University of Central Oklahoma

Jackson College of Graduate Studies

SURVEILLANCE OF MOLLUSCAN (GASTROPOD) INTERMEDIATE HOSTS FOR THE EMERGING INFECTIOUS DISEASE ANGIOSTRONGYLIASIS (*Angiostrongylus cantonensis*) IN OKLAHOMA

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

Degree of

MASTER OF SCIENCE IN FORENSIC SCIENCE – BIOLOGY/CHEMISTRY

By

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Edmond, Oklahoma

2018

SURVEILLANCE OF AN EMERGING INFECTIOUS DISEASE:
ANGIOSTRONGYLIASIS CAUSED BY *Angiostrongylus cantonensis*

By: Christopher M. Dunn

A THESIS

APPROVED FOR THE W. ROGER WEBB FORENSIC SCIENCE INSTITUTE

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ACKNOWLEDGMENTS

Thank you to my committee for helping with decision making along the way. Thank you to close friends who had invaluable insight in helping me past the many, many hurdles I came across. Thank you to Dr. Lord for this opportunity, guidance and mentorship, your ideas helped shape me into a better researcher and person. Thank you to Dr. Matlock Jeffries who helped me with methods and allowed me to use the instrumentation necessary to complete this project. A special thank you to Olivia Jane. Even though she completely halted my progress at one point, without her laughter this project would have been much more daunting. Finally, a very special thank you to my wife. She was there for all the complaints, for nothing working and for something finally working. Without her input, support and care none of this would have been possible.

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Abstract

Angiostrongylus cantonensis, the rat lungworm, is the etiologic agent of an emerging infectious zoonosis, angiostrongyliasis. To date there is relatively little known about this parasite from a public health perspective. Because snails play a critical role in the rat lungworm's life cycle, as intermediate hosts, it is paramount that snail species able to harbor this disease are identified to increase awareness of the geographic spread of this disease locally and globally. Our research aimed to identify the parasite within endemic Oklahoman molluscan intermediate hosts and to provide current geographic distribution within these intermediate hosts.

The most abundant aquatic snails and bivalves were collected by hand in southeast Oklahoma between the months of September 2016 and September 2017 (N=351). The sampling resulted in the collection of three species including *Physa spp.*, *Planorbella trivolvis* and *Corbicula fluminea*, the Asian clam. Total digestion of tissue was performed before extraction of DNA for each sample. Quantitative polymerase chain reaction (qPCR) was utilized for amplification of the internal transcribed spacer 1 (ITS1) region of *A. cantonensis*. Melt-curve analysis was implemented with qPCR for secondary confirmation. Further confirmation was performed by resolving all amplified products on an agarose gel via electrophoresis and identifying the presence or absence of a band at the target DNA size belonging to *A. cantonensis* (267bp fragment). Further optimization was conducted to ensure highest quality of results including temperature gradients, primer concentration gradients, different primer sets (including self-created sets), varying cycle conditions, different modes of PCR such as touchdown-PCR, utilizing intercalating dyes

as well as probe-based qPCR, and inclusion of more refined assay components such as a black hole quencher (BHQ). After performing the optimized assay on all 351 collected gastropod samples none yielded positive confirmation of *A. cantonensis* DNA.

These results indicate that Oklahoma creates bottlenecks in many forms such as geographic, climatic and biologic that inhibit the ability of this parasite to gain a foothold beyond southeast Oklahoma. The absence of rat lungworm DNA within the most abundant aquatic intermediate hosts in this region confirm the unsuitability of these species as intermediate hosts for this parasite.

Chapter 1: Introduction

Angiostrongylus cantonensis, the rat lungworm, is a filariform nematode commonly associated with both human and zoonotic eosinophilic meningitis. *A. cantonensis* is the cause of an emerging, potentially serious, infectious nematodiasis in the south-central United States (York *et al*, 2015). *Angiostrongylus cantonensis* poses significant public, veterinary, and wildlife health threats, due to its marked host plasticity and history of successful invasive establishment in novel geographic areas (Cowie, 2013). To date however, the parasite has not been well studied outside areas of initial endemic invasion due to low zoonotic frequency and minimal clinical incidence.

1.1 Parasites within an Ecosystem

Parasites play a pivotal role in nearly every ecosystem. Although past ecological studies often neglected the role of parasites, recent studies have begun to show their significance within these communities (Preston & Johnson, 2010). Traditionally there are two major classes of parasites, which are characterized by size, microparasites - those invisible to the naked eye such as viruses, protozoans and bacteria, and macroparasites - those visible to the naked eye such as nematodes and cestodes (De Mee & Renaud, 2002). These two groups take up more equity than all of predation in a standard food web and are believed to be one of the oldest survival strategies in nature (Wood *et al,* 2007).

The greatest, and perhaps most obvious, impact parasites have on an ecosystem is their ability to influence a food web (Lafferty *et al*, 2008). This is done through positively or negatively impacting competition. Parasites can positively affect competition by allowing an inferior species to thrive in the presence of a superior species when the superior species

is debilitated by heavy infection with a parasite. This is often the case with the nematode *Ascaris lumbricoides,* which weakens the immune system of a person and, coupled with many deleterious effects on humans, co-infection can occur with other parasites such as the protozoan *Giardia duodenalis* (Hagel *et al*, 2011). Negative impact occurs when a species is nearly driven to extinction due to heavy infection with a parasite. Examples of this type of infection include organisms like *Plasmodium falciparum* and *Taenia solium* that effects millions of people worldwide (CDC, 2017) and where parasite burden can become so ubiquitous that it often results in death.

The role of a parasite within an ecosystem becomes more impactful when an organism parasitizes a 'keystone' species. Keystone species are described as an abundant species that greatly modifies or has important ecological influence within a specific ecosystem (Paine, 1969). The effects of parasitism within these species causes a much broader effect than that of other species within an ecosystem. An example of parasitism of a keystone species within an ecosystem is the nematode *Howardula aoronymphium*. This organism can castrate specific *Drosophila* species thereby limiting these species to reproduce in certain instances (Thomas *et al,* 2005). Not only are parasites responsible for impacting host ecosystems, but research has also shown their effect on other parasite communities (Poulin, 1998). Co-infection with parasites can have both a positive and negative affect on parasite communities. It is also possible that one parasite can have both effects with different hosts. An example of this is the Acanthocephalan *Echinorhynchus truttae*, which causes a positive effect in one type of cephalopod, *Gammarus pulex*, by greatly increasing its predation rates but causes a negative effect in another cephalopod, *G. duebeni*, by halving its predation rates (Hatcher *et al*, 2014).

1.2 Emerging Infectious Diseases

Emerging infectious diseases (EIDs) can have a gravely detrimental role in the well-being of humans, domestic animals, and wildlife. These diseases can cause significant outbreaks which can lead to widespread morbidity and mortality, as well as facilitate local extinction of newly afflicted novel hosts. Examples of these conditions caused by an EID include AIDS, Legionnaires' disease and hantavirus pulmonary syndrome (NIH, 2007). EIDs are predominantly caused by ecologically adaptive, invasive zoonotic pathogens that are initially established in wildlife and/or domestic animals. To better detect, control and prevent such outbreaks from occurring, the methods by which we monitor and study these pathogens must be constantly investigated, revised and improved (Caliendo *et al*, 2013). Pinpointing geographic prevalence, distribution-enhancing parameters, host establishment mechanisms, and new-host pathogenicity allows us to better protect public health and to minimize adverse impacts on economically valuable animals, endangered/threatened species, and domestic pets. Angiostrongyliasis is a relatively novel disease, with human cases in the United States being reported in 1960 (CDC, 2016). When compared to diseases caused by other pathogenic, parasitic organisms such as Cestodes, which have parasitized humans for thousands of years (Cox, 2002), this is a nascent disease. Because of its recent arrival, disease potential, and propensity of native host invasion, it is essential that timely, cooperative research be undertaken to better elucidate regional, ecological and epidemiologic patterns.

1.3 Nematodes

Nematodes feed on almost anything and live on most everything. More than 25,000 nematode species have been identified with an estimated 500,000 species yet to be

discovered (Hodda, 2007). For this reason, it is proposed that nearly every organism has been parasitized by this phylum at some point. Nematodes are bilaterally symmetrical, which means if there was an axis down the middle of a nematode the two sides would be identical. This phylum is also typically vermiform, or worm-shaped. Nematodes form a pseudocoelom in development. A unique characteristic of nematodes is that they lack cilia and flagella in all stages of life (Farris *et al,* 2012). Less complex animals use these structures as a means of locomotion, but these parasites move through a motion called undulatory propulsion, which means they "wriggle" their longitudinal muscles. Nematodes must utilize these longitudinal muscles because they lack circular muscles. The outermost layer of a nematode is covered with a durable yet flexible cuticle that allows the worm to have a high internal pressure to maintain body fluids. To become mature, a nematode must shed its outer cuticle. Though it is extremely sturdy, the cuticle is semi-permeable which allows the worm to respire, this is necessary because they do not have respiratory organs but rather exchange gas through their epidermis. Nematodes also lack a true circulatory system but do have a well-developed nervous system composed of nerves, nerve chords, nerve rings, and ganglia. Nematodes use papillae organs as touch and taste receptors. These are typically located toward the anterior or cephalic region of a nematode. Nematodes also have well developed reproductive systems. The female reproductive system is made up of an ovary or ovaries, depending on the species, spermatheca, oviduct, uterus, and vulva. The spermatheca is used to store sperm and fertilize eggs as they pass. The male reproductive system is composed of testis, seminal vesicles, a cloaca, spicules, a gubernaculum and a bursa. The bursa is used to wrap around the female during copulation. Physical features used to classify nematodes are tail type, esophageal type, mouth type, spicule type and ovary location (Ash, 1970).

1.4 Nematode Life Cycle

There are many different diversities and complexities among nematode life cycles, but they are all composed of the same basic pattern characterized by four successive larval stages (L_1, L_2, L_3, L_4) and two adult stages, with the immature adult sometimes being denoted as L_5 (Johnstone, 2000). When an egg hatches it releases a first-stage larva (L_1) that must complete two molts before becoming infective as a third-stage larva (L_3). This can be accomplished in two ways, within an intermediate host or in the external environment. The intermediate host plays a critical role in the perpetuation of a nematode's life cycle, in fact, it is the method by which a definitive host acquires a disease and how the nematode continues to be infective. After being ingested by a definitive host, the nematode must undergo two additional molts (L_4 and L_5) before becoming sexually mature.

