THE DISTRIBUTION AND BIOLOGY OF THE INVASIVE EYEFLUKE OF WATERFOWL (*PHILOPHTHALMUS GRALLI*) IN TEXAS

by

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DEDICATION

I dedicated this work to the multitude of Veteran Service Members, past, present, and future, that find themselves looking into an uncertain future. May this provide assurance to any that find themselves facing an uncertain future that they can become something more than what they had planned when their plans for the future are forced to change.

ACKNOWLEDGEMENTS

I would like to thank the duck hunters of Texas for their cooperation with this endeavor. Without their willingness to participate in this work, the endeavor would not have even been possible. In particular I would like to thank the friends and family of Chris George, who shared not only their harvest but also their knowledge of waterfowl in Central Texas. I would also like to thank my family, and in particular my loving wife for their support and encouragement throughout this work.

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ABSTRACT

Philophthalmus gralli is an invasive trematode that primarily infects the conjunctival sac of waterfowl. Since its introduction in 1969, there has been little effort to determine the impacts of this parasite on the waterfowl of Texas. The purposes of this research were to explore how *P. gralli* may be impacting the eyes of Texas waterfowl, to study the COI and 18S genes for variation that may shed light on the invasion history of *P. gralli* in Texas, and to experimentally investigate the infection dynamics of the parasite to better understand its ecology. First, I investigated the current geographic distribution and infection status of the parasite in the eyes 1,362 hunter-harvested waterfowl wintering in Texas during the fall 2016 through winter 2018 hunting seasons, and rated the pathologies observed. I simultaneously studied COI and 18S sequences of some recovered worms for suggestions of invasion history. Then I investigated the substrate preferences and the behavior of the cercarial stage of *P. gralli* using multiple runs of a two-factor (substrate X depth) experimental design which included six substrate types, all placed at three depths within each of three replicate cylindrical glass aquaria. Lastly, I studied interactions between the intramolluscan larvae of *P. gralli* and those of co-infecting trematodes in the intermediate snail host. This experiment involved exposing wild-caught intermediate-host snails to miracidia of *P. gralli*. Experimental snails were divided into three groups: those shedding either of two invasive heterophyid cercariae, and those not shedding any cercariae. Each of the three groups was further subdivided into treatment and control subgroups, and snails in each treatment subgroup were

exposed to 5-10 *P. gralli* miracidia. After incubating for 120 d, the snails were crushed and examined for infection status. The distributional survey revealed *P. gralli* infections in ducks from every sampled location except the Panhandle counties, with a mean intensity of 5.34, prevalences ranging from 0% to 55%, and typical pathologies represented by mild irritation to conjunctivitis. The sequence analysis revealed an unexpectedly high variation, suggesting that the invasion history includes multiple introductions from several sources. The preferred substrate and depth for cercarial encystation were water stargrass at the surface. Hours of cercarial observations revealed two very different and novel behaviors not previously attributed to *P. gralli*. The co-infection study produced no experimental infections of *P. gralli*, but retrospective analysis revealed what may be a marked preference of specific *P. gralli* genotypes for specific genotypes of the snail host. This invasive parasite has now spread over much of Texas, and the pathologies revealed in the study suggest that the parasite is most likely impacting overwintering success of infected birds. The preference of water stargrass for encystment substrate by *P. gralli* cercariae indicates that many species of aquatic birds are at risk of infection. The infection dynamics are much more complicated than expected, and indicate that interactions between snail and parasite genotypes should be studied experimentally.

I. INTRODUCTION

Philophthalmus gralli

Taxonomy

Philophthalmus gralli (Mathis and Leger 1910) (Trematoda: Plagiiorchiida: Philophthalmidae: *Philophthalmus*, Looss 1899) was first described from the conjunctival sac of *Gallus gallus domesticus* in Vietnam (Mathis and Leger 1910). Several other species of *Philophthalmus* were subsequently described from East Asia, but most have now been synonymized with *P. gralli* (Nollen and Kanev 1995). The taxonomy of most of the genus *Philophthalmus* is still largely in disarray. One study in Peru has suggested, based on DNA analysis, that *P. gralli* is synonymous with *Philophthalmus lucipetus* Looss, 1899 (Literák et al. 2013); however, that assessment was challenged by other researchers based on life-cycle differences (Pinto and de Melo 2013). Since then, the GenBank sequences originally deposited by the Literák lab as *P. lucipetus* have been corrected to *P. gralli*.

Life Cycle

Unlike most trematodes, the lifecycles of philophthalmids do not have a second intermediate host. Adult worms reproduce sexually while living in the conjunctival sac of homeotherms, primarily birds (Alicata 1962, Díaz et al. 2002), but some mammals including humans are known hosts (Gutierrez et al. 1987). Eggs are shed directly into the surrounding water from the conjunctival sac when the definitive host submerges its head, and each egg quickly releases one miracidium which begins searching for a particular species of gastropod to serve as intermediate host (Alicata 1962, Díaz et al. 2002). The

miracidia have less than 6 h to find a suitable snail host (Alicata 1962). If the miracidium successfully locates a suitable snail, it will attach to the host's foot (Alicata 1962).

The miracidia of *Philophthalmus* spp. are unusual in that each contains within it a miniature, but fully formed, mother redia (Alicata 1962, Díaz et al. 2002). Upon attachment of the miracidium to the foot of the snail, the miracidium burrows into the skin of the snail and ejects the mother redia beneath the epidermis of the snail. The redia burrows through the tissues and migrates to the heart, where it attaches to the interior surface of the ventricle (Alicata 1962). After approximately 20 d post infection, the mother redia, still attached to the ventricle, contains developing daughter rediae (Alicata 1962, Díaz et al. 2002). Within 50 d post infection, daughter rediae are released from the mother redia into the lumen of the ventricle, enter the circulation, and eventually settle in the tissues of the digestive gland (Alicata 1962, Díaz et al. 2002). After 83-95 d post infection, megalurous cercariae emerge from the snail and begin swimming around in search of a suitable substrate on which to encyst (Alicata 1962, Díaz et al. 2002). Once a suitable substrate is located by a cercaria, it attaches by adhesive glands at the end of its tail and quickly begins to make a cyst around itself from material secreted from cystogenous glands in the body. Shortly after the cyst is formed, the tail detaches (Alicata and Noda 1960) and is eventually lost from the cyst. From this point on, the enclosed larva is usually referred to as a metacercaria, even though it does not undergo change into the next larval stage, as cercariae are generally expected to do when they encyst. The newly formed metacercarial cyst is flask shaped and is open at one end (Alicata 1962, Díaz et al. 2002), leaving the anterior end of the metacercaria exposed to the environment. After approximately 6 d post encystment, the majority of the metacercariae

will no longer be viable. However, some experimental metacercariae have been reported to be viable for up to 14 d post encystment (Nollen et al. 1985). If an encysted metacercaria is ingested by a homeothermic vertebrate while still viable, the sudden temperature increase that occurs when the host ingests the substrate stimulates the metacercaria to excyst (Nollen et al. 1985). Following excystment, the metacercaria enters the sinus cavity through the infundibular slit in the roof of the bird's mouth and begins migrating through the sinuses to the lacrimal ducts, and eventually settles in the conjunctival sacs (Alicata 1962, Díaz et al. 2002). The metacercaria then attaches to the proximal surface of the nictitating membrane (in the inner chamber of the conjunctival sac) for about 7 to 14 d until mature (Alicata 1962). When mature, the adults move to the outer chamber of the conjunctival sac, and may be found attached to the distal surface of the nictitating membrane or the proximal surface of the eyelid. Here the worms will mate or self-fertilize and release their eggs into the conjunctival chamber, thus completing the life cycle (Alicata 1962, Díaz et al. 2002).

The entire life cycle requires a minimum of approximately 120 d from egg to egg (Alicata 1962, Díaz et al. 2002), making it theoretically possible to complete three complete cycles per year under ideal conditions. Potentially, a snail infected with a *P. gralli* miracidium can shed cercariae for over 2 y (Heneberg et al. 2014), and an adult worm can remain gravid for at least 6 months post infection (Nollen and Kanev 1995). So, generational time for *P. gralli* has a theoretical minimum of 4 months, and a theoretical maximum exceeding 30 months.

Distribution in North America

Introduction and Dispersal in North America

The spread of *P. gralli* has been facilitated by the spread of its intermediate hosts; *Melanoides tuberculata* Müller, 1774 and *Tarebia granifera* Adams and Adams, 1854. Both thiarid snails are native to the tropics of Southeast Asia where *P. gralli* was first reported by Abbott (1952). Of these two snail species, *M. tuberculata* is the most frequently reported host for *P. gralli* in the Western Hemisphere (Murray 1964, Murray and Stewart 1968, Abbott 1973, Appleton and Nadasan 2002, Pinto and de Melo 2010, Krailas et al. 2014). It is unknown when *M. tuberculata* was first introduced into North America, but it was first reported at the San Antonio Zoo in 1964 (Murray 1964).

Periodical examination of both thiarids at the San Antonio Zoo (Bexar Co.) by the Murray lab revealed no trematode infections until 1968 (Murray and Stewart 1968), when a student worker discovered the intramolluscan larvae of what was later determined (Murray and Haines 1969, Nollen and Murray 1978) to be *Philophthalmus gralli* in 10 snails. A subsequent investigation revealed that birds that had been brought to the zoo from Hawaii had apparently carried the parasite in their eyes, and since the intermediate hosts were already established, *P. gralli* was able to become established at the zoo (Murray and Haines 1969, Nollen et al. 1985). Not long after the discovery of the parasite at the zoo, it was also discovered in *T. granifera* from the spring-fed Comal River in Comal County, Texas. (Kotrla 1975). It was speculated that the parasite was carried there prior to 1974 by birds donated to Landa Park (at the headsprings of the Comal River) by the San Antonio Zoo (Kotrla 1975). Both snail species had a relatively high prevalence of

P. gralli (up to 30%) in Bexar County (Murray and Haines 1969) and Comal County (Murray and Haines 1969, Kotrla 1975) of central Texas.

Philophthalmus gralli was reported in Florida in 1980 when captive-reared Ostriches (*Struthio camelus* Linnaeus, 1758) in a veld exhibit were found to be infected by the parasite (Greve and Harrison 1980). Because the infected birds had been reared in the exhibit and did not show symptoms for 5 months, and because other birds on the premises were negative, the initial introduction of the parasite there was probably from an unknown outside source. It is, however, unclear as to what that source was, and the authors did not report what snails were present at the exhibit.

