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Baylisascaris procyonis Prevalence and Impact in Raccoon (Procyon lotor) Populations

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(*Procyon lotor*) Populations

by

Matthew Ingle

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A Dissertation submitted in partial satisfaction of
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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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DEDICATION

I dedicate these works to my wife and children, who have sacrificed for me to accomplish this.

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ABSTRACT OF THE DISSERTATION

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Loma Linda University, September 2014
Dr. Stephen G. Dunbar, Chairperson

     Baylisascaris procyonis is a nematode parasite that infects the small intestines of

raccoons (*Procyon lotor*). Raccoons are the definitive host for this parasite, which can

have both an indirect and direct life cycle. In intermediate hosts, the parasite causes

serious pathology resulting from larvae migrating through host tissue to the brain.

Infection via exposure to *B. procyonis* eggs is fatal in over 90 vertebrate species that

serve as intermediate hosts, including humans. The zoonotic potential of *B. procyonis*

makes understanding factors that impact prevalence and pathology in raccoons important

for management strategies. Alimentary canals from 226 raccoons trapped in nine

townships of southwestern Ohio were collected and necropsied along the full length of

the intestines. All *B. procyonis* individuals found were collected, noting the presence and

abundance in each raccoon necropsied. Using these data, we compared the nine

townships, and noted that Beavercreek Township had significantly lower *B. procyonis*

prevalence than any other township. To determine how landscape features impact *B.

procyonis* prevalence, the proportion of urban versus agricultural land use and mean

patch size were calculated for each township. We found that the total proportion of

landscape modified by agriculture was the best predictor of *B. procyonis* prevalence. We

also isolated DNA from tissue of the greater omentum from the raccoons, and amplified

four loci. We used the sequences from NADH5 to build phylogenetic trees for the nine

townships, and used the other three loci to demonstrate evidence of genetic structuring

and determine the impact of *B. procyonis* on raccoon population genetics. There is

evidence of genetic structuring, and raccoons from areas with lower ($< 60\%$) *B.*

procyonis prevalence had more genetic variability than other raccoon populations.

Finally, we removed the stomach contents from each raccoon, noting the type of tissue

found and calculating the proportion of the total mass made up of plant tissue. Raccoons

from areas with lower *B. procyonis* prevalence had significantly less plant material in the

stomachs at necropsy than other raccoons. These data demonstrate that we can predict *B.*

procyonis prevalence from landscape features, and that *B. procyonis* impacts raccoon

genetics and behavior.

CHAPTER ONE

INTRODUCTION

Goals, Objectives and Hypotheses

- The first goal of this study was to assess the severity of *B. procyonis* parasitism of raccoons in southwestern Ohio, an area fragmented by both agriculture and urbanization.

  - The first objective to meet this goal was to assess the prevalence of *B. procyonis* in Southwest Ohio.

    - I hypothesized that the prevalence of *B. procyonis* would be higher in this area than in previously studied areas because of the intense agricultural and urban fragmentation, which provides reliable resources and denning sites.

  - The second objective to meet this goal was to determine the relationship between the landscape of a population of raccoons and the prevalence of raccoon roundworm.

    - I hypothesized that the prevalence of *B. procyonis* would be higher in populations that are from more fragmented landscapes, because of increased population densities and more interaction between host individuals.

- The second goal of this study was to determine the effects of *B. procyonis* on *P. lotor*.

o The first objective to meet this goal was to construct a phylogenetic tree of

the populations in the study area and determine the molecular distances

among the populations, using multiple nuclear and mitochondrial

segments.

- I hypothesized that the populations would be separated more than

can be accounted for by physical distance alone due to the

selective pressure exerted by *B. procyonis* on raccoons.

o The second objective to meet this goal was to determine whether the

populations of *P. lotor* are evolving.

- I hypothesized that the population in the areas fragmented by both

agriculture and urbanization would show signs of molecular

evolution, resulting from the coevolution of the raccoons and *B.*

procyonis worms.

o The third objective to meet this goal was to determine whether there are

ecological and behavioral differences between populations that can be

attributed to parasite prevalence.

- I hypothesized that populations in areas fragmented by both

agriculture and urbanization would have a different diet than

populations in more rural areas, and that these differences would

be attributed to high prevalence of *B. procyonis*.

Baylisascaris procyonis and Raccoons

Raccoons (*Procyon lotor*) are the definitive hosts for the raccoon roundworm,

Baylisascaris procyonis (Page et al., 2005). Raccoon roundworms are the leading cause

of the dangerous neurological disease larva migrans encephalopathy, infecting and

causing pathology in over 90 vertebrate species that serve as intermediate hosts in this

parasite's life cycle (Blizzard et al., 2010). Intermediate hosts are attracted to raccoon

latrines, where raccoons repeatedly defecate and roundworm eggs may be concentrated

(Page et al., 2001b). As the density of raccoon latrines increases, the percentage of

latrines containing *B. procyonis* increases (Roussere et al., 2003). Roussere et al. (2003)

also demonstrated that the density of raccoon latrines increased in areas with more

landscape fragmentation (creation of landscape discontinuity by converting native

landscape into patches of different landscape types). Therefore, landscape fragmentation

also increases the prevalence of *B. procyonis* parasites in intermediate hosts that use

raccoon feces as a source of food, leading to higher parasite prevalence in raccoons that

prey on some types of intermediate host species (Page et al., 2005). Comparisons of *B.*

procyonis prevalence in raccoons and intermediate hosts tend to group animals into areas

that have significant landscape fragmentation (Page et al., 2001a, 2001b). As the

prevalence of raccoon roundworm increases in raccoons, more intermediate hosts become

infected. *B. procyonis* is a common parasite in the white-footed mouse, *Peromyscus*

leucopis, which inhabits a wide variety of landscape types. Page et al. (2001a) found that

white-footed mice from fragmented landscapes with a high agricultural activity (greater

than 50% of total landscape) had significantly higher prevalence, intensity of infection (a

measure of the number of parasites per infected host individual), and abundance of *B.*

procyonis larvae than mice from homogenous, forested landscapes. Additionally, they

found that within heterogeneous landscapes, forest patch area and degree of isolation

were significant predictors of infection probability, intensity and average number of larvae.

Raccoons have responded to agriculturally-induced fragmentation of native forestland by moving closer together to produce high density populations (Page et al., 2001a). This has increased encounter rates between white-footed mice and other vertebrate intermediate hosts for *B. procyonis*) and the infective stages of *B. procyonis* (Blizzard et al., 2010; Page et al., 2001a). These interactions increase the transmission of the parasite to intermediate hosts, which in turn increases raccoon exposure to the parasite. Physical changes in native landscapes alter the abundance and distribution of species, which alters transmission of parasites among these species (Page et al., 2001a)

Parasite Prevalence

Prevalence

Prevalence refers to the number of individuals of the host species infected with a particular parasite, divided by the total number of hosts in the study (Margolis et al., 1982) (i.e. a proportion expressed as a percentage). Margolis et al. (1982) noted that multiplying the mean intensity of infection (mean number of parasite individuals per host individual in the study) by the prevalence, results in the abundance of a particular parasite (mean number of individuals of a particular parasite species per host individual). These terms are valuable tools for quantifying parasite infections, and provide insight into potential implications of infection.

Native animals can be negatively impacted by the parasites of closely related invasive species (Landaeta-Aqueveque et al., 2014; Romeo et al., 2014). Romeo et al. (2014) noted that invasive species also benefit in this exchange as they lose some of their

parasites to native species (this is termed "enemy release"). The European native red squirrel, *Sciurus vulgaris*, was affected by being introduced to new parasites from invasive squirrel species (spillover) and by coming into contact with more individuals of common parasites (spillback). Romeo et al. (2014) found 7 helminths and 4 parasitic arthropods in the alimentary canal of the invasive grey squirrels (*S. carolinensis*). Species richness was lower in the areas where the grey squirrel is invasive than in the native range, indicating that the hosts are benefitting from enemy release. The most common parasite was *Strongyloides robustus* (prevalence = 56.6%), which correlated positively with grey squirrel density.

Factors Contributing to Prevalence

Many ecological factors impact parasite prevalence, as host population dynamics, fitness, and densities change in response to changing landscapes (Page et al., 2005; Prange et al., 2011). For instance, helminth parasites influence population dynamics and herd sustainability in the caribou, *Rangifer tarandus groenlandicus*, with removal of intestinal helminth parasites associated with increased fat reserves and increased pregnancy rates (Steele et al., 2013). Additionally, geographical isolation contributes to differences in prevalence and intensity of infection of the same host by the same parasite (Steele et al., 2013). Steele et al. (2013) argued that these differences may explain variance in species diversity (when parasites cause local extinctions), host genetics, host health status, timing of sampling, or sampling years.

In their review of the literature, Young et al (2013) maintain that biodiversity reduces the prevalence of disease. This 'diffuse effect' is used to explain the link between

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high prevalence (60%) of zoonotic diseases (transmitted from animals to humans) in humans and decreasing biodiversity. When species become extinct, diseases become more prevalent in species that interact with humans, yet when the opposite happens, diseases will become less prevalent in individual species and communities (diffuse effect). However, others argue that increasing biodiversity leads to increased parasitism (Landaeta-Aqueveque et al., 2014). Young et al. (2013) presented an alternate view, arguing for a positive correlation between biodiversity and disease risk. Positive correlations between disease risk and biodiversity (amplification effect) lead to opposite predictions from those of the diffuse effect (Landaeta-Aqueveque et al., 2014). In support of this theory, Young et al. (2013) showed that 3 out of the 12 genera of parasites surveyed showed significant relationships between prevalence and host resilience to conversion of natural landscape into agriculture. Two of the genera (*Ascaris* and *Oesophagostomum*) had a positive relationship (prevalence increased with host resilience to landscape changing into agriculture). However, habitat disturbance and parasite prevalence are related. Seven of 14 studies showed negative effects of disturbance on prevalence, six showed positive effects and one showed no effects (Young et al., 2013) indicating that many other factors affect parasite prevalence in hosts.

The degree of domestication (feral introduced mammals, livestock, and pets) of introduced mammals is correlated with the amount of spillover of parasites into native species (Landaeta-Aqueveque et al., 2014). Domestic mammals have higher numbers of introduced parasites and spillover parasites than wild mammals, with pets having the highest of both types of parasites. These animals, especially pets, spread new parasites to native animals more often than introduced feral mammals. Parasites and hosts are

intimately related; thus, when prevalence increases, so do the effects of the parasite on the host.

Hosts and parasites exert selective pressures on each other, allowing parasites to influence host life-history characteristics (Cooper et al., 2012). Cooper et al. (2012) tested two opposing hypotheses: (1) if parasite burdens cause increased mortality, host longevity should correlate negatively with parasite species richness; (2) if long-lived hosts present a more stable environment, host longevity should correlate positively with parasite species richness. Ungulate host longevity provides evidence for the first hypothesis, but carnivore and primate longevity did not relate to parasite species richness (Cooper et al., 2012).

There is little support for the hypothesis that conserving biodiversity reduces the risk of infectious disease in primates (Young et al., 2013). Young et al. (2013) also found that primates that are more resilient to disturbance are not more susceptible to parasites than other primates that are not as resilient to disturbance. The extent of infection by *Plasmodium* in chimpanzees supports the amplification effect, with prevalence higher in areas with high mammal species richness (Young et al., 2013).

Prevalence of particular parasites varies within different populations of the same host species based on many factors (Romeo et al., 2014; Steele et al., 2013). Steele et al. (2013) contended that major differences in prevalence based on life history stages of the host indicated that two different species of parasites are infecting the hosts. For instance, caribou in some areas were infected as adults by some species of nematodirines, but only as juveniles in other areas. Even genetically and geographically related species had qualitative and quantitative differences in parasite loads, with variation in prevalence,

mean intensity, and distribution across age classes. Grey squirrels lost many of their

normal parasites and picked up several novel parasites when invading Palearctic areas,

(Romeo et al., 2014). Romeo et al. (2014) stated that when few individuals found a new

population or invade a new area, many of the parasite species from the native range are

not brought into the new area.

There is a weak relationship between parasite species richness and host longevity

(Cooper et al., 2012). Ungulate species with long average lifespans do have fewer

parasites than short-lived ungulate species. Cooper et al. (2012) noted that both

competing hypotheses introduced above (diffuse effect and amplification effect) could

explain the relationship between parasite species richness and host longevity. Increases in

parasite species richness may decrease longevity in some individuals, whereas other

individuals may live longer and provide a stable environment for several parasite species.

This is especially true in domesticated species (Landaeta-Aqueveque et al., 2014).

Landaeta-Aqueveque et al. (2014) noted that domestic animals pose a health risk to

native species as domestic animals are long-lived and provide a stable environment for

many parasite species. Long-lived animals do not invest as much in immune responses

against parasitic infection as shorter-lived animals. In fact, Cooper et al. (2012) also

found no relationship between host longevity and white blood cell count. They argue that

longer-lived mammal species invest more in behavioral defenses against parasite

infection than shorter-lived species do. Longer-lived species avoid contaminated areas,

invest in allogrooming, and ingest medicinal plants (Cooper et al., 2012). Many species

of mammals invest more in behavioral responses to parasitism than physiological

responses. This is especially true for parasites that cause little pathology when in small

numbers.

Calculating Prevalence

As noted above, parasite prevalence represents the percentage of host individuals infected with a particular parasite (Margolis et al., 1982). This is a useful measure of the relative extent of parasitic infection. We use prevalence to compare different populations, and make predictions about the potential zoonoses of parasites. When prevalence is high, more individuals of both the normal hosts and accidental hosts are exposed to the parasite. Schurer et al. (2013) collected trapped, hunted, and road killed wolves to study *Echinococcus* spp parasites prevalence in them. They removed all individual worms from wolf intestines and recorded both parasite presence and number. The researchers also used species-specific primers and PCR to amplify particular sequences from *Echinococcus multilocularis* or *Echinococcus canadensis* to calculate prevalence of each species of cestode. Steele et al. (2013) collected caribou during scientific hunting expeditions. The researchers removed rectal feces, processing them using a centrifugation-sugar flotation technique, and identifying the eggs or oocysts down to family or genus level based on morphology. In each study, the researchers were able to assess that populations with higher prevalence were most likely to be the source of parasite spillover into other animals and humans.

Page et al. (2001a) collected mice from 17 small farmland woodlots and three larger forested areas using Sherman[TM] live traps, and euthanized them using Methoxyflurane (Metofane[TM]). They removed the brains, pressed them between glass plates, and examined them for *B. procyonis* larvae. The researchers digested the rest of

the mouse tissue using acid-pepsin solution (1% pepsin and 1% hydrochloric acid and 0.85% saline), fixed all nematodes, identified *B. procyonis* larvae, and calculated prevalence for mice from each of the sample areas. This study demonstrated the effects on intermediate hosts when parasites are especially high in definitive hosts, with more intermediate hosts being infected with *B. procyonis* when prevalence was high in raccoons. This spillover is especially important in raccoons with *B. procyonis*, as the larvae of the worm are often fatal to intermediate hosts.

B. procyonis eggs are incredibly resilient even in temperate areas, where freezing cycles do little to reduce the viability of the larvae inside (Shafir et al., 2011). Because of the potential danger associated with this parasite infecting humans, proper techniques to estimate prevalence (percentage of host individuals with the parasite) are important. There are three major techniques to estimate *B. procyonis* prevalence in raccoons (Page et al., 2005). The first technique is to necropsy the raccoons, checking the intestines for *B. procyonis* worms (gut analysis). This technique is the most reliable, but is labor intensive and inefficient for large scale studies (Page et al., 2005). The second technique is to sample latrines; however this often underestimates prevalence (Page et al., 2005). The third technique is through fecal analysis, which provides a reliable method for estimating prevalence. Page et al. (2005) noted that fecal analyses do give false negatives for some raccoons, and found that fecal samples measured prevalence at 17.5%, whereas necropsies estimated prevalence at 44% (Page et al., 2005). They therefore suggested that fecal analyses might underestimate prevalence. Page et al. (2005) demonstrated that there are different methods for calculating prevalence with varying degrees of accuracy. It may be necessary to employ several methods to properly determine prevalence and

demonstrate the potential for zoonosis.

Future Work on Prevalence

Many parasites can infect humans, leading to zoonotic diseases (Schurer et al., 2013; Young et al., 2013), and illustrating why it is important to understand the potential animal and public health implications of various parasites. For instance, some parasites infect valuable animal populations (Schurer et al., 2013; Steele et al., 2013). Many of these parasites interact in complex ways with their hosts, with differing quantitative features (Steele et al., 2013), and constructing proper management strategies against particular parasite species may require determining quantitative differences. Schurer et al. (2013) advocate that more work needs to be done to understand all of the public health implications of cestode prevalence, and presence of foreign strains of common parasites, since prevalence is a strong indicator of zoonotic potential (Page et al., 2011; Schurer et al., 2013). As prevalence increases, the likelihood of humans coming into contact with infective eggs increases.

Dozens of populations of raccoons in the Midwestern United States maintain calculated *B. procyonis* prevalence close to 100% (Page et al., 2005). Because raccoon populations appear to be increasing in number in response to anthropogenic factors and land fragmentation (Prange et al., 2011), it is becoming more important to study transmission of *B. procyonis* to intermediate hosts (Page et al., 2005).

Phylogenetic Studies

Phylogenetics and Population Genetics

Phylogenetics is the study of evolutionary relatedness between taxa, and is critical when addressing evolutionary hypotheses (Kelly, 2005). A phylogeny, or phylogenetic tree, is a representation of these relationships, where each branch represents a lineage. A single branch can represent a higher taxon, such as Class Mammalia, or can represent a single population. Molecular data, such as amino acid and nucleotide sequence, morphological data, and chromosomal data are used as characters to construct phylogenies.

Comparative phylogenies illustrate co-evolutionary events between species living in a symbiotic relationship with each other (Krasnov et al., 2004; Srithayakumar et al., 2012; Srithayakumar et al., 2011; Wanntorp et al., 1990). The phylogenetic relationships between parasite populations explain how parasites exploit their hosts, and which hosts can be exploited (Krasnov et al., 2004). Host major histocompatibility complex (MHC) genes evolve in response to parasite loads in populations as parasites co-evolve along with their host (Srithayakumar et al., 2012; Srithayakumar et al., 2011).

Evolutionary trees have enhanced the study of animal ecology (Wanntorp et al., 1990). Phylogenetic studies provide a base that strengthens both problem formation and analysis in ecological studies. Researchers have provided regional explanations for local diversity, and explained when and how radiation events take place. Finally, phylogenies explain effects on the genome that happen during the evolution of a lineage (Aguileta et al., 2004; Holmes et al., 2008; Huxley-Jones et al., 2004; Wilkinson et al., 2005). Aguileta et al. (2004) used phylogenies to postulate where genes were duplicated, and what happened after the duplication event. They concluded that there are a series of

nonsynonomous mutations that follow a duplication event. Conventional theory holds that gene duplications and nonsynonomous mutations fuel evolution in a lineage. The collagen X family, containing C1q and TNF, may have radiated along with the evolution of the vertebrate lineage (Huxley-Jones et al., 2004), providing a possible sequence for some of the evolution of the extracellular matrix in vertebrates. Phylogenies have suggested positive selection as a possible explanation for the evolution of the family of relaxin-like peptides found in mammals and other vertebrates (Wilkinson et al., 2005).

There are some difficulties in constructing and interpreting phylogenies (Broughton et al., 2000; Davalos, 2004; Fulton & Strobeck, 2007; Koefli et al., 2007; Rutschmann, 2006; Welch et al., 2008). Broughton et al. (2000) determined that because functional constraints keep nucleotides at the first and second positions in a codon from changing, substitutions typically produce homoplasies (instances that look like common ancestry but are not), which are difficult to separate from true homologies in phylogenetic analyses. Davalos (2004) found that some of his conclusions about the phylogeny of raccoons did not match the geological history of the Caribbean Islands, necessitating some taxon-specific biogeography of the islands. Phylogenies constructed using nuclear DNA can differ from those using mitochondrial DNA (mtDNA) (Fulton & Strobeck, 2007). Molecular phylogenies can disagree with the fossil evidence and morphological phylogenies (Koefli et al., 2007), because genes evolve at different rates, even within the same organism (Rutschmann, 2006; Welch et al., 2008). Issues with correlating the results of many phylogenies, however, are outweighed by the benefits of the hypotheses resulting from constructing phylogenies, especially using molecular characters (Patterson et al., 1993).

Analyses of populations are another way to determine the relationships between animal groups. A population is a group of all individuals of a species that occupy the same area at the same time. These individuals have the potential to interact through both competition and mating, and thus have the potential to impact and change the relative frequencies of alleles present in the population (microevolution). In populations, some mutations are more likely to become fixed than others (Broughton et al., 2000). Transitions (single nucleotide substitutions involving the change from one purine to another or one pyrimidine to another) are more likely to become fixed than transversions (single nucleotide substitutions where a purine changes to a pyrimidine or a pyrimidine changes to a purine). This information is useful for weighting studies that compare the relatedness of the populations (Broughton et al., 2000). When constructing phylogenetic trees of raccoon populations, transversions are weighted differently than transitions. These are valuable tools in assessing the genetic relatedness of several populations of the same species, including raccoons.

