Documenting freshwater snail and trematode parasite diversity in the Wheeler Refuge Complex: baseline inventories and implications for animal health.

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Title: *Documenting freshwater snail and trematode parasite diversity in the Wheeler Refuge Complex: baseline inventories and implications for animal health.*

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ABSTRACT

The Wheeler National Wildlife Refuge (NWR) Complex includes: Wheeler, Sauta Cave, Fern Cave, Mountain Longleaf, Cahaba, and Watercress Darter Refuges that provide freshwater habitat for many rare, endangered, endemic, or migratory species of animals. To date, no systematic, baseline surveys of freshwater snails have been conducted in these refuges. Documenting the diversity of freshwater snails in this complex is important as many snails are the primary intermediate hosts of flatworm parasites (Trematoda: Digenea), whose infection in subsequent aquatic and terrestrial vertebrates may lead to their impaired health. In Fall 2015 and Summer 2016, snails were collected from a variety of aquatic habitats at all Refuges, except at Mountain Longleaf and Cahaba Refuges. All collected snails were transported live to the lab where they were identified to species and dissected to determine parasite presence. Trematode parasites infecting snails in the refuges were identified to the lowest taxonomic level by sequencing the DNA barcoding gene, 18s rDNA. Gene sequences from Refuge parasites were matched with published sequences of identified trematodes accessioned in the NCBI GenBank database. In total, 1600 individuals of 13 snail species were collected of which 120 individuals from six species were infected with trematodes. Results of the GenBank sequence comparisons showed that 21 species in 14 families of trematodes were found in the Wheeler NWR Complex. Of these, eight trematode species were found in snail species not listed as hosts in published literature. Although all trematodes have been reported in the US, the majority have not been reported in Alabama. In addition, the continued monitoring of *Physella* and *Planorbella trivolvus* that harbor trematodes that infect waterfowl and *Pleurocera spp*. that harbor trematodes that infect bats is recommended. The results of this study show that baseline surveys of freshwater mollusks and their trematodes not only increases an understanding of biodiversity but also detects potential pathogens of concern for wildlife.

INTRODUCTION

The highest diversity of freshwater snails in North America is found in the southeastern United States. Freshwaters in Alabama are considered a global hotspot of freshwater snail

diversity as they that are home to approximately 300 of the 600 described species in North America (Bogan et al. 2006) of which many are highly endemic (Johnson et al. 2013). Despite this high diversity, many of these species are imperiled and 36 species are extinct (Neves et al. 1997, IUCN 2006, Johnson et al. 2013) due to habitat degradation from dams and altered land use and water pollution, primarily from mines and wastewater effluent. Currently, two freshwater snail species are listed as federally endangered and four species are federally threatened. The striking imperilment of this fauna in Alabama creates a critical need to inventory the distribution of these species in order to monitor changes in biodiversity patterns of these sensitive species. Interestingly, recent collection efforts of taxonomic specialists in the Cahaba River resulted in the resurrection of two species presumed to be extinct, Hydrobiidae: *Somatogyrus cahawbensis* and Pleuroceridae: *Leptoxis compacta* (Whelan et al. 2012). Thus, categorizing the diversity of freshwater mollusks in Alabama, and across the southeast, is still very much in need of detailed survey work to create baseline biotic inventories.

Creating baseline biotic inventories of freshwater snails not only reflects more accurate distributions and diversity of native snails in the state, but it also aids in monitoring for the arrival of invasive species. The invasion of surface of waters by non-native species is a high priority to agencies involved with the conservation and protection of native wildlife. Yet, extensive surveys to document the extent of these invasions in most streams in Alabama have not occurred. Currently, two invasive species of snails that are popular in the aquarium trade are documented in Alabama. The Chinese mystery snail (*Cipanopaludina chinensis*) was reported from Smith Lake in Winston County in 2012, and the channeled apple snail (*Pomacea canaliculatum*) invaded Three Mile Creek in Baldwin County in the 1990s. As many of the introductions and successful establishment of freshwater invasive species is a result of aquaria release (Francis and Chadwick 2012) it is anticipated that more invasive snails will enter the state. This is of particular concern as invasive snails can negatively affect water quality, community composition and biomass of native fauna (Strayer and Dudgeon 2010).

Whatever their origin, freshwater snails are known to host a wide variety of parasitic flatworms known as digenean trematodes (Trematoda: Digenea). Parasite-host relationships are very complex as digenetic trematodes have multiphasic life cycles requiring separate host species for each stage of development (Esch and Fernandez 1994). Trematode life cycles start with adult worms which generally live in the intestinal tracts of terrestrial vertebrates (i.e., the definitive host) where eggs are produced and defecated into water bodies. Eggs hatch into miricidia which are consumed or enter into the mantle cavity of snails - the first intermediate host. Inside snails miricidia transform into redia that asexually produce thousands of larvae in the form of free swimming cercaria. Depending on the trematode species, cercaria transform and encyt into a metacercaria on aquatic substrates (e.g. macrophytes) or infect aquatic invertebrates or vertebrates known as second intermediate hosts. These second intermediate hosts are then ingested by the definitive host and the life cycle of the worms continue (Figure 1). Interestingly, trematode parasites will only persist in ecosystems if the snail hosts are present. The asexual reproductive stage found in the snail that produces larval worms in the form of motile cercaria is required to obtain sufficient numbers of individuals to infect other stages of the life cycle (Anderson and May 1979). As some trematode species are host specific to a single species of snail host (Wright 1973, Cribb et al. 2001), the potential diversity of these parasites in freshwater snails in Alabama may be enormous. Thus, investigations of this group should be included to better understand Alabama Diversity.

Another critical reason to understand the diversity of trematode parasites in Alabama

rivers is that trematodes are parasitic on each stage of the lifecycle. Thus, these parasites can impair the health of their vertebrate hosts. Yet, research on the health effects of trematode parasites in North America on wildlife is far less studied (except in fishes, see Hoffman 1999 and those species in livestock discussed in veterinary parasitology texts such as Soulsby 1965) than in Southeast Asia as these pathogens infect not only wildlife but humans that ingest undercooked or raw intermediate hosts (Vandemark et al. 2010). Thus, determining what trematode pathogens are carried by snails in Alabama freshwaters is necessary to evaluate potential disease threats to wildlife, particularly threatened and endangered fauna such as those that occur in the Wheeler National Wildlife Refuge Complex.

Determining if wildlife found in the Wheeler NWR complex are infected with parasites that can cause serious health concerns to vertebrate hosts is difficult as these animals must be sacrificed so that the worms can be removed and examined. However, the snail hosts can be sacrificed to remove cercaria that are used for parasite identification. Although taxonomic keys are generally not available for identifying these parasites during the snail stage, with the availability of molecular techniques, these parasites can be identified to lowest taxonomic level. Then, using published literature that describes the lifecycles of the parasites, the vertebrate hosts that are susceptible to particularly pathogenic Trematodes can be determined. Using this information, refuge managers can determine what strategies are most suitable for monitoring and eliminating disease spread.