Philophthalmus gralli and *M. tuberculata* have also been reported from a zoo in Phoenix, Arizona with no proposed mechanism for the introduction of the parasite (Church et al. 2013). Four Greater Rhea (*Rhea americana* Linnaeus, 1758) were found to be infected after being at the zoo after approximately 18 months. There had been no indications of infection at the end of the quarantine period (Church et al. 2013).

Philophthalmus gralli has also been reported from wild birds in North America. A comparative study of the parasite community of Blue-wing Teal (*Spatula discolor* Linnaeus, 1766) from different migratory pathways revealed infections in both Florida and East Texas (Garvon et al. 2011), while Gladden and Canaris (2009) reported the parasite from Buffleheads (*Bucephala albeola* Linnaeus, 1758) in West Texas. Since birds are the definitive hosts for this parasite and the intermediate hosts are successful invaders, it is possible that the parasite has spread across North America from a single introduction event, but multiple introduction events cannot be ruled out at this time.

Parasitism in the Definitive Host

Pathology

Reports on the effects of *P. gralli* on definitive hosts are highly varied. Manifestations of the infection range from asymptomatic, to excess lacrimation, to inflammation and blindness (Greve and Harrison 1980, Mukaratirwa et al. 2005, Rojas et al. 2013). (Díaz et al. 2002) reported few to no signs of pathology from experimentally infected chicks, even though two of the chicks had had 32 and 26 worms in their conjunctival sacs for 41 d. Reports of pathogenicity in captive Ostriches, Greater Rheas, and Great Tinamous (*Tinamus major* Gmelin, 1789) include blindness and eye irritation so severe that the eyes of some individuals were swollen shut (Greve and Harrison 1980, Church et al. 2013, Rojas et al. 2013). It is not clear what causes the extreme variation in signs. One explanation may be lengthy exposure time, but there are inconsistencies with this explanation since Díaz et al. (2002) reported only minor signs 41 d post infection. Another explanation may be intensity of infection, but there are some studies that report severe pathology when infection intensities are low (Alicata 1962).

Prevalence and Intensity

There have only been a few studies that examined the occurrence of *P. gralli* in wild populations. Studies in Florida examining parasite communities estimated 9% and 10% prevalence in Blue-winged Teal and Fulvous Whistling-Ducks (*Dendrocygna bicolor* Vieillot, 1816), respectively (Forrester et al. 1994, Garvon et al. 2011). In Brazil, prevalences of Brazilian Teal (*Amazonetta brasiliensis* Gmelin, 1789) and White-cheeked Pintail (*Anas bahamensis* Linnaeus, 1758) were estimated at 27.27% and 22.2%, respectively, with mean intensities of 8.3 and 3.25 (Muniz-Pereira and Amato 1993). In Texas, only two studies reported examining and discovering parasites from the eyes of

waterfowl. The first report was based on a parasite survey of Buffleheads in Hudspeth County, Texas, which estimated 7% prevalence (Gladden and Canaris 2009). In the second, researchers sampled Blue-wing Teal in East Texas and Louisiana and reported infection rates of 2.4% (Garvon et al. 2011).

The differences reported between prevalences of *P. gralli* in waterfowl populations from Texas and Florida vs. Brazil is possibly due to ambient water temperature. The thiarid intermediate hosts are both tropical species, and winter temperatures of surface-fed waterways in subtropical regions can sometimes fall below the reported lethal thermal minima for the snails (10C for *T. granifera* and 18C for *M. tuberculata*) (Chaniotis et al. 1980, Daniel et al. 2018). However, recent reports indicate that what appears to be genetic clones of *M. tuberculata* are able to survive through winter temperatures that remain well below the reported thermal minima for weeks at a time (Harding 2016). I have found wild-caught individuals of this same clone infected with *P. gralli*. Therefore, while the distribution of *M. tuberculata* in most of the continental US has been patchy due to it generally being restricted to thermally stable spring runs and powerplant cooling reservoirs, the detection of a clone with reduced cold-water sensitivity might indicate that the sources of future *P. gralli* infections may soon become more widespread. This situation could be exacerbated by the findings of Nollen et al. (1979), who reported that *P. gralli* miracidia have a longer half-life in water at temperatures lower than those reported as the lethal thermal minimum of *M. tuberculata* (18C). The combination of temperature adaptions by the intermediate host and the thermal tolerance of the *P. gralli* could increase exposure risk in populations of waterfowl in Texas and beyond.

Waterfowl in Texas

The U.S. Central Flyway is generally considered to include the states Colorado, Kansas, Montana, Nebraska, New Mexico, North Dakota, Oklahoma, Texas, and Wyoming (Dubovsky 2016). Mid-winter estimates of waterfowl numbers in the Central Flyway have exceeded $7x10^6$ birds (Dubovsky 2016). The same survey found that over $6x10^6$ of those birds wintered in Texas. Thus, about 85% of all waterfowl wintering within the Central Flyway will winter in Texas (Dubovsky 2016). Additionally, the same report estimated that there were more than $8x10^4$ waterfowl hunters in Texas with an average of 4.83 d afield/hunter in the 2015 waterfowl season. The number of hunters in Texas translates to large amounts of revenue generated in the sales of outdoor gear and clothing, and also fees for hunting licenses, migratory waterfowl stamps, ammunition, and firearms. Much of the revenue generated by these fees are used for conservation efforts. Thus, Texas is important for waterfowl, and waterfowl are important for Texas. Despite this well-known mutualistic relationship between Texas and migratory waterfowl, little is known about the impacts of the invasive parasite, *P. gralli*, on these birds in Texas, and there are no comprehensive baseline studies reporting the continued spread of the parasite into the Central Flyway of Texas since the original introduction. Given the severity of the pathology *P. gralli* can cause in the definitive host, a greater understanding of the parasite/waterfowl dynamic is needed to determine how this invasive parasite may be impacting this economically important group of birds.

Goals of this Study

This study had three goals.

- Goal 1 was to determine the impacts of *P. gralli* in waterfowl wintering in Texas. The associated objectives were:
  - to sample waterfowl from several areas in Texas for the parasite,
  - to determine the associated pathology, and
  - to estimate how long the bird had been infected.
- Goal 2 was to understand factors affecting the transmission dynamics of the parasite from the snail to the avian host. The associated objectives were:
  - to execute an experiment to determine if there is selection for encystment substrates by the cercarial stage of the parasite, and
  - to execute an experiment to determine if two other more common parasites of *Melanoides tuberculata* in Texas (*Haplorchis pumilio* and *Centrocestus formosanus*, both heterophyid trematodes) could interfere with the ability of *P. gralli* miracidia to successfully infect otherwise susceptible snails.
- Goal 3 was to explore various theories of origin for the invasion of the Americas by *P. gralli*. The associated objectives were:
  - to estimate the genetic diversity of the populations in the Americas, and
  - attempt to match recovered haplotypes with archived sequences from other naïve and native populations.

II. METHODS

Study Areas

Waterfowl from several areas throughout Texas were sampled during the 2016-2017 and the 2017-2018 Regular Waterfowl hunting seasons (Figure 1). Counties sampled in the 2016-2017 season were: Brazoria, Caldwell, Calhoun, Carson, Castro, Donley, Jefferson, Hutchinson, and Refugio. During the 2017-2018 season the counties sampled were: Caldwell, Calhoun, Live Oak, McMullen, and Refugio. These locations represent several of the ecoregions of Texas and were selected based on access to Public Lands, as well as cooperation by private landowners. Waterfowl from Jefferson County were sampled from J.D. Murphree Wildlife Management Area (JMWMA). JMWMA occurs along the coast in far-east Texas and is characterized as having saltwater, brackish, and freshwater marsh lands. Waterfowl from Brazoria County were collected from Justin Hurst Wildlife Management Area (JHWMA). JHWMA occurs in the Coastal Plains Ecoregion and is characterized as having saltwater, brackish, and freshwater marsh. Waterfowl from Refugio and Calhoun Counties were collected from Guadalupe Delta Wildlife Management Area (GDWMA) which is occurs in the Coastal Plains Ecoregion. The GDWMA has several areas and is characterized as saltwater, brackish, and freshwater marsh with hardwood bottoms also occurring in the WMA. Waterfowl from Carson, Castro, Donley, and Hutchinson Counties, henceforth referred to as Panhandle counties, were collected from various public and private lands. The Panhandle counties occur in the Panhandle Ecoregion and are characterized as grasslands and are interspersed with playa lakes. Waterfowl from Caldwell County were collected on private land. Caldwell County occurs in the Blackland Prairie Ecoregion and borders the Edward's Plateau ecoregion. The county is characterized as a mix of grassland and

hardwood forests with large patches of mesquite occurring in degraded grassland areas. Waterfowl from Live Oak and McMullen counties were collected from Choke Canyon Reservoir in James Daughtry WMA (JDWMA). Choke Canyon Reservoir occurs in the South Texas Ecoregion which is characterized as shrub land. The reservoir itself is used mostly for recreation but also serves as a water source for the surrounding area and as a back-up water source for the city of Corpus Christi, Texas.

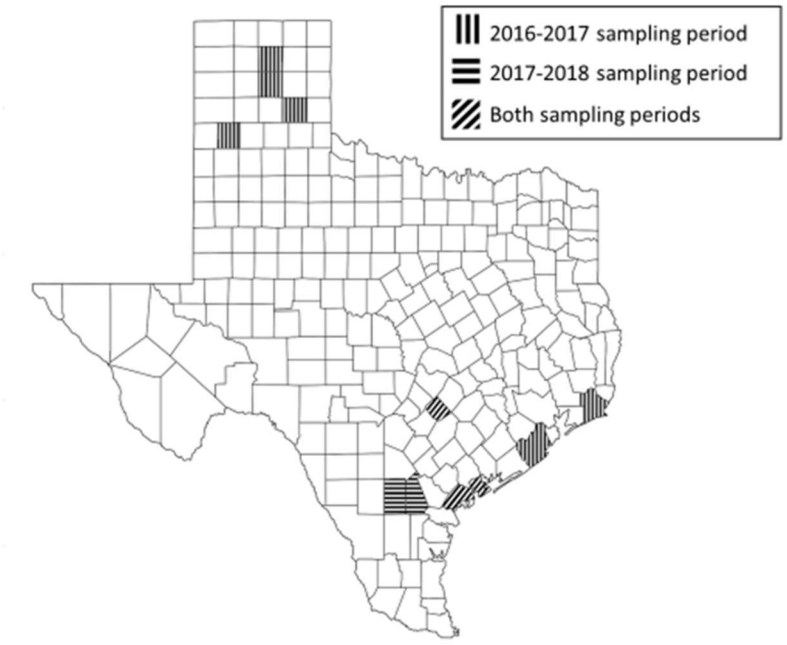

Figure 1. Map of counties sampled for *P. gralli*.

