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Angiostrongylus cantonensis: Epidemiologic Review, Location-Specific Habitat Modelling, and Surveillance in Hillsborough County, Florida, U.S.A.

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Angiostrongylus cantonensis: Epidemiologic Review, Location-Specific Habitat Modelling, and Surveillance in Hillsborough County, Florida, U.S.A.

by

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ABSTRACT

Angiostrongylus cantonensis is a parasitic nematode endemic to tropical and subtropical regions and is the leading cause of human eosinophilic meningitis. The parasite is commonly known as rat lungworm because the primary host in its lifecycle is the rat. A clinical overview of rat lungworm infection is presented, followed by a literature review of rat lungworm epidemiology, risk factors, and surveillance projects. Data collected from previous snail surveys in Florida was considered alongside elevation, population per square kilometer, median household income by zip code territory, and normalized difference vegetation index specific to the geographic coordinates from which the snail samples were retrieved. The parameters of interest were incorporated as possible predictor variables in a Poisson probability regression model and a negative binomial regression model. NDVI and population density were determined to be positively associated with number of snail samples positive for *A. cantonensis* in a given Miami-based location. A surveillance project was conducted in Hillsborough County, Florida, U.S.A.. Snail samples were collected and tested for *A. cantonensis* DNA via polymerase chain reaction (PCR) and gel electrophoresis. None of the samples tested positive for *A. cantonensis*.

INTRODUCTION

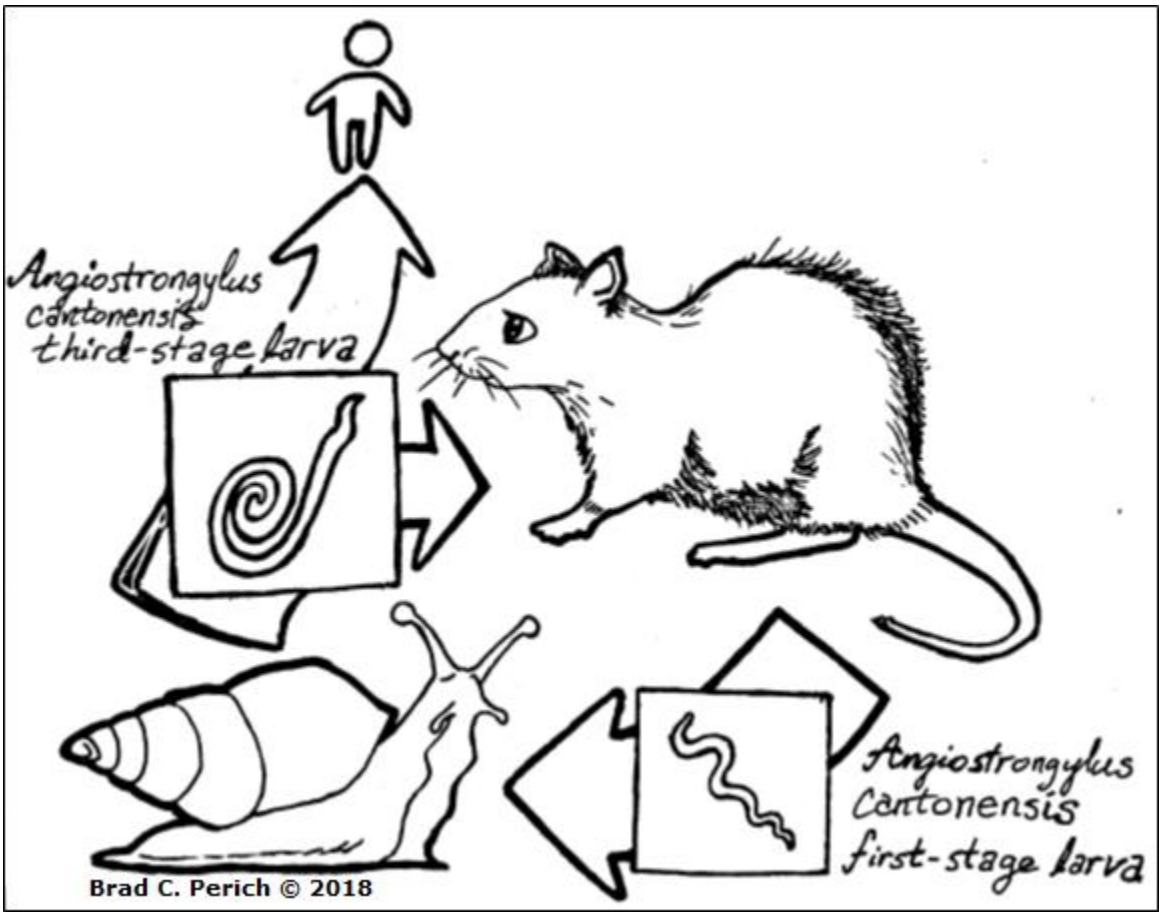

Figure 1. Rat Lungworm, Life Cycle

Life Cycle of Rat Lungworm

The parasite life cycle begins when first-stage *Angiostrongylus cantonensis* larvae are released in the feces of rats. Rats are definitive hosts of rat lungworm (Cowie, 2013a). There are four species of rat present in the United States of America and Caribbean that are confirmed to be hosts: *Rattus exulans, Rattus norvegicus, Rattus hispidus,* and *Rattus rattus*

(Aguiar, Morera, & Pascual, 1981; Andersen, Gubler, Sorensen, Beddard, & Ash, 1986; Graeff-Teixeira, da Silva, & Yoshimura, 2009; Qvarnstrom, Bishop, & da Silva, 2013; Stockdale-Walden et al., 2017; Yokogawa, 1937; York, Creecy, Lord, & Caire, 2015). Gastropods such as snails and slugs are the intermediate hosts of rat lungworm (Cowie, 2013a). There are at least 36 families in taxonomic class *Gastropoda* identified by research as natural hosts, and 10 additional families identified as experimental hosts; While only a limited number of host families have been discovered, it is possible that any species of snail or slug can be an intermediate host (Barratt et al., 2016; Yeung, Hayes, & Cowie, 2013a).

The first-stage *Angiostrongylus cantonensis* develops into third-stage larva while inside an intermediate host, which is then eaten by a rat (Cowie, 2013a). Third-stage *Angiostrongylus cantonensis* migrate through the rat digestive system into the small intestine, and move through the intestinal walls into the blood stream (Cowie, 2013a). The larvae travel through the circulatory system via passive transport until arriving at the central nervous system and brain of the rat (Cowie, 2013a). The third-stage larvae become sub-adult worms, enter the venous circulatory system again and then travel through active transport to the right ventricle of the heart and to pulmonary arteries, where the parasite will mature and procreate (Cowie, 2013a). The parasites produce eggs which enter the circulatory system, travel to the lungs, and hatch into first-stage larvae (Cowie, 2013a). First-stage larvae

migrate into the trachea of the rat where they are swallowed and are expelled in feces (Cowie, 2013a).