Documenting freshwater snail and trematode diversity in the Wheeler Refuge Complex was divided into three objectives: 1) Create a baseline inventory of freshwater snails for the Wheeler Refuge Complex, 2) Identify trematode parasites associated with freshwater snails to evaluate potential disease threats to wildlife utilizing refuges within the Wheeler Refuge and 3) Identify target areas that contain at-risk, threatened, or endangered species to consider for long-term monitoring for pathogens and invasive species Complex.

STUDY AREA

The Wheeler National Wildlife Refuge (NWR) Complex occupies about 17,000 hectares among the seven refuges that are a part of the complex (Figure 2). These lands are home to numerous threatened and endangered species such as federally endangered species including the Watercress Darter (*Etheostoma nuchale*) at the Watercress Darter NWR, the Whooping Crane (*Grus Americana*) at Wheeler NWR, the round rock snail (*Leptoxis compacta*) at the Cahaba NWR, large populations of bats (*Mytilus spp.)* at Fern Cave, Sauta Cave, and Key Cave NWR. In addition, Key Cave is home to the federally endangered Alabama cavefish (*Speoplatyrhinus poulsoni*) and two species of endangered crayfish: *Procambarus pecki* and *Cambarus jonesi*.

Surveys of freshwater snails and their parasites took place in water bodies within or proximate to Refuges within the Wheeler NWR complex (Figure 2). The Wheeler NWR Complex includes: Wheeler NWR near Decatur, AL, Key Cave NWR, near Muscle Shoals, AL, Fern Cave near Paint Rock, AL, Sauta Cave near Scottsboro, AL, Cahaba River NWR near West Blocton, Al, an Moutain Longleaf NWR near Anniston, AL. The Cahaba River NWR was excluded from this study as previous extensive surveys of freshwater gastropods in the Refuge area have already been conducted (Bogan and Pierson 1993, Tolley-Jordan 2008, Whelan et al. 2012). Key Cave NWR was also excluded as potential disruption of the bat populations and endangered freshwater fauna in Key Cave prevented surveying here.

In July and August 2015 a site selection survey was conducted throughout the ~14,500

hectare Wheeler NWR in Decatur, AL that borders the Tennessee River. Twenty-five sites encompassing streams, springs, sloughs, dikes, and a swamp (Figure 3a) were evaluated based on access, initial assessments of snail presence, and provided a representation of the types of aquatic habitats found on the refuge (spring, stream, river, slough, dike, swamp). From these sites, 13 were selected for this study (Figure 3b). Cave Spring is a spring-fed pool and it represented the spring habitat. Ginhouse Branch and Black Branch represented the small stream habitats. Beaverdam Creek, Piney Creek, and Flint Creek sites represented river habitats as they are large, mainstem tributaries into the Tennessee River. Arrowhead landing, Garth Slough Banding Site, Dinsmore Slough 1 and 2 represented backwater slough habitats. White Springs and Harris-Sweetwater Control valves represented the Dike (canal) habitats, and Blackwell Swamp represented the swamp habitat (Figure 4). It was important to sample a variety of habitats, particularly the sloughs and dikes as the water levels in these systems change drastically twice a year. In the spring water levels are high as parts of the refuge are flooded for waterfowl use. Conversely, in the summer and fall the water levels are lowered to allow row-crop farming on much of the refuge.

Watercress Darter NWR is a much smaller refuge (~ 1 ha) with two main aquatic habitats, a spring-fed pool where the watercress darter is found and a small stream that transects the property. At Fern Cave NWR (80.5 ha), a small, unnamed spring-fed tributary to the Paint Rock River was sampled along with the main stem of the Paint Rock that was adjacent to the property, and at Sauta Cave NWR (106 ha), the spring-fed Sauty Creek that runs along the outer edge of the Refuge was sampled for snails (Figures 1 and 4).

METHODS

Creating a baseline inventory of freshwater snails for the Wheeler Refuge Complex.

Only the habitat edges (wading into the water until hip deep) were sampled at the majority of locations, except for the small streams (Ginhouse Branch, Black Branch, Sauty Creek, and un-named stream at Watercress Darter NWR) that could be easily waded. All sites were sampled haphazardly using dipnets or hand collection for at least 1 hour or if 100 individuals of a species were obtained in less time. At Wheeler NWR, all sites were sampled at least once in Fall (September through November) 2015 and Summer (June and July) except Dinsmore Sloughs 1 and 2, Harris-Sweetwater Dike, Beaverdam and Piney Creeks that were sampled only in Summer 2016. At Watercress Darter NWR, both the pond and the stream were sampled in September and November 2015, and June 2016. Fern Cave NWR and Sauta Cave NWR were each visited once in June 2016. All snails collected from each location were transported live, in aerated water from the habitat where they were collected, to Jacksonville State University. Here, snails were identified to the lowest taxonomic level (usually species), according to Burch and Tottenham (1980) using nomenclature according to Johnson et al. (2013). Ranges of snail species were confirmed according to Burch and Tottenham (1980) and Mirarchi (2004). Identifications of representative snails from each location were verified by Jeff Garner, Mussel Management Supervisor, Division of Wildlife and Fisheries, Alabama Department of Conservation and Natural Resources. Individuals of each species from each location were vouchered at the Auburn Museum of Natural History (AUMNH).

Identification of Trematode parasites associated with freshwater snails to evaluate potential disease threats to wildlife utilizing refuges within the Wheeler Refuge Complex.

In general, the goal was to select at least 50 individuals of each snail species from each location. Tolley-Jordan (unpubl. Data) has found that a false negative for parasite presence in a snail population can occur if if less than 50 individual snails are collected. Parasite presence was detected in a live snail by removing the gonads, smearing the tissues onto a microscope slide, and viewing the smear at 4x or 10x magnification with a compound light microscope. Trematode presence is visually evident as the rediae and cercariae generally move and contrast sharply with the snail tissues. All snail tissues found to be positive for trematode presence were preserved in 95% molecular grade ethanol for subsequent molecular work.