Parasite Sampling

Host Collection

Hunters were asked for the heads of their harvested waterfowl at check-stations in JHWMA, GDWMA, and JDWMA. Donating hunters were required to fill out a Wildlife Resource Document in accordance with Texas Parks and Wildlife regulations. The heads of the waterfowl were removed using shears, placed in resealable bags labeled with the

locality and date of harvest, and placed on ice for transport the same day to Texas State University. The bags were then placed in a freezer until examination. Waterfowl from Caldwell County, the Panhandle Counties, and JMWMA were collected by hunters who volunteered to store of the heads of their harvested ducks. The hunters were instructed to follow the same protocol as was used when the researchers collected heads, except the heads were stored in the hunter's personal freezer until the end of the hunting season and then shipped to Texas State University and all duck heads that were shipped arrived still frozen.

Necropsy

Prior to examination, the heads were allowed to thaw in a refrigerator for 48 h. The sinus cavity, nasolacrimal ducts, and both conjunctival sacs of each bird were then examined under magnification. The number and location of each parasite was recorded, and any detected pathology was noted. Recovered worms were then fixed in either 95% EtOH for genetic analysis or in AFA (alcohol, formalin, and acetic acid) for morphometric analysis.

Parasite Identification

Morphometric characters recorded included length, width (at the half-way point between the ventral sucker and the posterior end of the worm), and ratio of the width of the oral to ventral sucker. The worms were then provisionally identified to species. A subset of sampled worms were fixed in AFA, stained with Semichon's Acetic Carmine, and counter-stained with Fast Green. The stained specimens were then processed through a dehydration sequence to 100% EtOH, cleared using Terpineol or Xylene, and mounted in either Permount® or Canada balsam.

Assessing Pathology and Age of Infection

Pathology Categories

Ocular pathology of the ducks was assessed and assigned to one of five categories. Category 1 pathology was assigned to an infected individual that was not manifesting any pathology. Category 2 pathology was assigned if the individual exhibited excess mucus accumulation but no obvious irritation. Category 3 pathology was assigned if the individual exhibited more conjunctivitis. Category 4 pathology was assigned if lesions were detected on the conjunctival sac and/or nictitating membrane. Category 5 pathology was assigned if the individual presented with lesions on the conjunctival sac and/or nictitating membrane, and also presented with additional damage to the conjunctival membrane such as callouses and/or discolorations of the nictitating membrane (Table 1). The eyes of each bird were assigned to pathology categories independently.

Table 1. Abbreviated description of the worm age and host pathology categories used to assess individual impacts on waterfowl infected with *Philophthalmus gralli* .

Age Category		Pathology Category	
Category	Range	Category	Description
1	≤ 9d	1	No observable pathology
2	10d to 14d	2	Minor irritation/lacrimation
3	15d to 20d	3	Conjunctivitus
4	21d to 28d	4	Conjuntivitus with lesions
5	29d to 35d	5	Partial damage of tissue
6	36d to 50d		
7	> 51d		

Age of Infection

The age of infection was estimated categorically using Nollen's (1983) length/age growth curve for adult worms from experimentally infected chicks (Table 1). When

multiple worms of approximately the same lengths were recovered from a host, the mean length of the parasites were used to calculate the age of the infection.

Molecular Analysis

DNA Extraction and Purification

Worms used for DNA analysis were stored in 95% ethanol at -20C until extraction was performed. Extraction and purification were carried out using a modified protocol from GeneJET Plasmid Miniprep Kit® from Thermo Fisher Scientific. Prior to extraction, residual ethanol was allowed to evaporate off the worm for 10 to 15 min. Then 20 µL of Proteinase K and 180 µL of digestion solution were added to whole worms. The worms were then incubated at 56C and mixed at 650 rpm on a vibration table between 4 and 24 hours. After incubation the product was either stored at -20C, or the process of extraction and purification was immediately continued. Twenty µL of RNase and 200 µL of lysis solution were added to the incubated product. The solution was then vortexed for 15 s and allowed to incubate for 10 min at room temperature. 400 µL of 50% ethanol was then added to the solution. The solution was poured into a spin column and centrifuged at 6,000 g for 1 min. The solution that passed through the column was discarded and the column was transferred to a new collection tube. 500 µL of wash buffer containing 50% ethanol was added and the spin column was centrifuged at 8,000 g for 1 min. After the 1 minute, the wash buffer solution was discarded and 500 µL of a second wash buffer containing 50% ethanol was added to the spin column and centrifuged at over 12,000 g for 3 min. Then the spin column was transferred to a new centrifuge tube and 50 µL of elution buffer was added to the spin column which was allowed to incubate for 2 min at room temperature. The column and centrifuge tube were centrifuged at 8,000

g for 1 min. The elution buffer was then retrieved from the centrifuge tube, returned to the spin column, and centrifuged again at 8,000 g for 1 min. The purified product was then stored at -20C until PCR amplification could be completed.

PCR Amplification

Amplification was conducted using a master mix comprised of 3.125µL of DreamTaq® Master Mix, 0.25µL each of forward and reverse 10µM primer resuspension, and 1.00 µL of 25mM $MgCl_2$. 4.625µL of the master mix, 0.50µL of tDNA, and 19.875µL of nuclease-free reagent grade H_2O were combined to make a 25µL reaction for PCR amplification. The reaction consisted of 36 cycles with 15 s denaturation at 94C, 2 min annealing at 53C, and 2 mins extension at 72C. The cycling was terminated by 5 min of incubation at 72C. Forward primer NSF4/18 (CTG GTT GAT CCT GCC AGT) and reverse primer 18S-1192R/20 (CAG GTG AG TTT CCC GTG TT) were used to amplify the 18S ribosomal region of the nuclear genome. COI-R (CAA CAA ATC ATG ATG CAA AAG G) reverse primer and JB3 (TTT TTT GGG CAT CCT GAG GTT TAT) forward primer were used to amplify the COI mitochondrial regions.

PCR Clean-up and DNA Sequencing

PCR product was processed through an ExoSAP® procedure. The master mix for the procedure consisted of 1.56µL of NFH_2O, 0.4µL of 1 unit/µL shrimp alkaline phosphatase, and 0.04µL of 10 units/µL exonuclease. The final product was incubated for 30 minutes at 37C and finished at 95C for 5 minutes. DNA strands were then prepared for sequencing with a 5µL reaction consisting of 1µL of cleaned PCR product, 0.5µL of Big Dye Terminator 5X Sequence Buffer®, 1.0µL of Big Dye 3.1®, 0.12µL of primer, and 2.38µL of NFH_2O. The mix was then amplified with 33 cycles starting with 2

minutes of denaturing at 96C. Each cycle consisted of 10 seconds of denaturing at 96C, followed by 5 seconds of annealing at 54C and ending with 4 minutes of extension at 60C. The sequenced product was then purified with 300µL of Sephadex^{TM} G-50 Superfine (GE Healthcare) gel filtration medium. The purified product was dehydrated overnight in an incubator before 12µL of formamide were added to the product. The formamide and sequencing product were then incubated at 95C for 3 min. The sequencing was carried out with an Applied Biosystems 3500 Genetic Analyzer.

Sequence Analysis

Sequence editing, alignments, and phylogenetic establishments were done using Geneious Pro v5.5.9 Software (http://www.geneious.com, (Kearse et al. 2012). The ends of the sequences were first trimmed to remove unresolvable ambiguities and aligned with the primer sequence to remove the primer region as well. De novo contigs were then assembled for individual trematodes using the forward and reverse sequences for each respective trematode and gene segment. This was done to allow for finer scale editing of ambiguities for individual trematodes and sequences. All sequences of each respective region were then aligned with one another and a reference sequence to further refine the editing of ambiguities. Once the ambiguities were resolved, consensus sequences were generated for each trematode and gene region (544 bp for the COI and 456 bp for the 18S) and aligned with the ClustalW algorithm (Facon et al. 2003)

Phylogenetic Analysis

Phylogenetic relationships were established by building neighbor-joining trees with the methods from PAUP* plugin version 4.0b10. Model selection was done with MODELTEST function in the PAUP* Geneious plugin. MODELTEST revealed that the

HKY85 + Γ was the best fit for the data. Bayesian Inference with posterior probabilities was obtained using MrBayes v3.2.6.

Substrate Selection Experiment

Collection of Experimental Organisms

Infected Snails

Melanoides tuberculata to be used as a source of cercariae were collected with dip nets from Landa Park Lake, New Braunfels, Texas (29.714780°, -98.134324°) and stored in aerated 5-gallon buckets for transport back to Texas State University campus. To determine which snails were infected with *P. gralli*, snails were individually placed aperture-up in 2.5 cm X 25-cm test tubes filled with spring water for 24 h. Snails found to be shedding megalurous cercariae were placed together in an aerated 5-gallon bucket until they were needed for testing. Several cysts were removed from the sides of the test tube and examined as wet mounts to confirm that they were *Philophthalmus* cercariae (*P. gralli* is the only species of the genus that is known to use *M. tuberculata* as host). Cercarial source snails were used for a single trial until all snails were used, and then the snails were recycled for a second use.

Substrate Specimens

Six different substrate types were used to determine selection by the cercariae. The six substrates used were crayfish (*Procambarus* sp.), dragonfly larvae (Odonata), mayfly larvae (*Ephemera* sp.), uninfected host snails (*M. tuberculata*), approximately 9 cm of water stargrass (*Heteranthera dubia*), and a native spring-associated snail (*Elimia comalensis*). These substrates were chosen because they have been reported as food items for waterfowl and other aquatic birds. Because no single species of odonate was found in

sufficient numbers to populate all aquaria in a run of the experiment, multiple odonate species were used in most runs of the experiment.

Specimens were sorted by type into aerated 5-gallon buckets with water from the collection site and transported back to Texas State University. Once at the lab, the specimens were examined for naturally occurring *Philophthalmus* sp. cysts, and all cysts found were noted and removed. All substrate types other than water stargrass were collected from Landa Lake on the Comal River, while water stargrass was collected from the headwaters of the San Marcos River, Hays County, Texas. When possible, an individual substrate specimen was only used once; however, some individual crayfish and dragonfly larvae had to be used in the experiment more than once. When this happened, all cysts were removed from the individual and those individuals were separated from the other substrates of that type for at least 72 hours before being reused.