Phylogenetics and Population Genetics of Mammals

Phylogenies enhance the understanding of mammalian evolution (Alroy, 1999; Davalos, 2004; Kelly, 2005; Kemp, 2007; Waddell et al., 1999; Welch et al., 2008). Mammals first appear in the fossil record during the Mesozoic Era, with modern mammals arising out of lineages that began diversifying intensely during the Cenozoic (Alroy, 1999). Phylogenies imply the evolution of more specialized carnivores as mammals diversify with ecological opportunity (Kemp, 2007). Kemp (2007) maintains that this has resulted in the evolution of new taxa within Class Mammalia. For instance,

morphological and molecular phylogenies explain evolution within a mammalian order (Lapointe et al., 1999; Lim, 2008; Stoner et al., 2003; Symonds, 1999). Phylogenies revealed that some members of Order Chiroptera arose from sympatric speciation, without a vicariance event (Lim, 2008). The subspecies of raccoons seem to have followed a similar mechanism for genetic isolation (Cullingham et al., 2008).

Morphological and molecular phylogenies can also explain evolution within mammalian families (Barrow & Macleod, 2008; Ferguson et al., 2006; Nash et al., 2001; Robinson & Matthee, 2005; Sacks & Louie, 2008; Tellgren-Roth et al., 2008). Barrow and Macleod (2008) used traits in the dentary bone of members of Family Talpidae (moles) to construct a phylogeny of the family. They contend that shape is an important tool for classification and for constructing phylogenies, and that it is important to consider substructures in order to construct an accurate phylogeny (Barrow & Macleod, 2008). Phylogenetic studies revealed that delayed implantation in mustelids (Mustelidae: Mammalia) evolved several times, with the delayed implantation of the zygote not correlating with the evolution of a smaller body size (Ferguson et al., 2006). Phylogenies, using chromosomes as characters, revealed the fission and fusion of chromosomes in the lineage of canids (Canidae: Mammalia) (Nash et al., 2001). Robinson and Matthee (2005) used phylogenies to demonstrate monophyly in 11 leporid (Leporidae: Mammalia) genera, and explained the lineage that produced each genus. In another example, two subfamilies of Bovidae, Bovinae and Antilopinae, diverged shortly following the divergence of Cervidae and Bovidae (Tellgren-Roth et al., 2008). Phylogenies have enhanced the understanding and study of many mammalian families, serving to build a foundation of mammal phylogeny that studies of raccoon genetic structures have been

based upon.

Mammal populations can be ranked on a slow-fast continuum with respect to growth rate (Gaillard et al., 2005; Oli & Dobson, 2005). Many life-history variables affect the position of a population along this continuum. Oli and Dobson (2005) argue that no life history variable has the largest effect on the elasticity of the growth rate in all mammal populations, whereas Gaillard et al. (2005) maintain that age of first reproduction is the main index for the position on the continuum. Nearly 60% of the variation in importance of the variable on the growth rate is accounted for by allometry and taxonomic position (Gaillard et al., 2005). Faster growth rates correspond to more rapid opportunities for molecular evolution, indicating that growth rate of a population should be known before going through phylogenetic analyses of the populations. Raccoons have a moderate growth rate, with males and females reaching sexual maturity at 2 years and 1 year, respectively (Prange et al., 2011).

Phylogenetics and Population Genetics of Raccoons

Phylogenies confirm two subfamilies within the raccoon family (Procyonidae: Carnivora) based on both nuclear and mtDNA (Fulton & Strobeck, 2007). Raccoons and other procyonids have a poor fossil record, with procyonids appearing in the Oligocene, and raccoons splitting off and diversifying during the Pleistocene (Cullingham et al., 2008; Fulton & Strobeck, 2007; Koefli et al., 2007). Procyonidae split during the Miocene, producing the two subfamilies: Procyoninae and Potosinae. Fulton and Strobeck (2007) discovered three lineages within the family (with *Nasua* forming a sister group to the rest of Procyoninae), and argue that future phylogenies can explain the

genetic change that created the split in the lineage to produce the two subfamilies.

Raccoon genetic structure plays a significant role in how the populations diverge from each other, and in how diseases move through these populations (Cullingham et al., 2008; Santonastaso et al., 2012). Cullingham and colleagues (2008) discovered three lineages that contain all four subspecies of the raccoon, *Procyon lotor*, with limited barriers to gene flow. The Mississippi River prevents contemporary gene flow in the southern portion of the range, and genetic isolation prevents some gene flow between two lineages east of the Mississippi. Most of the variation in mtDNA occurs within populations, demonstrating some of the genetic structure between these populations, which affects how rabies and other diseases move through these populations (Cullingham et al., 2008).

Nuclear DNA from raccoons contains large amounts of variation within and between populations, these variable sequences allow determination of genetic structure and separation (Cullingham et al., 2006; Dharmarajan et al., 2009; Fike et al., 2007; Santonastaso et al., 2012). Cullingham et al. (2006) discovered that polymorphic tetranucleotide (containing four nucleotides) microsatellites from the nuclear DNA of raccoons contained between 6 and 25 alleles, with heterozygosity ranging from 0.674 to 0.925. These microsatellites were not presently evolving, as all alleles were at Hardy-Weinberg Equilibrium (HWE) (Cullingham et al., 2006).

Raccoons thrive in anthropogenically-fragmented landscapes, which function to further construct genetic structure between "neighborhoods" within a population (Dharmarajan et al., 2009; Santonastaso et al., 2012). Dharmarajan et al. (2009) demonstrated that there was genetic structure between the 29 spatially distinct land

patches forming these neighborhoods, where 30% of the variation in the genome was

accounted for by variation in patch size and percentage of land represented by forest. The

landscape from the study by Dharmarajan et al. (2009) is similar to that in southwestern

Ohio, providing a possible explanation for any genetic differences found in this area.

Dharmarajan et al. (2009) found approximately 13 alleles per locus and a heterozygosity

of 0.749, with two alleles out of HWE. Fike et al. (2007) found between 4 and 18 alleles

per locus in microsatellites, with heterozygosities ranging from 0.31 to 1.0. Linkage

disequilibrium indicates the non-random association between alleles (Fike et al., 2007).

These data provide a foundation for the current genetic study in raccoons from Clark and

Greene Counties, Ohio.

In order to construct a useful phylogenetic study of raccoon populations, raccoon

population dynamics need to be considered. For instance, raccoons often form male

social groups that can spend long periods of time travelling and living together (Gehrt et

al., 2008; Hauver et al., 2010). Kin selection does not explain the origin of these groups,

but more closely related males may associate for longer periods than unrelated males

(Gehrt et al., 2008). Neither do these groups help males sequester access to females

(Hauver et al., 2010). Hauver et al. (2010) argue that these groups form because males

must rely on mutual tolerance in dense populations to reduce male-male competition.

They also found that these coalitions kept females from mating with other groups of

males, although a female may mate with multiple males from the same group. Prange et

al. (2011) called this a fission-fusion society, marked by many short-term acquaintances

and few long-term associations (other than the large migratory male groups that often

form). Most of the groups consisted of two individuals (dyads), with male-male dyads

most common (Prange et al., 2011). These social groups travel between populations, creating enough gene flow to preserve genetic similarities throughout the population (Santonastaso et al., 2012).

Populations of raccoons are also promiscuous (Hauver et al., 2010; Nielsen & Nielsen, 2007), with female and male raccoons mating with multiple partners within the same season. Nielsen and Nielsen (2007) argue that this is due to the male-biased sex ratios and high densities of reproductive individuals found in most raccoon populations. They also discovered that most raccoon litters show multiple paternity, with greater than two paternal alleles present. This multiple paternity is explained by raccoon promiscuity and by the fact that raccoons interact more during winter and spring than during other seasons, overlapping the typical raccoon breeding season (Prange et al., 2011).

Future Work on Phylogenetics

Hauver and colleagues (2010) revealed the difficulty with sampling all adults, and identifying when juveniles disperse from their natal areas. Additional work is needed to improve genetic sampling of all members of a population, and improve observation techniques. They proposed that juveniles might make exploratory movements outside their natal area even during their first winter. This could impact gene flow and genetic structure in fragmented areas. More work should be done to verify that raccoons do not maintain polygyny instead of promiscuity, as fathers seem to interact more with their offspring than other males (Hauver et al., 2010). Hauver et al. (2010) advocate for further study into the links between social relationships and genetic parentage, especially the adaptive reasons for den sharing, consortship, and male-male coalitions that reduce

competition. These factors all affect genetic structures and resulting phylogenies.

Prange et al. (2011) revealed that females might become temporary members of male social groups, indicating a more complex social system. Additional work is needed to understand the social structure of raccoon populations. Prange et al. (2011) call for additional work with proximity-detecting collars in both fragmented areas and contiguous forest. Raccoon behavior is flexible enough that we need to understand how they behave in dense populations, as well as in dispersed groups.

Diet analyses

Use of Diet Analyses

Diet analyses help uncover the natural history of a species, food webs, and impacts of species on the environment (Rulison et al., 2012), while also helping to devise management strategies and assess a species potential impact on prey items (Glen et al., 2010). Along these lines, diet analyses can help identify ecological features of extinct species from both terrestrial and aquatic ecosystems, and test ecological inferences made from the fossil record (Clementz, 2012). When food resources are clumped, the home range of the species using these resources changes shape in response to resource alteration (Wehtje & Gompper, 2011). Meloro (2011) determined that biomorphometric characteristics of the mandible can be used to predict diet in extant and extinct species. For example, many carnivorous species have jaws shaped to catch and tear at their specific food item. Carnivores that prey on large animals illustrate this, having more rigid and robust mandibles. Many carnivorous mammals have adapted well to opportunistic food items made available by anthropogenic modification of native landscapes. Crab-eating raccoons, *Procyon cancrivous*, and other mammal species use anthropogenic

sources for food almost exclusively (Aguiar et al., 2011). Other mammals have a more varied diet that requires a more general mandible shape (Meloro, 2011). Aguiar et al. (2011) found that coatis (*Nasua* spp.) have diets that overlap with foxes, and include fruit pulp biomass, soft-bodied animals, and soil. Diet analyses revealed that both coatis and foxes might rely on scavenging and geophagy (eating soil). Diet analyses can also provide insight into foraging strategies that depend on the relative, temporal availability of food types (Zhou et al., 2011). As food items decrease in availability, diet analyses reveal that predators will consume different items.

Raccoon and other Mammal Diets

There is high variability in feeding adaptation amongst predators, even within poorly studied species. (Meloro, 2011). Wild boar in central Punjab, Pakistan, are important agricultural pests that rely heavily on cultivated crops (Hafeez et al., 2012). In each season, cultivated crops made up at least 58% of the diet of the boars by mass, with wheat being the most heavily consumed crop. Hafeez et al. (2012) used diet analyses to help devise management strategies for this species.

Animal and plant tissues are equally frequent in coati diets (Aguiar et al., 2011). Coatis forage more heavily on invertebrates than they do vertebrates, relying especially on coleopterans, dipterans, and annelids. In the crab-eating raccoon, animal tissue had the highest frequency of occurrence, but plant tissues had the highest relative volume (Aguiar et al., 2011). Aguiar et al. (2011) also found that invertebrate and vertebrate tissues were present in equal frequencies in crab-eating raccoons, which they defined as opportunistic hypocarnivores.

Opportunistic carnivores, such as the crab-eating raccoon, are generalists that can switch between different food sources in response to fluctuations in food availability (Zhou et al., 2011). Many carnivores subsisting on an omnivorous-frugivorous diet display a temporal dietary switch. For example, when fruit abundance was at its peak, martens utilized small mammals least (Zhou et al., 2011). These authors determined that a marten's diet is a function of the relative abundance of food items, and inferred availability of alternative food types. The western quoll (*Dasyurus geoffroyi*) is another generalist predator that may be a frequent scavenger. The western quoll's diet is dominated by both mammals and invertebrates (Glen et al., 2010).

The composition of a species' diet may fluctuate from season to season (Hafeez et al., 2012; Parsons et al., 2011; Rulison et al., 2012). Hafeez et al. (2012) demonstrated that much of the variation in diet choices within a given season were due to variation in available choices, as well as how long the particular item had been available in a given area. Raccoons become more selective as availability of reliable food sources increases, and shift their diet to whichever plant is producing ripe fruit (Rulison et al., 2012). Predators, however, will not rely entirely on a single resource, even when extremely abundant (Storm & Whitaker, 2007). Parsons et al. (2011) note that earthworms and boned meat are difficult to detect in raccoon feces, and may be underrepresented in raccoon diet analyses. Parsons et al. (2011) demonstrated that the seasonal variation in diet led to variations in physical condition, with raccoons in human dominated areas being healthier than raccoons in rural areas. The poor body condition of the raccoons suggests that despite the abundance of food sources in human dominated areas, the population was near the carrying capacity.

In addition to seasonal changes in diet related to variation in the abundance of different foods, species' diets may also change when nutritional demands change due to parasitic infections (Rulison et al., 2012). Learned behavior may also play a significant role in determining the relative consumption frequency of various food items (Hafeez et al., 2012). Individuals or groups may exploit certain food resources they have learned to to use that other members of the same species or different species with overlapping dietary niches have not learned to use (Storm & Whitaker, 2007). For instance, diets vary between populations of primates, due in part to naïve individuals learning from different groups. While primates are phylogenetically distinct from raccoons, they are often the dominant omnivore in the native range like raccoons are in the Midwestern United States.

There is a link between the size of a species and diet (Robb et al., 2012). Larger species require less food per unit of mass than smaller species, having lower metabolic rates and relatively lower nutritional demands than smaller species. Robb et al. (2012) found that the larger the mole-rat species, the more general was its diet. This may be due to competition, as competition was inferred from common diet items used most often in each species. The generalist nature of the diet in the larger species may buffer against starvation. When the availability of one food item drops, the individuals of a generalist species will rely on different items. Other features may also account for diet differences within a species, such as sex ratios and symbionts.

Different populations have different sex ratios that should also be accounted for when studying diet (Barton & Roth, 2007). Barton and Roth (2007) reasoned that male raccoons dominate areas undergoing predator removal because male raccoons disperse further on average than females. Males are more likely to inhabit areas outside of the

maternal home range than are females. As such, it may be necessary to modify the sex ratio of the source population in order to properly understand and manage the population being studied.

Raccoons are highly adaptive omnivorous feeders that have responded well to anthropogenic landscape modification. Many individuals make diet choices based on availability, seasonal variation in foods, body size, body condition and other factors. When infected with a parasite, the nutritional demands of the host change and may override other physical determinants of diet choice (Rulison et al., 2012).

Future Work on Diet Analyses

We need to understand the relationships between diet flexibility and other ecological features in order to predict how animals might shift diet choice in response to environmental changes. Body size, condition, sex ratios, and symbionts all can affect the diet of omnivores, such as raccoons. It is important to determine the diet of predators across their entire range to assess whether there are significant fluctuations in diet composition (Glen et al., 2010). We need to determine what factors contribute the most to diet choice in order to elucidate the role parasitism might play in shaping host diet.

Parasite and Host Interactions

Parasites are important symbionts that play a large role in the evolution of their hosts. These symbionts can affect selection to such a degree that they should be considered direct agents of selection (Thomas et al., 2000). Parasitism is one the main sources of biotic stress facing all organisms (Lozano, 1998), with parasitic organisms imposing fitness costs comparable to those from predators, and influencing the

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adaptability of behavioral traits (Kortet et al., 2010). Helminth parasites affect the fitness of the host by reducing reproductive output and potentially shortening host lifespan (Hutchings et al., 1999). Lozano (1998) defines the stress caused by parasites as an aversive stimulus that affects homeostasis in the host. There are several behavioral traits, such as vigilance and foraging, under strong selective pressure from even small parasite loads. In addition, there are physiological and metabolic alterations, such as production of eosinophils and IgE antibodies, that come into force due to parasitic infections (Kyriazakis et al., 1998).

Most parasites have aggregated populations, and hosts with large numbers of parasites often behave oddly (Barber et al., 2000). Barber et al. (2000) reasoned that behaviors of the host depend on the genotypes of both the parasite and the host, with the parasite selecting for improved transmission success. Parasites, with a direct life cycle, benefit when hosts come into contact with other potential hosts (conspecific and others). Barber et al. (2000) found that an ectoparasite (*Entobdella soleae*) of sole (*Solea solea*) hatches during the first few hours of daylight when sole are resting in the substrate and in close contact with each other.

Parasites with direct life cycles do not greatly alter host behavior in order to increase predation of the host by other animals. It appears that individuals infected with parasites with direct life cycles may avoid predators better than uninfected individuals (Barber et al., 2000), and thus survive better than uninfected individuals. Sticklebacks infected with *Glugea anomala* fed further away from potentially predatory cichlids.

Many parasites have indirect life cycles, requiring multiple intermediate hosts before being exposed to their final host (Barber et al., 2000). Because parasites often

cannot reproduce inside of intermediate hosts, it is important for the parasite juveniles to be trophically transmitted in an efficient way. Parasites often cause morphological and behavioral changes in the intermediate host that increase the likelihood of predation by the final host (Aeby, 2002; Barber et al., 2000; Moore, 1995). Many parasites manipulate host behavior for their own interests (Lozano, 1998). Parasites with an indirect life cycle can increase the likelihood of predator encounters by altering the habitat choice and risk sensitivity of the intermediate host (Barber et al., 2000). Ants (*Leptothorax nylanderi*) infected with the cestode (*Anomotaenia brevis*) become yellow, have stunted growth, and do not flee when the nest is disturbed, increasing the exposure of the final host (various woodpecker species) to infected intermediate hosts. These infected individuals were described as a separate species until further studies revealed the immature cestode. A species of acanthocephalan (*Acanthocephalus dirus*) causes its intermediate host isopod (*Asellus intermedius*) to become hyperactive and crawl up the sides of an aquarium and over leaf litter. Moore (1995) showed that significantly more of infected isopods than uninfected isopods were eaten by the fish, *Semotilus atromaculatus*. The behavioral changes in the isopods did not correlate with the need for more food. Similarly, terrestrial isopods (*Armadillidium vulgare*) spent more time on light-colored substrate in areas of relatively low humidity when they were infected with *Plagiorhynchus cylindraceus*, and were preyed on more by European starlings (Moore, 1995). Seabirds were 30 times more likely to eat California killifish (*Fundulus parvipinnis*) infected with the brain-encysting trematode, *Euhaplorchis californiensis*, than uninfected killifish (Aeby, 2002; Barber et al., 2000). Moore (1995) found that a species of trematode (*Microphalius papillorobustus*) will encyst in the central nervous system of two species of *Gammarus*,

causing them to become photophilic. This behavioral change makes them twice as likely to be eaten by the gull, *Larus cachannans*. Another trematode species (*Maritema subdolum*) can occur with *M. papillorobustus* and benefit from the behavioral changes caused by the latter. When the cestode (*Eubothrium salvelini*) reaches the infective stage for its final host (the brook trout *Salvelinus fontinalis*), it causes its copepod host (*Cyclops vernalis*) to swim more often, increasing the risk of predation. Larvae of the mosquito (*Aedes aegypti*) become less active and get eaten more readily by the meadow mouse, *Microtus pennsylvanicus,* when infected with more than three immature individuals of the trematode, *Plagiorchis noblei* (Moore, 1995).

Parasites often affect the escape performance, sensory performance and swimming performance of the intermediate host, which enables predators to capture the intermediate host (Barber et al., 2000). Heavy infections with plerocercoid cestodes distends the abdomens of fishes, which alters swimming efficiency (Barber et al., 2000). When the amphipod crustacean, *Gammarus lacustris,* is infected with the acanthocephalan, *Polymorphus paradoxus,* cystacanths, it clings to vegetation or potential predators when disturbed. Moore (1995) noted that this behavior leads to significantly more predation. The parasitic trematode, *Dicrocoelium* causes ants to latch their mandibles onto blades of grass, increasing consumption of the ants by the final host (sheep) of the trematode. The fungus, *Entomophthora* causes a similar behavioral change in ants, but does not benefit when the ant is consumed by sheep (Moore, 1995). Barber et al. (2000) hypothesized that some parasites may alter the post-capture behavior and palatability of the intermediate hosts, affecting the ingestion by the predator.

The nutritional value of some intermediate hosts increases when the host becomes

infected by a parasite (Aeby, 2002; Barber et al., 2000), leading to more intermediate

hosts being consumed than uninfected individuals. Selection could favor predators that

feed on infected prey as long as the benefits outweigh the costs of infection (Aeby, 2002).

Aeby (2002) found that coral-feeding butterflyfish (*Chaetodon multicinctus*)

preferentially feed on corals infected with trematode metacercariae that have obvious,

swollen nodules on the coral colony. Corals are a nutritionally poor food source, and the

parasite causes an infection that is not costly to the fish. Aeby (2002) argued that fish that

eat the infected polyps obtained more coral tissue per bite than fish that did not feed on

the infected corals.

Parasites can cause behavioral changes that increase transmission without the

intermediate host being consumed by the next host of the parasite (Moore, 1995). When

parasitized by the trematode, *Gynaecotyla adunca*, the snail, *Ilyanassa absolete*, spends

significantly more time in the high intertidal zone during nocturnal low tides, making the

larval trematodes able to infect the next intermediate host (a semiterrestrial crustacean).

Some parasites that rely on insect vectors to transmit immature individuals to the next

host cause the insect vectors to feed more often. Moore (1995) pointed out that this

increases the transmission of the parasite.

