply to the general public. However, persons in other occupations or activities (e.g., zoo keepers, wildlife biologists) may have similar exposure risks. Domestic dogs, other wildlife species (e.g., skunks, bears), and some exotic pets (e.g., kinkajous) are hosts for *Baylisascaris* spp. parasites and may present exposure risks (1). Although the assay we used has a sensitivity of 88% and specificity of 98%, it is time-consuming and not ideal for large-scale epidemiologic studies (7). Development of a high-quality ELISA would facilitate larger epidemiologic studies on the risk for baylisascariasis among different demographic groups and help further elucidate specific risk factors.

Our study had several limitations. We used a convenience sampling, so not all regions were well represented, and sample size was relatively small. Our prevalence estimate may be

inflated because positive predictive value is reduced in populations in which prevalence is low. The assay we used is the reference standard for clinical diagnosis but has not been used to test asymptomatic persons. Although an association between human *B. procyonis* exposure and seroconversion has not been established, asymptomatic seropositive infections would be expected because clinical disease probably occurs only when larvae cause damage to neural tissue or eyes (1). An estimated 95% of migrating larvae enter muscle or visceral organs, where they may stimulate an immune response but not cause clinical disease (1). In support of this presumption, the assay we used indicated that experimental infections of Peromyscus rodents with low numbers of *B. procyonis* parasites resulted in no clinical disease with seroconversion (S.G.H. Sapp, unpub. data). Last, participants were primarily licensed rehabilitators who belonged to professional organizations, and many practiced rehabilitation in large, dedicated facilities. Such facilities generally have safety protocols that may encourage more consistent PPE use and awareness of zoonotic diseases, so the risk for infection may be greater in smaller or informal rehabilitation settings.

To prevent infection with *B. procyonis* parasites, proper PPE and hand hygiene practices should be used consistently when handling animals and when contact with animal feces might occur. Education materials and outreach efforts discussing PPE use, infection control, and zoonotic pathogens should be directed to wildlife rehabilitators to increase awareness of potential occupational risks.

BIOGRAPHICAL SKETCH

Ms. Sapp is a doctoral student in the Department of Infectious Diseases at the University of Georgia. Her research interests include the epidemiology of parasitic zoonoses and other emerging zoonotic diseases.

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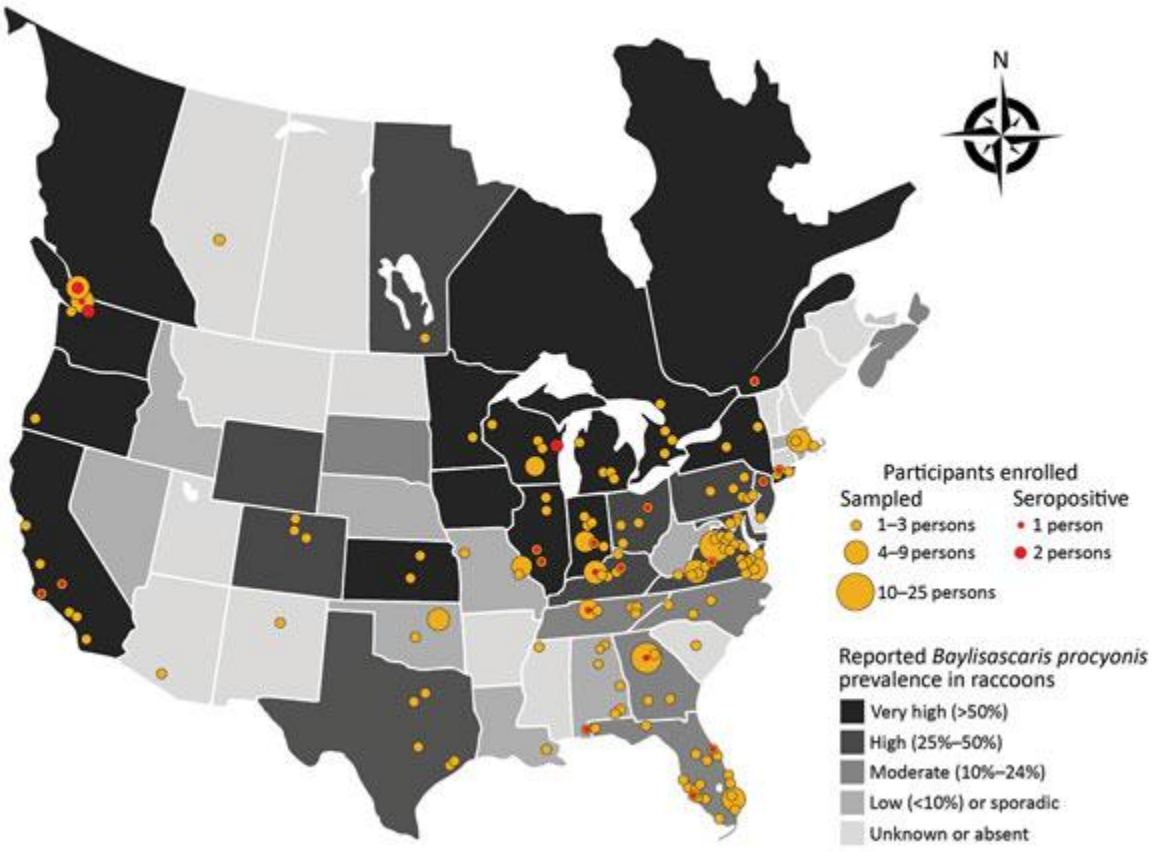

Figure 2.1. Locations for participant sampling in a study of *Baylisascaris procyonis* roundworm seroprevalence among wildlife rehabilitators, United States and Canada, 2012–2015. Yellow dots indicate counties (USA) or township/municipality (Canada) in which enrolled persons reported practicing wildlife rehabilitation. Red dots indicate locations of seropositive persons. Shading of states/provinces indicates general state/province level prevalence of *B. procyonis* in raccoons based on published reports (1,8–11).

Table 2.1. Demographic characteristics of participants in a study of *Baylisascaris procyonis* roundworm seroprevalence among wildlife rehabilitators, United States and Canada, 2012–2015.

Variable		No. respondents (% total)	No. seropositive (% category)
Gender			
	Female	299 (86.2)	21 (7.0)
	Male	48 (13.8)	3 (6.3)
Race			
	Asian	6 (1.7)	0 (0)
	American Indian or Alaska Native	1 (0.3)	0 (0)
	Black or African American	1 (0.3)	0 (0)
	White	327 (94.2)	23 (7.0)
	Other	2 (0.6)	0 (0)
	Multiracial	10 (2.9)	1 (10.0)
Ethnicity			
	Hispanic	5 (1.4)	0 (0)
	Not Hispanic	315 (90.8)	19 (6.0)
	Declined to state	27 (7.8)	5 (18.5)
Geographic region of rehabilitation activities*			
	Northeastern	106 (30.5)	4 (3.9)
	Midwestern	74 (21.3)	8 (12.5)
	Central	23 (6.6)	0 (0)
	Southern	110 (31.7)	5 (4.7)
	Western	34 (9.8)	7 (25.9)

*Geographic regions are defined as follows: Northeastern: Delaware, Maryland, Massachusetts, Maine, New Jersey, New York, Pennsylvania, and Virginia, USA, and Quebec Province, Canada; Midwestern: Illinois, Indiana, Kentucky, Michigan, Minnesota, Missouri, Ohio, and Wisconsin , USA, and Manitoba and Ontario Provinces, Canada; Central: Arizona, Colorado, Kansas, Oklahoma, and Texas, and Alberta, Province, Canada; Southern: Alabama, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Tennessee, USA; and Western: California, Oregon, and Washington, USA, and British Columbia Province, Canada.

Table 2.2. Rehabilitation characteristics and experience of wildlife rehabilitators enrolled in the present study, 2012 – 2015.

Variable	No. respondents (% total)	No. seropositive (% category)
Rehabilitation status		
Currently involved in wildlife rehabilitation	314 (90.5)	22 (7.0)
Formerly involved in wildlife rehabilitation	19 (5.5)	0 (0)
No rehabilitation activity (peripheral involvement, other wildlife contact, etc.)	14 (4.0)	2 (14.3)
Rehabilitation experience		
< 2 years	48 (14.9)	2 (4.2)
2 – 4.9 years	96 (29.8)	7 (7.3)
5 – 9.9 years	67 (20.8)	1 (1.5)
10 – 20 years	64 (19.9)	8 (12.5)
> 20 years	47 (14.6)	3 (6.4)
Raccoon rehabilitation		
Rehabilitated raccoons in past year	222 (64.0)	16 (7.2)
Rehabilitated raccoons historically	41 (11.8)	2 (4.9)
Never rehabilitated raccoons	84 (24.2)	6 (7.1)
General raccoon contact		
Had contact in past year	266 (80.9)	19 (7.1)
Had contact ever	36 (10.9)	3 (8.3)
Never had contact	27 (8.2)	2 (7.4)
State/province level raccoon *B.p.* prevalence*		
Very High (>50%)	79 (22.8)	14 (21.5)
High (25-49%)	127 (36.6)	5 (4.6)
Medium (10-24%)	92 (26.5)	4 (4.3)
Low (<10%), Sporadic, or Unknown	49 (14.1)	1 (2.1)

B.p. = Baylisascaris procyonis

* See Figure 2.1 for state/province level prevalence categorization.

TECHNICAL APPENDIX

Materials and Methods

Subject enrollment and sample acquisition

From 2012 to 2015, attendees at regional or national wildlife rehabilitation professional meetings were asked to participate in the study (n=303). The participants recruited at the professional meetings represented approximately 30-85% of the total attendance at these events; however, some individuals attended multiple meetings. A limited number of individuals (n=44) who wanted to enroll in the study but were unable to attend the meetings obtained the consent form, questionnaire, and a sampling kit from the study staff and later provided serum for testing. Healthy, non-pregnant adults (at least 18 years of age) reporting contact with any wildlife species were eligible for inclusion. A 31-item questionnaire was administered assessing wildlife rehabilitation history and experience for all participants, and demographic information. Participants reporting raccoon contact in the past twelve months provided responses regarding the nature of their raccoon contact, husbandry practices, and personal protective equipment (PPE; including gloves, hand hygiene, and masks) use frequency in different scenarios. This questionnaire is available upon request.

Approximately 20 mL of blood was collected into blood collection tubes (Becton Dickinson, Franklin Lakes, New Jersey) from each participant by a trained phlebotomist. Samples were allowed to clot and then centrifuged at 1,500 x g for 15 minutes. Serum was collected and stored at -20 C until testing. Serum samples and questionnaires were coded with a numerical identifier and the identification key was only available to one of the researchers (MJY). The

recruitment and enrollment procedures and sample collection methods were reviewed and approved by the UGA IRB (MOD00002218).

Serologic testing and data analysis

Sera were tested as previously described (1), with the following modifications: a control sample of anti-*Toxocara* sera from confirmed visceral toxocariasis cases was run concurrently with each batch of samples tested to ensure that cross-reaction with *Toxocara* was not occurring, and commercially-produced (GenScript), E. coli-expressed, GST-tagged rBpRAG-1 was used for sample testing (n=68) after December 2014 instead of in-house produced antigen. A positive reaction was defined as a single band present at 37 KDa (63 KDa for GST-tagged antigen). Positive or ambiguous samples were tested in triplicate and read independently by two individuals for confirmation. An adjusted seroprevalence estimate considering assay performance characteristics and the associated confidence interval were calculated using R statistical software version 3.2.1 (2,3).

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CHAPTER 3.1

RACCOON ROUNDWORM (*BAYLISASCARIS PROCYONIS*) AS AN OCCUPATIONAL HAZARD: 1. KNOWLEDGE OF *B. PROCYONIS* AND ATTITUDES TOWARDS IT AND OTHER ZOONOSES AMONG WILDLIFE REHABILITATORS.[1]

[1] Sapp, S.G.H, Murray, B.A., Hoover, E.R., Green, G.T., and M.J. Yabsley. 2018. *Zoonoses and Public Health* 65 (1): 130-142. Reprinted here with permission of publisher.

SUMMARY

Wildlife rehabilitators are at risk for zoonotic diseases because they often have prolonged contact with many species of wildlife and their bodily fluids. Raccoon roundworm (*Baylisascaris procyonis*) is a common zoonotic parasite of raccoons that has the potential to cause severe or fatal neurologic disease in a broad variety of hosts if the eggs within raccoon feces are ingested. We administered an online survey to wildlife rehabilitators to assess their knowledge regarding aspects of transmission, biology, and disease caused by *B. procyonis,* and also to evaluate attitudes towards wildlife diseases and *B. procyonis* as an occupational hazard. Knowledge was assessed using multiple choice and true–false questions; attitudes were measured using Likert type items. A total of 659 complete or near complete responses (missing fewer than three knowledge or attitudes items and/or non-response to some demographic fields) were collected. The median knowledge score was 7/14 questions correct (range: 0-14 correct). Generally, individuals with higher levels of education and rehabilitation experience, veterinary professionals, and those who are members of professional wildlife rehabilitation groups scored above the median significantly more often ($p<0.01$). Significantly more rehabilitators who were located in the Southeast and those with part-time or infrequent commitments scored below the median overall knowledge score. There was general agreement that *B. procyonis* is a health risk for rehabilitators and that measures should be taken to control transmission to people and animals. Some factors explaining differences in attitudes include setting of rehabilitation (home vs. animal care facility), veterinary profession, region, membership in a wildlife rehabilitation group, and rehabilitation of raccoons. Findings emphasize the importance of awareness and mentorship to inform rehabilitators on the potential risks of *B. procyonis* and other potential zoonoses within captive wildlife settings, and

the important role of professional wildlife rehabilitator groups in disseminating educational materials.

IMPACTS

- Exposure to *Baylisascaris procyonis* (raccoon roundworm) may present an occupational health risk to wildlife rehabilitators.
- In this survey we assessed knowledge of *B. procyonis* and attitudes towards it and other wildlife diseases as a health risk among wildlife rehabilitators.
- Overall, many knowledge gaps exist among rehabilitators regarding *B. procyonis* biology and transmission and patterns in knowledge performance exist based on professional memberships, education, and experience.

INTRODUCTION

Human-wildlife conflicts have rapidly increased in the face of growing urbanization and the expansion of urban wildlife populations (Soulsbury and White, 2016). In response, the practice of wildlife rehabilitation has also increased to accommodate diseased, injured, and abandoned wildlife which may result from these anthropogenic conflicts. Zoonotic diseases represent a potential occupational hazard for individuals working with wildlife, especially wildlife rehabilitators, given their frequent contact with diseased and stressed wildlife. Furthermore, zoonotic pathogens with potentially severe clinical consequences such as rabies, West Nile virus, *Campylobacter* spp., *Salmonella* spp., *Klebsiella pneumoniae,* and *Giardia* are

frequently detected in wildlife and in environment at rehabilitation centers, indicating a risk for exposure among wildlife rehabilitators (Jijon et al., 2007; Siembieda et al., 2011; Steele et al., 2005).

The raccoon roundworm *Baylisascaris procyonis* is one zoonotic pathogen of a common urban species, the raccoon (*Procyon lotor*). This parasite infects numerous paratenic host species, many of which may develop fatal neurologic disease if migrating larvae invade the central nervous system (Kazacos, 2016). Baylisascariasis in humans, while rare, is important because the majority of reported cases in the medical literature were either fatal or resulted in permanent neurologic and/or ocular sequellae (Graeff-Teixeira et al., 2016, Kazacos 2016, Sircar et al. 2016). However, the true prevalence of infection is likely underestimated, as the full spectrum of disease is not recognized (Sapp et al., 2016). Also, there is some evidence that *B. procyonis* prevalence is increasing in some regions of North America and the parasite is now recognized in new regions such as the Piedmont and coastal regions of the Southeast and arid western regions (Blizzard et al., 2010b; Hernandez et al., 2013; Roug et al., 2016). In addition, *B. procyonis* has been introduced and is spreading throughout Europe, Japan, and China (Miyashita, 1993; Popiołek et al., 2011; Xie et al., 2014). Finally, other wildlife species that may be rehabilitated are hosts for *Baylisascaris* species capable of producing larva migrans (e.g., *B. columnaris* in skunks, *B. melis* in badgers, and *B. transfuga* in bears), although their zoonotic potential is unknown (Sapp et al., 2017).

The wildlife rehabilitation community is a diverse group that have different educational and training backgrounds, and as such, their safety practices and awareness of potential occupational hazards may vary. Recently, we demonstrated that 7% (24/347) of healthy, adult wildlife rehabilitators were positive for antibodies to *Baylisascaris*, indicating prior infection, possibly associated with occupational activities (Sapp et al., 2016). Positive individuals generally

lacked consistent hand-washing and glove use while handling live raccoons, dead raccoons, and after potential fecal contact (Sapp et al., 2016). Furthermore, a small study of rehabilitated raccoons found that 37% and 56% of raccoons at two centers had patent *B. procyonis* infections (Kimball et al., 2003). Even with appropriate treatment at intake, young raccoons may become reinfected from eggs persisting in the environment and develop patent infections while still in care (Yabsley, unpub. data).

An important consideration in the prevention of pathogen transmission is knowledge about risks and possible prevention strategies. In addition, the attitudes of a group of people can provide insight into the likelihood of adopting new prevention strategies. If risk factors for exposure are modifiable, improving the awareness and knowledge about this zoonotic parasite may help reduce future risk of infection.

METHODS

Survey design

An online survey was developed by subject matter experts and administered through Qualtrics© (Qualtrics, Provo, Utah, USA) to a convenience sample of individuals involved with the rehabilitation of wildlife species. Two versions of the survey were administered, one for participants who rehabilitated raccoons (51-54 questions) and one for those who did not rehabilitate raccoons (38-39 questions). Knowledge questions relating to *B. procyonis* transmission, biology, and clinical aspects included multiple choice and disagree/agree, and attitudinal questions were assessed using a five-point Likert scale ranging from "strongly disagree" to "strongly agree." Standard socio-demographic data, including age, sex, location, and

education were collected, along with rehabilitation-specific questions. The survey was administered to a pilot group of approximately 40 individuals via email. Data analysis using Cronbach alphas examined the questions for internal reliability. Any items with poor internal reliability (<0.40) were modified for clarity based on pilot group feedback or eliminated.

After pilot testing and modification, a convenience sample of respondents was enrolled through electronic means from June 2014 to February 2015 through various mechanisms including emails to state, regional and national wildlife rehabilitation organization member listservs (e.g. National Wildlife Rehabilitation Association, etc.) postings to social media groups, and targeted emails to rehabilitation centers or hospitals. Also, a limited number of respondents were enrolled in person at regional and national wildlife rehabilitation meetings and were given paper copies of the survey. Inclusion criteria for respondents included age (18+) and current involvement in wildlife rehabilitation.

The survey and distribution methods were reviewed and approved by UGA's Institutional Review Board (MOD00002218).

Data analysis

Knowledge scores were calculated as the total number of questions answered correctly. Further sub-scores were calculated for questions pertaining to the areas of clinical attributes ("Clinical"), disease transmission ("Transmission"), and *B. procyonis* biology ("Biology"). For analysis, knowledge score and sub-score outcomes were condensed into a binary outcome variable based on scoring above the median score or equal to/below the median. Fisher's exact test was used for univariate analysis to derive odds ratios of individual demographic predictors

on knowledge score outcome. Due to the large sample size and number of predictors, a more conservative p-value ($p<0.01$) was considered significant. Multivariate analysis for overall knowledge score stepwise selection of predictors with $p<0.05$ with the best-fitting final model selected based upon Aikeke's Information Criterion (AIC). Two separate models were generated for all rehabilitators and those who rehabilitate raccoons.

Mean attitude scores for each individual question were calculated on 1-5 point Likert type scaling (i.e. 1=strongly disagree; 2=disagree; 3=neither agree nor disagree; 4=agree; 5=strongly agree). Attitudinal responses were analyzed using one-way ANOVA to evaluate differences in mean attitude score among groups within demographic predictors. P-values of <0.01 were considered significant. Significant predictors with more than two levels were further evaluated using post-hoc testing (Tukey's Honestly Significant difference) to determine which groups differed. All statistical analysis was performed in R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria), using the base package *stats* or the package *epitools* (Aragon, 2012; R Core Team, 2014).

RESULTS

Demographics and rehabilitation characteristics

A total of 659 complete or near-complete questionnaires were collected. Because the survey was administered through electronic means to various lists, a response rate could not be determined as we do not know how many people received the survey. Socio-demographic characteristics of study respondents are shown in Table 1. The majority of respondents were white (92.5%) and female (88.3%). The median age was 51 years (mean=48.6) ranging from 18 to 85 years.

Most respondents had either some college education or a college degree and approximately 22% were either registered medical professionals or had a professional medical degree (Table 3.1.1). All respondents were from either the United States or Canada with the exception of two individuals (Hungary, Trinidad and Tobago).

Experience levels were relatively equally represented, and more than half (63.1%) were current members of a professional wildlife rehabilitation group/organization. Most (68%) respondents reported that they rehabilitated raccoons. Among the 212 rehabilitators who reported not rehabilitating raccoons, the reasons provided were not having proper facilities to care for raccoons (44%), not rehabilitating rabies vector species (34%), concerns about *B. procyonis* (23%), and few/no raccoons admitted in area (6%). In addition, half (51%) provided a reason for not rehabilitating raccoons that we had not listed (e.g., most of these individuals stated it was not legal to rehabilitate in their area or they had made a personal choice to focus on non-raccoon species). When asked about the *primary* reason for not rehabilitating raccoons, the majority (45%) listed their "other" response, and only 3% of non-raccoon rehabilitators were primarily concerned with *B. procyonis*.

Knowledge

The mean overall score was 6.8 (median 7/14 correct). The proportion of correct responses to individual questions was variable [e.g., 83.7% answered the question "*B. procyonis* transmission occurs from raccoons to other animals by what route? (Ingestion of eggs)"correctly whereas only 9.6% answered "At what age can raccoons begin passing eggs of *B. procyonis*? (8 weeks)" correctly]

(Figure 3.1.1). The overall knowledge section had good internal reliability characteristics (α=0.733).

Factors associated with scoring above the median were determined for subsets of questions categorized for "transmission," "clinical," and "biology" as well as for the overall score (Table 3.1.2). Significantly more respondents with higher levels of education, as well as veterinary professionals, scored above the median overall and also in each individual category (Table 3.1.2 and Supplemental Table 3.1). Significantly fewer respondents in the southeastern region scored above the median overall and in individual categories ($p<0.001$). Among wildlife rehabilitation characteristics, significant differences existed between members of rehabilitation professional groups versus non-members, and greater number of years of experience was associated with a higher likelihood of scoring above the median (Table 3.1.2). Scores for individual categories generally followed the same trends as the overall scores (Supplemental Table 3.1). Among those individuals who rehabilitated raccoons, prior confirmed knowledge of *B. procyonis* infections in raccoons from their facility was significantly associated with an above-median score. Importantly, those individuals who worked in a facility with raccoons but without direct contact (i.e., may have cleaned cages) were less likely to score above the

median (Table 3.1.3).

The final regression model for knowledge section performance among all respondents included region, veterinary professional training, rehabilitation group membership, and rehabilitation of raccoons as significant or near-significant predictors. A separate model was generated for raccoon rehabilitators, and included similar predictors with the addition of a history of *B. procyonis* in the facility (Table 3.1.4).

Attitudes

The breakdown of responses to attitudes questions is summarized in Figure 3.1.2. In general, most respondents agreed that *B. procyonis* is a potential health risk to humans. Significant factors contributing to differences in mean attitude scores among respondents for *B. procyonis*-related items were diverse but generally included rehabilitation of raccoons and professional veterinary training (Table 3.1.5). In regards to the general wildlife disease related questions, rehabilitation setting (home vs. dedicated facility) explained differences in attitude scores across all items (Table 3.1.6). Home-based rehabilitators were less likely to agree that wildlife rehabilitation presents a health risk to humans, and also slightly less likely to disagree that release of raccoons outside of state or county origin is acceptable (Table 3.1.6). Individuals rehabilitating >100 raccoons per year were also more likely to agree that wildlife rehabilitation presents a health risk to humans than those rehabilitating ≤50 per year (Table 3.1.6).

DISCUSSION

The knowledge of *Baylisascaris procyonis* among wildlife rehabilitators was highly variable, but in general there were clear gaps in knowledge for most individuals. Overall, over half of the questions were answered incorrectly by ≥50% of respondents and three of the questions that were answered incorrectly most (≥87% of the time) were related to host range for patent infections and earliest age of patency in neonatal raccoons. The former answer may have been due to a misunderstanding of the question as many of the other responses (e.g. bears, skunks) are hosts for other *Baylisascaris* spp. (Sapp et al., 2017). Although these parasites are not known to be zoonotic, they can cause visceral and neural larva migrans in a variety of naturally- and

experimentally-infected hosts so feces from these hosts should be considered a possible risk (Sato et al., 2004; Sprent, 1955; Tiner, 1953). If data from these known *Baylisascaris* hosts are combined, 23% respondents knew that these animals are hosts for *Baylisascaris* spp., but incorrectly stated that these hosts could be infected with *B. procyonis*. Regardless, the frequency at which this question was answered incorrectly emphasizes a need for education about the life cycles and potential hosts of *B. procyonis* and related parasites (Sapp et al., 2017). The latter question ("At what age can raccoons begin passing eggs of *B. procyonis*?") was the most incorrectly answered (only 9.6% answered correctly). Given that rehabilitators often take in very young, orphaned raccoons, it is important to know that anthelmintic treatment should begin at an early age to prevent the raccoons from shedding eggs. Both of these questions were related to the biology of this parasite, and because the biology category scores were generally low, rehabilitation centers and organizations should not only emphasize treatment and control aspects for this parasite, but also general biology to improve infection control practices. This lack of understanding of the biology of *B. procyonis* is similar to what was observed in a survey of the general public, which revealed very limited knowledge among respondents regarding host range (Ogdee et al., 2016).

Over half (52%) of rehabilitators believed that fresh feces (which would contain unembryonated, non-infectious eggs) represent a risk of infection and while this false belief may encourage more stringent PPE use when in contact with fresh feces, it may also explain why less than half of the individuals did not know that old feces poses the greatest risk of infection. Importantly, most (65%) respondents answered correctly that common disinfectants were not sufficient to kill *B. procyonis* eggs; yet few raccoon rehabilitators reported using heat-based sanitization (35%) and most reported using bleach (73%) or disinfectants (45%), neither of which

are effective (Sapp et al., companion paper). It is unknown if the failure to use heat-based sanitization is because respondents do not know it should be used or if they do not use it because of inconvenience. In contrast, 85% of general public participants in another study believed that bleach and detergents were sufficient for decontamination (Ogdee et al. 2016). This is an important knowledge gap to address as many questions regarding concerns about raccoon roundworm come from the general public when the observe raccoons or their feces in their yards, on their decks, in the pools, etc. (Yabsley, unpublished data).

As expected, higher levels of education and rehabilitation experience were significantly associated with a higher knowledge score. Also, respondents with active membership in rehabilitation groups had higher knowledge scores which highlights the importance of professional organizations in providing educational materials and ensuring competency through continuing education events. The fact that rehabilitators in the Southeast scored scores that were at the median or below is likely multifactorial. Since *B. procyonis* has only relatively recently been recognized in the Southeast and it remains relatively uncommon in many areas, awareness appears to be more limited in this region compared to regions where the parasite is well-established and occurs at a high prevalence (Blizzard et al., 2010a, 2010b; Hernandez et al., 2013). Also, several states in the Southeast do not have state professional rehabilitation organization so ability of rehabilitators in this region to attend annual meetings may be limited. The Southeast region had the lowest percentage of members in professional groups (53%) although this was not significantly different than other regions. Veterinary professionals, who have required parasitology courses, unsurprisingly had higher odds of scoring above the median in the multivariate model. Many wildlife rehabilitation centers have veterinary professionals on staff

and these individuals can assist with infection control protocols; however, individuals operating out of their homes may not have ready access to veterinarians who can provide information on zoonoses and risk reduction., We had believed that knowledge would have been higher among individuals that work in rehabilitation centers but we did not detect differences between knowledge scores for home-based rehabilitators verses those who practice at dedicated centers; however, there were differences in attitude scores in questions related to risk perception.

While most responses to attitudes questions were in agreement that *B. procyonis* is a potential occupational risk and that measures should be taken to prevent zoonotic transmission of it and other pathogens to humans and animals, significant differences existed among various groups. Veterinary professionals perceived wildlife rehabilitation and *B. procyonis* as risks to human health more frequently than non-veterinary professionals. Of note is that several individuals had advanced research degrees (MS or PhDs) but the field of study was not known. It is possible that some of these individuals had extensive training in infectious diseases but samples sizes were too low to specifically analyze knowledge scores for this group.

Interestingly, those individuals who rehabilitated raccoons tended to believe that *B. procyonis* was not as great of a threat to wildlife rehabilitators as non-raccoon rehabilitators. This difference in risk perception could be due to the fact that some non-raccoon rehabilitators chose not to work with raccoons specifically due to *B. procyonis* concerns. Alternatively, raccoon rehabilitators may believe that they take appropriate precautions to avoid infection; however, a companion study on general practices showed that more education on the importance of personal protective equipment and hand sanitation is needed (Sapp et al., companion paper). Despite the general agreement that *B. procyonis* is a potential occupational hazard, the majority of respondents

agreed correctly that simple measures can help prevent the transmission of *B. procyonis* in the captive setting.

To assess general attitudes to wildlife diseases, some questions were asked about broader wildlife disease topics beyond *B. procyonis*. The vast majority of respondents agreed that rehabilitation of wildlife, in general, poses a human health risk. This result suggests that these individuals would be open to adopting risk-mitigating behaviors should they be provided with educational materials. Also, most individuals agreed that the release of rehabilitated raccoons outside of their location of origin posed a risk of pathogen transmission, although home-based rehabilitators were slightly less likely to agree with this statement.

These data indicate that many individuals that rehabilitate raccoons would benefit from additional education related to the parasite, especially those that are new rehabilitators, those that have lower levels of education, or who do not belong to a professional rehabilitation organization. We have several specific recommendations to facilitate education and outreach to these individuals. Many state wildlife agencies require rehabilitators to be permitted or certified, especially for raccoons which are rabies virus hosts in some regions, and some require annual continuing education (CE) credits to renew certifications. One possible way to improve knowledge of *B. procyonis*, as well as other zoonotic pathogens would be to introduce additional zoonoses educational efforts within this framework. Another consideration may be a requirement for rehabilitators to belong to one or more professional organizations to obtain permits or certification; however, we recognize that this may prove financially limiting for some rehabilitators as not all states have professional organization. In addition, in many cases, volunteers that clean cages would have equal or more risk of exposure compared to the person

responsible for the feeding and general care of the animal and these volunteers generally do not require permitting or certification. Thus, rehabilitators that utilize volunteers should provide specific and specialized training. As rehabilitators who lack experience generally performed worse on knowledge measures in this survey, a mentorship system involving a senior member with zoonoses training may be useful for more hands-on, directed education of new rehabilitators. Among rehabilitation facilities, efforts to raise consciousness and "visibility" of zoonoses (e.g. posters, pamphlets) should be employed and larger centers could employ guest speakers with expertise on public health and zoonoses to provide lectures on biosafety and disease issues. Future focus groups and additional targeted surveys of rehabilitators may help reveal the optimal strategies for disseminating relevant information.

Overall, despite a less than ideal knowledge on *B. procyonis* among wildlife rehabilitators participating in this study, attitudes towards it and other wildlife diseases topics were generally "correct." However, this survey may have been biased to those wildlife rehabilitators with an interest or concern about diseases and so those who did not participate in the survey may have even poorer knowledge and differing attitudes than those who dedicated time and effort to answering our questionnaire. Regardless, the data indicate that ongoing efforts are necessary to educate wildlife rehabilitators to ensure that knowledge gaps are not contributing to unnecessary health risks when rehabilitating wildlife.

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Table 3.1.1. Demographic characteristics of participating wildlife rehabilitators (n=659) this survey.

Characteristic	No.	% total
Gender		
Female	582	88.3
Male	75	11.4
Not specified	2	0.3
Race / Ethnicity		
American Indian or Alaska Native	15	2.2
Asian	6	0.9
Black / African American	2	0.3
Hispanic / Latino	18	2.7
Pacific Islander	1	0.1
White	627	92.5
Multiracial / Other / Declined to state	9	1.3
Education		
Less than high school	6	0.9
High school	45	6.8
Some college	132	20.0
College degree	235	35.7
Master's degree (MS, MA, etc.)	86	13.1
Doctoral degree (PhD)	7	1.1
Registered medical professional (RN, RVT, etc.)	70	10.6
Professional medical degree (MD, DVM, etc.)	78	11.8
Veterinary profession		
Professional veterinary degree, incl. DVM or equivalent or veterinary technical degree	96	14.6
No veterinary degree	563	85.4
Region (USA and Canada)*		
Northeast	161	24.7
Southeast	141	21.6
Midwest	173	26.5
Mountain / Central	75	11.5
West	103	15.8
Rehabilitation experience (years)		
0 to 2.9	94	14.5
3 to 5.9	114	17.6
6 to 9.9	107	16.5
10 to 19.9	172	26.5
≥20	162	25.0
Time commitment to rehabilitation		
Full time job	232	35.3
Full time volunteer	119	18.1
Part time job or volunteer	253	38.5
Volunteer infrequently	53	8.1

Membership in a professional rehabilitation group		
Non-member	241	36.9
Member	412	63.1
Rehabilitation facility type		
At home exclusively	451	68.4
Dedicated animal care facility (partly to fully)	208	31.6
Rehabilitation of raccoons		
No	212	32.2
Yes	447	67.8

* Regional categories defined as follows: Northeast (Connecticut, Delaware, Maine, Maryland, Massachusetts, New Brunswick, New Hampshire, New Jersey, New York, Nova Scotia, Pennsylvania, Prince Edward Island, Quebec, Rhode Island, Vermont, Virginia, West Virginia); Southeast (Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee); Midwest (Illinois, Indiana, Iowa, Kentucky, Manitoba, Michigan, Minnesota, Missouri, Ohio, Ontario, Wisconsin); Mountain/Central (Alberta, Colorado, Kansas, Montana, Nebraska, New Mexico, North Dakota, Oklahoma, Saskatchewan, South Dakota, Texas, Wyoming); Western (Arizona, British Columbia, California, Idaho, Nevada, Oregon, Utah, Washington). States and provinces not listed did not have any participants.

Table 3.1.2. Demographic and wildlife rehabilitation characteristics associated with median knowledge score.

Factor	Percent scoring above median (%)	cOR (95% CI)	p-value
Gender			
Female	56.6	Ref.	-
Male	45.3	0.64 (0.39-1.03)	0.0833
Region			
Northeast	63.0	Ref.	-
Southeast	41.0	0.42 (0.26-0.66)	0.0003*
Midwest	56.0	0.70 (0.45-1.09)	0.1173
Mountain / Central	53.3	0.67 (0.39-1.17)	0.1962
Western	66.0	1.14 (0.68-1.93)	0.6908
Education			
High school or less	37.3	Ref.	-
Some college to college graduate	50.7	1.73 (0.95-3.18)	0.0075*
Masters degree or PhD	56.0	2.14 (1.06-4.34)	0.0037*
Professional medical degree or registered medical professional	73.0	4.54 (2.32-8.92)	<0.0001*
Veterinary profession			
No veterinary degree	49.6	Ref.	-
Professional veterinary degree	81.3	4.16 (2.43-7.14)	<0.0001*
Rehabilitation experience (years)			
0 to 2.9	37.6	Ref.	-
3 to 5.9	49.1	1.60 (0.92-2.80)	0.1215
6 to 9.9	60.0	2.49 (1.40-4.41)	0.0018*
10 to19.9	58.6	2.34 (1.39-3.94)	0.0013*
≥20	64.0	2.94 (1.73-4.99)	<0.0001*
Rehabilitation setting			
At home exclusively	55.7	Ref.	-
Dedicated animal care facility	54.9	0.97 (0.69-1.35)	0.8655
Time commitment			
Full time job	60.4	Ref.	-
Full time volunteer	64.4	1.19 (0.75-1.89)	0.4854
Part time job or volunteer	50.8	0.68 (0.47-0.97)	0.4280
Volunteer infrequently	36.5	0.38 (0.20-0.71)	0.0030*
Member of wildlife rehabilitation professional organization			
Non-member	45.0	Ref.	-
Member	61.7	1.97 (1.43-2.72)	<0.0001*

* Significant p-value at alpha=0.01; cOR = crude odds ratio.

Table 3.1.3. Characteristics pertaining to raccoon rehabilitation associated with scoring above the median knowledge score.

	Overall Score			Transmission			Clinical Aspects			Biological Aspects		
Factor	**Percent scoring above median (%)**	**cOR (95% CI)**	**p-value[#]**	**Percent scoring above median (%)**	**OR (95% CI)**	**p-value[#]**	**Percent scoring above median (%)**	**OR (95% CI)**	**p -value[#]**	**Percent scoring above median (%)**	**OR (95% CI)**	**p-value[#]**
Rehabilitation of raccoons												
No	50.7	Ref.	-	70.0	Ref.	-	49.5	Ref.	-	24.3	Ref.	-
Yes	54.6	1.32 (0.95-1.84)	0.1085	66.5	0.85 (0.59-1.21)	0.4179	50.6	1.04 (0.75-1.45)	0.8676	36.0	1.75 (1.21-2.53)	0.0031*
Type of raccoon rehabilitation												
Active rehabilitation until release	61.4	Ref.	-	71.0	Ref.	-	53.8	Ref.	-	39.4	Ref.	-
Work in facility but no direct contact with raccons	27.5	0.24 (0.12-0.46)	<0.0001*	33.3	0.20 (0.11-0.38)	<0.0001*	21.6	0.24 (0.12-0.47)	<0.0001*	18.6	0.29 (0.13-0.63)	0.0009*
Transfer to other facility within 24 hrs	64.0	1.12 (0.48-2.60)	0.8356	68.0	0.87 (0.36-2.08)	0.8207	60.0	1.29 (0.56-2.94)	0.6793	31.6	0.48 (0.19-1.24)	0.1417
Approximate number of raccoons/year												
1 to 50	57.9	Ref.	-	66.1	Ref.	-	50.0	Ref.	-	35.4	Ref.	-
51 to 100	62.5	1.12 (0.70-2.09)	0.5812	70.8	1.29 (0.72-2.32	0.3927	58.9	1.43 (0.84-2.45)	0.2245	33.8	0.95 (0.54-1.66)	0.8885
≥101	69.2	1.63 (0.94-2.83)	0.0814	75.6	1.66 (0.92-2.98)	0.0927	62.8	1.69 (1.00-2.87)	0.0647	50.0	1.82 (1.08-3.07)	0.0308
***B. procyonis* diagnosis in raccoons under care**												
No	50.7	Ref.	-	61.0	Ref.	-	46.0	Ref.	-	26.1	Ref.	-
Yes	77.1	3.26 (2.08-5.15)	<0.0001*	84.0	3.4 (2.05-5.65)	<0.0001*	64.6	2.14 (1.42-3.24)	0.0003*	57.6	3.84 (2.52-5.87)	<0.0001*

* Significant p-value at alpha=0.01; [#] Based on Fisher's Exact Test.

Table 3.1.4. Multiple logistic regression models associated with an above-median knowledge score among all participating wildlife rehabilitators and those who rehabilitate raccoons.