In order to extract DNA from the trematodes, each sample was centrifuged to form a pellet. Ethanol was decanted and the pellet was used in all molecular procedures. DNA extraction was completed using the manufacturer's protocol for the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Quantity of DNA was measured using a nanodrop spectrophotometer, and dilutions were made using sterile deionized water to ensure that the DNA concentration was less than 100 ng / µl. We targeted an 800 bp region of the 18S rDNA gene using the forward primer 5'-ATGGCTCATTAAATCAGCTAT-3' and the reverse primer 5'-TGCTTTGAGCACTCAAATTTG-3' developed by Routtu et al. (2014). Polymerase chain reaction (PCR) was carried out in 25µL volumes using 12.5 µL of BioMix Red (Bioline, Taunton, MA), 2.5 µl of forward primer (10mM), 2.5 µL of the reverse primer (10mM), 5µl of sterile deionized water, and 5 µl of DNA. The PCR cycling conditions were the following: denaturing at 94^{o}C for 2 minutes, followed by 30 cycles of denaturation at 94^{o}C for 15S, annealing at 60^{o}C for 15S, and elongation at 72^{o}C for 15s, followed by a final elongation step of 5 minutes at 72^{o}C. PCR amplicons were run on a 1% agarose gels stained with SYBR Green (ThermoFisher Scientific). Successful PCR amplicons were cleaned-up using ExoSAPIT (ThermoFisher Scientific) following the manufacturer's protocol. DNA sequencing was completed at Molecular Cloning Laboratories (MCLAB; South San Francisco, CA). Consensus sequences were created using the computer program Geneious v. 7.1 (Biomatters Limited) and aligned using BioEdit (URL: http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

In order to molecularly identify each trematode based on the 18S rDNA barcoding sequence, we used the Basic Local Alignment Search Tool (BLAST) to obtain the best matched sequence. Each parasite was classified to the species-level at a 99%-100% sequence match, to the genus-level at a 98.9%-97% sequence match, and to the family level at a 95%-97% sequence match (Appendix 1).

Once parasites were identified, comparisons of snail hosts reported in published literature, primarily from Schell (1985) were compared against the parasite snail hosts in this study. As snails from this region are rarely evaluated for parasites, and snail assemblages at the genus and species level tend to display a remarkable degree of endemism in Alabama, only family level comparisons were used as all snail families found in Alabama are represented throughout the US (Burch and Tottenham 1980).

Identification of target areas that contain at-risk, threatened, or endangered species to consider for long-term monitoring for pathogens and invasive species.

Areas of greatest concern for parasite pathogens at Wheeler NWR are those that have

high diversity and densities of bird use. These areas were determined prior to sampling with assistance of Wheeler NWR refuge staff. High Bird Use areas were designated according to the presence of a large number of resident and migratory birds, where as Low Bird Use were areas not used by as many resident and migratory wading birds (Figure 6). These designations were used to determine if specific pathogens that harm waterfowl are present in high waterfowl use areas. In addition, while sampling of Blackwell Swamp in Fall 2015, a population of federally threatened spring pygmy sunfish (*Elasssoma alabamae*) was discovered making this swamp a priority habitat for identifying potential trematode pathogens that can impair fish health. In addition, surveying the 13 sites at Wheeler NWR allowed for baseline data to determine if any invasive snails were present and/or if invasive Trematode pathogens are present in Wheeler NWR. Pathogens or invasive species that could affect bat populations were deemed a priority at Fern and Sauta Caves, while at Watercress Darter NWR, determining the presence of trematode pathogens that could reduce fitness in the endangered Watercress darter (*Etheostoma nuchale*) was a priority. As with Wheeler NWR, these refuges were also surveyed to evaluate if any non-native snails and/or Trematodes had invaded these systems.

Based on the vertebrate hosts recorded in published literature, habitats with snails infected by trematodes that were pathogens of target taxa (waterfowl, federally endangered or threatened fishes, or bats) were considered areas to be monitored in future surveys. These monitoring efforts would focus on changes in percent infection of a pathogen parasite in high risk areas to alert Refuge staff to monitor target taxa for signs of illness.

RESULTS

Creating a baseline inventory of freshwater snails for the Wheeler Refuge Complex.

The greatest densities and diversities of snails were sampled in Wheeler NWR. The greatest snail diversity occurred at Arrowhead Landing (which is at the confluence of Limestone, Piney, and Beaverdam Creeks in Wheeler NWR) with 7 species. All other sites among the Refuges had between 1 and 3 species of snails (Tables 1&2).

Although the goal was to collect at least 50 individuals of each species, few individuals of pond snails were collected at Harris Sweetwater Dike and Blackwell Swamp at Wheeler, and in the ponds at Watercress Darter NWR, despite lengthy sampling efforts. Part of this was due to access as these swampy habitats were difficult to wade on the margins. Secondly, the snails were not abundant along the margins as compared to other sites. In addition, few individuals of Viviparidae were collected were collected at Wheeler NWR as they were only found in the fall when the sloughs were drained and large mud flats leading to creek channels were exposed (Table 1).

All snails found in this study are native to Alabama (Burch and Tottenham 1980) and can be described as three main groups: Riverine, Pond, and Semi-Aquatic. Riverine snails have gills (Gastropoda: Cerithoidea) that tend to be restricted to stream, river, or spring environments (Burch and Tottenham 1980). Riverine snails found in our study included species in Pleuroceridae, Hydrobiidae, and Viviparidae all of which are native and endemic to Alabama. The distribution of these riverine species showed a large separation in species found in the Tennessee drainage as compared to those found in the Black Warrior Basin (Watercress Darter NWR- (Table 1). Pond snails (Gastropoda: Bassomotophora) have lungs and are often found

around pond and stream margins as well as in temporary pools. The pond snails found in this study included four species in Lymnaeidae, Physidae, and Planorbidae. Pond snail species found in this survey are widespread throughout the eastern US (Burch and Tottenham 1980). Only a few individuals of the semi-aquatic snail species *Potamiopsis lapidaria* (Potamiopsidae) were recovered at the spring fed stream the on moss margins at Fern Cave NWR (Table 1). These snails were not dissected as all were preserved as voucher specimens.

Identification of trematode parasites associated with freshwater snails to evaluate potential disease threats to wildlife utilizing refuges within the Wheeler Refuge Complex.

Approximately 1,600 individuals of the ten aquatic snail species were dissected. Of these, 120 individuals were infected with trematodes. In addition to having the highest diversity of snails, Arrowhead Landing at Wheeler NWR had the highest diversity of snails infected with Trematode Parasites (Table2). For sites that had infected snails, percent infection, regardless of snail species, ranged between 8% (Cave Spring) to 54% (pond at Watercress Darter NWR) (Table 3).

In general, despite locality, the pond snails *Planorbella trivolvus* and *Physella sp*. had at least one infected individual (Table 1). Despite the large numbers of individuals of *Elimia bellacrenata* and *E. carinifera* dissected at Watercress Darter NWR no snails infected with trematodes were found. However, these snails were heavily infected with unknown Nematodes. Nematodes were also common in other Pleurocerids collected at all refuges. No other snail families were infected with Nematodes. Another worm found living within all groups of snails collected from Wheeler in the fall sampling period were Annelids known as *Chaetogaster sp.* (Oligochaeta: Naididae).