Several parasite species affect the diet composition of their hosts in different ways

(Barber et al., 2000). Many animals have evolved behaviors to avoid exposure to

parasites (Lozano, 1998). Because of these adaptations, animals are capable of making

complex foraging decisions, enabling them to select more nutritionally rich diets and

diets devoid of parasites (Hutchings et al., 1999). Hutchings et al. (1999) found that

patches of animal feces can lead to healthier patches of food, but also lead to increased

exposure to helminth parasites. Consequently, isopods tended to avoid eating starling

feces that contained acanthocephalan eggs (Moore, 1995). Parasites alter foraging

behavior in sticklebacks, causing them to evolve behaviors to avoid *Schistocephalus*

(Barber et al., 2000). Moore (1995) noted that oystercatchers (*Haemotopus ostralegus*)

rejected heavily infected bivalves (*Macoma balthica*).

Many animals have developed behaviors to avoid potential sources of parasites

(Lozano, 1998). Black-spotted sticklebacks (*Gasterosteus wheatlandi*) spend more time

in open waters when the parasitic branchiuran, *Argulus canadensis*, is present in

vegetated areas. Lambs avoided contaminated areas, especially swards contaminated by

parasitized animals (Hutchings et al., 1999). Hutchings et al. (1999) maintained that the

risks of grazing fecal-contaminated areas outweigh the benefit of nitrogen rich food

sources, and parasitized sheep grazed further from the soil surface and at slower rates

than uninfected sheep. Hosts may evolve behaviors to avoid habitats associated with

infection risk (Barber et al., 2000). Ungulates change foraging behaviors to avoid insect

pests (Moore, 1995). Birds selectively choose intermediate sized cockles that are a trade-

off between energy benefit and large trematode load, as large cockles have

disproportionately high parasite loads (Thomas et al., 2000). Starlings use nesting

materials that repel ectoparasites (Moore, 1995).

Some hosts have evolved therapeutic behaviors that help combat parasites (Barber

et al., 2000; Kyriazakis et al., 1998; Lozano, 1998; Moore, 1995). Hosts have developed

diets that select for prophylactic substances, as well as diets that select for therapeutic

substances (Lozano, 1998). Lozano (1998) indicated that the mechanisms behind the two

different types of diets differ. The freshwater snail, *Biomphalaria glabrata*, moves to

areas of lower water temperature when infected with *Schistosoma mansoni*. Moore (1995) illustrated that this behavior inhibits the development of the parasite. Bumblebees (*Bombus terrestris*) exhibit similar behaviors when parasitized by conopod flies, moving to less favorable microenvironments when infected with the parasites.

Some animals undergo anorexia during parasitic infections (Kyriazakis et al., 1998), which has a functional basis and serves a beneficial purpose. It is not solely a pathological response to parasite infection. The researchers hypothesized that anorexia could be due to the following: parasite induced for the parasite's benefit; host induced to starve the parasite; host induced to promote an effective immune response; or host induced to allow a more specific diet. The last two hypotheses were the only two to survive consistency comparisons with physiological, metabolic, and behavioral changes resulting from infection by protozoans or helminthes.

The reproductive behavior of many hosts experiences selective pressure from parasites (Barber et al., 2000). Parasites often affect the reproductive development or reduce reproductive effort so more host energy is available to the parasite. *Schistocephalus* causes gonads in sticklebacks to develop for a shorter period of time, leading to few infected sticklebacks being reproductively active (Barber et al., 2000). Male deep-snouted pipefish (*Syngnathus typhle*) avoided mating with females parasitized by the trematode, *Cryptocotyle*. Barber et al. (2000) stated that these infected females have a lower fecundity than uninfected females. Guppy and stickleback males show morphological evidence of previous episodes of surviving parasitism, and have greater fitness in increased reproductive success (Thomas et al., 2000).

Parasites can also affect social behaviors of their hosts. Parasites can castrate

hosts, and influence mate choice (Moore, 1995). *Toxoplasma* infections in mice favored

dominance and increased aggression in infected mice. Moore (1995) noted that mice

infected with the nematode, *Trichinella spiralis*, are more subordinate than uninfected

mice. It is proposed that xenophobia in primates may be a parasite avoidance behavior

(Thomas et al., 2000).

Parasites and Host Behavior

Changes in host behavior can be the result of hosts adapting to infection

(Manipulation hypothesis), the parasites increasing transmission, pathology (not being

advantageous to the host or the parasite), or a battle between host genetic interests and

parasite genetic interests (Barber et al., 2000). Barber et al. (2000) contend that the

manipulation hypothesis is difficult to support in some taxa. For instance, heavily

infected fishes often have to spend greater proportions of time foraging.

Parasites can also reduce the hosts' ability to forage efficiently (Figure 1). Dace,

Leuciscus leuciscus, infected with the trematode, *Diplostomum*, make more failed attacks

whilst foraging than uninfected individuals. The host's ability to encounter prey is often

limited by parasites affecting habitat choice and time spent foraging (Barber et al., 2000).

Parasites can also affect the ability of a predator to detect prey by altering the sensory

performance of the host. When parasites affect risk sensitivity or nutritional condition of

the host, they can affect the type of prey selected. Barber et al. (2000) argued that

parasites can alter the host's ability to capture prey by affecting swimming performance,

attack efficiency, and competitive ability. The trematode, *Ascocotyle pachycystis*, encysts

PREY ENCOUNTER

PREY DETECTION

PREY SELECTION

PREY CAPTURE

DIET COMPOSITION

Habitat choice

Time spent foraging

Sensory performance

Risk sensitivity

Nutritional condition

Competitive ability

Attack efficiency

Swimming performance

3, 4, 8, 29, 30

8, 9, 12

15

8, 9, 23

11, 13 19

16, 17, 22, 24, 32

10, 18, 25

2, 20

8, 10

1, 5, 6, 7, 14, 21, 26, 27, 28

15, 31
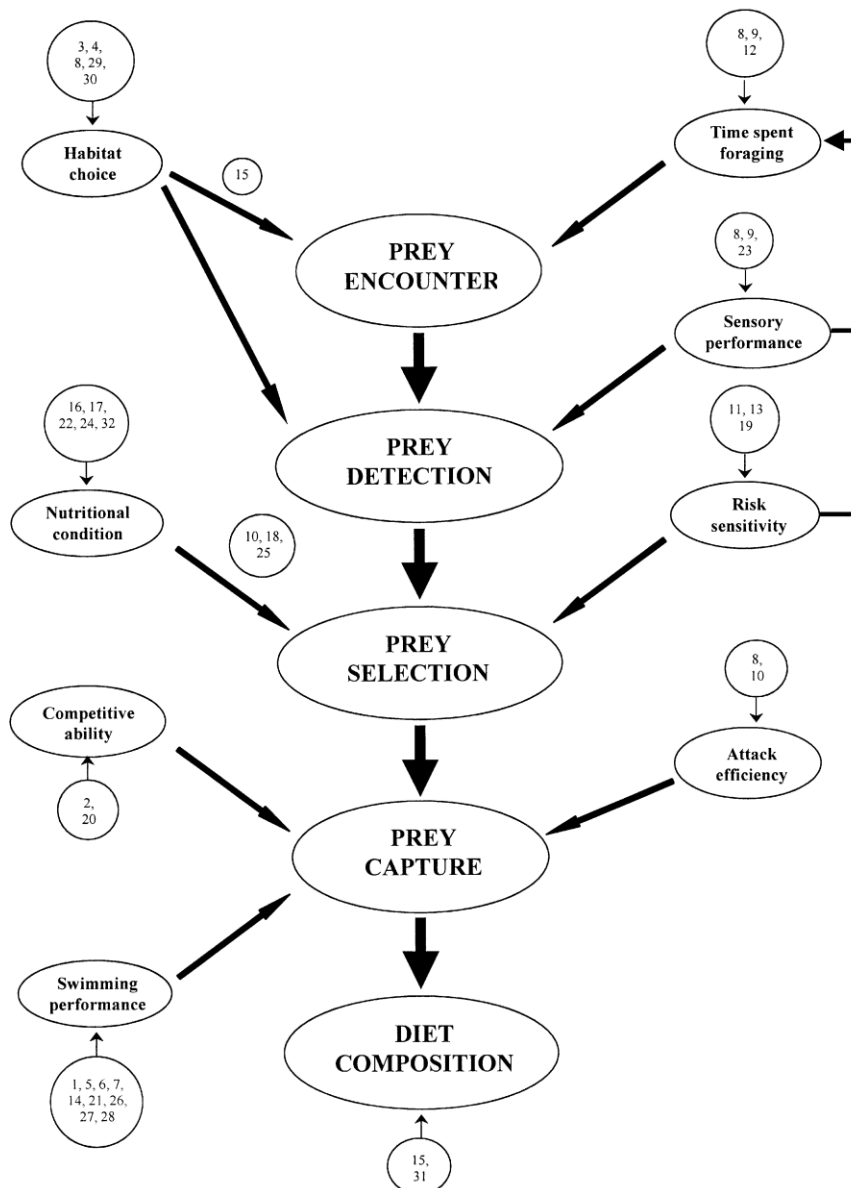
(1) Baldwin *et al.*, 1967 (2) Barber and Ruxton 1998 (3) Bean and Winfield 1989 (4) Bean and Winfield 1992 (5) Brassard *et al.*, 1982 (6) Butler and Millemann 1971 (7) Coleman 1993 (8) Crowden and Broom 1980 (9) Crowden 1976 (10) Cunningham *et al.*, 1994 (11) Giles 1983 (12) Giles 1987 (13) Godin and Sproul 1988 (14) Harris and Wheeler (15) Jakobsen *et al.*, 1988 (16) Lemly and Esch 1984 (17) Lester 1971 (18) Milinski 1984 (19) Milinski 1985 (20) Milinski 1990 (21) Moles and Heifetz 1998 (22) Orr and Hopkins 1969 (23) Owen *et al.*, 1993 (24) Pascoe and Mattey 1977 (25) Ranta 1995 (26) Smith 1973 (27) Smith and Margolis 1970 (28) Sprengel and Luchtenberg 1991 (29) Sproston and Hartley 1941 (30) Szidat 1969 (31) Tierney 1994 (32) Walkey and Meakins 1970

Figure 1 – From Barber et al., 2000. The figure represents a simplified illustration of predation and where parasites may affect predation in their host.

on the bulbus arteriosis of the infected sheepshead minnow, *Cyprinodon variegates*, and restricts blood flow. This decreases the time spent swimming and foraging (Barber et al., 2000). Moore (1995) pointed out that the beetle, *Tribolium confusum*, has behaviors to increase exposure to parasites, and was more attracted to rat feces that contained cestode eggs. Evolving parasite avoidance behaviors should be less costly than evolving immune responses against parasites, especially at high population densities (Hutchings et al., 1999). When the nutritional benefits outweigh the cost of increased infection, the animals should make the trade-off (Hutchings et al., 1999). However, Hutchings et al. (1999) found that even parasite immune sheep did not significantly select for more nutritious fecal contaminated foods.

A significant proportion of parasite activity may have no noticeable effect on the host (Lozano, 1998). Lozano (1998) illustrated that parasites with a direct life cycle are often opportunistic and increase in numbers to overwhelm a weakened host. More research needs to be done about the life cycles of these parasites within a single host to protect fragile populations of infected hosts. Thomas et al. (2000) argue that we need to consider all methods and markers of selection when determining the effects of a parasite. If direct costs and indirect consequences of parasitism act in opposite directions, parasitized individuals can have increased fitness over uninfected individuals. Because parasites do not systematically reduce host fitness to zero, if a parasite induces avoidance by predators or cannibals, it would increase fitness (Thomas et al., 2000).

Thomas et al. (2000) argued that parasites are not always detrimental, and can be somewhat beneficial. Parasites can also increase fitness when less harmful parasites outcompete more harmful parasitic species for hosts (Hutchings et al., 1999).

Oropendulas allow cowbirds to parasitize their nests so that the cowbirds will groom immature stages of botflies away before the oropendula young become infected (Thomas et al., 2000). When the oropendulas nest near wasps that chase botflies away, the oropendulas become much more hostile towards cowbirds.

If parasite presence produces acquired immunity in a species that has more detrimental effects from the parasite as the host ages, the parasite can increase fitness (Hutchings et al., 1999). Intestinal parasites may accumulate harmful toxins (such as heavy metals) more than the surrounding host tissues (Thomas et al., 2000). Thomas et al. (2000) noted that under deficient trophic conditions, the parasite, *Trypanosoma ostospermophili*, increases the fitness of the host, but not when the host is subject to a normal diet. During deficient conditions, hosts will sometimes select for food sources containing parasites, indicating that the parasites are beneficial under these conditions (Moore, 1995).

A geographic view of host-parasite interactions is important to understanding the coevolution of parasites and hosts (Thomas et al., 2000). Thomas et al. (2000) contended that when we understand the relationship of both symbionts with the ecosystem as a whole, we can estimate the net selective pressure exerted by the parasite on the host.

Future Work on Parasite and Host Interactions

There is a need to elucidate the role that parasites play in helping determine the population genetic features of hosts. We also need to determine how much of the diet of the host can be predicted by high parasite prevalence. These two features will provide additional evidence regarding the extent to which parasites exert significant pressure on

hosts, and should be accounted for when studying ecological features of a species.

Chapter 2 Summary

Chapter 2 focuses on the first goal of my dissertation. This chapter explores the impact that landscape features, especially the amount of landscape that has been modified by urbanization and agriculture, have on *B. procyonis* prevalence. We note that these landscape features do impact *B. procyonis* prevalence, and help provide a basis for predicting *B. procyonis* presence and abundance in individual raccoons.

Chapter 3 Summary

This chapter explores the relationship between *B. procyonis* prevalence and the population genetics of raccoons. It addresses the first and second objectives of the second goal of my dissertation. In this chapter, we group raccoons into two categories: those that come from populations with high *B. procyonis* prevalence ($> 60\%$), and those that come from populations with low prevalence ($< 60\%$). We also explore some phylogenies of raccoons collected from the townships we surveyed. We note that the two groups differ from each other genetically at all loci studied, and that some of these differences can be attributed to *B. procyonis* prevalence.

Chapter 4 Summary

We explore the final objective of the second goal in this chapter. We investigate the relationship between *B. procyonis* prevalence and diet choice. Again, we grouped the raccoons into two groups based on *B. procyonis* prevalence. The two groups differed significantly from each other based on the proportion of raccoons with plant material

found in the stomach at necropsy. In this chapter, we also build some models for predicting the amount of plant material expected from a raccoon based on the township in which the raccoon was found. *B. procyonis* prevalence is the best predictor of this feature.

Chapter 5 Summary

The final chapter of this dissertation provides some concluding thoughts of the study. It also recommends some areas for future work which include assessing the relationship between *B. procyonis* prevalence and landscape features, further assessing the genetic distances among the raccoons from the nine townships we surveyed from Clark and Greene Counties, OH, and exploring the home range features of raccoons from these townships to understand the role *B. procyonis* plays in influencing home range size and shape.

Appendix I Summary

This appendix provides preliminary data and analyses on an area that naturally developed from the study, but was not included under the initial two goals. In the appendix, we assess different techniques of identifying *B. procyonis* in nine townships of Clark and Greene Counties, OH.

Appendix II Summary

The second appendix follows the format of the first. It also explores an additional area that extends from the research done in chapter 3. This appendix explores the relationship between the genetic structuring in raccoons from nine townships of Clark

and Green Counties, OH and the genetic structuring in *B. procyonis* worms found in raccoons from the same nine townships.

CHAPTER TWO

PREDICTING *BAYLISASCARIS PROCYONIS* ROUNDWORM PREVALENCE,

PRESENCE AND ABUNDANCE IN RACCOONS (*PROCYON LOTOR*) OF

SOUTHWESTERN OHIO USING LANDSCAPE FEATURES

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Abstract

Raccoon roundworm is a leading cause of a neurological disease known as larva migrans encephalopathy in vertebrates. We determined that roundworm prevalence is significantly lower in Beavercreek Township than other townships surveyed, and that mean patch size and proportion of landscape modified by urbanization or by agriculture are good predictors of roundworm prevalence and abundance in raccoons. The proportion of landscape modified by urbanization was the best predictor of roundworm presence. These data will facilitate predictions regarding roundworm prevalence in areas that have not been previously sampled, and contribute to devising management strategies against the spread of raccoon roundworm.

Introduction

The raccoon, *Procyon lotor*, has the ability to adapt to utilizing anthropogenic resources, and is distributed across North and Central America (Parsons et al., 2011). It ranges throughout rural and urban areas of North America (Parsons et al., 2011). Parsons et al. (2011) also noted that raccoons thrive in areas where there are human developments and the absence of large predators. Prange et al. (2003) reported that raccoons in urbanized landscapes had increased survival, reproductive and recruitment rates than in rural settings. Urban areas with the highest breeding success have large forest patches fragmented by urbanization (Soga & Koike, 2013), where human developments appear to provide reliable food sources and multiple forms of shelter for the species. Anthropogenic resources available in urban and agricultural landscapes have allowed raccoon populations to reach higher densities compared with purely rural landscapes (Prange et al., 2003). In urban and suburban landscapes, raccoon densities can be

estimated to be as high as 90 raccoons/km^2, whereas densities rarely exceeded 15

raccoons/km^2 in rural settings of North America (Prange et al., 2003). Raccoon home

ranges decrease in highly fragmented landscapes, being as small as 25 hectares in some

areas, yet exceeding 100 hectares in more rural areas (Beasley et al., 2007). Southwestern

Ohio is a region with many areas of human developments, and few large predators. Most

of the natural landscape has been converted into cultivated cropland (Table 1). Within the

landscape mosaic, corn is the most common crop, and provides a reliable food source for

raccoons in the late summer and autumn months. The native landscape exists as small

patches surrounded by agriculture and urbanization. Page et al. (2005) noted that raccoon

densities are higher in urban settings, but that fewer of these raccoons are estimated to

have *Baylisascaris procyonis*.

The Raccoon, *P. lotor*, is the definitive host for the raccoon roundworm,

Baylisascaris procyonis (see Page et al., 2005). Raccoon roundworms are the leading

cause of the dangerous neurological disease, known as larva migrans encephalopathy in

vertebrates, found in over 90 vertebrate species (Blizzard et al., 2010b). The intermediate

hosts (usually small mammals) are attracted to raccoon latrines, where raccoons

repeatedly defecate and eggs can concentrate (Page et al., 2001b). As the density of

raccoon latrines increases, the possible transmission of *B. procyonis* increases (Roussere

et al., 2003). Smyser et al. (2010) noted that individual scats within a latrine rather than

the entire latrine should be used to assess exposure risk for intermediate hosts. Landscape

fragmentation also affects the prevalence of *Baylisascaris procyonis* parasites in

intermediate hosts that use raccoon feces as a source of food, leading to higher

prevalence among raccoons preying on these hosts (Page et al., 2005). Comparisons of *B.*

procyonis prevalence in raccoons and intermediate hosts tend to group the animals into areas that have drastically different landscapes and degrees of landscape fragmentation (Page et al., 2001a, 2001b). *B. procyonis* prevalence seems to change as a function of landscape (Page, 2013). Page et al. (2001a) reported that *B. procyonis* prevalence was higher in smaller, more isolated fragments in an agricultural landscape. Page et al. (2005) found that *B. procyonis* prevalence was lower in urban portions of Chicago than in rural areas. However, Blizzard et al. (2010a) reported higher *B. procyonis* among raccoons in an urban landscape than in a rural landscape. As the prevalence of raccoon roundworm increases in raccoons, more intermediate hosts become infected.

The purpose of this study was to investigate if landscape features are useful predictors of both presence and abundance of *B. procyonis* among definitive hosts, and to help determine the relationship between increased anthropogenic landscape and *B. procyonis* prevalence. By testing the ability to predict the presence and abundance of parasites in a raccoons from landscape features, we aim to provide valuable information for researchers assessing the potential impact of *B. procyonis* in areas that have not yet been sampled, and for those investigators interested in the potential for zoonoses.

Materials and Methods

We investigated raccoons from nine townships from Greene and Clark Counties in Southwest Ohio. We chose townships as the sampling scale so as to have areas larger than a typical raccoon home range in a fragmented agricultural landscape like that found in southwestern Ohio. The largest mean patch size in a township is less than 20 hectares (1 hectare = 10000 m^2). With a home range of 92 ± 6 hectares for males and 58 ± 7 for females (Beasley et al., 2007), raccoons would likely use habitat in multiple patches

within a sample area. We collected raccoons from Beavercreek, Xenia, and Miami Townships in Greene County, and from German, Green, Harmony, Mad River, Moorefield, and Springfield Townships in Clark County. Municipalities were included in their respective townships for analyses (Figure 1).

We accessed and downloaded the 2006 National Land Cover Dataset (NLCD) from mrlc.gov website. The dataset classifies land cover of each 30 x 30 m grid cell as belonging to one of 16 classes in eight categories: water, developed, barren, forest, shrubland, herbaceous, planted/cultivated and wetlands. Using ESRI ArcGIS software, we imported shapefile layers of Greene and Clark County townships in order to clip the NLCD. This resulted in individual land cover maps for each township. We then used Patch Analyst (http://www.cnfer.on.ca/SEP/patchanalyst/) to evaluate various landscape, class and patch metrics.