Human Rat Lungworm Infection

Humans are probably infected by rat lungworm through biological transmission. People can be infected by consuming raw or undercooked gastropod hosts either by intention or by accident and therefore expose themselves to third-stage larvae (Cowie, 2013b). Other possible modes of transmission are accidental consumption of snail mucus containing the parasite, water contaminated by host species, through open wounds, or consuming either raw or undercooked paratenic hosts such as shellfish, frogs, and lizards (Ash, 1968; Cowie, 2013b; Thiengo, Simões Rde, Fernandez, & Maldonado, 2013). The accidental consumption of raw hosts and host feces is suspected to happen when people eat unwashed vegetables and fruits (Cowie, 2013b; Saulo, 2009; Waugh et al., 2005).

Once ingested, *Angiostrongylus cantonensis* larvae enters the bloodstream and travels to the meninges and subarachnoid spaces, causing inflammatory responses characteristic of eosinophillic meningitis (Hochberg et al., 2007; Sawanyawisuth et al., 2009). While these are likely modes of transmission, the pathway of infection in humans is not clear.

Clinical symptoms of angiostrongyliasis cantonensis include common effects such as headache, fever, fatigue, neck stiffness, and typical

meningitis symptoms like Kernig's sign and Brudzinski's sign (Tseng et al., 2011). Other symptoms are mature larvae in the spinal cord, vomiting, sensitivity to light, hyperesthesia and paresthesia, facial palsy, severe pain, and ocular paralysis (Kliks, Kroenke, & Hardman, 1982; Murphy & Johnson, 2013). After entering the brain, *Angiostrongylus cantonensis* may attempt migration to pulmonary arteries in humans and damage brain tissues, leading to development of the fatal encephalitic angiostrongyliasis (Sawanyawisuth et al., 2009). Eosinophillic encephalitis is a fatal coma-inducing outcome that occurs in between 5 and 10 percent of cases and there is no known treatment (Chotmongkol & Sawanyawisuth, 2002; Sawanyawisuth & Sawanyawisuth, 2008; Tseng et al., 2011). Subpleural pulmonary nodules and ground-glass opacity lesions have been detected in patients infected by rat lungworm (Cui, Shen, & Meng, 2011). Rat lungworm infection is the foremost cause of eosinophillic meningitis worldwide (Graeff-Teixeira, da Silva, & Yoshimura, 2009).

Preventative Measures

Avoiding angiostrongyliasis is best done through diet. Avoid eating the primary, intermediate, and paratenic hosts of the parasite raw or undercooked (Saulo, 2009). Wash and rinse raw fruits and vegetables before eating them (Saulo, 2009). Remove any insects, dirt, debris, or any visible damage or contamination by gastropods (Saulo, 2009). A study

conducted by Yeung, Hayes, and Cowie (2013) shows that washing lettuce in drinkable water virtually eliminates risk of angiostrongyliasis from larvae or gastropods that may be on the vegetable surface (Yeung, Hayes, & Cowie, 2013). Wash the preparation area and cooking/eating utensils with hot soapy water, and rub the produce under running water with clean hands or a vegetable brush (Saulo, 2009).

Screening

Angiostrongyliasis cantonensis is the clinical term for disease caused as the result of infection by the parasite *Angiostrongylus cantonensis*. Since the symptoms associated with angiostrongyliasis are general and common to other diseases, laboratory-based criteria must be met for confirmed diagnosis. The only test currently considered definitive is the discovery of *Angiostrongylus cantonensis* inside a patient's body, often searched for in the cerebrospinal fluid (CSF) (Wilkins et al., 2013). Other diagnostic techniques are available in the absence of parasite discovery. Examination of the cerebrospinal fluid for abnormally high concentration of eosinophils (AEC) is a common test for possible infection. CSF is obtained via lumbar puncture spinal tap for the purposes of angiostrongyliasis cantonensis screening. Immunological diagnosis techniques of angiostrongyliasis cantonensis are being developed and refined. Research performed on serum samples from suspected patients examined by Enzyme-linked

Immunosorbent Assay (ELISA) determined that serum antibody levels were similar to the positive control samples from confirmed patients (Cross & Chi, 1982). Numerous immunological studies have since refined ELISA as an effective diagnostic tool for angiostrongyliasis cantonensis (Akao, Kondo, Ohyama, Chen, & Sano, 1992; Chye, Chang, & Yen, 2000; Chye, Yen, & Chen, 1997; Eamsobhana, Mak, & Yong, 1995; Eamsobhana, Ongrotchanakun, Yoolek, Punthuprapasa, Monkong, & Dekumyoy, 2006; Eamsobhana & Yong, 2009); Eamsobhana, Yong, Mak, & Wattanakulpanich, 1997; Eamsobhana, Yoolek, & Kreethapon, 2003; Eamsobhana, Yoolek, Suvouttho, & Suvouttho, 2001; Intapan, Maleewong, Polsan, Sawanyawisuth, & Chotmongkol, 2002; Intapan, Maleewong, Sawanyawisuth, & Chotmongkol, 2003; Maleewong, Sombatsawat, Intapan, Wongkham, & Chotmongkol, 2001; Morassutti, Levert, Perelygin, da Silva, Wilkins, & Graeff-Teixeira, 2012; Nuamtanong, 1996; Sawanyawisuth et al., 2011; Slom et al., 2002; Yen & Chen, 1991). Polymerase chain reaction (PCR)-based molecular detection techniques have shown success in detecting the presence of *Angiostrongylus cantonensis* DNA in suspected patients (Lima et al., 2009; Thyssen, Mitchell, Qvarnstrom, Rao, Benke, & Glodé, 2012). In a study of laboratory-confirmed patient cases of angiostrongyliasis who were infected at most two months prior, Computer Tomography (CT) scans have detected nodular ground-glass opacity lesions in all of the patients observed (Cui, Shen, & Meng, 2010). Their results

indicate that CT scanning may be an effective diagnostic technique. Magnetic Resonance Imaging (MRI) examination can detect minor hemorrhaging and granuloma characteristic of *Angiostrongylus* infection in brain and lung tissues of patients.

EPIDEMIOLOGIC REVIEW OF RAT LUNGWORM

History and Geography of Rat Lungworm Infection

While the extent of the historical burden of *Angiostrongylus cantonensis* is unknown, the parasite begins to appear in published literature during the 1930s. During an ecological survey in 1933, researcher H. T. Chen examined a Norwegian brown rat, *Rattus norvegicus*, sampled from Canton, China. Upon examining the lungs, Chen found a microscopic organism. Chen became the first researcher to publish the morphology of this new organism, which he assigned to the genus *Pulmonema,* species name *cantonesis* (Chen, 1935). Shortly after, researcher Yokogawa identified the organism inside the body of a rat as *Haemostrongylus ratti* (Yokogawa, 1937). The first recorded case of human infection was found in a Taiwanese meningitis patient and this finding was published by Nomura and Lin in 1945 (Beaver & Rosen, 1964). Nomura and Lin clinically established *Angiostrongylus cantonensis* as a cause of eosinophillic meningitis (Beaver & Rosen, 1964).