Ninety Trematode samples from the 120 infected snails were successfully sequenced while nine samples could not be sequenced for unknown reasons. Thus, trematode species identifications Blackwell Swamp, Beaverdam Creek, trematodes infecting *Pseudosuccinea sp*. in White Springs Dike and Harris-Sweetwater Dike are unknown. In all, 22 species in 14 families of Trematodes were found within 11 snail species in the Wheeler NWR Complex (Table 3). Trematode diversity was markedly higher in the pond snails than in the riverine snails. *Planorbella trivolvus* and *Physella sp* were each infected by 7 trematode species (Table 4). Species composition between riverine and pond snails were markedly different. Collyriclidae, Heterophyidae, Microphalloidea (Superfamily level), Opisthorchiidae, Pronocephalidae infected only riverine snails while Cephalagonimidae, Diplostomatidae, Echinostomatidae, Paramphistomatidae, Haemoatoloechidae, Macroderoididae, and Telorchiidae infected only pond snails. Only one family, Spirochiidae was found in both pond snails (Planorbidae, Physidae), and riverine Snails (Pleuroceridae).

In total, novel snail hosts were reported in eight species of trematodes found in this study. In general, novel snail hosts were in the same order as the known host snails (e.g. Bassomotophora -Pond snails or Cerithoidea-Riverine Snails) with the exception of the . However, parasites in the family Spirochiidae (Blood flukes) are reported only in pond snails according to Schell (1985) but were found in *Pleurocera striata* in this study. Additionally, parasites in Microphalloidea are generally reported in marine Cerithoideans (Schell 1985). In this study, an unknown species of Microphalloidea was reported in two species of Pleuroceridae at two different locations in Wheeler NWR (Table 3).

Identification of target areas that contain at-risk, threatened, or endangered species to consider for long-term monitoring for pathogens and invasive species.

Three parasite families were found to be potential pathogens of concern for the Wheeler NWR complex (Table 5). Diplostomatidae: *Posthodiplostomum sp*. and Echinostomatidae: *Echinostoma sp*. infects the intestinal tracts of waterfowl while *Lecithodendriidae spp.* generally infects intestinal tracts of bats.

DISCUSSION

Creating a baseline inventory of freshwater snails for the Wheeler Refuge Complex.

Although there is a concensus among researchers that the highest diversities of many groups of mollusks are found in Albama, very few recent distribution records are published native snails (but see Burch and Tottenham1980, Bogan and Pierson 1993a, Bogan and Pierson 1993b, Tolley-Jordan 2008, Whelan et al. 2012). Given the high degree of endemism by many of these groups, particularly within the Pleuroceridae and Hydrobiidae (Brown et al. 2008, Lysne et al. 2008), published detailed locality information on these taxa is critical for future conservation efforts. In addition, documenting habitats where endemic snails are found is necessary as these organisms likely have a very narrow niche breadth and are particularly susceptible to changes in habitat and water quality (Crowl and Schnell 1990). As the Wheeler NWR complex has a wide range of habitats (large tributaries, small creeks, backwater sloughs, swamps, springs, and dikes) snail diversity will vary according to the unique conditions of each habitat type. This is particularly important for the Hydrobiids and Pleurocerids that display a high degree of endemism and are at much greater risk of extirpation than wide-spread pond snails due to habitat loss (Brown et al. 2008). Our study showed that the *Marstonia arga* (Hydrobiidae) and *Pleurocera pyrenella* (Pleuroceridae) are fairly widespread in refuges within the Tennessee Basin (Wheeler and Fern Cave) which corresponds to the ranges described in Burch and Tottenham (1980) and Mirarchi 2004. *Pleurocera striata* was restricted to only Cave Spring. This species is not listed in Burch and Tottenham (1980) or in Mirarchi (2004) and is listed only in (Tryon 1873). In addition, *Elimia bellacrenata* is a rare endemic in the Cahaba Basin (Mirarchi 2004) and the documentation of this snail in the WatercressDarter NWR refuge can be an important finding for conserving this snail. Small, isolated populations of spring endemics are particularly at risk of extirpation from small scale habitat alterations (e.g. bulldozing an area for road expansion). As the likelihood of habitat destruction springs on Refuge property is low, *E. bellacrenata* and *P. striata* populations will persist.

Although the results of this survey clarify specific habitat use and distributions of freshwater snails to better understand molluscan diversity, future amendments to these records will be necessary. Hydrobiids and pleurocerids are exceptionally difficult to identify and the true taxonomic status of many species within this groups are uncertain (Brown et al. 2008). There is currently an ongoing, concerted effort by state, national and international malocologists to systematically review and update the taxonomic status of pleurocerids by incorporating phylogenetics and morphological traits. Yet, this is a complex task (Whelan and Strong 2015) and will be ongoing for the next several years. It is anticipated that the generic names of many groups in the Tennessee and Mobile basins will change so that an update on the distribution of snails in the Wheeler NWR complex will be required. Hydrobiid taxonomy is also in flux and currently named taxa are subject to future change. Also, although this survey systematically

surveyed refuges to document snail diversity it is possible that pond snails such as those found in Lymnaeidae, Physidae, and Planorbidae are found at all locations but are not present at all locations during the entire year. In general, pond snails have many life-history adaptations that allow them to thrive in temporary aquatic habitats (Brown 1979) which change as water levels are managed for waterfowl use and row crops at Wheeler NWR and may result in dynamic parasite diversity and distributions in this refuge.

In addition to documenting native species, another goal of this research was to document the occurrence of invasive snails. More aquatic snails have been introduced into the southeastern US than any other group of animals (Fuller et al. 2012) so there is a strong likelihood of future snail invasions into the Wheeler NWR complex. Although no invasive snails were found during this research, it is likely that snails not originally found in the Tennessee Basin in North Alabama will move into Refuge properties, albeit these invasive snails may be native to other regions of the US. For instance, *Viviparus georgianus* and *V. subpurpurea*, snails that are native to the Tennessee and Mobile Basins, and were found in the Wheeler Refuge during this study, have invaded lakes North Carolina (Fuller et al. 2012). Thus, continued monitoring of the different habitats within the Refuges is recommended as it is likely that snail invasions will begin in isolated patches. Once invasive snails are established, eradication is generally not successful. Monitoring and preventing establishment of an invasive species is the most effective way to deter successful invasions (Strayer et al. 2010).

Identification of trematode parasites associated with freshwater snails to evaluate potential disease threats to wildlife utilizing refuges within the Wheeler Refuge Complex.