We calculated the proportion of landscape modified by urbanization (*Turb*) and the proportion of landscape modified by agriculture (*Tag*) according to the formulas: $Turb = (Do + Dl + Dm + Dh)/TA$, and $Tag = (P + C)/TA$, where land areas are defined as: developed-open (*Do*), developed-low (*Dl*), developed-medium (*Dm*), developed-high (*Dh*), pasture/hay (*P*) and cultivated crops (*C*) for a township, and where TA is the total land area in the township (Table 1).

We worked with six fur trappers to gather raccoons for the study. The trappers recorded only the township, where the raccoon was trapped. We collected the viscera from trapped raccoons at two different work sites: one located in Xenia Township and one located in Harmony Township, OH (Figure 1). We dissected out the viscera, and placed them into two freezer bags marked with the date of collection, the trapper

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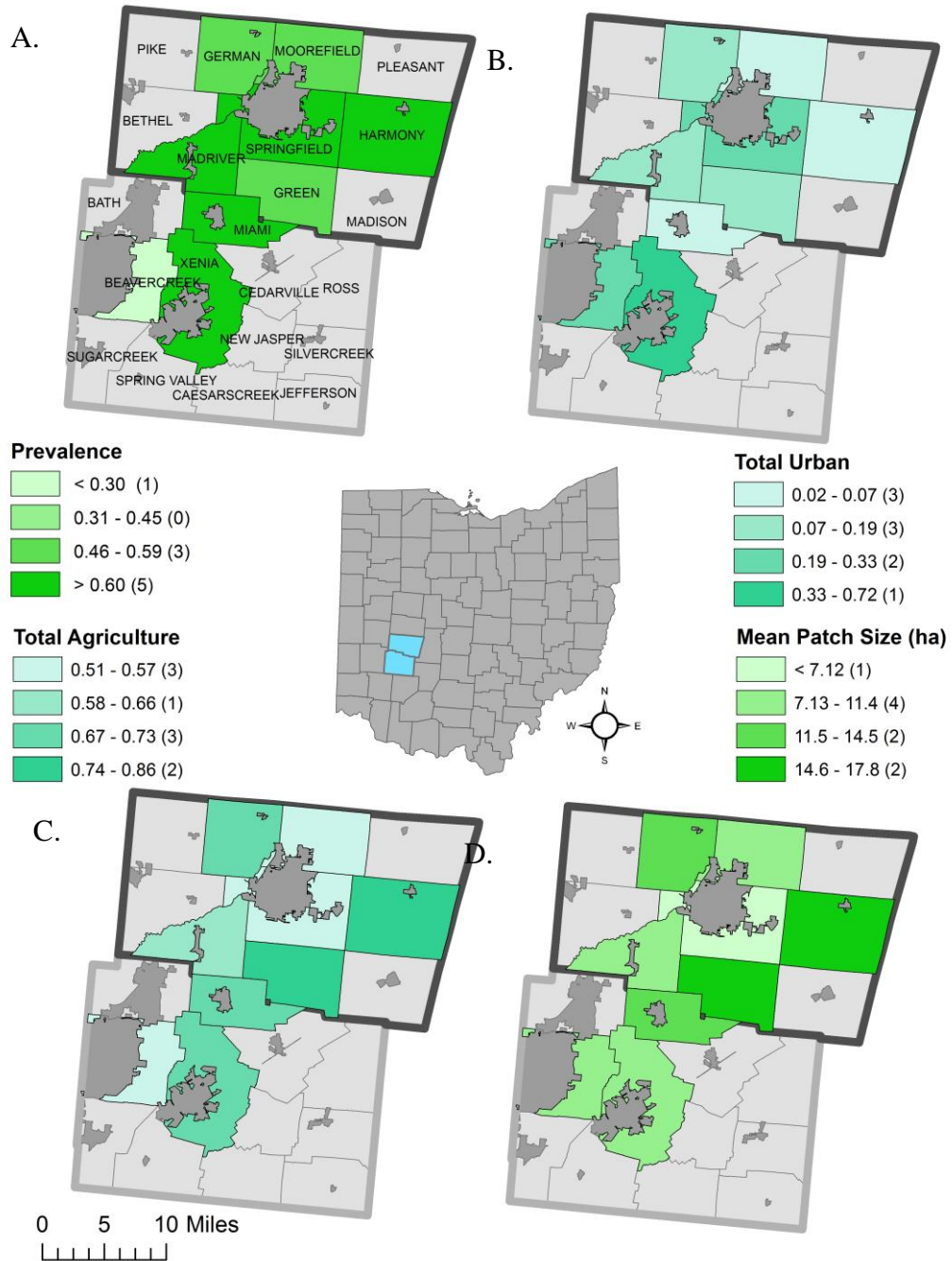
Figure 1. Map of the townships of Greene and Clark Counties Ohio. The data represent the proportion of raccoons from an individual township that had raccoon roundworms when necropsied (A.). This map also demonstrates the proportion of landscape modified by urbanization (B.), the proportion of landscape modified by agriculture (C.), and the mean patch size (D.) for the nine townships. The proportion of landscape modified by urbanization was highest in Beavercreek and Springfield Townships, the proportion modified as agriculture was lowest in the same two townships, and mean patch size was lowest in Springfield Township.

responsible for the raccoon, the township or city where the raccoon was trapped, and the county that the township or city resides in. All samples were stored at -20 ºC until they were necropsied. Collections were made from November 10 through December 9, 2012. The skinned carcasses were necropsied, and we examined sections of the gut for *B. procyonis*. We used the term abundance to refer to the number of *B. procyonis* worms present in a single raccoon whether or not it is infected (Margolis et al, 1982; Rozsa et al., 2000). We noticed that many of the necropsied raccoons also contained cestodes, so we also collected and recorded any cestodes found in the intestinal tract, but were unable to identify them to species or determine the number present in the intestines of any of the raccoons sampled. We ran a Chi-Squared equality of distributions test on the *B. procyonis* prevalence data, by combining Springfield Township with the adjacent Mad River Township (Figure 1).

We developed three models to test the correlation of *B. procyonis* features with three landscape features (*Turb*, *Tag* and mean patch size (*M*)), and the predictive capabilities of these landscape features. The first model we developed was the following linear regression: parasite prevalence $= \beta_0 + \beta_1(Turb) + \beta_2(Tag) + \beta_3(M)$, where β_0 is the intercept, and β_1, β_2 and β_3 are coefficients for the explanatory variables. We used the data from the nine townships surveyed (Table 2) for the first model. The next model tested the relationship between the three landscape features and presence of *B. procyonis*: presence $= \beta_0 + \beta_1(Turb) + \beta_2(Tag) + \beta_3(M)$, where β_0 is the intercept, and β_1, β_2 and β_3 are coefficients for the explanatory variables. This logistic regression had a binary dependent variable (either the roundworm was present or not). We ran this model on each of the 226 raccoons necropsied. The final model tested the ability of the three landscape

features to predict *B. procyonis* abundance: parasite abundance = $\beta_0 + \beta_1(Turb) + \beta_2(Tag)$ + $\beta_3(M)$. We also ran this model on each of the 226 raccoons necropsied.

After noting that many raccoons infected with *B. procyonis* were also infected with at least one cestode, we tested the ability of these landscape features to predict the cestode features: cestode prevalence in the nine townships and cestode presence in each raccoon. We used the same models as above with the cestode feature as the dependent variable. We also constructed a model to test the correlation between *B. procyonis* presence and cestode presence: roundworm presence = $\beta_0 + \beta_1(cestode)$, with *B. procyonis* presence as the dependent variable and cestode representing the presence of at least one cestode at necropsy.

After preliminary analyses, we decided to drop the intercept from all of the models as the dependent variables should all be zero when all of the independent variables are zero. We added the independent variables stepwise in the linear regressions, and added the variables conditionally in the binary logistic regressions to generate the best models without introducing unnecessary variation with additional independent variables.

Results and Discussion

We calculated the proportion of landscape modified by agriculture, and the proportion of landscape modified by urbanization for each of the nine townships (Table 1). The values for *Tag* ranged from 0.5144 in Beavercreek Township to 0.8603 in Harmony Township (Mean ± Standard Error, 0.6799 ± 0.1167). The proportion of landscape modified by agriculture exceeds 0.8 (or 80%) in two of the nine townships: Harmony and Green. We also calculated the proportion of landscape modified by

Table 1. Areas of landscape features for the nine townships surveyed in hectares (ha)*.

Township	Do	Dl	Dm	Dh	P	C	TA	Turb	Tag	M
Beavercreek	1294	325	116	22	1332	1627	5752	0.3055	0.5144	9.2 ± 80.5
Xenia	863	365	47	9	1727	6425	11391	0.1127	0.7157	11.4 ± 64.6
Miami	403	28	7	2	736	4013	6683	0.0658	0.7106	14.5 ± 73.3
German	669	238	88	39	1415	4891	8641	0.1197	0.7298	13.0 ± 83.6
Green	815	85	37	8	830	6600	9243	0.1022	0.8039	16.8 ± 169.8
Harmony	640	213	32	5	1033	10083	12921	0.0689	0.8603	17.8 ± 329.0
Mad River	1055	450	66	12	1014	4562	8415	0.1881	0.6626	9.8 ± 52.6
Moorefield	729	603	168	71	1674	3270	8657	0.1815	0.5711	9.6 ± 49.5
Springfield	1912	778	140	58	936	3926	8835	0.3269	0.5503	7.1 ± 63.6

* (*Do*) developed-open, (*Dl*) developed-low, (*Dm*) developed-medium, (*Dh*) developed-high, (*P*) pasture/hay, (*C*) cultivated crop, (*TA*) total area, (*Turb*) proportion of landscape modified by urbanization, (*Tag*) proportion of landscape modified by agriculture and (*M*) mean patch size.

urbanization (Table 1). This value ranged from 0.0689 in Harmony Township to 0.3055

in Beavercreek Township (0.1635 ± 0.0965). Beavercreek Township had the highest

proportion of landscape modified by agriculture and had the lowest *B. procyonis*

prevalence.

We calculated the prevalence of *B. procyonis* and cestodes for all of the nine

townships and both counties (Table 2). We collected 135 raccoons total from Greene

County. We found *B. procyonis* individuals in 71 of the raccoons during necropsies

(52.6%). The mean prevalence (55.8 ± 4.3%) is slightly higher in Clark County, although

this difference was not significant ($\chi^2 = 0.737$, df = 1, p = 0.39). Cestode prevalence

ranges from 0% in Moorefield Township to 0.5 in Harmony Township with a mean of

21.5% ± 0.053 S.E. As with *B. procyonis* prevalence, cestode prevalence is higher in

Clark County (24.7%) than in Greene County (21.5%) although this difference was not

significant ($\chi^2 = 0.320$, df = 1, p = 0.57). The only townships with *B. procyonis*

prevalence below 50% (Beavercreek, German and Moorefield Townships) also have the

three lowest cestode prevalence calculations (4.2%, 6.7% and 0% respectively).

The prevalence of *B. procyonis* from each of the various townships was

determined (Table 2). The prevalence of *B. procyonis* from Beavercreek is significantly

lower than the rest of the townships ($\chi^2 = 25.19$, df = 7, p-value = 0.0007). Beavercreek is

the only township with *B. procyonis* prevalence below 45%. Of the 49 raccoons we

necropsied, only 12 (24.5%) had raccoon roundworm.

B. procyonis prevalence increases as the proportion of landscape modified by

agriculture increases ($F = 264.44$, p-value < 0.001). This finding is consistent with that

reported by Page et al. (2001a) and Page et al. (2005). The agricultural landscape in

Table 2. Prevalence, mean intensity of infection and range of *B. procyonis* and prevalence of cestodes in *Procyon lotor* sampled from Greene and Clark Counties, Ohio.

County	Township	Number of Raccoons	Prevalence of *B. procyonis*	Mean Intensity of *B. procyonis*	Range of *B. procyonis*	Prevalence of Cestodes
Greene						
	Beavercreek	49	24.5%	7.00	0 - 52	4.2%
	Xenia	37	67.6%	27.40	0 - 176	35.1%
	Miami	51	68.6%	26.34	0 - 210	25.5%
Clark						
	German	15	46.7%	13.71	0 - 47	6.7%
	Green	23	56.5%	18.08	0 - 64	21.7%
	Harmony	26	73.1%	16.37	0 - 50	50.0%
	Mad River	8	62.5%	8.00	0 - 31	25.0%
	Moorefield	13	46.1%	3.33	0 - 4	0%
	Springfield	4	50.0%	9.50	0 - 15	25.0%
		226*	54% ± 14%**	14.41± 8.45**	0 - 210	22% ± 5%**

*   Total number of raccoons necropsied from Greene and Clark Counties, Ohio
** Mean and standard error

southwestern Ohio is highly fragmented with small patches of agriculture and native landscape. Page et al. (2001a) found that *B. procyonis* prevalence was higher in these landscapes, and decreases as you move from rural to urban landscapes. Blizzard et al. (2010a) found the opposite to be true in Georgia, but the differences were not significant (12% in urban and 10% in rural). The urban areas in this study were more similar to non-urban areas and in close proximity to one another (Page, 2013). Blizzard et al. (2010a) were also studying *B. procyonis* in an area only recently documented to contain *B. procyonis* (only present in a single county of the 25 the researchers sampled from. Beavercreek Township has the lowest *B. procyonis* prevalence and the second lowest recorded *Tag*. *Tag* predicts *B. procyonis* rather well (R^2 = 0.97). The logistic regression designed to predict *B. procyonis* presence predicted presence better than no predictor (48% of raccoons negative and 65.5% overall vs 0% and 54.9% for no predictor). Both mean patch size and the proportion of landscape modified by urbanization contributed to the logistic regression (Table 3). *B. procyonis* becomes more likely to be present when mean patch size increases, and less likely to be present as *Turb* increases. Beavercreek Township has the second lowest mean patch size (9.2 hectares), the second highest *Turb* (0.3055), and the lowest *B. procyonis* prevalence. This supports our model. The abundance of *B. procyonis* in the 226 raccoons sampled correlated significantly with *Turb* and *Tag* (*F* = 28.382, p-value <0.001). These two explanatory variables account for a small portion of the variation in abundance (R^2 = 0.20). The presence of cestodes at necropsy contributed significantly to the logistic regression predicting *B. procyonis* prevalence (score = 10.373, p-value = 0.001), and predicts *B. procyonis* presence better than no predictor (86.3% of raccoons negative and 55.3% overall vs. 0% and 54.9% for

Table 3*: Regressions testing the correlation between parasite prevalence, presence and abundance and several landscape features.

Dependent Variable	Independent Variables added stepwise or conditionally	Model Independent Variables**	β	S.E.	Test Statistic	*p*-value
Roundworm Prevalence	*Turb*	*Tag*	0.814	.050	16.262	<0.001
	Tag					
	M					
Roundworm Presence	*Turb*	*Turb*	-5.079	1.192	18.184	<0.001
	Tag	*M*	0.073	0.016	21.621	<0.001
	M					
Roundworm Abundance	*Turb*	*Turb*	22.726	3.491	6.511	<0.001
	Tag	*Tag*	-33.892	13.776	-2.460	0.015
	M					
Cestode Prevalence	*Turb*	*Tag*	0.381	0.093	4.075	0.004
	Tag					
	M					
Cestode Presence	*Turb*	*Turb*	-9.690	1.323	53.605	<0.001
	Tag					
	M					
Roundworm Presence	Cestode Presence	Cestode	0.972	0.314	9.593	0.002

* All of the independent variables were added stepwise to determine the final model. *Tag* = Proportion of landscape modified by agriculture; *Turb* = Proportion of landscape modified by urbanization; *M* = Mean patch size. The final model was determined to be the model that explained the most variation in the dependent variable without adding additional variation. The two models for prevalence were linear regressions, and the remaining models were logistic regressions.

** These were the independent variables that contributed to the final model.

no predictor). Many of the raccoons positive for *B. procyonis* at necropsy were also positive for cestodes.

Cestode prevalence also increases with *Tag* ($F = 16.605$, p-value = 0.004). Much of the variation in cestode prevalence is predicted by variation in *Tag* ($R^2 = 0.63$). While all three landscape features contributed significantly to the model, only *Turb* remained after the independent variables were all added conditionally for predicting cestode presence. *Turb* predicted cestode presence better than no predictor (100% of raccoons negative and 77.4% vs 0% and 22.6% for the model with no predictor).

We found raccoon roundworm prevalence ranged from 24.5% to 73.1% in the area of the current study. Prevalence of raccoon roundworm in this area is lower than many areas in the midwestern United States. Suggesting the need for further research to determine reasons for the lower prevalence in the Ohio region. Page et al. (2005) reported a documented prevalence range of 68 – 82%. Raccoon resistance to *B. procyonis* is believed to increase with age, possibly lowering prevalence and mean intensity of infection (Owen et al., 2004). We did not estimate the age of raccoons we sampled, thus age cannot be ruled out as a contributing factor to the lower prevalence numbers. Additional factors should be explored that may predict the presence of *B. procyonis* in raccoons, as well as the overall load raccoons carry, and that may reduce the potential impact caused by the roundworm.

While the most reliable method to calculate prevalence of *B. procyonis* in *P. lotor* is to necropsy individual raccoons, this method is labor intensive, time consuming, and potentially dangerous due to zoonosis caused by *B. procyonis* (Blizzard et al., 2010b). The most convenient (although still dangerous) method to estimate prevalence of the

parasite is to sample latrines used by raccoons, yet these can underestimate prevalence (Page et al., 2005). Each female worm can produce hundreds of thousands of eggs each day, which can remain viable at latrine sites for several years (Page et al., 2011). The current study demonstrates that we can predict the prevalence, presence and abundance of roundworms found in a raccoon based on the amount of landscape that is modified as agriculture or urban, and the mean patch size. This study also demonstrates evidence that *B. procyonis* prevalence increases as you move from urban to non-urban landscapes.

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CHAPTER THREE

THE PREVALENCE OF RACCOON ROUNDWORM (*BAYLISASCARIS*

PROCYONIS) IN THE NORTH AMERICAN RACCOON (*PROCYON LOTOR*),

IN RELATION TO GENETIC STRUCTURING

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Abstract

Baylisascaris procyonis is an intestinal roundworm that parasitizes raccoons (*Procyon lotor*) as a final host. *B. procyonis* often develops using an indirect life cycle, requiring an intermediate host. Several vertebrate species, including humans, can serve as intermediate hosts for *B. procyonis,* with infection causing visceral larva migrans, one of the leading causes of larva migrans encephalopathy in humans. Parasites often reduce relative fitness and exert selective pressures on host individuals and populations. In this study, we aimed to determine how *B. procyonis* affects the genetic structuring of raccoon subpopulations. We removed the alimentary canal from 226 raccoons obtained from fur trappers from Clark and Greene Counties, Ohio. We obtained parasite prevalence data for raccoons from nine townships within these counties. DNA from the greater omentum was isolated and we then amplified a portion of exon 2 from MHC II, two microsatellite loci, and the mitochondrial gene for NADH5. The DNA was amplified using real-time PCR, calculating heterozygosity for the nuclear locus, using this to determine the amount of genetic structuring in the raccoons from Clark and Greene Counties. Townships of these counties were treated as subpopulations, and differences in *B. procyonis* prevalence between these subpopulations were noted. Heterozygosity for exon 2 of MHC II differed among raccoons from these counties, and raccoons from subpopulations with *B. procyonis* prevalence above 0.60 had significantly lower heterozygosity than raccoons from subpopulations with prevalence below 0.60. Phylogenetic trees using NADH5 showed genetic structuring, with raccoons from the same subpopulation grouping closer than raccoons from other subpopulations. Raccoons from townships with higher *B. procyonis* prevalence also had lower heterozygosity in the microsatellite loci. Our data

suggest that at least some of the genetic structuring in raccoons may be explained by differences in *B. procyonis* prevalence among raccoons from these counties.

Introduction

Phylogenetics is the study of evolutionary relatedness between taxa. A phylogeny, or phylogenetic tree, is a representation of these relationships, where each branch represents a lineage. These trees are critical when addressing evolutionary hypotheses (Kelly, 2005). The branches can represent a higher taxon, such as Class Mammalia, or can represent a single population. Phylogenies help resolve the evolutionary relationships between taxa and the characters used to construct the phylogenies. Molecular data, such as amino acid and nucleotide sequence, morphological data, and chromosomal data are used as characters to construct the phylogenies used to illustrate relationships between organisms.

Phylogenies illustrate co-evolutionary events between species living in a symbiotic relationship with each other (Krasnov et al., 2004; Srithayakumar et al., 2011; Srithayakumar et al., 2012; Wanntorp et al., 1990). The phylogenetic relationships between parasite populations provide an explanation on how the parasites exploit their hosts, and which hosts can be exploited (Krasnov et al., 2004). Some genetic loci are under selective pressure from symbionts. Trees illustrate these relationships. The genes coding for the host major histocompatibility complex (MHC) co-evolves with its parasites (Srithayakumar et al., 2011; Srithayakumar et al., 2012).