In 1946, E. C. Dougherty of the Helminthological Society of Washington published a taxonomic update of many members of phylum *Metastrongylidae*, and determined genus *Pulmonema* to be synonymous to

the genus *Angiostrongylus,* and the species *ratti* became synonymous with *cantonensis* (Dougherty, 1946). *Angiostrongylus* is assigned to superfamily *Metastrongyloidae*: characterized as nematodes that inhabit the lungs of vertebrates and typically develop into late-stage larvae within a molluscan intermediate host (Drozdz, 1970). Species *cantonensis*, among other members of *Angiostrongylus*, inhabit the bodies of rodent definitive hosts, and all first-stage larvae migrate through the gastrointestinal system of their hosts and exit with feces (Drozdz, 1970; Dougherty, 1946).

Since the initial discovery of *Angiostrongylus cantonensis* and its capacity for zoonosis, Angiostrongyliasis has been identified in tropic and subtropic regions all over the world. Rosen, Laigret, and Bories (1961) recognized that hundreds of Tahitians experienced an unusual meningitis characterized by white blood cell pleocytosis. Testing indicated that most of the patients had more than 500 cells per cubic millimeter of cerebrospinal fluid, and at least 25 percent of these cells were eosinophils (Rosen, Laigret, & Bories, 1961). Common symptoms included neck stiffness, localized paresthesias, and facial paralysis (Rosen, Laigret, & Bories, 1961). Examination of this fluid by culture and animal inoculation failed to reveal any causative organisms (Rosen, Laigret, & Bories, 1961). Although the Tahitian outbreak investigation was officially unsolved, similar outbreaks of eosinophillic meningitis continued to appear across the Pacific.

Horio and Alicata (1961) identified a case of parasitic meningoencephalitis in Hawaii. The patient reported consuming raw *Veronicella leydigi* harvested in Honolulu (Horio & Alicata, 1961). A sample of *V. leydigi* from the area tested positive for *Angiostrongylus cantonensis* (Horio & Alicata, 1961). A year later, L. Rosen (1962), with a different team of researchers, performed a postmortem examination of two mental patients from Hawai'i. They were both clinically diagnosed with eosinophillic meningitis (Rosen, Chappell, Laqueur, Wallace, & Weinstein, 1962). *Angiostrongylus cantonensis* was isolated from the brain of one patient, and remnants discovered in the brain of the second patient suggested a similar infection (Rosen, Chappell, Laqueur, Wallace, & Weinstein, 1962). This early finding established a connection between *Angiostrongylus cantonensis* and the eosinophillic meningitis epidemic of the Pacific Islands.

During the 1950s, Human *Angiostrongylus cantonensis* infection was identified on New Caledonia, the Philippines, Rarotonga, Saipan, Sumatra, Tahiti, and Taiwan (Kliks & Palumbo, 1992). During the 1960s, cases were identified in Cambodia, Guam, Java, New Hebrides, Sarawak, Thailand, and Vietnam (Kliks & Palumbo, 1992). From the 1970s to today, cases of *Angiostrongylus cantonensis* infection has been clinically confirmed in mainland China, Japan, Indonesia, India, Egypt, Nigeria, Cote d'Ivoire, Madagascar, Brazil, Cuba, Jamaica, the Bahamas, Puerto Rico, and in the United States of America (Aguiar, Morera, & Pascual, 1981; Kliks & Palumbo,

1992; Lv et al., 2011; Wang, 2012). This list is not exhaustive and *Angiostrongylus cantonensis* infection should be expected wherever the parasite is present in rats and mollusks (Kliks & Palumbo, 1992). There are over 2800 worldwide documented cases of angiostrongyliasis (Wang, Wu, Wei, Owen, & Lun, 2012).

Angiostrongylus cantonensis has firmly established itself in the United States of America, including the states of Hawai'i, Louisiana, Alabama, Texas, Florida, and the territory Puerto Rico (Andersen, Gubler, Sorensen, Beddard, & Ash, 1986; Campbell & Little, 1988; Hammoud et al., 2017; Hochberg et al., 2011; Hochberg et al., 2007; Kim, Stewart, Bauer, & Mitchell, 2002; Teem et al., 2013). Within the state of Florida, the first local rat lungworm infection was detected inside a mammalian accidental host in 2003. A white handed gibbon (*Hylobates lar*) from the Metrozoo in Miami, Florida, developed a neurological disorder (Duffy, Miller, Kinsella, & de Lahunta, 2004). Post-mortem examination revealed *Angiostrongylus cantonensis* present in the meninges of the brain and spinal cord and in the subarachnoid space of the cervical spinal cord, cerebellum, and cerebrum (Duffy, Miller, Kinsella, & de Lahunta, 2004). Since then multiple environmental surveys have been conducted in Florida establishing the presence of *Angiostrongylus cantonensis* in species of snail and rat (Iwanowicz et al., 2015; Stockdale-Walden et al., 2015; Stockdale-Walden et al., 2017; Teem et al., 2013). The most recent of these published studies

was published by Stockdale-Walden et al. (2017). Stockdale-Walden et al. (2017) conducted a survey of 171 *Rattus rattus* and 1,437 gastropods representing 32 different species. Among *Rattus rattus*, 39 (22.8%) tested positive, and among the gastropods, 27 (1.9%) tested positive. The counties from which positive samples were retrieved from were Alachua, Hillsborough, Leon, Orange, and Saint Johns (Stockdale-Walden et al., 2017).

In addition to establishing the presence of *Angiostrongylus cantonensis* in the state of Florida, Stockdale-Walden et al. (2017) identified new intermediate host species of *Angiostrongylus cantonensis* in their study: *Bradybaena similaris, Paropeas achatinaceum, Succinea floridana, Ventridens demissus, Zachrysia provisoria,* and *Zonitoides arboreus.* Although rat lungworm is present in Florida, there has yet to be a confirmed case of Florida-local human angiostrongyliasis.

Risk Factors for Rat Lungworm Infection

Risk of angiostrongyliasis cantonensis primarily depends on the ingestion of an infected host or fomite. There have been few published epidemiologic investigations employing methods such as cohort and case-control studies for assessment of potential risk factors.