All rehabilitators			
Factor	**Estimate**	**aOR (95% CI)**	**p-value**
Intercept	0.30593	1.36 (1.22-1.50)	<0.0001
Region			
Northeast	Ref.	-	-
Central	-0.10807	0.90 (0.79-1.02)	0.1014
Midwest	-0.09691	0.91 (0.82-1.01)	0.0665
South	-0.25268	0.78 (0.70-0.87)	<0.0001
West	0.03795	1.04 (0.92-1.17)	0.5316
Veterinary profession	0.30521	1.36 (1.22-1.51)	<0.0001
Member of rehabilitation group	0.15526	1.17 (1.08-1.26)	<0.0001
Raccoon rehabilitation	0.10369	1.11 (1.02-1.20)	0.011
Raccoon rehabilitators			
Factor	**Estimate**	**aOR (95% CI)**	**p-value**
Intercept	0.39877	1.49 (1.31-1.69)	<0.0001
Region			
Northeast	Ref.	-	-
Central	-0.14822	0.87 (0.73-1.02)	0.0942
Midwest	-0.1687	0.84 (0.74-0.96)	0.0134
South	-0.22165	0.80 (0.69-0.93)	0.0036
West	-0.03417	0.97 (0.83-1.13)	0.6625
Veterinary profession	0.24106	1.27 (1.11-1.46)	0.0007
Member of rehabilitation group	0.12175	1.13 (1.02-1.25)	0.0195
Bp diagnosis in facility	0.29207	1.34 (1.20-1.49)	<0.0001

aOR = adjusted odds ratio; *B.p. = Baylisascaris procyonis*

Table 3.1.5. Factors influencing differences in mean attitude scores for *Baylisascaris procyonis* specific questions.

	Feeding domestic animals outside increases risk of B.p. exposure		Treatment of raccoons for B.p. during rehabilitation		Simple prevention measures can prevent transmission of B.p.		B.p. as a health threat to rehabilitators	
	Mean score	**p-value†**	**Mean score**	**p-value†**	**Mean score**	**p-value †**	**Mean score**	**p-value †**
Overall Mean Score#	**4.12**	**--**	**4.36**	**--**	**4.40**	**--**	**3.83**	**--**
DEMOGRAPHICS								
Region		0.101		0.0269		0.901		0.0004*a
Northeast	4.20		4.18		4.36		3.95	
Southeast	4.01		4.27		4.45		3.71	
Midwest	4.04		4.43		4.36		3.61	
Mountain/Central	4.11		4.36		4.39		3.92	
Western	4.30		4.50		4.37		4.09	
Education		0.0739		0.732		0.984		0.0107
High school or less	4.00		4.35		4.37		3.71	
Some college to college graduate	4.05		4.37		4.39		3.74	
Masters degree or PhD	4.22		4.28		4.42		3.87	
Professional medical degree or registered medical professional	4.27		4.28		4.41		4.05	
Veterinary professional		0.0018*		0.051		0.165		<0.0001*
No	4.07		4.36		4.38		3.76	
Yes	4.41		4.17		4.45		4.23	
REHABILITATION (GENERAL)								
Rehabilitation experience (years)		0.25		0.0019*b		0.264		0.134
0 to 2.9	4.03		4.09		4.48		3.91	
3 to 5.9	4.01		4.15		4.37		3.62	
6 to 9.9	4.07		4.43		4.5		3.85	
10 to 19.9	4.17		4.45		4.37		3.80	
≥20	4.23		4.43		4.47		3.92	
Rehabilitation setting		0.0944		0.193		0.252		0.0086*
Animal care facility	4.03		4.30		4.42		3.90	
At home only	4.16		4.40		4.35		3.68	
Time commitment		0.0545		<0.0001*c		0.054		0.759
Full time job	4.26		4.44		4.44		3.86	
Full time volunteer	4.03		4.55		4.54		3.81	
Part time job or volunteer	4.06		4.21		4.32		3.79	
Volunteer infrequently	4.08		3.98		4.3		3.92	
Member of wildlife rehabilitation professional organization		0.871		<0.0001*		0.0349		0.138
Non-member	4.12		4.09		4.32		3.76	
Member	4.13		4.48		4.5		3.88	

REHABILITATION (RACCOONS)								
Rehabilitation of raccoons		0.162		<0.0001*		0.19		<0.0001*
No	4.20		4.11		4.34		4.16	
Yes	4.09		4.44		4.43		3.67	
Type of raccoon rehabilitation		0.126		0.0107		0.606		0.125
Active rehabilitation until release	4.11		4.46		4.44		3.66	
Work in facility but no direct contact	3.84		4.67		4.35		3.59	
Transfer to other facility within 24 hrs	4.28		4.44		4.32		4.08	
Approximate number of raccoons/year		0.0044*d		0.159		0.163		0.0519
1 to 50	3.95		4.50		4.38		3.60	
51 to 100	4.36		4.20		4.6		3.85	
≥101	4.22		4.12		4.4		3.88	
***B. procyonis* diagnosis in raccoons under care**		0.112		0.0013*		0.122		0.0253
No	4.05		4.38		4.4		3.60	
Yes	4.21		4.66		4.53		3.84	

* Significant p-value at alpha=0.01.[#] Mean attitude scores based on 1-5 Likert scale.

[†] Omnibus p-value derived from ANOVA.

a. Significant between West and Midwest (p=0.0009).
b. Marginally significant between "0-2.9" and "≥20" (p=0.01701).
c. Significant between "full time job" and "volunteer infrequently" (p=0.0056); "full time volunteer" and "part time job or volunteer" (p=0.0041); "full time volunteer" and "volunteer infrequently" (p=0.0008).
d. Significant between "1 to 50" and "51 to 100" (p=0.0071).

Table 3.1.6. Factors influencing differences in mean attitude scores for general wildlife diseases questions.

	Rehabilitation of wildlife is a human health risk for diseases.		In regards to diseases, release of rehabilitated raccoons outside the **county** of origin is acceptable.		In regards to disease, release of rehabilitated raccoons outside the **state** of origin is acceptable.	
	Mean score	**p-value**[†]	**Mean score**	**p-value**[†]	**Mean score**	**p-value**[†]
Overall Mean Score[#]	**3.87**	**--**	**2.10**	**--**	**1.58**	**--**
Demographics						
Region		0.131		0.113		0.144
Northeast	3.76		2.13		1.64	
Southeast	3.89		2.02		1.58	
Midwest	3.78		2.27		1.67	
Mountain/Central	4.04		2.07		1.52	
Western	4.03		1.93		1.42	
Education		0.0013*[a]		0.0491		0.0472
High school or less	3.57		2.08		1.65	
Some college to college graduate	3.78		2.2		1.64	
Masters degree or PhD	4.01		2.01		1.60	
Professional medical degree or registered medical professional	4.11		1.91		1.41	
Veterinary professional		<0.0001*		0.122		0.192
No	3.80		2.12		1.60	
Yes	4.31		1.94		1.48	
Rehabilitation (general)						
Rehabilitation experience (years)		0.997		0.428		0.875
0-2.9	3.93		2.01		1.65	
3 to 5.9	3.87		2.23		1.62	
6 to 9.9	3.86		2.18		1.59	
10 to 19.9	3.85		2.1		1.54	
≥20	3.91		2.01		1.57	
Rehabilitation setting		0.0006*		0.0078*		0.0038*
Animal care facility	3.97		2.02		1.52	
At home only	3.66		2.26		1.73	
Time commitment		0.319		0.177		0.57
Full time job	3.92		2.19		1.54	
Full time volunteer	3.72		2.17		1.58	
Part time job or volunteer	3.92		2.02		1.64	
Volunteer infrequently	3.77		1.91		1.51	

Member of wildlife rehabilitation professional organization		0.0309		0.745		0.0701
Non-member	3.75		2.11		1.66	
Member	3.94		2.08		1.53	
Rehabilitation (raccoons)						
Rehabilitation of raccoons		0.857		0.0032*		0.441
No	3.88		1.91		1.55	
Yes	3.87		2.18		1.60	
Type of raccoon rehabiliation		0.0111		0.044		0.782
Active rehabilitation until release	3.81		2.24		1.61	
Work in facility but no direct contact	3.94		1.98		1.65	
Transfer to other facility within 24 hrs	4.48		1.76		1.48	
Approximate number of raccoons/year		<0.0001*[b]		0.247		0.0382
1 to 50	3.69		2.22		1.68	
51 to 100	4.26		1.99		1.37	
≥101	4.23		2.27		1.62	
***B. procyonis* diagnosis in raccoons under care**		0.011		0.508		0.0994
No	3.79		2.15		1.64	
Yes	4.07		2.22		1.49	

* Significant p-value at alpha=0.01.

[#] Mean attitudes scores based on 1-5 Likert scale.

[†] Omnibus p-value derived from ANOVA.

a. Significant between "Master's degree or PhD" and "high school or less" (p=0.0078); marginally significant between "professional medical degree" and "high school or less" (p=0.0101).

b. Significant between"1 to 50" and "51 to 100" (p=0.0002) and "1 to 50" and "≥101" (p=0.0003)

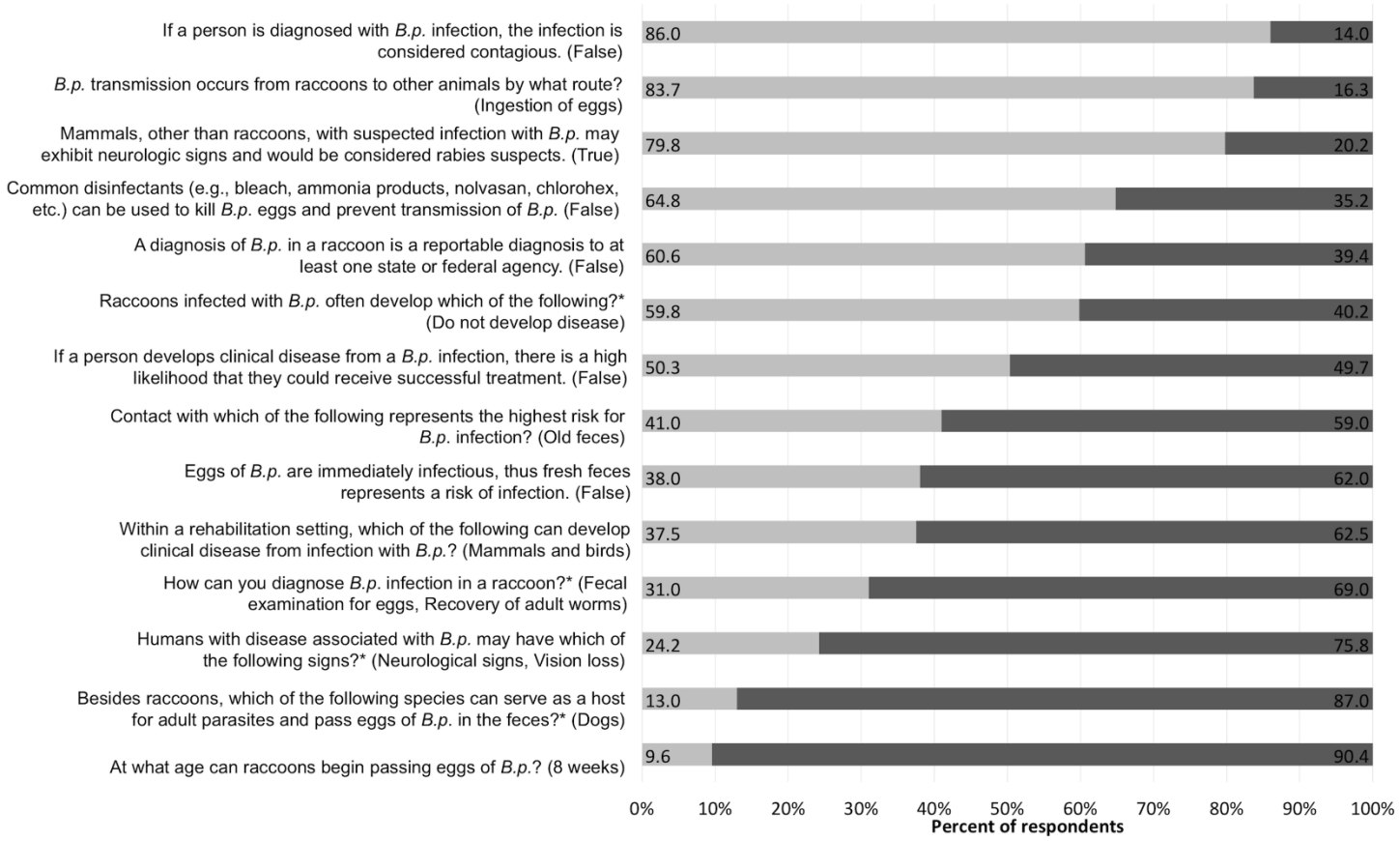

Figure 3.1.1. Knowledge questions, answers, and percent of participants answering correctly (light gray) and incorrectly (dark gray). Correct answer(s) provided in parentheses. * Multiple response item; only one combination of answers was considered a correct response. *B.p. = Baylisascaris procyonis*

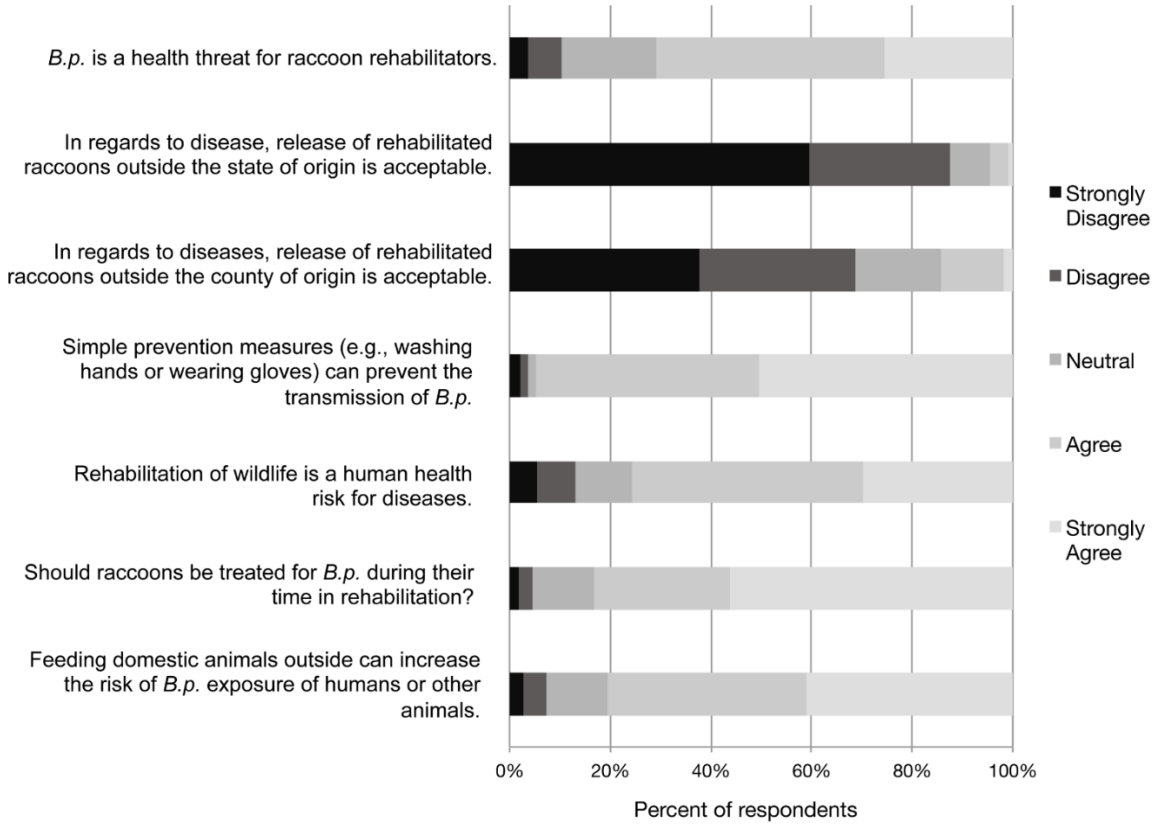

Figure 3.1.2. Likert scale responses to attitudes questions. *B.p.* = *Baylisascaris procyonis*

Supplemental Table 3.1. Demographic and wildlife rehabilitation characteristics associated with scoring above the median score within individual categories of the knowledge section of the survey.

	Transmission			Clinical Aspects			Biological Aspects		
Factor	**Proportion scoring above median (%)**	**OR (95% CI)**	**p -value**	**Proportion scoring above median (%)**	**OR (95% CI)**	**p -value**	**Proportion scoring above median (%)**	**OR (95% CI)**	**p -value**
Sex									
Female	68.2	Ref.	-	51.7	Ref.	-	31.3	Ref.	-
Male	62.7	0.78 (0.47-1.29)	0.3594	38.7	0.59 (0.40-0.96)	0.0371	38.7	1.38 (0.84-2.27)	0.2366
Region									
Northeast	75.0	Ref.	-	57.8	Ref.	-	35.4	Ref.	-
Southeast	55.7	0.42 (0.26-0.69)	0.0006*	38.3	0.45 (0.29-0.72)	0.0008*	17.9	0.40 (0.23-0.68)	0.0007*
Midwest	64.7	0.61 (0.38-0.99)	0.0533	50.3	0.74 (0.48-1.14)	0.1882	34.1	0.94 (0.60-1.48)	0.818
Mountain/Central	66.7	0.67 (0.36-1.22)	0.2089	41.3	0.52 (0.30-0.90)	0.0248	40.0	1.21 (0.69-2.14)	0.5616
Western	78.6	1.22 (0.68-2.23)	0.5493	60.2	1.11 (0.67-1.83)	0.703	38.8	1.16 (0.69-1.93)	0.6012
Education									
High school or less	49.0	Ref.	-	29.4	Ref.	-	21.6	Ref.	-
Some college to college graduate	66.7	2.08 (1.15-3.76)	0.0185	46.0	2.05 (1.08-3.87)	0.0034*	26.8	1.33 (0.66-2.70)	0.4985
Masters degree or PhD	66.7	2.08 (1.03-4.20)	0.0493	50.5	2.45 (1.19-5.07)	0.0022*	30.8	1.62 (0.72-3.60)	0.3272
Professional medical degree or registered medical professional	77.0	3.49 (1.76-6.81)	0.0003*	67.6	5.00 (2.50-10.01)	<0.0001*	50.0	3.64 (1.73-7.63)	0.0004*
Veterinary profession									
No veterinary degree	48.8	Ref	-	45.8%	Ref.	-	28.3	Ref.	-
Professional veterinary degree	84.4	2.94 (1.65-5.24)	<0.0001*	76.0%	3.75 (2.28-6.17)	<0.0001*	55.2	3.13 (2.01-4.87)	<0.0001*

Rehabilitation Experience (years)									
0-2.9	55.4%	Ref.	-	36.2%	Ref.	-	25.0%	Ref.	-
3-5.9	60.5%	1.23 (0.71-2.15)	0.4803	46.5%	1.53 (0.88-2.68)	0.1582	24.6%	0.98 (0.52-1.85)	1
6-9.9	68.3%	1.73 (0.96-3.10)	0.0769	49.5%	1.73 (0.98-3.05)	0.0644	27.4%	1.13 (0.60-2.14)	0.7479
10-19.9	75.7%	2.51 (1.46-4.31)	0.0012*	55.2%	2.18 (1.30-3.65)	0.0032*	33.3%	1.50 (0.85-2.65)	0.2058
≥20	70.8%	1.95 (1.14-3.32)	0.0193	56.8%	2.32 (1.36-3.91)	0.0018*	44.4%	2.40 (1.36-4.22)	0.0029*
Rehabilitation setting									
At home only	67.1%	Ref.	-	51.4%	Ref.	-	33.0%	Ref.	-
Animal care facility	68.8%	1.08 (0.75-1.54)	0.7184	47.6%	0.86 (0.62-1.19)	0.402	35.6%	0.89 (0.62-1.26)	0.53
Time commitment									
Full time job	77.0%	Ref.	-	59.9%	Ref.	-	38.4%	Ref.	-
Full time volunteer	72.9%	0.80 (0.48-1.34)	0.4288	49.6%	0.66 (0.42-1.03)	0.0697	31.1%	0.72 (0.45-1.16)	0.1959
Part time job or volunteer	61.4%	0.47 (0.31-0.71)	0.0002*	43.9%	0.52 (0.36-0.75)	0.0005*	30.2%	0.69 (0.47-1.01)	0.0672
Volunteer infrequently	46.2%	0.26 (0.13-0.48)	<0.0001*	41.5%	0.47 (0.26-0.87)	0.0207*	18.9%	0.37 (0.18-0.78)	0.0066*

Member of wildlife rehabilitation professional organization									
Non-member	57.4%	Ref.	-	37.8%	Ref.	-	27.1%	Ref.	-
Member	73.6%	2.08 (1.48-2.91)	<0.0001*	57.8%	2.25 (1.63-3.12)	<0.0001*	35.7%	1.50 (1.05-2.12)	0.0243*

* Significant p-value at alpha=0.01

CHAPTER 3.2

RACCOON ROUNDWORM (*BAYLISASCARIS PROCYONIS*) AS AN OCCUPATIONAL HAZARD: 2. USE OF PERSONAL PROTECTIVE EQUIPMENT AND INFECTION CONTROL PRACTICES AMONG RACCOON REHABILITATORS[1]

[1] Sapp, S.G.H, Murray, B.A., Hoover, E.R., Green, G.T., and M.J. Yabsley. 2018. Accepted by *Zoonoses and Public Health.* Reprinted here with permission of publisher, Mar. 2018.

SUMMARY

Baylisascaris procyonis, the raccoon roundworm, is a zoonotic ascarid of importance to human and animal health. Wildlife rehabilitators who care for raccoons may be at an increased risk for exposure to the parasite, especially if proper precautions are not taken. In a wider effort to evaluate awareness regarding *B. procyonis* in the wildlife rehabilitation community, an online survey (38-39 questions) including questions about *B. procyonis* knowledge and attitudes was developed and administered to wildlife rehabilitators but to assess precautions taken among raccoon rehabilitators, participants who rehabilitated raccoons (n=447) answered additional questions about use of personal protective equipment (PPE) and infection control practices (ICP). Reported use of gloves was variable but hand hygiene was generally consistent. Masks and gowns were seldom used. Part-time or infrequent volunteers, and rehabilitators located in the Central, Midwest, and Southeast were significantly less likely to report consistent use of PPE. A total knowledge score from the survey was used to predict the likelihood of reporting the use of particular ICP/PPE. Knowledge score had a highly significant but small effect on the likelihood of prophylactic use of anthelmintics, anthelmintics use for *B. procyonis* specifically, cleaning appropriately, and using species-dedicated housing. Risk factor analysis was performed on data from a prior serologic survey to evaluate factors associated with exposure to *B. procyonis* and inconsistent hand-washing after contact with live raccoons and their feces, practicing rehabilitation in *B. procyonis* hyperendemic regions, and practicing rehabilitation in the western region were significant risk factors for being seropositive. These data further demonstrate that correct PPE/ICP are critical in mitigating the risk of *B. procyonis* exposure among raccoon rehabilitators and among other captive species.

IMPACTS

- Proper precautions when caring for captive raccoons may reduce risk for exposure to the zoonotic roundworm *Baylisascaris procyonis.*
- Factors such as experience, education, and professional group membership influence the use of personal protective equipment and infection control practices among raccoon rehabilitators.
- Inconsistent hand hygiene after handling live raccoons or after potential fecal contact were significant risk factors associated with the presence of antibodies to *Baylisascaris* in wildlife rehabilitators enrolled in a previous study, demonstrating the importance of appropriate precautionary measures among individuals in contact with captive raccoons.

INTRODUCTION

Raccoons (*Procyon lotor*) are a ubiquitous species highly adapted to urban environments. As their populations and densities increase, potential raccoon-human conflicts may arise, resulting in more raccoons being admitted to wildlife rehabilitation centers. Raccoons are a reservoir for many potentially serious viral, bacterial, and viral zoonoses, so contact with raccoons may present a risk for exposure to these pathogens.

Among these zoonoses, *Baylisascaris procyonis,* the raccoon roundworm, is widespread across North America. Larval migrans can occur following the ingestion of eggs containing infective stage larvae, and can produce severe neural and ocular disease. Ocular and cerebral baylisascariasis has been reported about 50 times in people, and the number of reported cases

annually appears to be increasing (Kazacos, 2016; Sircar et al., 2016). This is likely due to better diagnostics and awareness of the disease, although factors such as increasing raccoon densities, geographic expansion of the parasite, and involvement of alternate definitive hosts (dogs, some exotic pets) may be involved (Blizzard et al., 2010; Lee et al., 2010; Page et al., 2009; Taira et al., 2013; Yabsley and Sapp, 2017). While clinical reports in humans are rare, the consequences are severe as the case fatality rate is >40% and full recoveries are uncommon (Kazacos, 2016). Early and aggressive treatment has been successful in reducing serious clinical outcomes; however, treatment of advanced cerebral baylisascariasis is complicated as most anthelmintics do not easily cross the blood-brain barrier (Peters et al., 2012).

Given the varied clinical presentations and difficulty in diagnosis, prevention of exposure to *B. procyonis* is critical. Many symptomatic clinical cases have been associated with direct contact with captive/pet raccoons (and therefore their feces) and recently, evidence for covert or subclinical infection in wildlife rehabilitators was found (Sapp et al., 2016; Sircar et al., 2016). Though the significance of antibodies in asymptomatic individuals is not known, it suggests the spectrum of disease is greater than previously assumed, and there may be health impacts outside of overt ocular and neurologic disease. A similar paradigm has been demonstrated with covert toxocariasis, which may be associated with urticaria, occult seizures, and developmental delays (Sharghi et al., 2000).

Thus, prevention of exposure to *B. procyonis,* especially among those individuals in contact with captive raccoons, should be of utmost importance in reducing risk. Since *B. procyonis* transmission is dependent on fecal-oral contact, appropriate personal protective equipment (PPE) and adequate infection control practices (ICP) in facilities housing raccoons should be the primary

interventions. Many PPE items such as gloves, gowns, face masks, etc. and strict hand-washing are commonplace in rehabilitation facilities that have established and enforced safety protocols for their employees; however, compliance in smaller or more informal settings (especially home) may be less common. In a small survey on PPE compliance among wildlife rehabilitators working with birds of prey, only 45% reported washing hands after contact with animals, and 12% did not use gloves (Saito and Shreve, 2005). Additionally, many significant zoonotic pathogens (e.g. *Giardia, Cryptosporidium, Salmonella, Campylobacter*, *Leptospira*) have been recovered from both captive animals environmental samples from rehabilitation facilities (Greig et al., 2014; Jijon et al., 2007; Siembieda et al., 2011). Environmental contamination with these pathogens presents a risk for transmission to both personnel and captive wildlife.

Baylisascaris procyonis eggs are extremely environmentally persistent and may remain viable for extended periods of time; they are only inactivated by high heat (>62 C) (Ogdee et al., 2016; Shafir et al., 2011). Infected raccoons, particularly juveniles, can shed copious numbers of eggs (mean 26,000 eggs/gram feces) and easily contaminate enclosures or equipment as eggs have a sticky proteinaceous coat (Kazacos, 2016). Therefore, *B. procyonis* should be considered an occupational hazard for individuals rehabilitating raccoons or those in contact with captive raccoons for extended periods (e.g. zookeepers, raccoon pet owners). Utilization of PPE (e.g., gloves and masks) provides physical barriers to exposure while ICP, such as proper hand hygiene, frequent removal of feces from enclosures, sterilizing enclosures with heat based methods, using species-dedicated housing, and anthelmintic treatment of raccoons upon intake and throughout their stay in captivity reduce risk to personnel and other wildlife in the facility.

The goals of this study were to 1) Assess patterns of PPE use and ICP among raccoon rehabilitators and 2) Determine risk factors associated with seropositivity to *Baylisascaris.* Additionally, knowledge and awareness of *B. procyonis* may play a role in how well PPE are used and ICP are implemented. We analyzed responses in an extensive survey of wildlife rehabilitators, and then performed risk factor analysis on questionnaire items associated with a previous serologic study to assess risk factors for exposure to *B. procyonis.*

METHODS

Survey design

An online survey administered through Qualtrics (Qualtrics, Provo, Utah, USA) was developed and administered to a convenience sample of individuals involved with the rehabilitation of wildlife species. Details on the survey design, testing and dissemination are provided in Sapp et al (companion paper in this issue). Only participants who rehabilitated raccoons are included in this study except where indicated otherwise. Items in the raccoon rehabilitator survey included basic demographic information (e.g. age, gender, location, education level, and years of rehabilitation experience), a set of true/false and multiple choice questions assessing overall knowledge of *Baylisascaris procyonis*, and questions assessing raccoon husbandry, ICP, and PPE use.

Survey data analysis

Fisher's Exact Tests were used to analyze the association between various demographic factors (e.g. education, experience) and reporting of adequate infection control and PPE use. The category with greatest number of observations was assigned as a reference category for factors with no apparent control group (e.g. geographic region). Strata for PPE use frequency were collapsed into "always" and "inconsistent" for glove use and hand washing due to a low number of responses in particular frequency categories. Given the large sample size and multitude of tests performed, an alpha value of 0.01 was used throughout analysis unless otherwise stated.

Knowledge scores were calculated as the number knowledge questions (out of 14 total) answered correctly. Binomial logistic regression models were generated to assess the impact of knowledge score on the likelihood of reporting particular practices. All statistical analyses were performed in R statistical software, version 3.1.2 and Fisher's Exact Tests were performed using the *epitools* package (Aragon, 2012; R Core Team, 2014).

Serologic data and risk factors

Serologic data from wildlife rehabilitators who were enrolled in a prior surveillance study were obtained as described (Sapp et al., 2016). Risk factors for seropositivity were determined for raccoon rehabilitators in this study based on responses to a questionnaire with similar questions. Fisher's exact tests were used to identify significant risk factors and calculate unadjusted odds ratios and 95% confidence intervals. For the purpose of risk analysis, states and provinces were assigned to approximate predicted risk categories based on reported overall *B. procyonis* prevalence in raccoons (see Sapp et al. 2016). Multiple logistic regression was performed using

the *glm* function with a binomial distribution in the R package *stats* to assess which factors were independently associated with risk and to calculate adjusted odds ratios. Variables of epidemiologic interest in the univariate analysis were included in candidate models, and a final model was selected based on Akaike's Information Criterion (AIC) comparison.

RESULTS

Demographics

A total of 447 raccoon rehabilitators participated in the online survey. Demographic characteristics are given in Table 3.2.1. Respondents were primarily female (89.5%) and white (92%); ages ranged from 18 to 80 years (median 51). Most respondents had completed at least a college degree, and among these, 11.6% were registered medical professionals and 9.6% held professional medical degrees (primarily DVM). Among rehabilitators, 83% actively rehabilitated raccoons until release; a smaller proportion worked in a facility housing raccoons but did not have contact with the raccoons or raccoons were transferred to another facility within 24 hours. Rehabilitation experience levels were relatively equally represented and more than half reported membership in a professional rehabilitation organization. Over half described their time commitment as either a full-time job or full-time volunteer effort (Table 3.2.1). Each rehabilitator had contact with an average (median) of 20 (6) neonatal raccoons, 30 (10) juvenile raccoons, and 14 (1) adult raccoons per year.

Infection control practices (ICP)

The majority of respondents (71%) reported housing raccoons in dedicated housing that was never used for other species. These enclosures were most commonly cleaned once to twice daily, using either bleach (73%), water (47%), disinfectants (e.g. Chlorohex, Roccal, Nolvasan; 45%), and/or heat (35%) and combinations of these were common (Table 3.2.2). The construction materials of adult and juvenile housing were similar, typically a combination of steel (47-57%, percentage of cages reported to have steel components), wood (26-31%), and plastic (18-45%) caging. Juveniles were more commonly housed indoors compared with adults.

Most (72%) rehabilitators also reported regular prophylactic anti-parasitic treatment of raccoons while in care, while 15% did not and 13% reported occasional or as-needed treatment (Table 3.2.2). About three-quarters of rehabilitators reported deworming for *B. procyonis* specifically (Table 3.2.2). Pyrantel formulations were the most commonly utilized anthelminthic drugs, followed by benziamidazoles (e.g. fenbendazole) and macrocyclic lactones (e.g. ivermectin, moxidectin). Use of non-anthelmintic substances (e.g. anti-protozoals, diatomaceous earth, herbal products) was less commonly reported. About a third (32%) of rehabilitators used multiple drugs. Despite the high rate of treatment, 34% (114/429) reported a confirmed *B. procyonis* diagnosis in their captive raccoons (range: 1-4 animals).

We also asked if rehabilitators had confirmed, by a pathologist or parasitologist, infections in any non-raccoon hosts. Including responses from non-raccoon rehabilitators, 11% (68/662) reported *B. procyonis* infections in at least one species of non-raccoon host. Many of the reported species are known to be common paratenic hosts that can develop clinical disease (e.g., woodchuck (*Marmota monax*), squirrels, rabbits, birds); however, several species have only

recently been associated with disease due to *B. procyonis* (e.g., beavers) or are new reports (Table 3.2.3) (Desprez et al., 2017).

Generally, we found respondents with longer experience times, time commitment and involvement in professional societies related to rehabilitation were more likely to report use of the most appropriate "correct" ICP while working with raccoons (Table 3.2.5). Individuals with >6 years of experience, members of rehabilitation groups ($p<0.0001$; OR=2.86), and those practicing rehabilitation at dedicated centers ($p=0.0046$; OR=2.00) were significantly more likely to report use of prophylactic anti-parasitics in raccoons. Those reporting rehabilitation as a part time job/volunteering ($p<0.0001$; OR=0.36) and those that volunteer infrequently ($p<0.0001$; OR=0.07) were significantly less likely to use anti-parasitics in raccoons. Similar patterns were observed when asked if they use anti-parasitics specifically for *B. procyonis.* Interestingly, rehabilitators located in the Southeast were less likely to report use of anti-parasitics specifically for this parasite ($p=0.0003$; OR=0.26), but region was an insignificant predictor for prophylactic anti-parasitic use overall. Veterinary professionals ($p=0.0009$; OR=2.64), individuals with >6 years of experience, and members of rehabilitation groups ($p=0.0067$; OR=1.82) were more likely to utilize heat based methods to clean enclosures whereas part-time volunteers/employees ($p=0.0011$; OR=0.47) and infrequent volunteers ($p=0.0088$; OR=0.09) were less likely to use heat. Members of rehabilitation groups were also more likely to use species-dedicated housing than non-members ($p=0.0002$; OR=2.56) (Table 3.2.5). Details on statistical analysis of all factors associated with correct practices are provided in Supplemental Tables 3.2.1 and 3.2.2.

Use of Personal Protective Equipment (PPE) and hand hygiene

The self-reported frequency of PPE use or hand hygiene during contact with live raccoons, dead raccoons, and raccoon feces are presented in Table 4. Generally, compliance with glove use and hand hygiene was relatively high in all situations, whereas masks and gowns were only sporadically utilized. For statistical analysis, glove use and hand washing categories were condensed to "always" vs. "less than always" (containing the often, sometimes, rarely, and never data). A summary of significant ($p<0.01$) predictors for glove use and hand-washing with live raccoons, dead raccoons, and fecal exposure is given in Table 3.2.5. Details on statistical analyses of glove use and hand hygiene are given in Supplemental Tables 3.2.3 and 3.2.4.

Influence of knowledge on ICP/PPE

Knowledge score was a highly significant predictor of the several infection control practices although the magnitude of the effect was relatively small. Knowledge score was significantly associated with the following ICP: prophylactic anti-parasitic use ($p<0.0001$; OR=1.23 (95% CI: 1.14-2.33)), anti-parasitic use for *B. procyonis* ($p<0.0001$; OR=1.05 (95% CI: 1.03-1.06)), cleaning enclosures with heat-based methods ($p<0.0001$; OR=1.04 (95% CI: 1..03-1.06)), and using dedicated housing for raccoons ($p<0.001$; OR=1.02 (95% CI: 1.01-1.04)) (Figure 3.2.1). A lower knowledge score was also significantly associated with the probability of inconsistent hand washing after handling live raccoons ($p<0.0001$; OR=0.97 (95% CI: 0.96-0.98)) and after potential fecal contact ($p<0.0001$; OR=0.98 (95% CI: 0.98-0.99)) and marginally associated with inconsistent hand washing after contact with dead raccoons ($p=0.0032$; OR=0.99 (95% CI: 0.98-1.00)) (Figure 3.2.2). Glove use in any situation was not significantly associated with knowledge score.

Risk factors for exposure

Of individuals tested in the prior serologic surveillance study, 24/347 (7%) tested positive for anti-*Baylisascaris* IgG antibodies via Western blot (Sapp et al., 2016). With the exception of two individuals, all had contact (but not necessarily active rehabilitation) with raccoons and 18 were active raccoon rehabilitators (Table 3.2.6).

Significant risk factors ($p<0.05$) for being seropositive in the univariate analysis included practicing wildlife rehabilitation in the Western region, location in a "very high" prevalence state/province, hand-washing on an inconsistent basis when in contact with live raccoons or feces, and using gloves on an inconsistent basis when in contact with raccoon feces (Table 3.2.6). Factors related to raccoon husbandry practices, including contact frequency, contact type, feces removal frequency, cleaning methods, anti-parasitic use, observation of nematodes in raccoon feces, or a *B. procyonis* diagnosis in the facility, were not significantly associated with being seropositive.

As the majority of significant variables in the univariate analysis were only applicable to individuals reporting raccoon contact within the past year, multivariate analysis was only carried out for this group. Adjustment for demographic factors of age, sex, and race/ethnicity was not necessary due to the relative homogeneity of the population. Variables of epidemiologic interest were used to generate a set of candidate models, including geographic region, hand hygiene when in contact with live and/or dead raccoons or feces, and glove use when in contact live and/or dead raccoons or raccoon feces (Supplemental Table 3.2.5) To address multicollinearity with geographic region, state/province level *B. procyonis* prevalence was excluded from the final multivariate analysis.

The final multivariate model ($p<0.0001$) included geographic region, hand washing when in contact with feces, and glove use when handling dead raccoons (Table 3.2.7). Region and inconsistent hand washing practices when handling raccoon feces were both significantly associated with serologic evidence of exposure to *Baylisascaris* with slightly higher odds of being seropositive in the Western region compared to the referent group. Inconsistent glove use when handling dead raccoons also had a significant association with this outcome, but only a very small increase in odds of being seropositive (Table 3.2.7).

DISCUSSION

This study evaluated raccoon rehabilitators for their knowledge of *B. procyonis* and their typical ICP and PPE use. We defined "correct" *B. procyonis* ICP and PPE use as routine anthelmintic treatment upon intake to prevent shedding of eggs and contamination of the environment, using housing that is specifically designated for raccoons and never used for other species, cleaning enclosures with heat-based methods (e.g. very hot water, steam), and consistently using gloves and washing hands during/after contact with raccoons and their feces.

We identified differences between ICP and PPE use across groups and detected risk factors for exposure to *Baylisascaris* spp. Most respondents reported consistent hand washing in all situations assessed, and about 75% used gloves in all situations, similar to the results for wildlife biologists reported by Bosch et al. (2013). As expected, inconsistent hand hygiene after handling raccoon feces was significantly associated with exposure to *B. procyonis* (seroconversion), with a modest increase in odds of seropositivity after adjusting for region.

Inconsistent hand hygiene after handling live raccoons was also a significant risk factor in the univariate analysis. It is not clear if this is due to contamination of raccoon fur with *B. procyonis* eggs, as has been shown with *Toxocara canis* eggs on canid fur, or simply indicative of a lack of caution in general (Sapp et al. 2016).