1.5 Lungworms (Metastrongylidae)

Lungworms are nematodes that infect the pulmonary arteries of a definitive host. They are the cause of a variety of diseases such as ill thrift and pneumonia, as seen in the *Metastrongylus* genera (Conolea *et al*, 1999), coughing and dyspnea, as seen with *Aelurostrongylus* (Taubert *et al*, 2009), and eosinophilia of the brain with concomitant nerve damage as seen in the *Angiostrongylus* genus (Ji *et al*, 2017). Not all lungworms possess the same pathogenic potential and parasite burden, or load, is often the determining factor for severity of a lungworm infection, which can range from being asymptomatic to causing mortality based on these levels of parasitemia. Severity of disease is also influenced by synergistic effects with coinfections of other parasites. Lungworm pathogenesis also varies significantly when infection occurs within an

accidental host. These types of infections are responsible for greater clinical pathology, as they often involve humans, and can cause symptoms such as eosinophilic meningitis as well as meningoencephalitis (Cowie, 2011).

Angiostrongylus cantonensis belongs to the lungworm superfamily Metastrongylidae, which are species of nematodes that preferentially live in the lungs of terrestrial mammals, although some have been found in marine mammals (Schmidt, Roberts, Janvoy Jr., 2009). Another organism in this superfamily is *Metastrongylus elongatus* that infects the lungs and intestines of pigs (Lewis, 1926) rather than the pulmonary arteries of rats. *Angiostrongylus* is the genus given to Metastrongylids that specifically infect carnivores (Ubelaker, 1986). The rat lungworm was previously categorized within the *Parastrongylus* genus, but this nomenclature is not widely used (Cowie, 2013). The *Parastrongylus* genus also infects rats, but the murids infected by this genus differ from those infected by *Angiostrongylus*.

1.6 Identification of Lungworms

Lungworms are traditionally identified by two methods, diagnostic morphology and molecular detection (Ash, 1970 & Qvarnstrom *et al*, 2007). The third-stage larvae of lungworms (metastrongylids) can be identified based on morphologic characteristics such as the presence and shape of a bursa on the male as well as the dimorphism between sexes with females typically being larger (Ash, 1970). Positive identifications can also be made by targeting specific molecular regions of lungworms. While 18S rRNA is one of the most often used targets for phylogenetic determination (Yang *et al*, 2002; Hewett *et al*, 2003; Rougemont *et al*, 2004), it is not commonly used amongst nematodes due to this region being highly conserved within the phylum. The internal transcribed spacers (ITS),

for its ability to exhibit high interspecific sequence variability, as well as the mitochondrial cytochrome oxidase subunit 1 gene (CO1) are two regions that have been heavily involved in making species-specific identifications of nematodes (Qvarnstrom *et al*, 2016). Traditional methods involved in species identification, such as ELISAs and other serum antibody assays are not typically employed when identifying lungworms due to a high rate of cross-reactivity with closely related species, as seen with *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis* (Voller *et al*, 1976).

1.7 Purpose of This Study

The objective of this study was to collect conspicuous intermediate hosts within the southeast region of Oklahoma and investigate the presence or absence of *Angiostrongylus cantonensis* DNA within these hosts. Geographic distribution is an important factor to determine the rat lungworm's current range within the United States.

This project aimed to explore possible new-host pathogenicity by selecting novel, endemic aquatic gastropod species through field sampling, and to further define the distribution of *Angiostrongylus cantonensis* in Oklahoma within these intermediate hosts. By understanding host/parasite dynamics in species of endemic wildlife, we will be better able to understand the ecology of the parasite, identify its vulnerabilities, and develop plans of action targeted at preventing zoonotic outbreaks, minimizing public health threats, and curtailing spread.

Chapter 2: Literature Review

2.1 Nomenclature

Angiostrongylus cantonensis was first described in 1935 in the Chinese province of Canton, what is now Guangzhou (Chen, 1935). H.T. Chen originally named the nematode *Pulmonema cantonensis* because he believed, as it was later confirmed, it infected the pulmonary system of its definitive host. Shortly after its discovery, the rat lungworm was accidentally named *Haemostrongylus ratti* by Yokogawa in 1937 because he was not aware of the same species previously being described by Chen. The genus *Pulmonema* was later synonymized with *Angiostrongylus* and the name *Angiostrongylus cantonensis* became the rat lungworm's globally accepted nomenclature. In 1986, J.E. Ubelaker split the *Angiostrongylus* genus into five genera based on morphology as well as preferential definitive hosts: *Angiostrongylus* - infecting carnivores such as dogs, cats, and raccoons, *Parastrongylus* - infecting murids such as mice and rats, *Angiocaulus* - infecting mustelids such as otters and badgers, *Gallegostrongylus* - infecting gerbils and *Stefanskostrongylus* - infecting insectivores such as moles and shrews. However, this nomenclature for the rat lungworm is less often used and *Angiostrongylus cantonensis* remains the most widely accepted classification (Cowie, 2013).

2.2 Geographic Distribution of *Angiostrongylus cantonensis*

As mentioned previously, the rat lungworm was first described by Chen in China in 1935 and then reported in Taiwan by Yokogawa in 1937. The next major event in the timeline of the *A. cantonensis* was in Brisbane, Australia where the life cycle was identified in rodent definitive hosts (Mackerras & Sandars, 1955). By 1960, epidemiological studies

had identified *A. cantonensis* as a significant public health problem within the tropics and subtropics (Wallace, 2013), including numerous Pacific islands such as Guam, Tahiti and Fiji. Since then the parasite has been identified globally in places such as the Canary Islands (Martin-Alonso *et al*, 2011), Japan (Tokiwa *et al*, 2013), Brazil (Caldeira *et al*, 2007; Thiengo *et al*, 2010), Egypt (Ibrahim, 2007), Thailand (Pipitgool *et al*, 1997), and Spain (Martin-Alonso *et al*, 2015). It is believed that the expanding global distribution of this parasite has been accomplished via oceanic travel and commerce involving ships harboring infected rodents within their cargo (Martin-Alonso et al., 2011). Covert transport and commercial distribution of infected snails and other intermediate hosts may have also facilitated global range expansion.

More recently *A. cantonensis* has begun to be described in the United States. Identification has been made within definitive hosts in Louisiana (Kim *et al*, 2002), Oklahoma (York *et al*, 2015) and Florida (Walden *et al*, 2017) as well as within intermediate hosts in Hawaii (Cowie, 2013), Florida (Teem *et al*, 2013) and Louisiana (Qvarnstrom *et al*, 2013). *Angiostrongylus cantonensis* is of marked local interest because of the large population of snails found in Oklahoma that have the potential to serve as intermediate hosts. Also of interest is its recent discovery in a native rodent species in Oklahoma. Recent University of Central Oklahoma-based research has documented the geographic expansion of the parasite into southeastern Oklahoma, verified the establishment in a novel native rodent (*Sigmodon hispidus*), and developed enhanced molecular detection and diagnostic techniques (York *et al*, 2015). Additionally, this research has highlighted the potential impact of global climate change and associated alterations in regional hydrological patterns as important predictors of future disease

distribution. These findings render this disease of considerable investigative importance, especially locally within Oklahoma.

2.3 *Angiostrongylus cantonensis* Ecology

A. cantonensis first-stage larva (L_1) typically infect snails or slugs, which serve as intermediate hosts. This occurs when the snail or slug ingests the first-stage larva, harbored in rodent feces. Intermediate host infection can also be accomplished transdermally via first-stage larva penetration. Several species of mollusks, as well as freshwater prawns, crabs, frogs and fish (Lv *et al,* 2009) can serve as paratenic hosts; paratenic hosts assist in transportation but are not required for the successful development of the parasite. In the case of the rat lungworm, a frog that eats an infected snail may harbor the parasite until a rodent ingests the frog. After two molts within the intermediate host, the parasite is infective to mammals as a third-stage larva (L_3). Once a mammalian definitive host ingests an intermediate or paratenic host, the parasite continues development. When ingested by a definitive host, several species of rodents, the worm penetrates the small intestines of the rodent and utilizes the venous system to migrate to the central nervous system (CNS) where it matures to a young adult. The young adult worm of *A. cantonensis* returns to the venous system and migrates to the pulmonary arteries of the definitive host where it becomes sexually mature. Adult worms lay eggs in the pulmonary arteries then the eggs migrate to the alveoli of the lungs and are coughed up, effectively expelling the eggs into the oral cavity. The expelled eggs are then swallowed and passed in the feces after traveling through the digestive system. According to Cowie (2011), this entire process takes approximately 45 days on average, but other studies have

shown that the life cycle can take from one week to several months to complete depending on host species (Capinera & Walden, 2013).

Humans are accidental hosts and become infected when they accidentally or intentionally ingest the third-stage larva of *A. cantonensis*. The primary means for infection with the rat lungworm occurs through ingestion of intermediate hosts or paratenic hosts. More rarely, ingestion of the third-stage larva occurs when sanitary measures are not practiced with food, such as the washing of salads before consumption. Other means by which humans can become infected involve ingestion of larvae that exist in slime trails left by snails or slugs, although this method is believed to be far less frequent (Cowie, 2013). When a human becomes infected, third-stage larvae are transported to the CNS, via the venous circulation. The third-stage larva penetrates the blood-brain barrier and forms space occupying lesions in neural tissue. The nematode fails to reach sexual maturity within human neural tissue and will expire, causing a potentially severe inflammatory response. In rare cases, the rat lugworm can migrate to the eye where it causes ocular neuritis (Sinawat *et al*, 2008) but ultimately it is, again, unable to reach sexual maturity and expires.

2.4 Clinical Significance of *Angiostrongylus cantonensis*

Although it is likely that humans have been in close contact with and infected by *Angiostrongylus cantonensis*, it wasn't until 1945 that the parasite became an important human pathogen when it was discovered in the CNS of a Taiwanese man and identified as a fatal infection (Beaver & Rosen, 1945). The first major outbreak of the parasite occurred in Southeast Asia in the 1960's (Tsai *et al,* 2013). Due to high rates of helminthiasis in that region, it is not believed that this was the first major outbreak but

rather the first one to be recognized. While distribution seems to have reached many places throughout the globe, the United States has only recently documented the emergence of this disease in humans, with the first case taking place in Hawaii in 1960 (Cowie, 2011). Although the disease has largely been contained in the United States, an outbreak of angiostrongyliasis did occur in nearby Jamaica in 2000 (Slom *et al*, 2002), which poses as a warning sign of the parasite's ability to spread in neotropic regions, not unlike Florida's climate.