Sawanyawisuth et al. (2009) conducted a comparison study among patients in Thailand to determine clinical factors for an encephalitis outcome

compared to a meningitis outcome as a result of *Angiostrongylus cantonensis* infection. Encephalitic angiostrongyliasis is fatal but rarer than meningitic angiostrongyliasis (Chotmongkol & Sawanyawisuth, 2002; Sawanyawisuth & Sawanyawisuth, 2008). The researchers worked with adult angiostrongyliasis patients from a hospital located in an endemic area of Thailand. Inclusion criteria were used to determine angiostrongyliasis infection. These were history of ingesting freshwater snails or paratenic hosts, cerebrospinal fluid samples with a white blood cell count greater than 10 cells/mm^3, cerebrospinal fluid samples in which eosinophils make up more than 10% of total white blood cell count, negative cerebrospinal fluid gram, acid-fast, india ink staining cryptococcal antigen and culture test results. Exclusionary criteria were designed to exclude other possible sources of eosinophillic response such as diet of raw fish and medical history of related diseases.

With the parameters desired power of 80%, meningitis to encephalitis ratio of 6:1, and a two-sided significance level of 0.05, they determined an approximate necessity of 14 encephalitis patients and 86 meningitis patients. Among a group of 94 angiostrongyliasis patients enrolled, 14 were diagnosed with encephalitis and 80 were diagnosed with meningitis. Baseline characteristics collected were sex, age in years, season during which the patient was admitted, number of days since last exposure to snails/paratenic host up to first day of symptom development (incubation

period), duration of headache in days, presence of paresthesia, vomiting, fever, seventh cranial nerve palsy, papilledema, and stiff neck.

Stepwise multivariate logistic regression analysis was used to determine odds ratios for the development of encephalitis with meningitis as a reference group. The final model included fever (adjusted odds ratio 37.05, confidence interval 1.59-862.35), prolonged headache duration (adjusted odds ratio 1.26, confidence interval 1.03-1.55), and older age (adjusted odds ratio 1.22, confidence interval 1.05-1.42) (Sawanyawisuth et al., 2009). Each variable with a significance value less than 0.2 in a first univariate analysis was added to the variables included in the multivariate analysis. Variables with a significance value 0.15 or less were included in a stepwise approach to obtain a final model.

These odds ratios indicate differences between encephalitis and meningitis patients among these variables. The median headache duration is 18.5 days among encephalitis patients and 7 days among meningitis patients. 71% of encephalitis patients had a fever compared to 10% of the meningitis patients. This study is the first to show evidence that old age is a risk factor for encephalitic angiostrongyliasis (Sawanyawisuth et al., 2009).

A case of autochthonous eosinophillic meningitis was discovered in a Jamaican patient in 1994, and although there was no confirmed presence of *Angiostrongylus cantonensis*, it was an indicator that the parasite may be endemic in Jamaica (Barrow, St. Rose, & Lindo, 1994). The first confirmed

infection by *Angiostrongylus cantonensis* in Jamaica was documented in 1998 when an autopsy revealed the parasite in a child's lungs and meninges (Lindo et al., 2004). Jamaican *Angiostrongylus cantonensis* received epidemiological attention in 2000 during the largest documented outbreak of eosinophillic meningitis in the Americas, when 12 out of 23 U.S. travelers came down with an outbreak after sharing a meal (Slom et al., 2002). The researchers conducted a retrospective cohort study to identify risk factors for eosinophillic meningitis. The 23 travelers were all in Jamaica from April 2 through April 9, and were assessed according to a clinical description. The clinical definition included acute onset headache within 35 days after returning from Jamaica, in addition to at least one of the following symptoms: paresthesia, hyperesthesia, neck pain, photophobia, visual disturbances, and nuchal rigidity (Slom et al., 2002). Lumbar puncture was performed on all 9 of the hospital patient cases and 4 had peripheral blood tests. Eosinophilia was diagnosed in 8 of the 9 hospitalized patients. Abnormally high eosinophil count was set at 10 percent or greater eosinophil in total white blood cell count of cerebrospinal fluid obtained by lumbar puncture, or 600 eosinophils per cubic millimeter or greater in samples of peripheral blood (Slom et al., 2002). *Angiostrongylus cantonensis* infections were confirmed by presence of parasite specific antibody via western blot (Slom et al., 2002).

Each traveler was interviewed twice with questionnaires designed to get information about restaurants visited and items ordered in Jamaica, other potential exposures, and symptoms experienced. There was only one restaurant patronized and one meal that was eaten by all 12 patients in this study, Caesar salad. 12 of 13 travelers who attended this restaurant and ate a Caesar salad became ill, compared to none of the 3 who attended this restaurant and did not eat the Caesar salad. The researchers reported this risk ratio as undefined but the resultant significance value was 0.007 (Slom et al., 2002).

In addition to studying risk factors for angiostrongyliasis in humans, it is also beneficial to determine the extent of infection among primary and paratenic hosts. The outbreak among travelers in Jamaica generated interest in the extent of *Angiostrongylus cantonensis* presence in Jamaica. Waugh, Lindo, Lorenzo-Morales, and Robinson (2016) assessed Jamaican rodents to survey the extent of the parasite presence in the local primary and intermediate host populations. 297 *Rattus rattus*, 140 *Rattus norvegicus*, and 777 terrestrial molluscs were harvested from 14 parishes around Jamaica (Waugh, Lindo, Lorenzo-Morales, & Robinson, 2016). Among the 777 terrestrial molluscs collected, 12.5% were infected by third-stage larvae. Waugh, Lindo, Lorenzo-Morales, and Robinson (2016) reported that *Angiostrongylus cantonensis* was identified in 35.4% *R. rattus* (n=297, 95% C.I. 29.6-40·7) with a mean intensity of 16.8 (14.19-19.7).

A. cantonensis was identified in 25.0% R. norvegicus (n=140, 18.7-33.8) with a mean intensity of 11.3 (8.31–15·9). The rat collection sites on the northeastern section of Jamaica had the highest rat sample size (n=151) and had the highest prevalences of infected rats, ranging from 47% to 60%. Western Jamaica collection sites had the lowest prevalence among rats, ranging from 4% to 11% (Waugh, Lindo, Lorenzo-Morales, & Robinson, 2016).

Rat Lungworm Habitat Modelling

Angiostrongylus cantonensis is endemic to tropic and subtropic areas and is expected to expand into new territories as a result of climate change, exposing more human, rodent, and gastropod populations and increasing the frequency of outbreaks (Lv et al., 2011; York, Butler, & Lord, 2013). It is therefore beneficial to develop techniques for predicting *Angiostrongylus cantonensis* habitats. Effective forecast habitat modelling can identify locations to monitor and respond to in case of an epidemic. Predictive models are often used to find parasite habitats. (Jacob et al., 2007; Jacob et al., 2009; Jacob et al., 2010; Jacob et al., 2013; York, Butler, & Lord, 2013). The research presented in this thesis contains a Florida-specific habitat model for presence of *Angiostrongylus cantonensis* in snail vector populations.