Experimental Design

The experiment consisted of ten trials, each involving three replicate aquaria, with each aquarium containing six types of substrates arranged radially around the aquarium. The ten trials were executed at irregular intervals from January 7 through March 3, 2019.

Setup of Experimental Aquaria

The experimental environment was a 13-L cylindrical glass aquarium (TOPFIN™ bettaflo™ 5262074) measuring 26.4 X 27.1 cm (diameter X height). Each aquarium was marked on the outside with six vertical lines at 60° intervals. These lines were used as guides to determine the substrate positions. Three equidistant substrate positions were established on each line (top, middle and bottom, Figure 2). This provided a total of 18 experimental substrate positions divided into six columns (representing substrate types)

and three levels. This arrangement allowed the positioning of three individual substrates of one type in one column at three levels.

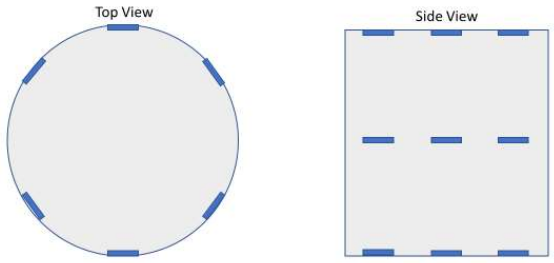

Figure 2. Schematic of specimen layout in experimental aquaria.

For each aquarium, 18 segments of cotton string were cut to length, and a slip knot ("overhand knot with draw-loop," https://en.wikipedia.org) was prepared at one end. At each substrate position, the standing end of the cotton string was secured to the inside of the aquarium using aquarium-grade silicone sealant and allowed to cure 24h, after which the loose ends of the strings were trimmed and discarded. Wooden pegs were then cut to 4 cm long from 3/8 in. dowel rod. These pegs provided a reusable detachment mechanism allowing the same string segments to be used for multiple runs of the experiment. The pegs were soaked in DI water for 24 h to remove any residual tannins or processing chemicals. Water was replaced for several days until it was clear after 24 h soaking. Segments of monofilament fishing line (14 lb. nylon, about 0.35 mm diam.) were cut to about 30 cm, and one end was secured to the middle of a peg using a Trilene knot. A "uni" knot was then fashioned at the tag end of the monofilament to hold a substrate. Each substrate was then secured to a wooden peg with the uni knot, and the wooden peg was then secured in place by tightening the adjustable loop of one of the pre-positioned cotton strings to the middle of the peg.

Prior to being secured in an aquarium, each substrate item was examined under magnification to count and remove any naturally occurring *P. gralli* cysts. The six substrates used in each aquarium were three crayfish (Cambaridae), three dragonfly larva (Odonata), three mayfly larva (Ephemeroptera), three uninfected *M. tuberculata*, three segments of approximately 9 cm of *Heteranthera dubia* (water star grass), and three *Elimia comalensis*.

The source of fresh cercariae for each aquarium was three *Melanoides tuberculata* collected from Landa Lake and known to be shedding *Philophthalmus* cercariae. The three snails were suspended together at the center of the aquarium using a cotton string tied to a metal rod resting across the top of the aquarium. This provide a cercarial source equidistant from all substrate types. The outside of each aquarium was wrapped all the way around with black plastic film from top to bottom so as to prevent light sources in the lab from influencing cercarial orientation after leaving the source snail. A 40-watt incandescent bulb was positioned at about 15 cm above the center of the open aquarium. The purpose of the light was to induce cercarial shedding by the source snails and to simulate a bright sky.

Experimental Trials

Replicate aquaria #1, #2, and #3 were always located at the same designated spot in the lab for all ten trials. Radial position #1 (of 6) was always consistently assigned to the same position in the aquaria relative to its orientation in the lab, and the other five positions were assigned sequentially clockwise. Assignment of substrates to radial positions for all trials was determined by randomly sorting the numbers 1-6 in one column of a table (representing the radial positions in an aquarium) and randomly sorting

the letters A-F in the adjacent column (representing the six substrate types). The substrate type randomly appearing in the first row of the table then became the starting point for setting up that aquarium, and that substrate was assigned to all three depths at the radial position randomly appearing in the same row of the table, and so on, until all three individuals of all six substrates had been so assigned. Once all substrates had been assigned a position in the aquaria, well water was poured into the aquaria to a sufficient depth to cover all the substrates at the bottom depth. Once the positions at the bottom depth were filled, a sufficient amount of water was added to submerge the substrates at the middle depth and the mid-level substrates were placed. The surface level depth was treated in the same manner as the bottom and middle depths.

Each trial was initiated when the lights above the three aquaria were turned on sometime between mid-morning and noon, and terminated 24 h later. At the end of 24 h, source snails were removed from the aquaria, and each aquarium was examined for swimming cercariae. If swimming cercariae were found, the light was left on over that aquarium and the next aquarium was checked for swimming cercariae, etc. If an aquarium did not have swimming cercariae, the light was turned off and the process of removing and examining substrates began. The substrates were removed one at a time, starting with those at the top level, then the middle and bottom levels until all substrates in an aquarium had been removed and examined. Then the next aquarium was processed, etc. Processing time for trials (from first light off to last substrate examined) ranged from about 1 to 1.5 h.

Removed substrates were photographed from dorsal view and immediately examined for the cysts under a dissection microscope. If cysts were present, the cysts

were counted and removed. After the last substrate from an aquarium had been processed, the aquarium was emptied of water and the inside surfaces of the aquarium were examined for cysts from the outside with a handheld magnifier.

Surface Area Estimation

Surface areas of substrates were estimated from digital photographs using SketchandCalc® software (https://www.sketchandcalc.com/).

Water Stargrass

The plant was treated as a two-dimensional object, so the surface area of every plant specimen was estimated from a photograph of the specimen while flattened out (including both stems and leaves). That area was then doubled, since cercariae had an equally probable chance of encysting on the opposite side of the plant.

All Other Substrate Types

Surface area estimates for crayfish, mayfly larva, dragon fly larva, and both gastropod species were based on models derived from surface analyses of three prototype specimens (chosen to represent the largest length, the smallest length, and a typical length) from each substrate type. Each prototype specimen was first photographed in dorsal, lateral, and frontal profile views, and the area of each profile view was estimated by SketchAndCalc®. The total area of a specimen was then estimated by doubling the three profile areas (to accommodate both surfaces of each profile) and then summing these products across all three profiles get an overall area of the specimen.

Rather than assume that the growth of all of these substrate types is isometric, the total areas of the three prototype specimens were then regressed against their total lengths (photographed while relaxed) using quadratic regression. The resulting substrate-specific

coefficients were then used to estimate the areas of all other specimens of that substrate type based solely on their total lengths (photographed while relaxed).

Estimation of odonate areas required special consideration. It proved virtually impossible to always acquire enough individual odonates of the same morphotype for all the trials, and I included several odonate species in the experiment as they became available. Unfortunately, these species represented four obviously different morphotypes, and each morphotype required a separate area-estimation model. Therefore, three prototype specimens were chosen for each morphotype as above, and separate area-estimation models were developed to estimate the areas of experimental specimens of odonates after classifying their photographs into one of the four morphotypes.

Cyst Density Determination and Analysis

The number of cysts found on an experimental individual was divided by the estimated surface area of that individual to determine the density of cysts in cm^2. The assumptions of normality and homoscedasticity could not be met, so a randomization test with a two-factor ANOVA using the factors substrate type and depth with an interaction term was conducted to determine significance of the factors. In accordance with Manly (2006), 10,000 complete randomizations were performed. To visualize differences between factors, the mean cyst density and 95% confidence intervals were graphed.

Snail Co-infection Experiment

During the investigation of the impacts of *P. gralli* on the native fauna of Texas, it became apparent that understanding the relationship of this trematode with the other invasive parasites found in Texas was necessary to even begin to consider a management plan for *P. gralli*. As such, an experiment was designed with the goal of exploring how *P.*

gralli interacted with *Centrocestus formosanus* and *Haplorchis pumilio* in the intermediate snail host. It was hypothesized that the larval stages of *P. gralli* would be able to infect snails already infected with *C. formosanus* and *H. pumilio* and perhaps to clear those infections.

Collection of Experimental Snails

Snails were collected from Landa Park Lake, Comal County Texas on 5 October 2018 and 10 October 2018. Landa Park Lake is located in a city park at the spring head of the Comal River and *Melanoides tuberculata* infected with *Haplorchis pumilio* and *Centrocestus formosanus* occur in abundance there. Snails were collected at random locations using dip nets. Collected snails were placed in aerated 5-gallon buckets filled with lake water and transported to Texas State University campus.

Infection Status and Estimation of Shedding Rates

Categorizing Snails by Infection Status

Individual snails were placed aperture up in 25 x 200 mm test tubes filled with approximately 60 mL of well water and left under a 60-watt incandescent light source for 24 hours. A fiber optic light was then used to examine the test tube for the presence of cercariae. If cercariae were detected, the cercariae were identified and the snails were sorted into aerated 5-gallon buckets based on their infection status. Infection status categories were: *Cf*-Shed if shedding *C. formosanus* cercariae, *Hp*-Shed if shedding *H. pumilio* cercariae, and No-Shed if uninfected. Snails were then fed dried algal tablets *ad libitum* and maintained in their appropriate bucket. Snails designated as uninfected were re-examined every 24 h for the up to three times or until the snail shed cercariae, whichever came first. Categorized snails were maintained in separate aerated 5-gallon

buckets, fed algal tablets, and the water was changed in each bucket at least every two days. Figure 3 provides a schematic of the procedure for determining the infection status of a snail.