The name given to the inflammatory response resulting from CNS invasion by the parasite, and subsequent host immunological response, is meningoencephalitis. The clinical symptoms of this disease include: nerve damage, edema, debilitating migraines and in rare instances death (2018). The current process for diagnosing meningoencephalitis involves conducting a lumbar puncture and noting abnormal edema of the meninges, the membrane surrounding neural tissue. *Angiostrongylus cantonensis* also causes an increase in eosinophils within the cerebral spinal fluid (CSF), known as eosinophilic meningitis that is difficult to treat and just as difficult to diagnose. A normal distribution of eosinophils for leukocytes is between 0-6% in blood and 0% in CSF for adults (Liess, 2014). Individuals infected with this parasite have displayed up to 50% eosinophilia within the CSF (Tsai *et al,* 2001). Other symptoms of rat lungworm infection include: nuchal rigidity, torticollis, acute severe headache, emesis, pyrexia, and death. Some patients have reported pain caused by nerve damage 2-3 years after exposure to *Angiostrongylus cantonensis* (Martins *et al*, 2016). Eosinophilia does not always occur in the CSF of infected patients which is a major obstacle for differential diagnosis. Tsai et

al. (2001) found that only 30% of their 17 cases demonstrated high eosinophilia in patients.

An assay has not yet been created to specifically target *A. cantonensis* for rapid detection. This is difficult to create for many reasons, one of which is that the rat lungworm, like many parasites, is able to change its outer protein layer, disguising itself once it is inside the human body (Lee *et al*, 2017). A variety of pharmacological agents, such as high doses of acetaminophen, albendazole, mebendazole, and corticosteroids, have been tested as treatments for *A. cantonensis* infection. A major limitation of these treatments is that they are palliative remedies as opposed to a means of eliminating the parasite. The reason for this is due to the lungworm's propensity to end its travel in humans within the meninges. With limited background knowledge, doctors may prescribe patients with a class of anthelmintic called benzimidazoles. This class of antihelmintics works by interfering with the worm's energy metabolism, which ultimately kills the parasite. These types of antihelmintics are not only used because of their ability to kill parasites but also for their general safety to humans. This treatment becomes less beneficial, however, if it infects the nervous system. If a patient is given an anthelmintic such as albendazole or mebendazole, both of which are benzimidazoles, to kill *A. cantonensis* in the brain, the worm will produce a toxin upon death that results in substantive inflammation of the region. This inflammation will cause swelling on the brain that can result in serious migraines, impaired motor skills, possible coma, and death, as mentioned previously. Unfortunately, the parasite can cause an inflammatory response even without death. Although there is not an effective, asymptomatic method of treating the disease, it has been shown that eosinophilic meningitis, induced by *A. cantonensis* infection, frequently

resolves within weeks of initial infection. In the same Thai study by Tsai *et al* (2001) seventeen adults infected with *Angiostrongylus cantonensis* were monitored through recovery. The most common symptom amongst the group was severe headache and none of the adults died; however, children display a more defined set of symptoms that include anorexia, constipation, abnormalities in motor function, and asthenia (Lindo *et al*, 2004). It is also reported that there is a higher death rate in children, likely due to the absence of a well-developed immune system and a child more frequently being in contact with, or near, intermediate hosts.

Other parasites have been known to cause similar symptoms to rat lungworm infection, Such as *Gnathastoma spinigerum*. This nematode also causes eosinophilia in an infected person. Like some lungworms, *Gnathastoma spinigerum* infects people when they consume undercooked or raw fish; although this parasite is often absent in nervous tissue since it does not preferentially select neural tissue (Schmidt *et al,* 2009). In its definitive host, *Gnathastoma* matures in muscle tissue unlike *Angiostrongylus cantonensis*. *Baylisascaris procyonis*, the raccoon roundworm, is another parasite that can cause eosinophilic meningitis, however, this parasite does not specifically target the brain either, and it often manifests as an ocular debilitation. While it typically inhabits raccoons, humans can acquire this parasite by accidental ingestion of raccoon feces. For this reason, infection of children is a major concern due to their nature of playing on the ground in close proximity to where raccoon feces are often deposited.

TECHNIQUE REVISION, ENHANCEMENT AND REVIEW OF SPECIES IDENTIFICATION IN GASTROPOD INTERMEDIATE HOSTS OF *ANGIOSTRONGYLUS CANTONENSIS*

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ABSTRACT

Angiostrongylus cantonensis is the etiologic agent of eosinophilic meningitis in humans and neurologic debilitations in a wide range of other domestic and wild mammalian species. *A. cantonensis* offers a unique opportunity for disease surveillance and reconnaissance due to its marked host plasticity. Eosinophilic meningitis caused by this helminth is often misdiagnosed as many health care professionals have little to no exposure to this emerging infectious disease. For this reason, a more reliable and rapid diagnostic assay is needed. Joint research employed current molecular identification assays as well as epidemiologic field survey techniques to illustrate distribution of *A. cantonensis*. The molecular assays were optimized by several means including temperature and primer gradients. 351 of the most abundant aquatic intermediate hosts, snails, were collected and used for analysis of optimized protocols. To date, few groups have investigated the most efficient method in detecting parasite DNA within invertebrate intermediate hosts. This study was concentrated and targeted on snails, as they play a critical role as an intermediate host required for the perpetuation of the *A. cantonensis* life cycle. It is necessary for the revision, enhancement and review of these assays to maximize chances of

identifying disease within invertebrate intermediate hosts since their tissue largely differs from vertebrate hosts. This also serves to identify the most accurate test to be considered for clinical diagnostics.

Keywords: Species Identification, Nematodes, *Angiostrongylus cantonensis,* Rat Lungworm

INTRODUCTION

Species identification assays are regularly performed in the laboratory but are seldomly optimized after implementation. This can be the result of limited time due to throughput or lack of desire due to infrequent use, amongst other factors. It is important to tune diagnostic assays as they serve a vital function in molecular laboratories. In this study we attempted to identify *Angiostrongylus cantonensis*, an emerging infectious disease within intermediate hosts. To achieve the best results, we optimized several steps in processing our sample tissues. This allowed us to confirm the most efficient real-time polymerase chain reaction (qPCR) profiles, tissue input, method of digestion, etc. Once optimized, this assay provided a unique opportunity to identify foreign DNA within an organism.

Angiostrongylus cantonensis is the etiologic agent of eosinophilic meningitis in humans and neurologic debilitations in a wide range of other domestic and wild mammalian species. *A. cantonensis* offers a unique opportunity for disease surveillance and reconnaissance due to its marked host plasticity. Joint research conducted in this study employed current molecular identification assays as well as epidemiologic field survey techniques to illustrate distribution of *A. cantonensis*. Genetic identification assays and field survey techniques were also used to

identify current geographic distribution of *A. cantonensis*. Snails, which serve as an intermediate host of *A. cantonensis*, were collected in southeast Oklahoma and analyzed using qPCR as well as gel electrophoresis. This study aimed to optimize current methodology to better identify the presence of *Angiostrongylus cantonensis* DNA within a gastropod intermediate host.

SUBJECTS AND METHODS

Study Areas

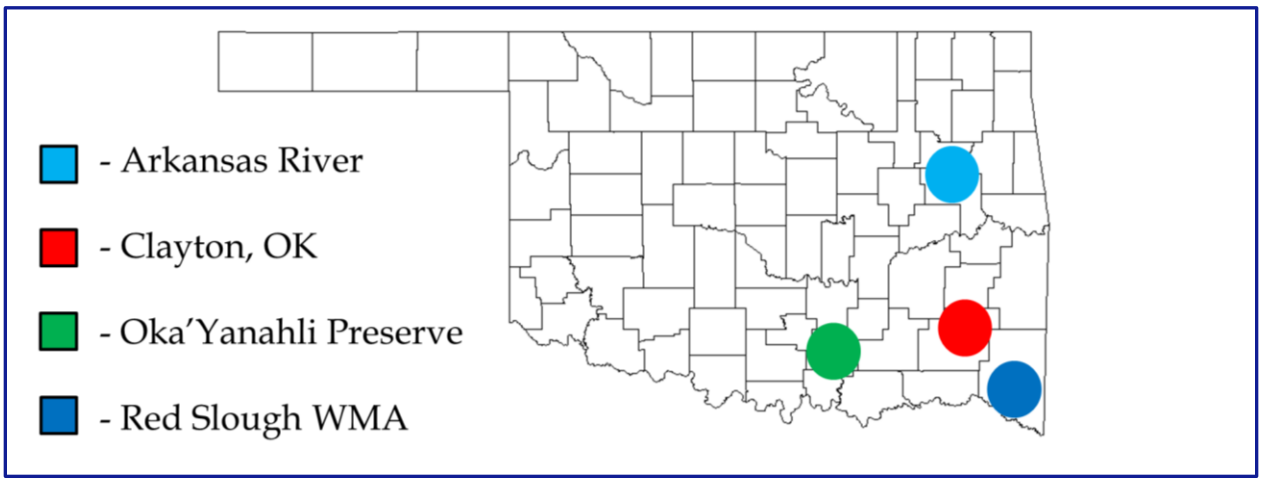

Figure 1. Collection areas

Sampling was performed in the southeast region of Oklahoma. A majority of samples (N=246) were collected at the Oka'Yanahli Preserve (34° 26' 40.2" N, 96° 39' 7.1" W), located one mile southwest of Connerville, Oklahoma. The preserve is owned and operated by the Nature Conservancy and consists of 3,600 acres, some of which runs along the Blue River. This river is one of two free-flowing rivers within Oklahoma that serves as a source of water for local wildlife as well as surrounding cities. The land is largely made up of karst topography, which includes eroded limestone and consequent sinkholes. This area was selected based on proximity to areas of confirmed *Angiostrongylus cantonensis* infection (SE OK and LA) and presence of aquatic bodies that serve as habitats for the critical intermediate host of the rat lungworm, snails. A large

number of samples (N=105) were also collected at the Red Slough Wildlife Management Area (RSWMA), which is owned and operated by the Oklahoma Department of Wildlife Conservation (33° 42' 23.1" N, 94° 40' 56.4" W) and located on 5,814 acres, six miles south of Haworth, Oklahoma. The RSWMA is composed of many habitats including: wetlands - 2,400 acres, shrub habitat - 1,600 acres, hardwood forest - 1,100 acres, reservoirs - 414 acres, and woodlands - 300 acres (ODWC, 2017). This area was chosen for sampling because of its wetlands, which serve as a natural habitat for *Angiostrongylus cantonensis* intermediate hosts. It was also chosen based on previous research that documented the rat lungworm within definitive hosts within this specific area (York *et al*, 2015).