Rat lungworm habitat modelling has previously been attempted. York, Butler, and Lord (2013) collected data from 86 locations where *Angiostrongylus cantonensis* presence is documented. The researchers identified 20 possible explanatory variables, incorporated them into Maximum Entropy Model, and determined which variables, when included, minimized their Akaike's information criterion for small sample correction (AICc) score (York, Butler, & Lord, 2013). They identified three environmental variables: mean diurnal temperature, minimum temperature of coldest month, and precipitation of the warmest quarter (York, Butler, & Lord, 2013). They postulated that probability of occurrence increases with increasing precipitation during the warmest quarter and increasing temperature of the coldest month, predicting that the probability of occurrence hovers between 80-90% at the highest values (York, Butler, & Lord, 2013). Increasing mean diurnal temperature range was suggested to increase probability of occurrence up to near 7 degrees Celsius, and then decrease to 0 past that temperature (York, Butler, & Lord, 2013).

LOCATION-SPECIFIC HABITAT MODELLING IN MIAMI, FLORIDA, USA

Study site

Miami-Dade County is the furthest southeastern county of Florida state, and the furthest southeastern part of mainland United States of America. Miami-Dade County has a population exceeding 2.7 million, the highest of all counties in Florida. It is the third largest county in Florida by land area measuring 5,040 km^2. Miami-Dade County is bordered by Broward County to the north, Monroe County to the west, and the Atlantic Ocean on the east and west. The altitudinal range of Miami-Dade County ranges from sea level up to 13 meters. The mean annual temperature range is between 15 and 31.7 degrees Celsius. Miami-Dade has a Köppen tropical monsoon climate. The Florida Everglades cover much of the western region while urban development covers the eastern coast in a longitudinal direction. *Angiostrongylus cantonensis* is established in snail populations located at various collection sites throughout urban Miami-Dade (Iwanowicz et al., 2015; Stockdale-Walden et al., 2017; Stockdale-Walden et al., 2015; Teem et al., 2013).

Subjects and Setting

Data was acquired from 2 of 4 published studies conducted in Florida in which snail populations were sampled and tested for *Angiostrongylus cantonensis* DNA (Iwanowicz et al., 2015; Teem et al., 2013). Researchers in each study using identical molecular detection techniques developed by Qvarnstrom et al. (2010). Each of the 10 snail sample sites included in this research are located in Miami-Dade County. 9 sites are in urban-residential properties and 1 sample site is in Everglades Park, located outside limits. Sample site geographic coordinates are listed in Table 1.

Relevant studies were recently published by Stockdale-Walden et al. in 2015 and 2017. Snail sample data was published without reporting geographic coordinates. Geographic data specific to sample sites could not be retrieved and therefore it is not possible to incorporate the data from the 2015 study nor from the 2017 study.

Materials and Methods

Regression models are often used in public health studies to explore the relationship between a measurable phenomenon and possible predictive factors (Chao, Zhao, Kupper, & Nylander-French, 2008). Poisson regression and negative binomial regression are performed in public health data analysis when the desired outcome is a count variable (Kim & Kriebel, 2009). These tests examined total count of positive samples gathered from

each specific geographic location. Number of positive snail samples was chosen as the outcome variable rather than proportion of positive snails since the true total population of snails per respective location is unknown. Identical predictor variables were used for the negative binomial regression.

The Poisson Regression Model: $g(\mu)=\beta_0+\beta_1x_1+\beta_2x_2+\ldots+\beta_kx_k$

$g(\mu)$ refers to the mean value of Y given predictor value X, β_0 indicated the Y-intercept, β_1 is the beta-coefficient also known as slope for a designated linear predictor X1. β_kx_k indicates a variable amount of beta-coefficients and their respective linear predictors where one exists for each predictor in the *Angiostrongylus cantonensis* habitat model.

The Poisson model carries two assumptions. One is the random component assumption, that $y_i \sim \text{Poisson}(\mu_i)$ for $i=1,...,N$ where y is equal to the mean (Kim & Kriebel, 2009). The other is the systematic component, that any set of x variables are predictor variables. Kim and Kriebel (2009) state that the random component assumption is often violated due to natural data overdispersal in public health models. Negative binomial regression models compensate for the overdispersion through the use of a non-homogeneous gamma distributed mean, forcing y equal to the mean and therefore correcting for the Poissonian random component assumption violation (Dinh & Jacob, 2016; Haight, 1967; Hosmer & Lemeshow, 1980).

The Negative Binomial Regression Model: $\ln(\mu)=\beta_0+\beta_1x_1+\beta_2x_2+\ldots+\beta_pX_p$ Where the X variables refer to the predictors, and the β are the estimators. Regressions were performed using statistical programming software package SAS® 9.1.3 (SAS Inc. Cary, North Carolina).

The independent variables of interest were elevation in meters, median household income by zip code territory, population per square kilometer by zip code territory and normalized difference vegetation index. Elevation raster data at 1/3 arc-second resolution was retrieved from the United States Geological Survey National Elevation Dataset and point elevation was determined for each sample site using Esri ArcMap® 10.5. The 1/3 second resolution is the most detailed available for the area and thus selected for study. Zip code geodatafiles were retrieved from Esri company (2017). Their data is based on postal information and was most recently updated March 2016. Median household income and population by zip code territory was obtained from the US Census FactFinder American Community Survey 2011-2015 5-year estimate. The values for each site are listed in Table 1.

Table 1. Location-Specific Snail Habitat Data

Latitude	Longitude	NDVI	Population per square kilometer*	Median Household Income**	Number of Positive Snail Samples	Zip Code	Elevation (meters)	Author
25.723147	-80.38674	0.354096	4243.41	$53,206	7	33175	1.328	Iwanowicz et al 2015
25.818961	-80.19848	0.234684	6118.65	$28,909	5	33127	4.2531	Iwanowicz et al 2015
25.67175	-80.42833	0.177484	3573.53	$67,137	4	33186	2.12939	Teem et al 2013
25.779903	-80.26017	0.150878	5205.98	$46,867	2	33126	1.82866	Iwanowicz et al 2015
25.734603	-80.34776	0.202358	4487.06	$56,149	2	33165	2.428	Iwanowicz et al 2015
25.671156	-80.42676	0.171409	3573.53	$67,137	1	33186	2.43728	Iwanowicz et al 2015
25.608558	-80.38694	0.249562	2808.57	$75,163	1	33177	3.44045	Iwanowicz et al 2015
25.609297	-80.39788	0.288629	2808.57	$75,163	0	33177	2.537	Teem et al 2013
25.522064	-80.40888	0.285561	1079.06	$34,088	0	33032	2.35354	Iwanowicz et al 2015
25.762197	-80.76639	0.194149	58.6	$43,620	0	33196	0.85733	Teem et al 2013

* Population density was obtained from census.gov Factfinder service

** Median Household Income (2011-2015 ACS 5-year estimate)

Remote Sensing Data and Environmental Parameters

Spectral reflectance of plants reveal photosynthetic activity in an area (Huete & Tucker, 1991) and is used to calculate a Normalized Difference Vegetation Index (NDVI). Visible and near-infrared (NIR) spectral response of bare soil varies in brightness associated with magnitude of reflected flux and in spectral curve form due to mineral/organic composition and moisture (Huete & Tucker, 1991).