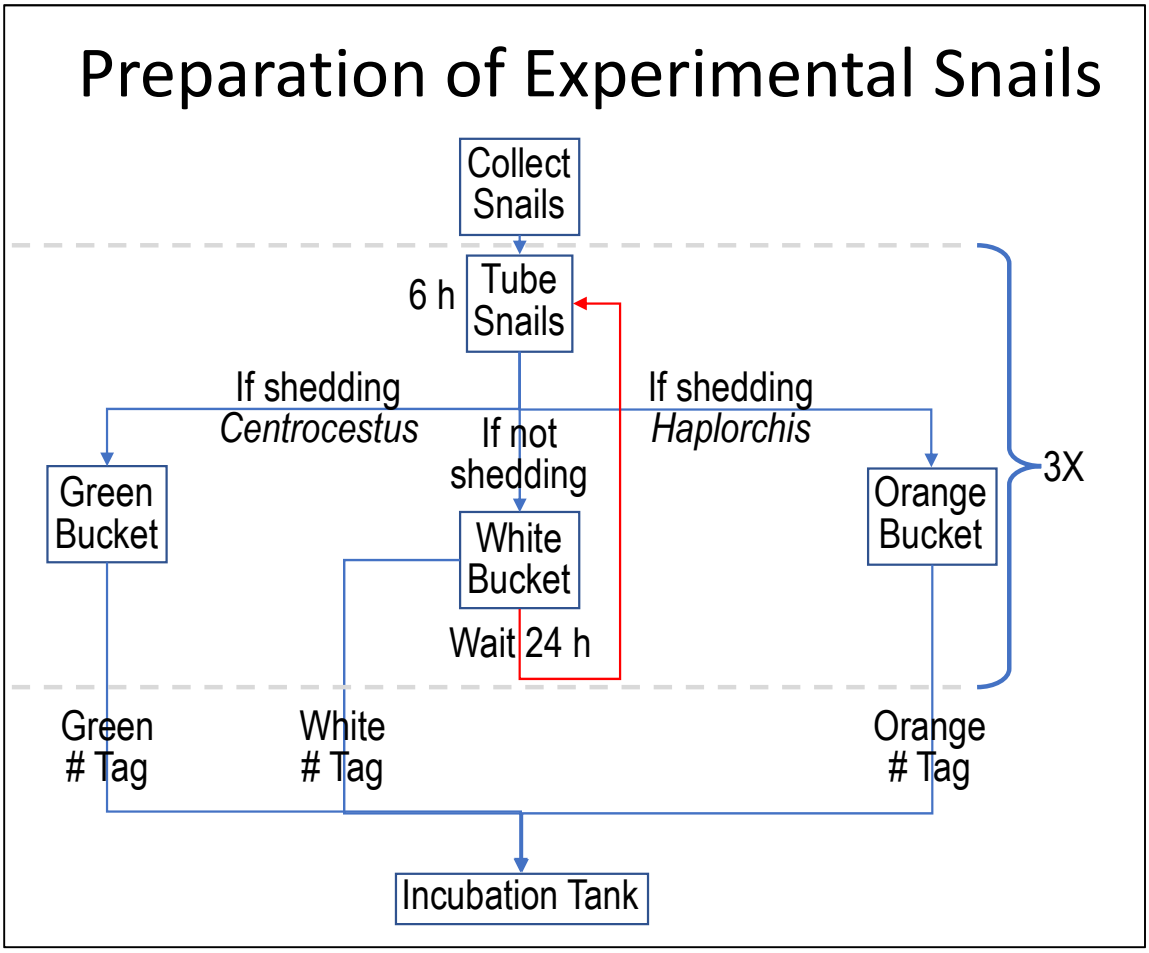

Figure 3. Schematic diagram for determining infection status.

Selecting and Marking Experimental Snails

Once the infection status of the snails had been determined, 50 snails were selected at random from the *Cf*-Shed group, 50 from the *Hp*-Shed group, and 80 from the No-Shed group. The lengths of the snails were then measured and tested with a simple one-way ANOVA to ensure that the mean lengths of the snails used in the three groups were not statistically different ($p[F_{\alpha(1),df=2,177} \geq 1.687]=0.188$). Individual snails were then

assigned a unique code and tagged using bee marking tags (www.BetterBee.com) secured to the shell with cyanoacrylate glue (dorsal side of body whorl after scrubbing with toothbrush and drying with paper towel). Once marked, the snails were then pooled together and maintained in a flow-through holding tank at constant temperature of 23° C with a 12 h light-dark cycle and fed *ad libitum* with dried algal pellets.

While being maintained in the flow-through holding tank, snails were checked every other day for mortalities. This was done by moving all the snails to one end of the holding tank. Any snail found at the end of the holding tank two days later was examined by gently poking the operculum of the snail to elicit a reflex response by the snail to close its operculum. Any snail that did not respond was removed from the holding tank and stored in 70% EtOH. Once every week, all snails were removed from the holding tank and placed in a 5-gallon bucket of well water. Snails were checked individually for viability to ensure none of the snails had lost their tag, died, or could not be found in the holding tank, and status was recorded on a roll-call sheet. This sheet was examined periodically to make sure that treated snails were not dying more rapidly than untreated snails, in which case an unplanned cercariometry procedure would have been scheduled.

Cercarial-counting Procedure

Pre-exposure shedding rates for individual snails were estimated at about 30 d prior to exposing the snails to *P. gralli* miracidia. The estimates were accomplished by placing the snails in 25 x 200 mm test tubes marked with the snail's accession number and filled with 50 mL of well water. The tubes were then placed under incandescent light for 6 h (to induce shedding) starting about 0800 hrs. Snails were then removed from the tubes with forceps and returned to the flow-through holding tank. Once the snails were

removed from the tubes, the water in each tube was gently mixed and 10 mL of water from each test tube was poured off into separate watch glasses. EtOH (10 mL, 70%) was then added to each of the watch glasses and allowed to mix naturally for approximately 5 min to fix any cercariae. After the 5 min had passed, 5 to 7 drops of 1% Rose Bengal solution was added to the watch glass and allowed to mix for another 5 min. The watch glass was then placed over a 1-mm grid and all cercariae were counted. The number of cercariae counted was multiplied by 5 to estimate the total number of cercariae in the test tube. This cercarial-counting procedure was repeated two more times, with a 48-h period between successive shedding estimation events. The mean of the three cercarial counts for each snail was then calculated and recorded as the estimate for each snail's pre-exposure shedding rate. The same procedure was conducted on the snails designated as uninfected to ensure that they did not have latent infections that had been missed earlier. If a snail designated as uninfected began shedding cercariae, it was removed from the experiment.

Once the pre-exposure rates had been determined, the 50 *Cf*-Shed snails were split into two equal groups at random, one of which would later be exposed to *P. gralli* miracidia the "*Cf*-Shed-Treat" group, and the other 25 snails would serve as controls for the *P. gralli* X *C. formosanus* co-infection group ("*Cf*-Shed-Control" group). The 50 Hp-Shed snails were likewise split into the "*Hp*-Shed-Treat" and the "*Hp*-Shed-Control" groups, and the No-Shed snails were split into the No-Shed-Treat and No-Shed-Control groups (Figure 4).

The cercarial-counting procedure was repeated 60 d and 120 d post-exposure to *P. gralli* miracidia. After the 120 d post-exposure shedding period, snails were crushed to

confirm their status of infection with *P. gralli*. The larval stages of any co-infected snails were separated by species and a ratio of any *P. gralli* larval stages to heterophyid larval stages was calculated.

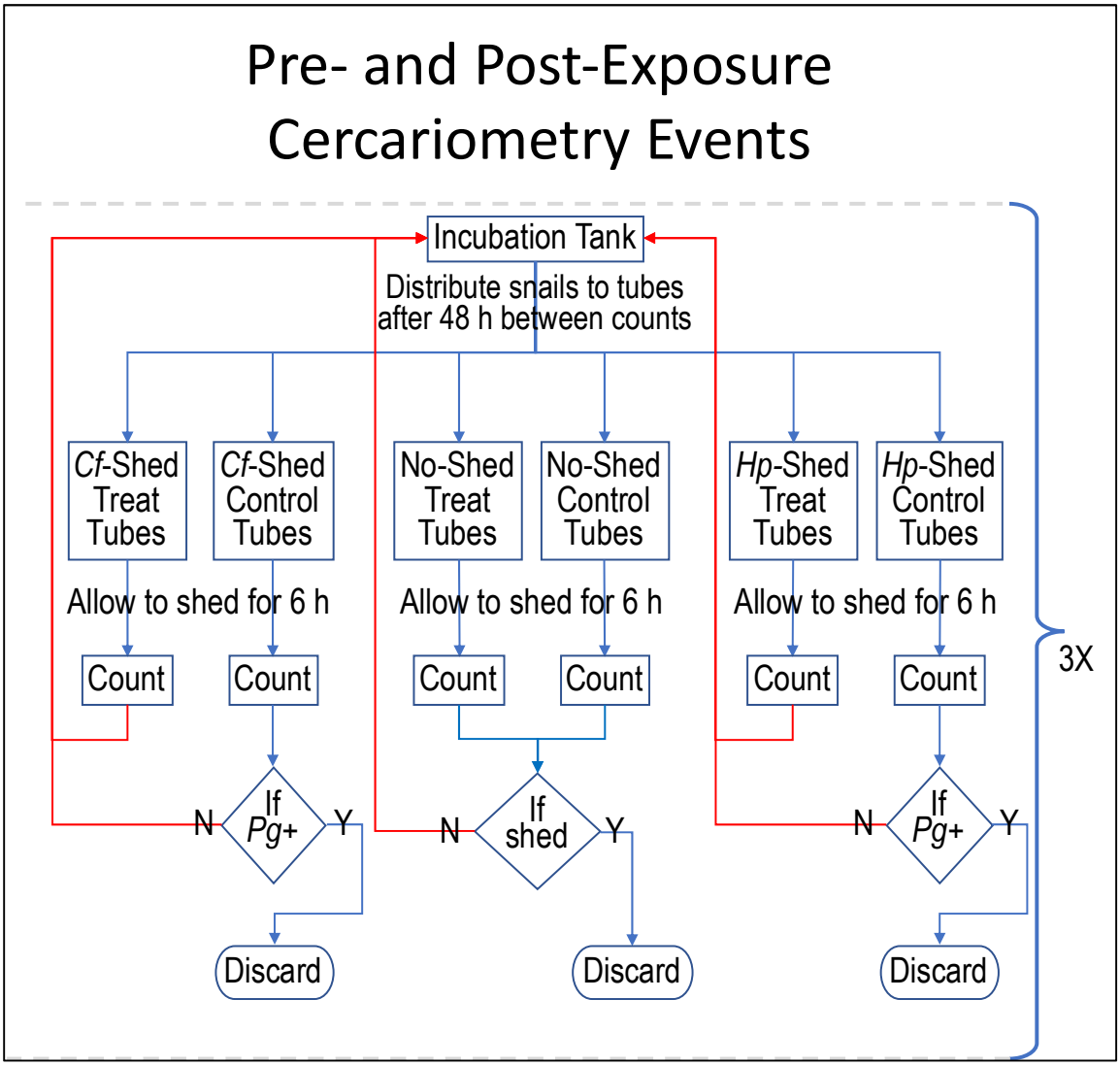

Figure 4. Schematic diagram of the process for estimating shedding rates.