Data Collection

From September 2016 to September 2017, 351 of the most abundant, most represented mollusks (*Planorbella trivolvis, Physa spp., Corbicula fluminea*) were collected by hand. Mollusks were taken from freshwater habitats and stored in 50mL conical tubes. Their collection location and date were recorded, and they were placed on ice until they could be placed in a -20°C freezer for long term storage. Species were identified with the help of Dr. Mike Mather, an ecologist with a specialization in malacology.

DNA Extraction

DNA was extracted from snail tissue using two techniques. The first technique utilized the standard DNeasy Blood and Tissue chemistry protocol (QIAGEN 69506). Snails were removed from their shells prior to extraction. After placing the tissue in a 2mL centrifuge tube, 180µL of Buffer ATL (provided) and 20µL of Proteinase K was added and mixed by vortexing. Samples were incubated at 56°C for two hours in order to completely digest the tissue. Samples were then

briefly vortexed and 200µL of Buffer AL (provided) was added, followed by brief vortexing. A total of 200µL of 100% ethanol was added and the samples were vortexed once more before being placed onto a DNeasy Mini spin columns in a 2mL collection tubes (provided). Samples were then centrifuged at 8,000 rpm for one minute and the flow-through was discarded. The spin columns were placed in new 2mL collection tubes and 500µl of Buffer AW1 (provided) was added. The samples were centrifuged at 8,000 rpm for one minute with the flow-through being discarded again. The spin columns were placed in new 2mL collection tubes and 500µl of Buffer AW2 (provided) was added. The samples were centrifuged for three minutes at 14,000 rpm to dry the DNeasy membrane. Flow-through was discarded once more and the spin columns were placed in new 2mL centrifuge tubes. Finally, 200µl of Buffer AE (provided) was pipetted directly onto the DNeasy membrane. The samples were incubated at room temperature for one minute then centrifuged at 8,000 rpm for one minute to elute. Extracted DNA was then quantitated using a Nanodrop (ThermoFisher ND-2000).

The second method involved digesting the tissue in 0.7% hydrochloric acid and 1.0% pepsin. After incubation at 37°C for 48-60 hours, the tissue had not totally digested, and this method was no longer utilized for optimization.

Real-time Polymerase Chain Reaction

All reactions involving intercalating dyes (SYBR) were performed on a Rotor-Gene Q thermal cycler (QIAGEN, Hilden, Germany). To reduce primer dimer formation, a Luna Universal One-Step Reaction Mix (New England BioLabs, Ipswich, MA) qPCR assay was conducted to screen for optimal concentration and annealing temperature of previously published rat lungworm primers (AcanITS1F1 & Angio58SR4). A total of 10ng of DNA from a standard containing *Angiostrongylus cantonensis* DNA was loaded and reaction volumes were 15µL total. Primer

concentrations were tested at 10µM, 5µM, 2.5µM, 1.25µM and 0.625µM. Cycling conditions were 95°C for 5 minutes followed by 45 cycles of 95°C for 30s, 56°C for 30s and 72°C for 60s. Products were resolved on a 1% agarose electrophoresis gel in Tris acetate EDTA (TAE) buffer for fifteen minutes at 125 volts, stained with 3X GelRed Nucleic Acid Stain (Biotium, Fremont, CA). Bioluminescence was detected using a transilluminator (IO Rodeo, Pasadena, CA) and a digital image was obtained with a digital camera.

Optimal primer concentration (2.5µM) was chosen by the PCR product that produced the brightest band and cleanest resolution of the target amplification region (267bp) and the dimmest representative of the unwanted primer dimer band (150bp). To find optimal temperature, 10ng of DNA from a standard containing *Angiostrongylus cantonensis* DNA was loaded and reaction volumes were 15µL total with a primer concentration of 10µM. Samples were then placed in a thermal cycler with a temperature gradient of 51.5, 53.2, 55.5, 58.4, 61.8, 64.6, 66.8 and 68.4°C. The cycling conditions were 95°C for 5 minutes followed by 45 cycles of 95°C for 30s, specific temperatures mentioned above for 30s and 72°C for 60s. The PCR products from the temperature gradient were resolved on a 1% agarose electrophoresis gel in Tris acetate EDTA (TAE) buffer for fifteen minutes at 125 volts, stained with 3X GelRed Nucleic Acid Stain (Biotium, Fremont, CA). Bioluminescence was detected using a transilluminator (IO Rodeo, Pasadena, CA) and a digital image was obtained through a digital camera. The optimal temperature was chosen by the PCR product (55.5°C) that produced the brightest band of the target amplification region (267bp) with the dimmest representative of the primer dimer band (150bp).

The same methods were used with probe-based qPCR of all samples as mentioned above. The probe (AcanITS1P1) utilized was previously published by Qvarnstrom *et al* (2010). These primers were also used in the intercalating optimization assays. All the attributes (temperature,

concentration, etc) were determined to be the same as the previous method. Gel electrophoresis was also carried out on all these samples. Several primers sets were tested for this assay to determine the most optimal one, defined as creating the least amount of primer dimer, most consistent amplification of the target region, etc. These included AcanITS1F1, AcanITS1R1, Angio58SR4 and a set that we created using IDTDNA (5'-TTCATGGATGGCGAACTGATA & 5'-GGCGCCCATTGAAACATTATA).

A melt curve analysis was used for both techniques to find specific melting temperatures of all qPCR products. To determine the specific melting temperature of 10ng of DNA from a standard containing *Angiostrongylus cantonensis*, DNA was loaded, and reaction volumes were 15µL total with a primer concentration of 10µM. The cycling conditions were 95°C for 5 minutes followed by 45 cycles of 95°C for 30s, 56°C for 30s and 72°C for 60s. This was followed by a melt curve created by ramping the temperature up from 72 to 95°C stepwise, 1 degree each step, with 5s between each step. Based on the melt curve analysis, the DNA product from *Angiostrongylus cantonensis* melted at 82.3 and a temperature of 82.3 (±1°) was used to determine if an unknown sample was putatively positive before sequencing. It should be noted that other types of PCR were implemented in this testing, such as touchdown-PCR, but were determined to be inferior to the qPCR methods.

Optimized qPCR Assay

Based on my results and well as previous studies, the probe-based method was chosen as the most optimized of the two methods. A total of 10ng of DNA was analyzed from each sample. The DNA was loaded into reactions containing 10µM of primers AcanITS1 and Angio58SR4, 7.5µL of Luna Universal One-Step Reaction Mix and brought to 15µL with nuclease-free water.

The cycling conditions were 95°C for 5 minutes followed by 45 cycles of 95°C for 30s, 56°C for 30s and 72°C for 1 minute. The qPCR assay was finished with a melt curve to determine the melting temperature of the amplified products. All series of samples were run with a positive control containing *Angiostrongylus cantonensis* DNA and negative controls containing water. Samples with a melting point of 82.3°C (±1°) were considered putatively positive and were resolved on a 1% agarose electrophoresis gel in Tris acetate EDTA (TAE) buffer for fifteen minutes at 125 volts, stained with 3X GelRed Nucleic Acid Stain (Biotium, Fremont, CA). All samples that were non-negative were also resolved on a gel for secondary confirmation. Bioluminescence was detected using a transilluminator (IO Rodeo, Pasadena, CA) and a digital image was obtained through a digital camera.

DNA Sequencing

Sequencing was performed on known positive standards, which were confirmed as containing *Angiostrongylus cantonensis* DNA using BLAST. A series of collected samples were also sequenced to ensure they were truly negative.

RESULTS AND DISCUSSION

None of the 351 extracted DNA samples were positive for *A. cantonensis* after analyzing melt curves and gel resolution, with no collected samples having a band near the 267bp mark during electrophoresis. Although our sampling did not yield any snails with a positive indication of *A. cantonensis* DNA, the types of methods used in this study have been utilized as a confirmation method for the rat lungworm in other studies. The optimized qPCR profile is similar to the profile that was used at the same university as an assay that positively identified *A. cantonensis* within a definitive host (York *et al*, 2015). The primers used in this experiment have also been

used in several studies to illustrate the presence of DNA in both definitive and intermediate hosts (Qvarnstrom *et al,* 2010, (Kim *et al*, 2014, Walden *et al,* 2017). This encourages us in our choice of methodology and the belief that a larger sample size, in conjunction with a wider selection of snail species, would bolster any negative or positive finding in the future and may be required for comprehensive results. It is difficult to identify parasite DNA within intermediate host DNA; the optimization of assays is paramount for disease surveillance as intermediate hosts are critical for the perpetuation, transmission and expansion of these diseases and we have composed the most extensive and accurate method for clinical diagnostics.

ACKNOWLEDGEMENTS

The respective study was primarily supported by the University of Central Oklahoma. We would like to thank the Nature Conservancy and the Oklahoma Department of Wildlife Conservation for allowing us to collect snails on their property.

SURVEILLANCE OF MOLLUSCAN (GASTROPOD) INTERMEDIATE HOSTS FOR THE EMERGING INFECTIOUS DISEASE ANGIOSTRONGYLIASIS (*ANGIOSTRONGYLUS CANTONENSIS)* IN OKLAHOMA

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ABSTRACT

Angiostrongylus cantonensis is the etiologic agent of eosinophilic meningitis in humans and neurologic debilitations in a wide range of other domestic and wild mammalian species. *A. cantonensis* offers a unique opportunity for disease surveillance and reconnaissance due to its marked host plasticity. Joint research employed current molecular identification assays as well as epidemiologic field survey techniques to illustrate distribution of *A. cantonensis*. Rodents were sampled in Louisiana and southeast Oklahoma. Tissues were analyzed in the lab to identify the presence of the pathogen. Of 34 samples, three were found to be positive in *Sigmodon hispidus* and two in *Rattus norvegicus. S. hispidus* was a previously undocumented native novel mammalian host for *A. cantonensis*. In the current study genetic identification assays and field survey techniques were also used and assisted in identification of current geographic distribution of *A. cantonensis*. Snails, an intermediate host of *A. cantonensis*, were collected in southeast Oklahoma and analyzed using quantitative-PCR (qPCR). This study aimed to illustrate how hydrodynamic pulsivity, specifically host-parasite relationship within refugia, affects the dispersal of this parasite. It was expected that snails collected in the southeast quadrant of

Oklahoma will contain some level of parasitemia to coincide with data on previously identified, positive mammalian, definitive host species in this region. Rat lungworm introduction is likely to have occurred in Louisiana, as well as other port cities within the United States, and spread inland to areas such as southeast Oklahoma. This type of dynamic distribution led us to believe *A. cantonensis* could be found northwest of current distribution models.