The Normalized Difference Vegetation Index:

$$NDVI = (NIR - RED)/(NIR + RED)$$

NIR is the near-infrared wavelength reflectance and RED is visible red wavelength reflectance.

Topographic imagery of Florida was obtained from the LANDSAT satellite EarthExplorer database. LANDSAT 8 multispectral images provide four spectral band layers: green, red, blue, and near-infrared. NDVI was determined for each location sampled for *Angiostrongylus cantonensis* using Esri ArcMap® 10.5 Normalized Difference Vegetation Index Tool (Rhew et al., 2011). NDVI values corresponding to each coordinate point were added to the regression model. The sample sites and corresponding NDVI values are listed in Table 1. See Appendix A for a NDVI map of the Miami, Florida, study area created in ArcMap® 10.5.

Results

Negative binomial regression and Poisson regression determined the likelihood of each predictor variable explaining the outcome (number of rat-lungworm positive snails) under the assumption that the observed results happened due to chance (p-value). Each predictor variable: elevation, NDVI, median household income by zip code, and population by zip code, has a probability value attached to it between 0 (0% chance of happening if the predictor variable has no significant effect) and 1 (100% chance).

Table 2.1 Negative Binomial Model, NDVI and Population Density

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
NDVI	6.3623	3.2174	0.0563	12.6683	3.91	**0.0480**
Population Density	0.0005	0.0002	0.0002	0.0009	10.95	**0.0009**

Table 2.2 Negative Binomial Model, all predictors included

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
NDVI	6.4392	3.5774	-0.5724	13.4508	3.24	0.0719
Population Density	0.0011	0.0007	-0.0004	0.0025	2.05	0.1521
Median Household Income	0.0000	0.0001	-0.0001	0.0001	0.43	0.5104
Elevation	-0.2328	0.2484	-0.7197	0.2540	0.88	0.3486

Table 2.3 Negative Binomial Model, NDVI only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
NDVI	3.7703	4.9434	-5.9185	13.4591	0.58	0.4456

Table 2.4 Negative Binomial Model, Population Density only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
Population Density	0.0006	0.0002	0.0001	0.0010	5.72	0.0168

Table 2.5 Negative Binomial Model, Median Household Income only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
Median Household Income	0.0000	0.0000	-0.0001	0.0000	0.38	0.5361

Table 2.6 Negative Binomial Model, Elevation only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
Elevation	0.0653	0.3418	-0.6046	0.7353	0.04	0.8484

Table 3.1 Poisson Model, NDVI and Population Density

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
NDVI	6.3623	3.2174	0.0563	12.6683	3.91	**0.0480**
Population Density	0.0005	0.0002	0.0002	0.0009	10.95	**0.0009**

Table 3.2 Poisson Model, all predictors included

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
NDVI	6.4392	3.5773	-0.5723	13.4506	3.24	0.0719
Population Density	0.0011	0.0007	-0.0004	0.0025	2.05	0.1521
Median Household Income	0.0001	0.0001	-0.0001	0.0001	0.43	0.5104
Elevation	-0.2328	0.2484	-0.7197	0.2540	0.88	0.3486

Table 3.3 Poisson Model, NDVI only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
NDVI	4.7284	3.3099	-1.7588	11.2156	2.04	0.1531

Table 3.4 Poisson Model, Population Density only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
Population Density	0.0005	0.0002	0.0002	0.0008	9.57	0.0020

Table 3.5 Poisson Model, Median Household Income only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
Median Household Income	0.0000	0.0000	0.0000	0.0000	0.92	0.3366

Table 3.6 Poisson Model, Elevation only

	Beta estimate	Standard Error	95% Confidence Limits (Wald)		Wald X^2	p-value
Elevation	0.0829	0.2282	-0.3644	0.5301	0.13	0.7165

The variables NDVI (p=0.0480) and Population per square kilometer (p=0.0009) were both significant when regressed together in both negative binomial and Poisson models. A post-hoc Pearson correlation test was performed using SAS® 9.1.3 (SAS Inc. Cary, North Carolina) to evaluate if the two predictors for multicollinearity. Significant correlation would suggest redundancy in the predictor-outcome relationship.

Pearson correlation: $r_{ab} = covariance(a, b) \,/\, ([\sqrt{variance(a)}][\sqrt{variance(b)}])$ covariance(a, b) is the covariance of a and b, variance(a) is the sample variance of a, and variance(b) is the sample variance of b. Pearson correlation values range from -1 to 1. Values approaching -1 or 1 indicate a negative/positive relationship respectively, and values close to 0 indicate no correlation. The Pearson correlation coefficient between NDVI and Population Density is equal to -0.12417, p=0.7325.

Interaction was further evaluated post-hoc by including an interaction term in the negative binomial and Poisson models. The interaction term was insignificant and thus removed.

Discussion

The negative binomial regression and Poisson regression reveals NDVI and Population per square kilometer as potential positive predictors when regressed together. Population per square kilometer is still significant in

both models when regressed by itself. Pearson correlation between NDVI and population density does not suggest multicollinearity. These results provide evidence that the number of snails positive for *Angiostrongylus cantonensis* in any given snail habitat in Miami, Florida can be predicted by location-specific NDVI values and population density in a positive direction. P-values returned for the other variables were considered too large to be reliable predictors. The available data was somewhat limited. Future studies would benefit from more detailed collection methods. The data published by these studies does not include the date and time of sample collection. Adding these would allow researchers to add more variables such as precipitation, humidity, season, and other data specific to that date and time. Further research could attempt a thorough collection methodology, including as many details as possible, and reveal more significant predictors for number of infected snails. Such a project may thereby establish a standard for all similar sampling projects. In addition to lack of detail, there is not yet much relevant research conducted and published. More sampling projects should be conducted on confirmed host populations to determine the extent of *Angiostrongylus cantonensis* presence in Florida. Further analysis using similar regression tests performed on larger sample sizes and incorporating more covariates can further explore the significance of NDVI, population density, and other potential covariates.