Collection of Adult Philophthalmus gralli

Adult *P. gralli* (to serve as a source of eggs) were collected from hunter-donated ducks harvested on Choke Canyon Reservoir, Live Oak and McMullen Counties, Texas on the 5th, 12th, and 26th of January 2019. Choke Canyon Reservoir was created by

damming the Nueces and Frio Rivers as a source of municipal drinking water, and is used for recreation, including waterfowl hunting. As hunters left the reservoir at boat ramps, a researcher would ask the hunter for the heads of their harvested ducks. Any hunter that agreed to donate the heads of their ducks was required to fill out a wildlife resource document, stating that the animal was harvested legally, and the duck head was donated by the hunter to the researcher. The heads would then be removed using pruning shears, placed in a resealable bag, and stored in a cooler with cold gel packs to keep the heads cool but not sufficiently cold as to kill any *P. gralli* that may be infecting the duck. The heads were transported to Texas State University and examined the same day for adult *P. gralli*. If an adult worm was found, it was examined as a wet mount under coverslip pressure to verify the worm as *P. gralli*. The worm was then placed in warm physiological saline to encourage the release of eggs.

Exposure of Snails to P. gralli Miracidia

Snails were exposed to *P. gralli* miracidia by placing both the treatment and control snails in 25 x 200 mm test tubes with 10 to 15 mL of well water. Adult *P. gralli* were placed in warm physiological saline and allowed to shed eggs, and the miracidia were allowed to emerge. Five to ten miracidia were pipetted into each of the test tubes containing treatment snails until there were no remaining active miracidia being produced by the adult worm. The control snails were paired at random with a treatment snail and both the treatment and paired control remained in their individual test tubes for 6 h (Figure 5). Once no more miracidia were available for exposures, the treatment snails that had not received any miracidia and their corresponding control snails were immediately returned to the holding tank. At subsequent exposure events, only the treatment snails

that had not been exposed to miracidia and the control snails that had not been previously paired with treatment snails were placed in the test tubes. On the final exposure event, we lacked sufficient miracidia to expose the remaining treatment snails at the prescribed minimum rate of 5 miracidia/snail. So, the remaining treatment snails and all remaining control snails were left in their individual test tubes for 6 h, regardless of whether they were exposed to miracidia or not, and any treatment snail that was not exposed to miracidia was re-designated as a control. The exposure phase of the experiment was then terminated after the third exposure event because the 2018-2019 waterfowl season had closed in Texas and I was unable to acquire any more infected waterfowl from which adult *P. gralli* could be recovered.

Within 24 h of removing the snails from the test tubes, the water from each test tube was poured into separate watch glasses, fixed with 10 mL of 70% EtOH, and stained with 5 to 7 drops of 1% Rose Bengal. The watch glass was then placed over a 1 mm grid and examined under magnification for the presence of any residual miracidia that had failed to penetrate the targeted snail.

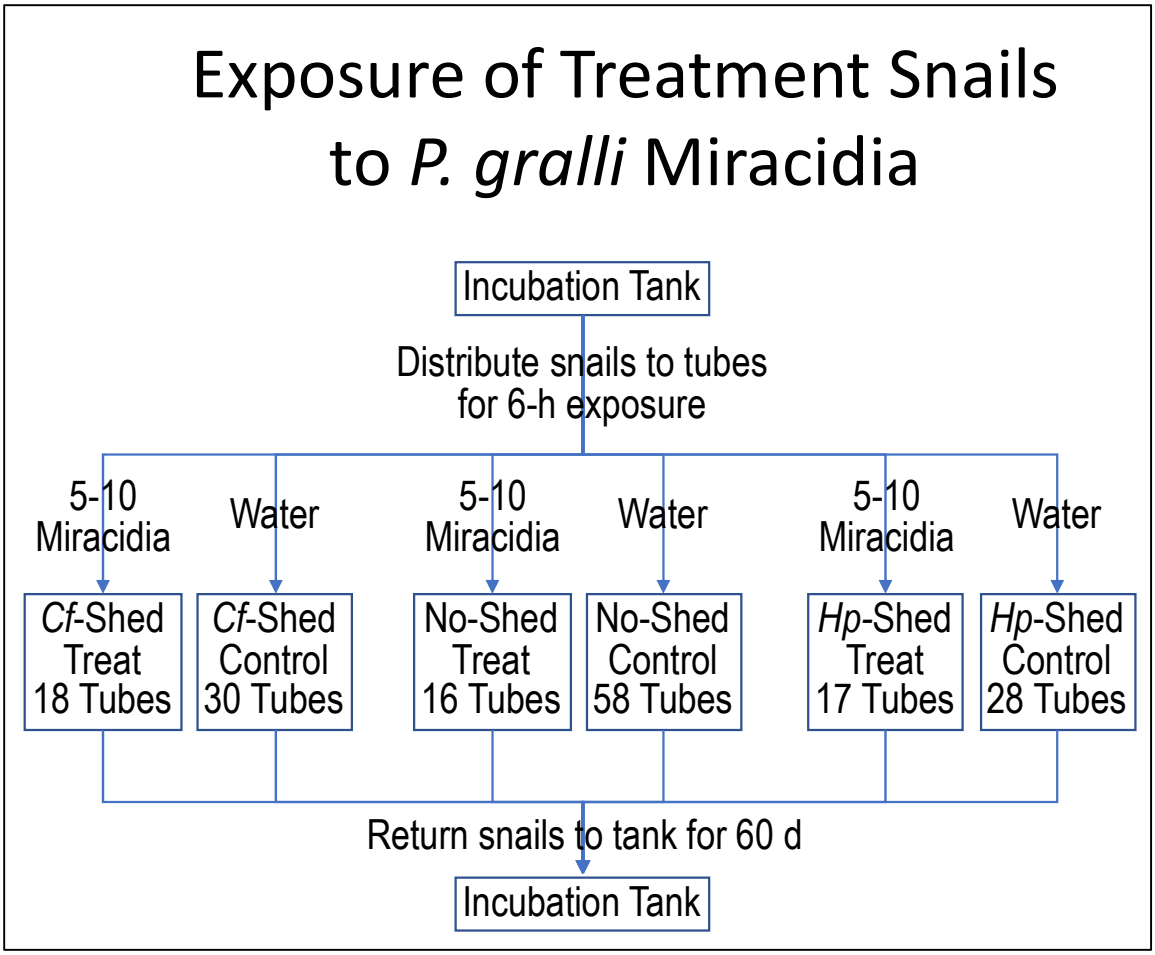

Figure 5. Schematic of the protocol for exposing the designated snails to *P. gralli* miracidia.

III. RESULTS

Parasitism and Morphometry

I sampled a total of 1,362 waterfowl representing 18 species and 2 orders (Table 2 and Table 3), and recovered two species of eye-flukes (*Philophthalmus gralli* and *Philophthalmus lacrymosus* Braun, 1902). Overall prevalence, mean intensity, and standard deviation of philophthalmid infections was 12.5%, 5.34 worms, and 8.8, respectively.

Philophthalmus gralli

During the 2016-2017 season, I sampled a total of 756 waterfowl representing 17 species. Prevalence was 5.7% with mean intensity of 3.4 (Table 2). During the 2017-2018 season, I sampled 606 waterfowl representing 18 species, with a prevalence of 21.1%, a mean intensity of 6.0 (Table 3).

I measured morphometric characters on n=8 adult worms that had been thawed in the refrigerator, fixed in AFA under coverslip pressure, stained with Semichon's Acid Carmine, and counter-stained with Fast Green. I then dehydrated them in an ethanol series, cleared them with xylene, and mounted them in Canada balsam. Measurements are in µm (unless otherwise noted) and expressed as mean (min-max). I derived the qualitative descriptions from 40 other specimens studied as wet mounts under coverslip pressure.

General body shape elongate with anterior end attenuated; body length 3.75 (2.52-4.98) mm; width half-way between posterior margin of acetabulum and posterior end of body 1.19 (0.59-1.93) mm; minute tegumental spines observed in wet-mounted individuals between acetabulum and oral sucker, but were not detectable in permanent

mounts; oral sucker subterminal, 386 wide (301-458) and 321 long (261-458); prepharynx observable in wet mounts but not in permanent mounts; muscular pharynx 315 wide (290-359) by 323 long (229-412); esophagus short but not distinguishable in some permanent mounts; conspicuous caeca ending blindly near excretory bladder; muscular acetabulum 473 wide (377-595) by 512 long (460-563) and pre-equatorial; ratio of acetabulum to oral sucker 1.22 (1.15-1.26); excretory bladder elongate and arranged transversely; excretory pore terminal; two testes ovoid or lobed, in tandem or oblique, anterior 356 wide (363-533) by 313 long (269-483), posterior 362 wide (288-469) by 293 long (185-472); efferent seminal ducts emerging laterally and converging anteriorly into single duct before entering cirrus pouch which was frequently obscured by the uterus; cirrus pouch, without cirrus extruded, 902 long (798-1027); genital pore along midline just posterior to esophageal bifurcation; ovary sub-globular, 248 wide (205-314) and 218 long (166-293), anterior to testes and ventral to uterus; oviduct appearing muscular, originating at posterior margin of ovary, and extending posteriorly to Laurer's canal; vitellaria tubular to follicular, vitelline fields mostly bisymmetric; uterus long and coiled, between anterior testis to just posterior of the acetabulum; eggs in various developmental stages observable throughout uterus.

Philophthalmus lacrymosus

I found only 16 ducks that were infected with *P. lacrymosus* (0.8% prevalence, 7.2 mean intensity, 5.9 SD). In the 2016-2017 waterfowl season, 4 out of the 756 examined waterfowl were infected (0.5% prevalence, 4.0 mean intensity, 6.0 SD), all from Redheads (*Aythya americana* Eyton, 1838). One of the infected birds came from Brazoria County, one from Refugio/Calhoun Counties, and 2 from Caldwell County. In

the 2017-2018 waterfowl season, I examined 606 waterfowl and only 12 of were infected with *P. lacrymosus* (1.3% prevalence, 7.9 mean density, 6.8 SD). Infected species included Redheads and Gadwalls (*Mareca strepera* Linnaeus, 1758) (10 and 2 infected individuals, respectively). Two infected Redheads were from Caldwell County, two infected individuals from Calhoun/Refugio Counties, and 6 infected individuals from Live Oak/McMullen Counties. One duck, a Gadwall, was concurrently infected with a single *P. gralli* in the right eye and a single *P. lacrymosus* in the left.

I derived morphometry from four adults that had been stained and mounted following the protocols used for *P. gralli,* and all measurements are in µm unless otherwise stated. Qualitative descriptions were derived from and additional 15 wet-mounted individuals.