Raccoons (*Procyon lotor*) have adapted well to changes in landscape associated with human developments (Prange et al., 2003). Parsons et al. (2011) noted that raccoon populations throughout North and Central America are benefitted by anthropogenic

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factors, such as developed landscape and predator removal. Human developments provide diverse denning sites, and reliable resources for raccoons, which do best in urban areas surrounded by large patches of forest (Prange et al., 2003; Soga & Koike, 2013). As raccoon populations become more dense, disease transmission and epidemiology become important factors of raccoon population dynamics (Roussere et al., 2003). As the density of raccoon latrines increases, the possible transmission of *B. procyonis* increases (Roussere et al., 2003). Smyser et al. (2010) noted that individual scats within a latrine, rather than the entire latrine, should be used to assess exposure risks for intermediate hosts. Landscape fragmentation also affects the prevalence of *Baylisascaris procyonis* parasites in intermediate hosts that use raccoon feces as a source of food, leading to higher prevalence among raccoons preying on these hosts (Page et al., 2005). Comparisons of *B. procyonis* prevalence in raccoons and intermediate hosts tend to group the animals into areas that have drastically different landscapes and degrees of landscape fragmentation (Page et al., 2001a, 2001b). *B. procyonis* prevalence appears to change as a function of landscape (Page, 2013). Page et al. (2001a) reported that *B. procyonis* prevalence was higher in smaller, more isolated fragments in an agricultural landscape. Page et al. (2005) found that *B. procyonis* prevalence was lower in urban portions of Chicago than in rural areas. However, Blizzard et al. (2010a) reported higher *B. procyonis* among raccoons in an urban landscape than in a rural landscape.

　　Baylisascaris procyonis roundworm is a common intestinal parasite of raccoons, whose prevalence ranges from 68 to 82% in the Midwestern United States (Page et al., 2005). Unless there are several dozen *B. procyonis* individuals in a single juvenile raccoon, the parasite causes little to no pathology (Roussere et al., 2003). However, eggs

can accumulate in raccoon feces and remain viable for years (Shafir et al., 2011). Raccoons often defecate in the same location each time, establishing latrines (Roussere et al., 2003), which can be dangerous sites of infection for potential intermediate hosts of the parasite (Page et al., 2011; Page et al., 2001a, 2001b; Roussere et al., 2003). *B. procyonis* causes significant damage in tissues of intermediate hosts, including encephalopathy due to migrating worms (Blizzard et al., 2010). Infected intermediate hosts likely become easier prey for raccoons, further increasing prevalence. Parasites, especially highly prevalent parasites, account for one of the strongest selective forces on hosts (Thomas et al., 2000).

Raccoon genetic structure plays a significant role in how populations diverge from each other, and in how diseases move through these populations (Cullingham et al., 2008; Santonastaso et al., 2012). Cullingham and colleagues (2008) discovered three lineages that contain all four subspecies of the raccoon, *Procyon lotor*, with limited barriers to gene flow. The Mississippi River prevents contemporary gene flow in the southern portion of the range, and genetic isolation prevents some gene flow between two lineages east of the Mississippi. Most of the variation in mtDNA occurs within populations; demonstrating some of the genetic structure between these populations, which effects how rabies and other diseases move through these populations (Cullingham et al., 2008).

Nuclear DNA from raccoons contains large variation within and between populations, indicating genetic structure and separation (Cullingham et al., 2006; Dharmarajan et al., 2009; Fike et al., 2007; Santonastaso et al., 2012). Cullingham et al. (2006) discovered that polymorphic tetranucleotide (containing four nucleotides)

microsatellites from the nuclear DNA of raccoons contained between 6 and 25 alleles, with heterozygosity ranging from 0.674 to 0.925. These microsatellites were not presently evolving, as all alleles were at Hardy-Weinberg Equilibrium (HWE) (Cullingham et al., 2006).

Raccoons thrive in anthropogenically-fragmented landscapes, which influence genetic structure between "neighborhoods" within a population (Dharmarajan et al., 2009; Santonastaso et al., 2012). Dharmarajan et al. (2009) demonstrated that there was genetic structure between the 29 spatially distinct patches forming these neighborhoods, where 30% of the variation in the genome was accounted for by variation in patch size and percentage of land represented by forest. The landscape from the study by Dharmarajan et al. (2009) conducted in Central Indiana is similar to that in southwestern Ohio, providing a possible explanation for any genetic differences found in the area of the current study. Dharmarajan et al. (2009) found approximately 13 alleles per locus and a heterozygosity of 0.749, with two alleles out of HWE. Fike et al. (2007) found between 4 and 18 alleles per locus in microsatellites, with heterozygosities ranging from 0.31 to 1.0. Linkage disequilibrium indicates the non-random association between alleles (Fike et al., 2007). These data provide a foundation for the current genetic study in raccoons from Clark and Greene Counties, Ohio.

In order to construct a useful phylogenetic study of raccoon populations, raccoon population dynamics need to be considered. For instance, raccoons often form male social groups that can spend long periods of time travelling and living together (Gehrt et al., 2008; Hauver et al., 2010). Kin selection does not explain the origin of these groups, but more closely related males may associate for longer periods than unrelated males

(Gehrt et al., 2008). Neither do these groups help males sequester access to females (Hauver et al., 2010). Hauver et al. (2010) argue that these groups form because males must rely on mutual tolerance in dense populations to reduce male-male competition. They also found that these coalitions kept females from mating with other groups of males, although a female may mate with multiple males from the same group. Prange et al. (2011) called this a fission-fusion society, marked by many short-term acquaintances and few long-term associations (other than the large migratory male groups that often form). Most of the groups consisted of two individuals (dyads), with male-male dyads most common (Prange et al., 2011). These social groups travel between populations, creating enough gene flow to preserve genetic similarities throughout the population (Santonastaso et al., 2012).

Populations of raccoons are also promiscuous (Hauver et al., 2010; Nielsen & Nielsen, 2007), with female and male raccoons mating with multiple partners within the same season. Nielsen and Nielsen (2007) argue that this is due to the male-biased sex ratios and high densities of reproductive individuals found in most raccoon populations. They also discovered that most raccoon litters show multiple paternity, with greater than two paternal alleles present. This multiple paternity is explained by raccoon promiscuity and by the fact that raccoons interact more during winter and spring than during other seasons, overlapping the typical raccoon breeding season (Prange et al., 2011).

The purpose of the current study was to determine if raccoons from populations with high (>60%) *B. procyonis* prevalence were genetically different than raccoons from other populations. We hypothesized that groups of raccoons with higher *B. procyonis* prevalence would differ genetically from groups with lower prevalence.

Materials and Methods

We collected the alimentary canal from raccoons from nine townships of Clark and Greene Counties, OH. For a detailed description about the trapping sites, collection techniques, necropsies and calculations of prevalence see Ingle et al. (2014).

DNA Isolation and Amplification

We isolated DNA from greater omentum tissue of raccoons from Clark and Greene Counties using the Qiagen DNA Mini Kit using the published protocol. Real-time PCR of exon 2 from MHC II and the entire sequence of NADH5 was performed using the following ratios: 2 µL of 1 µM of each primer (forward and reverse), 6 µL of DNA eluate and 10 µL of Sybr Select Master Mix (Life Technologies). We also amplified two microsatellite loci found to be out of HWE by Fike et al. (2007). We sent the amplified DNA to the Plant-Microbe Genomics Facility (PMGF) at The Ohio State University for sequencing. The PMGF conducted fragment analyses using fluorescent primers and a 3730 DNA Analyzer (Life Technologies) on the two microsatellite loci: Plot-12 and Plot-14.

DNA Analyses

We calculated the heterozygosities for exon 2 of MHC II for the nine townships studied from Clark and Greene Counties. These heterozygosities were used to assess genetic diversity and determine genetic structuring, by comparing the heterozygosities of raccoons from townships with *B. procyonis* prevalence above 60% and townships with prevalence below 60%. We used the NADH5 sequences from the raccoons to construct the following three phylogenetic trees of raccoons from the nine townships in these

counties: maximum likelihood (ML), neighbor-joining (NJ) and maximum parsimony (MP). Geneious 7.1.2 was used to construct the neighbor joining, while PAUP 4.0 was used to construct the maximum likelihood and maximum parsimony trees. Finally, we calculated heterozygosities and allele frequencies, and tested for departure from HWE for the two microsatellite loci using CERVUS 3.0 (Fike et al., 2007). The heterozygosities and allele frequencies for the two microsatellites were compared in the same way as for exon 2 of MHC II, which we also used as evidence of genetic structuring.

Results

DNA Analyses

Rates of heterozygosity in exon 2 of MHC II differed among townships (Table 1). Heterozygosity ranged from 0.50 in Springfield Township to 1.00 in Harmony Township. Raccoons from townships with prevalence above 60% had significantly lower heterozygosity than other raccoons from townships with prevalence below 60% ($\chi^2 =$ 13.13, $p < 0.001$). Xenia Township had the lowest heterozygosity of any township (0.60) with more than five raccoons trapped, and had the highest recorded *B. procyonis* abundance (18.51 worms per raccoon).

The ML tree shows strong support for grouping Springfield, German, and Green Townships together (Figure 1). These townships are close in proximity, and all have *B. procyonis* prevalence below 60%. Maximum likelihood analysis also supports grouping Moorefield and Harmony Townships apart from the other seven townships. Harmony has

Table 1: Observed heterozygosities for three loci from raccoons from nine townships of southwestern Ohio

Township	Number of Raccoons Sampled for MHC II	OH* for MHC II	Number of Raccoons Sampled for Plot-12	OH* for Plot-12	Number of Raccoons Sampled for Plot-14	OH* for Plot-14
Beavercreek	20	0.90	36	0.33	32	0.94
Xenia	20	0.60	25	0.32	25	0.80
Miami	27	0.93	44	0.32	42	0.93
German	6	0.83	7	0.57	7	0.86
Green	15	0.89	21	0.33	21	0.91
Harmony	10	1.00	13	0.31	12	0.92
Mad River	8	0.75	9	0.57	9	0.89
Moorefield	12	0.83	14	0.29	14	1.00
Springfield	4	0.50	3	0.67	4	1.00
> 60%**	65	0.80	91	0.34	88	0.89
< 60%***	57	0.86	81	0.36	78	0.94

* Observed heterozygosity
** Townships with *B. procyonis* prevalence above 60%: Xenia, Miami, Harmony and Mad River
*** Townships with *B. procyonis* prevalence below 60%: Beavercreek, German, Green, Moorefield and Springfield
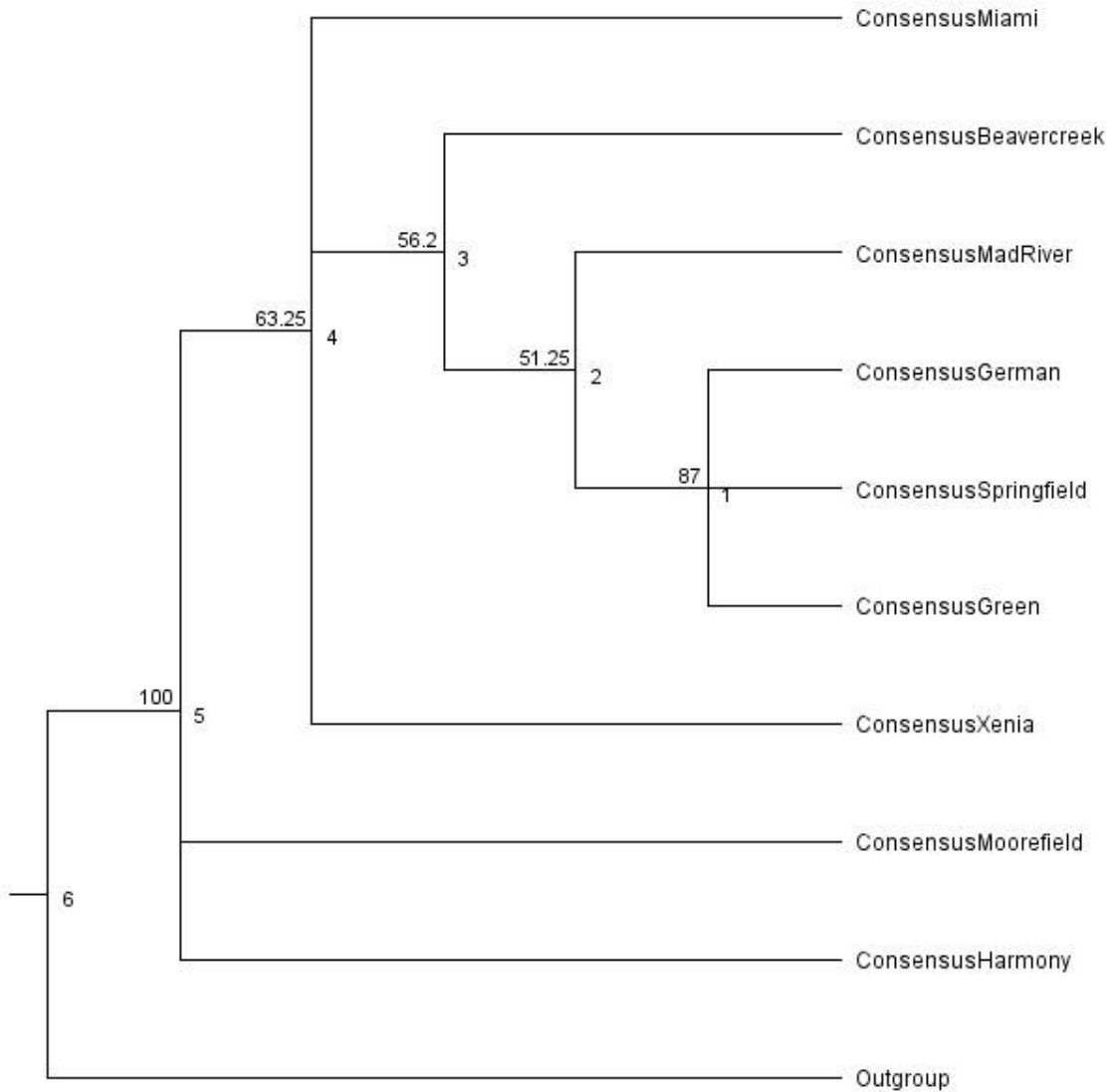
Figure 1: Maximum likelihood tree for the raccoons from the nine townships of Clark and Green Counties, OH. The branch labels represent the bootstrap support from 1000 pseudoreplicates. The node labels represent the node heights at each node, and the tip labels are the consensus sequences for the corresponding township. We calculated the consensus sequences using a strict method (50% consensus for each base in the sequence). The outgroup is the published sequence for NADH5 from a raccoon from Indiana.

the highest *B. procyonis* prevalence (73.1%), but Moorefield Township has the second lowest recorded prevalence at 46.1%. Both these townships are in Clark County, along with Mad River, Springfield, German, and Green Townships. The NJ tree also supports grouping Springfield, German, and Green Townships together, but without as high a bootstrap value as the ML tree (63 and 87, respectively). Harmony and Moorefield Townships grouped away from the rest in the NJ tree as well, with Moorefield Township grouping closer to the other townships than Harmony (Figure 2). The three townships surveyed from Green County, (Beavercreek, Xenia and Miami Townships) grouped along with Mad River Township from Clark County, with Beavercreek and Xenia Townships forming a clade apart from the other two. Beavercreek has the lowest recorded *B. procyonis* prevalence (24.5%), and grouped with three townships with *B. procyonis* prevalence above 60%. Finally, the MP tree also shows support (81.5 bootstrap value) for grouping Springfield, German and Green Townships together (Figure 3). Maximum parsimony analysis also showed strong support for grouping Moorefield and Harmony Townships apart from the other seven. Beavercreek, Xenia, Miami and Mad River Townships also grouped together in the MP tree.

Heterozygosities for both Plot-12 and Plot-14 differed between townships (Table 3). Plot-12 had lower heterozygosity than Plot-14 in every township surveyed. The heterozygosities for Plot-12 ranged from 0.29 in Moorefield Township to 0.67 in Springfield Township. Raccoons from townships with *B. procyonis* prevalence above 60% had the same heterozygosity as did raccoons from townships with prevalence below 60% ($\chi^2 = 0.06$, p = 0.812).

Figure 2: Neighbor-joining tree for the raccoons from nine townships of Clark and Green Counties, OH. The branch labels represent the bootstrap support from 1000 pseudoreplicates. The node labels represent the node heights at each node, and the tip labels are the consensus sequences for the corresponding township. We calculated the consensus sequences using a strict method (50% consensus for each base in the sequence).
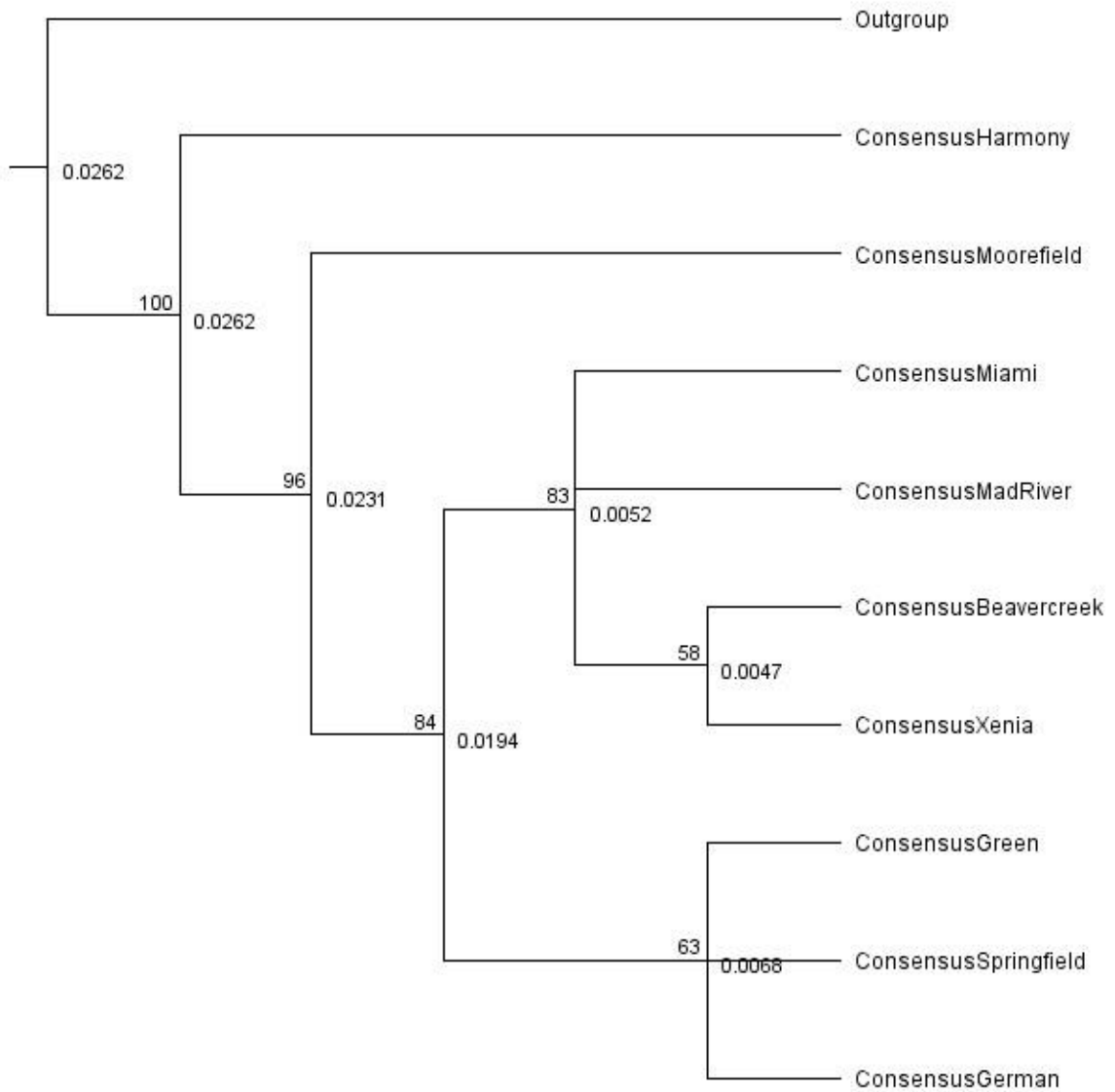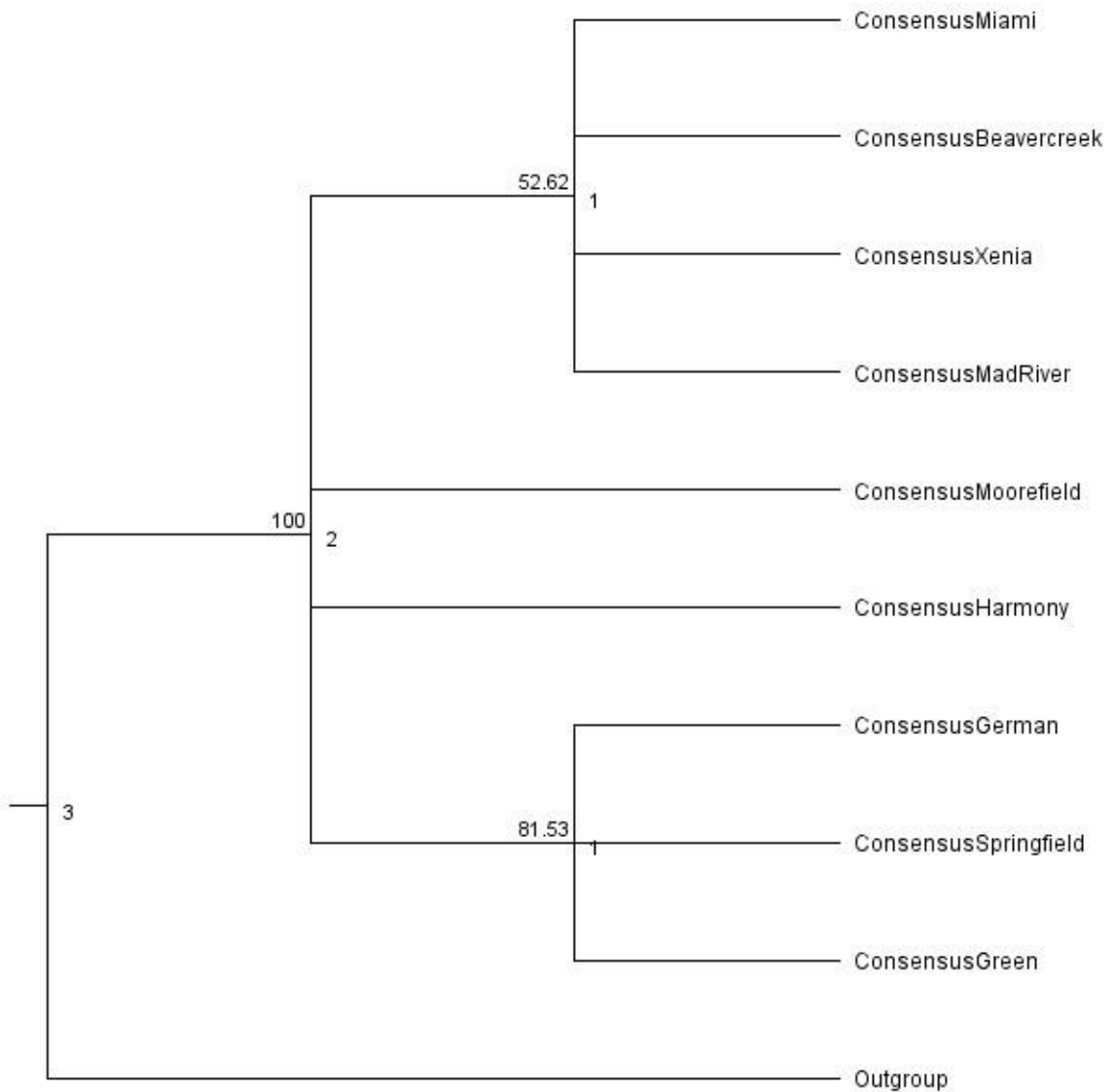
Figure 3: Maximum parsimony tree for the raccoons from nine townships of Clark and Greene Counties, OH. The branch labels represent the bootstrap support from 1000 pseudoreplicates. The node labels represent the node heights at each node, and the tip labels are the consensus sequences for the corresponding township. We calculated the consensus sequences using a strict method (50% consensus for each base in the sequence).