Results from this study showed that no specific habitat conditions were more likely to promote infection than others which suggests that trematode diversity and densities were most dependent on host snail densities and distributions. A high parasite diversity was found in the study as 22 species of trematodes were found in 11 snail species. Although the purpose of this study was to document trematodes as potential pathogens of wildlife in the refuges, healthy ecosystems are often ones that have a high diversity of parasites. These parasites often strongly affect the dynamics of host population size which, in turn, effects other interactions within the ecosystem (Hudson et al. 2006). Previous research has shown that ecosystems with high bird biodiversity are correlated with high trematode biodiversity (Huspeni and Lafferty 2004, Hechinger and Lafferty 2005). About 300 species of birds have been observed on Refuge property, including 35 species of waterfowl, 11 species of herons, and 1 cormorant (https://www.fws.gov/wheeler/observation/BirdsofWheelerNWR) that all serve as hosts to a wide variety of trematode parasites given the strong association of these birds with water where snails occur. Although this study focused on targeting habitats with known high bird use versus those with low bird use, parasite diversity in Wheeler NWR was highest in Arrowhead landing (10 species of trematodes), which was designated as low bird use. However, this site also had the highest diversity of snails that harbor a wide diversity of parasites. In addition, these parasite lifecycles may persist as many of the parasite species found in this study have definitive hosts that are amphibians, reptiles and/or mammals which may be abundant at Arrowhead Landing. In general, high bird use sites in Wheeler NWR are those that are primarily dominated by pond snails. Given that pond snails are very patchy in distribution, with fast life-cycles (Brown 1979), the opportunity for these snails to harbor a wide diversity of parasites that have adapted to the shorter life spans of these snails also allows these parasite communities to be very dynamic

through time (Gerard et al. 2008). Thus, a targeted, repetitive sampling strategy is necessary to capture these dynamics.

The general lack overlap in trematode species diversity between riverine (Cerithoidea) and pond (Bassomotophora) snails occurred suggests that some level of host specificity occurs within these parasites. Host specificity varies highly among parasite groups (Cribb et al. 2001), and is due, in part, to the evolutionary relationships of trematodes with their snail hosts. A review of the lifecycles of trematodes found in this study based on Schell (1985) showed that eight species of trematodes were not documented in the snail hosts that were found in this study. This result may be due, in part, to the paucity of research on snail hosts of trematodes as identification of worms in this stage is difficult as compared to identification of adult worms. In fact, the majority of worm sequences in GenBank came from adult worms found in definitive, vertebrate hosts. In general, the snail hosts in the literature corresponded to this study at a coarse scale (pond snail vs. riverine snail) with the exception of a species of Spirochiidae that was found in *P. striata* along with known pond snail hosts. Further investigation of this species determination using additional molecular markers will be necessary to verify this finding. In addition, additional molecular markers and the generation of phylogenetic relationships will further clarify if trematode families that have multiple, unknown species are genetically divergent. Another interesting result of this study is the identification of parasite *Collyriclum sp*. This species is the dominant parasite species found in *P. pyrenella* in the Paint Rock River and has also been found in pleurocerid snails in other river basins in Alabama (Tolley-Jordan, pers. comm.). *Collyriclum* is a relatively common endoparasite of song birds whose lifecycle is poorly understood in North America and the snail host is unknown (Blankenspoor et al. 1981). In Slovakia this parasite is reported in a Hydrobiidae snail (Heneberg 2015), but to our knowledge this is the only record of a snail intermediate host. The identification of an intermediate host for this parasite gives a greater understanding of the lifecycle of this parasite in North America.

There were no exotic trematodes found in this study. It is unlikely that exotic trematodes will be permanent residents unless there are established invasive snails in this system. To date, no native snails have been recorded harboring invasive trematodes. Yet, continued efforts to monitor for invasive snails in the different habitats identified in this study (riverine, creek, spring, swamp, dikes, and sloughs) are necessary to prevent the arrival of invasive pathogenic trematodes into the Wheeler NWR complex.

Identification of target areas that contain at-risk, threatened, or endangered species to consider for long-term monitoring for pathogens and invasive species.

A review of published literature, primarily in Schell (1985), described the life-cycles of the parasites found in this study (Table 5). Based on these described hosts, three parasite families were found to be potential pathogens of concern for the Wheeler NWR complex. Diplostomatidae: *Posthodiplostomum sp*. and Echinostomatidae: *Echinostoma sp*. infects the intestinal tracts of waterfowl. However, only two High Bird Use sites were infected with *Posthodiplostomum* and no High Use sites were infected with *Echinostoma*. In general, pathogenic effects of these infections are generally density dependent and may only be problematic to vertebrate hosts during seasons with high host snail densities and habitat conditions that facilitate increased densities of infected snails. Thus, continued monitoring of bird health by refuge staff and bird enthusiasts will be an effective way to determine if any pathogenic issues are occurring. Lecithodendriidae spp., that infect bats, are only carried by

pleurcerids and these snails do not occur at Sauta Cave so bats at this site should not be at risk of impaired health from these parasites. Also no fish pathogens were found at Watercress Darter NWR during this survey. However, only a few pond snails were collected and additional surveys may yield other pathogens to the watercress darter. Parasites in the family Heterophyidae and Macroderioididae are known to be pathogenic to many fishes. In general, heterophyids are only found in riverine snails which were not infected with trematodes in this study. Also, Macroderioididae occur in *P. trivovlus* which were not collected at Watercress Darter NWR. Based on the results of this study, the parasites found in the Watercress Darter NWR seem to be a natural component of these ecosystems and no control efforts are recommended at this time.

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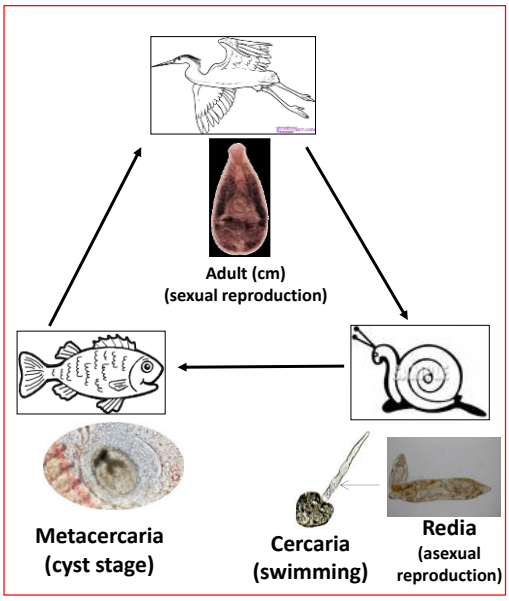

Figure 1. Generalized life cycle of a Digenetic Trematode (Trematoda: Digenea). Note the definitive host is a terrestrial vertebrate while the first intermediate host (snail) and second intermediate host (other invertebrates or vertebrates) are aquatic.