Rehabilitators practicing in regions of very high *B. procyonis* prevalence were significantly more likely to be seropositive. Therefore, rehabilitators practicing in areas where *B. procyonis* occurs in high prevalence in raccoons should take extra care to use appropriate ICP and PPE. Rehabilitation in areas of known very high (>50%) *B. procyonis* prevalence in raccoons was also a significant risk factor. However, until more comprehensive surveillance for *B. procyonis* in raccoons occurs, it is difficult to make accurate inferences about risk based on location only. A lack of reports should not be interpreted as absence; *B. procyonis* is likely found throughout the range of raccoons in North America and continues to be detected in previously negative locations such as the Southeast, Southwest, and even Latin America (Baldi et al., 2016; Blizzard et al., 2010; Roug et al., 2016; Yabsley et al., unpublished). An interesting finding in this study was that while rehabilitators in the Southeast reported prophylactic anti-parasitic use at the same frequency as other regions, they were significantly less likely to report that they treated for *B. procyonis* specifically, suggesting that awareness of this parasite may be more limited in this region. This is supported by our finding in Sapp et al. (companion article in this issue) that rehabilitators in the Southeastern region were less likely to score above the median knowledge score compared to those in other regions. Therefore, education on *B. procyonis* and its management and prevention aspects should be emphasized in the Southeast where this parasite appears to be an emerging concern.

Professional rehabilitators and those with more experience more frequently reported correct practices while part-time rehabilitators, or those that volunteer infrequently, less frequently reported correct ICP and PPE use. These findings emphasize the importance of wildlife rehabilitation professional groups in education on correct practices for all species/risks. Additionally, less experienced rehabilitators and/or part time volunteers would likely benefit from a mentorship system by a more experienced rehabilitator who is educated on correct practices. Given that knowledge score did have a significant, albeit small, impact on the likelihood of reporting particular correct practices, educating newer members on the potential risks of *B. procyonis* and other zoonoses should be a training priority. However, a study on prevention of zoonoses among veterinarians in the United Kingdom revealed that greater knowledge was not associated with greater compliance with risk-mitigating practices (Robin et al., 2017). Therefore more in-depth investigation involving personal interviews and/or focus groups may be necessary to fully understand decisions behind risk perception and corresponding action.

While most rehabilitators reported routine prophylactic anti-parasitic treatment, the doses and schedules used were not reported. Most of the frequently reported anti-parasitic drugs are effective against *B. procyonis*, however, a few individuals (8, 2.1%) reported regimens that are not efficacious for nematodes (coccidiostats, praziquantel) or are not known to be effective treatments (diatomaceous earth, herbal products) (Bauer and Gey, 1995). Frequent anthelminthic use and frequent enclosure cleaning are critical for preventing reinfections. Currently there is no evidence for drug resistance in *Baylisascaris* spp., so presumed treatment failures in rehabilitation facilities are likely due to reinfections. Additional data on the prevalence of patent infections and longitudinal testing of captive raccoons are needed to better inform ICP.

Infection control is critical to preventing transmission to both personnel and other animals in the facility. Eleven percent of rehabilitators (including non-raccoon rehabilitators) reported *B. procyonis* infections in paratenic hosts. Scurids (e.g. groundhogs, squirrels) and columbid birds (e.g. doves, pigeons), common *B. procyonis* hosts in the wild, were the most commonly reported (Kazacos 2016). Some reports included species known to be definitive hosts for other *Baylisascaris* species (e.g. black bear with *B. transfuga* and skunks with *B. columnaris*); these may have been due to misinterpretation of the question since we only asked if *B. procyonis* had been identified in a non-raccoon host although our intent was to provide information on cases of *Baylisascaris* larva migrans in paratenic hosts only. This confusion is also highlighted in Sapp et al., (companion manuscript in this issue) in which participants commonly listed skunks and bears as definitive hosts for *B. procyonis*. Since diagnosis of *B. procyonis* larva migrans can be difficult, and although we did give guidance on what a confirmed case would be, these cases were self-reported with no case details and thus some may not be accurate. Cases could also be missed by rehabilitators who are not aware of the clinical features of *B. procyonis*, as clinical signs are similar to other common neurologic diseases (rabies, canine distemper, angiostrongyliasis, etc.). It is unknown whether these cases were acquired in the facility, or if these animals arrived to the facility already infected. Nonetheless, preventing environmental contamination is important to minimize risk of infection while in care. Outbreaks of other infectious diseases, including some zoonotic pathogens (e.g. *Leptospira* spp., *Chlamydophila psittaci*) in captive wildlife facilities have been documented on multiple occasions and are likely perpetuated by poor husbandry practices (Miller et al., 1991; Raso et al., 2004; Szonyi et al., 2011; Vanstreels et al., 2014).

Collectively, this component of the survey, along with the other discussed in the companion paper, reveals important aspects of knowledge, attitudes, and practices of wildlife rehabilitators in the context of *B. procyonis.* Given that the majority of participants in this study are likely licensed rehabilitators with an interest in *B. procyonis,* knowledge, attitudes, and practices may differ for those individuals that did not complete our survey. Further investigation is warranted to reveal differences in these parameters in the groups that may have a more limited awareness or those who practice rehabilitation without permits/licensure/training. Since "professionalism", including time commitment, education, experience, and involvement in professional groups, was a predominant factor influencing knowledge/attitudes/practices, groups with limited access to these resources may be at increased risk for exposure to *B. procyonis* and other zoonotic pathogens.

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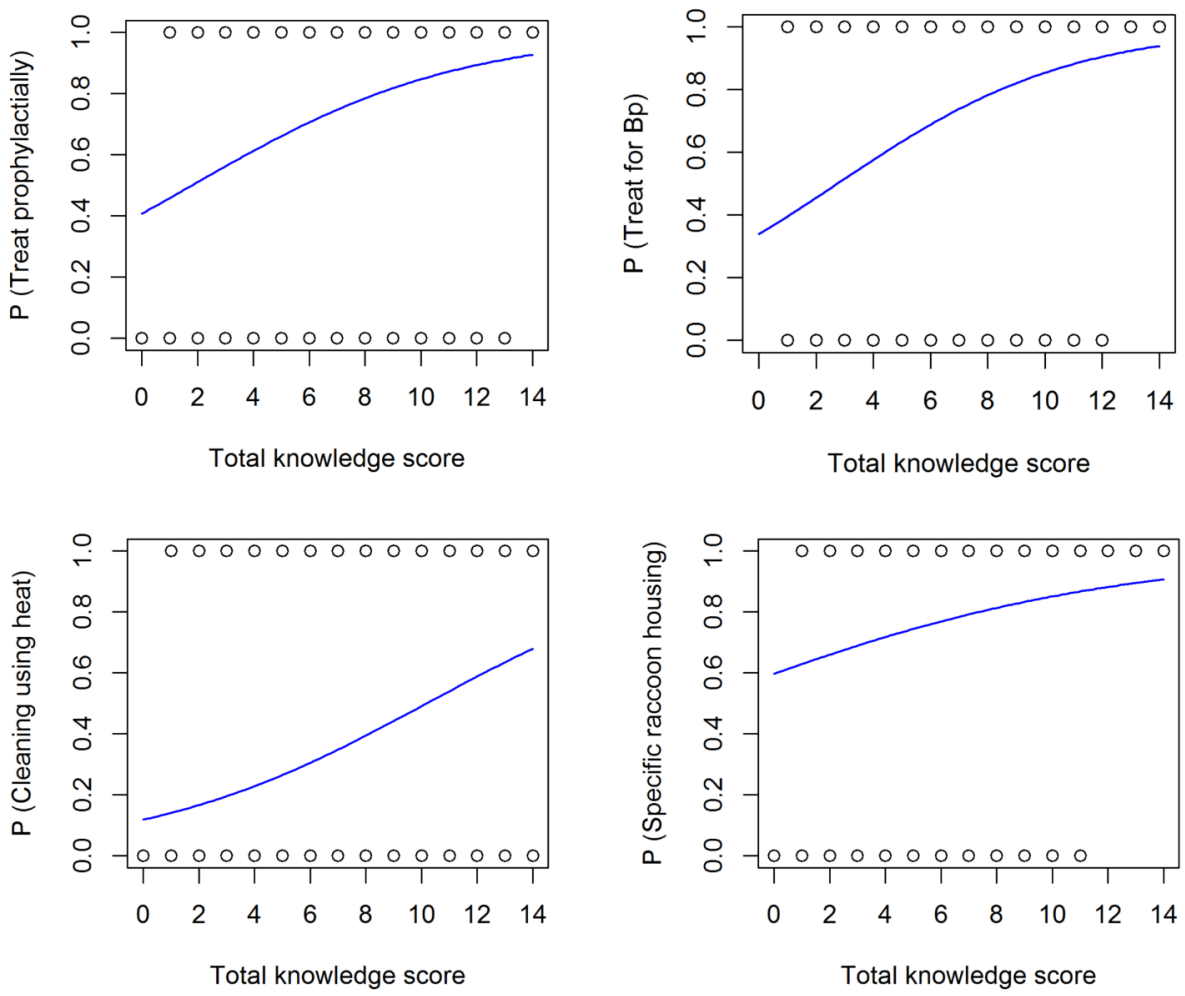

Figure 3.2.1. Logistic regression models for infection control practices where association with knowledge score was significantly ($p<0.01$) associated with probability of reporting a particular practice.

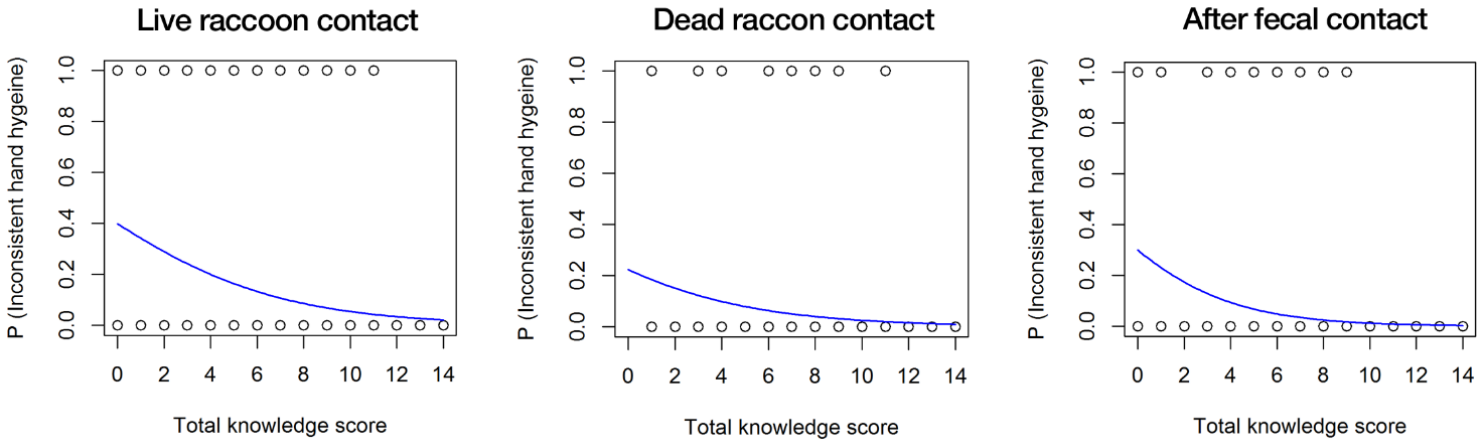

Figure 3.2.2. Logistic regression models for the association of knowledge score with the probability of inconsistent hand hygiene following contact with live raccoons, dead raccoons, and fecal contact.

Table 3.2.1. Demographic characteristics of raccoon rehabilitators in this survey.

Characteristic	No.	% total
Gender		
Female	400	89.5
Male	46	10.3
Not specified	1	0.2
Race / Ethnicity		
American Indian or Alaska Native	1	0.2
Asian	2	0.5
Black / African American	1	0.2
Hispanic / Latino	7	1.6
White	411	92.0
Multiracial / Other	25	5.6
Region (USA and Canada)*		
Northeast	89	20.0
Southeast	98	22.0
Midwest	132	29.7
Mountain / Central	50	11.2
West	76	17.1
Education		
Less than high school	3	0.7
High school	30	6.7
Some college	100	22.4
College degree (BS, BA)	170	38.0
Master's (MS, MA, etc.) degree	44	9.8
Doctor of philosophy (PhD) degree	5	1.1
Registered medical professional (LVT, LPN, etc.)	52	11.6
Professional medical degree (MD, DVM, etc.)	43	9.6
Rehabilitation experience (years)		
0 to 2.9	53	11.9
3 to 5.9	95	21.3
6 to 9.9	79	17.6
10 to 19.9	113	25.4
20+	105	23.6
Time commitment to rehabilitation		
Full time job	172	38.5
Full time volunteer	90	20.1
Part time job or volunteer	171	38.3
Infrequent volunteer	14	3.1
Type of raccoon rehabilitation		
Active rehabilitation until release	370	83.0
Work in facility with raccoons, but no direct contact	51	11.4
Transfer to other facility within 24 hrs	25	5.6
Membership in a professional rehabilitation group		
Non-member	157	35.5
Member	285	64.5

* Regional categories defined as follows: Northeast (Connecticut, Delaware, Maine, Maryland, Massachusetts, New Brunswick, New Hampshire, New Jersey, New York, Nova Scotia, Pennsylvania, Prince Edward Island, Quebec, Rhode Island, Vermont, Virginia, West Virginia); Southeast (Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee); Midwest (Illinois, Indiana, Iowa, Kentucky, Manitoba, Michigan, Minnesota, Missouri, Ohio, Ontario, Wisconsin); Mountain/Central (Alberta, Colorado, Kansas, Montana, Nebraska, New Mexico, North Dakota, Oklahoma, Saskatchewan, South Dakota, Texas, Wyoming); Western (Arizona, British Columbia, California, Idaho, Nevada, Oregon, Utah, Washington). States and provinces not listed did not have any participants.

Table 3.2.2. Reported infection control practices among raccoon rehabilitators.

Parasite Control Practices	**No.**	**% respondents**
Treat with anti-parasitic prophylactically		
No	68	15.2
Yes	322	72.0
Sometimes	56	12.5
Treat specifically for *B.p.*		
No	101	27.5
Yes	267	72.5
Anti-parasitic drugs used		
Pyrantel	237	84.8
Macrocyclic Lactones	68	21.1
Benzimidazoles	87	27.0
Coccidiostats	20	6.2
Other	28	8.7
Unknown	34	10.6
Housing and sanitation	**No.**	**% respondents**
Species-dedicated housing		
Utilized	316	70.9
Not utilized	42	19.7
Not sure	88	9.4
Frequency of enclosure cleaning		
Twice daily	205	45.9
Daily	211	47.2
Weekly	19	4.3
Monthly	2	0.5
Less than monthly	10	2.2
Cleaning method		
Bleach	328	73.4
Disinfectants	199	44.5
Heat	156	34.9
Water	195	43.6

B.p.=Baylisascaris procyonis

Table 3.2.3. Non-raccoon captive species reportedly diagnosed with *Baylisascaris procyonis* infections in wildlife rehabilitation centers.

Host	**No. times reported**
Woodchuck (*Marmota monax*)	16
Squirrel species (unspecified)	7
Mourning dove (*Zenaida macroura*)	5
American beaver (*Castor canadensis*)	4
Cottontail rabbit (*Sylvilagus* spp.)	4
Eastern gray squirrel (*Scuirus carolinensis*)	4
Muskrat (*Ondrata zibethicus*)	3
Rock pigeon (*Columba livia*)	3
Bird species (unspecified)	2
Skunk species (unspecified)†	2
Black bear (*Ursus americanus*)†	1
California ground squirrel (*Otospermophilus beecheyi*)†	1
Chipmunk (*Tamias* sp.)	1
Domestic cattle (*Bos taurus*)*	1
Domestic dog (*Canis familiaris*)†	1
Emu (*Dromaius novaehollandiae*)	1
Jack rabbit (*Lepus californicus*)*	1
Mountain beaver (*Aplodontia rufa*)*	1
Rabbit species (unspecified)	1
Reindeer (*Rangifer tarandus*)*	1

* Currently not known/reported as *Baylisascaris procyonis* paratenic host.

† Known definitive hosts for *Baylisascaris* spp.

Table 3.2.4. Self-reported frequency of personal protective equipment/behavior use by raccoon rehabilitators.

PPE	Live Animals		Dead Animals		Fecal Contact	
	No.	% total	No.	% total	No.	% total
Gloves						
Never	15	3.5%	19	5.5%	16	3.8%
Rarely	29	6.8%	6	1.7%	13	3.1%
Sometimes	71	16.7%	26	7.6%	45	10.8%
Often	83	19.5%	28	8.1%	48	11.5%
Always	227	53.4%	265	77.0%	296	70.8%
Hand Hygiene						
Never	1	0.2%	7	2.0%	4	1.0%
Rarely	0	0.0%	0	0	0	0
Sometimes	4	0.9%	0	0	3	0.7%
Often	31	7.3%	13	3.8%	16	3.8%
Always	389	91.5%	323	94.2%	398	94.5%
Mask						
Never	185	49.5%	144	50.7%	167	46.5%
Rarely	79	21.1%	46	16.2%	64	17.8%
Sometimes	61	16.3%	26	9.2%	49	13.6%
Often	27	7.2%	24	8.5%	17	4.7%
Always	22	5.9%	44	15.5%	62	17.3%
Gown						
Never	163	43.1%	148	50.7%	170	48.4%
Rarely	61	16.1%	41	14.0%	55	15.7%
Sometimes	70	18.5%	41	14.0%	46	13.1%
Often	31	8.2%	15	5.1%	18	5.1%
Always	53	14.0%	47	16.1%	62	17.7%

PPE = Personal protective equipment

Table 3.2.5. Summary of factors with significant ($p<0.01$) association with correct infection control practices and use of personal protective equipment/behaviors.

Practice	More likely to report	Less likely to report
Prophylactic anti-parasitic treatment	Rehabilitation experience >6 yrs Member of rehabilitation group Rehabilitation at animal care facility	Part time or volunteer infrequently
Treat for *B.p*. specifically	Rehabilitation experience >3 yrs Full time volunteer Member of rehabilitation group Rehabilitation at animal care facility	Rehabilitation in Southeast Part time or volunteer infrequently
Clean with heat	Veterinary professional Rehabilitation experience >6 yrs Member of rehabilitation group	Part time or volunteer infrequently
Maintain specific housing	Member of rehabilitation group	n/a
Use gloves consistently when handling live animals	Having 51-100 raccoons/year	Rehabilitation in Central or Midwest Full time volunteer or part time Rehabilitation at home only
Use gloves consistently when handling dead animals	n/a	Rehabilitation in Midwest
Use gloves consistently during fecal contact	n/a	Rehabilitation in Central, Midwest, or Southeast
Wash hands consistently after handling live animals	Medical degree Member of rehabilitation group	Volunteer infrequently Having 100+ raccoons/yr
Wash hands consistently after handling dead animals	n/a	n/a
Wash hands consistently after fecal contact	n/a	Having 100+ raccoons/yr

Note: Based on Fisher's Exact Tests with alpha=0.01.
n/a = no significant factors.

Table 3.2.6. Risk factors for *Baylisascaris procyonis* exposure in wildlife rehabilitators with raccoon contact enrolled in a previous study (Sapp et al., 2016)

Variable	No. respondents (% total)	No. seropositive (% category)	cOR (95% CI)	p-value**
Frequency of contact with raccoons in past year				
Daily	132 (38.2)	13 (9.0)	1.20 (0.32 – 4.45)	0.747
Weekly	64 (18.5)	1 (1.6)	0.23 (0.03 – 2.02)	0.244
Monthly or less	54 (15.6)	4 (7.4)	1.63 (0.59 – 4.44)	0.468
Not applicable / never	96 (27.7)	6 (6.3)	Ref.	-
Type of raccoon contact				
Touched live animal				
No	19 (7.5)	1 (5.3)		
Yes	234 (92.5)	17 (7.3)	1.41 (0.26 – 26.12)	1.000
Bitten				
No	179 (71.0)	12 (6.7)		
Yes	73 (29.0)	6 (8.2)	1.25 (0.42 – 3.35)	0.788
Scratched				
No	121 (48.0)	8 (6.6)		
Yes	131 (52.0)	10 (7.6)	1.17 (0.45 – 3.16)	0.810
Feces or bodily fluid contact				
No	33 (13.0)	1 (3.1)		
Yes	221 (87.0)	18 (8.1)	2.84 (0.55 – 51.92)	0.483
Touched dead animal				
No	89 (35.3)	4 (4.5)		
Yes	163 (64.7)	14 (8.6)	2.00 (0.69 – 7.22)	0.309
Performed necropsy				
No	185 (73.4)	14 (7.6)		
Yes	67 (26.6)	4 (6.0)	0.77 (0.21 – 2.25)	0.787
PPE Usage - Live Raccoons				
Gloves				
Utilized always	114 (59.0)	5 (4.4)		
Utilized less than always	130 (41.0)	12 (9.2)	2.22 (0.79 – 7.15)	0.207
Hand Washing				
Utilized always	214 (87.7)	12 (5.6)		
Utilized less than always	30 (12.3)	5 (16.7)	3.37 (1.00 – 9.93)	0.043*
PPE Usage - Dead Raccoons				
Gloves				
Utilized always	128 (73.6)	7 (5.5)		

Utilized less than always	46 (26.4)	7 (15.2)	3.10 (1.01 – 9.60)	0.053
Hand Washing				
Utilized always	162 (97.0)	11 (7.3)		
Utilized less than always	7 (3.0)	2 (28.8)	5.50 (0.73 – 28.96)	0.093
PPE Usage - Feces Contact				
Gloves				
Utilized always	156 (65.5)	9 (5.8)		
Utilized less than always	82 (34.5)	9 (11.0)	2.01 (0.76 - 5.37)	0.196
Hand Washing				
Utilized always	198 (89.6)	10 (5.0)		
Utilized less than always	23 (10.4)	5 (21.7)	5.22 (1.50 - 16.50)	0.012*

CI = confidence interval; PPE = personal protective equipment; cOR= crude odds ratio;
* = Significant p-value at alpha=0.05.
** Fisher's Exact Test

Table 3.2.7. Multivariate analysis of risk factors for *Baylisascaris procyonis* exposure (seropositivity) in participants reporting raccoon contact within the past year.

Variable	Crude Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)	p-value**
Region[#]			**0.005**
Northern	0.55 (0.08 – 2.62)	1.02 (0.91 – 1.13)	0.761
Central	†	†	-
Midwestern	2.00 (0.58 – 7.24)	1.09 (0.99 – 1.21)	0.087
Western	4.74 (1.21 – 18.66)	1.29 (1.11 – 1.48)	<0.001
Southern	Ref.	Ref.	-
PPE Usage - Raccoon feces contact			
Hand washing – utilized always	Ref.	Ref.	-
– utilized less than always	5.22 (1.50 - 16.50)	1.29 (1.12 – 1.50)	<0.001
PPE Usage - Contact with dead raccoons			
Gloves – utilized always	Ref.	Ref.	-
– utilized less than always	3.10 (1.01 – 9.60)	1.11 (1.01 – 1.21)	0.032

CI = confidence interval; *B.p. = Baylisascaris procyonis*.

** Fisher's Exact Test

†Odds ratio and confidence interval undefined due to zero-value cell.

[#]Geographic regions are defined as practicing rehabilitation in the following areas: South (Alabama, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee); Northeast (Delaware, Maryland, Massachusetts, Maine, New Jersey, New York, Pennsylvania, Quebec, Virginia); Midwest (Illinois, Indiana, Kentucky, Manitoba, Michigan, Minnesota, Missouri, Ohio, Ontario, Wisconsin); and Central: (Alberta, Arizona, Colorado, Kansas, Oklahoma, Texas); Western: (British Columbia, California, Oregon, Washington)

Supplemental Table 3.2.1. Factors influencing the likelihood of treating raccoons with anti-parasitic drugs prophylactically and for *Baylisacaris procyonis* specifically.

	Practice									
Factor	**Prophylactic anti-parasitic use**					**Treat for *B.p.* specifically**				
Education	No	Yes	OR	CI	p	No	Yes	OR	CI	P
High school or less	8	25	Ref	-	-	11	16	Ref	-	-
Some college to college graduate	80	189	0.7560	(0.32-1.75)	0.6851	60	155	1.7760	(0.78-4.05)	0.1821
Masters degree or PhD	13	36	0.8862	(0.32-2.45)	1	15	30	1.3750	(0.513-3.688)	0.6149
Professional medical degree or registered medical professional	23	72	1.0017	(0.39-2.52)	1	15	66	3.0250	(1.169-7.826)	0.0352
Region	No	Yes	OR	CI	p	No	Yes	OR	CI	P
Northeast	22	66	Ref	-	-	15	60	Ref	-	-
Central	12	38	1.0555	(0.47-2.37)	1	10	33	0.8250	(0.33-2.04)	0.8153
Midwest	37	95	0.8559	(0.46-1.58)	0.6446	28	80	0.7140	(0.35-1.45)	0.3806
Southeast	38	60	0.5263	(0.28-0.99)	0.0591	37	38	0.2570	(0.12-0.53)	0.0003*

West	13	63	1.6155	(0.75-3.48)	0.2543	10	56	1.4000	(0.58-3.37)	0.5121
Veterinary professional	No	Yes	OR	CI	p	No	Yes	OR	CI	p
No	109	281	Ref	-	-	92	228	Ref	-	-
Yes	15	41	1.0602	(0.56-1.99)	1	9	39	1.7490	(0.81-3.75)	0.1679
Experience category (years)	No	Yes	OR	CI	p	No	Yes	OR	CI	p
0-2.9	25	28	Ref	-	-	22	13			
3 to 5.9	33	61	1.6504	(0.83-3.28)	0.1636	24	48	3.385	(1.46-7.86)	0.0006*
6 to 9.9	17	62	3.2563	(1.52-6.97)	0.0024*	13	52	6.769	(2.71-16.92)	<0.0001*
10 to 19.9	22	91	3.6932	(1.81-7.53)	0.0004*	24	77	5.429	(2.38-12.39)	<0.0001*
20+	26	79	2.7129	(1.35-5.45)	0.0066*	18	75	7.051	(2.99-16.62)	<0.0001*
Time commitment	No	Yes	OR	CI	P	No	Yes	OR	CI	p
Full time job	34	138	Ref	-	-	39	111	Ref	-	-
Full time volunteer	10	79	1.9464	(0.92-4.15)	0.0847	20	65	1.1419	(0.71-2.12)	0.755
Part time job or volunteer	69	102	0.3642	(0.22-0.59)	<0.0001*	37	88	0.8356	(0.49-1.42)	0.5883
Volunteer infrequently	11	3	0.0672	(0.02-0.25)	<0.0001*	5	3	0.2108	(0.04-0.92)	0.0387

Professional rehabilitator group membership	No	Yes	OR	CI	p	No	Yes	OR	CI	p
Nonmember	65	91	Ref			44	71	Ref	-	-
M	57	228	2.8571	(1.86-4.40)	<0.0001*	57	193	2.0983	(1.30-3.39)	0.0026*
Raccoons contacted/year	No	Yes	OR	CI	p	No	Yes	OR	CI	p
1 to 50	56	169	Ref	-	-	44	145	Ref	-	-
51 to 100	15	58	1.2889	(0.68-2.45)	0.528	10	53	1.6083	(0.76-3.42)	0.2872
101+	21	57	0.9048	(0.50-1.62)	0.7638	23	46	0.0607	(0.33-1.11)	0.1109
Rehabilitation setting	No	Yes	OR	CI	p	No	Yes	OR	CI	p
At home only	97	207				76	163	Ref.	-	-
Animal care facility	27	115	1.9960	(1.23-3.24)	0.0046*	25	104	1.9396	(1.16-3.24)	0.0141

Based on Fisher's Exact Test; *significant at alpha=0.01; CI = 95% confidence interval; *B.p. = Baylisascaris procyonis*.

Supplemental Table 3.2.2. Factors influencing the likelihood of using species-dedicated housing and cleaning enclosures using heat.

Factor	Specific housing					Heat-based cleaning				
Education	No	Yes	OR	CI	p	No	Yes	OR	CI	p
High school or less	8	22	Ref			24	9	Ref		
Some college to college graduate	55	191	1.2628	(0.53-2.99)	0.6454	190	80	1.1228	(0.50-2.52)	0.8425
Masters degree or PhD	7	34	1.7662	(0.56-5.56)	0.3853	31	18	1.5484	(0.59-4.05)	0.474
Professional medical degree or registered medical professional	18	69	1.3939	(0.53-3.64)	0.611	46	49	2.8406	(1.20-6.75)	0.0247
Region	No	Yes	OR	CI	p	No	Yes	OR	CI	p
Northeast	13	68	Ref			54	35	Ref		
Central	9	31	0.6585	(0.25-1.70)	0.4544	31	19	0.9456	(0.46-1.93)	1
Midwest	34	87	0.4892	(0.24-1.00)	0.0612	96	36	0.5786	(0.32-1.03)	0.0777
Southeast	21	70	0.6373	(0.30-1.37)	0.2582	68	30	0.6807	(0.37-1.25)	0.2226
West	10	59	1.1279	(0.46-2.76)	0.8242	40	36	1.3886	(0.75-2.58)	0.345
Veterinary professional	No	Yes	OR	CI	p	No	Yes	OR	CI	p
No	74	278	Ref			266	125	Ref		
Yes	14	38	0.7225	(0.37-1.40)	0.3682	25	31	2.6387	(1.50-4.66)	0.0009*
Experience category (Years)	No	Yes	OR	CI	p	No	Yes	OR	CI	p
0-2.9	14	33	Ref			44	9	Ref		
3 to 5.9	26	59	0.9627	(0.44-2.09)	1	67	28	2.0431	(0.88-4.74)	0.1143

6 to 9.9	13	58	1.8928	(0.79-4.51)	0.1808	46	33	3.5072	(1.51-8.16)	0.0039*
10 to 19.9	14	85	2.5758	(1.11-5.98)	0.0411	73	40	2.6788	(1.19-6.05)	0.0176*
20+	21	79	1.596	(0.73-3.51)	0.2996	59	46	3.8117	(1.69-8.60)	0.0008*
Time commitment	No	Yes	OR	CI	p	No	Yes	OR	CI	p
Full time job	32	124	Ref			95	77	Ref		
Full time volunteer	12	71	1.5269	(0.74-3.15)	0.295	59	31	0.6482	(0.38-1.10)	0.1446
Part time job or volunteer	42	109	0.6697	(0.39-1.13)	0.144	124	47	0.4676	(0.30-0.73)	0.0011*
Volunteer infrequently	2	12	1.5484	(0.33-7.27)	0.7385	13	1	0.0949	(0.01-0.74)	0.0088*
Professional rehabilitator group membership	No	Yes	OR	CI	p	No	Yes	OR	CI	p
Nonmember	46	97	Ref			115	42	Ref		
Member	40	216	2.5608	(1.57-4.17)	0.0002*	171	114	1.8254	(1.19-2.79)	0.0067*
Raccoons contacted / year	No	Yes	OR	CI	p	No	Yes	OR	CI	p
1 to 50	37	167	Ref			141	83	Ref		
51 to 100	14	52	0.8229	(0.41-1.64)	0.5899	44	29	1.1200	(0.65-1.92)	0.6793
101+	20	50	0.5539	(0.30-1.04)	0.0868	50	28	0.9513	(0.56-1.63)	0.8922
Rehabilitation setting	No	Yes	OR	CI	p	No	Yes	OR	CI	p
Animal care facility	75	200	Ref			201	104			
At home only	13	116	3.3461	(1.78-6.29)	<0.0001	90	52	1.1167	(0.73-1.69)	0.67

Based on Fisher's Exact Test; *significant at alpha=0.01; CI = 95% confidence interval.

Supplemental Table 3.2.3. Factors associated with inconsistent glove use in raccoon rehabilitation activities.

	Implementation of gloves in different situations														
Factor	**Live raccoon contact**					**Dead raccoon contact**					**After fecal contact**				
Education	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p
High school or less	23	10	Ref			20	3	Ref			23	8	Ref		
Some college to college graduate	131	139	2.44 05	(1.12-5.32)	0.026 4	154	56	2.42 42	(0.69-8.47)	0.208 3	175	78	1.28 14	(0.55-2.99)	0.6807
Masters degree or PhD	22	27	2.82 27	(1.11-7.17)	0.041 2	29	8	1.83 90	(0.43-7.79)	0.505 7	31	17	1.57 67	(0.58-4.28)	0.4605
Professional medical degree or registered medical professional	51	44	1.98 40	(0.85-4.62)	0.151 8	71	14	1.31 46	(0.34-5.03)	0.505 1	68	19	0.80 33	(0.31-2.08)	0.6283
Region															
Northeast	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p
Central	54	35	Ref			64	10	Ref			71	14	Ref		
Midwest	17	33	2.99 50	(1.45-6.17)	0.002 8*	28	13	2.97 14	(1.16-7.58)	0.027 9	30	19	3.21 19	(1.43-7.23)	0.0006 *
Southeast	51	81	2.45 04	(1.41-4.25)	0.001 6*	62	35	3.61 29	(1.64-7.92)	0.000 9*	71	54	3.85 71	(1.97-7.56)	<0.000 1*
West	47	51	1.67 41	(0.93-2.99)	0.105 9	64	12	1.20 00	(0.48-2.97)	0.818 4	56	28	2.53 57	(1.22-5.27)	0.0013 *
Veterinary professional	56	20	0.55 10	(0.28-1.07)	0.097 7	55	11	1.28 00	(0.51-3.24)	0.641 6	67	7	0.52 99	(0.20-1.39)	0.2429
No															
Yes	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p
Experience Category (Years)	192	199	Ref			233	73	Ref			257	112	Ref		
0-2.9	35	21	0.57 89	(0.33-1.03)	0.064 6	41	8	0.62 28	(0.28-1.39)	0.276 4	40	10	0.57 37	(0.28-1.19)	0.1394
3 to 5.9															
6 to 9.9	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p
10 to 19.9	31	22	Ref			31	6	Ref			37	10	Ref		
20+	49	46	1.32 28	(0.67-2.61)	0.492 3	58	15	1.33 62	(0.47-3.79)	0.797 8	58	31	1.97 76	(0.87-4.51)	0.1186
Time Commitment	48	31	0.91	(0.45-1.85)	0.857	52	12	1.19 23	(0.41-3.50)	0.794 8	56	18	1.18 93	(0.50-2.86)	0.8258
Full time job	45	68	2.12 3	(1.10-4.13)	0.030 4	62	31	2.58 33	(0.97-6.85)	0.055 6	71	38	1.98 03	(0.89-4.42)	0.1298
Full time volunteer	53	52	1.38 2	(0.71-2.69)	0.399 6	71	17	1.23 71	(0.45-3.44)	0.803 2	73	25	1.26 71	(0.55-2.92)	0.6802
Part time job or volunteer															
Volunteer infrequently	No	Yes	OR	CI	p	No	Yes	OR	CI	p	No	Yes	OR	CI	p
Professional rehabilitator group membership	195	67	Ref			126	27	Ref			130	36	Ref		
No	38	52	2.14 45	(1.27-3.61)	0.004 1*	50	24	2.24 00	(1.18-4.25)	0.017 1	59	28	1.71 37	(0.96-3.06)	0.0934
Yes	79	92	1.82 50	(1.19-2.80)	0.006 8*	91	29	1.48 72	(0.82-2.68)	0.227	101	55	1.96 64	(1.20-3.22)	0.0091
Raccoons contacted/year	5	9	2.82 10	(0.91-8.77)	0.088 8	7	1	0.66 67	(0.08-5.64)	1	7	3	1.54 80	(0.38-6.29)	0.6942
1 to 50															

51 to 100 101+	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p	Consistent use	Inconsistent use	OR	CI	p
Rehabilitation setting	81	76	Ref			91	30	Ref			99	43	Ref		
Animal care facility	143	142	1.05 83	(0.72-1.56)	0.8425	1182	50	0.83 33	(0.50-1.40)	0.505	194	78	0.92 57	(0.59-1.44)	0.7341
At home only															

Based on Fisher's Exact Test; *significant at alpha=0.01; CI = 95% confidence interval.

Supplemental Table 3.2.4. Factors associated with inconsistent hand hygiene implementation in raccoon rehabilitation activities.

	Implementation of hand hygiene in different situations														
Factor	**Live raccoon contact**					**Dead raccoon contact**					**After fecal contact**				
Education	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
High school or less	26	7	Ref			19	2	Ref			28	3	Ref		
Some college to college graduate	228	42	0.684	(0.28-1.68)	0.4510	184	16	0.826	(0.18-3.87)	0.683	237	17	0.669	(0.18-2.43)	0.4656
Masters degree or PhD	43	6	0.518	(0.16-1.71)	0.3584	36	1	0.263	(0.02-3.10)	0.546	45	3	0.622	(0.12-3.30)	0.6745
Professional medical degree or registered medical professional	92	3	0.121	(0.03-0.50)	0.0029*	84	1	0.113	(0.01-1.20)	0.099	89	0	NA	NA	NA
Region	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
Northeast	81	8	Ref			67	4	Ref			80	4	Ref		
Central	48	2	0.421	(0.09-2.07)	0.3301	37	4	1.810	(0.42-7.66)	0.461	47	3	1.276	(0.27-5.95)	1.0000
Midwest	109	23	2.136	(0.91-5.02)	0.1129	89	5	0.941	(0.24-3.64)	1	119	9	1.512	(0.45-5.08)	0.5717
Southeast	78	20	2.596	(1.08-6.24)	0.0392	68	4	0.985	(0.24-4.10)	1	79	5	1.265	(0.33-4.89)	1.0000
West	71	5	0.713	(0.22-2.28)	0.7732	61	3	0.823	(0.18-3.83)	1	72	2	0.555	(0.10-3.12)	0.6853
Veterinary professional	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
No	336	55	Ref			275	19				348	23			
Yes	53	3	0.345	(0.10-1.15)	0.0872	48	1	0.301	(0.04-2.31)	0.330	51	0	NA	NA	NA
Experience Category (Years)	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
0-2.9	43	10	Ref			30	5	Ref			44	5	Ref		
3 to 5.9	81	14	0.743	(0.30-1.81)	0.6424	63	6	0.571	(0.16-2.02)	0.501	81	8	0.869	(0.27-2.82)	1
6 to 9.9	68	11	0.695	(0.27-1.78)	0.4743	58	3	0.310	(0.07-1.39)	0.136	69	5	0.637	(0.17-2.33)	0.5173
10 to 19.9	100	13	0.559	(0.23-1.37)	0.231	88	2	0.136	(0.03-0.74)	0.018	106	3	0.249	(0.06-1.09)	0.1084
20+	96	9	0.403	(0.15-1.06)	0.0724	84	4	0.285	(0.07-1.13)	0.116	98	2	0.179	(0.03-0.96)	0.039
Time Commitment	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
Full time job	155	17	Ref			145	7	Ref			157	10	Ref		
Full time volunteer	84	6	0.651	(0.25-1.71)	0.4927	68	4	1.218	(0.35-4.30)	0.748	87	1	0.180	(0.02-1.43)	0.1035
Part time job or volunteer	143	28	1.785	(0.94-3.34)	0.0806	104	8	1.593	(0.56-4.53)	0.426	148	10	1.060	(0.43-2.62)	1
Volunteer infrequently	7	7	9.117	(2.85-29.12)	0.0005*	6	1	3.452	(0.36-37.72)	0.308	7	2	4.485	(0.82-24.47)	0.1175
Professional rehabilitator group membership	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
No	123	34	Ref			111	7	Ref			131	12	Ref		

Yes	262	23	0.3176	(0.18-0.56)	<0.0001*	210	12	0.9061	(0.35-2.37)	0.8094	264	10	0.4135	(0.17-0.98)	0.0621
Raccoons contacted/year	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
1 to 50	209	15	Ref			161	9	Ref			216	5	Ref		
51 to 100	66	7	1.4778	(0.58-3.78)	0.442	66	2	0.5421	0.1141	0.7332	68	3	1.9059	(0.44-8.18)	0.4075
101+	62	16	3.5957	(1.68-7.68)	0.0018*	62	4	1.1541	0.349	0.76	63	7	4.8000	(1.47-15.64)	0.0099*
Rehabilitation setting	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p	Consistent	Inconsistent	OR	CI	p
Animal care facility	258	47	Ref			227	14				265	18	Ref		
At home only	131	11	0.4609	(0.23-0.92)	0.024	96	6	1.0134	(0.38-2.71)	1	134	5	0.5493	(0.20-1.51)	0.3611

Based on Fisher's Exact Test; *significant at alpha=0.01; CI = 95% confidence interval.