Both heterozygosities and allele frequencies of Plot-14 differed among townships (Table 2). Xenia Township had the lowest heterozygosity for Plot-14 (0.80) and Moorefield and Springfield had the highest (1.00). Beavercreek had the lowest *B. procyonis* prevalence, and was the only township that had allele frequencies that differed from expected values. The rest of the townships were all in HWE. The most common alleles for plot-14 had 15, 16, and 17 repeats of the repeated section. The most common allele in every township, except Springfield Township, had 16 repeats. The allele with 18 repeats was also common in Xenia, German, and Moorefield Townships. The three most common alleles accounted for between 71 and 88% of all alleles in the townships. There is no significant difference between the heterozygosities at Plot-14 in raccoons from populations with (>60%) high *B. procyonis* prevalence when compared to other raccoon populations ($\chi^2 = 1.23$, p = 0.267). The heterozygosity at Plot-14 was significantly lower in Xenia Township than the rest of the townships surveyed ($\chi^2 = 4.30$, p = 0.038).

Discussion

We found evidence of genetic structuring in two of the loci we investigated. Rates of heterozygosity were different among townships for exon 2 of MHC II and Plot-14. The most extensive variations in heterozygosity were in the portion of exon 2 from MHC II. Srithayakumar et al. (2011) found this locus varied with influence from raccoon rabies virus, while some alleles corresponded with resistance to the virus. All of the observed heterozygosities for Plot-12 departed from HWE. Fike et al. (2007) found that the observed heterozygosities for female raccoons did not depart from HWE, indicating that this locus is found on the X chromosome.

Table 2: Analysis of the genetic structure of raccoons from nine townships of southwestern Ohio according to data from Plot-14

Township	Number of Raccoons Sampled for Plot-14	OH* for Plot-14	EH** for Plot-14	Departure from HWE	3 most common alleles***	Frequency of 3 most common alleles
Beavercreek	32	0.94	0.74	Yes	15, 16, 17	0.86
Xenia	25	0.80	0.72	No	15, 16, 18	0.88
Miami	42	0.93	0.77	No	15, 16, 17	0.76
German	7	0.86	0.81	Not Run	15, 16, 18	0.79
Green	21	0.91	0.79	No	15, 16, 17	0.76
Harmony	12	0.92	0.83	No	14, 16, 17	0.71
Mad River	9	0.89	0.78	No	15, 16, 17	0.83
Moorefield	14	1.00	0.79	No	15, 16, 18	0.79
Springfield	4	1.00	0.86	Not Run	17, 18, 19	0.75
> 60%**	88	0.89	0.77	No	15, 16, 17	0.76
< 60%***	78	0.94	0.77	Yes	15, 16, 17	0.77

*   Observed heterozygosity
**   Expected heterozygosity
***The number of repeats of repeated segment represents alleles

The most common alleles for Plot-14 varied among townships as well. The most common three alleles had 15, 16, and 17 repeats, yet some of the townships had other alleles that were more common. The most interesting of these variations occurred in Xenia Township (18 repeats instead of 17) and Harmony (14 repeats instead of 15). Xenia and Harmony townships have the two highest measures of *B. procyonis* prevalence. Beavercreek Township had the lowest recorded prevalence, and was the only township that had observed allele frequencies out of HWE.

The phylogenetic trees from NADH5 also demonstrated evidence of genetic structuring that was more complex than geographic distance alone can explain. Adjacent townships were more likely to group together, although some townships did not group with adjacent townships. Moorefield and Harmony townships always grouped apart from other, even adjacent, townships. There is need to explore features that can account for the relationships seen among townships of Clark and Greene Counties.

Genetic structuring represents the presence of some barriers to gene flow (Cullingham et al., 2008), and can be present even while no physical barriers or significant distances exists between populations (Dharmarajan et al., 2009). Many factors contribute to genetic structuring by limiting the amount of gene flow between populations or subpopulations. Geographic isolation provides mechanisms that limit gene flow and lead to genetic structuring (Cullingham et al., 2008). Dispersal distances and dispersal rates are important population dynamics, increasing the amount of interaction between populations that are partially geographically isolated and thus also impact genetic structuring (Cullingham et al., 2006). Additionally, social structure plays a large role in producing the genetic structuring present within raccoon populations (Dharmarajan et al.,

2009; Gehrt et al., 2008; Hauver et al., 2010).

Dispersal is a major contributor to both social and genetic structure. Typically males move away from the home range of their mother and thus account for much of the gene flow among raccoon populations (Dharmarajan et al., 2009). Thus, there is a sex-linked bias to dispersion in raccoons (Dharmarajan et al., 2009). Dispersing males often travel in groups, the members of which are usually unrelated (Gehrt et al., 2008), and contribute to nearly half of all offspring produced with resident males accounting for the other offspring (Hauver et al., 2010). Raccoon dispersal accounts for some of the fragility of raccoon associations, where few long-term associations exist (Prange et al., 2011).

Parasites function, along with competition, predation, and sexual selection, in creating selective pressures on individuals by making some alleles contributing more to fitness than others (Thomas et al., 2000). This selective pressure can vary from ecosystem to ecosystem, as environmental factors change (Thomas et al., 2000). Pathogens and parasites exert selective pressure that can be seen in haplotype associations with the major histocompatibility complex in raccoons (Srithayakumar et al., 2011). Srithayakumar et al. (2011) found an association between raccoon rabies virus and the major histocompatibility complex in raccoons.

In the current study, we found differences in prevalence of *B. procyonis* that appear to explain some of the genetic structuring seen as differences of heterozygosities and allele frequencies among townships. This genetic structuring could also be the result of behavioral patterns and other differences among the populations. Allele frequencies from Plot-14 and exon 2 of MHC II indicate that *B. procyonis* may be applying some stabilizing selection on raccoons. Individuals with successful alleles for exon 2 of MHC

II may present the roundworm antigen to lymphocytes better than other raccoons. These raccoons may develop a resistance to *B. procyonis* as they age, resulting in large litters and more reproductive years. The sequences from Plot-14 may be linked to a portion of the genome under selective pressure from *B. procyonis.* Fike et al. (2007) also noted that some populations of raccoons had allele frequencies at Plot-14 that differed significantly from expected values. As more landscape in Beavercreek Township has become urbanized, it appears as though *B. procyonis* prevalence has dropped and the stabilizing selection *B. procyonis* provided may have subsided.

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CHAPTER FOUR

RACCOON (*PROCYON LOTOR*) DIETS ARE IMPACTED BY

BAYLISASCARIS PROCYONIS ROUNDWORM INFECTION

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Abstract

Raccoons (*Procyon lotor*) are the definitive host for raccoon roundworms (*Baylisascaris procyonis*), and cause a dangerous neurological disease known as larva migrans encephalopathy. Raccoons are omnivorous animals and rely on various food items, demonstrated by dietary analyses that help determine how a raccoon changes its diet in response to environmental features. Raccoons often eat whatever food resource is most convenient and abundant. Heavily parasitizes animals often have to alter eating habits to attain food that requires less effort to acquire, but that is also of lower energy value. In this study, we analyzed the diets of necropsied raccoons from nine townships of Clark and Greene Counties by examining their stomach contents, and categorized stomach contents by separating out plant material, vertebrate tissue, and invertebrate tissue. We measured the total stomach content mass and the mass of plant material alone in order to compare and obtain a percentage of plant material in the raccoons' diet. Townships with low roundworm prevalence had significantly lower percentages of plant prey material than townships with high prevalence. Parasite prevalence explained variation in percent plant material in the raccoon diet better than any other independent variable. These data will help us understand the relationship between *B. procyonis* and raccoon diet and further understand how parasites can impact host ecology.

Introduction

The raccoon (*P. lotor*) has the ability to adapt to a wide variety of resources, and is distributed across North and Central America (Parsons et al., 2011), and is free-ranging throughout rural and urban areas of North America (Roussere et al., 2003). Parsons et al. (2011) noted that the effects that raccoons have on their ecosystem are dependent on the

characteristics of the environment. They also noted raccoons thrive in areas where there are human developments, and the absence of large predators, with the result that raccoons in urbanized landscapes have increased survival and reproductive rates (Prange et al., 2003). Prange et al. (2003) found that urbanized areas had higher annual recruitment rates than rural areas, indicating that raccoons disperse into areas with human developments. Urban areas with the highest breeding success also have large forest patches fragmented by urbanization (Soga & Koike, 2013). Human developments provide reliable food sources and shelter for the raccoons. Southwestern Ohio is an area with many areas of human developments and few large predators.

P. lotor is the definitive host of *B. procyonis* (Page et al., 2005). Several populations of raccoons have prevalence of raccoon roundworm close to 100%. Prevalence is measured as the number of individuals that show signs of parasitism divided by the number of total individuals in the population. Page et al. (2005) discovered that necropsies were the most reliable measure of prevalence, but are not feasible in large scale prevalence investigations. They advocate for fecal samples conducted at capture. Because raccoon populations seem to be increasing in number in response to anthropogenic factors, including landscape fragmentation, it is becoming more important to study the transmission of the parasite to intermediate hosts (Page et al., 2005).

Several populations of small vertebrates are negatively affected by the transmission of *B. procyonis* (Owen et al., 2004; Page et al., 2001a, 2001b). *B. procyonis* can infect many intermediate hosts, often with devastating effects. Page et al. (2001a) found that the prevalence of *B. procyonis* in the white footed mouse, *Peromyscus leucopis*, and potentially other small vertebrates is related to the fragmentation of the

landscape. Larva migrans, an infection of *B. procyonis* in intermediate hosts, has been found in almost 90 species of birds and mammals, and can lead to death (Blizzard et al., 2010). Page et al. (2001b) found that, in agriculturally fragmented landscapes, small vertebrates are exposed to more *B. procyonis* eggs than in urban landscapes. The intermediate hosts are attracted to raccoon latrines, where eggs can concentrate.

Raccoons often defecate in the same area, called a latrine, and multiple raccoons may use the same latrine. Because a single raccoon can shed millions of *B. procyonis* eggs in a single defecation, sites of contamination can be very dangerous for potential intermediate hosts, including humans (Page et al., 2011; Roussere et al., 2003; Shafir et al., 2011). Roussere et al. (2003) found that as many as 50% of the areas sampled in urban California included at least one latrine positive for *B. procyonis* eggs.

Raccoons are highly adaptive meso-carnivores (Parsons et al., 2011). They are successful carnivores in many ecosystems because they are ecological generalists that eat a wide variety of fauna and flora (Rulison et al., 2012), changing their behavior when confronted by a new environment. Parsons et al. (2011) found that raccoons foraged primarily in the most abundant landscape type, but shifted movements in relation to seasonal resource conditions. While raccoons are one of the most important predators of eggs from water fowl and sea turtles, few individual raccoons were responsible for the total predation on the eggs (Parsons et al., 2011; Rulison et al., 2012). Parsons et al. (2011) found feathers in less than 10% of the raccoons surveyed. In the Parsons study, the raccoons relied most heavily on fruits in the fall and winter months and crustaceans in the spring and summer.

Raccoon diets vary by environment, as food choices are determined primarily by

availability (Rulison et al., 2012). Rulison et al. (2012) found that suburban raccoons eat mainly plant material, whereas raccoons in areas close to bodies of water rely most heavily on crustaceans (the most abundant prey item). Raccoons in areas subject to predator removal have more prey items to consume than raccoons in natural areas, and thus rely less on plant material (Barton & Roth, 2007). Barton and Roth (2007) found that stable isotope ratios differed significantly between the site under predator removal and the natural site. In both sites, the raccoons relied on the same abundant resources, but in different ratios that could be detected by stable isotope studies.

The purpose of this study was to investigate if landscape features and prevalence of *B. procyonis* in the intestines of *P. lotor* are useful predictors of both presence of plant material in raccoon diet, and the proportion of plant material found at necropsy based on mass. By testing the ability to predict the presence and proportion of plant material, we aim to provide valuable information for researchers assessing the potential impact of fragmentation and *B. procyonis* on *P. lotor* diet choice, and help determine the role fragmentation and parasites play in host dietary behavior.

Materials and Methods

Sites of Surveys

We conducted this study on raccoons from Greene and Clark Counties in Southwest Ohio (Table 1). These counties are made up mostly of agricultural lands as well as many dense population centers. Native landscape, without development, makes up the smallest portion of all but three of the townships surveyed. We collected the raccoons from Bath, Beavercreek, Xenia and Miami townships in Greene County, as well

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as from German, Greene, Harmony, Mad River, Moorefield and Springfield townships, and the City of Springfield in Clark County.

Trapping Season and Trappers

We worked with six fur trappers to gather raccoons for the study. Trapping season for raccoons in Ohio extends from November 10 to January 31. However, trappers usually cease trapping at the beginning of January as weather prevents raccoons from travelling great distances and interacting with traps. Raccoons were trapped on private property with the permission of landowners and traps were set in areas frequented by raccoons and readily accessible by trappers. We collected the entrails from trapped raccoons in Xenia on Mondays, Wednesdays and Fridays, and in Harmony Township on Tuesdays and Saturdays, and from Harmony Township. Raccoons trapped between collection days were stored in a -20 degree Celsius freezer by the trappers until we were able to collect them. The last day we harvested entrails was December 9. In total 232 raccoon entrails were collected during the trapping season.

Collection of Samples

We made a longitudinal incision along the ventral surface of the raccoon, cutting the esophagus just anterior to the stomach, and the rectum just anterior to the anus. We extracted the entrails, and placed them into two freezer bags (doubled up to prevent leakage) marked with the collection date, the trapper responsible for the raccoon, the township or city where the raccoon was trapped, the county where the township or city resides and a unique number to facilitate tracking of each individual raccoon. Samples

were then placed in a -20 ^{0}C freezer until necropsied.

Necropsies

Uniquely numbered thawed, entrails were placed into a dissection tray and stomachs and large intestines were removed then refrozen for future analysis. The mesentery and any attached organs were cut away and disposed of. There was at least one person with uncontaminated gloves present at all times in order to ensure that any eggs were not spread outside the dissection trays.

The full length of the small intestines were dissected, collecting any *B. procyonis* worms found, as well as any other intestinal parasites. Parasites were placed into 70% ethanol and stored in cups marked with the same number as the raccoon for future analyses and verification. We saved the small intestines from all raccoons that did not have any *B. procyonis* worms, to verify they were negative for *B. procyonis* infection and recorded the number of *B. procyonis* worms found in each raccoon (Table 1). We also recorded whether or not tapeworms were found in the intestines. After each necropsy session, all trays and dissection equipment were autoclaved at 121 ^{0}C for at least 5 minutes to destroy any contaminating eggs. Six entrails were disposed of because they were too decomposed to necropsy.

Prevalence and Abundance Analyses

We computed the overall prevalence of *B. procyonis* by dividing the total number of raccoons that contained at least one roundworm by the total number of raccoons necropsied. We repeated this process for raccoons from each township in Greene County

Table 1: Nine townships surveyed from Clark and Green Counties, OH*

Township	Population	Large Population Centers	Total Area (ha)	Developed Area (ha)	Agricultural Area (ha)	Rural Area (ha)
Beavercreek	41745	Beavercreek City, Fairborn City, Xenia City and Wright-Patterson Air Force Base	5752	1758	2958	955
Xenia	6117	Xenia City and Wilberforce Village	11391	1284	8152	1929
Miami	5106	Clifton and Yellow Springs Villages	6683	440	4748	1478
German	7336	Springfield City	8641	1033	6306	1276
Greene	2736	Clifton Village	9243	945	7430	845
Harmony	3548	South Vienna Village	12921	891	11117	883
Mad River	11828	Enon Village and Springfield City	8415	1583	5576	1132
Moorefield	11402	City of Springfield	8657	1571	4944	1329
Springfield	13424	Springfield City	8835	2888	4862	1013

* The population numbers are from the 2000 census. All of the values for area are given in hectares (ha).

and Clark County.

We computed the overall abundance of *B. procyonis* in raccoons from each location by dividing the total number of roundworms by the total number of raccoons necropsied. We also computed the mean number of worms and standard deviation for each of the townships involved in the study.

Diet Analyses

We conducted diet analyses on 190 raccoons from 9 townships of Clark and Greene Counties, OH using the stomachs that were earlier separated and refrozen from the small intestines. Once thawed, a small piece of mesentery connected to the stomach was removed to use for future genetic analyses. We recorded the mass of the stomachs, then opened the stomach and preserved the contents in 70% ethanol in a cup that was marked with the same unique number as the raccoon that the stomach came from. We disposed of any stomachs that did not contain any food, making note of such. We analyzed the contents by separating the material according to type. All plant material was grouped together, and we made note of the different types of plants that were found and identified them to the level of Family, when possible. We grouped all vertebrate material together, and all invertebrate material together, identifying the different types to the level of Class, when possible. We recorded the presence of each type (vertebrate, invertebrate, and plant) of diet material for each raccoon sampled. We noted that vertebrate tissue was present when we found juvenile *B. procyonis* in the stomach. We did not analyze fur found in the gut to determine whether or not the fur came from the trapped raccoon. We separated all plant material from other stomach contents, and allowed them to dry. We

then took the mass of the plant material, and compared it to the total wet mass of the stomach contents.

Landscape Analysis

ESRI ArcGIS software was used to edit the 2006 National Land Cover Dataset (NLCD) (downloaded from mrlc.gov), trimming it to the township level. We used Patch Analyst (http://www.cnfer.on.ca/SEP/patchanalyst/) to evaluate the metrics of 16 landscape classes in eight categories: water, developed, barren, forest, shrubland, herbaceous, planted/cultivated and wetlands.

Statistical Analyses

We analyzed the diets of the raccoons from different townships by comparing the prevalence of the different types of diet material for each township. We conducted two χ^2 tests for equality of distributions. The first χ^2 tested the null hypothesis that there was no difference in the proportion of raccoons with vertebrate tissue found in the stomach at necropsy whether they came from a township with roundworm prevalence above 0.60 or below 0.60. The second χ^2 tested the same null hypothesis replacing vertebrate tissue with plant tissue. We tested the correlation of this value to numerous landscape values and the prevalence of the roundworm.

We assessed landscape, class, and patch metrics as predictors of prevalence using simple linear regression and stepwise linear regression (forced intercept) with SPSS Version 21. Additionally, we analyzed relationships of these landscape metrics with diet (% plant) using step-wise linear regressions in order to determine what landscape metrics

were significant ($\alpha = 0.05$) predictors of diet. Based on this analysis we identified six

factors, five landscape variables and prevalence that significantly ($p < 0.05$) predicted the

raccoons' percentage of plant diet. In order to be parsimonious we eliminated two

redundant landscape metrics from these six. Eliminated variables were crop area and

mean crop patch size, while we retained percentage crop cover in landscape and total

crop patch edge, respectively.