Keywords: Surveillance, Nematodes, *Angiostrongylus cantonensis,* Rat Lungworm

INTRODUCTION

Angiostrongylus cantonensis is the etiologic agent of eosinophilic meningitis in humans and neurologic debilitations in a wide range of other domestic and wild mammalian species. *A. cantonensis*, the rat lungworm, offers a unique opportunity for disease surveillance and reconnaissance due to its marked host plasticity. This study employed current molecular identification assays as well as epidemiologic field survey techniques to illustrate distribution of *A. cantonensis*. 351 of the most abundant aquatic snails, an intermediate host of *A. cantonensis*, were collected in southeast Oklahoma and were analyzed using qPCR. This study aimed to investigate how hydrodynamic pulsivity, specifically host-parasite relationship within refugia, affects the dispersal of this parasite. As rodents, a definitive host of the rat lugworm, have already been positively identified to harbor the disease in southeast Oklahoma this region was targeted for sampling.

A. cantonensis first-stage larva (L_1) typically infect snails or slugs, which serve as intermediate hosts. This occurs when the snail or slug ingests the first-stage larva, often contained in rodent feces. Intermediate host infection can also be accomplished via transdermal first-stage larva

penetration. Several species of mollusks, as well as freshwater prawns, crabs, frogs and fish can serve as paratenic hosts (Lv *et al,* 2009). Paratenic hosts assist in transportation but are not required for the successful development of the parasite. Once a mammalian definitive host ingests an intermediate or paratenic host, the parasite continues development. In the case of the rat lungworm, a frog that eats an infected snail may harbor the parasite until a rodent ingests the frog. After two molts within the intermediate host, the parasite is infective to mammals as a third-stage larva (L3). When ingested by a definitive host, normally a rodent, the worm penetrates the small intestines and utilizes the venous system to migrate to the central nervous system where it matures to a young adult. The young adult worm of *A. cantonensis* returns to the venous system and migrates to the pulmonary arteries of the definitive host where it becomes sexually mature and lays its eggs. The eggs migrate to the alveoli of the lungs and are coughed up, effectively expelling the eggs into the oral cavity. The expelled eggs are then swallowed and passed in the feces after traveling through the digestive system. According to Cowie (2011), this entire process takes an average of 45 days, but other studies have shown that the life cycle can take anywhere from one week to several months to complete depending on host species (Capinera & Walden, 2013).

SUBJECTS AND METHODS

Collection

Sampling was performed in the southeast region of Oklahoma. A majority of samples (N=246) were collected at the Oka'Yanahli Preserve (34° 26' 40.2" N, 96° 39' 7.1" W), located one mile southwest of Connerville, Oklahoma. The preserve is owned and operated by the Nature Conservancy and consists of 3,600 acres, some of which runs along the Blue River. This river is one of two free-flowing rivers within Oklahoma that serves as a source of water for local wildlife

as well as surrounding cities. The land is largely made up of karst topography, which includes eroded limestone and consequent sinkholes. This area was selected based on proximity to areas of confirmed *Angiostrongylus cantonensis* infection (SE OK and LA) and presence of aquatic bodies that serve as habitats for the critical intermediate host of the rat lungworm, snails. A large number of samples (N=105) were also collected at the Red Slough Wildlife Management Area (RSWMA), which is owned and operated by the Oklahoma Department of Wildlife Conservation (33° 42' 23.1" N, 94° 40' 56.4" W) and located on 5,814 acres, six miles south of Haworth, Oklahoma. The RSWMA is composed of many habitats including: wetlands - 2,400 acres, shrub habitat - 1,600 acres, hardwood forest - 1,100 acres, reservoirs - 414 acres, and woodlands - 300 acres (ODWC, 2017). This area was chosen for sampling because of its wetlands, which serve as a natural habitat for *Angiostrongylus cantonensis* intermediate hosts. It was also chosen based on previous research that documented the rat lungworm within definitive hosts within this specific area (York *et al*, 2015).

From September 2016 to September 2017, 351 of the most abundant, most represented mollusks in southeast Oklahoma (*Planorbella trivolvis, Physa spp., Corbicula fluminea*) were collected by hand, unless a net was required to reach them underwater. Mollusks were collected from freshwater habitats and stored in 50mL conical tubes. Their collection location and date were recorded, and they were placed on ice until they could be placed in a -20°C freezer for long term storage. Species were identified with the help of Dr. Mike Mather, an ecologist with a specialization in malacology.

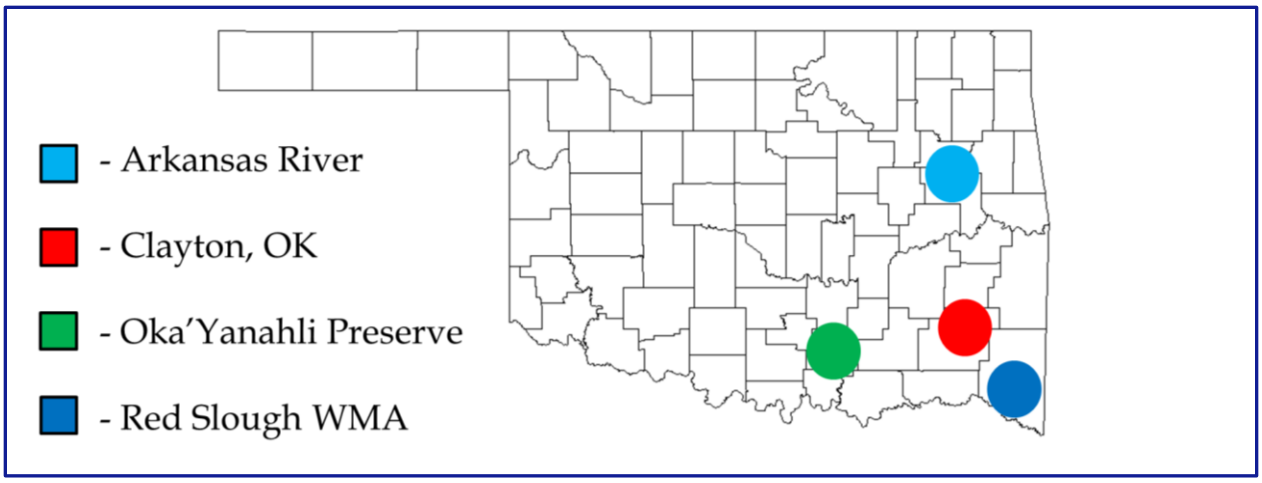

Figure 1. Collection areas

Methods

DNA was extracted using a DNeasy Blood and Tissue Kit according to the Qiagen protocol for complete tissue digestion. To determine putative positives, qPCR was performed using previously published primers (Qvarnstrom *et al,* 2010) to amplify a 267bp region of the internal transcribed spacer 1 (ITS1). All samples resolving a band around 267bp after gel electrophoresis were analyzed by sequencing. Sequencing was performed on known *A. cantonensis* DNA, known negatives and a group of collected samples as a secondary confirmation method. Known positives were matched to *Angiostrongylus cantonensis* DNA using BLAST. The known negative samples and the sample group were confirmed negative for the presence rat lungworm DNA.

RESULTS & DISCUSSION

Given the recent emergence of *A. cantonensis* in the cotton rat, it is critical to look for the parasite in potential intermediate hosts within Oklahoma. This study targeted the most abundant snails in SE Oklahoma with no positive findings. The absence *A. cantonensis* DNA within our

collected samples is an interesting finding but does not necessarily imply that the emerging infectious disease is not present in Oklahoma, in fact it has been documented in definitive hosts, as mentioned previously. It also does not mean it cannot be found in intermediate hosts. More sampling on a much larger scale, over a much larger area, may be required for a more thorough survey, possibly with more targets (i.e. more snail species). It is also possible that the parasite is not present in intermediate hosts beyond southeast Oklahoma, as previously mentioned. Both abiotic and biotic limiting factors should be taken into consideration when determining suitability of an intermediate host within Oklahoma.

Abiotic factors would include Oklahoma's climate, which is comprised of a vast number of biomes. Due to high variance in temperature, rainfall, and elevation, Oklahoma is a composition of several microhabitats that are capable of greatly influencing the survival and dispersal of a snail, which influences the transmission rate and eventual establishment of the rat lungworm. Climate alone is enough to limit suitable habitats for the intermediate host, with temperature fluctuations varying up to 10°C on a given day (Oklahoma Climatological Survey, 2018). These temperature fluctuations also affect parasites, especially obligate internal species such as the rat lungworm, that require very specific temperatures to function appropriately.