Figure 2. The Wheeler National Wildlife Refuges Complex including: Key Cave, Wheeler, Fern Cave, Sauta Cave, Mountain Longleaf, Watercress Darter, and Cahaba River Refuges in Alabama, USA.

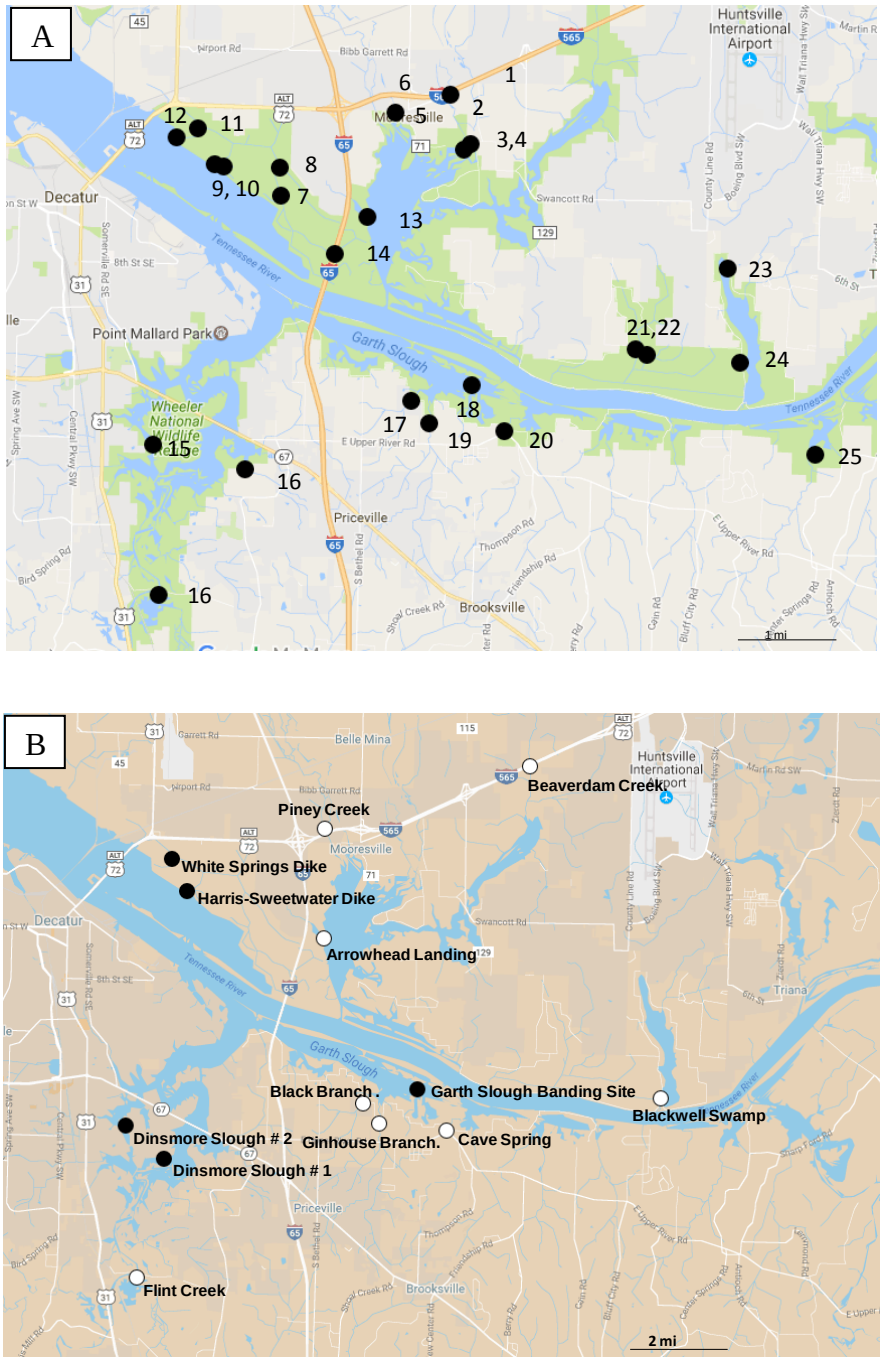

Figure 3. Panel A shows the 25 sites surveyed in Summer 2015 in aquatic habitats at Wheeler NWR and panel B shows the 13 sites sampled in Fall 2015 and Summer 2016. Open circles in panel B indicate sites with low bird use and closed circles indicate sites with high bird use.

Cave Spring

Beaverdam Creek

Dinsmore Slough 1 & 2

Black Branch

PineyCreek

Harris-Sweetwater Dike

Ginhouse Branch

Arrowhead Landing

White Springs Dike

Flint Creek

Garth Slough Banding Site

Blackwell Swamp

Sauta Cave NWR

Fern Cave NWR

Watercress Darter NWR

Figure 4. Photos of the sites sampled in Wheeler, Sauta Cave, Fern Cave, and Watercress Darter National Wildlife Refuges.

Table 1. The total number of individuals per snail species dissected at each site in the 2015-2016 survey. Sites 1-13 correspond to Wheeler NWR and sites 14-18 correspond to other Refuges surveyed in the Wheeler Complex. Bold, underlined values indicate snail species infected with Trematode parasites while plain fonts indicate no infections. * represent individuals that were collected but not dissected.

Locations	Pleuroceridae					Hydrobiidae	Viviparidae		Planorbidae		Lymnaeidae	Physidae	Potamiopsidae
		Pleurocera		*Elimia*		*Marstonia*	*Viviparus*	*Viviparus*	*Micromenetus*	*Planorbella*	*Pseudosuccinea*	*Physella*	*Potamiopsis*
	striata	*excurata*	*pyrenella*	*carinifera*	*bellacrenata*	*arga*	*georgianus*	*subpurpurea*	*dilitata*	*trivolvis*	*sp*	*sp*	*lapidaria*
1 Cave Springs	**146**					50						**40**	
2 Beaverdam Creek			**40**										
3 Piney Creek			43										
4 Flint Creek			62			25*							
5 Black Branch										**63**		**50**	
6 Ginhouse Branch												**75**	
7 Arrowhead Landing		**64**				27	2		40	**37**	4	**75**	
8 Garth Slough Banding Site		18						**4**				**50**	
9 Dinsmore Slough 1										**15**		**34**	
10 Dinsmore Slough 2										**50**	**41**	7	
11 Blackwell Swamp									8	**4**		6	
12 Harris Sweetwater Dike										**2**		**9**	
13 White Springs Dike										**14**	**50**	**7**	
14 Pond @ WD NWR				50							**7**	**22**	
15 Creek @ WD NWR				175	120								
16 Sauty Creek @ SC NWR						20*						**36**	
17 Paint Rock River @ FC NWR			**50**										
18 Unnamed Spring @ FC NWR												50	4*

Table 2. Survey dates, Bird Use as designated by Wheeler NWR staff as areas highly used by waterfowl or less used by waterfowl) Snail Species Richness, Number of infected snails, and parasite richness of each of the 18 sites surveyed within the Wheeler NWR Complex. N/A refers to Not Applicable for bird use in Watercress Darter NWR, Sauta Cave NWR, and Fern Cave NWR which are not managed for waterfowl.