Supplemental Table 3.2.5. Candidate models compared for multivariate risk analysis of *Baylisascaris procyonis* exposure (seropositivity) in wildlife rehabilitators reporting raccoon contact within the past year, 2012-2015, enrolled in a prior study (Sapp et al. 2016).

Model	AIC	n parameters	delta
Region + FecesHW + DeadGloves	50.857	7	-
Region + FecesHW + LiveGloves	51.154	7	0.297
Region + FecesGloves + DeadHW	51.284	7	0.427
Region + FecesHW + FecesGloves	51.903	7	1.046
Region + DeadHW	52.128	6	1.271
Region + FecesGloves + LiveHW	53.166	7	2.309
Region + FecesHW	54.039	6	3.182
Region + DeadGloves	54.371	6	3.514
Region + LiveHW	55.149	6	4.292
Region + LiveGloves	56.263	6	5.406

AIC = Akaike's Information Criterion; Region = geographic region in which individual practices wildlife rehabilitation; FecesHW = hand washing frequency after raccoon feces contact; DeadHW = hand washing frequency after dead raccoon contact; LiveHW = hand washing frequency after live raccoon contact; FecesGloves = glove use frequency during raccoon feces contact; DeadGloves = glove use frequency during dead raccoon contact; LiveGloves = glove use frequency during live raccoon contact.

CHAPTER 4

VARIABLE INFECTION DYNAMICS IN FOUR *PEROMYSCUS* SPECIES FOLLOWING EXPERIMENTAL INOCULATION WITH *BAYLISASCARIS PROCYONIS*[1]

[1] Sapp, S.G.H., Weinstein, S.B., McMahan, C.S. and Yabsley, M.J. 2016. *Journal of Parasitology*, 102(5): 538-544. Reprinted here with permission of publisher.

ABSTRACT

Wild rodents such as *Peromyscus* spp. are intermediate hosts for the zoonotic ascarid *Baylisascaris procyonis* (raccoon roundworm), and previous studies indicate *Peromyscus leucopus* (white-footed mouse) likely serves an important role in parasite ecology. Natural infections have been sporadically identified in a few *Peromyscus* spp., but no data are available on differences in susceptibility among the many other species. We compared survival and infection dynamics of *B. procyonis* in 4 species (*P. leucopus, Peromyscus maniculatus* [deer mouse], *Peromyscus californicus* [California mouse], *Peromyscus polionotus* [Oldfield mouse]) from regions of varying habitat types as well as *B. procyonis* prevalence in raccoons. Six captive-bred mice of each species were inoculated per os with 1 of 3 biologically-relevant doses of embryonated *B. procyonis* eggs (~10, ~50, or ~500). Animals were monitored twice daily for clinical signs and behavioral abnormalities and were euthanized at the onset of neurological signs or extensive (≥20%) weight loss, or at 45 days post-infection if no disease developed. Larvae were counted in the brain via microscopic examination and in skeletal *Mus*cle and visceral organs via artificial digestion. In the high-dose group, all but 1 mouse developed severe neurologic disease and were euthanized. In the medium-dose group, survival was variable and ranged from 33–85% across species. Little to no disease was observed in the low-dose group, although 1 *P. maniculatus* developed disease and was euthanized. Survival analysis reveals *P. leucopus* had a longer time until clinical disease onset versus the other species, which did not differ significantly from each other. Interestingly, larval recovery relative to dose was nearly identical across species and doses; however, larvae were differentially distributed in skeletal *Mus*cle, visceral organs, and brain among species. These data indicate that *P. leucopus* may be more resilient toward severe baylisascariasis compared to the

other species and that even closely-related rodents may experience differential mortality. This variation in tolerance may have ecological implications for the different species as *B. procyonis* intermediate hosts, although more work is needed to put these experimental findings into context.

INTRODUCTION

The raccoon roundworm, *Baylisascaris procyonis*, is an important pathogen of humans and numerous wildlife species. Raccoons (*Procyon lotor*) and occasionally domestic dogs (*Canis* familiaris) serve as the definitive host for adult, intestinal-stage *B. procyonis*. In other host species, infection with larval-stage *B. procyonis* can cause larva migrans syndromes including visceral larva migrans (VLM), neural larva migrans (NLM), and/or ocular larva migrans (OLM). To date, there have been approximately 30 documented cases of baylisascariasis in humans, with most cases being very severe or fatal (Graeff-Teixeira et al., 2016). In addition, severe or fatal NLM has been documented in over 150 species of birds and mammals (Page, 2013; Graeff-Teixeira et al., 2016) and, as intermediate hosts, these species may influence the maintenance and transmission of this parasite. *Peromyscus* spp. are likely common intermediate hosts for *B. procyonis* due to their wide geographic distribution, high population densities, and feeding behavior (Page et al., 2001a). Rodents forage in raccoon feces, and caching and storing undigested seeds and plant material from raccoon feces allows *B. procyonis* eggs within the feces to become larvated and infectious, and the consumption of feces-contaminated seeds may result in infection (Logiudice, 2001; Vander Wall et al., 2001). Even if seeds are foraged from fresh feces, which would contain non-larvated eggs, larvated eggs in raccoon latrines could adhere to fur and later be ingested during

grooming. Natural infections in the white-footed mouse (*Peromyscus leucopus*), deer mouse (*Peromyscus* maniculatus), and brush mouse (*Peromyscus* boylei) have been documented; however, only *P. leucopus* has been extensively investigated as a natural host of *B. procyonis* (Tiner, 1954; Kazacos, 2001; Page et al., 2001b; Evans, 2002; Beasley et al., 2013).

In this study, we experimentally inoculated 4 *Peromyscus* spp. with *B. procyonis* eggs to characterize differences in infection dynamics and survival among these species. We selected 4 species (*P. leucopus*, *P. maniculatus* ssp. *bairdii*, *Peromyscus californicus* ssp. *insignis*, and *Peromyscus polionotus* ssp. *subgriseus*) that differed in size, habitat use, and endemic range. Both the white-footed mouse, *P. leucopus*, and deer mouse, *P. maniculatus*, are broadly distributed across North America and sympatric through much of this range, with the exception of the southeastern region (*P. leucopus*) and the western United States and Canada (*P. maniculatus*). The Oldfield mouse (*P. polionotus*) inhabits coastal plain and sand dune habitats throughout the southeastern United States and is the smallest species in this study (Carleton, 1989). The California mouse (*P. californicus*) inhabits chaparral and woodland habitats from mid California through the Baja Peninsula and is the largest species included in the study (Merritt, 1974).

These 4 selected species are endemic to regions that have variable *B. procyonis* prevalence in raccoons (Kazacos, 2001; Blizzard et al., 2010a). *Baylisascaris procyonis* infects more than 50% of raccoons throughout much of the upper Midwest, Northeast, Mid-Atlantic, and Pacific Northwest (Tiner, 1954; Kazacos, 2001). In contrast, the parasite was historically absent in the piedmont and coastal plain regions of the Southeast and, although now documented in some counties, remains rare (generally <15% prevalence in raccoons) throughout the region (Babero and Shepperson, 1958; Harkema and Miller, 1964; Eberhard et al., 2003; Blizzard et al., 2010b; Hernandez et al.,

2013). Data on historical trends of *B. procyonis* prevalence in southern California are not available prior to 2000 (Evans, 2002; Moore et al., 2004); however, raccoon population density is very low in scrub/chaparral habitats where *P. californicus* is found (Parker et al., 2015).

We hypothesize that species native to areas where *B. procyonis* was historically endemic (i.e., *P. leucopus* and *P. maniculatus*) will demonstrate higher survival rates when challenged with *B. procyonis* due to a longer evolutionary history and thus possible selection for tolerance. By extension, species where *B. procyonis* is historically absent or rare (*P. polionotus* and perhaps *P. californicus*) should be more susceptible to severe baylisascariasis. These selected species were inoculated with 3 different doses of *B. procyonis* eggs based on either biologically-plausible doses or doses previously used in experimental infections (Tiner, 1953; Sheppard and Kazacos, 1997) with the parasite (500, 50, and 10 eggs) in order to characterize host survival and parasite distribution in host tissues.

MATERIALS AND METHODS

Experimental infections

Baylisascaris procyonis eggs were obtained from the feces of a naturally infected raccoon from West Linn, Oregon. Feces were suspended in potassium dichromate and held at room temperature with occasional stirring for approximately 3 wk, at which time most eggs were completely larvated. The egg suspension was washed 3 times in phosphate-buffered saline (PBS) and the number of larvated, viable (as indicated by larval movement) eggs per milliliter of suspension was determined microscopically. Aliquots of eggs in 3 doses (low [~10 eggs], medium

[~50 eggs], and high ~500 eggs]) were prepared and centrifuged and the pellet was re-suspended in a small amount of sucrose solution prior to inoculation.

Adult (~1 yr old) male mice of 4 species (*P. californicus* ssp. insignis, *P. leucopus*, *P. maniculatus* ssp. bairdii, and *P. polionotus* ssp. subgriseus) were purchased from captive breeding colonies (*Peromyscus* Genetic Stock Center, University of South Carolina, Columbia, South Carolina). Mice of the same species were housed in standard cages in groups of 6 (except *P. californicus*, which were housed in groups of 3), with food (commercial, nutritionally-complete laboratory rodent diet) and water available ad libitum, under a standard 12-hr light cycle at 23 C. Six mice of each species were assigned randomly to a dose group and weighed. Each mouse was inoculated per os with a plastic pipette. Two sentinel mice of each species were sham-inoculated with PBS, housed separately from inoculated mice, and observed until the end of the study. Animals were observed twice daily for behavioral abnormalities and weighed weekly. If severe neurologic signs (e.g., profound ataxia, torticollis, seizures, partial to full paralysis, etc.) developed or if mice lost more than 20% of their starting weight, they were anesthetized via isoflurane inhalation and euthanized by cervical dislocation (American Veterinary Medical Association, 2013). All surviving animals were euthanized at 45 days post-inoculation and processed. All animal procedures were reviewed and approved by the UGA's IACUC (#A2014 01-007).

Larval recovery and enumeration

Immediately following euthanasia, mice were skinned and necropsied, and grossly visible granulomas or lesions were noted. To enumerate larvae in brain tissue, whole brains were placed

on round glass plates (12.7 mm diameter), a top glass plate was added, and tissue was flattened with gentle pressure. The flattened brain tissue was examined immediately for migrating larvae using a dissecting microscope (×30) (Kazacos, 2001). Visceral organs and skeletal muscle were separated for artificial digestion. Tissues were comminuted with 100–200 mL of 1% HCl/1% pepsin in 0.85% saline solution using a blender. The homogenate was gently stirred on a heated plate with a magnetic stir bar at 37 C until completely digested (1–2 hr). The homogenate was then poured into conical glasses and allowed to settle for 30 min, after which the top two-thirds of supernatant was decanted and replaced with cold water. This process was repeated and the settled material examined under a dissecting microscope at full magnification (×30), and all larvae were enumerated in the entire skeletal muscle and visceral organ solutions.

Data analysis

Statistical analysis was conducted using the "stats" and "survival" packages in R statistical software, version 3.1.2 (R Core Team, 2014). Poisson regression models were used to assess the association between dose group and the number of larvae recovered in the different tissue groups, while adjusting for mouse species. The effect of species and dose on survival was analyzed under a parametric Weibull model. This analysis explicitly accounted for the right censoring introduced by mice that survived to the end of the study.

RESULTS

Clinical disease and survival

Mouse survival was strongly dose dependent. All but 1 mouse inoculated with the highest dose (500 eggs) developed severe neurologic disease and were euthanized (Figure 4.1). For mice inoculated with 50 eggs, mortality ranged from 67% in *P. leucopus*, *P. polionotus*, and *P. californicus* to 83% in *P. maniculatus*. Of the mice inoculated with 10 eggs, only 1 individual (*P. maniculatus*) developed clinical disease and was euthanized (Table 4.1). Common clinical signs observed among all species included extensive weight loss (>20% of body weight), poor body condition, lethargy, unresponsiveness, torticollis, lateral recumbency, circling, ataxia, abnormal posturing, partial to full paresis, paw and facial tremors, seizures, and apparent blindness. At necropsy, granulomas were observed frequently in the heart (44%), lungs (48%), and small intestine (40%) in most individuals receiving 50 or 500 eggs. Weight loss was more pronounced in high-dose mice of all species and in some medium-dose mice that experienced neurological disease. No weight loss was evident in any mice in the low-dose group (Figure 4.2).

Survival also varied by species. In the high-dose group *P. leucopus* had a significantly longer time ($P < 0.0001$) until onset of central nervous system (CNS) disease than did *P. maniculatus*, *P. californicus*, and *P. polionotus*, while survival did not differ among the latter 3 species. Therefore, for modeling survival, *P. leucopus* data were compared to collated data from the other 3 species. In the final model, increasing dose was strongly associated with decreased survival time ($P < 0.0001$) as was being a species other than *P. leucopus* ($P < 0.01$). Excluded from analysis was the sole *P. californicus* that did not develop CNS disease; this likely was due to an inoculation error because the total number of larvae recovered was lower than other individuals

in the high-dose group, and it represents a statistically significant outlier (data not shown). No association between survival time and starting body weight or number of larvae recovered from the different tissue groups was detected.

Larval recovery

Mean numbers of larvae recovered from brain, skeletal muscle, and visceral organs of inoculated mice across dose groups are presented in Figure 4.3 and Table 4.1. Larval recovery percentages were similar across species and doses, generally averaging approximately 7–10% of the dose given (Table 4.1). No larvae were recovered from control mice.

In all tissue types, there was a highly significant association between dose and larvae recovered; i.e., the Poisson regression model estimated that the mean number of larvae recovered increased with dose (Table 4.2). Within brain tissue, the estimated mean number of larvae recovered was higher for *P. maniculatus* ($P < 0.0001$) than for the other 3 species at all dose levels. The estimated mean number of larvae recovered from skeletal muscle was significantly greater for *P. californicus* ($P < 0.0001$) and marginally but non-significantly less for *P. leucopus* ($P = 0.0561$) when compared to the other 2 species at all dose levels. In visceral organs, the estimated mean number of larvae recovered from *P. leucopus* was significantly higher ($P < 0.0001$) and significantly lower for *P. californicus* ($P = 0.0369$) when compared to the other 2 species at all dose levels. No differences among species were observed for total larvae recovered.

DISCUSSION

Both field and experimental studies have investigated the potential role of *P. leucopus* as an intermediate host of *B. procyonis*; however, little data are available on infection dynamics in other *Peromyscus* species. Our survival analysis suggests that, compared to *P. maniculatus*, *P. polionotus*, and *P. californicus*, *P. leucopus* may be more tolerant of *B. procyonis* infection. Although our mice came from parasite-free captive stocks, we found that survival of our *P. leucopus* was relatively consistent with previously published experimental infections that used wild-captured animals which may have had some degree of pre-existing or cross-protective immunity (Tiner, 1953; Sheppard and Kazacos, 1997). Survival varied in these captive-bred mice, and this variation was not due to the considerable size differences between the species.

As expected, dose was a significant factor for the development of clinical disease. The highest dose (500 eggs) caused severe neurologic disease and extensive weight loss in almost all mice, regardless of species. The medium dose (50 eggs) produced severe clinical disease in 4–5 of the 6 mice in each species. This result was similar, but higher, to what Sheppard and Kazacos (1997) found in *P. leucopus* inoculated with 50 eggs (3 of 10 mice developed clinical signs). Only a single mouse in the low-dose group (10 eggs), a *P. maniculatus*, developed clinical signs. Additionally, larvae were not recovered from approximately 60% of the mice in this dose group. Thus, it is difficult to ascertain whether the low dose was unable to establish infection consistently, if the dose was not administered completely, or if larvae were simply not recovered via the given method. Also, some mice in this dose group may have cleared all migrating larvae prior to processing.

The overall mean numbers and percentages of larvae recovered across species at each dose were similar, a finding similar to other experimental ascarid infections that inoculated with varying doses (Havasiová-Reiterová et al., 1995; Cox and Holland, 2001). Although parasite establishment was similar across species, the larval distribution in host tissues varied by species. In *P. leucopus*, significantly more larvae were recovered from the viscera. A possible mechanism behind this observation is that *P. leucopus* may have a greater capacity to restrict movement of larvae in the intestinal *Mus*cularis and visceral organs, possibly slowing migration to the central nervous system. This is consistent with our observation of delayed onset of severe neurologic disease in *P. leucopus* compared to the other 3 species. In contrast to *P. leucopus*, *P. californicus* had both higher mortality and relatively more larvae in the skeletal *Mus*cle, suggesting that *P. californicus* was less able to slow larval migration. This observation mirrors that of Sheppard and Kazacos (1997), who found a higher proportion of granulomas in the GI tract, liver, and lungs of experimentally infected *P. leucopus* as well as superior survival compared to *Mus musculus* (house mouse). However, their study also found significant differences in total number of larvae recovered between *P. leucopus* and *M. musculus*, suggesting that susceptibility between those 2 distantly-related rodents differs more dramatically than among *Peromyscus* species (Sheppard and Kazacos, 1997).

The number of larvae recovered from brain tissue was significantly greater in *P. maniculatus* compared to other species; however, *P. maniculatus* did not differ from *P. californicus* or *P. polionotus* in terms of overall survival or time to disease onset. A single larva in the brain was always sufficient to cause severe neurologic disease. However, neurologic disease was also observed in mice in which no larvae were recovered from brain tissue. The brain tissue squash

method may have missed larvae that had already died, were difficult to observe, or had migrated out of the brain prior to sampling. Alternatively, some neurological signs such as ataxia and paralysis could have been due to larvae in the spinal cord, and these larvae would have been included in counts from the musculature.

The species-specific responses to *B. procyonis* infection could be due to historical differences in exposure risk. Host species that have co-evolved with a particular parasite may evolve tolerance or resistance as the most-susceptible individuals are removed from the population (Roy and Kirchner, 2000). Earlier studies comparing infection outcomes in *P. leucopus* and *M. musculus* found that survival was significantly higher in *P. leucopus*, which is from the endemic parasite range (Sheppard and Kazacos, 1997). Here we hypothesized that mice from regions with endemic *B. procyonis* would be less susceptible to severe disease, and thus predicted that survival would be poorest in *P. polionotus* and perhaps *P. californicus*. Although we found no difference in the onset of neurologic signs among *P. polionotus*, *P. californicus*, and *P. maniculatus*, we did find that clinical disease took longer to develop in *P. leucopus*. This suggests that ancestral overlap with the parasite may contribute to the evolution of tolerance; however, results from *P. maniculatus* suggest that these patterns are driven by more than co-occurrence.

Habitat partitioning may influence selection for *B. procyonis* tolerance by altering exposure to raccoon latrines. In the Midwest United States, most latrine sites are found on fallen logs, stumps, and at the base of trees, which may be more consistent with *P. leucopus* microhabitat preferences than with *P. maniculatus* (McMillan and Kaufman, 1995; Page et al., 1998). *Peromyscus maniculatus* favors arboreal microhabitats with above-ground refuges whereas *P. leucopus* preferentially chooses brushy areas with low-lying refuges of fallen logs and stumps (Wolff and

Hurlbutt, 1982; Bucker and Shure, 1985; Parren and Capen, 1985). In an experimental setting, *P. maniculatus* also displaces *P. leucopus* from elevated nesting areas when co-housed in a simulated habitat (Stah, 1980). Thus, even though these species are sympatric, *P. leucopus* may undergo more selection pressure for tolerance toward *B. procyonis* infection as compared to *P. maniculatus* due to more frequent encounters with *B. procyonis*-contaminated latrines.

Several field studies have investigated the natural prevalence and intensity of *B. procyonis* infection in wild rodents. Although these studies were conducted in areas where both *P. maniculatus* and *P. leucopus* occur (Tiner, 1954; Page et al., 2001b, 2011; Kellner et al., 2012; Beasley et al., 2013), all focused on *P. leucopus*, and none included data from *P. maniculatus*. The 2 species can be challenging to distinguish morphologically, and so it is unknown if both species were trapped and not confirmed at species level or if *P. leucopus* was selectively targeted. At least 1 study states that *P. maniculatus* and *P. leucopus* were not distinguished, and thus both species could have been included in analysis (Kellner et al., 2012). Field studies that distinguish *P. maniculatus* from *P. leucopus* are needed in order to assess whether experimental survival differences correspond to differences in infection among wild populations.

Experimental infections are a critical tool for testing hypotheses about wildlife disease dynamics, but have several inherent limitations. Our use of captive-bred mice controls for acquired immunity due to past *B. procyonis* and other helminth exposure but could potentially introduce foundation bias from the original mice used to establish the captive colony. For example, the *P. polionotus* stock is noted to have a high inbreeding coefficient; however, this may be somewhat representative of natural populations of this species (Brewer et al., 1990; Wooten, 2011). Additionally, inoculating mice with a single dose may not mimic natural exposure patterns.

It is likely that *Peromyscus* are exposed to many smaller doses of eggs over time; however, the dynamics of acute versus chronic exposure, or infections acquired slowly over time, have not been investigated for *B. procyonis*.

The geographic expansion of *B. procyonis* into previously naïve areas may present a population-level risk for native rodent populations. Particularly, the recent occurrence of *B. procyonis* in the Southeastern United States Gulf regions may threaten native *P. polionotus* populations, especially since many ecologically-important *P. polionotus* subspecies are critically endangered (Blizzard et al., 2010b; Oli et al., 2001). The study of *B. procyonis* in different species of *Peromyscus* and other native rodent species will not only be important in further understanding the parasite's ecology but may also be important in future conservation efforts.

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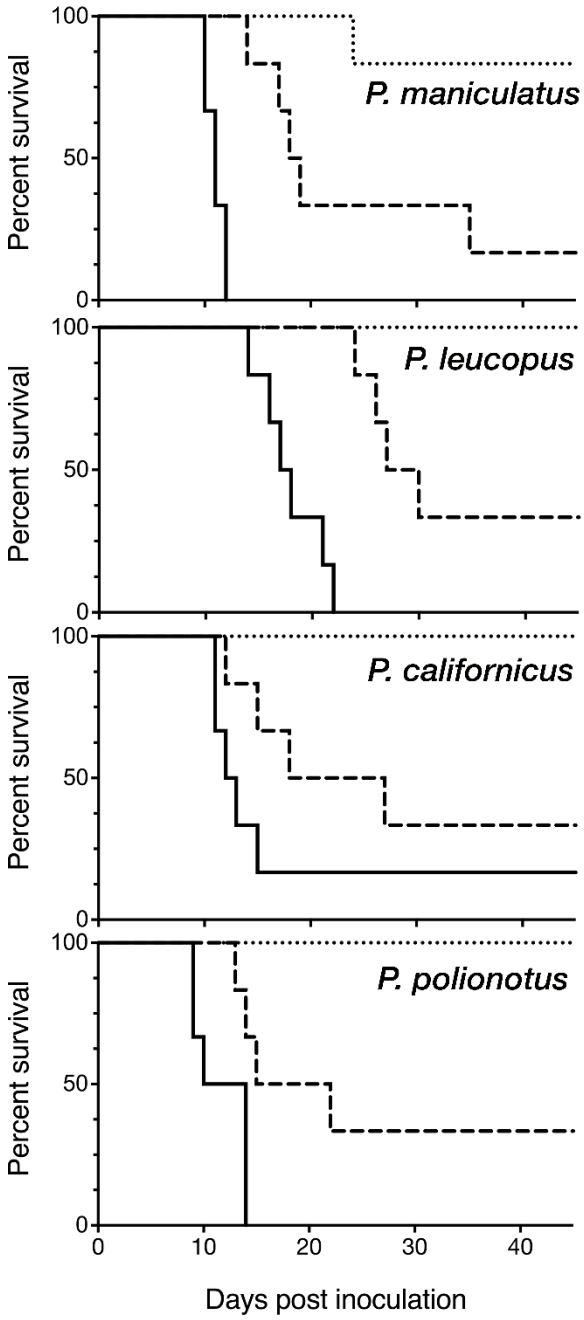

Figure 4.1. Survival plots for 4 species of *Peromyscus* spp. inoculated with *Baylisascaris procyonis*. Solid line – high dose (500 eggs); dashed line – medium dose (50 eggs); dotted line – low dose (10 eggs).

Table 4.1. Average numbers of *Baylisascaris procyonis* larvae recovered from brain tissue, skeletal muscle, and visceral organs of inoculated mice by dose group.

	Avg. no. larvae recovered (SD)				Avg. larval recovery*
	Brain	**Muscle**	**Viscera**	**Total**	
P. californicus					
10 eggs	0.0 (0)	0.7 (1.2)	0.0 (0)	0.7 (1.2)	6.7%
50 eggs	0.5 (0.5)	2.3 (1.0)	0.3 (0.5)	3.2 (1.0)	6.3%
500 eggs	2.0 (1.3)	37.4 (14.6)	3.5 (1.0)	43.2 (16.3)	8.6%
P. leucopus					
10 eggs	0.0 (0)	0.7 (1.2)	0.3 (0.5)	1.0 (1.5)	10.0%
50 eggs	0.8 (0.8)	3.2 (2.4)	1.5 (1.6)	5.5 (2.7)	11.0%
500 eggs	3.0 (0.6)	24.0 (7.9)	12.0 (7.0)	39.0 (13.4)	7.8%
P. maniculatus					
10 eggs	0.0 (0)	0.7 (1.2)	0.2 (0.4)	0.8 (1.2)	8.3%
50 eggs	1.0 (0.9)	3.3 (1.2)	0.8 (1.0)	5.2 (2.3)	10.3%
500 eggs	6.3 (2.8)	28.2 (9.9)	4.7 (2.9)	39.2 (9.2)	7.8%
P. polionotus					
10 eggs	0.0 (0)	0.3 (0.5)	0.0 (0)	0.3 (0.5)	3.3%
50 eggs	0.5 (0.5)	2.3 (2.6)	0.3 (0.5)	3.2 (3.1)	6.3%
500 eggs	2.3 (1.8)	30.7 (6.3)	7.3 (3.3)	40.3 (8.8)	8.1%

* Larval recovery percentage = total number of larvae recovered/dose x 100

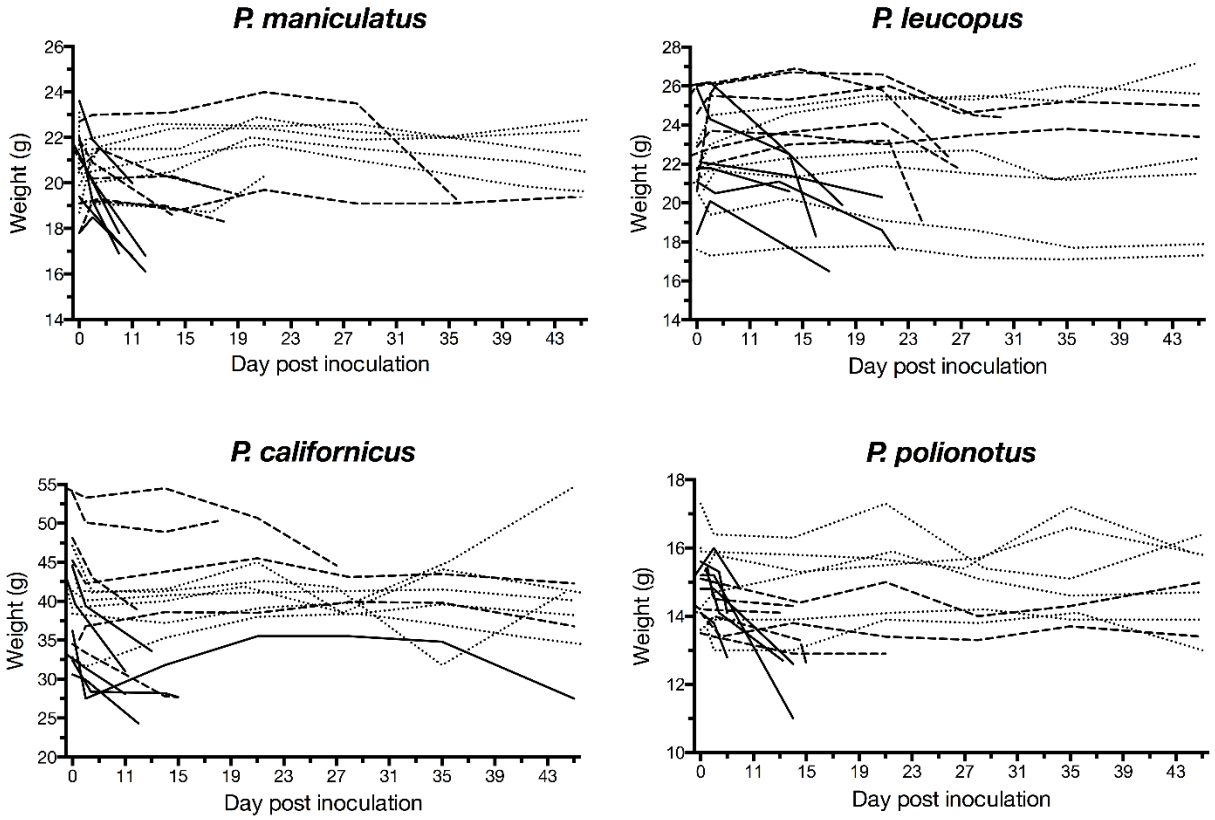

Figure 4.2. Body weights of individual *Peromyscus* spp. following inoculation with *Baylisascaris procyonis*. Weights were recorded at the beginning of the study, at weekly intervals, and prior to euthanasia. Solid line – high dose (500 eggs); dashed line – medium dose (50 eggs); dotted line – low dose (10 eggs).

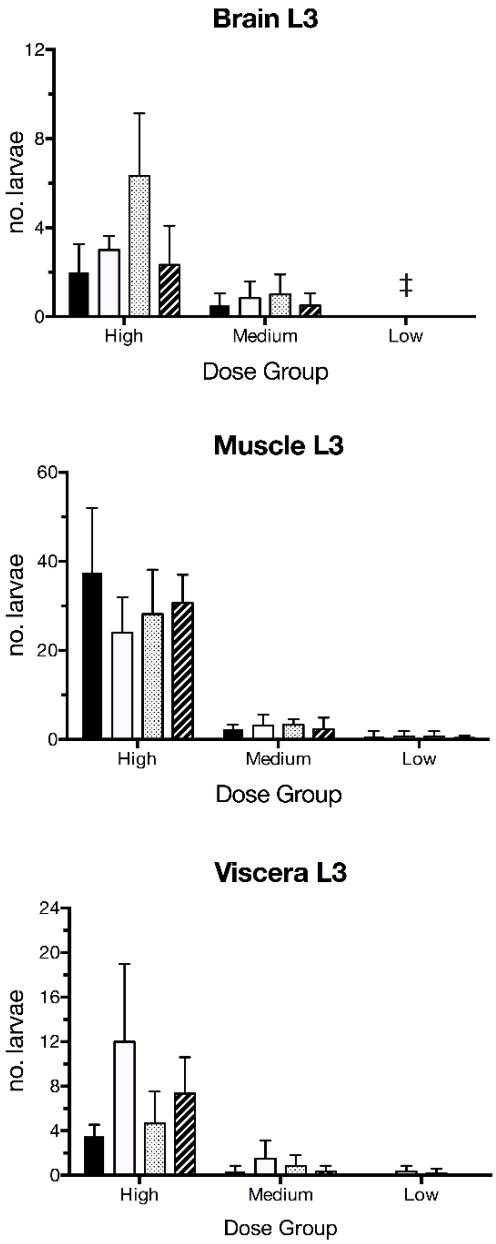

Figure 4.3. Mean number of *Baylisascaris procyonis* larvae recovered from tissues in experimentally infected *Peromyscus* spp. (black – *Peromyscus californicus*; white – *Peromyscus leucopus*; dotted – *Peromyscus maniculatus*; hatched – *Peromyscus polionotus*). Error bars indicate standard deviation. ‡ = no larvae recovered.

Table 4.2. Poisson regression models for larval counts.

Parameter	Estimate	SE	Z-value	p-value
Brain L3				
Intercept	-4.02408	0.61044	-6.592	<0.0001
Log(Dose)	0.80567	0.10508	7.652	<0.0001
Log(Dose):Species-Pm	0.14170	0.03425	4.138	<0.0001
Muscle L3				
Intercept	-3.00369	0.26682	-11.258	<0.0001
Log(Dose)	1.02764	0.04480	22.936	<0.0001
Log(Dose):Species-Pc	0.05884	0.01468	4.008	<0.0001
Log(Dose):Species-Pl	-0.02956	0.01547	-1.911	0.0561
Viscera L3				
Intercept	-4.13603	0.52668	-7.850	<0.0001
Log(Dose)	0.95191	0.08928	10.662	<0.0001
Log(Dose):Species-Pc	-0.08737	0.04187	-2.087	0.0369
Log(Dose):Species-Pl	0.11793	0.02618	4.504	<0.0001
Total L3				
Intercept	-2.53289	0.22173	-11.42	<0.0001
Log(Dose)	1.00702	0.03707	27.17	<0.0001

CHAPTER 5

DETECTION AND EVALUATION OF ANTIBODY RESPONSE TO A *BAYLISASCARIS*-SPECIFIC ANTIGEN IN RODENT HOSTS USING WESTERN BLOTTING AND ELISA[1]

[1] Sapp S.G.H., Handali S., Weinstein, S.B., and Yabsley, M.J. Submitted to *Journal of Parasitology*, Mar 2018.

ABSTRACT

Diagnosis of parasitic diseases that involve tissue stage larvae is challenging, and serology remains the most effective ante-mortem test for detecting these infections. *Baylisascaris procyonis,* the raccoon roundworm, is a zoonotic ascarid that can use >150 species of birds and mammals as paratenic hosts. Migratory larvae in paratenic hosts tissues can produce severe to fatal neurologic disease and ocular disease but not all infected hosts develop signs. In human clinical practice, a sensitive and specific Western blot (WB) based on a recombinant *Baylisascaris*-specific antigen (rBpRAG-1) is the primary diagnostic assay. In this study, we sought to use this antigen to detect *Baylisascaris* spp. infections in rodent paratenic hosts, which play an important role in the transmission and maintenance of this parasite in nature. Using four species of *Peromyscus* mice (*P. californicus, P. leucopus, P. maniculatus, P. polionotus*), we developed a species-adapted WB and ELISA. These assays revealed species-level differences in seroconversion and terminal antibody concentrations, with *P. leucopus* having significantly greater antibody concentrations than *P. californicus* and *P. polionotus* at all dose levels, and *P. maniculatus* at the low dose. Interestingly, some *P. californicus* and *P. polionotus* failed to seroconvert despite the recovery of larvae from their tissues. WB and ELISA results were correlated; however, the WB demonstrated higher sensitivity than the ELISA (72.2% versus 63.9% respectively). Using experimental samples, specificity was also good for both platforms (WB: 100%; ELISA: 94.1%). A WB was also used to test *Mus* and *Rattus* samples, and although numbers were too limited to evaluate sensitivity and specificity, all animals known to be infected by tissue digestion were WB positive and all uninfected animals were negative. Finally, the *Peromyscus*-adapted WB and ELISA were used to test a set of serum samples from wild-trapped *P. maniculatus* and *Rattus rattus.* Both assays were generally sensitive,

but specificity was equivocal. This emphasizes the challenge of using serology for investigation of wildlife diseases, in which hosts have unknown exposure histories. Nevertheless, serologic methods have utility in the study of *Baylisascaris* spp. in paratenic hosts, either wild or captive, and have advantageous attributes (non-lethal, high-throughput) as long as results are interpreted carefully.

INTRODUCTION

Baylisascaris procyonis, the raccoon (*Procyon lotor*) roundworm, is increasingly recognized as a potential cause of neurologic disease in a broad variety of paratenic host species, including humans (Kazacos 2016). Eggs from the infected definitive hosts (raccoons, occasionally dogs and possibly other procyonids) are shed in the feces, become infectious after ~10-14 days in the environment, and are able to infect >150 species of mammals and birds. Following ingestion of infectious eggs, larvae hatch and penetrate the wall of the small intestine of the paratenic host and undergo somatic migration. These migrating larvae can cause severe or fatal neural larva migrans if they invade the central nervous system or ocular larva migrans if they invade the eye. Baylisascariasis has been implicated in paratenic host species declines and local extinctions, for example in the Allegheny Woodrat (*Neotoma magister*) (Page 2013).

This pathology associated with cerebral bayliascariasis is thought to be an adaptation to increase transmission, as incapacitated animals likely become easier prey for raccoons (Kazacos, 2016; Page et al., 2001). Infection prevalence can exceed 50% in some rodent populations and raccoons readily scavenge rodent carcasses (Beasley et al. 2013, Weinstein 2017), suggesting that these small mammals contribute to the transmission and maintenance of *B. procyonis*. Among

rodents, deer mice (*Peromyscus* spp.) are likely common hosts for *Baylisascaris* spp. due to their caching-foraging feeding strategy, which involves scavenging plant material from raccoon feces and storing for later consumption (Logiudice, 2001; Page et al., 2001). *B. procyonis* likely infects wild *Peromyscus* wherever they overlap with raccoons, however, infection status in these and all other paratenic hosts can only be ascertained with lethal sampling techniques due to the fact that larvae are within tissues.

The current "gold standard" method for diagnosing *B. procyonis* in wildlife or exotic species involves digesting tissue to recover migrating larvae, visualization of larvae in tissue squashes or molecular detection. Larvae may also be identified morphologically in cross-section of histological samples, however, the probability of observing a migrating larva in a small section of tissue is low, especially in light infections (Kazacos, 2016). Currently, ante-mortem diagnosis of *Baylisascaris* spp. infections in free-ranging paratenic hosts, like *Peromyscus* spp., is not possible, however, such assays have been recently developed for diagnosing human infections. A Western blot based on a recombinant excretory-secretory (ES) antigen specific to *Baylisascaris* (rBpRAG-1) has been developed and validated, and it is currently used in clinical diagnosis of suspected human cases (Rascoe et al., 2013). This assay is both sensitive (88%) and specific (96%), and has been used in epidemiologic studies on subclinically infected human populations (Rascoe et al., 2013; Sapp et al., 2016a; Sircar et al., 2016; Weinstein et al., 2017a). Similar sera based diagnostics would greatly expand our ability to test wildlife, and might be the key to understanding the strong species specific differences in *B. procyonis* induced pathology.

Even among similarly sized rodent species, *B. procyonis* exposure often results in significantly different parasite loads, pathology, and survival. For example, a previous infection

trial on wild-caught mice found that *P. leucopus* are more resistant to infection than *M. musculus* based on lower larval recovery and a longer survival time (Sheppard and Kazacos, 1997). Recently, we conducted a *B. procyonis* experimental infection trial on four species of captive-bred *Peromyscus* (*P. leucopus, P. maniculatus, P. californicus, P. polionotus*) (Sapp et al., 2016b) and we noted differences in survival and tolerance towards infection. A significantly longer time until onset of neurologic disease onset was noted for *P. leucopus* compared with the other three species differ despite no differences in the numbers of larvae recovered (Sapp et al., 2016b). Detection and quantitation of the anti-*B. procyonis* humoral response can provide insight as to if these differences in tolerance are attributable to species level differences in immunity, given that humoral immunity is increasingly recognized as an important component of host defense against helminths (Harris and Gause, 2011).