Another factor to consider is rainfall within Oklahoma. The state is largely separated in its climate by northwestern and southeastern regions. For instance, the northwest acquires approximately 30 inches less rain than the southeast region. This is important for rat lungworm surveillance because snails are often found near aquatic bodies, especially the freshwater snails examined in this study. One idea of how intermediate hosts, and by association parasites, migrate is through hydrodynamic pulsivity. When rainfall is intense enough in a specific area, transient bodies of water can form in a short amount of time. This spattering of ponds, in correlation to

traveling storms, may serve as aquatic refugia that allow an intermediate host to migrate by affording it a suitable habitat in areas it may not be able to otherwise reach. With such a large discrepancy in rainfall between the northwest and southeast corners of Oklahoma, hydrodynamic pulsivity may not be an efficient means of travel/establishment for the parasite in this region. In fact, it is possible that the inverse occurs, which is targeted areas of drought. If this occurs, it would facilitate the concentration of snails. This aggregation of intermediate hosts would seem beneficial for *A. cantonensis*; however, it is likely that the hosts examined in this study are not suitable intermediate hosts which creates a species bottleneck for the rat lungworm. These punctate, arid regions also impact intermediate host migration because associated risk would keep them from migrating. If they were to egress their aquatic safe havens without the promise of another they would likely desiccate or die along the way, thereby eliminating the parasite. This wide array of abiotic variance might also support the biotic factors that inhibit a parasite's establishment through limiting suitable habitat for a definitive or intermediate host. Intermediate and definitive host availability plays an important role in survival of a parasite by inhibiting its transmission efficiency. To our knowledge *Planorbella trivolvis* has not been recorded as a suitable intermediate host of *A. cantonensis* (Kim *et al*, 2014, Walden *et al*, 2017). *Physa spp.* has been reported as an intermediate hosts of *A. cantonensis* in one study (Richards & Merritt, 1967); however, the snails were exposed/infected experimentally as opposed to naturally. Another study (Spratt, 2015) showed that *Physa* were a far less suitable intermediate host than other snails and this has been corroborated in several other studies (Kim *et al*, 2014; Walden, 2017). It is believed that *Angiostrongylus cantonensis* is highly plastic due to its ability to infect a wide berth of not only intermediate and definitive hosts, but also a plethora of paratenic hosts; however, we found conflicting data in our study. There are several reasons why this might be,

and it might further illustrate the importance of intermediate host suitability. This also gives merit to the idea that the parasite is plastic in one host but not necessarily as plastic in another. *Planorbella* and *Physa* species were the only snail species identified during sampling in all areas (*Corbicula*, which were also sampled, are a group of bivalves). This overwhelming representation poses two problems. First, if these two species do not serve as suitable intermediate hosts, they are creating a bottleneck for the rat lungworm by out-competing other, more suitable, intermediate snail hosts within SE Oklahoma. Host specificity is a critical factor for a parasite and the invasion or introduction of a new species, such as *Corbicula spp.*, might have downstream ecological effects on the parasite (Giblin-Davis *et al*, 2013). It is known that *Corbicula spp.*, the Asiatic clam, is highly invasive and destructive. Their ability to reproduce in large numbers and cause obstructions to suitable habitats could have an impact on intermediate hosts (Foster *et al*, 2012). Second, this could be the effect of limited species diversity within this region.

Future surveillance studies should aim to collect more snails than the 351 collected in this study. In a recent study conducted in Florida, a group collected 1,437 gastropods with 27 (1.9%) of the sample testing positive for rat lungworm DNA (Walden *et al*, 2017). If this infection rate is applied to our study, we would have seen 3 infected snails optimally. This study also shows that a larger number of species is more desirable for disease surveillance, when possible. Although only two species of snails were collected, there are many other species that inhabit Oklahoma, such as *Polygyra spp.*, a land snail, but the literature on snail distribution within Oklahoma is lacking with the most extensive body of knowledge written in the early 1900's (Lutz, 1949). A larger search area, not restricted to the southeast, may prove invaluable in collecting more species of snails and increasing the odds of finding *A. cantonensis* within intermediate hosts.

Future studies on *A. cantonensis* would be served well to include secondary intermediate hosts in their surveys. Access to an accurate, current distribution of snails within Oklahoma would also greatly benefit future studies of this kind. This study illustrates that the lack of suitable intermediate hosts in SE OK serve as a bottleneck that slows the migration of an emerging infectious disease through climatic, ecologic and biologic barriers.

ACKNOWLEDGEMENTS

The respective study was primarily supported by the University of Central Oklahoma. We would like to thank the Nature Conservancy and the Oklahoma Department of Wildlife Conservation for allowing us to collect snails on their property.

Chapter 5: Discussion

5.1 Discussion of Results

While the absence of *A. cantonensis* DNA within our collected samples cannot rule out the presence of rat lungworm disease within Oklahoma, as it has been documented in definitive hosts as mentioned previously, it does imply that no suitable intermediate gastropod hosts exist in southeast Oklahoma. More sampling on a much larger scale, over a much larger area, should be conducted for a more complete survey, possibly with more targets (i.e. more snail species); however, limiting factors both abiotic and biotic should be taken into consideration when determining suitability of an intermediate host within Oklahoma.

5.2 Oklahoma's Diverse Biomes

Oklahoma is comprised of a vast array of biomes, in fact it has the most variation in biomes per mile in the US according to the Environmental Protection Agency (EPA, 2018). Reasons for this vast diversity are largely attributed to abiotic factors, such as elevation, precipitation, and temperature within Oklahoma. Northwest Oklahoma has a drastically higher elevation, an average of 4,977.53 above sea level, compared to the southeast portion of Oklahoma, an average of 272.31 feet above sea level (Oklahoma Climatological Society, 2018). Precipitation follows a similar pattern by major differences in far northwest, with an average rainfall of only twenty inches or less a year, and southeast regions, with average rainfalls of fifty or more inches a year. Again, temperature ranges are largely divided by northwest, average temperature of ≤55°F, and southeast regions, average temperature of ≥ 63°F. These vast differences amongst

geographic locations allows Oklahoma to have microhabitats with unique biodiversities; however, this can also serve as a limiting factor by inhibiting what types of wildlife are able to live in such a specific climate and their ability to travel between them. Microhabitats not only serve as a limiting factor for some species but can also isolate them from adjacent habitats that may vary significantly. For instance, a highly aquatic species, such as the freshwater snails collected in this research, may not be able to migrate a from a specific location if it is surrounded by arid biomes.

5.3 Dispersal within Oklahoma

5.3.1 Life cycle

Along with the abiotic factors mentioned, it is possible there are many biotic factors contributing to the absence of *Angiostrongylus cantonensis* within Oklahoma, such as life cycle patterns. With no positive findings, coupled by the fact that there is a species bottleneck created with unsuitable hosts, such as *Physa* and *Planorbella*, in Oklahoma, we are left wondering how the parasite can continue its life cycle if not through endemic snails in this region. It is important to elucidate the method by which the rat lungworm is spreading, if at all, so future studies can focus on organisms known to perpetuate the parasite's life cycle. Although we cannot illustrate that the entire state of Oklahoma does not contain suitable hosts with this study, it is certainly a starting point for other studies and could help to show why we were unable to identify rat lungworm DNA within our collected gastropod samples. This also allows other groups to discredit *Physa spp*. and *Planorbella trivolovis* as suitable intermediate hosts.

5.3.2 Intermediate Host Specificity

Previous studies have demonstrated both land and freshwater snails as suitable intermediate hosts of the rat lungworm; however, an exhaustive list of studies focusing on these intermediate hosts shows an overwhelmingly preference for the parasite to be in freshwater snails with several studies illustrating an absence of *A. cantonensis* DNA involves analyses of land snails (Walden *et al*, 2017, Spratt, 2015, Carvalho *et al*, 2012, Campbell, 1988). This is true in all but one land snail species where groups document an absence of *A. cantonensis* DNA in *Planorbella* snails (Walden *et al*, 2017), the genus in our research. This is an interesting finding that strengthens our lack of positive findings by illustrating that *P. trivolvis* is not a suitable intermediate host. To date, there has also not been a positive identification of *A. cantonensis* DNA within *Physa spp*. (Kim *et al*, 2014). In fact, there was a group that experimentally infected several different species of snails and were able to show that *Physa* was less likely to harbor the parasite thereby limiting the facilitation of its spread (Spratt, 2015). Even with this information these two species were collected as they were the most abundant and, in our experience, only snails within a region known to harbor the disease within definitive hosts (York *et al*, 2015).

5.3.3 Effects of Slower Transmission

The absence of suitable intermediate hosts within Oklahoma causes a species bottleneck for the rat lungworm which drastically slows its transmission rate. Due to this slower transmission rate, and difficulty infecting domestic snails, *Angiostrongylus cantonensis* is unable to establish a strong foothold beyond the regions where it has been previously identified. This difficulty in establishment is a major stressor and likely forces the rat

lungworm to utilize novel intermediate hosts and adapt to thrive in a new ecosystem, which is difficult for a parasite (Ebert, 2005).

5.3.4 Future Surveillance

Future surveillance projects should attempt to collect more snails than the 351 in this study. In a recent study conducted in Florida, a group was able to collect 1,437 gastropods where only 27 (1.9%) of the samples were positive for rat lungworm DNA (Walden *et al*, 2017). Applying this infection rate to our research, we would have seen 3 snails infected optimally. This illustrates that with a higher population size there is a greater probability of encountering the organism if it is present. This study also shows that a larger number of species is required in surveillance, when possible. Although we were only exposed to three species of mollusks during collection, there are many other that inhabit Oklahoma, such as *Polygyra* further northwest, and a larger search area, not restricted to the southeast, may prove invaluable in collecting more species of snails thereby yielding more robust results.

Supplemental Tables

Reference Location	Location Coordinates
Side-house Pond	34.444285, -96.650569
Front-house Pond	34.439662, -96.644867
Barn Pond	34.440512, -96.661706
Two Ponds	34.438983, -96.669646
Blue River	34.450953, -96.654002
Haworth, OK	33.7064102, -94.6823394

Supplemental Table 1 – Sampling Locations

Sample #	Location	Date Collected	Date Processed
1	Side-house Pond, OYP	9/10/16	2/5/17
2	Side-house Pond, OYP	9/10/16	2/5/17
3	Side-house Pond, OYP	9/10/16	2/5/17
4	Side-house Pond, OYP	9/10/16	2/5/17
5	Side-house Pond, OYP	9/10/16	2/5/17
6	Side-house Pond, OYP	9/10/16	2/5/17
7	Side-house Pond, OYP	9/10/16	2/5/17
8	Side-house Pond, OYP	9/10/16	2/5/17
9	Side-house Pond, OYP	9/10/16	2/5/17
10	Side-house Pond, OYP	9/10/16	2/5/17
11	Blue River, OYP	9/10/16	2/8/17
12	Blue River, OYP	9/10/16	2/8/17
13	Blue River, OYP	9/10/16	2/8/17
14	Blue River, OYP	9/10/16	2/8/17
15	Blue River, OYP	9/10/16	2/8/17
16	Blue River, OYP	9/10/16	2/8/17
17	Blue River, OYP	9/10/16	2/8/17
18	Blue River, OYP	9/10/16	2/8/17
19	Blue River, OYP	9/10/16	2/8/17
20	Blue River, OYP	9/10/16	2/8/17
21	Blue River, OYP	9/10/16	2/8/17
22	Blue River, OYP	9/10/16	2/8/17
23	Blue River, OYP	9/10/16	2/8/17
24	Blue River, OYP	9/10/16	2/8/17
25	Blue River, OYP	9/10/16	2/8/17
26	Side-house Pond, OYP	11/5/16	2/5/17
27	Side-house Pond, OYP	11/5/16	2/5/17
28	Side-house Pond, OYP	11/5/16	2/5/17
29	Side-house Pond, OYP	11/5/16	2/5/17