ANGIOSTRONGLYUS CANTONENSIS SURVEILLANCE IN HILLSBOROUGH COUNTY, FLORIDA, USA

Materials and Methods

Sample Collection

80 samples of *Zachrysia provisoria* were collected in total from 2 locations in Hillsborough County. Both locations were in urban residential areas, where *Z. provisoria* has potential to interact with humans. Identical land-use characteristics and similar weather conditions on the day of collection were chosen for the two sites to control for confounding. Sampling was conducted after rainfall. Location 1 and 2 are approximately 21.2 km apart and are chosen as surveillance sites for the respective surrounding communities. Location 1 (28.070615, -82.4187421) is adjacent to an apartment complex near University of South Florida. Location 2 (27.90570639, -82.52797611) is adjacent to an apartment complex and Tampa Bay.

40 *Z. provisoria* samples were collected early in the summer season, 20 from location 1 and 20 from location 2, and 40 samples were collected 1 month later, 20 from location 1 and 20 from location 2, to explore potential temporal variability in results. 20 samples were collected from Location 1 on

May 20th, 2017. 20 samples were collected from Location 2 on May 24th, 2017. 20 samples were collected from Location 1 on June 30th, 2017. 20 samples were collected from Location 2 on June 30th, 2017. *Z. provisoria* was identified based on morphology. *Z. provisoria* samples were individually stored in 50 mL centrifuge tubes and frozen at -20°C. Average temperature and total precipitation for each collection day was retrieved from National Oceanic and Atmospheric Association monthly weather summary observed climate report.

Table 4. Location-Specific *Zachrysia provisoria* Habitat Data

Date	Location	Coordinates, Latitude/Longitude	*Z. provisoria* samples collected	Temperature between min and max on this day (°C)	Total Precipitation on this day (cm)
5/20/2017	1	28.070615, -82.4187421	20	30	Trace
5/24/2017	2	27.90570639, -82.52797611	20	27.22	0.41
6/30/2017	1	28.070615, -82.4187421	20	28.61	0.64
6/30/2017	2	27.90570639, -82.52797611	20	28.61	0.64

Figure 2. Images of Collected Snails

DNA Extraction

Protocol established by Qvarnstrom et al. 2010 was followed as closely as possible. Foot sections weighing approximately 0.25 mg were cut with a sterile razor and placed in individual 1.5 mL microfuge tubes. The DNA extraction procedure was performed with guidance from the DNAeasy®: Animal Tissue spin column protocol provided in the Qiagen DNAeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). Stock solution buffers ATL, AL, AW1, and AW2 were created on the day the procedure was performed. 180 µL ATL was added to each snail sample. 20 µL Proteinase K was added to each snail sample and the tubes were vortexed for 5 seconds. The centrifuge tubes were then incubated at 56°C and vortexed for 5 seconds at 10 minute intervals over 2 hours, after which the snail tissues were fully lysed. The tubes were then centrifuged for 5 minutes at 5000 rpm, 21°C. The supernatant from each centrifuge tube was transferred to new tubes, the pellets and old tubes were discarded. 200 µL Buffer AL was added to each new centrifuge tube and vortexed for 5 seconds. The centrifuge tubes were centrifuged for 2 minutes at 5000 rpm, 21°C. 200 µL of 100% ethanol was added to each centrifuge tube, and then the tubes were vortexed for 5 seconds and centrifuged for 2 minutes at 5000 rpm, 21°C. The contents of the centrifuge tubes were transferred into individual 2 mL spin columns and centrifuged for 1 minute at 8000 rpm, 21°C. The spin columns were transferred into new 2 mL collection tubes. 500 µL of Buffer AW1 was added

to each spin column and centrifuged for 1 minute at 8000 rpm, 21°C. The spin columns were then transferred into new 2 mL collection tubes. 500 µL of Buffer AW2 was added to each spin column and centrifuged for 3 minutes at 10,000 rpm, 24°C. Spin columns were transferred again into new 2 mL collection tubes, and centrifuged for 1 minute at 10,000 rpm, 24°C, to ensure that all solution was removed. The spin columns were inserted into 1.5 mL microfuge tubes, and 100 µL AE Buffer was added to elute the DNA. The microfuge tubes with inserted spin columns were incubated at room temperature for 1 minute, and then centrifuged for 1 minute at 8000 rpm, 18°C. To elute any remaining DNA from the spin columns, an additional 100 µL AE Buffer was added to the tubes, incubated at room temperature for 1 minute, and then centrifuged for 1 minute at 8000 rpm, 18°C.

The purity of extracted DNA was tested with Thermo Fisher Scientific Nano-Drop® 1000 spectrophotometer and software package. Water was used as a blank for the spectrophotometer, and 2 µL DNA was measured to ensure that the 260 nm to 280 nm sample absorbance ratio was above 1.5, indicating good quality results. For reference, a ratio of 1.8 is considered to be pure DNA, while a ratio near 1 indicates contamination.

Polymerase Chain Reaction (PCR)

DNA samples were amplified via polymerase chain reaction. Protocol established by Qvarnstrom et al. 2010 was followed as closely as possible.

Stock reaction solution was created for 81 reactions, 80 samples and one blank. The stock reaction solution was briefly vortexed and centrifuged upon creation. Reaction solution for each individual sample consisted of 10.75 µL water, 2.5 µL 2 mM dNTP mix, 2.5 µL 10x Buffer I (minus $MgCl_2$), 1.5 µL 50 mM $MgCl_2$, 1.5 10 µM primer AcanITS1F1, 1.5 µL 10 µM primer AcanITS1R1, and 0.25 µL Taq polymerase, for a total of 20.5 µL reaction solution for each DNA sample. 20 µL aliquots of reaction solution were added to 81 positions of a 96-well PCR plate. 5 µL from each DNA sample and 5 uL from a blank were added to each of the 81 aliquots. A seal was applied to the plate and placed in a Biorad MyCycler® Thermocycler. The procedure was set as follows:

1. 1 cycle of 94°C for 3 minutes.
2. 35 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds.
3. 1 cycle of 72°C for 7 minutes.
4. 4°C until removed.

DNA Gel Electrophoresis

Gel electrophoresis was performed to separate a visual band of the desired amplified PCR product. A 1.5% agarose gel solution was created with quick dissolve LE Agarose® (Apex Bioresearch Products (Genesee Scientific, San Diego, CA) and 0.05% TAE buffer. Gels were created from

the solution. 2 µL of each of the 81 PCR samples were added to 8 µL loading dye, consisting of 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 3% glycerol, in water. Each 10 µL DNA/dye mixture was pipetted into an individual well in the gel. A well in each gel was reserved for a 100 base-pair ladder for reference. The gel electrophoresis was run at 400 mAMP at 120 Volts for 50 minutes. The gels were soaked in 0.5 µg/mL ethidium bromide solution for 15 minutes and viewed with ultraviolet light.