Summer 2016	Fall 2015	Bird Use		Sites	# Snail Species	# Infected Snail Species	Trematodes	
							Families	Genera
1	1	Low	1	Cave Springs	3	2	5	5
1		Low	2	Beaverdam Creek	1	1	?	?
1		Low	3	Piney Creek	1	0	0	0
1	1	Low	4	Flint Creek	1	0	0	0
1	1	Low	5	Black Branch	2	2	3	4
1	1	Low	6	Ginhouse branch	1	1	5	5
1	1	High	7	Garth Slough Banding Site	3	2	2	2
1	1	Low	8	Arrowhead Landing	7	4	10	10
1		High	9	Dinsmore Slough 1	2	1	2	2
1		High	10	Dinsmore Slough 2	3	2	3	3
1	1	Low	11	Blackwell Swamp	3	1	?	?
1		High	12	Harris Sweetwater Dike	2	2	1?	1?
1	1	High	13	White Springs Dike	3	2	2	2
1	1	N/A	14	Pond @ WD NWR	1	0	0	0
1	1	N/A	15	Creek @ WD NWR	3	1	1	2
1		N/A	16	Sauty Creek @ SC NWR	2	1	?	?
1		N/A	17	Paint Rock R. @ FC NWR	1	1	3	4
1		N/A	18	Unnamed Spring @ FC NWR	2	0	0	0

Table 3. The % infection (# infected individuals/total individuals) of each Trematode species per snail species from each of the 18 sites surveyed in the Wheeler NWR Complex in 2015-2016. Letters A-N correspond to Trematode families and species given in the legend below. Infected snail species are indicated as follows: PT - *Planorbella trivolvus*, PY-*Physella sp.*, PSS-*Pseudosuccinea sp.*, PS- *Pleurocera striata*, PYR-*P. pyrenella*, and VS- *Viviparus subpurpurea*. * indicate sites with infected snails but DNA could not be sequenced. ** indicate sites where no infected snails were found.

Sites	Trematode Species																														Total
	A		B	C		D1		D2	E	F	G1	G2	H1	H2		H3	I	J		K	L	M1	M2	M3			N1			N2	
	PT	PY	PYR	PY	PT	PT	PY	PT	PT	PY	PYR	VS	PY	PS	PYR	PYR	PT	PS	PYR	PYR	PS	PT	PS	PY	PSS	PT	PT	PSS	PY	PY	
1 Cave Springs										2%				1%				1%			4%		1%								8%
2 Beaverdam Creek *																															0%
3 Piney Creek																															0%
4 Flint Creek																															0%
5 Black Branch	19%	2%																						2%					6%	2%	31%
6 Ginhouse Branch				1%						1%			1%											1%					9%		15%
7 Garth Slough Banding Site				2%								29%																			31%
8 Arrowhead Landing	14%			3%			1%		3%		3%				2%		3%		3%			3%					3%		5%		42%
9 Dinsmore Slough 1	4%				20%																										24%
10 Dinsmore Slough 2						2%											16%								2%						20%
11 Blackwell Swamp*																										50%					50%
12 Harris- Sweetwater Dike																															0%
13 White Springs Dike*								7%																					25%		32%
14 Pond @ WD NWR																												33%	18%		52%
15 Creek @ WD NWR																															0%
16 Sauty Creek @ SC NWR																															0%
17 Paint Rock R. @ FC NWR			48%												2%	2%				2%											54%
18 Spring @ FC NWR																															0%

Trematode Species Legend

A Cephalogonimidae, *Cephalogonimus sp.*	F Haematoloechidae, *Unknown sp.*	I Macroderoididae, *Macroderoides typicus*	M1 Spirorchiidae, *Unknown sp. 1*
B Collyriclidae, *Collyriclum sp.*	G1 Heterophyidae, *Euryhelmis sp.*	J Microphalloidea,Unknown sp.	M2 Spirorchiidae, *Unknown sp. 2*
C Diplostomatidae, *Posthodiplostomum sp.*	G2 Heterophyidae, *Unknown sp.*	K Opisthorchiidae, *Unknown sp.*	M3 Spirorchiidae, *Unknown sp. 3*
D1 Echinostomatidae, *Echinostoma sp.*	H1 Lecithodendriidae, *Unknown sp. 1*	L Pronocephalidae, *Macrovestibulum sp.*	N1 Telorchiidae, *Telorchis sp.*
D2 Echinostomatidae, *Drepanocephalus spp.*	H2 Lecithodendriidae, *Unknown sp. 2*		N2 Telorchiidae, *Opisthioglyphe sp.*
E Paramphistomidae, *Paramphistomum*	H3 Lecithodendriidae, *Unknown sp. 3*		

Table 4. Trematode Family and Genus Richness for each snail species collected among all Refuges in the Wheeler NWR Complex in Fall 2015-Summer 2016. N/A indicates that no individuals of *P. lapidaria* were dissected.

Snail Species	Trematode Richness	
	Families	Genera
Riverine Snails	**10**	**12**
Pleuroceridae	8	10
Pleurocera		
striata	4	4
excurata	0	0
pyrenella	4	6
Elimia		
carinifera	0	0
bellacrenata	0	0
Hydrobiidae	0	0
Marstonia arga	0	0
Viviparidae	1	1
Viviparus		
georganius	0	0
subpurpurea	1	1
Pond Snails	**16**	**18**
Planorbidae	7	8
Micromenetus sp.	0	0
Planorbella trivolvus	7	8
Physidae	7	8
Physella sp.	7	8
Lymnaeidae	2	2
Pseudosuccinea sp.	2	2
Semi-Aquatic Snails		
Potamiopsidae	**N/A**	
Potamiopsis lapidaria		

Table 5. The known snail, secondary intermediate, definitive hosts, for each Trematode species according to Schell (1985). Parasites that are of concern to conservation efforts on the Refuge are those that correspond to highlighted hosts. Letters A-N correspond to Trematode families and species given in the legend below. Infected snail species are indicated as follows: PT - *Planorbella trivolvus*, PY-*Physella sp.*, PSS-*Pseudosuccinea sp.*, PS- *Pleurocera striata*, PYR-*P. pyrenella*, and VS- *Viviparus subpurpurea*.