Morphometry: Body shape elongate with a constriction at the acetabulum, 3.77 mm long (2.96-5.24 mm) and 1.01 mm wide (0.83-1.17 mm); tegument with minute spines ventrally between oral and ventral suckers; oral sucker subterminal and muscular, 383 wide (325-418) and 273 long (244-329); prepharynx short and usually not discernable; pharynx muscular, 360 wide (324-385) and 333 long (301-381); esophagus bifurcated beyond pharynx, 106 long (72.9-152); caeca conspicuous, each ending in a blind sac at near the posterior end; acetabulum muscular, 551 wide (467-622) and 553 long (433-649); ratio of acetabulum width to oral sucker width 1.44 (1.37-1.48); testes round or ovoid, tandem, sometimes touching and sometimes separate; anterior testis 233 wide (204-281) and 270 long (226-310), posterior testis 233 wide (208-307) and 281 long (212-343); efferent ducts arising laterally to form a single duct connecting to cirrus pouch; cirrus pouch sometimes partially obscured by acetabulum, 457 long (425-519)

with the cirrus retracted; genital pore at or just posterior to bifurcation of esophagus; ovary globular, 172 wide (130-268) and 181 long (136-256) and anterior to and sometimes oblique to, the anterior testis, sometimes obscured by uterus; oviduct arising posteriorly from the ovary and then extended anteriad to Laurer's canal; vitellaria follicular, in bilateral fields of 6-8 follicles lateral to caeca; uterus extensive and coiled, filling space from posterior testis to acetabulum in gravid specimens; eggs in different developmental stages could be observed throughout the uterus with more mature eggs anteriad.

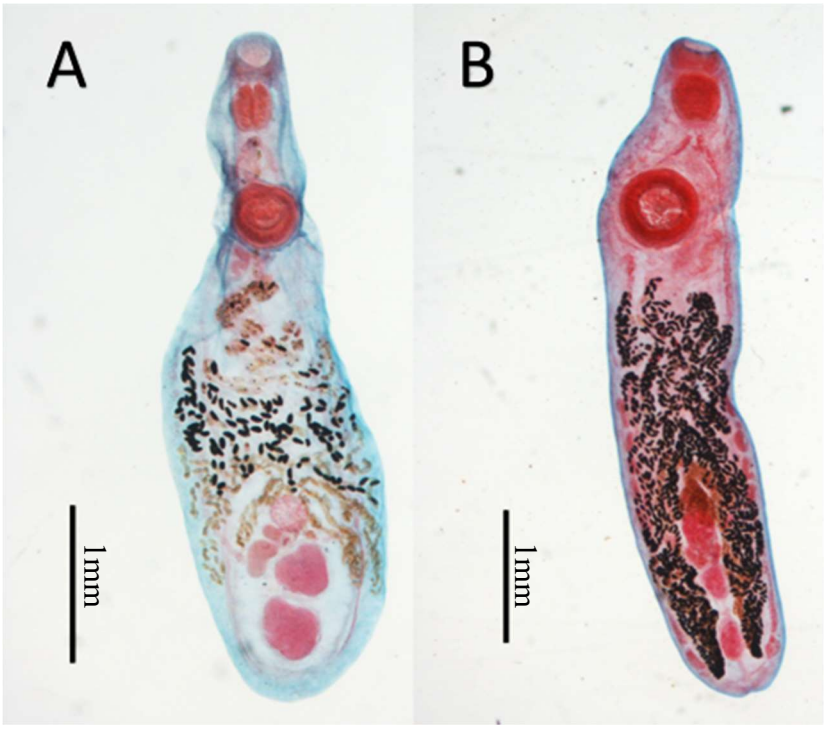

Figure 6. Representative specimens of *Philophthalmus gralli* (A) and *P. lacrymosus* (B) from waterfowl in Texas.

Table 2. Host-species accounts of *Philophthalmus gralli* by county for samples collected in the 2016-2017.

	Infection Stats During 2016-2017 Waterfowl Season, By Species & County																			
	Brazoria				Refugio & Calhoun				Caldwell				Jefferson				Panhandle Cos.			
Species	*n* [1]	Prev.[2]	Mean Inten.[3]	SD[4]	*n*	Prev.	Mean Inten.	SD	*n*	Prev.	Mean Inten.	SD	*n*	Prev.	Mean Inten.	SD	*n*	Prev.	Mean Inten.	SD
Anas discolor	216	0.06	1.5	0.7	35	0.17	2.5	2.3	--	--	--	--	28	3.6	2	--	--	--	--	--
Anas carolensis	80	0.01	22	--	23	0	--	--	--	--	--	--	--	--	--	--	5	0	--	--
Anas clypeata	91	0.03	1	0	33	0.09	1.7	1.2	--	--	--	--	--	--	--	--	--	--	--	--
Anas strepera	66	0.06	1.5	1.0	21	0.05	3	--	3	0	--	--	--	--	--	--	--	--	--	--
Anas acuta	30	0.07	6	7.1	5	0.2	33	--	--	--	--	--	--	--	--	--	5	0	--	--
Anas americana	17	0.1	4	--	4	0	--	--	2	0	--	--	--	--	--	--	5	0	--	--
Anas platyrhynchos	--	--	--	--	2	0	--	--	--	--	--	--	--	--	--	--	18	0	--	--
Anas fulvigula	2	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Aix sponsa	--	--	--	--	--	--	--	--	2	0	--	--	--	--	--	--	--	--	--	--
Aythya americana	7	0.00	--	--	3	0.0	--	--	2	0	--	--	--	--	--	--	--	--	--	--
Aythya collaris	2	0	--	--	2	0	--	--	11	0.2	1.00	0	--	--	--	--	--	--	--	--
Aythya affinis	--	--	--	--	1	0	--	--	2	0	--	--	--	--	--	--	--	--	--	--
Bucocephela clangula	--	--	--	--	1	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Bucocephela albeola	--	--	--	--	2	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Dendrocygna bicolor	1	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Oxyura jamaicensis	5	0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Fulica americana	24	0.04	2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Totals	541	0.05	2.7	4.4	132	0.1	4.8	9.1	22	0.1	4	6	28	3.6	2	--	33	0	--	--

1=Number of indiviuals sampled

2=Prevalence the parasite

3=Mean intensity

4=Standard deviation of mean intensity

Table 3. Host-species accounts of *Philophthalmus gralli* by county for samples collected in the 2017-2018 Waterfowl hunting season.

	Infection Stats for 2017-2018 Waterfowl Season											
	Caldwell				Live Oak & McMullen				Refugio & Calhoun			
Species	*n* [1]	Prev.[2]	Mean Inten.[3]	SD[4]	*n*	Prev.	Mean Inten.	SD	*n*	Prev.	Mean Inten.	SD
Anas discolor	--	--	--	--	46	0.74	6.4	8.20	1	0	--	--
Anas carolensis	11	0	--	--	17	0.29	4	5.6	7	0	--	--
Anas clypeata	10	0	--	--	21	0.48	7.5	4.0	12	0	--	--
Anas strepera	154	0.07	1.5	0.7	64	0.47	4.4	5.2	32	0.09	2.7	2.1
Anas acuta	6	0.17	2	--	5	0.60	5	3.6	1	0	--	--
Anas americana	65	0.05	1.3	0.6	18	0.72	4	2.7	11	0	--	--
Anas platyrhynchos	1	0	--	--	--	--	--	--	--	--	--	--
Anas fulvigula	--	--	--	--	1	100	1	--	--	--	--	--
Aix sponsa	4	0	--	--	--	--	--	--	--	--	--	--
Aythya americana	5	0.2	1	--	6	0.67	12.8	7.3	2	0	--	--
Aythya valisineria	--	--	--	--	1	0	--	--	1	0	--	--
Aythya collaris	70	0.04	29	45.0	4	0.3	1	--	--	--	--	--
Aythya affinis	9	0	--	--	--	--	--	--	9	0	--	--
Bucocephela clangula	--	--	--	--	--	--	--	--	1	0	--	--
Dendrocygna autumnalis	--	--	--	--	2	100	31	15.6	--	--	--	--
Dendrocygna bicolor	--	--	--	--	2	100	12.5	3.5	--	--	--	--
Oxyura jamaicensis	1	0	--	--	--	--	--	--	--	--	--	--
Fulica americana	--	--	--	--	1	0	--	--	5	0	--	--
Totals	336	5.7	0.0	0.0	188	0.56	6.2	7.3	82	4.9	1.5	2.1

1=Number of indiviuals sampled

2=Prevalence the parasite

3=Mean intensity

4=Standard deviation of mean intensity

Pathology

The mean of the pathology categories for all infected birds (*n*=206 infected eyes) was 2.3±0.08, indicating that the typical pathology associated with infection is mild irritation to conjunctivitis. Table 4 shows the mean pathology by host species. Mean pathology related the amount of time the host was infected is shown in Table 5.

Table 4. Pathology category associated with the species that had infected individuals during this study.

Pathology of Host Species			
Species	Mean[1]	SE[2]	Mean Inten[3]
Anas carolensis	2.0 (*n* =7)	0.31	7.0
Anas acuta	2.8 (*n* =12)	0.46	8.9
Anas fulvigula	1 (*n* =1)	--	1.0
Mareca strepera	2.4 (*n* =64)	0.14	3.4
Mareca americana	2.3 (*n* =24)	0.23	3.5
Spatula discolor	2.3 (*n* =64	0.13	4.7
Spatula clypeata	2.5 (*n* =24)	0.25	5.2
Aythya americana	2.7 (*n* =14)	0.46	7.0
Aythya collaris	3.0 (*n* =5)	0.84	15.0
Dendrocyagna bicolor	4.5 (*n* =4)	0.29	12.5
Dendrocyagna autumnalis	5.0 (*n* =4)	0.00	31.0
Fulica americana	2.0 (*n* =1)	--	2.0

1=Mean pathology for the species with the number of infected eyes
2=Standrd error of the pathology
3=Mean intensity of the infected eyes

Table 5. Pathology associated with each age category.