Results

Noticeable DNA banding was absent in 80 of the DNA samples. Habitat modelling and data analysis was not conducted due to absence of positive samples.

Discussion, Research Needed, and Future Directions

Among the *Zachrysia provisoria* collected as part of the Tampa Bay rat lungworm surveillance project, none appeared to test positive for *Angiostrongylus cantonensis*. There was no positive control available confirm protocol. The results suggest that rat lungworm may not yet be endemic in snail populations residing near Tampa Bay apartment complexes. This research has not identified a risk of infection to residents near the locations studied. While researchers Stockdale-Walden et al. (2017) established the presence of rat lungworm in Hillsborough County, Florida,

the exact geographic locations were not reported and the extent of its presence is yet to be discovered. The researchers reported total counts from samples harvested throughout the entire county. More surveillance projects should be conducted to learn about the extent of rat lungworms' presence in Hillsborough County, Florida. Since human infections occur via contaminated food, places such as farms, food processing facilities, and produce markets are critical sites to collect rats and gastropods.

The sample collection methodology in this thesis serves as a foundation for all for future projects. The specific geographic coordinate location of each sample site and the calendar date of each collection should be recorded. Collecting this data allows for variables such as rainfall, humidity, and geographic features to be included in constructing a habitat model. Rat lungworm habitat modelling can be done if future Tampa-local surveillance projects discover positive samples. While the research presented in this thesis establishes Population per square kilometer and NDVI in tandem as possible significant predictors of positive snail samples in Miami-Dade County, this association may be location-specific and the strength of association may be different outside of Miami-Dade.

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APPENDICES

APPENDIX A

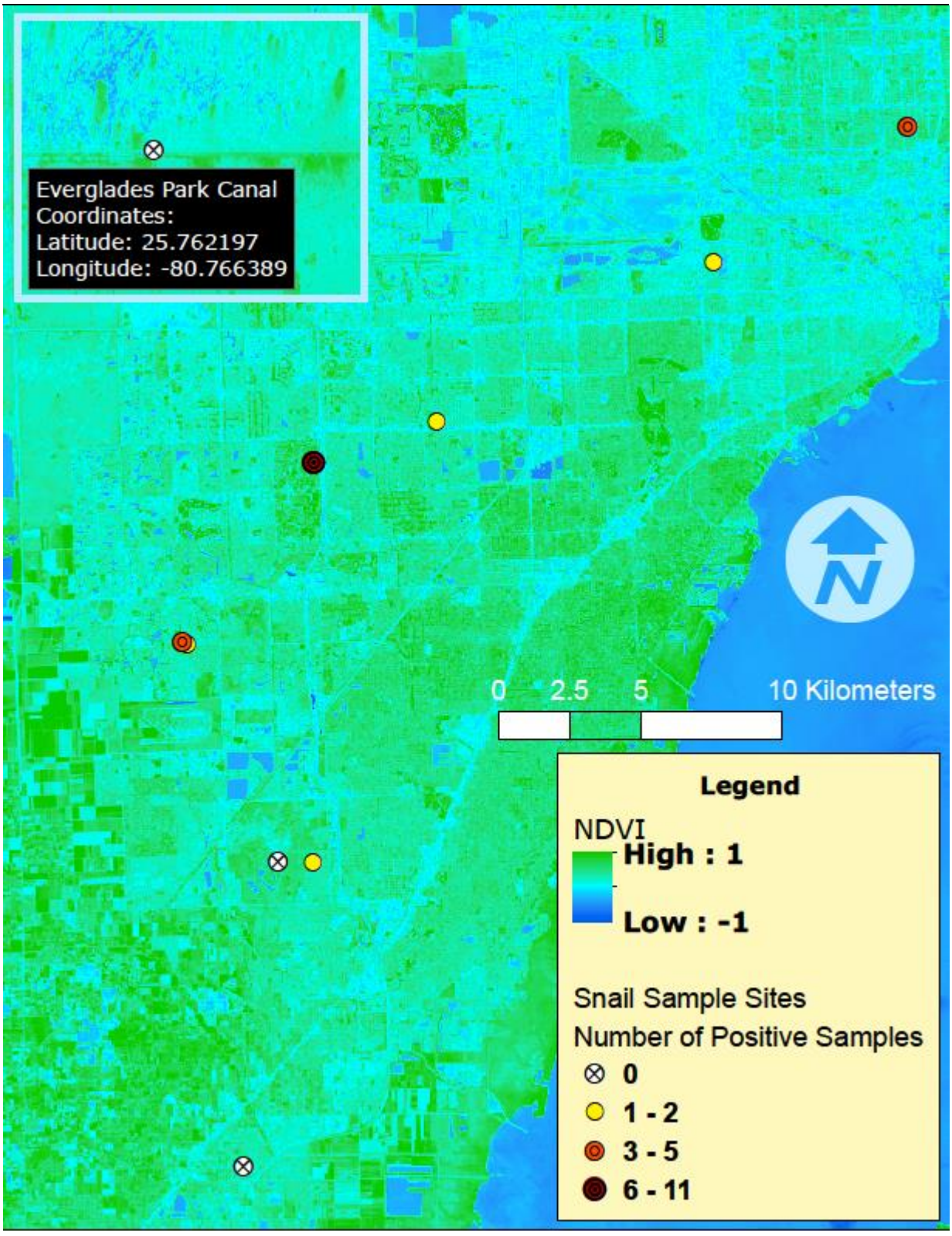

Figure A1. NDVI Map of Miami, FL

APPENDIX B

USF
UNIVERSITY OF
SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX(813)974-7091

7/20/2017

Brad Perich
Epidemiology and Biostatistics
Tampa, FL 33612

RE: **Not Human Subjects Research Determination**
IRB#: Pro00031675
Title: Presence of Angiostrongylus cantonensis in samples of Zachrysia provisoria in Hillsborough County

Dear Mr. Perich:

The Institutional Review Board (IRB) has reviewed your application. The activities presented in the application involve methods of program evaluation, quality improvement, and/or needs analysis. While potentially informative to others outside of the university community, study results would not appear to contribute to generalizable knowledge. As such, the activities do not meet the definition of human subject research under USF IRB policy, and USF IRB approval and oversight are therefore not required.

While not requiring USF IRB approval and oversight, your study activities should be conducted in a manner that is consistent with the ethical principles of your profession. If the scope of your project changes in the future, please contact the IRB for further guidance.

If you will be obtaining consent to conduct your study activities, please remove any references to "research" and do not include the assigned Protocol Number or USF IRB contact information.

If your study activities involve collection or use of health information, please note that there may be requirements under the HIPAA Privacy Rule that apply. For further information, please contact a HIPAA Program administrator at (813) 974-5638.

Sincerely,

VJorgensen MD

E. Verena Jorgensen, M.D., Chairperson
USF Institutional Review Board