Host-Trematode Relationship	**A**		B	C		D1		D2	E	F	G1	G2
	PT	PY	PYR	PY	PT	PT	PY	PT	PT	PY	PYR	VS
Trematode Native to US	Yes		Yes	Yes		Yes		Yes	Yes	Yes	Yes	Yes
Previously reported in Snail Family	Yes	No	***No***	***No***	Yes	Yes	Yes	Yes	***No***	Yes	Yes	Yes
2° Vertebrate Intermediate Hosts	Tadpoles		None	Sunfish		amphibians		Catfish	None	None	tadpoles	?
2° Inverterate Intermediate Hosts	Snails		Aquatic insect Larvae	None		snails		None	None	Dragonfly larva	None	?
Definitive Host (intestine)	Adult Frogs		Songbirds	**Herons**		**Ducks**		**Cormorants**	Ruminants	None	Mammals	?
Definitive Host (lung)	None		None	None		None		None	None	Frogs	None	?
Definitive Host (blood)	None		None	None		None		None	None	None	None	?

Host-Trematode Relationship	H1	H2		H3	I	J		K	L	M1	M2	M3			N1		N2
	PY	PS	PYR	PYR	PT	PS	PYR	PYR	PS	PT	PS	PY	PSS	PT	PT	PSS	PY	PY
Trematode Native to US	Yes				Yes	Yes		Yes	Yes	Yes					Yes			No
Previously reported in Snail Family	No	Yes	Yes	Yes	Yes	***?***	***?***	Yes	Yes	Yes	***No***	Yes	***No***	Yes	***No***	***No***	Yes	***No***
2° Vertebrate Intermediate Hosts	Variable				fish/tadpoles	None		None	Fish	Variable					Tadpoles			Tadpoles
2° Inverterate Intermediate Hosts	None				None	Crustaceans		Snails	None	Variable					None			None
Definitive Host (intestine)	**Bats**				Gar	Birds		Turtle	Turtle	Variable					Turtles			Mammals
Definitive Host (lung)	None				None	None		None	None	None					None			None
Definitive Host (blood)	None				None	None		None	None	Yes					None			None

Trematode Species Legend

A Cephalogonimidae, *Cephalogonimus sp.*	F Haematoloechidae, *Unknown sp.*	I Macroderoididae, *Macroderoides typicus*	M1 Spirorchiidae, *Unknown sp. 1*
B Collyriclidae, *Collyriclum sp.*	G1 Heterophyidae, *Euryhelmis sp.*	J Microphalloidea,Unknown sp.	M2 Spirorchiidae, *Unknown sp. 2*
C Diplostomatidae, *Posthodiplostomum sp.*	G2 Heterophyidae, *Unknown sp.*	K Opisthorchiidae, *Unknown sp.*	M3 Spirorchiidae, *Unknown sp. 3*
D1 Echinostomatidae, *Echinostoma sp.*	H1 Lecithodendriidae, *Unknown sp. 1*	L Pronocephalidae, *Macrovestibulum sp.*	N1 Telorchiidae, *Telorchis sp.*
D2 Echinostomatidae, *Drepanocephalus spp.*	H2 Lecithodendriidae, *Unknown sp. 2*		N2 Telorchiidae, *Opisthioglyphe sp.*
E Paramphistomidae, *Paramphistomum*	H3 Lecithodendriidae, *Unknown sp. 3*		

Appendix 1. Percent match of sequences from Trematodes collected in this study to Trematode sequences accessioned in the NCBI Genbank Database. Percent match values in the table are represented for each infected snail for each of the 18 sites sampled in the Wheeler NWR Complex in 2015-2016. Letters A-N correspond to Trematode families and species given in the legend below. Infected snail species are indicated as follows: PT - *Planorbella trivolvus*, PY-*Physella sp.*, PSS-*Pseudosuccinea sp.*, PS- *Pleurocera striata*, PYR-*P. pyrenella*, and VS- *Viviparus subpurpurea*. * indicate sites with infected snails but sequencing of DNA was unsuccessful.

Sites	Trematode Taxa																														
	A		B	C			D1		D2	E	F	G1	G2	H1	H2		H3	I	J		K	L	M1	M2	M3			N1		N2
	PT	PY	PYR	PY	PYR	PT	PT	PY	PT	PT	PY	PYR	VS	PY	PS	PYR	PYR	PT	PS	PYR	PYR	PS	PT	PS	PY	PSS	PT	PT	PY	PY
1 Cave Springs											95.6%				99.7%				96.1%			98.7%		98.2%						
2 Beaverdam Creek *																															
3 Piney Creek**																															
4 Flint Creek**																															
5 Black Branch**	98.3%	98.2%																							99.4%			97.9%		97.6%	
6 Ginhouse Branch**				99.3%							95.4%			99.4%											99.6%			97.9%			
7 Garth Slough Banding Site				98.4%									96.1%																		
8 Arrowhead Landing	98.4%			99.5%	98.8%			97.8%		99.5%		98.3%				99.3%		99.7%		95.9%			95.9%					98.4%	97.7%		
9 Dinsmore Slough 1				99.2%		99.4%																									
10 Dinsmore Slough 2							98.7%											99.7%								99.6%					
11 Blackwell Swamp*																															
12 Harris- Sweetwater Dike																											99.5%				
13 White Springs Dike									97.9%																				97.7%		
14 Pond @ WD NWR																												98.4%	98.1%		
15 Creek @ WD NWR**																															
16 Sauty Creek @ SC NWR																															
17 Paint Rock R. @ FC NWR																															
18 Unnamed Spring @ FC NWR**			97.0%													99.7%	99.3%				98.1%										

Trematode Species Legend

A Cephalogonimidae, *Cephalogonimus sp.*	F Haematoloechidae, *Unknown sp.*	I Macroderoididae, *Macroderoides typicus*	M1 Spirorchiidae, *Unknown sp. 1*
B Collyriclidae, *Collyriclum sp.*	G1 Heterophyidae, *Euryhelmis sp.*	J Microphalloidea,Unknown sp.	M2 Spirorchiidae, *Unknown sp. 2*
C Diplostomatidae, *Posthodiplostomum sp.*	G2 Heterophyidae, *Unknown sp.*	K Opisthorchiidae, *Unknown sp.*	M3 Spirorchiidae, *Unknown sp. 3*
D1 Echinostomatidae, *Echinostoma sp.*	H1 Lecithodendriidae, *Unknown sp. 1*	L Pronocephalidae, *Macrovestibulum sp.*	N1 Telorchiidae, *Telorchis sp.*
D2 Echinostomatidae, *Drepanocephalus spp.*	H2 Lecithodendriidae, *Unknown sp. 2*		N2 Telorchiidae, *Opisthioglyphe sp.*
E Paramphistomidae, *Paramphistomum*	H3 Lecithodendriidae, *Unknown sp. 3*		