30	Side-house Pond, OYP	11/5/16	2/5/17
31	Side-house Pond, OYP	11/5/16	2/5/17
32	Side-house Pond, OYP	11/5/16	2/5/17
33	Side-house Pond, OYP	11/5/16	2/5/17
34	Side-house Pond, OYP	11/5/16	2/5/17
35	Side-house Pond, OYP	11/5/16	2/5/17
36	Side-house Pond, OYP	11/5/16	2/5/17
37	Side-house Pond, OYP	11/5/16	2/5/17
38	Two Ponds, OYP	9/10/16	2/19/17
39	Two Ponds, OYP	9/10/16	2/19/17
40	Two Ponds, OYP	9/10/16	2/19/17
41	Two Ponds, OYP	9/10/16	2/19/17
42	Two Ponds, OYP	9/10/16	2/19/17
43	Two Ponds, OYP	9/10/16	2/19/17
44	Two Ponds, OYP	9/10/16	2/19/17
45	Two Ponds, OYP	9/10/16	2/19/17
46	Two Ponds, OYP	9/10/16	2/19/17
47	Two Ponds, OYP	9/10/16	2/19/17
48	Two Ponds, OYP	9/10/16	2/19/17
49	Two Ponds, OYP	9/10/16	2/19/17
50	Two Ponds, OYP	9/10/16	2/19/17
51	Two Ponds, OYP	9/10/16	2/19/17
52	Two Ponds, OYP	9/10/16	2/19/17
53	Barn Yard Pond, OYP	11/5/16	2/22/17
54	Barn Yard Pond, OYP	11/5/16	2/22/17
55	Barn Yard Pond, OYP	11/5/16	2/22/17
56	Barn Yard Pond, OYP	11/5/16	2/22/17
57	Barn Yard Pond, OYP	11/5/16	2/22/17
58	Barn Yard Pond, OYP	11/5/16	2/22/17
59	Barn Yard Pond, OYP	11/5/16	2/22/17
60	Barn Yard Pond, OYP	11/5/16	2/22/17
61	Barn Yard Pond, OYP	11/5/16	2/22/17
62	Barn Yard Pond, OYP	11/5/16	2/28/17
63	Barn Yard Pond, OYP	11/5/16	2/28/17
64	Barn Yard Pond, OYP	11/5/16	2/28/17
65	Barn Yard Pond, OYP	11/5/16	2/28/17
66	Barn Yard Pond, OYP	11/5/16	2/28/17
67	Barn Yard Pond, OYP	11/5/16	2/28/17
68	Barn Yard Pond, OYP	11/5/16	2/28/17
69	Barn Yard Pond, OYP	11/5/16	2/28/17
70	Barn Yard Pond, OYP	11/5/16	2/28/17
71	Barn Yard Pond, OYP	11/5/16	2/28/17
72	Barn Yard Pond, OYP	11/5/16	2/28/17

73	Two Ponds, OYP	9/10/16	3/6/17
74	Two Ponds, OYP	9/10/16	3/6/17
75	Two Ponds, OYP	9/10/16	3/6/17
76	Two Ponds, OYP	9/10/16	3/6/17
77	Two Ponds, OYP	9/10/16	3/6/17
78	Two Ponds, OYP	9/10/16	3/6/17
79	Two Ponds, OYP	9/10/16	3/6/17
80	Two Ponds, OYP	9/10/16	3/6/17
81	Two Ponds, OYP	9/10/16	3/6/17
82	Two Ponds, OYP	9/10/16	3/6/17
83	Two Ponds, OYP	9/10/16	3/6/17
84	Two Ponds, OYP	9/10/16	3/6/17
85	Two Ponds, OYP	9/10/16	3/6/17
86	Side-house Pond, OYP	11/5/16	3/16/17
87	Side-house Pond, OYP	11/5/16	3/16/17
88	Side-house Pond, OYP	11/5/16	3/16/17
89	Side-house Pond, OYP	11/5/16	3/16/17
90	Side-house Pond, OYP	11/5/16	3/16/17
91	Side-house Pond, OYP	11/5/16	3/16/17
92	Side-house Pond, OYP	11/5/16	3/16/17
93	Side-house Pond, OYP	11/5/16	3/16/17
94	Side-house Pond, OYP	11/5/16	3/16/17
95	Side-house Pond, OYP	11/5/16	3/16/17
96	Side-house Pond, OYP	11/5/16	3/16/17
97	Side-house Pond, OYP	11/5/16	3/16/17
98	Side-house Pond, OYP	11/5/16	3/16/17
99	Side-house Pond, OYP	11/5/16	3/16/17
100	Side-house Pond, OYP	11/5/16	3/16/17
101	Side-house Pond, OYP	11/5/16	3/16/17
102	Side-house Pond, OYP	11/5/16	3/16/17
103	Side-house Pond, OYP	11/5/16	3/16/17
104	Side-house Pond, OYP	11/5/16	3/16/17
105	Side-house Pond, OYP	11/5/16	3/16/17
106	Side-house Pond, OYP	11/5/16	3/16/17
107	Barn Yard Pond, OYP	11/5/16	4/3/17
108	Barn Yard Pond, OYP	11/5/16	4/3/17
109	Barn Yard Pond, OYP	11/5/16	4/3/17
110	Barn Yard Pond, OYP	11/5/16	4/3/17
111	Side-house Pond, OYP	11/5/16	4/11/17
112	Side-house Pond, OYP	11/5/16	4/11/17
113	Side-house Pond, OYP	11/5/16	4/11/17
114	Side-house Pond, OYP	11/5/16	4/11/17
115	Side-house Pond, OYP	11/5/16	4/11/17

116	Side-house Pond, OYP	11/5/16	4/11/17
117	Side-house Pond, OYP	11/5/16	4/11/17
118	Side-house Pond, OYP	11/5/16	4/11/17
119	Side-house Pond, OYP	11/5/16	4/11/17
120	Side-house Pond, OYP	11/5/16	4/11/17
121	Side-house Pond, OYP	11/5/16	4/11/17
122	Side-house Pond, OYP	11/5/16	4/11/17
123	Side-house Pond, OYP	11/5/16	4/12/17
124	Side-house Pond, OYP	11/5/16	4/12/17
125	Side-house Pond, OYP	11/5/16	4/12/17
126	Side-house Pond, OYP	11/5/16	4/12/17
127	Side-house Pond, OYP	11/5/16	4/12/17
128	Side-house Pond, OYP	11/5/16	4/12/17
129	Side-house Pond, OYP	11/5/16	4/12/17
130	Side-house Pond, OYP	11/5/16	4/12/17
131	Side-house Pond, OYP	11/5/16	4/12/17
132	Side-house Pond, OYP	11/5/16	4/12/17
133	Side-house Pond, OYP	11/5/16	4/12/17
134	Side-house Pond, OYP	11/5/16	4/12/17
135	Side-house Pond, OYP	11/5/16	4/12/17
136	Side-house Pond, OYP	11/5/16	4/12/17
137	Front-house Pond, OYP	3/11/17	4/19/17
138	Front-house Pond, OYP	3/11/17	4/19/17
139	Front-house Pond, OYP	3/11/17	4/19/17
140	Front-house Pond, OYP	3/11/17	4/19/17
141	Front-house Pond, OYP	3/11/17	4/19/17
142	Front-house Pond, OYP	3/11/17	4/19/17
143	Front-house Pond, OYP	3/11/17	4/19/17
144	Front-house Pond, OYP	3/11/17	4/19/17
145	Front-house Pond, OYP	3/11/17	4/19/17
146	Front-house Pond, OYP	3/11/17	4/19/17
147	Front-house Pond, OYP	3/11/17	4/19/17
148	Front-house Pond, OYP	3/11/17	4/19/17
149	Front-house Pond, OYP	3/11/17	4/19/17
150	Front-house Pond, OYP	3/11/17	4/19/17
151	Front-house Pond, OYP	3/11/17	4/19/17
152	Front-house Pond, OYP	3/11/17	4/19/17
153	Front-house Pond, OYP	3/11/17	4/19/17
154	Front-house Pond, OYP	3/11/17	4/19/17
155	Front-house Pond, OYP	3/11/17	4/19/17
156	Front-house Pond, OYP	3/11/17	4/19/17
157	Front-house Pond, OYP	3/11/17	4/19/17
158	Front-house Pond, OYP	3/11/17	4/19/17

159	Front-house Pond, OYP	3/11/17	4/19/17
160	Barn Yard Pond, OYP	3/11/17	5/17/17
161	Barn Yard Pond, OYP	3/11/17	5/17/17
162	Barn Yard Pond, OYP	3/11/17	5/17/17
163	Barn Yard Pond, OYP	3/11/17	5/17/17
164	Barn Yard Pond, OYP	3/11/17	5/17/17
165	Barn Yard Pond, OYP	3/11/17	5/17/17
166	Barn Yard Pond, OYP	3/11/17	5/17/17
167	Barn Yard Pond, OYP	3/11/17	5/17/17
168	Barn Yard Pond, OYP	3/11/17	5/17/17
169	Barn Yard Pond, OYP	3/11/17	5/17/17
170	Two Ponds, OYP	3/11/17	5/17/17
171	Two Ponds, OYP	3/11/17	5/17/17
172	Barn Yard Pond, OYP	3/11/17	5/17/17
173	Barn Yard Pond, OYP	3/11/17	5/17/17
174	Barn Yard Pond, OYP	3/11/17	5/17/17
175	Blue River, OYP	9/10/16	5/17/17
176	Blue River, OYP	9/10/16	5/17/17
177	Blue River, OYP	9/10/16	5/17/17
178	Clayton, OK	9/2/17	5/17/17
179	Side-house Pond, OYP	3/11/17	5/25/17
180	Side-house Pond, OYP	3/11/17	5/25/17
181	Side-house Pond, OYP	3/11/17	5/25/17
182	Side-house Pond, OYP	3/11/17	5/25/17
183	Side-house Pond, OYP	3/11/17	5/25/17
184	Side-house Pond, OYP	3/11/17	5/25/17
185	Side-house Pond, OYP	3/11/17	5/25/17
186	Side-house Pond, OYP	3/11/17	5/25/17
187	Side-house Pond, OYP	3/11/17	5/25/17
188	Side-house Pond, OYP	3/11/17	5/25/17
189	Side-house Pond, OYP	3/11/17	5/25/17
190	Side-house Pond, OYP	3/11/17	5/25/17
191	Side-house Pond, OYP	3/11/17	5/25/17
192	Side-house Pond, OYP	3/11/17	5/25/17
193	Side-house Pond, OYP	3/11/17	5/25/17
194	Side-house Pond, OYP	3/11/17	5/25/17
195	Side-house Pond, OYP	3/11/17	5/25/17
196	Side-house Pond, OYP	3/11/17	5/25/17
197	Side-house Pond, OYP	3/11/17	5/25/17
198	Side-house Pond, OYP	3/11/17	5/25/17
199	Side-house Pond, OYP	3/11/17	5/25/17
200	Side-house Pond, OYP	3/11/17	5/25/17
201	Side-house Pond, OYP	3/11/17	5/25/17