Utilization of serology for detection of *Baylisascaris* spp. infections in free-ranging wildlife would minimize the need for time-consuming and labor-intensive tissue digestions and sera could be obtained non-lethally, allowing for long term monitoring or studies on sensitive populations that cannot be lethally sampled. A high performance, species-adapted Western blot or ELISA would therefore aid in studies on the exposure of rodents to *Baylisascaris* spp. and improve our understanding on the ecological implications of *B. procyonis* in wild rodent populations. In this study, we adapted the rBpRAG-1 Western blot for use on *Peromyscus, Mus and Rattus,* and developed and optimized an indirect ELISA for the quantitation of anti-rBpRAG-1 IgG in experimentally infected Peromyscus. We then test these assays onwild *P. maniculatus* with and without *B. procyonis* infection.

MATERIALS AND METHODS

Experimental infections and sample acquisition

Experimental infections of *Peromyscus* spp. were conducted in a previous study (Sapp et al. 2016b). Whole blood was collected via cardiac puncture from mice immediately following CO_2 euthanasia. Blood samples were centrifuged at 1500x g for 10 minutes and serum was collected and stored at -20 C until processing. All procedures involving the experimentally infected rodents were reviewed and approved by the University of Georgia's IACUC committee (A2016 10-009).

Wild rodents (*Peromyscus maniculatus, Rattus rattus*) from California were trapped and processed for *B. procyonis* as described in Weinstein (2017b). Blood was collected via cardiac puncture and processed as described above. All field captures were reviewed and approved by the University of California, Santa Barbara IACUC protocol (850.1) and California DFG permit #11188.

Antibody detection

Western blotting

Sera from rodents were tested for anti-*Baylisascaris* antibodies via Western blotting using a recombinant antigen currently used in the diagnostic assay for human baylisascariasis (rBpRAG-1) (Rascoe et al., 2013). The Western blotting procedure was conducted as previously described, with the following modifications: commercially produced, GST-tagged, *Escherichia coli*-expressed antigen (GenScript, Piscataway, New Jersey) was used at a concentration of 0.25 μg/mL after titration to optimize signal, and a genus-specific secondary antibody (goat-anti-mouse IgG-HRP, goat-anti-rat IgG-HRP, or goat-anti-*Peromyscus* IgG-HRP; Kirkegaard & Perry

Laboratories, Inc.) diluted 1:5,000 was used following serum incubation. Pooled sera from eight laboratory C57BL/6J *Mus musculus* orally inoculated with 50 larvated *B. procyonis* eggs and euthanized 20 days later were used as a positive control for wild-caught *Mus musculus.* Sera from *Peromyscus* spp. orally inoculated with 50 or 500 *B. procyonis* eggs in the prior study were pooled and used as a positive control. Positive control from *Rattus* spp. consisted of pooled sera from two wild-caught individuals with high *B. procyonis* larval burdens. Pooled sera from uninfected, captive *Peromyscus*, *Mus* or *Rattus* were used as a negative control for each species. The presence of a single band at 63 kDa was considered a positive.

Peromyscus-adapted ELISA

An ELISA was developed and optimized for the quantitation of anti-*Baylisascris* humoral responses in infected *Peromyscus*. Briefly, optimal antigen (rBpRAG-1) concentration, serum dilutions, and secondary antibody (goat-anti-*Peromyscus* IgG-HRP) concentrations were determined via standard checkerboard titration protocols on Immulon 2HB 96-well plates (Thermo Scientific, Rochester, New York) and selected based on optimal signal-to-noise ratio using the same controls as the Western blot. Substrate (3, 3', 5, 5'-Tetramethylbenzidine (TMB); Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) reaction time was determined using a kinetic ELISA to maximize signal-to-noise ratio. Intra- and interplate coefficients of variation were determined via twenty independent runs (inter-), and 50 replicates (intra-). Antibody concentrations were expressed in arbitrary units (AU) based on a standard curve of pooled sera testing strongly positive via Western blot serially diluted in uninfected *Peromyscus* sera to create standard curve points.

The optimized ELISA protocol was carried out as follows: microwell plates were sensitized with 100 μL sensitization buffer (0.05M Tris-HCl pH 8.0, 1M KCl, 2 mM Ethylenediaminetetraacetic acid (EDTA))/well containing 1.25 μg/mL rBpRAG-1 antigen overnight at 4 degrees C, after which plates were washed 4x with phosphate buffered saline (PBS)/Tween 0.3%. 100 μL of diluted sera (1:100 in PBS/0.3% Tween/5% nonfat dry milk) were applied to each well and incubated for 30 minutes at room temperature on a plate shaker set at half maximum speed. Plates were washed as described, and 100 μL of conjugate antibody diluted 1:1,000 in PBS/0.3% Tween was added to each well and incubated for 30 minutes at room temperature on a plate shaker. Plates were washed and 100 μL substrate was applied and allowed to develop for 3 minutes, shaking at room temperature. The reaction was stopped by the addition of 100 μL 1N H_2SO_4, and the plate was read immediately at $A_{450\,nm}$ with a VersaMax Kinetic ELISA Microplate Reader using SoftMax Pro v5.4 (Molecular Devices Corporation, California, USA). Samples with antibody concentrations above the standard curve range were diluted in pooled negative sera and re-tested, and results adjusted for dilution.

Statistical analysis

Cutoff values, sensitivity, and specificity and associated 95% confidence intervals for the ELISA were determined using the package *pROC* (Robin et al., 2011) for R statistical software (v. 3.1.4) (R Foundation for Statistical Computing, Vienna, Austria) with 2,000 stratified bootstrap replicates. For statistical analysis, antibody concentrations were log transformed (log (AU+1)), and species-level differences in serologic responses within dose levels were determined using pairwise t-tests with Bonferroni correction for multiple comparisons. The overall relationship

between antibody concentration, species, and total number of larvae recovered was assessed via multiple regression. Agreement between Western blot and ELISA results was calculated using Cohen's Kappa (κ). All statistical analysis was carried out in R statistical software (R Core Team, 2014).

Experimental rodents

Western Blotting

Serum samples from experimentally infected *Mus* and *Peromyscus* produced a positive band of the expected size using the BpRAG-1 Western blot assay. Using an antigen concentration of 0.25 μg/mL, the optimal secondary antibody dilutions were 1:2,000 for anti-*Mus* and 1:3,000 for anti-*Peromyscus.* Sera from these genera were diluted 1:50 for Western blotting. Under these conditions, all uninfected rodents were negative. All inoculated *Mus* produced positive Western blot reactions; results varied by species and dose in inoculated *Peromyscus* (Table 5.1). At the highest and medium egg doses (500 and 50 eggs, respectively), all *P. leucopus* and *P. maniculatus* samples produced positive reactions on Western blot (Table 5.1). However, not every *P. californicus* or *P. polionotus* individual had a detectable signal at any dose (Table 5.1; Figure 5.1). At the lowest dose (10 eggs), none of the *P. californicus* and only a single *P. polionotus* sample showed evidence of seroconversion. Generally, the Western blot signal was stronger (i.e. darker bands) for *P. leucopus* and *P. maniculatus.* Seroconversion was evident as early as nine days post inoculation (dpi). All of the sentinel negative controls were consistently negative on the Western blot. Based on samples collected from experimentally inoculated *Peromyscus* spp. (inoculated vs. not inoculated regardless of larvae recovery), the sensitivity and specificity for the optimized

Peromyscus-adapted Western blot were 72.2% (95% CI: 60.4-82.1%) and 100% (95% CI: 63.1-100%), respectively. Based on the recovery of larvae, sensitivity was 83.9% (95% CI: 71.7-92.3%) and specificity was 82.6% (95% CI: 61.2-95.1%) (Table 5.2).

ELISA

Serum samples from all experimentally-infected mice were tested for anti BpRAG-1-IgG using the optimized ELISA protocol described above. For negative controls, eight uninfected mice (~2x per species) from the experimental trials were used, as well as sera from eight uninoculated *P. maniculatus* purchased from the supplier (*Peromyscus* Genetic Stock Center, Columbia, South Carolina). The sensitivity and specificity of this assay were 63.9 (52.8-73.6%) and 94.1% (82.3-100%) respectively, and the area under the curve was 0.815 (Figure 5.2). The optimal minimum threshold value for a positive result was 8.27 AU. The inter-plate coefficient of variation (CV) was 6.82 and the intra-plate variability was 7.18. Overall, the agreement between the Western blot and ELISA was very good (Cohen's $\kappa = 0.843$) (Table 5.3).

Significant associations between antibody concentration and species were detected when stratified by exposure dose (Table 1). At the high and medium dose, *P. leucopus* had significantly greater antibody concentrations than *P. californicus* (high dose: p=0.0057; medium dose: p=0.0285) and *P. polionotus* (high dose: p=0.0110; medium dose: p=0.0045). However, antibody concentrations between *P. leucopus* and *P. maniculatus* did not differ significantly at these dose levels. At the low dose, *P. leucopus* had significantly greater antibody concentrations compared with the other three species (*P. californicus*: p<0.0001; *P. maniculatus*: p=0.0187; *P. polionotus*: p=0.0002) (Figure 5.3). There was a linear positive correlation between the total number of larvae

recovered and antibody response (r=0.679) (Figure 5.4). In the linear model including species and total number of larvae as predictors of antibody concentration, both factors were highly significant ($p<0.0001$) and together explained 73.2% (r^2) of variation in antibody concentration observed.

Field Samples

Serum samples from wild *P. maniculatus* (n=28) from California were tested using the Western blot and ELISA. Based on previous necropsies and tissue examination, 8 of these mice were positive for *Baylisascaris* sp. larvae and burdens ranged from 1-17 larvae. All but one of these samples were positive on both Western blot and ELISA (Table 5.4). The single discordant sample, which had 4 *B. procyonis* larvae, tested positive on Western blot but negative on ELISA, but was very close to the cutoff value (7.89 AU) (Table 5.5). The number of larvae recovered and antibody concentration were positively and linearly correlated (r=0.886) and total larvae recovered explained 71.6% (adjusted r^2) of variation in antibody concentration. Data from wild *P. maniculatus* where no larvae were recovered were equivocal. Of 20 larvae-negative mice, 9 and 11 produced a positive reaction on Western blot and ELISA, respectively; 6 of these larvae-negative samples were positive on both serologic platforms. Samples fromhe three wild *R.* rattus (infected with intensities of 287-793 larvae) also tested strongly positive on the Western blot using sera diluted 1:100 and secondary antibody diluted to 1:5,000. Pooled sera from uninfected laboratory rats used as a negative control tested negative under these conditions. An ELISA was not developed for *Mus* or *Rattus* due thelow numbers of samples for full development and validation.

DISCUSSION

Serologic assays are a promising new tool for the diagnosis of *Baylisascaris* larva migrans in rodents. In experimentally-infected *Peromyscus* spp., our rBpRAG-1 Western blot generally had high specificity and sensitivity. However, for animals inoculated with the lowest dose of eggs (10 eggs), the sensitivity and specificity varied based on how "positive" was defined (i.e., inoculation status vs. detection of larvae) as well as exposure dose with higher exposures and worm burdens having increased sensitivity. The most discordant results were observed in the low dose group (10 eggs), which in some cases some mice had positive WB but no larvae recovered, while otherswere infected but WB negative. This suggests both that there is a minimum level of exposure needed to develop infection and detectable antibodies and that some mice might be able to clear infection prior to sampling. Thus, while these assays might be accurate for heavily infected individuals, sensitivity and specificity are reduced in animals exposed to fewer than 10 eggs. Animals exposed to low doses may not have been successfully infected, may have been cleared larvae prior to sampling, or the recovery method may simply not be sensitive enough to recover very low numbers of larvae present. For this reason, we chose to report sensitivity and specificity for both inoculation status and recovery of larvae status separately. We also observed differences in seroconversion by *Peromyscus* species. Several *P. californicus* and *P. polionotus* individuals failed to seroconvert despite some of them being inoculated with high numbers of eggs and had similar total numbers of larvae recovered from these hosts compared to the other *Peromyscus* spp. While the mechanism is not clear, these two species are native to areas in which *B. procyonis* is believed to be historically absent and therefore have a shorter evolutionary history with the parasite, and perhaps have undergone less selection for an effective response against

infection (Sapp et al., 2016b). This demonstrates that assay performance estimates can be variable, even among congeneric species and is an important consideration in interpreting sensitivity and specificity characteristics.

The ELISA had somewhat inferior performance characteristics compared to the Western blot but agreement was generally good, which is often expected in comparing these platforms (due to alterations in epitope conformation during antigen coating, lower detection threshold and ability to separate cross-reactive fractions on Western blot, etc.) (Cortes et al., 2006; Fillaux and Magnaval, 2013; Frey et al., 2009; Jitsukawa et al., 1989). Despite lower sensitivity and specificity, the ELISA has a utility as it provides a quantitative result compared with the qualitative Western blot and is a more rapid test for large samples sizes.

The most important finding using the quantitative ELISA output was the species-level differences among the experimentally infected *Peromyscus* spp. *P. leucopus* had highly significantly greater mean anti-BpRAG-1 IgG concentrations than *P. californicus* and *P. polionotus* at the high and medium dose, and against all other species at the low dose. It is plausible that the anti-BpRAG-1 IgG response serves to slow or prevent larva migration through somatic tissue, and therefore delay entry of *B. procyonis* into the CNS. In addition to a significantly longer time until neurologic disease onset, we found *P. leucopus* significantly higher numbers of larvae in visceral organs in our previous study (Sapp et al., 2016b). Survival time (i.e. length of infection) did not have a significant association with antibody concentration after adjustment for dose (data not shown), suggesting these observed differences are a result of the differential host responses among species and not due to longer survival time and exposure to antigens. Evidence for this exists in other ascarid species as well. For example, laboratory mice inoculated with a

recombinant *Ascaris suum* ES product mounted a strong IgG response, and following challenge had a 54% reduction in the number of larvae recovered from lungs (Tsuji et al., 2003). While that study did not attempt to recover larvae from other organs in the carcass, it seems likely that larvae become trapped in the livers of these immunized mice. This blocking of liver-lung migration could be possibly be analogous to *B. procyonis* larva migration from viscera to the CNS.

The rBpRAG-1 antibody concentrations, as measured by ELISA, were dose-dependent which is similar to data from studies on *Toxocara*. A very similar pattern was observed for ES IgG in laboratory mice inoculated with near-equivalent, graded doses of *Toxocara canis* eggs (500, 50, and 5 eggs) (Rodrigues e Fonseca et al., 2017). In that study, antibodies were still detectable by 170 days post infection. Another study on laboratory mice also revealed dose-dependent patterns was following small, graded doses of *T. canis* and *T. cati* eggs, with titers reaching a plateau after ~50 days (Havasiová-Reiterová et al., 1995). Although our experimental rodent study was not long-term, we had detectable antibodies out to 45 DPI.

This study has some important limitations to note. First, we used a single antigen target, BpRAG-1 which is a well-characterized *Baylisascaris*-specific ES antigen (Dangoudoubiyam et al., 2010). While ES antigens are frequently used in diagnosis of helminthic diseases due to their immunogenicity, the role of anti-ES antibodies in immunity to larvae survival or migration is complex and it is not known if antibodies to BpRAG-1 represent a protective response. However, the longer survival time (Sapp et al., 2016) and higher anti-BpRAG-1 IgG concentrations observed in *P. leucopus* versus other species suggests that a more robust immune response may confer tolerance towards infection, or at least serves as an indicator of effective control of the parasite (even if not directly larvicidal). Furthermore, immunization with ES antigens can confer some

degree of protection and/or resistance in laboratory mice experimentally infected with other ascarids, including *T. canis, T. vitulorum,* and *A. suum,* and *T. canis* monoclonal anti-ES IgG binds directly to the cuticular surface of larvae (Abo-Shehada et al., 1991; Bowman et al., 1987a; Nicholas et al., 1984; Tsuji et al., 2003). Secreted proteins also induce protective responses in mice infected with non-ascarid, tissue-dwelling helminths, such as *Trichinella spiralis* (Silberstein and Despommier 1984). However, generalization about ES antigens is difficult due to the high diversity of proteins secreted by helminths, which will all have variable impacts on the host. Evaluation of additional ES antigen targets, immunization or challenge studies, and analysis of other immune effectors are necessary for further insight to this question.

Another limitation is that we did not collect serial blood samples from the experimentally exposed *Peromyscus.* Thus, we were only able to assess terminal antibody concentrations from the serum collected at the time of euthanasia and cannot assess immune response kinetics. However, it is interesting to note that strongly positive IgG reactions were observed on WB and ELISA in *Peromyscus* euthanized as early as 9 days post inoculation, and the sample with the greatest antibody concentration (14,700 AU) was from a *P. leucopus* euthanized at 14 dpi. This contrasts with findings from *T. canis* studies in laboratory mice, in which a significant IgG response in infected animals was not evident until ≥2 weeks post inoculation (Bowman et al., 1987b; Fan et al., 2003; Pinelli et al., 2007). Antisera from *B. procyonis*- and *B. melis*-infected laboratory mice that were euthanized at 11 dpi due to neurologic disease also reacted strongly on immunoblots with a crude *B. procyonis* ES antigen fraction (Boyce et al., 1988). Perhaps *B. procyonis* antigens are more immunogenic and *Peromyscus* spp. may be able to mount a response more rapidly than laboratory mice. We were only able to extend our study to 45 days post inoculation,

so we also cannot assess antibody persistence or changes over time. In *T. canis* infected laboratory mice, anti-ES IgG peaked at 5-6 weeks post inoculation and remained at that level until the end of the 26-week study, so it is possible that the antibody concentrations observed in surviving *Peromyscus* that were euthanized at the end of our study indicate maximum values (Bowman et al., 1987b).

While assay performance was favorable among experimental rodents, based on our results on wild rodents, it seems using either the WB or ELISA on field samples may yield results that are hard to interpret. On our sample of wild-trapped *P. maniculatus,* all but one animal positive for *Baylisascaris* larvae had a positive result on WB or ELISA, and the WB+/ELISA- animal had an AU value very close to the cutoff. However, there were also serologic-positive wild rodents that did not have any larvae recovered which is difficult to interpret as it is impossible to distinguish between past infection that has cleared (true positive) or cross-reactivity with other helminth fauna of wild rodents (false positive). No wild *P. maniculatus* were larvae positive and negative on both serologic assays, but the possibility for this situation exists as demonstrated in our findings on low-level infections (especially in *P. polionotus* and *P. californicus*) and may further complicate interpretation. Even with these limitations, these assays still may provide a sensitive method of detecting infections in wild rodents versus larval digestion or tissue squashes. Too few *Mus* or *Rattus* samples were available for further validation of the respective species-adapted WB and development of an ELISA; however, the WB data indicated it should work on these rodent species as well.

Ultimately, serologic detection of infections in free-ranging wildlife has limitations and challenges. However, it does have advantages that can warrant use in some situations (e.g. non-

lethal, high-throughput, detection of exposure in a population vs. active infection or disease). However, application of novel assays in wildlife should be interpreted appropriately. Currently, serologic testing is the only ante-mortem method for diagnosing *Baylisascaris* larva migrans in paratenic hosts. However, further efforts to improve and refine serological assays as *Baylisascaris procyonis* now presents a serious disease risk to wildlife across the northern hemisphere.

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Table 5.1. Number of *Peromyscus* mice in each dose and species group testing positive on the rBpRAG-1-based Western blot and ELISA.

	No. Western blot positive/No. Infected	No. ELISA positive*/No. Infected	Mean antibody concentration (AU)	Mean antibody concentration (log AU+1) (SD)
High Dose (500 eggs)				
P. leucopus	6/6	6/6	4108	3.34 (0.54)
P. maniculatus	6/6	6/6	360.1	2.50 (0.27)
P. californicus	5/6	5/6	285.5	1.93(1.04)
P. polionotus	6/6	6/6	138.2	2.03 (0.36)
Medium Dose (50 eggs)				
P. leucopus	6/6	6/6	210.3	2.13 (0.51)
P. maniculatus	6/6	5/6	64.11	1.29 (0.80)
P. californicus	4/6	2/6	25.73	0.76 (0.89)
P. polionotus	5/6	3/6	13.04	0.41 (0.75)
Low Dose (10 eggs)				
P. leucopus	5/6	5/6	335.0	1.96 (0.94)
P. maniculatus	4/6	3/6	9.88	0.87 (0.37)
P. californicus	0/6	0/6	0.615	0.09 (0.14)
P. polionotus	1/6	1/6	2.83	0.22 (0.48)

AU = arbitrary units; * Using a cutoff value of >8.27 AU for positivity.

Table 5.2. Concordance of rBpRAG-1 based Western blot (WB) results for experimentally infected *Peromyscus* spp., versus infection status and larval recovery (L3) (n=81).

	WB +	WB -
Inoculated	52	20
Not Inoculated	0	8
L3 +	47	9
L3 -	4	19

Table 5.3. Concordance between rBpRAG-1 based Western blot (WB) and ELISA results for experimentally infected *Peromyscus* spp (n=82).

	WB +	WB -
ELISA +	48	0
ELISA -	6	28

Cohen's $\kappa = 0.843$

Table 5.4. Concordance of rBpRAG-1 based Western blot (WB) and ELISA results for wild-caught *Peromyscus maniculatus* versus larval recovery (L3) (n=28).

	L3 +	L3 -
WB +	8	9
WB -	0	11
ELISA +	6	11
ELISA -	2	9
	WB +	WB -
ELISA +	12	5
ELISA -	5	6

Table 5.5. Serologic test results of wild rodents from which *Baylisascaris* sp. larvae were recovered.

Species	Total L3 recovered	WB result	ELISA result (AU)
Peromyscus maniculatus	17	Positive	Positive (>2000)
Peromyscus maniculatus	6	Positive	Positive (1641)
Peromyscus maniculatus	5	Positive	Positive (282.8)
Peromyscus maniculatus	4	Positive	Suspect (7.89)
Peromyscus maniculatus	2	Positive	Positive (40.0)
Peromyscus maniculatus	2	Positive	Positive (106.2)
Peromyscus maniculatus	1	Positive	Positive (487.4)
Peromyscus maniculatus	1	Positive	Positive (23.8)
Rattus rattus	793	Positive	ND
Rattus rattus	123	Positive	ND
Rattus rattus	89	Positive	ND

WB= Western blot; AU = arbitrary units; ND = not done

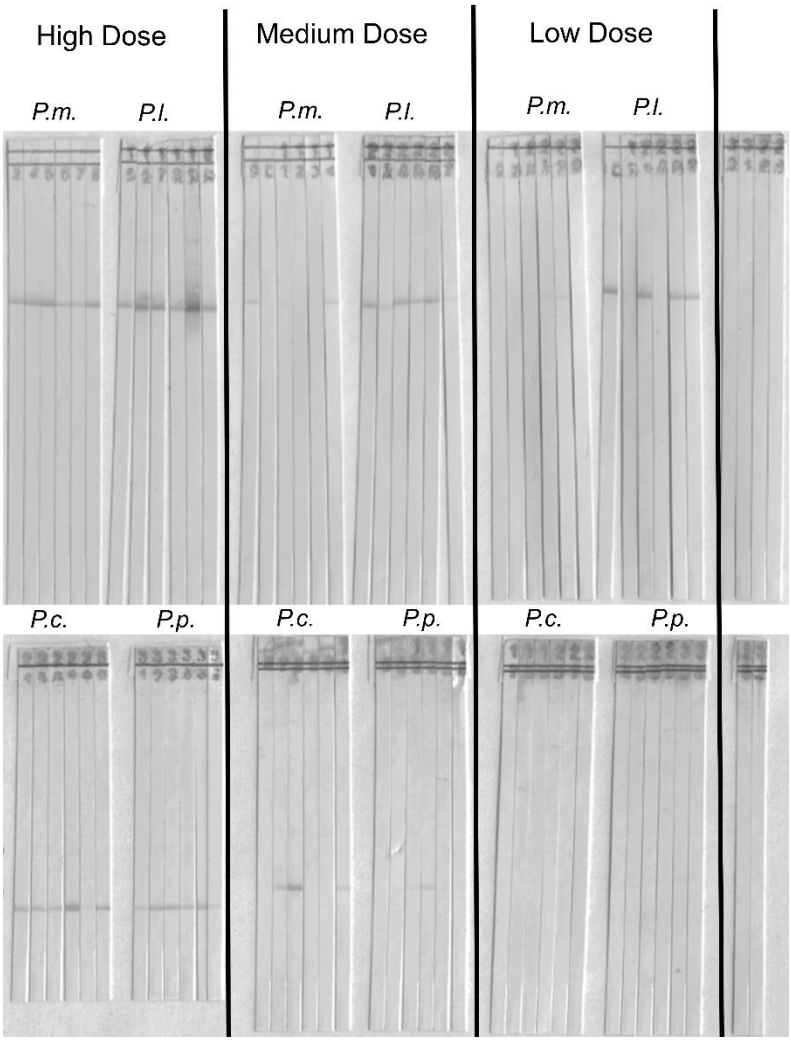

Figure 5.1. Western blot strips for experimentally infected *Peromyscus* spp. A band at 63 kDA (arrow) is considered a positive reaction. (*P.m. = P. maniculatus; P.l. = P. leucopus; P.c. = P. californicus; P.p. = P. polionotus*)

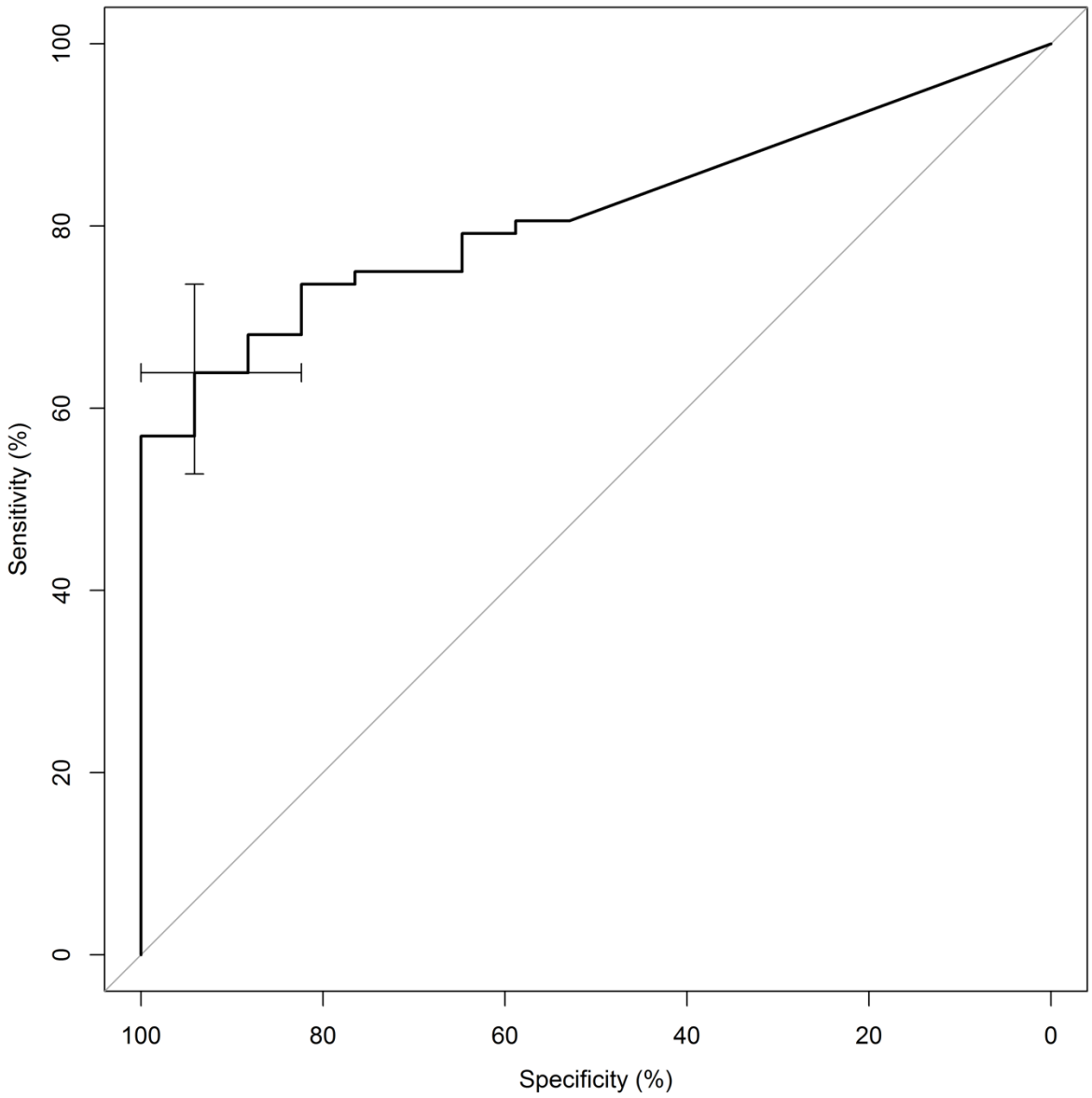

Figure 5.2. Receiver-operating characteristic curve for the *Peromyscus*-adapted rBpRAG-1 IgG ELISA. Area under the curve (AUC) = 0.815.

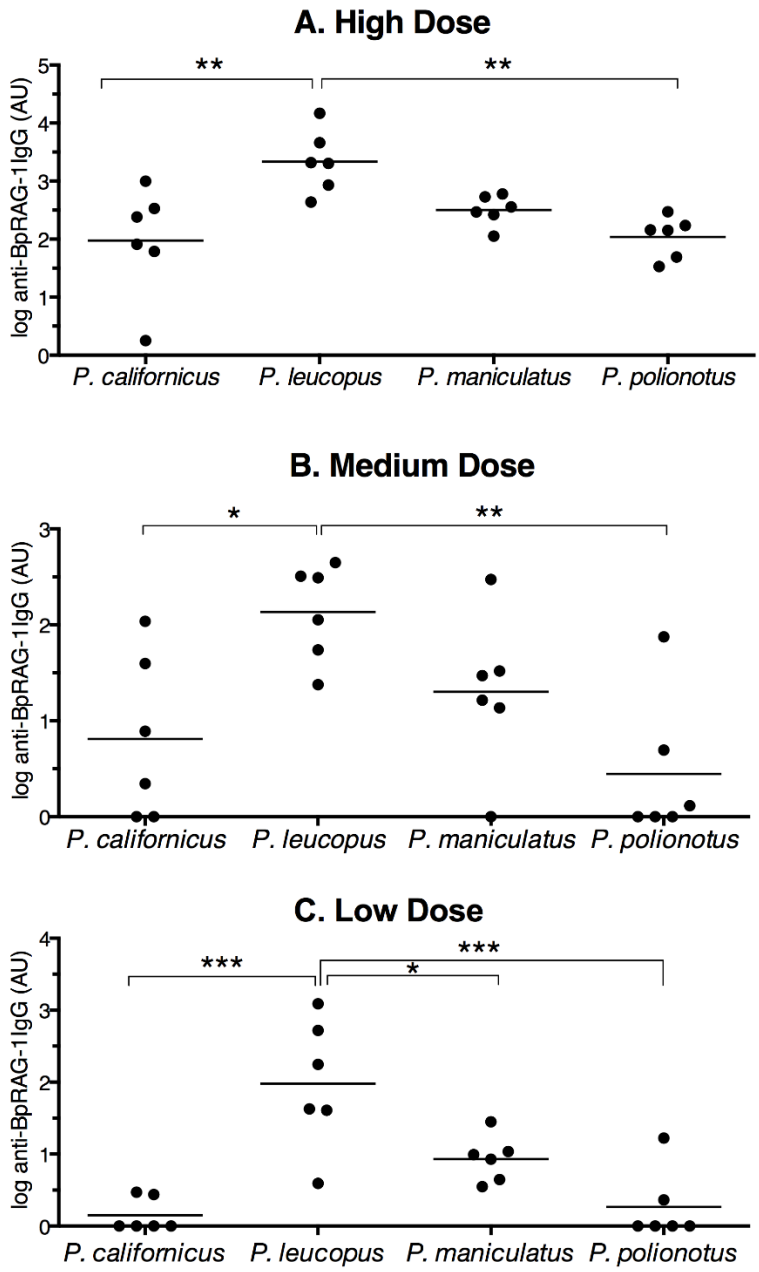

Figure 5.3. Anti-BpRAG-1 IgG concentrations (AU) among experimentally infected *Peromyscus* spp. given either 500 (high dose), 50 (medium dose), or 10 (low dose) larvated *Baylisascaris procyonis* eggs. * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (pairwise t-test with Bonferroni correction)

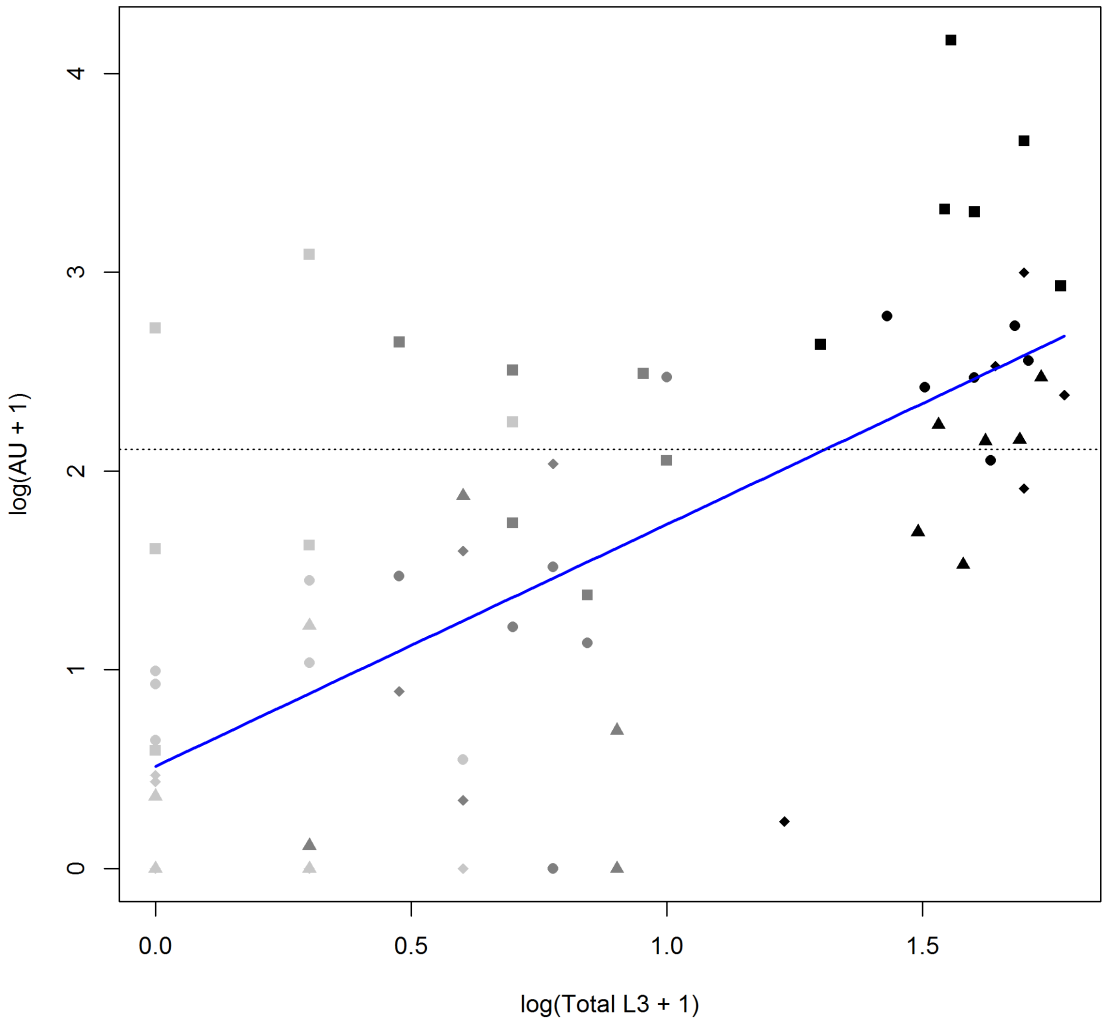

Figure 5.4. Linear model showing the relationship between the total numbers of *Baylisascaris procyonis* larvae recovered and anti BpRAG-1 IgG concentrations (in arbitrary units; AU) at time of euthanasia in experimentally infected *Peromyscus* spp. Cutoff value for positivity (8.27 AU, or 2.11 log(AU)) is indicated by the dotted line. Symbol represents species (diamond = *P. californicus*; square = *P. leucopus*; circle = *P. maniculatus*; triangle = *P. polionotus*) and shading represents dose group (black = 500 eggs; medium gray = 50 eggs; light gray = 10 eggs).

CHAPTER 6

PREVALENCE OF *BAYLISASCARIS* IN DOMESTIC DOG COPROLOGICAL EXAMINATIONS IN THE UNITED STATES, 2013-2016[1]

[1] Sapp S.G.H. & Yabsley M.J. 2017. *Veterinary Parasitology: Regional Studies and Reports.* 9 (Aug.): 65-69. Reprinted here with permission of publisher.

ABSTRACT

Dogs are alternative definitive hosts for *Baylisascaris procyonis*, the raccoon roundworm, but broad-scale prevalence and distribution of canine cases is not known. Based on a large dataset from nationwide reference laboratories, *Baylisascaris* spp. eggs were detected in 504/9,487,672 (0.005%) canine fecal samples. While many of the positive dog samples originated in areas of known high *B. procyonis* prevalence in raccoons, positives were also detected in 9 new states. Young dogs, large breeds, and certain regions had higher prevalence. Although overall prevalence was low, and some infections may be spurious, these results demonstrate that dogs may shed *Baylisascaris* spp. into domestic environments. Routine parasitic testing, rigorous preventive use, and restrictions on coprophagy should be encouraged to reduce risk of human or animal exposure to infectious eggs.

INTRODUCTION

Baylisascaris procyonis, an ascarid roundworm of raccoons (*Procyon lotor*), can cause fatal neural larva migrans (NLM) or ocular larval migrans (OLM) in numerous bird and mammal species, including humans (Kazacos, 2016). At least 54 human cases have been reported; however, additional cases may not have been recognized or reported especially OLM cases for which parasite identification is rare (Cortez et al., 2010; Cunningham et al., 1994; Kazacos 2016). The majority of fatal neurologic cases have occurred in children who likely ingested raccoon feces. The clinical presentation of NLM is severe and typically involves rapid degeneration to eosinophilic meningitis, paralysis, seizures, and coma. Furthermore, exposure to this parasite may be more common than previously anticipated and result in subclinical infections, as

antibodies have been found in healthy adults (Sapp et al. 2016). Treatment is difficult after onset of neurologic symptoms and often results in permanent neurologic sequelae (Kazacos, 2016).