We employed a likelihood approach to identify which models were most effective

at predicting diet. This approach allowed us to investigate the effect on the model of

including additional predictor variables (and associated error). The likelihood approach

allowed us to evaluate all possible model combinations of four variables. These included

mean nearest neighbor, total edge of crop patches, percent of the landscape as crop

patches, and prevalence of parasite infection. Using the sum of square error (SSE) from

the regression analyses of candidate models we calculated the Akaike Information

Criterion (AIC), and Akaike weights (w_i) for all possible combinations ($n = 15$) of these

variables while excluding the intercept only model.

Results

Necropsies

We necropsied 226 raccoons from Green and Clark Counties, and collected 2390

B. procyonis worms from 122 infected raccoons. We had 137 raccoons from Greene

County spread amongst the following townships: Miami (51 raccoons), Beavercreek (48

raccoons), Bath (1 raccoon grouped with Beavercreek) and Xenia (37 raccoons) (Table

2). There were 89 raccoons trapped in Clark County from the following townships:

Greene (23 raccoons), German (15 raccoons), Mad River (8 raccoons), Harmony (26 raccoons), Springfield (4 raccoons) and Moorefield (13 raccoons) (Table 2). Table 2 contains the number of raccoons from each township that were infected with roundworm. Trappers collected three raccoons in Springfield City that were added to the Springfield Township numbers. The raccoon with the highest number of roundworms (205) came from Miami Township. Raccoons with the highest average number of roundworms came from Xenia and Miami townships.

Prevalence and Abundance Analyses

We obtained prevalence data from these nine townships from Ingle et al. (2014). The abundance of roundworms from Clark and Green Counties was 10.58 worms per raccoon. The abundance of Green County was higher than that of Clark County (12.33 worms per raccoon compared with 8.10 worms per raccoon). The abundances for the individual townships ranged from 1.54 worms per raccoon in Moorefield Township to 18.51 worms per raccoon in Xenia Township (Table 2).

Diet Analyses

We found the following classes of vertebrates in our analyses of raccoon stomachs: Actinopterygii, Aves, and Mammalia. We also found materials from Class Oligochaeta and Coleoptera, in some of the raccoons. The only plant family we identified in the raccoons was Poaceae, with most raccoons having some *Zea mays* kernals in their stomach at necropsy (Table 3). Nearly 80% (150 out of 191) of the raccoons necropsied had plant material in the stomach. We calculated prevalence of vertebrate and plant tissue

Table 2: Prevalence of *B. procyonis* in *Procyon lotor* for the nine townships from Clark and Greene Counties, OH

County	Township	Number of Raccoons	Number of raccoons infected	Prevalence of *B. procyonis**	Abundance of *B. procyonis*	Largest number of *B. procyonis*
Greene		137	72	0.526	12.33	210
	Beavercreek	49	12	0.245	1.71	52
	Xenia	37	25	0.676	18.51	176
	Miami	51	35	0.686	18.08	210
Clark		89	52	0.584	8.10	64
	German	15	7	0.467	6.4	47
	Green	23	13	0.565	10.22	64
	Harmony	26	19	0.731	11.96	50
	Mad River	8	5	0.625	5	31
	Moorefield	13	6	0.461	1.54	4
	Springfield	4	2	0.500	4.75	15

* We obtained this data from Ingle et al (2014)

Table 3: Prevalence of vertebrate and plant tissues found in stomach contents for the nine townships from Clark and Greene Counties, OH

County	Township	Number of Raccoon stomachs	Vertebrate tissue*	Vertebrate tissue prevalence	Plant Tissue**	Plant tissue prevalence
Greene		119	110	0.924	87	0.731
	Beavercreek	42	41	0.976	24	0.571
	Xenia	27	25	0.926	19	0.704
	Miami	50	44	0.880	44	0.880
Clark		72	66	0.917	63	0.875
	German	9	8	0.889	8	0.889
	Green	22	21	0.955	18	0.818
	Harmony	16	15	0.938	16	1.000
	Mad River	8	8	1.000	8	1.000
	Moorefield	13	11	0.846	10	0.769
	Springfield	4	3	0.750	3	0.75
> 0.60 prevalence		101	92	0.911	87	0.861
< 0.60 prevalence		90	84	0.933	63	0.700

* The number of raccoon stomachs that contained vertebrate tissue at necropsy
** The number of raccoon stomachs that contained plant tissue at necropsy

by dividing the number of raccoons found with the material in the stomach at necropsy by the total number of raccoons necropsied. Plant tissue prevalence in the townships ranged from 0.571 in Beavercreek Township to 1.000 in both Harmony and Mad River Townships. Four out of the nine townships we surveyed had plant tissue prevalence below 0.8. Raccoons from townships with higher *B. procyonis* prevalence typically had higher plant tissue prevalence. We did not notice the same trend with vertebrate tissue prevalence. The vertebrate tissue prevalence from Springfield Township was the only value below 0.8, but we surveyed only four raccoons from this township. Vertebrate tissue prevalence had a much narrower range (0.750 in Springfield Township to 1.000 in Mad River Township).

Statistical Analyses

We calculated the χ^2 value for each of the tests for equality of distributions. We grouped all of the raccoons into two groups (Table 3): those from townships with *B. procyonis* prevalence above 0.6, and those from townships with prevalence below 0.6. Raccoons from townships where *B. procyonis* prevalence was above 0.6 had insignificantly lower vertebrate tissue prevalence than other raccoons (0.911 vs 0.933, χ^2 = 0.331, df = 1, p = 0.565). However, plant tissue prevalence did differ significantly between the two groups (χ^2 = 7.353, df = 1, p = 0.007), with raccoons from townships with high *B. procyonis* prevalence having significantly higher plant tissue prevalence (0.861 vs 0.700).

Landscape Analysis

Our analysis of linear relationships between prevalence and landscape variables revealed a strong correlation. The percent of the landscape (or township) that was considered crop had a significant impact on prevalence ($p < 0.0001$, $r^2 = 0.975$). Four of the five landscape variables were able to individually predict at least 90% of the variability in roundworm prevalence (Table 4).

Using a likelihood approach to evaluate diet, we discovered that each of the top five models included prevalence as a predictor of raccoon diet (Table 5). Interestingly, there were no models in the top 8 models that excluded prevalence. This suggests there is a strong influence that prevalence has in predicting raccoon diet relative to landscape variables. We investigated 15 model combinations of four variables and found that, when taken together, the top five models had an 81% chance of being the best model (w_r) given these data.

Discussion

The composition of a species' diet fluctuates from season to season (Hafeez et al., 2012; Parsons et al., 2011; Rulison et al., 2012). Hafeez et al. (2012) demonstrated that much of the variation in diet choices within a given season was due to variation in choices available, as well as how long the particular item had been available in a given area. Raccoons become more selective as availability of reliable food sources increases, and shift their diet to whichever plant is producing ripe fruit (Rulison et al., 2012). Predators will not rely entirely on a single resource, even when extremely abundant (Storm & Whitaker, 2007). Parsons et al. (2011) note that earthworms and boned meat are difficult to detect in feces, and may have been underrepresented in diet analyses.

Table 4. Simple linear regression used to predict prevalence from landscape metrics derived from Patch Analyst*.

Variable	r²	Regression Mean Square	β	F	p-value
% Landscape as Crop	0.975	2.843	0.987	313.837	< 0.0001
Mean Nearest Neighbor	0.957	2.790	0.978	177.755	< 0.0001
Total Edge of Crop Patches	0.965	2.814	0.982	220.255	< 0.0001
Crop Area (ha)	0.920	2.682	0.959	91.714	< 0.0001
Mean Crop Patch Size (ha)	0.688	2.005	0.829	17.600	0.003

* Landscape metrics include percent landscape under crop cover (Pct Landscape Crop), total edge of crop patches (Total Edge Crop) and Mean Nearest Neighbor of patches in townships.

Table 5. The top five models identified to predict percent of plant in raccoon diet*.

Model Rank	Model description	k	AIC	Δr	ζ	w_r
1	Prevalence	2	862.61	0.00	1.00000	0.305
2	MNN + Prevalence	3	863.67	1.06	0.58802	0.179
3	TotEdgeCrop + Prevalence	3	864.59	1.99	0.37027	0.113
4	% LandscapeCrop + Prevalence	3	864.59	1.99	0.37009	0.113
5	MNN + TotEdgeCrop + Prevalence	4	864.85	2.24	0.32575	0.099

* These models are ranked based on the calculated AIC value derived from the residual sum of squares and the number of parameters (k) in the model. These data were used to calculate the Likelihood of the particular model given the data and the Akaike weight (w_r) for each candidate model. Models were selected from all possible combinations of four variables; prevalence, mean nearest neighbor (MNN), Total Edge Crop (TotEdgeCrop), and % landscape crop.

A species' diet often changes when nutritional demands change (Rulison et al., 2012). As nutritional demands change, an animal may begin to behave differently in many ways. These behavioral changes may cause an animal to use certain resources not typically used by that particular species (Storm & Whitaker, 2007). For instance, animals that do not feed on the yearly cicadas will feed on the periodic cicadas. Learned behavior may play a significant role in determining the relative frequency of various food items (Hafeez et al., 2012).

There is a link between the size of a species and diet (Robb et al., 2012). Robb et al. (2012) found that the larger the mole-rat species, the more general was its diet. This may be due to competition, as competition was inferred by the researchers from common diet items used most often in each species. Even in the same species, there is a high level of variation in diet choice. Different populations have different sex ratios that need to be accounted for when studying diet (Barton & Roth, 2007). Barton and Roth (2007) argue that males dominate areas under predator removal because male raccoons disperse further on average than females. Males are more likely to be in areas not used by their mother than females are. As such, it may be necessary to modify the source population in order to properly understand and manage the population being studied.

B. procyonis prevalence correlated significantly with all of the landscape features we calculated. We were able to predict *B. procyonis* prevalence significantly well using each of the five landscape features, with percent landscape identified as crop, predicting prevalence better than any other variable. The best models for predicting the percent plant material in the gut all had parasite prevalence as one of the explanatory variables (Table 5). In fact, every model that contained parasite prevalence as one of the variables was

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better than every model that lacked prevalence. This relationship is important to note, as raccoon feces is an important resource for small mammals (Page et al., 2001a, 2001b). The best variable that we tested for explaining and predicting percent plant in the stomach at necropsy is *B. procyonis* prevalence. We cannot ignore the role symbionts play in helping to shape diet choice, and potentially other behaviors.

Most of the raccoons we necropsied had vertebrate tissue in the stomach. Raccoons appeared to rely more on mammals than any other type of vertebrate prey items. Raccoons from across Clark and Greene Counties were equally likely to have vertebrate tissue in their stomachs at necropsy. Corn is the most abundant agricultural item in the townships we surveyed. It was also the most abundant plant material we found at necropsy. Raccoons from townships with higher roundworm prevalence were more likely to have plant tissue in the stomach at necropsy. This indicates that raccoons from areas with higher roundworm prevalence are more likely to consume plant material than raccoons from areas with lower prevalence. This could be due to raccoons with increased nutritional demands selecting more readily available resources, or behavioral changes caused by the parasite to encourage the movement of larvae to intermediate hosts without depending on increased host density. While raccoons are opportunistic feeders, raccoon roundworm prevalence seems to impact diet choices.

The raccoons we sampled appear to be selecting food items based on more than what is most available. Raccoons from areas with high *B. procyonis* prevalence select for more plant material. This makes the raccoon feces more attractive to intermediate hosts that use the feces as a source of food (Page et al., 2001b). Raccoons in townships with higher roundworm prevalence are more likely to transmit roundworms to intermediate

hosts through the feces, but not more likely to eat intermediate hosts. The intermediate hosts in townships with higher roundworm prevalence would likely also have higher roundworm prevalence. While the raccoons were not more likely to eat intermediate hosts, they should be more likely to encounter infected hosts, and complete the indirect life cycle of *B. procyonis*. This appears to support that *B. procyonis* contribute to diet changes in raccoons that increase the likelihood of eggs being consumed by intermediate hosts. We need to study the role symbionts play in impacting host behavior. We need to determine what factors contribute the most to determining diet, and what role parasitism might play in the determination of diet.

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CHAPTER FIVE

CONCLUSIONS AND FUTURE WORK

Conclusions

First Goal

My hypothesis that *B. procyonis* prevalence would be higher in southwestern

Ohio than in previously studies areas was incorrect. We measured *B. procyonis*

prevalence at 55%, which is lower than the 68 - 80% found in other studies done in the

Midwestern United States. We did hear from trappers that the survival rate of juveniles

was low the year the raccoons were trapped for the current study, and most raccoons

trapped were adults. Because it has been suggested that raccoons gain resistance to *B.*

procyonis with age (Owen et al., 2004), our prevalence measures may have been lower

than what is typical in this area. I was also incorrect in hypothesizing that *B. procyonis*

prevalence would be higher in more fragmented landscapes (small mean patch size).

Instead, *B. procyonis* prevalence increased with mean patch size, due to the larger tracks

of agriculture that also correlated to higher *B. procyonis* prevalence. Beavercreek

Township has the second smallest mean patch size (highly fragmented landscape) and the

lowest *B. procyonis* prevalence we found. Townships with higher amounts of agriculture

relative to total area had higher *B. procyonis* prevalence.

Second Goal

My hypothesis that raccoons would be more genetically isolated than can be

accounted for by physical distance alone was supported by the data. Raccoons from

adjacent townships did not always group together in our phylogenies. We will need to explore the question further, comparing the genetic distances between the townships with the physical distances between the townships. We did notice that raccoons from adjacent townships may be quite different genetically (raccoons from Moorefield and Harmony Townships always grouped away from the adjacent townships). We were correct in predicting that raccoons from areas fragmented by both agriculture and urbanization would show signs of divergence. Beavercreek Township had the most landscape modified by urbanization (31%), and had 51% of landscape modified by agriculture. We found raccoons from this area departed from HWE in both microsatellite loci studied. My final hypothesis was correct as well. Raccoons from areas fragmented by landscape modification from both urbanization and agriculture have significantly lower amounts of plant material in the stomach at necropsy. These raccoons rely less on agriculture for food, have lower *B. procyonis* prevalence and more genetic variability than do raccoons from areas with less urbanization. The *B. procyonis* worms appear to encourage behavioral changes in the raccoons that lead to parasite eggs being exposed to intermediate hosts, such as small mammals. Also some raccoons survive better and reproduce better in the presence of high *B. procyonis* prevalence, leading to less genetic variation in townships with high prevalence when compared with townships with low prevalence.

Suggestions for Future Work

B. procyonis Prevalence and Landscape

- The goal of this study is to determine whether *B. procyonis* prevalence decreases as time increases since landscape modification.

  o The objective for this goal is to demonstrate that *B. procyonis* prevalence is low in areas where recent urban development has replaced agriculture.

    ▪ I hypothesize that areas with recent urbanization will have lower *B. procyonis* prevalence than areas with more agriculture, and that *B. procyonis* prevalence will decrease with the time since urbanization started.

B. procyonis Prevalence and Raccoon Genetics

- The purpose of this study is to assess the genetic distances between populations of raccoons, and determine if these distances correlate with differences in *B. procyonis* prevalence.

  o Meeting this goal will require calculating the genetic distances between populations of raccoons using at least 12 microsatellite loci, and compare the distances among populations with similar *B. procyonis* prevalence and among populations with greatly varying prevalence.

    ▪ Raccoon populations with low *B. procyonis* prevalence will be more genetically isolated from other populations.

B. procyonis Prevalence and Raccoon Home Range

- This study aims to determine the relationship between *B. procyonis* prevalence and raccoon home range.

  o In order to complete this goal, the researcher would run a linear regression with average raccoon home range size for an area as the dependent variable and *B. procyonis* as the independent variable.

    ▪ Raccoons from areas with higher *B. procyonis* prevalence will have significantly lower home range size than raccoons from areas with low *B. procyonis* prevalence. I hypothesize that *B. procyonis* prevalence will predict raccoon home range size better than habitat patch size.

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APPENDIX 1

COMPARING TECHNIQUES FOR ASSESSING PARASITE PREVALENCE

Comparing Prevalence Techniques

Introduction

The North American raccoon, *Procyon lotor*, can be found all over the continental

United States. Raccoons prefer to live in moist, wooded habitats but are very adaptable

and can survive in many other habitats (Parsons et al., 2011). Raccoons thrive in

suburban habitats associated with humans (Parsons et al., 2011; Prange et al., 2003; Soga

& Koike, 2013). Rulison et al. (2012) argue that raccoons are so successful in areas

where humans have altered the native landscape because they are highly adaptive

generalist omnivores. Plant material is the most common diet item in raccoons in

suburban areas (Rulison et al., 2012). Because raccoons thrive and accumulate in areas

associated with humans, they can become a serious health risk when considering

transmission of disease (Page et al., 2011; Prange et al., 2003; Roussere et al., 2003).

Raccoon roundworm, *Baylisascaris procyonis*, is a common raccoon parasite that

infects over 90 vertebrates as intermediate hosts (Blizzard et al., 2010). *B. procyonis*

poses a significant health risk to these species as larva migrate throughout tissues

(Blizzard et al., 2010). Intermediate hosts become infected with *B. procyonis* by

consuming infected eggs, which reach infective state after about 30 days and remain

infective for years (Page et al., 2011). Many animals are attracted to raccoon feces as a

source of food (Owen et al., 2004; Page et al., 2001a, 2001b). Raccoons often defecate in

the same area (latrine), where *B. procyonis* eggs can accumulate and large numbers of

intermediate hosts can become infected with the parasite (Page et al., 2011; Page et al., 2001a, 2001b). Humans can become infected with the parasite (zoonosis), where it can cause severe neurological trauma (Blizzard et al., 2010).

B. procyonis eggs are incredibly versatile even in temperate areas, where freezing cycles do little to reduce the viability of the larvae inside (Shafir et al., 2011). Because of the potential danger associated with this parasite infecting humans, proper techniques to estimate prevalence (percentage of host individuals with the parasite) are important. There are three major techniques to estimate *B. procyonis* prevalence in raccoons (Page et al., 2005). The first technique is to necropsy the raccoons, checking the intestines for *B. procyonis* worms (gut analysis). This technique is the most reliable, but is labor intensive and inefficient for large scale studies (Page et al., 2005). The second technique is to sample latrines, but this often underestimates prevalence (Page et al., 2005). Fecal analyses are the third technique, and provide a reliable method for estimating prevalence. Page et al. (2005) noted that fecal analyses do give false negatives for some raccoons and may therefore underestimate prevalence. These researchers found that fecal samples measured prevalence at 17.5%, when necropsies estimated prevalence at 44% (Page et al., 2005).

The purpose of this study was to investigate the use of fecal analyses in estimating *B. procyonis* prevalence. Specifically, we investigated if fecal analyses will produce false positives for *B. procyonis* presence in raccoons tested. By testing the ability of fecal analyses to estimate prevalence, we aimed to provide information for researchers attempting to estimate *B. procyonis* prevalence and assess potential zoonotic potential.

Materials and Methods

We obtained prevalence data from Ingle et al (unpublished data). We necropsied the intestines of 226 raccoons from nine townships of Clark and Green Counties, Ohio, recording the presence of *B. procyonis* worms in the intestines at necropsy and collecting them for further analyses. We collected a fecal sample from the large intestine of each raccoon that was negative for *B. procyonis* at necropsy. Seven of the 102 raccoons that were negative at necropsy did not have any feces to be collected, so we excluded them from our data.

We suspended the fecal matter in Sheather's sucrose solution, making the solution by mixing 1362 grams glucose and 24 milliliters of 37% formaldehyde with 1.065 liters of water. The solution and feces was placed into a fecalyzer (Vetoquinol USA). Using the fecalyzer insert, we mixed the feces with the solution until the insert could be locked in place. Adding additional Sheather's sucrose solution created a meniscus. After placing a glass slide on top of the meniscus, we let it sit for 10 minutes. Each slide was labeled with the number of the raccoon contributing the corresponding fecal sample. After 10 minutes, we coated one side of a coverslip with Kleermount and Xylene (Carolina Biological Supplies) and placed it over top of the slide sample.

We examined the slides under a microscope looking for *B. procyonis* eggs, recording all slides that contained *B. procyonis* eggs, and making note of the township the raccoon that contributed the fecal sample was from. One slide was labeled incorrectly and had to be discarded and excluded from our data. In total, four of our raccoons that were negative at necropsy had to be excluded from our data.

We grouped the raccoons into two groups to run a χ^2 test for equality of

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distributions. We tested the null hypothesis that raccoons from townships with *B. procyonis* prevalence above 50% have the same proportion of false positives as raccoons from townships with prevalence below 50%. We also ran a logistic regression to determine if parasite prevalence is a good predictor of whether or not an individual raccoon will get a false positive from a fecal analysis.