202	Side-house Pond, OYP	3/11/17	5/25/17
203	Side-house Pond, OYP	3/11/17	5/25/17
204	Side-house Pond, OYP	3/11/17	5/25/17
205	Side-house Pond, OYP	3/11/17	5/25/17
206	Side-house Pond, OYP	3/11/17	5/25/17
207	Side-house Pond, OYP	3/11/17	5/25/17
208	Side-house Pond, OYP	3/11/17	5/25/17
209	Side-house Pond, OYP	3/11/17	5/25/17
210	Side-house Pond, OYP	3/11/17	5/25/17
211	Side-house Pond, OYP	3/11/17	5/25/17
212	Side-house Pond, OYP	3/11/17	5/25/17
213	Side-house Pond, OYP	3/11/17	5/25/17
214	Side-house Pond, OYP	3/11/17	5/25/17
215	Side-house Pond, OYP	3/11/17	5/25/17
216	Side-house Pond, OYP	3/11/17	5/25/17
217	Side-house Pond, OYP	3/11/17	5/25/17
218	Side-house Pond, OYP	3/11/17	5/25/17
219	Side-house Pond, OYP	3/11/17	5/25/17
220	Side-house Pond, OYP	3/11/17	5/25/17
221	Side-house Pond, OYP	3/11/17	7/11/17
222	Side-house Pond, OYP	3/11/17	7/11/17
223	Side-house Pond, OYP	3/11/17	7/11/17
224	Side-house Pond, OYP	3/11/17	7/11/17
225	Side-house Pond, OYP	3/11/17	7/11/17
226	Side-house Pond, OYP	3/11/17	7/11/17
227	Side-house Pond, OYP	3/11/17	7/11/17
228	Side-house Pond, OYP	3/11/17	7/11/17
229	Side-house Pond, OYP	3/11/17	7/11/17
230	Side-house Pond, OYP	3/11/17	7/11/17
231	Side-house Pond, OYP	3/11/17	7/11/17
232	Side-house Pond, OYP	3/11/17	7/11/17
233	Front-house Pond, OYP	3/11/17	7/11/17
234	Front-house Pond, OYP	3/11/17	7/11/17
235	Front-house Pond, OYP	3/11/17	7/11/17
236	Front-house Pond, OYP	3/11/17	7/11/17
237	Front-house Pond, OYP	3/11/17	7/11/17
238	Front-house Pond, OYP	3/11/17	7/11/17
239	Front-house Pond, OYP	3/11/17	7/11/17
240	Front-house Pond, OYP	3/11/17	7/11/17
241	Front-house Pond, OYP	3/11/17	7/11/17
242	Front-house Pond, OYP	3/11/17	7/11/17
243	Front-house Pond, OYP	3/11/17	7/11/17
244	Front-house Pond, OYP	3/11/17	7/11/17

245	Front-house Pond, OYP	3/11/17	7/11/17
246	Front-house Pond, OYP	3/11/17	7/11/17
247	Red Slough WMA	9/2/17	9/23/17
248	Red Slough WMA	9/2/17	9/23/17
249	Red Slough WMA	9/2/17	9/23/17
250	Red Slough WMA	9/2/17	9/23/17
251	Red Slough WMA	9/2/17	9/23/17
252	Red Slough WMA	9/2/17	9/23/17
253	Red Slough WMA	9/2/17	9/23/17
254	Red Slough WMA	9/2/17	9/23/17
255	Red Slough WMA	9/2/17	9/23/17
256	Red Slough WMA	9/2/17	9/23/17
257	Red Slough WMA	9/2/17	9/23/17
258	Red Slough WMA	9/2/17	9/23/17
259	Red Slough WMA	9/2/17	9/23/17
260	Red Slough WMA	9/2/17	9/23/17
261	Red Slough WMA	9/2/17	9/23/17
262	Red Slough WMA	9/2/17	9/23/17
263	Red Slough WMA	9/2/17	9/23/17
264	Red Slough WMA	9/2/17	9/23/17
265	Red Slough WMA	9/2/17	9/23/17
266	Red Slough WMA	9/2/17	9/23/17
267	Red Slough WMA	9/2/17	9/23/17
268	Red Slough WMA	9/2/17	9/23/17
269	Red Slough WMA	9/2/17	9/23/17
270	Red Slough WMA	9/2/17	9/23/17
271	Red Slough WMA	9/2/17	9/23/17
272	Red Slough WMA	9/2/17	9/23/17
273	Red Slough WMA	9/2/17	9/23/17
274	Red Slough WMA	9/2/17	9/23/17
275	Red Slough WMA	9/2/17	9/23/17
276	Red Slough WMA	9/2/17	9/23/17
277	Red Slough WMA	9/2/17	9/23/17
278	Red Slough WMA	9/2/17	9/23/17
279	Red Slough WMA	9/2/17	9/23/17
280	Red Slough WMA	9/2/17	9/23/17
281	Red Slough WMA	9/2/17	9/23/17
282	Red Slough WMA	9/2/17	9/23/17
283	Red Slough WMA	9/2/17	9/23/17
284	Red Slough WMA	9/2/17	9/23/17
285	Red Slough WMA	9/2/17	9/23/17
286	Red Slough WMA	9/2/17	9/23/17
287	Red Slough WMA	9/2/17	9/23/17

288	Red Slough WMA	9/2/17	9/23/17
289	Red Slough WMA	9/2/17	9/23/17
290	Red Slough WMA	9/2/17	9/23/17
291	Red Slough WMA	9/2/17	9/23/17
292	Red Slough WMA	9/2/17	9/23/17
293	Red Slough WMA	9/2/17	9/23/17
294	Red Slough WMA	9/2/17	9/23/17
295	Red Slough WMA	9/2/17	9/23/17
296	Red Slough WMA	9/2/17	9/23/17
297	Red Slough WMA	9/2/17	9/23/17
298	Red Slough WMA	9/2/17	9/23/17
299	Red Slough WMA	9/2/17	9/23/17
300	Red Slough WMA	9/2/17	9/23/17
301	Red Slough WMA	9/2/17	9/23/17
302	Red Slough WMA	9/2/17	9/24/17
303	Red Slough WMA	9/2/17	9/24/17
304	Red Slough WMA	9/2/17	9/24/17
305	Red Slough WMA	9/2/17	9/24/17
306	Red Slough WMA	9/2/17	9/24/17
307	Red Slough WMA	9/2/17	9/24/17
308	Red Slough WMA	9/2/17	9/24/17
309	Red Slough WMA	9/2/17	9/24/17
310	Red Slough WMA	9/2/17	9/24/17
311	Red Slough WMA	9/2/17	9/24/17
312	Red Slough WMA	9/2/17	9/24/17
313	Red Slough WMA	9/2/17	9/24/17
314	Red Slough WMA	9/2/17	9/24/17
315	Red Slough WMA	9/2/17	9/24/17
316	Red Slough WMA	9/2/17	9/24/17
317	Red Slough WMA	9/2/17	9/24/17
318	Red Slough WMA	9/2/17	9/24/17
319	Red Slough WMA	9/2/17	9/24/17
320	Red Slough WMA	9/2/17	9/24/17
321	Red Slough WMA	9/2/17	9/24/17
322	Red Slough WMA	9/2/17	9/24/17
323	Red Slough WMA	9/2/17	9/24/17
324	Red Slough WMA	9/2/17	9/24/17
325	Red Slough WMA	9/2/17	9/24/17
326	Red Slough WMA	9/2/17	9/24/17
327	Red Slough WMA	9/2/17	9/24/17
328	Red Slough WMA	9/2/17	9/24/17
329	Red Slough WMA	9/2/17	9/24/17
330	Red Slough WMA	9/2/17	9/24/17

331	Red Slough WMA	9/2/17	9/24/17
332	Red Slough WMA	9/2/17	9/24/17
333	Red Slough WMA	9/2/17	9/24/17
334	Red Slough WMA	9/2/17	9/24/17
335	Red Slough WMA	9/2/17	9/24/17
336	Red Slough WMA	9/2/17	9/24/17
337	Red Slough WMA	9/2/17	9/24/17
338	Red Slough WMA	9/2/17	9/24/17
339	Red Slough WMA	9/2/17	9/24/17
340	Red Slough WMA	9/2/17	9/24/17
341	Red Slough WMA	9/2/17	9/24/17
342	Red Slough WMA	9/2/17	9/24/17
343	Red Slough WMA	9/2/17	9/24/17
344	Red Slough WMA	9/2/17	9/24/17
345	Red Slough WMA	9/2/17	9/24/17
346	Red Slough WMA	9/2/17	9/24/17
347	Red Slough WMA	9/2/17	9/24/17
348	Red Slough WMA	9/2/17	9/24/17
349	Red Slough WMA	9/2/17	9/24/17
350	Red Slough WMA	9/2/17	9/24/17
351	Red Slough WMA	9/2/17	9/24/17

Supplemental Table 2 – Sample Information

Primer	Direction	5' - 3' Sequence
AcanITS1F1	Forward	TTCATGGATGGCGAACTGATA
Angio58SR4	Reverse	TAGCTGCGTTTTTCATCGATA
AcanITS1R1	Reverse	GCGCCCATTGAAACATTATACTT
FWDSet1	Forward	TTCATGGATGGCGAACTGATAG
REVSet1	Reverse	GGCGCCCATTGAAACATTATAC

Supplemental Table 3 - Primers

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