Alternative definitive hosts of *B. procyonis* have been reported including non-raccoon procyonids (e.g., olingo, coati) and domestic dogs (Kazacos, 2016; Overstreet, 1970). Domestic dog infections are a concern because of their close association with people and indiscriminate defecation habits. However, while some case reports and smaller studies exist, broader-scale systematic surveillance for canine baylisascariasis is lacking. Patent *B. procyonis* infections have been reported from ~47 dogs from Iowa, Indiana, Michigan, and Missouri (USA), 14 dogs from Quebec (Canada), and a low prevalence (0.36%) was detected on Prince Edward Island (PEI) (Canada), (Conboy et al. 2010, Kazacos 2016). Experimental studies have confirmed that dogs are susceptible by exposure to eggs or larvae in non-definitive (paratenic) hosts (Bowman et al., 2005; Miyashita, 1993). Also, there have been several canine cases of NLM caused by *B. procyonis* so infection can result in disease, primarily in puppies (Kazacos 2016; Rudmann et al., 1996; Thomas, 1988). To better understand the ecology of this zoonotic parasite, we determined the prevalence of *Baylisascaris* spp. ova in fecal samples from domestic dogs from the United States.

THE STUDY

Results of fecal centrifugal flotation results for dog fecal samples (~1 gram) submitted to IDEXX Reference Laboratories from 2013-2016 were reviewed. For dogs that were positive for *Baylisascaris* spp. ova, the following information was extracted from the record: date of testing, zip code of customer, breed and age (in months) of dog, and other parasites diagnosed in that individual dog. Not all information was available for all positive dogs. In addition, a semi-

quantification of egg numbers was determined (rare=1-2 eggs, few (3-10), moderate (11-30) and many (>30) for *Baylisascaris* spp. and other ascarids (if present).

A total of 504/9,487,672 (0.005%) dog fecal samples were positive for *Baylisascaris* spp. ova. These positive fecal samples originated from dogs in 35 states and Washington D.C. Prevalence was significantly lower in the southern, central, and western regions compared to the northeastern and Midwestern regions (Table 6.1, Figure 6.1, Supplemental Table 6.1). Of positive dogs with a predominant breed indicated, 70% were large (>50 lbs) breeds with most being sporting group breeds (41%), followed by working (20%) and herding (15%) breeds (Table 6.2). Age of positive dogs ranged from 1 month to 15 years (mean 39 months; median 12 months); 35% were ≤6 months of age (Table 6.2). Interestingly, 48 positive dogs (10% of positive dogs) were ≤2 mo old (Table 6.2). Each semi-quantitative category was similarly represented (Table 6.2).

For the 498 dogs positive for *Baylisascaris* that had full fecal exam results available, numerous co-infecting parasites were noted including *Toxocara* spp. (61 dogs, 12%), another zoonotic ascarid, and other common dog parasites (e.g., *Giardia* spp., *Cystoisospora* spp., *Dipylidium*, *Strongyloides* spp., *Trichuris* spp., *Uncinaria* spp., *Ancylostoma* spp., capillarids, etc.). Importantly, spurious parasites of dogs were also detected, suggesting coprophagy: *Eimeria* spp. (75 dogs; 15%), common parasites of numerous hosts including raccoons and ruminants; *Moniezia* spp. (6 dogs; 1.2%), ruminant cestodes; and *Anoplocephala* spp. (1 dog; <0.01%), equine cestodes. It is also possible that some other parasites detected (e.g., hookworms, capillarids, etc) were spurious parasites; however, morphologically they were similar to canine-infecting species.

DISCUSSION

We detected *Baylisascaris* ova in the feces of domestic dogs across a wide geographic range within the US. The prevalence was low, but confirms that dogs are shedding *Baylisascaris* ova into the domestic environment, which may put people or other animals at risk of exposure. These results highlight the importance of testing, treatment, and preventive use. The Companion Animal Parasite Council (CAPC) recommends testing dogs for intestinal parasites at least four times during their first year and then at least two times per year afterwards (www.capcvet.org). Puppies should be given anthelmintics, many of which are efficacious for treating intestinal *Baylisascaris* infections, and treatment should be repeated until regular broad-spectrum parasite control begins, and adult dogs should receive year-round broad-spectrum parasite control (www.capcvet.org) (Bauer and Gey, 1995; Bowman et al., 2005). Many anthelmintics are proven efficacious against intestinal *B. procyonis* in raccoons, including fenbendazole, pyrantel pamoate, ivermectin, and moxidectin; milbemycin oxime has also been proven efficacious in naturally- and experimentally-infected dogs (Bowman et al. 2005; Kazacos 2016).

The data used in this study were obtained from veterinary reference laboratories so the reason of testing is unknown (routine exam or due to illness). These data are biased towards dogs taken to veterinarians for care and the prevalence of *Baylisascaris* spp. ova is thus expected to be higher in dogs that do not get veterinary care regularly. A more accurate prevalence would be obtained by testing unowned/feral dogs, shelter dogs, or dogs owned by individuals who do not seek routine or any veterinary care. For example, the prevalence of *Toxocara canis* in shelter dogs in the US was significantly higher than prevalence in owned dogs tested at Banfield veterinary hospitals or by IDEXX reference laboratories (Blagburn et al., 1996; Lucio-Forster et al., 2016;

Mohamed et al., 2009). Our prevalence was much lower than the only previous systematic survey of dogs for *B. procyonis* where 2 of 555 (0.36%) dogs were positive, but it is unknown if the difference was related to sampling strategy or if transmission is very common on Prince Edward Island, Canada (Conboy et al., 2010). Additionally, *Baylisascaris* spp. and *Toxocara* spp. eggs are morphologically similar, so it is possible that some infections were misidentified. Although it is impossible to determine how often this occurred, we believe that it is more likely for individuals to miss *Baylisascaris* infections due to perceptions that it is rare or absent in dogs. However, personnel at IDEXX Laboratories, Inc. reference laboratories have comprehensive technician training, thus test results may be more accurate compared to fecal exams conducted in veterinary offices where technician training may not be as standardized.

Positive dogs were detected in 35 states and Washington D.C. and prevalence was greater in the Northeast and Midwest compared to the South, Central, or Western regions. This corresponds with regions where the prevalence of *B. procyonis* is highest in raccoons (Sapp et al., 2016). Among these 35 states, 9 represent new state records for *Baylisascaris* (Alaska, Connecticut, Delaware, Maine, Montana, North Dakota, New Hampshire, Rhode Island, South Carolina); however, it is possible that some of these cases were acquired during travel to endemic states. Also, most of these positive dogs are presumed to be passing *B. procyonis* ova, but some dogs could be passing other species of *Baylisascaris* such as *B. columnaris*, a common parasite of skunks, or *B. transfuga*, a parasite of bears (especially the single positive dog in Alaska as raccoons are absent in Fairbanks).

A limitation of testing a single fecal sample is that actual infection status is unknown (i.e. true infection with adult parasites versus spurious "infections" of eggs passing through the digestive tract after coprophagy). A low percentage (15%) of the *Baylisascaris*-positive dogs also were passing *Eimeria* spp. ooycsts and/or *Moniezia* spp. ova and because these are not parasites of dogs, these positives could have been due to coprophagy (Nijsse et al., 2014). It is also possible that other genera of parasites detected were also acquired due to coprophagy. However, even if these spurious parasites are detected, this does not rule out patent infection with *Baylisascaris* spp. Testing a fecal sample acquired after a dog has been prevented from coprophagy activity can confirm if the dog is infected with *Baylisascaris*; however, our study design precluded subsequent sampling. Also, the development status of the eggs may also assist if coprophagy is suspected; if larvated eggs are detected on fecal exam of fresh feces, this would indicate coprophagy as *Baylisascaris* spp. eggs require at least 10-14 days in the environment to develop. A client could be asked if a dog had an opportunity to engage in coprophagy in the past 2-3 days; however, even owners who indicate there was no opportunity had dogs passing spurious parasites (Nijsse et al., 2014). Regardless, our data indicate that *Baylisascaris* spp. ova are being passed in the feces of these owned dogs which means they represent a public health risk even if they are obtaining the parasites by ingesting raccoon feces. In addition, ingesting raccoon feces is a transmission route for a dog to obtain an intestinal infection. This may be of importance to puppies, because experimental infections with various *Baylisascaris* spp. species suggest that young definitive hosts are much more susceptible to infections by the egg ingestion route (versus older animals, which are more likely to become infected by ingesting larvae in paratenic host tissues) (Kazacos 2016).

A proportion of *Baylisascaris*-positive dogs were very young, some as early as 8 weeks of age. In general, *Toxocara canis* infections in dogs are more common in puppies, because most are infected through vertical transmission (Mohamed et al., 2009; Schnieder et al., 2011). Although there are no data to confirm vertical transmission of *Baylisascaris* spp. in dogs, previous detections in young puppies combined with our findings suggest it can occur, similar to *T. canis* (Kazacos 2016). Also, infection of newborn lambs in Idaho suggest that vertical transmission can occur among paratenic hosts (Anderson, 1999). Typical early anthelminthic use in puppies will likely decrease the risk that puppies will develop patent infections. Expanding surveillance for canine *Baylisascaris* spp. infections is necessary to address knowledge gaps in its epidemiology and further assess risk for public health and veterinary health.

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Table 6.1. Prevalence of *Baylisascaris* spp. ova in dogs from regions of the United States, 2013-2016.

Region[#]	No. negative	No. positive	Prevalence (%)	OR	95% CI	p value[†]
Northeast	3,444,053	244	0.00708	Ref.	-	-
South	1,124,464	36	0.00320	0.452	(0.318-0.641)	<0.0001*
Midwest	2,239,605	176	0.00786	1.109	(0.913-1.346)	0.2950
Central	1,018,609	4	0.00039	0.055	(0.021-0.149)	<0.0001*
West	1,596,950	43	0.00269	0.380	(0.275-0.525)	<0.0001*
Total	9,423,681	503**	0.00531			

Regional categories are as follows: Northeast: Maine, New Hampshire, Massachusetts, Vermont, Connecticut, Rhode Island, New York, New Jersey, Pennsylvania, Maryland, Delaware, West Virginia, Virginia, Washington DC; South: Florida, Georgia, South Carolina, North Carolina, Tennessee, Alabama, Mississippi, Louisiana, Arkansas; Midwest: Ohio, Kentucky, Indiana, Michigan, Illinois, Wisconsin, Minnesota, Iowa, Missouri; Central: Texas, Oklahoma, Kansas, Nebraska, South Dakota, North Dakota, Montana, Wyoming, Colorado, New Mexico, Arizona, Utah; West: Washington, Oregon, Idaho, California, Nevada. Note: Alaska and Hawaii were excluded from regional analysis

* significant p-value at alpha=0.05

[†] Fisher's Exact Test.

Table 6.2. Attributes associated with positive *Baylisascaris* spp. samples.

Attribute	No. positive (% of attribute total)
Age	
≤2 months	48 (10.5)
>2 months	411 (89.5)
≤6 months	162 (34.7)
>6 months	305 (65.3)
Predominant Breed Class*	
Herding	33 (15.0)
Hound	18 (8.2)
Non-Sporting	9 (4.1)
Sporting	90 (40.9)
Terrier	10 (4.5)
Toy	17 (7.7)
Working	43 (19.5)
Breed Size**	
Small (<25 lbs)	29 (14.8)
Medium (25-50 lbs)	31 (15.8)
Large (>50 lbs)	136 (69.4)
Semi-quantitative egg burden	
Rare (1-2 eggs)	92 (18.1)
Few (3-10 eggs)	155 (30.5)
Moderate (11-30 eggs)	109 (21.5)
Many (>30 eggs)	152 (29.9)

* If known; classifications based on American Kennel Club standards.
** Approximate size if breed indicated.

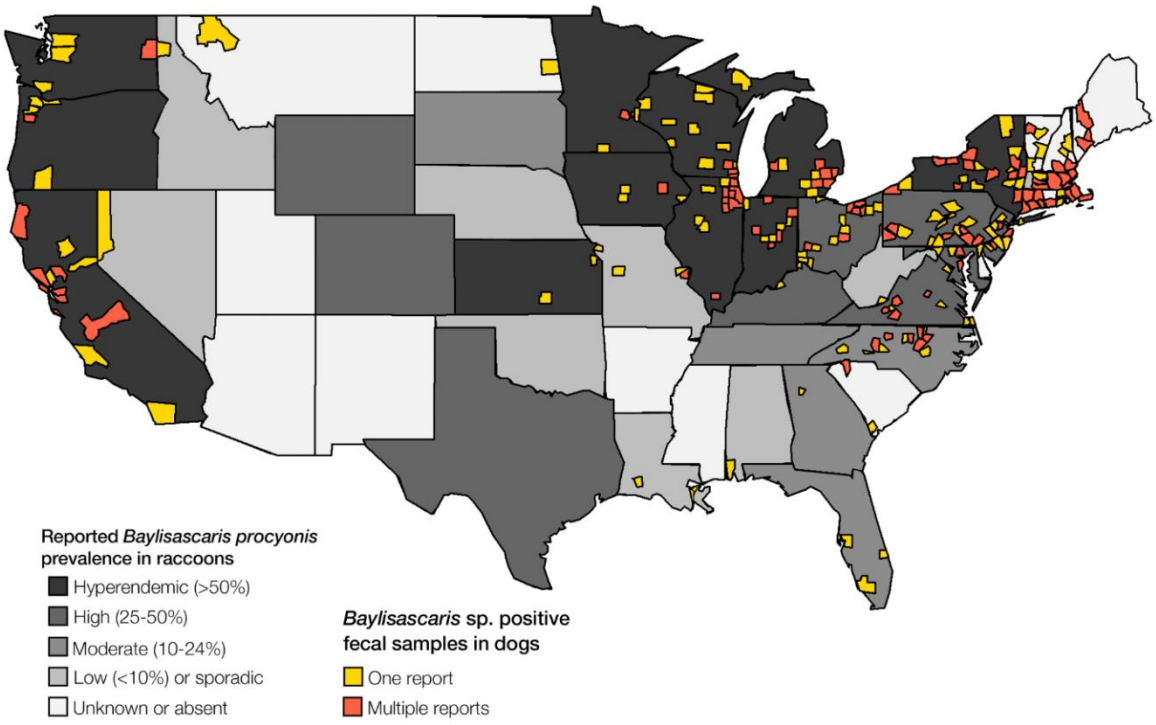

Figure 6.1. County-level locations of dogs with *Baylisascaris* spp. ova-positive fecal examinations. Shading of states indicates the prevalence of *B. procyonis* in raccoons based on published reports. Prevalence estimates derived from Kazacos 2016.

Supplemental Table 6.1. Prevalence of *Baylisascaris* spp. ova in feces of domestic dogs in the United States, 2013-2016

State	Year									
	2013		**2014**		**2015**		**2016**		**Total**	
	No. pos/No. tested	% pos	No. pos/No. tested	% pos	No. pos/No. tested	% pos	No. pos/No. tested	% pos	No. pos/No. tested	% pos
AK	0/1069	0	0/645	0	0/1062	0	1/1365	0.07326	1/4141	**0.02415**
AL	0/424	0	0/1179	0	1/1767	0.05659	0/2804	0	1/6174	**0.01620**
AR	0/1443	0	0/2089	0	0/1099	0	0/4349	0	0/8980	**0**
AZ	0/48172	0	0/51009	0	0/58076	0	0/60243	0	0/217500	**0**
CA	4/243744	0.00034	16/284082	0.00563	4/318477	0.00125	7/335691	0.00209	31/1181994	**0.00262**
CO	0/20207	0	0/23322	0	0/28593	0	0/35490	0	0/107612	**0**
CT	5/66355	0.0015	9/72539	0.01241	8/89088	0.00898	7102124	0.00685	29/330106	**0.00879**
DC	0/3023	0	0/5165	0	0/10324	0	0/13368	0	0/31880	**0**
DE	0/4910	0	0/5081	0	1/5227	0.019131	0/6404	0	1/21622	**0.00462**
FL	2/96229	0.00047	0/103940	0	1/110482	0.000905	1/110948	0.00090	4/421599	**0.00095**
GA	0/43470	0	0/49264	0	1/57614	0.001736	0/69262	0	1/219610	**0.00046**
HI	0/14220	0	0/14530	0	0/15723	0	0/14974	0	0/59447	**0**
IA	2/7601	0.0039	1/10804	0.00926	2/15091	0.013253	1/17408	0.00574	6/50904	**0.01179**
ID	0/3475	0	0/3640	0	0/5208	0	0/6437	0	0/18760	**0**

IL	16/124291	0.0028	14/134442	0.01041341	16/146704	0.010906	13/162037	0.00802	59/567474	**0.01040**
IN	2/28055	0.00137	2/32525	0.00615	8/42472	0.018836	3/43480	0.00690	15/146532	**0.01024**
KS	0/9062	0	0/13799	0	2/19779	0.010112	0/23473	0	2/66113	**0.00303**
KY	0/4272	0	0/8181	0	0/12412	0	2/14848	0.01347	2/39713	**0.00504**
LA	0/6074	0	0/8423	0	1/12742	0.007848	1/14639	0.00683	2/41878	**0.00478**
MA	7/123101	0.00569	17/145796	0.01167	25/167828	0.014896	15/180788	0.00830	64/617513	**0.01036**
MD	1/52439	0.00191	1/56935	0.00176	3/66151	0.004535	2/81004	0.00247	7/256529	**0.00273**
ME	1/17783	0.00562	0/21466	0	7/25798	0.027134	6/30918	0.01941	14/95965	**0.01459**
MI	7/110547	0.00633	9/128618	0.007	7/140851	0.00497	6/156780	0.00383	29/536796	**0.00540**
MN	1/23352	0.00428	1/28275	0.00354	6/39148	0.015326	3/43199	0.00694	11/133974	**0.00821**
MO	0/19898	0	3/23180	0.01294	3/29895	0.010035	0/36302	0	6/109275	**0.00549**
MS	0/2332	0	0/2605	0	0/3001	0	0/3434	0	0/11372	**0**
MT	0/3284	0	0/3432	0	0/4067	0	1/4676	0.02139	1/15459	**0.00647**
NC	7/45710	0.01531	5/50729	0.00986	7/61065	0.011463	6/76304	0.00786	25/233808	**0.01069**
ND	0/4462	0	1/3435	0.02911	0/3623	0	0/3846	0	1/15366	**0.00651**
NE	0/774	0	0/2830	0	0/7231	0	0/14234	0	0/25069	**0**
NH	0/29371	0	2/31482	0.00635	4/36461	0.010971	2/37733	0.00530	8/135047	**0.00592**
NJ	0/66656	0	12/75188	0.01596	1/84923	0.001178	0/94724	0	13/321491	**0.00404**
NM	0/4412	0	0/4240	0	0/4731	0	0/5837	0	0/19220	**0**
NV	1/15906	0.00629	0/17714	0	0/17639	0	0/16453	0	1/67712	**0.00148**
NY	5/103868	0.00481	6/139955	0.00429	19/173831	0.01093	12/189491	0.00633	42/607145	**0.00692**

OH	4/79764	0.00501	4/100088	0.004	8/108313	0.007386	14/117652	0.01190	30/405817	**0.00739**
OK	0/2374	0	0/3439	0	0/5099	0	0/5835	0	0/16747	**0**
OR	1/25310	0.00395	0/28354	0	2/30128	0.006638	3/30790	0.00974	6/114582	**0.00524**
PA	8/93726	0.00854	5/121460	0.00412	8/153724	0.005204	19/180443	0.01053	40/549353	**0.00728**
RI	0/10550	0	1/13470	0.00742	1/15859	0.006306	3/18505	0.01621	5/58384	**0.00856**
SC	0/20033	0	1/26805	0.00373	1/32096	0.003116	1/40379	0.00248	3/119313	**0.00251**
SD	0/2149	0	0/2434	0	0/3358	0	0/4437	0	0/12378	**0**
TN	0/7080	0	0/8640	0	0/9079	0	0/13229	0	0/38028	**0**
TX	0/113273	0	0/126103	0	0/135487	0	0/144378	0	0/519241	**0**
UT	0/5190	0	0/6079	0	0/5205	0	0/7264	0	0/23738	**0**
VA	4/71079	0.00563	3/80960	0.00371	5/95952	0.005211	3/112464	0.00267	15/360455	**0.00416**
VT	1/8722	0.01147	0/10018	0	1/11068	0.009035	3/13759	0.02180	5/43567	**0.01148**
WA	0/37244	0	1/45123	0.00222	3/58929	0.005091	1/72549	0.00138	5/213845	**0.00234**
WI	2/51704	0.00387	7/55162	0.01269	4/66812	0.005987	5/75618	0.00661	18/249296	**0.00722**
WV	0/2499	0	0/3334	0	1/3646	0.027427	0/5761	0	1/15240	**0.00656**
WY	0/565	0	0/632	0	0/1099	0	0/1612	0	0/3908	**0**
Total	**81/1881253**	**0.00431**	**121/2192640**	**0.00552**	**161/2554037**	**0.006304**	**141/2859742**	**0.00493**	**504/9487672**	**0.00531**

CHAPTER 7

COMPARISON OF INFECTION DYNAMICS OF *BAYLISACARIS PROCYONIS* IN DOMESTIC DOGS AND RACCOONS (*PROCYON LOTOR*) AFTER EXPOSURE TO INFECTIVE EGGS OR LARVAE FROM PARATENIC HOSTS. [1]

[1] Sapp, S.G.H., Elsemore, D.A, Hanna, R., and Yabsley, M.J. To be submitted to *International Journal for Parasitology*.

ABSTRACT

Domestic dogs can be either paratenic or definitive hosts for the zoonotic raccoon roundworm *Baylisascaris procyonis*. However, how often a dog develops a patent infection after exposure is poorly understood. Our goal was to compare the infection dynamics of *B. procyonis* in dog and raccoon hosts, including pre-patent periods, egg output, and infection efficiency. We tested fecal samples with centrifugal fecal flotation as well as a newly developed coproantigen ELISA that can detect canine and feline ascarid (*Toxocara* spp. and *Toxascaris leonina*) infections prior to patency based on egg shedding. Groups of 12 six-month-old dogs and three-month-old raccoons were orally inoculated with high (5,000) or low (500) doses of larvated *B. procyonis* eggs (n=3 per dose) or were fed laboratory mice that were orally inoculated with high (1,000) or low (250) doses of larvae *B. procyonis* eggs (n=3 per dose). Only two dogs, both from the low dose larvae group, developed patent infections. In contrast, all 12 raccoons inoculated with eggs or larvae became infected, although only 4/6 raccoons in the egg group developed patent infections. Prepatent periods were shorter in raccoons (avg. 33 days post inoculation (DPI)) receiving the low dose of larvae compared with the two dogs (44, 69 DPI) that became patent. Maximum eggs per gram of feces (EPG) was orders of magnitude greater in raccoons (up to ~9,000 EPG) compared with dogs, which did not exceed 600 EPG. The two dogs spontaneously lost infections after 20 and 66 DPI. The coproantigen ELISA successfully detected *B. procyonis* antigens in all 12 raccoons and the 2 infected dogs. The optical density values were markedly greater in the raccoons suggesting higher intensity infections. In summary, our results demonstrate that dogs are capable of becoming patently infected with *B. procyonis*; however, they are not ideal hosts and growth and fecundity are constrained compared to the natural raccoon host. However, it is

important to note that we exposed 6-month old dogs so it is possible that young puppies may be more susceptible to infection, similar to other ascarid species. Despite dogs having low host competence in this study, dogs can develop patent infections so measures should still be taken to minimize the exposure of dogs to *B. procyonis*.

INTRODUCTION

In the face of increasing urbanization, parasites of wildlife may have increased opportunities to spill into domestic animal hosts. This is especially a concern for zoonotic parasites, as domestic hosts may act as a "bridge" host and place people at risk of wildlife zoonoses that would otherwise be seldom encountered. Many helminth species are able to successfully infect multiple definitive host species; however, there can be variability in many factors observable in experimental infections (e.g., infectivity success rates, fecundity, longevity of infections, etc.) (Jaleta et al., 2017; Johnson et al., 2003; Schantz et al., 1976) . These differences in host competence may result in disparities in the relative epidemiological/ecological impacts among possible definitive host species (Gervasi et al., 2015). Therefore, comparative studies on infection dynamics between unusual/novel hosts and natural hosts are needed to understand relative roles of these hosts in maintenance and transmission of a parasite.

Bayliascaris procyonis, the raccoon roundworm, is an example of a zoonotic parasite that is known to utilize several possible definitive hosts including raccoons (primary host) and possibly other procyonids, domestic dogs, and possibly Virginia opossums (*Didelphis virginiana*) (Kazacos, 2016). This parasite has become ubiquitous across North America and is exceedingly prevalent in some regions (Upper Midwest, West Coast, etc.) and has been introduced into parts of Asia and

much of Europe (Kazacos, 2016). A wide variety of mammalian and avian species can serve as paratenic hosts and larval migration to the central nervous system (neural larva migrans (NLM)) or eyes (ocular larva migrans; OLM) can have fatal or disabling consequences. Of approximately 50 reported clinical baylisascariasis cases in humans, only two complete recoveries have been documented; all other survivors experienced permanent neurologic sequellae and/or vision loss (Kazacos, 2016; Pai et al., 2007; Sircar et al., 2016).

Domestic dogs are unusual hosts for *B. procyonis* as they may serve as a paratenic host and develop larva migrans, with associated disease, or develop patent intestinal infection with adult nematodes and shed eggs in feces as a definitive host (Conboy et al., 2010; Greve and O'Brien, 1989; Kazacos, 2016; Rudmann et al., 1996; Snyder, 1983; Yabsley and Sapp, 2017). The public health significance of a domestic definitive host for *B. procyonis* is obvious, especially given that dogs defecate indiscriminately instead of using defined areas ("latrines") like raccoons (Page et al., 1998). Furthermore, people, particularly children, are more likely to come into contact with dog feces than raccoons in domestic environments. In spite of the public health concern, little is known about the host competence and development of patent infections in dogs. Previous studies in which dogs were experimentally inoculated with *B. procyonis* proved they could develop infections, but these studies used small numbers of animals and did not quantify egg outputs (Bowman et al., 2005; Miyashita, 1993; Snyder, 1983).

In this study, our goal was to compare dogs and raccoons as definitive hosts for *B. procyonis.* We used two routes of exposure, oral inoculation with embryonated eggs and feeding of paratenic host tissues containing larvae, to assess the efficiency and success of these routes between the two host species, and quantified pre-patent periods and egg outputs. Also, we

evaluated the ability of a newly developed coproantigen ELISA (IDEXX Laboratories, Inc.) designed to detect antigens shed by adult canine and feline ascarids (*Toxocara* spp. and *Toxascaris leonina*) to detect intestinal *Baylisascaris* infections (Elsemore et al., 2017).

METHODS

Twelve six-month-old, purpose-bred, ascarid-free beagles of mixed sexes were pair-housed and fed standard kibble with water available ad libitum. After an acclimation period of one week, dogs were separated into individual runs. Six dogs were orally inoculated with either 500 or 5,000 embryonated *B. procyonis* eggs suspended in sucrose. Eggs were derived from the anterior uteri of several adult female *B. procyonis* from naturally infected raccoons. Eggs were dissected from uteri, transferred to an Erlenmeyer flask with 0.5% formalin in tap water, and allowed to develop at room temperature for four weeks. Fully developed eggs were decorticated for 10 minutes in 1:1 20% sodium hypochlorite, washed 4 x in phosphate buffered saline, and the number of embryonated eggs per milliliter was determined microscopically. Aliquots containing the appropriate number of eggs were stored at 4 C prior to inoculation (maximum of ~45 days). Immediately before administering to dogs, aliquots were centrifuged and the egg pellet suspended in sucrose solution for palatability.

For inoculation with *B. procyonis* larvae, outbred non-Swiss albino mice (Envigo, Somerset, NJ) were orally inoculated with either 250 or 1,000 *B. procyonis* eggs and euthanized via CO_2 asphyxiation at the onset of clinical disease (7-9 days post inoculation). Immediately following euthanasia, carcasses were skinned, and the feet, tail, and large bones were removed. The brain, eyes, masseter muscles, and tongue were removed from the skull and combined with the rest of

the carcass. These tissues were coarsely minced, mixed with a small amount of canned dog food, and immediately fed to six dogs. Each dog was fed one carcass, with the exception of one dog (DL-1) who was fed four mice inoculated with 250 eggs (for a total of ~1,000 larvae).

Twelve male, subadult (~12 week old), captive-bred raccoons (Ruby Fur Farms, New Sharon, IA) were group housed during quarantine but the transferred to individual housing before being inoculated using the same conditions and methods as described for the dogs. Oral inoculations of raccoons with eggs was facilitated by mild sedation using intramuscular injection of ketamine (5 mg/kg).

Sample collection and parasite detection

During quarantine, fecal samples were collected from each dog and group of raccoons. Fecal samples were collected from animal runs daily for three days following inoculation, and then weekly until 21 DPI, at which time daily sampling was initiated again to ensure that the first day of patency was detected. Due to variation in feces production, fecal samples were not available for every animal each day of sampling. Samples were stored at -20 C prior to processing.

For detection of eggs, 2-3 g of feces were subjected to standard centrifugal fecal flotation procedures using Sheather's flotation solution (specific gravity = 1.27) (Hendrix and Robinson, 2016). The number of eggs per gram of feces (EPG) was determined by dividing the total number of eggs counted on the slide by the sample weight. Samples with very high egg burdens were diluted, and EPG calculated accordingly.

Three to five grams of feces were submitted to IDEXX Laboratories (Westbrook, Maine, USA) for ascarid coproantigen detection using a commercially available proprietary ELISA. This

assay has been validated for the detection of *Toxocara canis* and *Toxocara cati* in domestic dogs and cats, respectively (Elsemore et al., 2017). Results were reported in optical density (OD_{650}) values; samples with OD_{650}>0.1 were considered positive. The maximum OD_{650} value for this assay is 4.000.

Animal monitoring and disposition

After inoculations, dogs and raccoons were monitored at least twice a day for 20 days post inoculation (DPI) to detect any behavioral abnormalities that may have developed. After 20 DPI, animals were monitored daily.

Raccoons were treated after a minimum of 60 days post patency with ivermectin (1 mg/kg) mixed into food. Fecal samples were collected for 3-4 days post treatment to ensure that animals became antigen and egg negative. Nematodes that were expelled were collected and counted. Animals that did not become negative on both assays were re-treated and resampled. After treatment raccoons were sedated via intramuscular injection of xylazine (0.5 mg/kg) and ketamine (5 mg/kg) and euthanized via intracardiac injection of sodium pentobarbital (1 ml/4 kg). The small intestine of raccoons was removed and examined for remaining nematodes. After dogs were negative for *B. procyonis* eggs, they were transferred to another experiment.

Statistical Analysis

Differences in the onset of antigen positivity and patency were analyzed via ANOVA followed by Tukey's Honestly Significant Difference to identify which groups differed

significantly. Statistical analysis was conducted using R statistical software version 3.1.2 (R Core Team, 2014).

Ethics statement

All procedures involving dogs, raccoons and mice were reviewed and approved by the University of Georgia's IACUC committee (A2016 10-009).

RESULTS

Patency and egg output

Only 2/12 dogs became patent following the original inoculation, both in the low-dose larval group. *Baylisascaris procyonis* eggs were first detected in the feces of dog DL-5 at 44 DPI and DL-6 at 69 DPI (Table 7.1). At peak shedding, DL-5 shed 583.9 EPG and DL-6 shed 217.7 EPG (Figure 7.1). These two dogs ceased shedding eggs after 66 and 20 days post-patency (DPP), respectively.

Ten of 12 raccoons became patent (Table 7.2). Egg shedding generally followed a pattern of a steep increase within the first week of patency, and then fluctuations which generally followed observed expulsion events as noted below (Figure 7.2). The two raccoons that did not become patent were RE-3 (5,000 eggs) and RE-4 (500 eggs). RE-3 intermittently shed unfertilized, empty eggs and expelled one small female nematode measuring 6 cm after treatment. RE-4 never became patent during the course of the study and was euthanized on 94 DPI for unrelated reasons. Two male worms measuring 5.5 cm were recovered from the small intestine. Overall, prepatent periods were significantly shorter ($p=0.0005$) in larvae inoculated raccoons (avg. 37

DPI) versus egg inoculated (avg. 58 DPI). Analysis by dose/route group revealed that prepatency in the low larval groups was significantly shorter than both the low and high egg groups (p=0.010, p=0.005) Prepatency in the high-dose larval group was also significantly shorter than the high-dose egg group (p=0.24). No other significant differences in patency among dose/route groups were found.

Eight raccoons were treated (RE-2, RE-3, RE-5, RE-6, RL-2, RL-3, RL-4, RL-5) and all but three of these became antigen- and egg-negative within 4 days of treatment (Figure 7.3). Raccoons RE-2, RE-5, and RL-5 failed to become egg and antigen negative in the four day timeframe, although RE-5 was egg-negative at 4 days post treatment. Intensity (number of worms passed) ranged from 6 to 18 among all treated raccoons; however, many raccoons spontaneously passed adult worms at various points during patency (Table 7.2). Most spontaneous passages occurred within the first 20 DPP. Worms expelled were usually of variable sizes, with average size increasing with the length of infection. While most expulsion events involved only single to a few worms, raccoons RE-5 and RL-2 passed large numbers (43 and ~60 respectively) between 50 and 55 DPI.

Coproantigen dynamics

Both dogs that became patent after the first exposure became antigen positive prior to egg shedding, on 38 DPI (Dog DL-5) and 41 DPI (DL-6) (Figure 7.3). Following the cessation of egg shedding in DL-6, antigen values fell to borderline levels, fluctuating above and below the cutoff threshold (OD_{650}>0.10) over the span of 10 days, and became consistently negative by 104 DPI.

Dog DL-5 was antigen negative on 115 DPI and was consistently negative until the conclusion of the study (Figure 7.3).

None of the dogs inoculated with eggs and only 3/7 fed larvae became antigen positive. A single sample (73 DPI) from DL-1 yielded a positive result, but all subsequent samples were negative. This dog was housed next to antigen-positive DL-6 and contamination of the sample could have occurred.

All but one raccoon became antigen positive at variable time points and OD_{650} values were considerably higher than the two antigen positive dogs (Figure 7.4). The onset of antigen positivity was significantly sooner ($p=0.0005$) for larvae-inoculated raccoons (avg. 17.5 DPI) versus egg inoculated raccoons (avg. 42.5 DPI). RE-4 had sporadic positive results on 39 dpi ($OD_{650}=0.132$), 44 dpi ($OD_{650}=0.132$), and 82 dpi ($OD_{650}=0.141$) and was negative at all other time points tested; as noted above this raccoon was only infected with two male worms.

Re-exposure of dogs

Dogs that did not become patent were randomized to new groups and re-inoculated with 100 larvae, 250 larvae, or 500 eggs (Table 7.1). These doses were chosen based on success of the prior low larval dose, and to maintain one egg-inoculated group for comparison. None of the re-inoculated dogs became patent or antigen positive by 85 days post inoculation.

DISCUSSION

Our data demonstrate that raccoons develop infections with *B. procyonis* more often after exposure, have higher worm burdens, and high egg output compared with domestic dogs. Only 2 of the 12 dogs inoculated became patently infected and none of the remaining 10 on re-exposure, compared to ten of the raccoons exposed to the same conditions. Both dogs that became infected were both inoculated with the low number of larvae (250) and both infections were abortive. Egg shedding ceased abruptly after only 20 DPP in dog DL-6 which only had borderline coproantigen OD_{650} suggesting a single-worm or single-sex infection that was eventually cleared. Dog DL-5 passed eggs for a longer period, but EPG levels decreased after the peak observed at 41 DPI with a number of empty, unfertilized eggs being passed on its final egg-positive fecal exam (66 DPP). Five days later, DL-5 became antigen negative. This pattern suggests male worms may have senesced early during the infection and that the remaining females were exhausting spermatozoa stores, and senesced shortly thereafter. Similarly, raccoon RL-5 began passing a mixture of unfertilized and fertilized eggs around 60 DPP and only female worms were recovered following treatment (suggesting males were expelled/lost previously). Egg counts in raccoons were frequently an order of magnitude greater than those observed in the two dogs, even after large numbers of worms were spontaneously purged, and all raccoons that developed patent infections remained positive throughout the course of the study. Among the raccoons inoculated with 250 larvae, peak EPG values were 337.5-1,056 compared to 583.6 and 217.7 EPG in the patent dogs which had received the same dose.

In this study, dogs which were inoculated with eggs did not establish patent *B. procyonis* infections which is consistent with previous studies (Dubey, 1982; Miyashita, 1993). Inoculation

of three 8-week old puppies with eggs was successful in establishing a patent infection only in the puppy receiving the most eggs (30,000), whereas 3/4 puppies that were fed mice infected with larvae became patent (Miyashita, 1993). This suggests an age resistance to infection may exist for dogs, similar to results observed with adult raccoons, which generally only become infected via ingestion of infected definitive hosts (Kazacos, 1983). Age resistance to patent infection with the related *Toxocara canis* is also well-documented. In dogs over 3-5 weeks of age, the vast majority, if not all larvae undergo somatic migration and become arrested in tissues (commonly kidney, liver, heart, and brain), never reaching the gastrointestinal tract for further maturation in a phenomenon termed "age resistance" (Greve, 1971; Sprent, 1958). This may be the case with dogs inoculated with *B. procyonis* eggs, with migrating larvae becoming arrested in somatic tissue instead of reaching the small intestine. Severe NLM and VLM have been observed in adult dogs inoculated with very high numbers of eggs (10,000 – 200,000), although clinical signs are likely absent or mild with smaller egg doses similar to what we have observed in paratenic hosts, including experimentally infected rodents and people occupationally exposed to low levels of *B. procyonis* eggs (Sapp et al., 2016a;. Sapp et al., 2016b; Snyder, 1983). We did not observe any clinical or behavioral abnormalities in dogs inoculated with eggs in our study.

During the course of the study, fluctuations in egg shedding by raccoons were commonly observed following purge events in which many adult nematodes were shed. Although the egg outputs of some animals appeared to stabilize after 30 DPP, this varied by individual and events of expulsion. In naturally infected raccoons, extremely high EPG values (up to 225,000) are commonly observed, but none of our raccoons reached levels above 10,000 EPG (Kazacos, 2016; Snyder and Fitzgerald, 1987). It is possible that multiple exposures are required to build infection

intensity to such a level required for these high EPG counts, or that the subadult raccoons in this trial do not shed eggs to the degree typical of naturally-infected juvenile raccoons from highly endemic areas (Snyder and Fitzgerald, 1987, 1985). A trial involving experimental infection of wild-caught raccoons found a higher mean EPG (~15,000 EPG), although the number of larvae those raccoons received is not clear (Reed et al., 2012). Further, the final intensity among our raccoons following treatment was not high (maximum of 18 worms recovered), which supports the notion that repeated exposure is required for very high intensity and high output infections.

An interesting trend was observed regarding dose and onset of either antigen positivity or patency. Raccoons inoculated with 500 eggs tended to became antigen positive and patent before ones inoculated with 5,000 eggs although this was not statistically significant ($p=0.09$). Among the raccoons receiving larvae, differences in the onset of antigen positivity were marginally non-significant ($p=0.06$), but lower dose raccoons did became patent sooner (33-35 days vs 37-47 days). Similarly, although only two dogs in our study became infected, these two dogs were also in a low dose group. In ascarid-naïve dogs inoculated with varying doses of *T. canis* eggs, a paradoxical effect occurred in response to dose as none of the dogs inoculated with a high dose of eggs (10,000) developed patent infections (Dubey, 1978). It is unknown why lower doses appear more successful, although it may be related to crowding stress on developing larvae and perhaps a strong non-specific immune reaction triggered by a large inoculum (compared to a smaller inoculum in which larvae may be able to evade host immunity more effectively). It is possible that a "carrying capacity" is reached following a large inoculum, after which growth may be negatively impacted by crowding stress, leading to delays in reproductive onset (i.e. patency). One previous study did not find evidence of crowding on egg output in naturally

infected raccoons, although that does not rule out crowding impacts on rate of development (Weinstein, 2016).