<div align="center">Results and Discussion</div>

We compared the data from the fecal analyses with the prevalence we obtained from Ingle et al (2014). We surveyed 6 townships in Clark County, OH and 3 townships from Greene County, OH (Table 1). The number of fecal samples per township ranged from 2 in Springfield Township to 29 in Beavercreek Township. Four of the six townships surveyed from Clark County had counts of 0 raccoons that tested positive for *B. procyonis* during the fecal analyses (German, Mad River, Moorefield and Springfield Townships). The highest percentage of raccoons negative for *B. procyonis* at necropsy and positive during the fecal analyses (28.6%) from Clark County came from Harmony Township. Harmony Township also had the highest *B. procyonis* prevalence recorded by Ingle et al. (2014) (73.1%). Every township from Greene County had at least 1 raccoon with a potentially false positive fecal analysis result, with Miami Township having the highest prevalence and percentage of raccoons with a positive fecal test after a negative necropsy (68.6% and 31.3%, respectively). We noticed that Green County had a higher percentage of raccoons demonstrating false positive fecal analysis results than Clark County (17.2% vs 8.1%). This difference was not significant ($\chi^2 = 0.162$, df = 1, p = 0.69).

Table 1: Raccoon roundworm presence in raccoons from Clark and Greene Counties, Ohio*

County	Township	+ Necropsy	- Necropsy	+ Fecal sample	- Fecal sample	Percentage positive
Clark		**52**	**37**	**4**	**30**	**8.1%**
	German	**7**	**8**	**0**	**6**	**0%**
	Green	**13**	**10**	**2**	**8**	**20%**
	Harmony	**19**	**7**	**2**	**4**	**28.6%**
	Mad River	**5**	**3**	**0**	**3**	**0%**
	Moorefield	**6**	**7**	**0**	**7**	**0%**
	Springfield	**2**	**2**	**0**	**2**	**0%**
Greene		**71**	**65**	**9**	**52**	**17.2%**
	Beavercreek	**12**	**37**	**3**	**26**	**8.1%**
	Miami	**35**	**16**	**5**	**15**	**31.3%**
	Xenia	**25**	**12**	**1**	**11**	**8.3%**
<50% prevalence**		**25**	**52**	**3**	**39**	**5.8%**
>50% prevalence***		**99**	**50**	**10**	**43**	**20%**

*Fecal analyses were only done with raccoons negative for *B. procyonis* at necropsy.
**Townships with <50% prevalence included German, Moorefield, and Beavercreek townships.
***Townships with >50% prevalence included Green, Harmony, Mad River, Springfield, Miami, and Xenia townships.

Page et al. (2005) noted that two thirds of raccoons positive for *B. procyonis* at necropsy had eggs in the feces during analysis. Because fecal analyses can result in false negatives, we wanted to determine if there was a relationship between *B. procyonis* prevalence and the percentage of raccoons demonstrating false positives during fecal analyses. It is possible that necropsy could give a false negative for *B. procyonis* prevalence if the individual expelled all worms, but was still producing eggs in the feces. It is also possible that worms could be overlooked and therefore undetected during the necropsy process. It is more likely that raccoons have consumed uninfected eggs, and have a false positive fecal test. Raccoons from townships with *B. procyonis* prevalence above 50% had a higher percentage of raccoons demonstrating false positive results during fecal analysis than raccoons from townships with lower prevalence (20% vs 5.8%). The observed numbers of raccoons testing positive or negative at fecal analysis departed from the expected values under the null hypothesis (Tables 2 and 3), but was not significant ($\chi^2 = 2.73$, df = 1, p = 0.098). While the difference was not significant, areas with higher prevalence (>50%) may be more likely to test positive falsely during fecal analyses.

We tested the ability of *B. procyonis* prevalence from the township where the raccoon was trapped, to predict whether a raccoon would test positive or negative during fecal analysis using the following binary linear regression: egg presence = B_1(*B. procyonis* prevalence), where B_1 is the coefficient for *B. procyonis* prevalence. We removed the intercept from the model because if *B. procyonis* prevalence is 0, no raccoons should ever be exposed to eggs (ratio of positive fecal analyses to negative would be zero). This model predicted egg presence significantly well (Nagelkerke R^2 = 0.499, $p < 0.001$). The model predicted 100% of the raccoons negative at fecal analysis

Table 2: Observed fecal analysis results between populations with prevalence above 50% and populations with prevalence below 50%

	With eggs	Without eggs
Prevalence below 50%	3	39
Prevalence above 50%	10	43

Table 3: Expected fecal analysis results between populations with prevalence above 50% and populations with prevalence below 50%*

	With eggs	Without eggs
Prevalence below 50%	5.75	36.25
Prevalence above 50%	7.25	45.75

correctly and 86.3% overall correctly (compared to 0% and 13.7% for the model with no predictor). False positives do correlate with *B. procyonis* prevalence. When predicting whether or not raccoons will have a false positive result from fecal analyses, *B. procyonis* prevalence is a good explanatory variable. Fecal analyses are more likely to generate false positives when *B. procyonis* prevalence is high. It is important to know whether the false negatives correlate with *B. procyonis* to determine how well this method correctly estimates prevalence. Page et al. (2005) noted that fecal analyses estimated prevalence nearly 30% lower than necropsies. Even with more false positives in areas with high *B. procyonis*, fecal analyses are likely to underrepresent prevalence as *B. procyonis* females vary egg production by season, but remain present throughout the year (Owen et al., 2004; Page et al., 2005).

APPENDIX II

THE GENETIC STRUCTURING OF *B. PROCYONIS* AND

RACCOONS

Genetic Structuring of *B. procyonis*

Introduction

Many species of economically valuable livestock and plants are devastated by parasites, many of which are resistant to common and established anthelmintic treatments (Demeler et al., 2013; Douda et al., 2013; Gutierrez-Gutierrez et al., 2013). Phylogenies constructed for nematode parasites can be effective tools to determine and illustrate relationships between conspecific individuals and individuals from separate species (Demeler et al., 2013; Gutierrez-Gutierrez et al., 2013; Kumari & Subbotin, 2012). Few species of nematode parasites have been studied at the molecular level (Li et al., 2012). Li et al. (2012) demonstrate that phylogenetic trees of nematode parasites of wild animals provide useful markers for epidemiological investigation of roundworms.

In this study, we aim to determine the phylogenetic relationship between raccoons (*Procyon lotor*) and raccoon roundworm (*Baylisascaris procyonis*). We aim to determine the degree of coevolution between the two species to better understand how *B. procyonis* helps shape raccoon genetics. We hypothesize that the phylogeny of *B. procyonis* from the nine townships of Clark and Green Counties, OH will have congruence to the raccoons from the same nine townships. These data will help us better understand the genetic structuring of *B. procyonis*, and better design management strategies to reduce *B. procyonis* prevalence in raccoons.

Methods

DNA Isolation and Amplification

We isolated DNA from a piece of the greater omentum of the raccoons from Clark and Greene Counties, OH using the DNA Mini Kit from Qiagen (using the published protocol from Qiagen). We amplified exon 2 from MHC II and NADH5 using Real-time PCR. We used the following ratios: 2 µL of each primer (forward and reverse), 6 µL of DNA eluate and 10 µL of Sybr Select Master Mix (Life Technologies). We sent the amplified DNA to the Plant-Microbe genomics Facility at The Ohio State University for sequencing.

We isolated DNA from sections removed from *Baylisascaris procyonis* worms from Clark and Greene Counties, OH using the DNA Mini Kit from Qiagen (using the published protocol from Qiagen). We cut the removed sections into pieces as small as possible to reduce the amount of time necessary for lysis. We sent the isolated DNA to the Plant-Microbe genomics Facility at The Ohio State University for amplification and sequencing. The facility amplified and sequenced cytochrome c oxidase subunit 1.

DNA Analyses

We used the sequences from NADH5 to construct a maximum parsimony phylogenetic tree of the raccoons from the nine townships we surveyed in these counties. We also constructed a maximum parsimony tree using the sequences from cytochrome c oxidase subunit 1 from the roundworms sampled from the same nine townships. We compared these two trees to determine any relationships between the genetic structuring of *B. procyonis* and the genetic structuring of raccoons.

Results

We found evidence of enough genetic differences among raccoon populations to construct a phylogeny using the sequences for NADH5 (Figure 1). We also detected genetic structuring in the roundworms (Figure 2). Differences in *B. procyonis* prevalence don't seem to explain the phylogenetic relationships among populations (Figure 3). Some adjacent townships group separately in both trees, illustrating congruence between the phylogenies of the raccoons and *B. procyonis*. The raccoons from Mad River Township are grouping with the raccoons from the townships of Greene County, and the raccoons from Harmony and Moorefield Townships are not grouping with the clade that represents raccoons from Green, Springfield and German Townships.

We found similarities in the phylogenies and genetic structures of the raccoons and *B. procyonis* roundworms, but little congruence. The only node that matched had raccoons from Moorefield Township as part of a polytomy with raccoons from the other townships. Xenia, Beavercreek and Mad River Townships group together in both the raccoons and roundworms. Springfield and German also group together in both trees. *B. procyonis* worms from Miami Township do not group with the worms from Mad River, Beavercreek and Xenia Townships (the raccoons from Miami Township group with raccoons from these townships). Green Township doesn't group with Springfield and German Townships in the *B. procyonis* phylogeny, but does in the raccoon phylogeny.

Discussion

We have found potential evidence of coevolution in *B. procyonis* and raccoons based on phylogenetic trees constructed for individuals from nine townships of Clark and
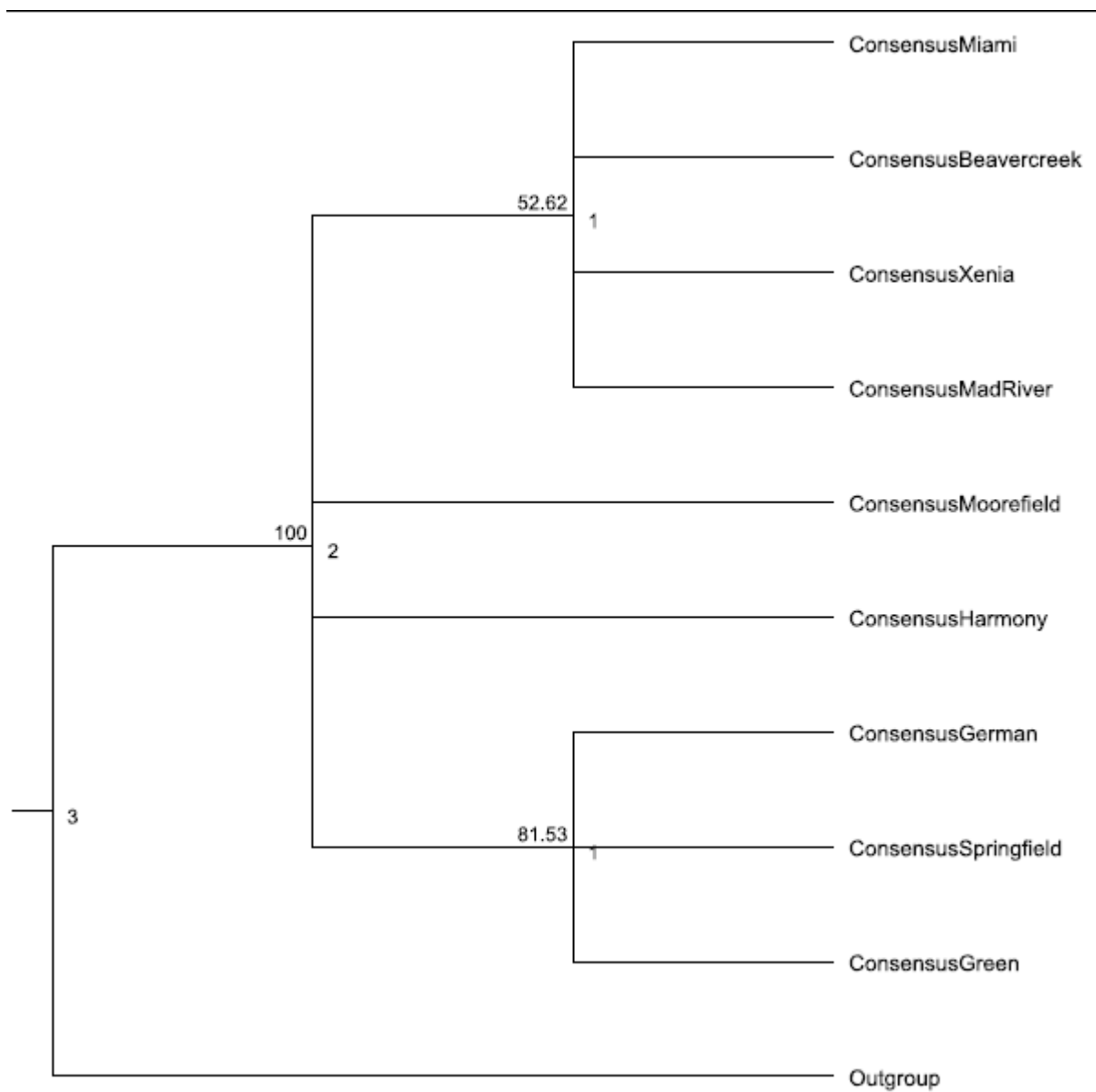
Figure 1: Maximum parsimony tree for raccoons (*Procyon lotor*) from southwestern Ohio based on NADH dehydrogenase subunit 5 (NADH5)
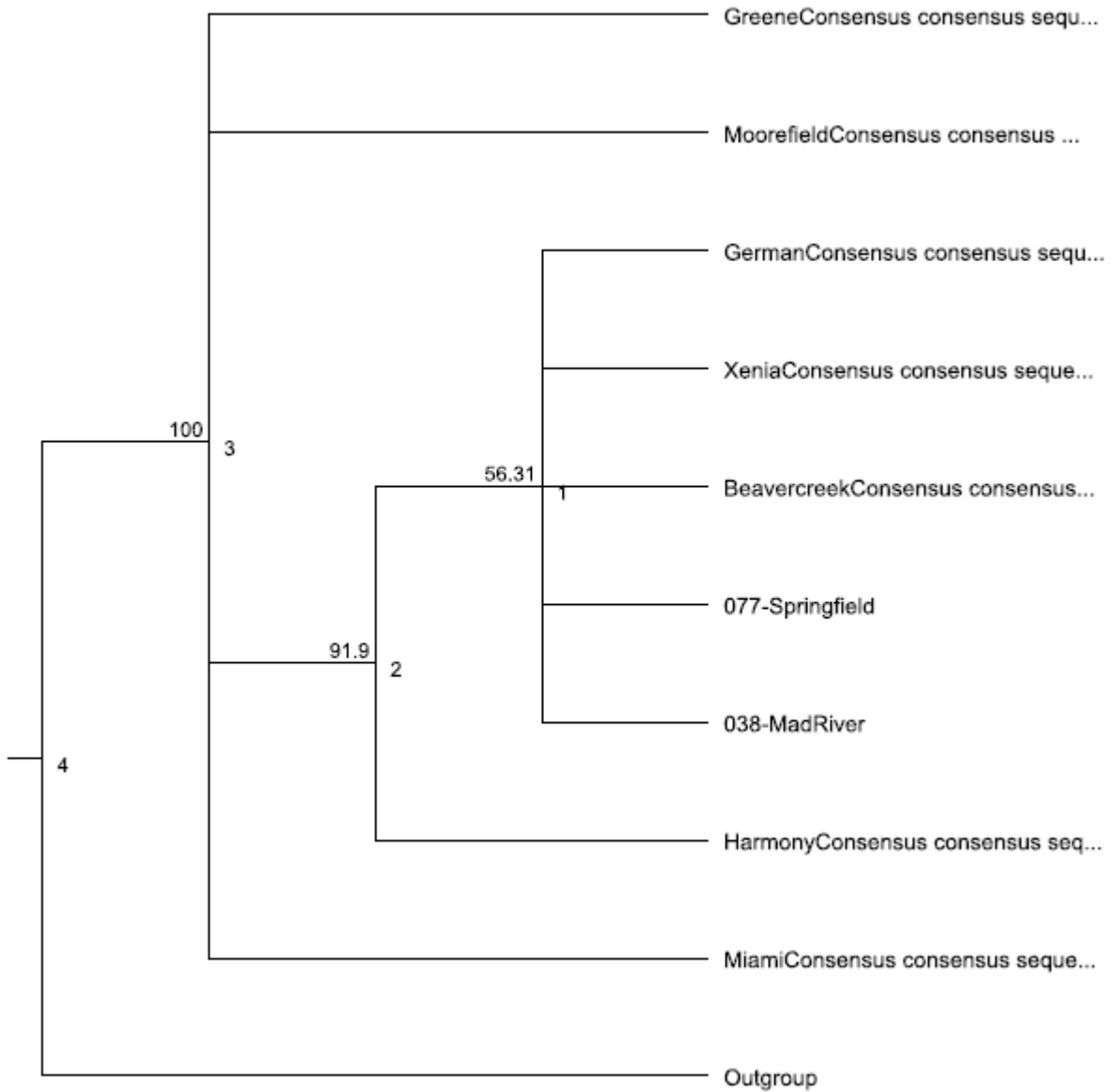
Figure 2: Maximum parsimony tree for raccoon roundworm (*Baylisascaris procyonis*) from southwestern Ohio based on cytochrome c oxidase subunit 1
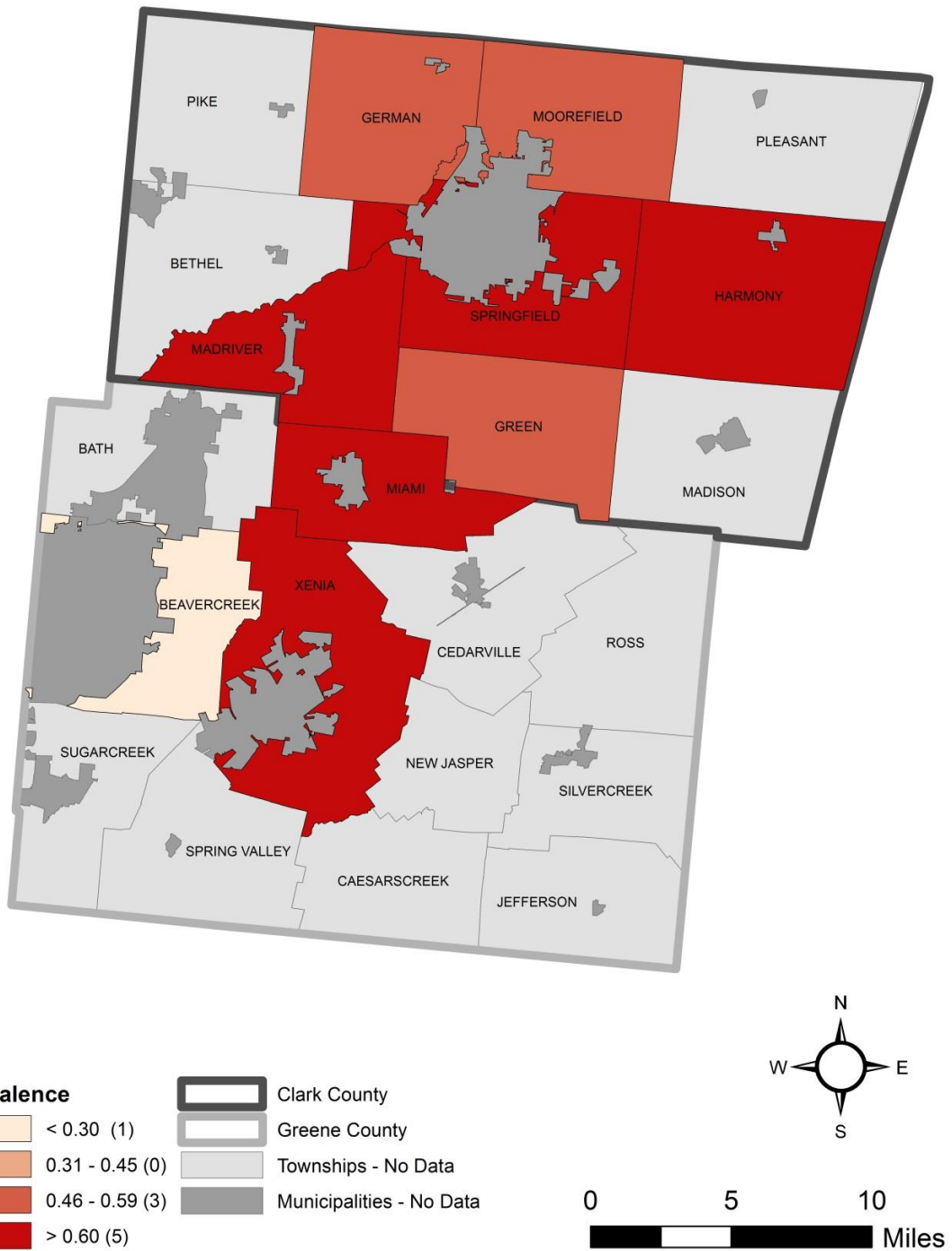
Figure 3: Map of the townships of Clark and Greene Counties, OH. The townships surveyed are highlighted, and *B. procyonis* prevalence for the township is shown.

Greene Counties, OH. Our hypothesis that the phylogeny of *B. procyonis* will have congruence to the phylogeny of raccoons is not supported by the data. There are some key differences that we need to explore further. We hypothesize that the incongruence between the phylogeny of raccoons and the phylogeny of *B. procyonis* will be due to landscape features and how raccoons move among townships. However, we need to isolate DNA from each roundworm found in a raccoon, and generate a consensus sequence for the *B. procyonis* roundworms from each raccoon from each township. This will eliminate one roundworm with a novel sequence from having too large of an influence in the overall phylogeny of the townships. As we understand more about how *B. procyonis* works in raccoons, we can better devise management strategies to reduce prevalence and potential spillover into human populations closely associated with raccoons.