As anticipated, raccoons inoculated via feeding of larvae in tissues became antigen positive and patent much sooner than egg inoculated raccoons. Our observations are consistent with prior experimental infections in raccoons (Kazacos, 2016). Infections in adult raccoons are thought to be achieved solely via ingestion of larvae (Kazacos 1983), whereas the subadult (approximately 12 weeks old) raccoons in our study and were susceptible via exposure to eggs or larvae.

The roundworm coproantigen ELISA used in this study was developed and validated for detection of *Toxocara* spp. antigens in domestic dogs and cats but our data demonstrates that it effectively cross-recognizes *B. procyonis* antigen. We expected this as many somatic and excretory-secretory antigens are conserved among *Ascaris, Toxocara,* and *Baylisascaris* spp. (Boyce et al., 1988; Dangoudoubiyam and Kazacos, 2009; Wang et al., 2008; Xie et al., 2013). While the resulting cross-reaction of these antigens complicates serodiagnosis of clinical disease when used for diagnosis, this is advantageous in the coproantigen ELISA as it can be used to detect any ascarid infection in a rapid and high throughput method. However, the primary advantage of this assay is the detection of luminal infections that are in the pre-patent period. In the original validation of this assay for *Toxocara*, dogs inoculated with 150-300 *T. canis* eggs became antigen-positive by 31 DPI but did not became patent until 38-41 DPI. In our study, we found much more variation in the time between the first antigen positive and patency, ranging from 5 to 29 days, but infected animals were consistently antigen positive before they became patent. This difference is could be due to differences in the growth and development of *B. procyonis* versus *Toxocara;* the marked size

variation among spontaneously expelled *B. procyonis* adults suggests growth rates of individual worms may vary widely in the same host following a single exposure. Importantly, assay characteristics (e.g. differences between the binding efficiency of *Toxocara* spp. and *B. procyonis* antigen to the monoclonal capture antibody) could lead to species-level differences in detection thresholds. For example, in one raccoon that was only intermittently antigen positive, two small (5.5 cm) male worms were recovered, which may have failed to shed antigen at a level that was consistently detectable. We are currently conducting studies to quantify the performance characteristics (i.e. sensitivity, specificity, predictive values) of this coproantigen ELISA for the detection of *Baylisascaris* spp. infections, and to characterize the antigenic homologue expressed by *B. procyonis*.

In summary, dogs do not appear to frequently develop patent infections after *B. procyonis* exposures; however, those that do develop patent intestinal infections represent a risk for egg shedding in a domestic environment. This apparent low level of adaptation to dogs may could indicate a recent host-switching event; increasing raccoon populations in urban and suburban areas allows greater opportunities for exposure of domestic dogs to *B. procyonis.* Our work suggests that for dogs over 6 months of age, consumption of low numbers of larvae in paratenic host tissue is the most likely route that may lead to intestinal infection. While this could partially explain the rarity of patent infections in dogs, it is unlikely that this route is the sole cause of all reported "patent" *B. procyonis* infections in dogs. Coprophagy is very common among pet dogs, and not all dogs have access to prey or the drive to hunt. For example, 8% of dogs with egg-positive fecal exams from 2013-2018 were toy group breeds, which would be unlikely to become infected through consuming paratenic hosts (Yabsley and Sapp, 2017). Further, the presence of

the *Eimeria,* a raccoon coccidian, in fecal samples confirmed that coprophagy was common (Yabsley and Sapp, 2017). Notably, no reports of intestinal *Baylisascaris* spp. infections exist in wild canids to our knowledge, despite the great potential for exposure via the predation of infected paratenic hosts. Further investigation of the host, parasite, and epidemiological factors leading to the establishment of intestinal *B. procyonis* infections in canids is still warranted, owing to the public health risk of pet dogs as hosts for this high-consequence zoonosis.

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Table 7.1. Routes of inoculation and days post inoculation (DPI) on which a positive antigen result was first obtained and when patency was observed in experimental dogs.

Dog ID	Route of exposure	Re-exposure route	First antigen positive - last antigen postive (DPI)	First patent – last patent (DPI)	Difference between antigen positivity and patency (days)
DE-1	5,000 eggs	100 L3	NA	NA	NA
DE-2	5,000 eggs	250 L3	NA	NA	NA
DE-3	5,000 eggs	500 eggs	NA	NA	NA
DE-4	500 eggs	100 L3	NA	NA	NA
DE-5	500 eggs	250 L3	NA	NA	NA
DE-6	500 eggs	500 eggs	NA	NA	NA
DL-1	1,000 L3[a]	100 L3	73[b]	NA	NA
DL-2	1,000 L3	250 L3	NA	NA	NA
DL-3	1,000 L3	500 eggs	NA	NA	NA
DL-4	250 L3	500 eggs	NA	NA	NA
DL-5	250 L3	ND	38-115	44 – 110	6
DL-6	250 L3	ND	44-104	69 – 89	25

a. Fed L3 larvae in tissue of infected mouse fed 1,000 or 250 eggs.

b. A single antigen-positive result; all other time points were negative.

Table 7.2. Routes of inoculation and days post inoculation (DPI) on which a positive antigen result was first obtained and when patency was observed in experimental raccoons.

Raccoon ID	Dose/Route of exposure	First antigen positive (DPI)	First patent (DPI)	Difference (days)	No. worms recovered after treatment	Size range (cm)
RE-1	5,000 eggs	52	57	5	0	NA
RE-2	5,000 eggs	40	63	23	0	NA
RE-3	5,000 eggs	59	NA	NA	2[c]	6-14
RE-4	500 eggs	39[b]	NA	NA	2[c]	5.5-6
RE-5	500 eggs	35	50	15	18	8-17
RE-6	500 eggs	39	62	23	12	5.5-19
RL-1	1,000 L3[a]	19	37	18	NA[d]	NA
RL-2	1,000 L3	13	39	26	15	5-13
RL-3	1,000 L3	18	47	29	14	4-16
RL-4	250 L3	20	35	15	6	6.5-13
RL-5	250 L3	21	33	12	11	12-18
RL-6	250 L3	14	31	17	NA[d]	NA

a. Fed L3 larvae in tissue of infected mouse fed 1,000 or 250 eggs.
b. Intermittent positive results recoded on 39, 44, and 82 DPI; all other time points negative.
c. Non-patent, single-sex infection.
d. Ongoing study; these individuals have not yet been treated.

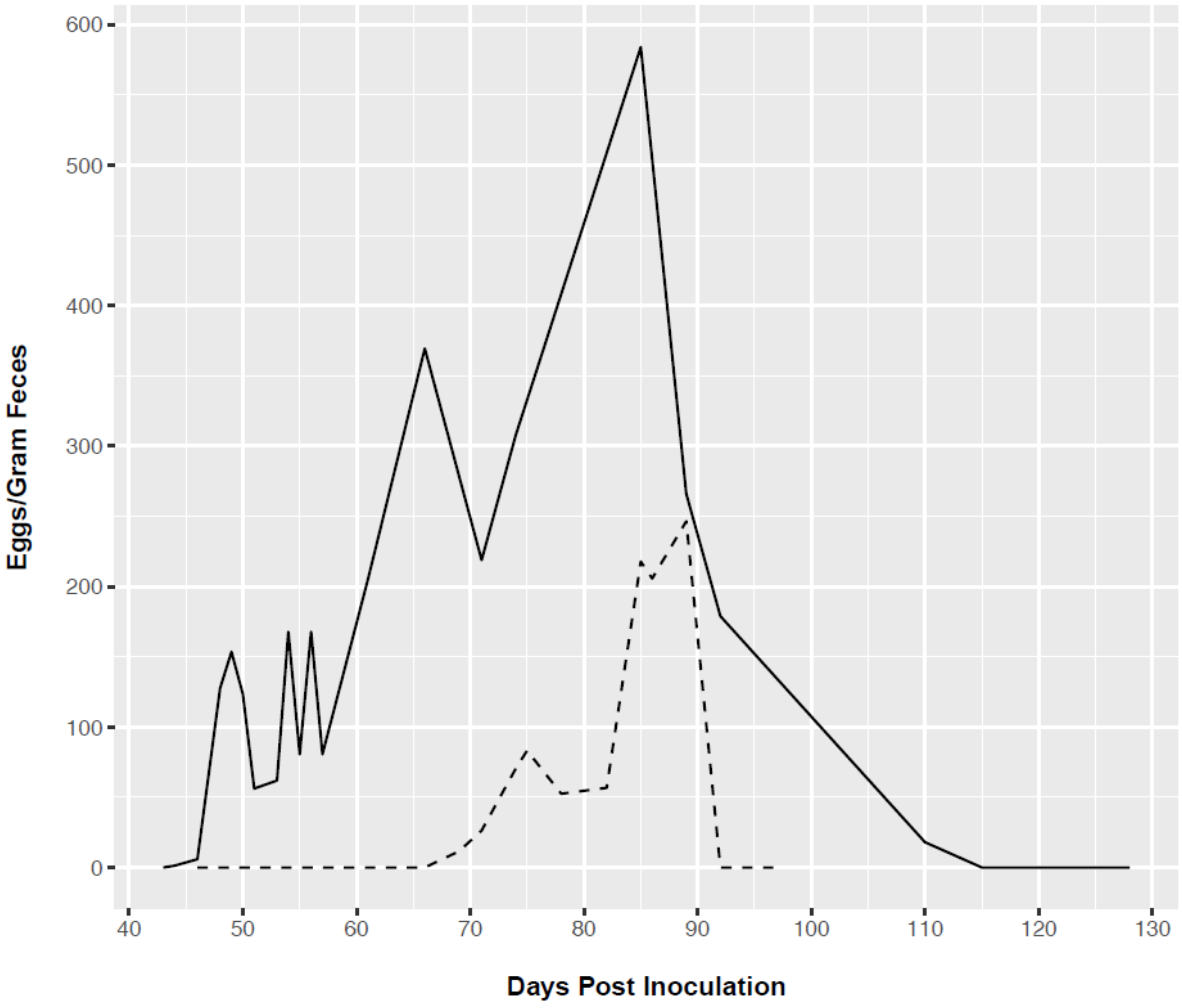

Figure 7.1. *Baylisascaris procyonis* egg outputs for the two dogs that developed patent infections. (Solid line = Dog DL-5; Dashed line = Dog DL-6).

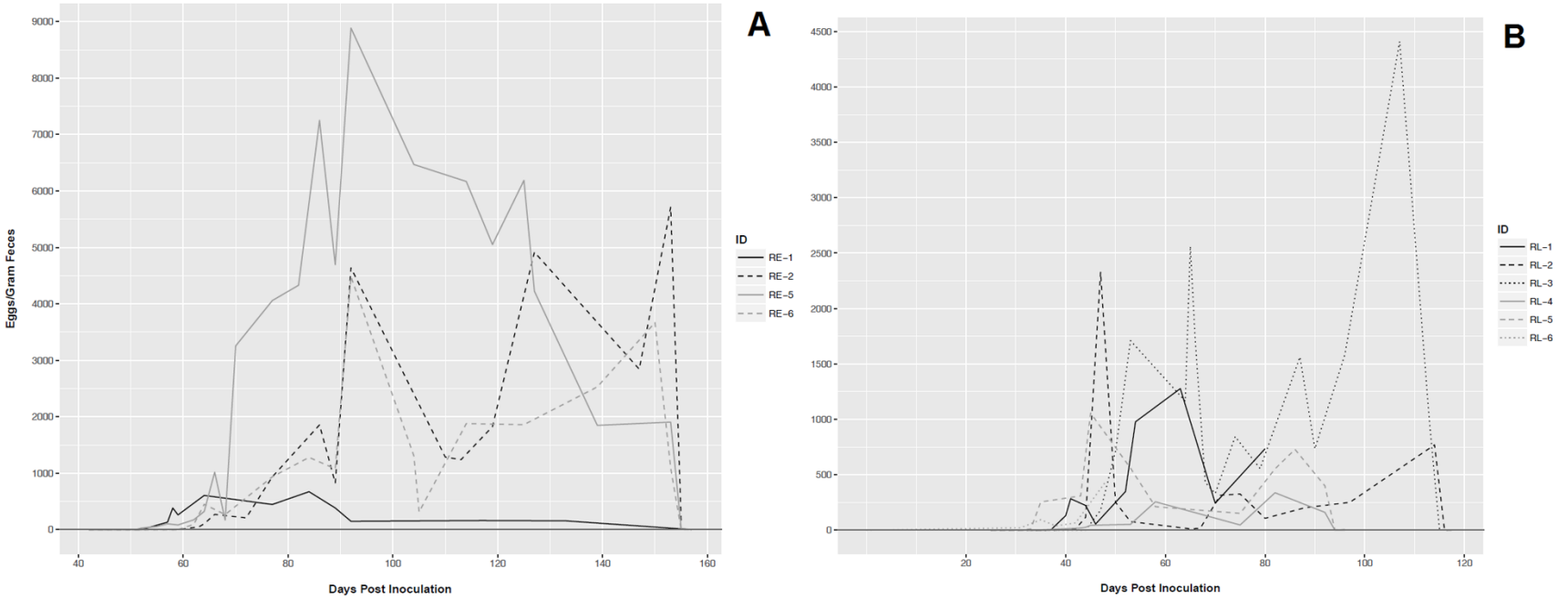

Figure 7.2. *Baylisascaris procyonis* egg outputs for patently-infected raccoons exposed to eggs (A) or larvae (B). Treatment occurred on 153 DPI for all raccoons in A, on 114 DPI for RL-2 and RL-3, and on 92 DPI for RL-4 and RL-5.

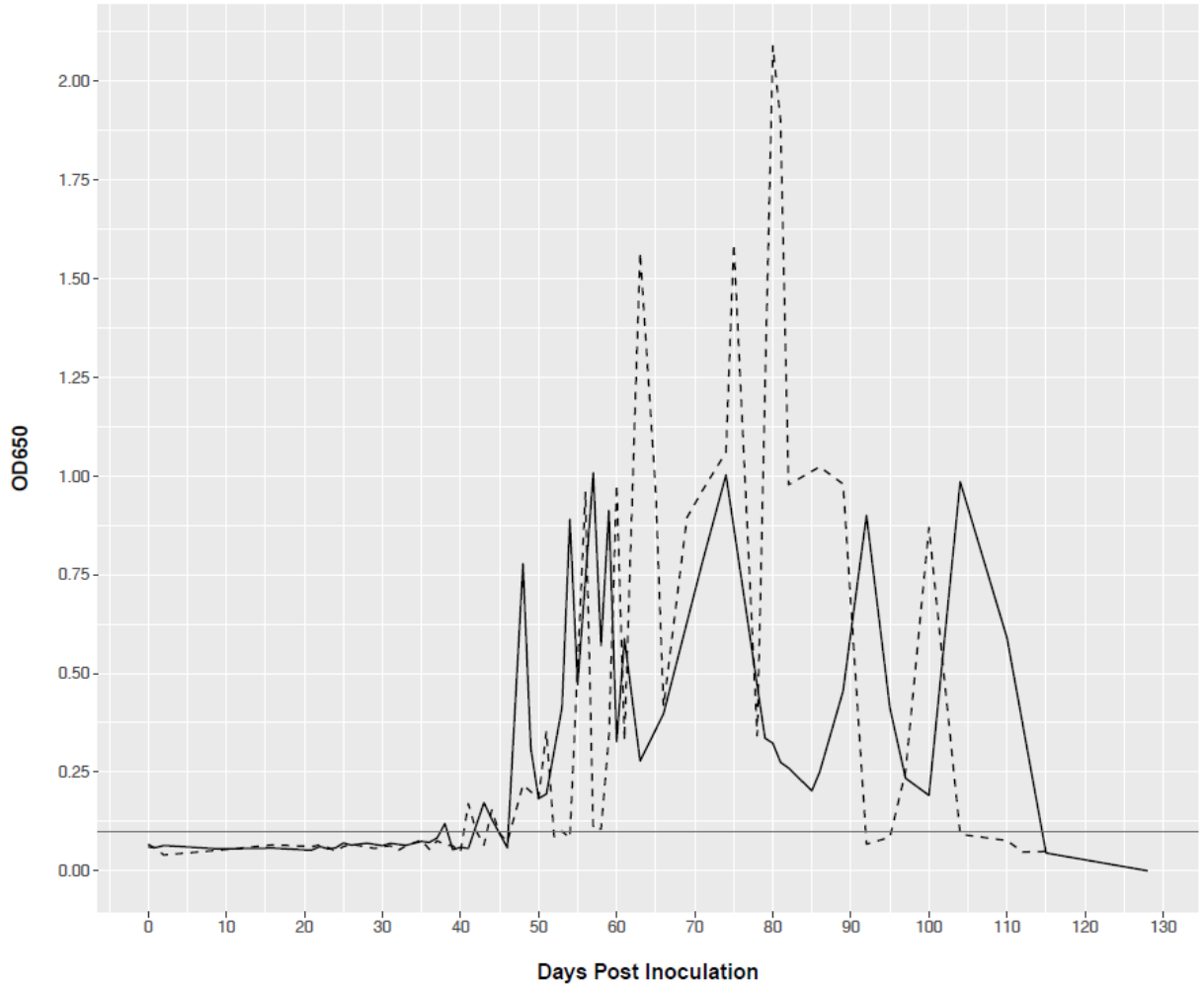

Figure 7.3. ELISA coproantigen values for two dogs that developed patent infections. (Solid line = Dog DL-5; Dashed line= Dog DL-6). Horizontal line indicates positive cutoff value (OD_{650}>0.1).

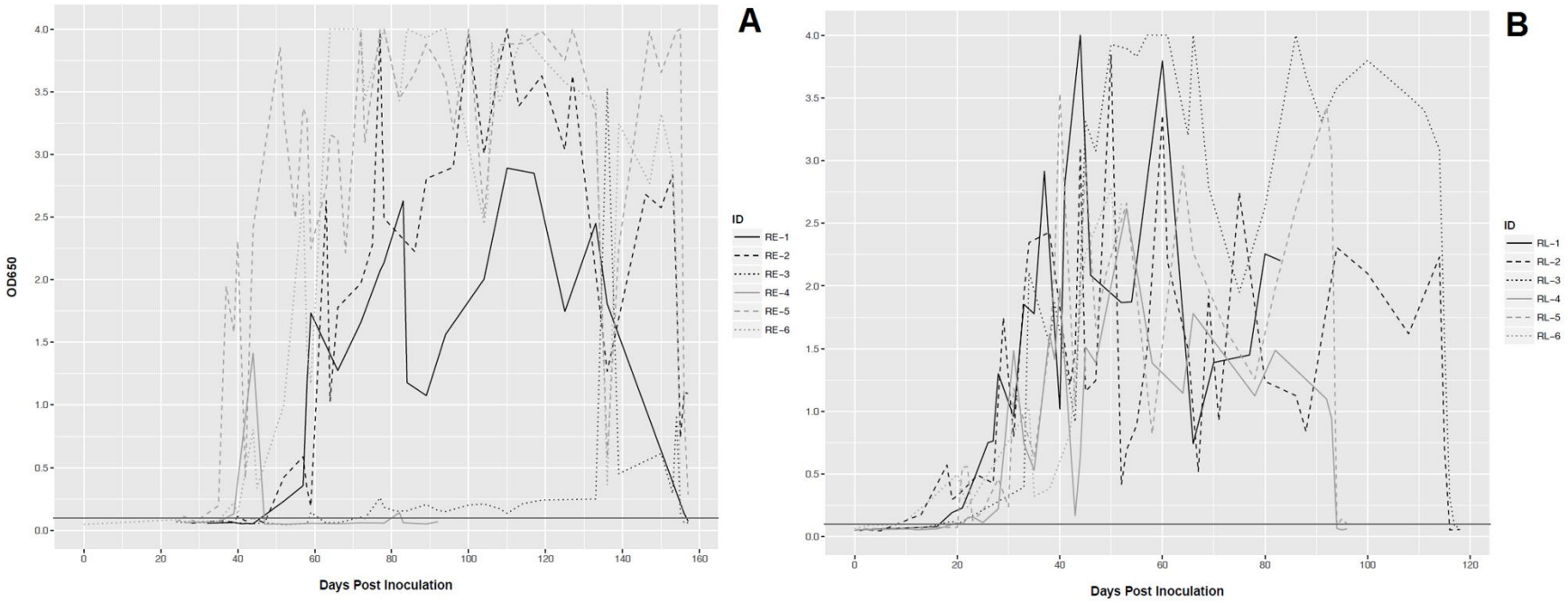

Figure 7.4. ELISA Coproantigen values for raccoons infected with eggs (A) or larvae (B). Horizontal line indicates positive cutoff value (OD_{650}>0.1). Treatment occurred on 153 DPI for all raccoons in A, 114 DPI for RL-2 and RL-3, and 92 DPI for RL-4 and RL-5.

CHAPTER 8
CONCLUSIONS

The ultimate goal of this dissertation was to investigate aspects of *Baylisascaris procyonis* epidemiology and transmission among humans, domestic, and wildlife. Zoonotic diseases, especially those of urban adapted species like raccoons, are at the forefront of public health and will continue to be so as the human-wildlife interface expands with urbanization. A One Health approach to disease investigation that incorporates human, veterinary, and environmental studies is especially well-suited to zoonoses and parasites in particular, many of which have complex life cycles and whose transmission is influenced by a myriad of host and environmental factors. An overview of the transmission and epidemiological factors of *B. procyonis* investigated in this dissertation is presented in Figure 8.1.

Study 1 (Chapter 2)

Prior to our investigation, the limited evidence for subclinical or covert baylisascariasis included a study in which individuals who handled raccoons were seropositive using an older, crude antigen-based assay and the finding of a *Baylisascaris* sp. larva in the brain of an elderly patient who died of unrelated causes (Conraths et al., 1996; Hung et al., 2012). We chose to investigate wildlife rehabilitators due to their likely elevated occupational risk of exposure, given their prolonged contact with raccoons and raccoon feces during husbandry. In our study, 24/347 (7%) of individuals tested were positive for antibodies to *Baylisascaris* spp. based on the current

recombinant antigen-based (rBpRAG-1) diagnostic Western blot. All but two of these individuals reported raccoon contact either currently or in the past. More research is warranted to understand the significance of covert infection with *Baylisascaris* spp., similar to how covert infection with related *Toxocara* spp. is known to be correlated with chronic conditions such as asthma, urticaria, and occult seizures (Hotez and Wilkins, 2009). Overall, this study provided further, more definitive evidence that *Baylisascaris procyonis* can infect people subclinically in demonstrating the presence of anti-*Baylisascaris* antibodies in otherwise healthy (i.e. without neurological disease) individuals, and also emphasizes the importance of proper hygiene and sanitation protocols within rehabilitation facilities to reduce the risk of exposure to *B. procyonis* eggs.

Study 2 (Chapter 3)

In this study, we investigated the knowledge of *B. procyonis* and attitudes towards it as an occupational hazard among wildlife rehabilitators using a comprehensive online survey. Knowledge was assessed using multiple choice and true-false questions, and attitudes were assessed using Likert-type items. The median overall knowledge score was 7/14 questions correct (range: 0/14 – 14/14 correct). Factors associated with an above-median knowledge score were related to education (higher education level, veterinary professional background) and experience/professionalism (membership in a wildlife rehabilitation professional group, and greater experience in years). Rehabilitators in the southeastern United States were more likely to score below the median in overall score and in sub-categories (questions related to transmission, clinical aspects, and biology of *B. procyonis*). Factors influencing attitudes towards *B. procyonis*

were diverse but also followed a similar pattern as knowledge, with greater risk perception among educated/experienced individuals.

Rehabilitators who worked with raccoons were also asked about their use of personal protective equipment (PPE) and infection control practices (ICP) during raccoon husbandry. Experience, education, and professional group membership influenced the likelihood of reporting appropriate PPE and ICP use. Knowledge score was a highly significant predictor of reporting correct ICP, albeit with a small effect size. Risk factor analysis on PPE use data from Study 1 (Chapter 2) revealed that inconsistent hand hygiene after handling live raccoons or fecal contact, and location in highly *B. procyonis*-endemic areas were significantly associated with seropositivity (i.e. exposure). These comprehensive surveys are important in that they further emphasize the occupational risk that *B. procyonis* and other zoonoses pose to wildlife rehabilitators. Responses to and the analysis of knowledge, attitudes, and practices sections identify potential misconceptions or knowledge gaps among the wildlife community, and characterize traits of those in the community who may require outreach and further education.

Study 3 (Chapter 4)

This study involved an experimental infection trial to evaluate differences in infection dynamics among four *Peromyscus* species, which may serve as important paratenic hosts. *P. leucopus* (white footed mouse) demonstrated a longer survival time after inoculation versus *P. maniculatus* (deer mouse), *P. polionotus* (Oldfield mouse), and *P. californicus* (California mouse). The highest dose of *B. procyonis* eggs (500) was nearly uniformly fatal, where many to all survived lower doses (50 eggs and 10 eggs). Differences in larval burdens among species were noted as

well; *P. leucopus* had a greater proportion of larvae recovered from visceral organs than other species, and *P. maniculatus* had a greater proportion in the brain. Total larval burdens did not differ among species, and represented ~10% of the dose given at all dose levels. Our findings suggest *P. leucopus* has a greater tolerance towards infection than the other *Peromyscus* species tested, as it is able to survive longer with an equal larval burden. This tolerance may be due to the ability to wall off larvae in viscera and slow migration to the brain, evidenced by the greater larval recovery in viscera in this species. Prior work has demonstrated that *P. leucopus* is more resistant towards *B. procyonis* than *Mus musculus,* indicating quantifiable differences in resistance and tolerance among paratenic species for this generalist parasite (Sheppard and Kazacos, 1997). These findings have implications for the role of these species as hosts in the wild. Some field studies have found larvae in free-ranging *P. leucopus,* but did not recover any sympatric *P. maniculatus,* perhaps due to the more rapid mortality of the latter species (Page et al., 2013, Beasley et al., 2013). These data imply that the paratenic host species composition and the inherent variations in tolerance may influence the transmission and maintenance of *B. procyonis* in its sylvatic cycle.

Study 4 (Chapter 5)

Using samples from the prior trial (Study 3) and field studies, we developed species-adapted serologic assays for the detection of anti-*Baylisascaris* IgG in various rodent genera. The antigen target utilized in human diagnostics (rBpRAG-1) was successfully adapted to a Western blot format for *Peromyscus, Mus,* and *Rattus,* and further into an ELISA for *Peromyscus* spp. Performance characteristics and concordance between the *Peromyscus*-adapted Western blot and

ELISA was good, and both assays revealed species-level differences in seroconversion and terminal antibody concentration. Several *P. californicus* and *P. polionotus* failed to mount a detectable antibody response at medium and low doses, despite having larvae recovered from their carcasses. *P. leucopus* had a significantly greater antibody concentration than *P. polionotus* and *P. californicus* at high and medium doses, and greater than those two and *P. maniculatus* at the lowest dose. These results further provide an explanation for the differences in survival and tolerance towards infection among *Peromyscus* species observed in Study 3 (Chapter 4). Overall, the data fit in the context of what is known regarding humoral responses to experimental ascarid infections in rodents, with response towards ES antigens being dose-dependent and correlated with survival (Bowman et al., 1987; Abo-Shehada et al., 1991; Tsuji et al., 2003; e Fonseca et al., 2017).

Additionally, the two serologic assays were used on a set of samples collected from wild *P. maniculatus* for another survey. The assay proved sensitive in detecting antibodies in mice that were positive for *Baylisascaris* sp. larvae. Specificity was equivocal, highlighting the challenges of using serology to investigate diseases in wildlife, which have unknown exposure histories. However, serology remains the only ante-mortem test for detection of *Baylisascaris* spp. infections in paratenic hosts, and thus has utility in field studies if results are interpreted with caution.

Study 5 (Chapter 6)

This chapter involved a characterization and descriptive epidemiology of the occurrence *Baylisascaris* in domestic dog fecal exams based on a large, nation-wide dataset. Previously, very few studies had investigated patent *Baylisascaris* spp. infections in dogs (Conboy et al., 2010;

Kazacos 2016). The prevalence was low (504/9,487,672 (0.005%)), as expected for a rare parasite, and in general, the distribution of positive dogs was found to overlap with the known distribution of *B. procyonis* in raccoons. Despite the low prevalence, some important points emerged from this study. Some positive dogs originated from some states where *B. procyonis* had not previously been reported, further emphasizing the range of *B. procyonis* may be expanding. Even if these cases were travel-related, this implies that dogs could play a role in the translocation of this parasite to novel areas. The dogs in this dataset were presumably receiving a high level of veterinary care; prevalence may be higher in dogs receiving a lower standard of care. Finally, spurious "infections" of dogs following coprophagy still represent a public health risk since *B. procyonis* eggs may be introduced into areas of greater human contact, potentially placing household members and other animals at risk. Coprophagy also would put dogs at risk of acquiring infections, which can have fatal consequences if larvae migrate to the central nervous system (Thomas, 1998; Ruddman et al., 1996; Windsor et al., 2009). This study provides a foundation for the continuing study on the role of dogs as domestic "bridge" hosts for *B. procyonis* and associated epidemiology. It also emphasizes the importance of routine use of anthelmintic preventives and restrictions on coprophagy in pet dogs to prevent infection.

Study 6 (Chapter 7)

Finally, an experimental infection trial was carried out to assess the definitive host competence of dogs for *B. procyonis* versus its natural raccoon host. A marked difference between infection dynamics in dogs versus raccoons was noted—importantly, that dogs are far inferior definitive hosts than raccoons. This is not surprising given that *B. procyonis* is adapted to the

raccoon host rather than the genetically distant canine host. Only two of twelve dogs developed patent infections, and both of these dogs spontaneously aborted infection after 66 and 20 days. Egg outputs and coproantigen levels were generally low. None of the dogs inoculated with eggs developed patent infections; in limited prior experimental infections, eggs were also a very inefficient route of infection and success has only been achieved in a single young puppy (Dubey 1982; Miyashita 1993). Infection via consumption of infected paratenic hosts (L3 larvae in tissue) appears more successful in older dogs, mirroring what is assumed to be true in raccoons (Kazacos 1983). However, among the subadult raccoons in this study, both egg and larval exposures were successful and all exposed raccoons became infected. Across the board, raccoons demonstrated markedly greater coproantigen levels than dogs and had egg outputs that were frequently an order of magnitude higher than the peak shedding by dogs, highlighting their superior host competence. Despite being a remarkable generalist with respect to paratenic host species, *B. procyonis* appears to be much more of a specialist for its definitive host. Although dogs were less effective hosts for *B. procyonis,* infections in dogs still remain a public health concern their indiscriminate nature of defecation within domestic environments and the serious consequences of human exposure.

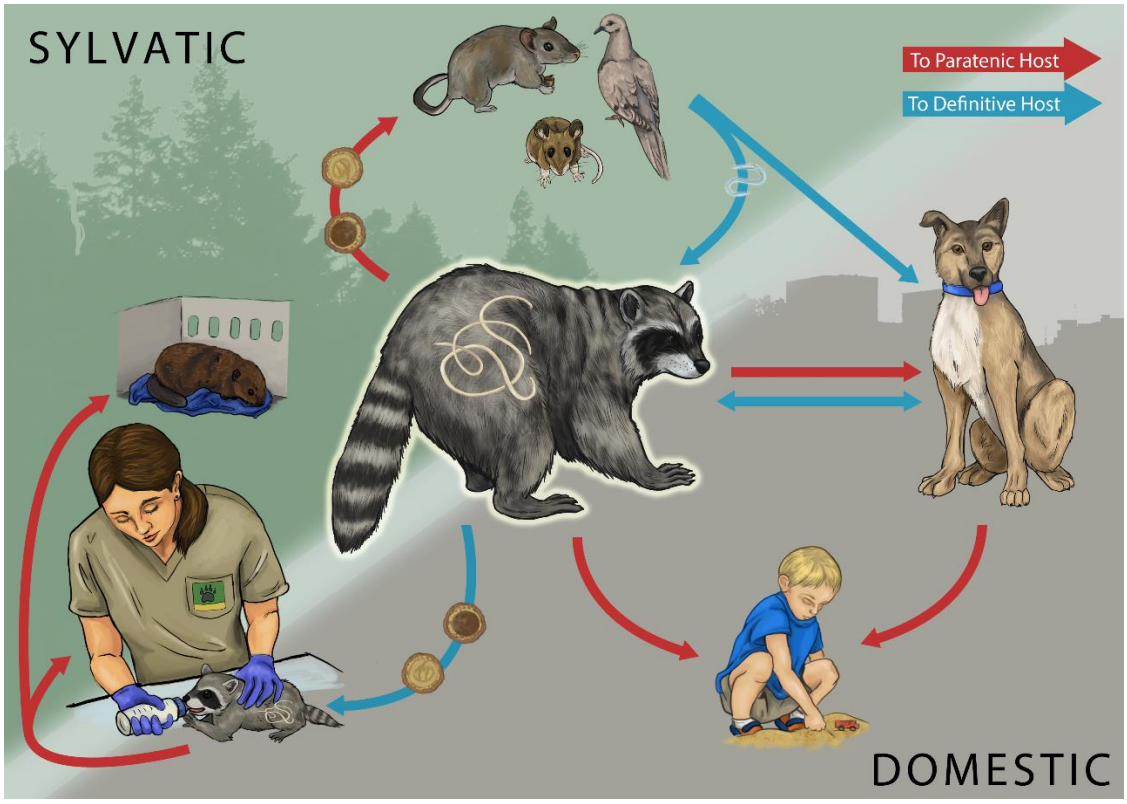

Figure 8.1. The "expanded life cycle" of *Baylisascaris procyonis*. Raccoons, well adapted to both sylvatic and domestic/urban environments, are definitive hosts for *B. procyonis*. Many mammalian and avian paratenic host species are susceptible to larva migrans-associated disease following ingestion of infectious eggs. Larvae in these paratenic hosts develop to adulthood after a definitive host consumes these infected tissues. Domestic dogs may serve as either paratenic or definitive hosts for *B. procyonis*, possibly acting as a bridge from the sylvatic or peridomestic environment into domestic environments in closer proximity to people. Finally, infected raccoons in captivity place wildlife professionals (i.e. rehabilitators) and other animals in the facility at risk for exposure if proper precautions are not taken. Illustration by S.G.H. Sapp.

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APPENDIX A

ATTEMPTED ADAPTATION OF THE BpRAG-1 WESTERN BLOT TO ELISA FORMAT FOR CLINICAL AND EPIDEMIOLOGICAL DIAGNOSIS OF BAYLIASCARIASIS IN HUMANS

INTRODUCTION

Currently, a Western blot based on a *Baylisascaris*-specific antigen (Bp-RAG1) is the standard diagnostic assay used for the diagnosis of baylisascariasis in clinical cases. This assay detects total anti-BpRAG1 IgG in both serum and cerebrospinal fluid. While this assay has good performance characteristics (Sensitivity 89%; Specificity 98%), Western blotting is labor-intensive, time-consuming, and generally requires experienced laboratory technicians as there are many steps that are error-prone (Rascoe et al. 2013). An ELISA platform allows faster turnaround, a simpler protocol, and is higher-throughput, which would be useful for both clinical diagnosis and broader epidemiological research applications. Converting the baylisascariasis diagnostic test to an ELISA platform has been attempted previously, however, there was an unacceptable degree of cross-reactivity with toxocariasis specimens (25%) (Dangodoubiyam et al. 2011). Here, additional optimization steps and modifications to the assay protocol were attempted in order to reduce this specificity issue and create an improved Bp-RAG1 ELISA.

EXPERIMENTAL SUMMARIES AND RESULTS

Antigen concentration optimization

To optimize antigen concentration and serum dilution, commercially produced (GenScript), GST-tagged antigen (rBpRAG-1) was serially diluted into coating buffer (50 mM tris-HCl, 1 M KCl, 2 mM EDTA pH 8.0). 100 μL/well of antigen was applied in decreasing concentrations (from 20 μg/mL to 0 μg/mL) across the twelve columns of a 96-well polystryrene microwell plate (Immulon 2HB). Plates were covered and incubated for 2 hours at room temperature, shaking, or at 4 degrees C overnight.

Positive control serum (from a baboon experimentally infected with 100,000 *B. procyonis* eggs) and negative control serum (pooled sera from healthy adults) were diluted serially four times from 1:50 to 1:200 into serum diluent (0.5% dry milk/0.3% Tween-20/PBS) in glass titer tubes. Diluted positive control sera (100 μl/well) were added to the top four rows of the plate and the negative control sera to the bottom four rows of the plate. The plate was incubated for 30 minutes, shaking, at room temperature, then washed four times with wash buffer (0.3% Tween-20/PBS) on a plate washer. Secondary antibody (goat-anti-human IgG-HRP) was diluted 1:8000 into wash buffer and 100 μL was applied to each well of the plate. The plate was again incubated and washed as described. 100 μL of substrate solution (3,3',5,5' – tetramethylbenzidine; KPL Inc.) was added to each well of the plate, which was shaken briefly, and placed in a plate reader. The optical density at 650 nm (OD) was measured until the top left well (i.e. highest concentration of antigen and positive serum) reached a value of ~2.0. Signal-to-noise (SN) ratios (OD positive control/OD negative control, at matched antigen and serum concentrations) were calculated for each

concentration of antigen used. The optimal antigen concentration was chosen based on the peak SN ratio.

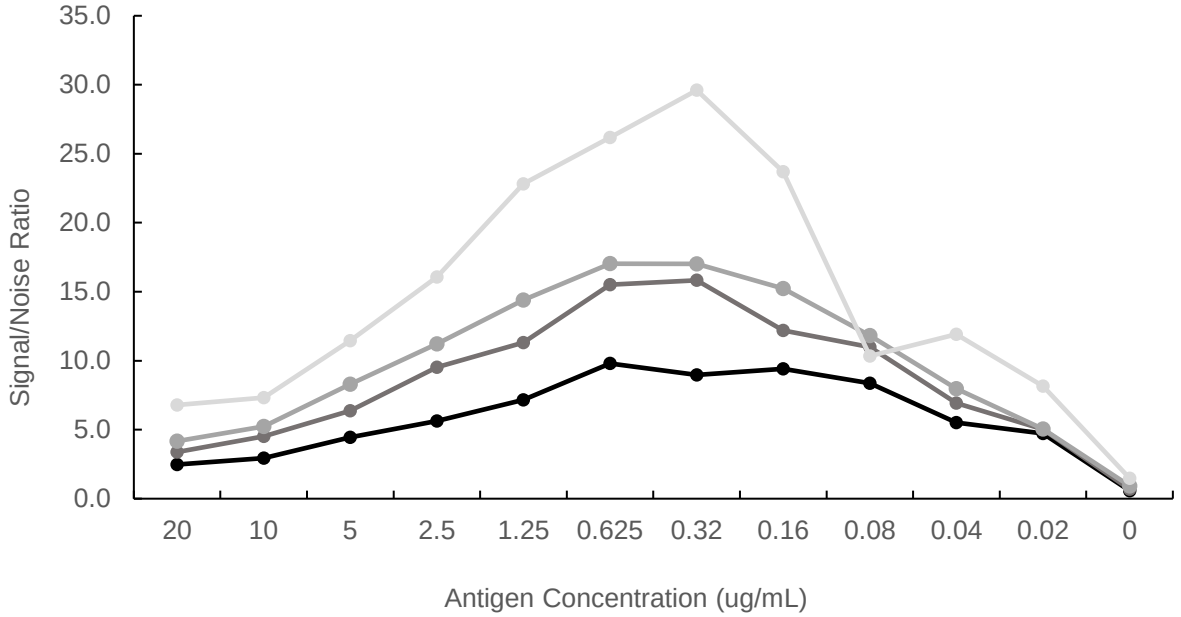

Figure A.1. Signal-to-Noise ratio (SN) of control sera OD values at decreasing concentrations of antigen (rBpRAG-1) in the human-adapted rBpRAG-1 ELISA. Lines represent SN values for serial dilutions of positive control sera (black = 1:50; dark gray = 1:100; medium gray = 1:200; light gray = 1:400).

Secondary antibody concentration optimization

A plate was coated with the optimal antigen concentration found in the previous experiment. Positive and negative control sera were serially diluted from 1:50 to 1:400 as described in the previous experiment, and applied to the top four (positive) and bottom four (negative) rows plate, incubated, and washed as previously described. The secondary antibody

was serially diluted six times from 1:500 to 1:16,000 into 0.3% Tween/PBS in glass titer tubes and applied in duplicate along the columns of the plate. The plate was processed as described and OD values were measured. The secondary antibody concentration yielding the highest SN ratio was considered optimal. The optimal serum dilution value was selected based on the results of both experiments while avoiding extreme values.

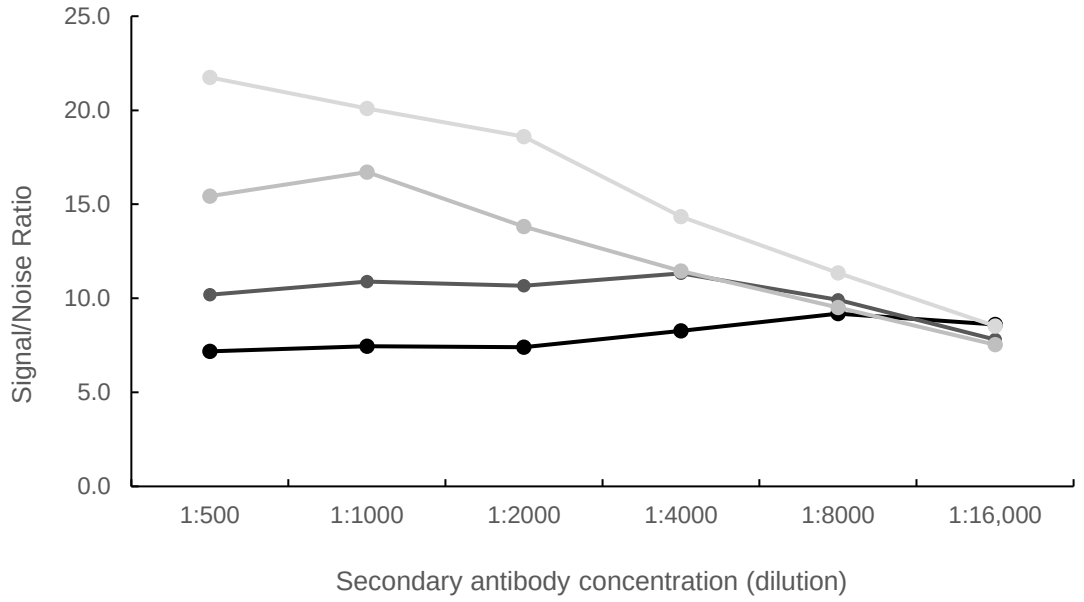

Figure A.2. Signal-to-Noise ratio (SN) of control sera OD values at increasing dilutions of secondary antibody (goat-anti-human IgG-HRP) in the human-adapted rBpRAG-1 ELISA. Lines represent SN values for serial dilutions of positive control sera (black = 1:50; dark gray = 1:100; medium gray = 1:200; light gray = 1:400).

Reaction time optimization

A plate was prepared using the optimal antigen concentration. Positive test sera yielding signals of varying strength on Western blot were selected and diluted to the optimal concentration. Negative control serum and potentially cross-reactive serum (toxocariasis specimens) were also prepared as described. The ELISA was performed using the standard protocols, except immediately after the application of the substrate, the OD values were measured in a kinetic ELISA format. Readings were recorded every 20 seconds at 650 nm for 20 minutes. Resulting SN ratio values over time were plotted, and the time point at which the SN values leveled off was chosen for the reaction time.

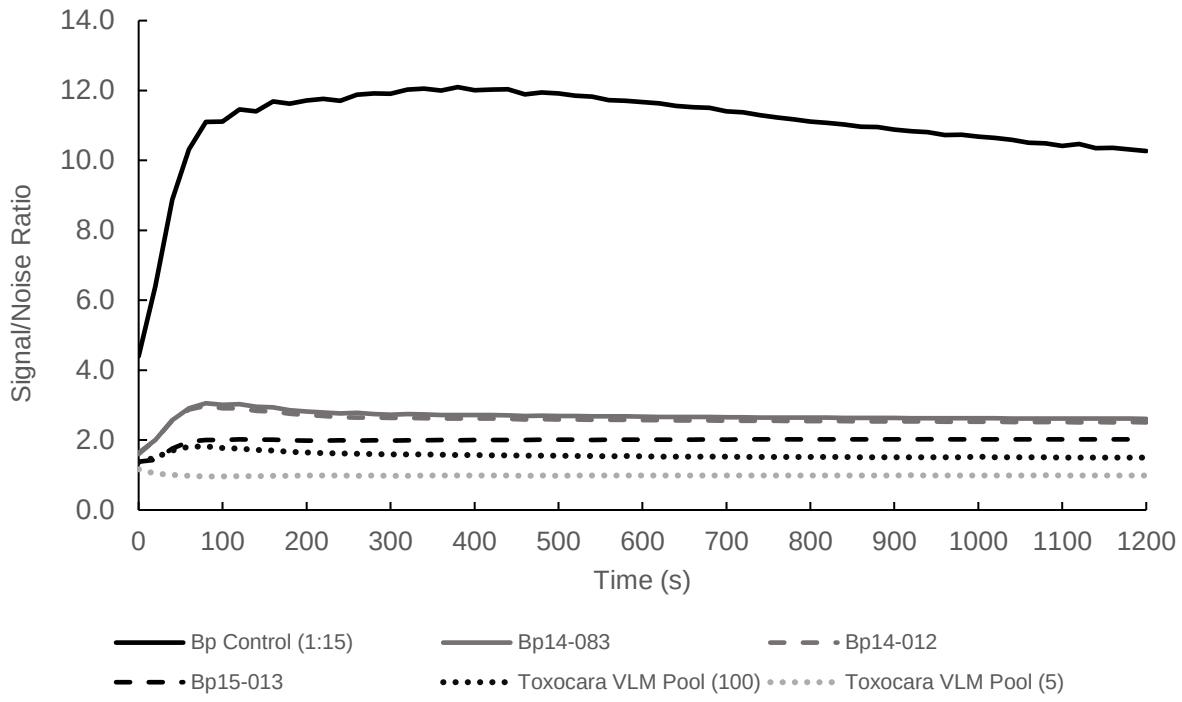

Figure A.3. Kinetic ELISA results using test sera under optimized antigen and secondary antibody concentration conditions. Lines represent signal-to-noise (SN) of OD values ratios over time. Test sera are as follows: Bp control (1:15) (solid black line) represents positive control sera diluted 1:15 in normal human sera. Bp14-083 (solid gray line), Bp15-012 (dashed gray line), and Bp15-013 (dashed black line) are sera from wildlife rehabilitators enrolled in a previous study that tested positive on Western blotting. Diluted pooled serum from toxocariasis cases (*Toxocara* VLM Pool) with 100 (dotted black line) and 5 (dotted gray line) arbitrary units (as defined on TcCTL-1 Luminex assay; see Anderson et al. 2015) were used to assess cross-reactivity.

Standard curve optimization

To create an internal standard curve in arbitrary units (AU), positive control serum (at a 1:15 dilution) was serially diluted into pooled negative sera from 1:5 to 1:40, which were then diluted 1:200 into serum diluent in plastic titer tubes. A plate was coated, washed, and this dilution series was applied in triplicate along rows of the plate. The ELISA was carried out using the optimized conditions. Substrate was applied and allowed to react (shaking, at room temperature) for the optimal time derived from the previous experiment. The reaction was then stopped with the addition of 100 μL of 1.0 N sulfuric acid and the OD values were measured at 450 nm. The dilution with an OD value closest to 2.0 was assigned 100 AU and selected for the "top" of the standard curve.

Table A.1. Conditions of ELISA after initial optimization steps.

Condition	Optimal value
Antigen concentration	0.32 ug/mL (in 50 mM tris-HCl, 1 M KCl, 2 mM EDTA pH 8.0)
Serum dilution	1:200 (in 0.5% dry milk/0.3% Tween-20/PBS)
Secondary antibody concentration	Goat-anti-human IgG-HRP; 1:500 (in 0.3% Tween-20/PBS)
Reaction time	4 minutes
Standard curve	100 AU = OD of positive control serum (1:15) diluted 1:100 into negative pooled sera.

* arbitrary units

Intraplate variation

To assess well-to-well variability, positive control serum at a concentration of 50 AU ("calibrator sample") was diluted 1:200 in serum diluent and applied to 50 wells alongside a standard curve (100, 50, 40, 30, 20, 10, 5, and 0 AU) in the first column of the plate. The optimized protocol (Table X) was carried out, and values in AU of the calibrator samples were derived from the standard curve. The three greatest and three lowest values were eliminated, and the intraplate coefficient of variation was determined (CV = (mean AU / standard deviation)*100). The intraplate coefficient of variation for this assay was 8.9%.

Specificity and sensitivity assessment

Seventy-nine samples from the Centers for Disease Control employee serum bank (EMP sera) were used to assess specificity. 11/79 (14%) of samples yielded AU values of ≥8.0. This AU value represents a temporary working cutoff value based on the mean for all samples run thus far; a more precise cutoff value was not calculated at this point as sensitivity was not evaluated. Two of the 11 "positive" EMP samples were weakly positive on a follow-up Western blot; the rest were negative.

Defined sera from individuals with other parasitic diseases were also included in this specificity analysis. Cross-reactivity to toxocarasis was a special consideration so 29 toxocariasis clinical samples (defined as positivity on TcES ELISA) were included. A high degree of cross-reactivity was observed as 8/29 (28%) samples had an AU of ≥8.0. Among other parasitosis samples, 3/8 strongyloidiasis samples, 1/10 echinococcosis samples, and 1/5 schistosomasis samples yielded an AU of ≥8.0, again

Due to a lack of adequate specificity performance, and the fact that access is limited to the scarce and valuable baylisascariasis clinical case-derived positive controls, true sensitivity was not evaluated. However, Western blot positive sera from a previous study (Sapp et al. 2016) were tested on ELISA. 14/23 (61%) WB-positive samples had an AU of ≥8.0 (above mean) on ELISA; 12/66 (18%) WB-negative samples from the same study had an AU of ≥8.0 on ELISA.

Further assay modifications

Further modifications to the ELISA were attempted to improve performance (i.e. create a larger difference between signal-to-noise ratios between baylisascariasis and toxocariasis samples), which are detailed in the following figures.

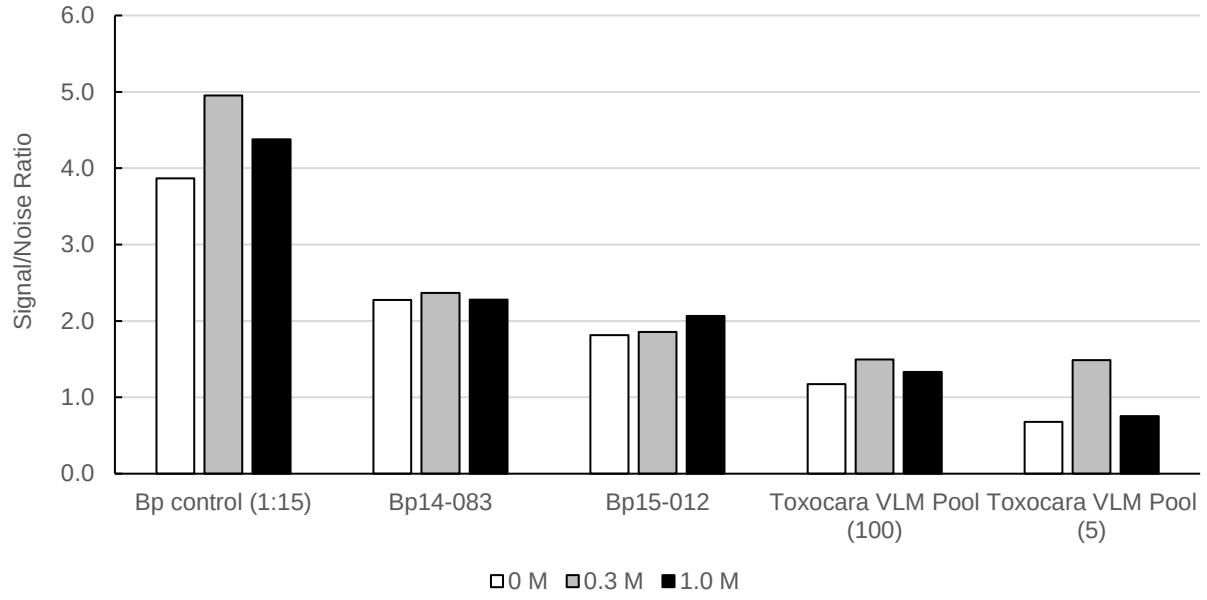

Figure A.4. Signal-to-noise ratio of OD values of various *Baylisascaris* and *Toxocara* positive test sera on ELISA plates coated using buffers with different concentrations of potassium chloride (KCl). Shading of bars represents KCl concentration (White = 0.0 M; Gray = 0.3 M; Black = 1.0 M) in coating buffer (50 mM tris-HCl, 2 mM EDTA, pH 8.0). Test sera are as follows: Bp control (1:15) represents positive control sera diluted 1:15 in normal human sera. Bp14-083, Bp15-012, and Bp15-013 are sera from wildlife rehabilitators enrolled in a previous study that tested positive on Western blotting. Diluted pooled serum from toxocariasis cases (*Toxocara* VLM Pool) with 100 and 50 arbitrary units (as defined on TcCTL-1 Luminex assay; see (Anderson et al., 2015) were used to assess cross-reactivity.

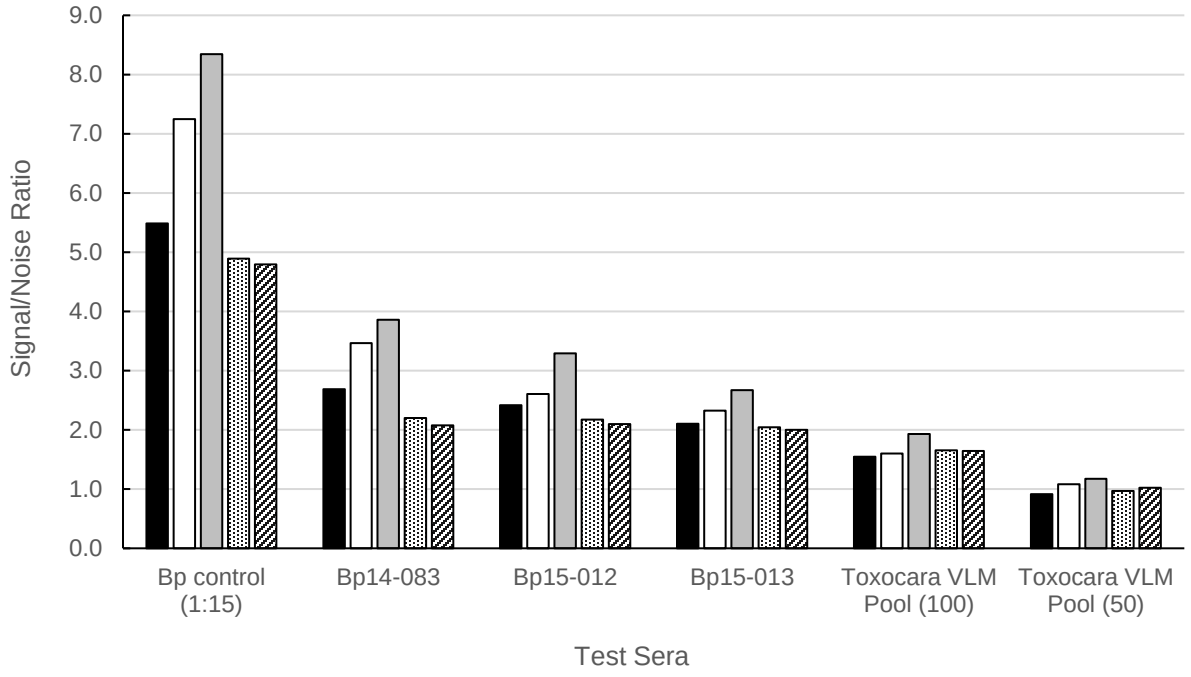

Figure A.5. Signal-to-noise ratio of OD values of various *Baylisascaris* and *Toxocara* positive test sera under different blocking conditions. Shading of bars represents blocking condition (Black = None (PBS); white = 5% dry milk/0.3% PBS-Tween; gray = 10 mM nickel chloride in 5% dry milk/0.3% PBS-Tween; dotted = 1% bovine serum albumin in PBS; hatched = 5% fetal bovine serum in PBS). Test sera are as follows: Bp control (1:15) represents positive control sera diluted 1:15 in normal human sera. Bp14-083, Bp15-012, and Bp15-013 are sera from wildlife rehabilitators enrolled in a previous study that tested positive on Western blotting. Diluted pooled serum from toxocariasis cases (*Toxocara* VLM Pool) with 100 and 50 arbitrary units (as defined on TcCTL-1 Luminex assay; see Anderson et al. 2015) were used to assess cross-reactivity.

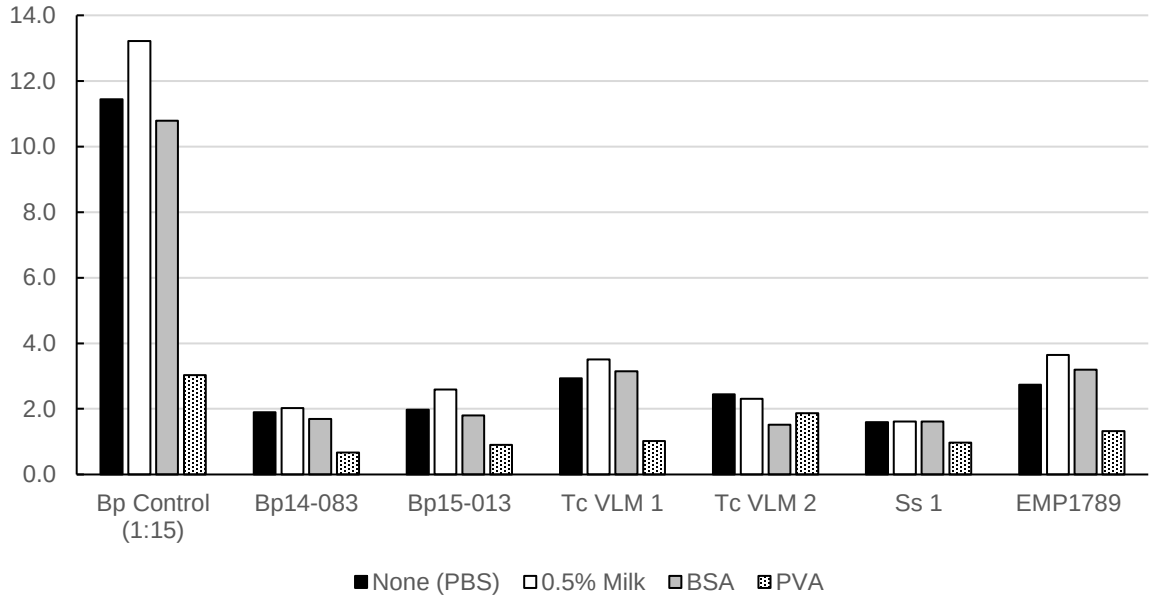

Figure A.6. Signal-to-noise ratio of OD values of *Baylisascaris*-positive and other cross-reactive sera under a second set of blocking conditions, including a non-protein blocking agent (polyvinyl alcohol (PVA)). Shading of bars represents blocking condition (Black = None (PBS); white = 5% dry milk/0.3% PBS-Tween; gray = 5% bovine serum albumin (BSA) in PBS; dotted = 1% PVA). Test sera are as follows: Bp control (1:15) represents positive control sera diluted 1:15 in normal human sera. Bp14-083 and Bp15-013 are sera from wildlife rehabilitators enrolled in a previous study that tested positive on Western blotting. The remainder are all selected sera that were cross-reactive on the optimized ELISA; samples "Tc VLM 1" and "Tc VLM 2" are sera from clinical toxocariasis cases, "Ss 1" is a sample from a clinical strongyloidiasis, and EMP1789 is a sample from a healthy adult.

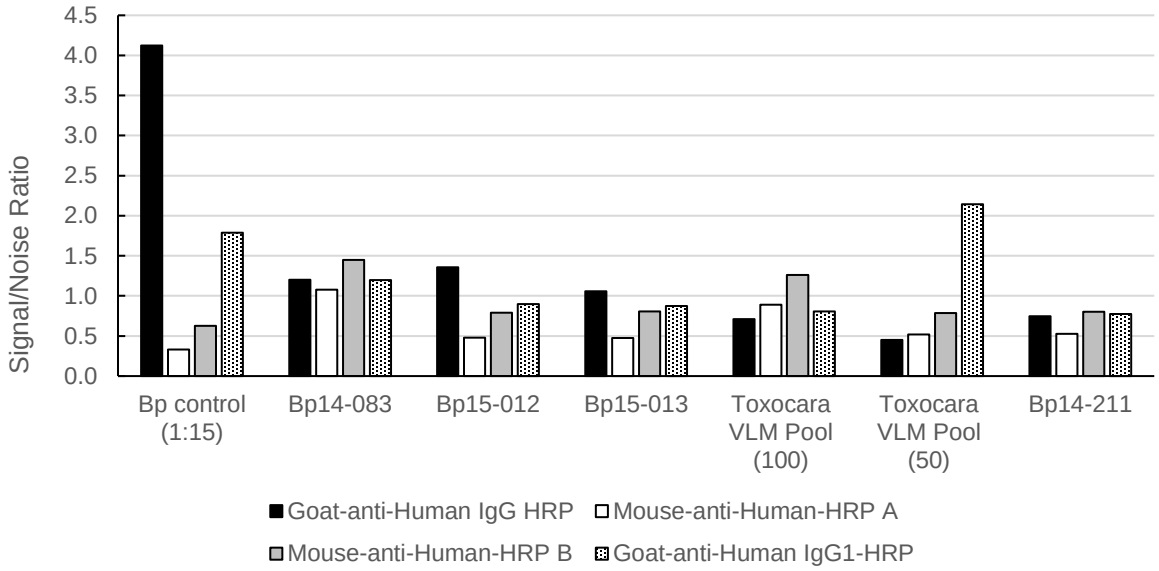

Figure A.7. Signal-to-noise ratio of various *Baylisascaris procyonis* and *Toxocara* antisera on ELISA using different commercially-produced and "house"-produced secondary antibodies. Shading of bars represents which secondary antibody was used [black = Goat-anti-Human IgG-HRP; white = Mouse-anti-Human IgG-HRP A (Jackson Laboratories); gray = Mouse-anti-Human IgG-HRP B (SouthernBiotech); dotted = Goat-anti-Human IgG1]. Test sera are as follows: Bp control (1:15) represents positive control sera diluted 1:15 in normal human sera. Bp14-083, Bp15-012, and Bp15-013 are sera from wildlife rehabilitators enrolled in a previous study that tested positive on Western blotting. Diluted pooled serum from toxocariasis cases (*Toxocara* VLM Pool) with 100 and 50 arbitrary units (as defined on TcCTL-1 Luminex assay; see Anderson et al. 2015), and a serum sample from an individual testing negative on Western blot (Bp14-211) were used to assess cross-reactivity and non-specific recognition.

In an attempt to reduce cross-reactivity of toxocariasis specimens, an adsorption procedure using *Toxocara canis* excretory-secretory (ES) antigen fraction was attempted. ES antigen was obtained previously in standard *Toxocara canis* culture protocols (De Savigny, 1975). Test sera were diluted 1:100 in 0.5% dry milk/0..3% Tween/PBS with either 0, 0.3, or 1.0 μg/mL of *T. canis* ES antigen fraction and incubated for 1 hour, shaking, at room temperature, before use in the ELISA. No reduction in the signal-to-noise ratio of toxocariasis control OD values was observed (Figure A1.8).

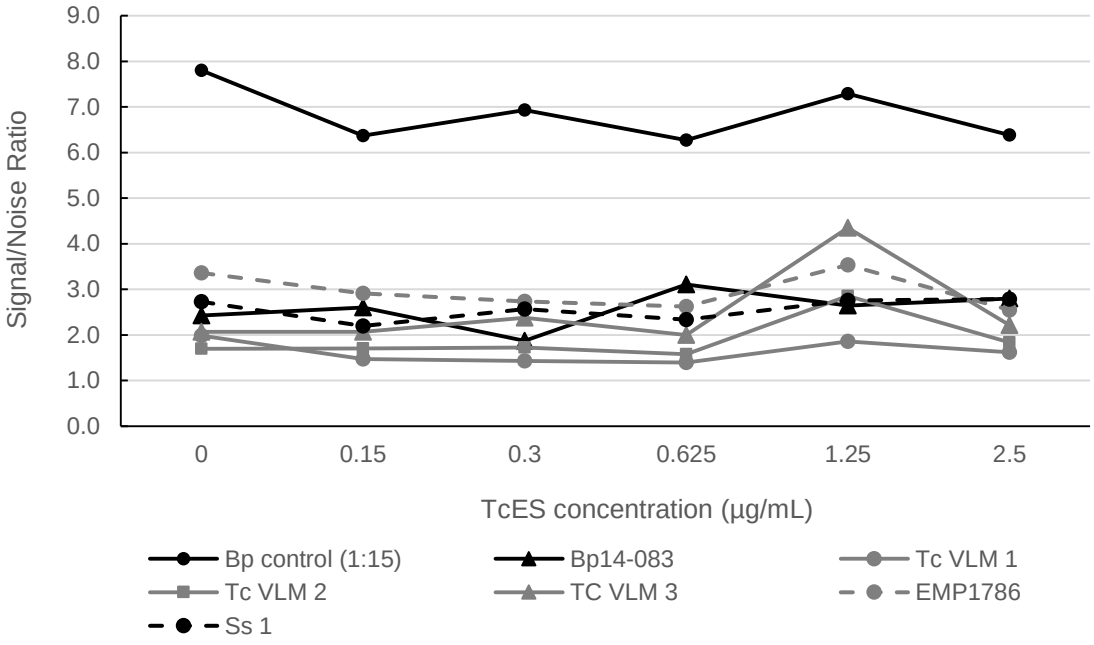

Figure A.8. Signal-to-noise ratio of various *Baylisascaris procyonis* and *Toxocara* antisera on ELISA after one-hour incubation with purified *Toxocara canis* ES antigen. Test sera are as follows: Bp control (1:15) represents positive control sera diluted 1:15 in normal human sera. Bp14-083 and Bp15-012 are sera from wildlife rehabilitators enrolled in a previous study that tested positive on Western blotting. The remainder are all selected sera that were cross-reactive on the optimized ELISA; samples "Tc VLM 1" and "Tc VLM 2" are sera from clinical toxocariasis cases, "Ss 1" is a sample from a clinical strongyloidiasis, and EMP1789 is a sample from a healthy adult.

DISCUSSION

Despite numerous modifications, assay performance remained woefully inadequate. The rBpRAG-1 ELISA developed for use on experimentally infected rodents had generally good performance characteristics, laboratory animals have a very limited exposure to pathogens and thus the chance for unknown cross-reactivity is lower than for humans or animals in natural settings. Our results mirror that of Dangodoubiyam et al. (2011), despite many modifications that were not utilized in that previous attempt (including using a commercially-produced, quality controlled antigen, pre-adsorption with *Toxocara* ES, numerous variations in blocking and coating buffers, and secondary antibody subclass (IgG1 vs total IgG)).

A high proportion of ES antigens are seemingly conserved among ascarids. Western blotting experiments using antisera *Toxocara* spp. and *Baylisascaris* spp. infections to probe ES antigen fractions from both reveal that a great number of antigenic components are cross-recognized (Boyce et al., 1988; Dangoudoubiyam and Kazacos, 2009). BpRAG-1 represents an antigen unique to *Baylisascaris* spp., and in pilot studies, showed very minimal to no cross-reactivity with toxocariasis specimens (Dangoudoubiyam and Kazacos, 2009). However, these pilot studies all utilized western blotting and not ELISA. Even with an identical antigen, assay performance may vary based on the platform of the test. In the BpRAG-1 ELISA initially developed by Dangoudoubiyam et al., 25% cross-reactivity with toxocariasis specimens was observed (versus 0% using the Western blot) (Dangoudoubiyam et al., 2011; Rascoe et al., 2013). Despite our success in using rBpRAG-1 in the rodent ELISA (See Chapter 5), this antigen may not be appropriate for an ELISA platform for human diagnostics. When converting a Western blot to an ELISA format, binding of the antigen to the polystyrene walls of the well may influence which

epitopes of the protein are exposed to the sample (versus a Western blot, in which the porous nitrocellulose strip is submerged in sample and the antigen is presumably completely exposed). Furthermore, antigen conformation may be altered in binding to polystyrene wells which can influence reactivity and performance characteristics (Jitsukawa et al., 1989). Modification of salt concentration in the buffer used in coating may help stabilize the antigen, although this was not successful in the present study (Table A1.4). In summary, BpRAG-1 may not be a suitable antigen for a human diagnostic ELISA. Additional antigen targets or isolation and utilization of specific epitopes from BpRAG-1 may aid in future attempts to develop a diagnostic ELISA.

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APPENDIX B

OPTIMIZATION OF BpRAG-1 ELISA FOR DETECTION OF ANTI-*BAYLISASCARIS* ANTIBODIES IN *PEROMYSCUS* SPP.

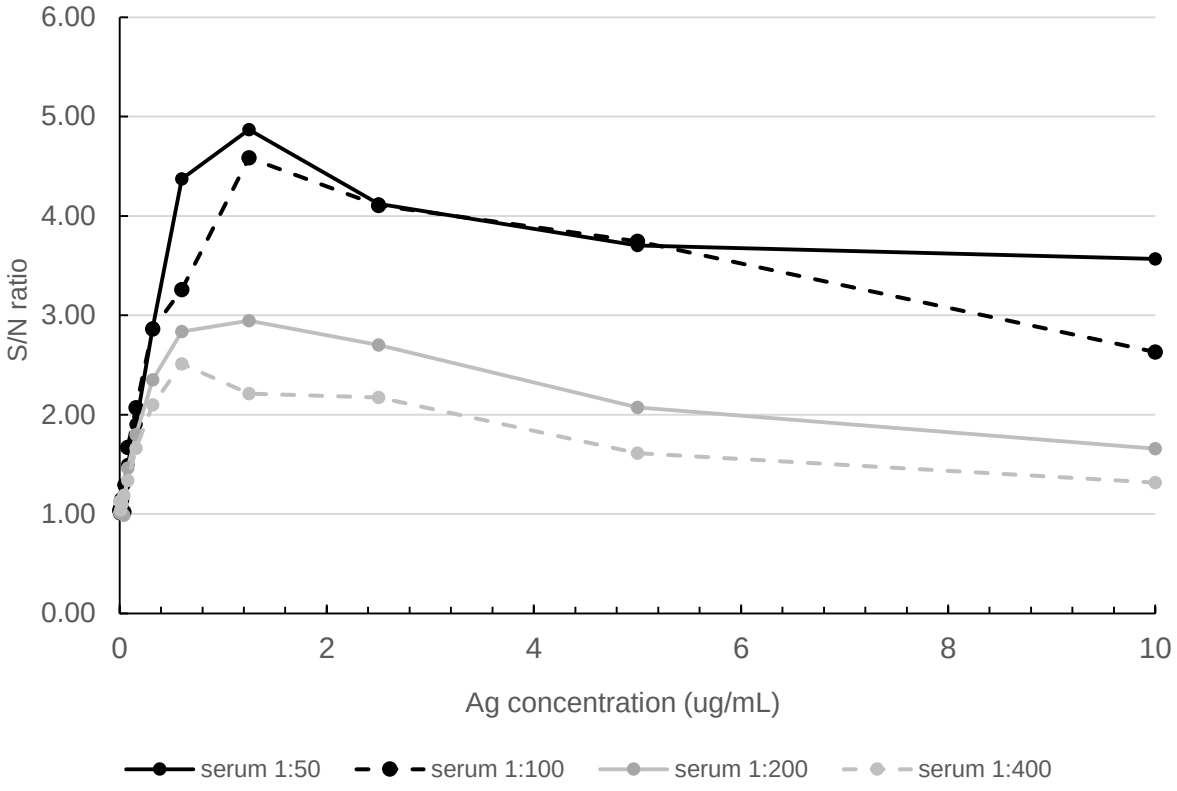

Figure B.1. rBpRAG-1 antigen (Ag) titration for optimization of signal-to-noise (S/N) ratio of optical density for detection of total *Baylisascaris procyonis*-specific IgG in experimentally infected *Peromyscus* spp. Curves represent different dilutions of positive (signal) and negative (noise) control sera. Samples testing strongly positive on Western blot were pooled to create the positive control serum.

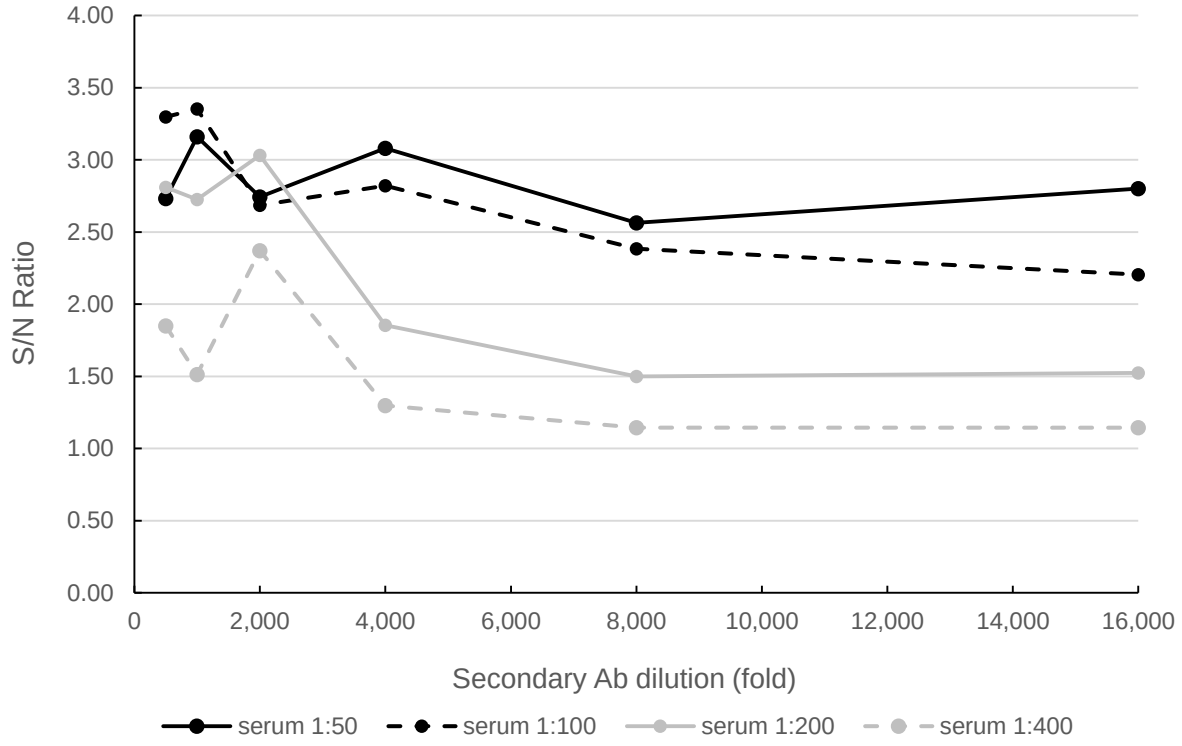

Figure B.2. Secondary antibody (Ab) (goat-anti-*Peromyscus* IgG HRP) titration for optimization of signal-to-noise (S/N) ratio of optical density for detection of total *Baylisascaris procyonis*-specific IgG in experimentally infected *Peromyscus* spp. Curves represent different dilutions of positive (signal) and negative (noise) control sera.

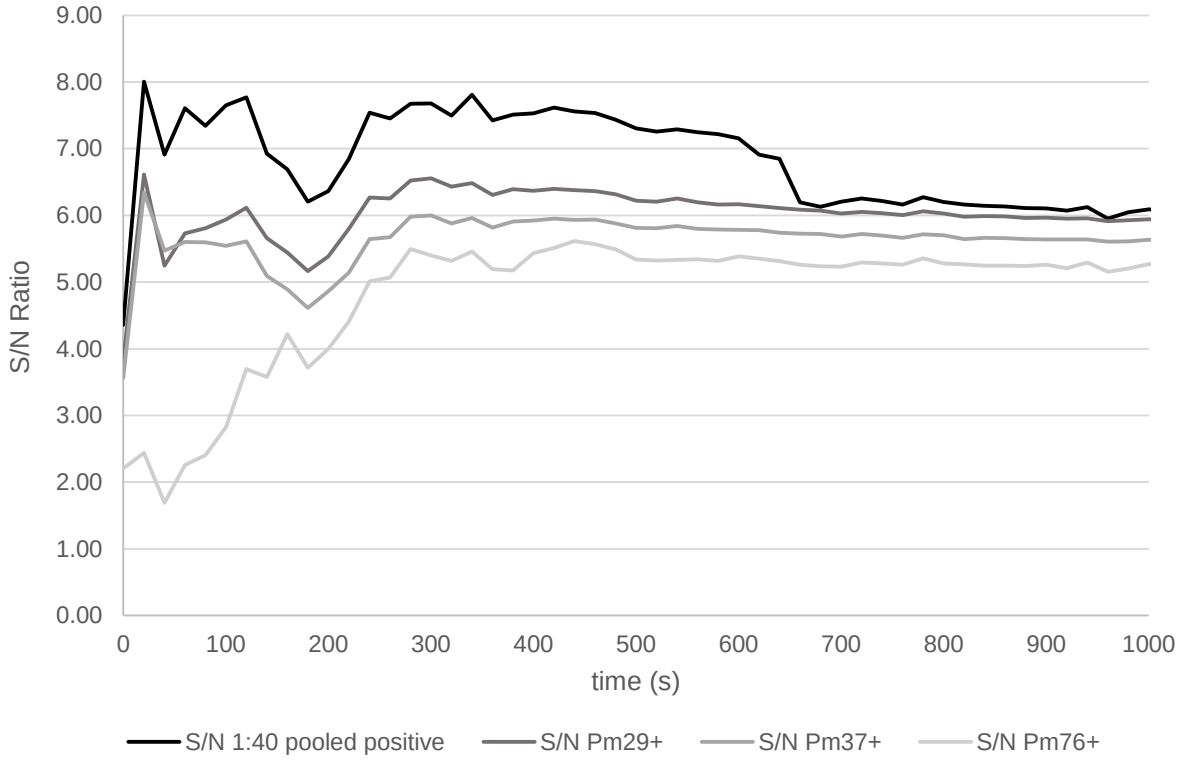

Figure B.3. Kinetic ELISA for determination of optimal reaction time based on of signal-to-noise (S/N) ratio of optical density (OD) for detection of total *Baylisascaris procyonis*-specific IgG in experimentally infected *Peromyscus* spp. Curves represent different dilutions of sera from the pooled positive control diluted 1:40 in control sera (black line) and individual mice with moderate signal positives (determined via Western blotting; gray lines) versus negative (noise) control sera. OD values were read and logged every 20 seconds.