Pathology by Infection Duration			
Age Category[1]	Mean[2]	SE[3]	Mean Inten[4]
1	1.3 (*n* =20)	0.2	6.0
2	1.9 (*n* =34)	0.1	6.1
3	2.9 (*n* =25)	0.2	8.8
4	2.7 (*n* =20)	0.2	5.1
5	2.8 (*n* =10)	0.4	3.5
6	3.4 (*n* =14)	0.3	6.9
7	2.4 (*n* =17)	0.3	2.0

1=If more than one age category could be detected in the bird, the oldest age category was used for that bird
2=Mean pathology category and number of birds in that category
3=Standard error of the mean pathology category
4=Mean intensity of infection for the birds in that age category

Phylogenetic Analysis

Fifteen worms identified as *P. gralli* were successfully sequenced for the COI mitochondrial gene, and among those 15, 9 sequences for the 18S region were recovered from individuals identified as *P. gralli*. Seven unique haplotypes were identified from the COI region and one unique sequence from the 18S region. Four worms identified as *P. lacrymosus* were sequenced for the COI region and 18S region with two unique haplotypes identified from the COI region and two unique sequences from the 18S region. Figure 7 shows the relationship between COI sequences from this study and sequences from GenBank that are described as belonging the genus *Philophthalmus*, and Figure 8 shows the phylogenetic relationships for the 18S sequences.

Figure 7. Phylogenetic tree from dereplicated *Philophthalmus* spp. from NCBI GenBank and this study. *Philophthalmus s*equences from GenBank include the GenBank accession number with the names and locations the trematodes were collected.

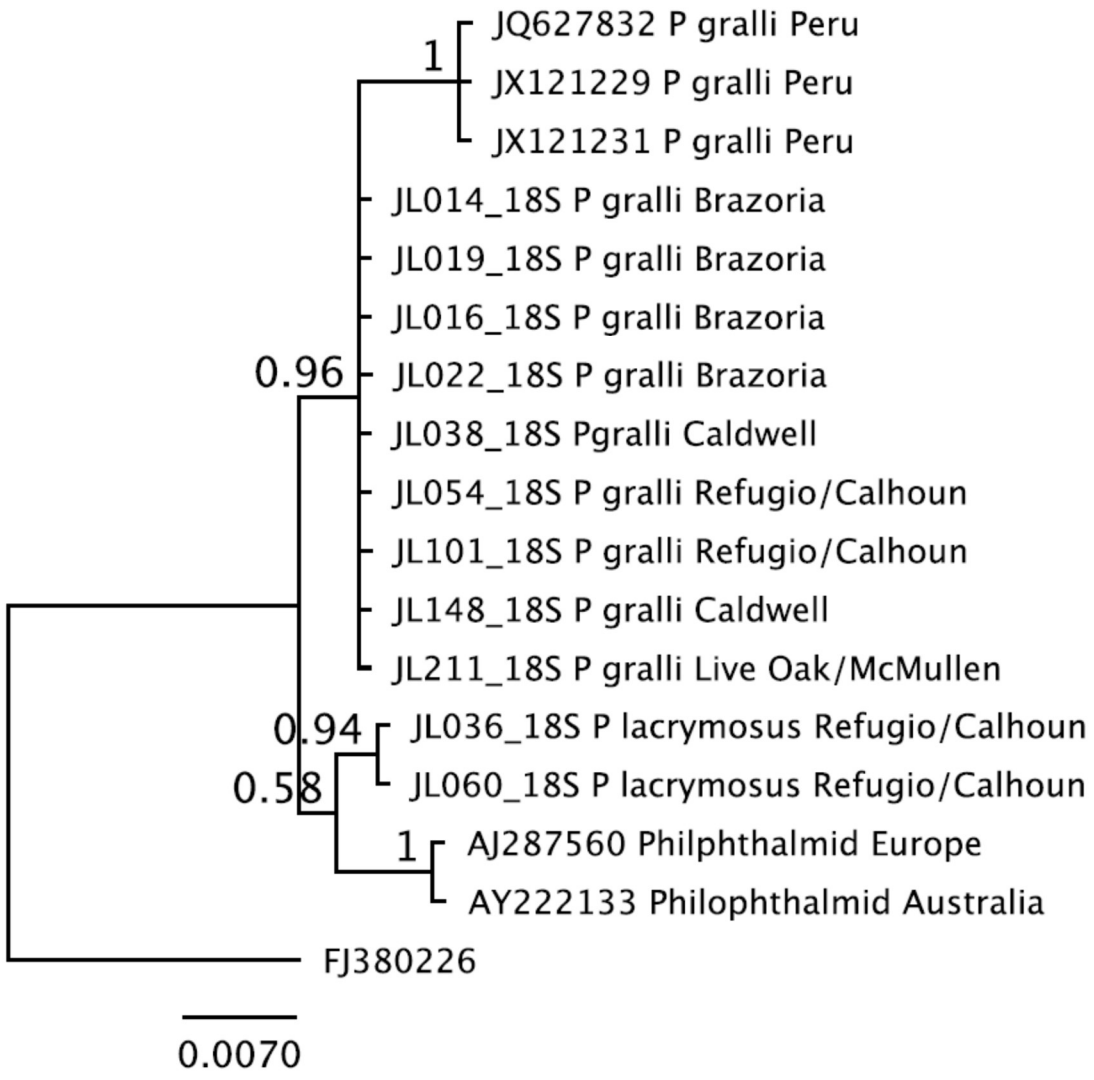

Figure 8. Phylogenetic relationship of *Philophthalmus* for the 18S nuclear region using *Acanthoparyphium* sp. as the outgroup.

Substrate Selection Experiment

Naturally Occurring Cysts

We found *Philophthalmus* metacercariae naturally occurring on substrates collected from the Comal River: 6 of 42 crayfish (14%), 4 of 148 mayfly larva (8.3%), 3 of 57 dragonfly larva (2.9%), 3 of 104 *E. comalensis* (2.2%), and 12 of 378 *M. tuberculata* (8.1%). Since water stargrass (one of the experimental substrates) did not

occur in the substrate-collections sites of the Comal River, *Sagittaria* spp. was used as a surrogate to look for naturally occurring cysts in the Comal River, and no cysts were found on any of the hundred or so leaves examined among the 12 plants collected from the Comal River.

Experimental Cysts

I recovered a total of 335 cysts from the experimental substrates and only 22 from the aquaria walls. Before correcting for surface area, *M. tuberculata* had the highest number of cysts (147), followed by water stargrass (129), crayfish (41), dragonfly larvae (11), mayfly larvae (4), and *Elimia comalensis* (3). The top level of the aquaria had the most cysts (216) and the middle and bottom levels both had 60 cysts.

After correcting for surface area and estimating the density of cysts, the 2-Factor ANOVA calculated the following F-rations from the observed data; Depth = $F_{\alpha(1),2,520}$=18.315, Substrate Type = $F_{\alpha(1),5,520}$ = 10.468, and Interaction = $F_{\alpha(1),10,520}$ = 7.16. The *p*-values estimated from the randomizations were; Depth $p(F_{\alpha(1),2,520} \geq 18.315)$ = 0.0001, Substrate Type, and Interaction = $p(F_{\alpha(1),10,520} \geq 7.16)$ = 0.0001. Since depth and substrate type interacted significantly, mean densities with the confidence intervals for each substrate type at each depth are displayed in Figure 9 and show that water stargrass at the top of the tank had the highest cyst density (mean=0.81/cm^2, SE=1.07).

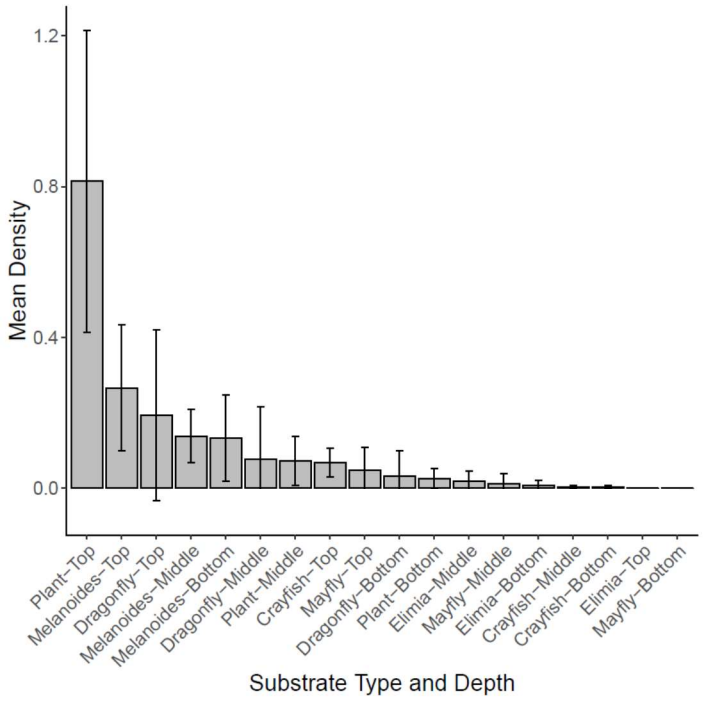

Figure 9. Type and depth of substrates rank-ordered by mean density of cysts.

Cercarial Behavior

When cercariae first emerge from a snail, they refused to attach to anything at first, and engage in one of two behavioral patterns. The most common behavior observed (35 of the 42 studied cercariae) was an alternating Hanging/Undulating behavior (Figure 10). This pattern involved the cercaria first swimming to the surface of the aquarium. Once at the surface, the cercaria would hang upside-down with the tail apparently attached to the surface film, presumably by the adhesion glands on the tail. Then, the cercariae would begin to undulate horizontally in one direction for a few seconds, return to rest hanging vertically for a few seconds, then undulate horizontally again for a few seconds in a different radial direction, etc.

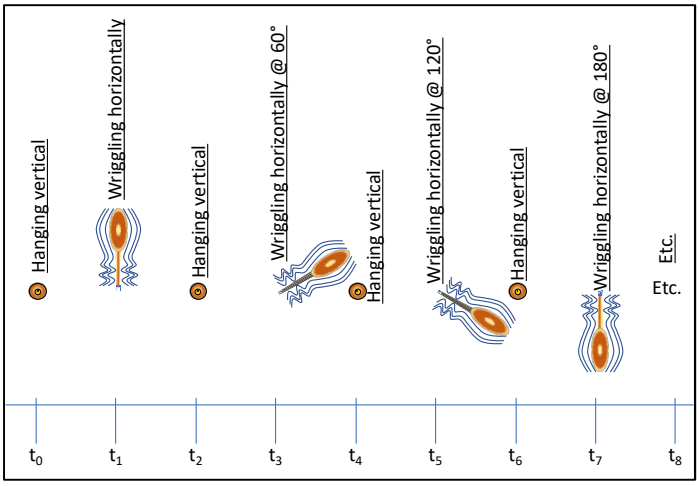

Figure 10. Vertical view of Hanging/Undulating type of cercarial behavior showing how cercariae hang for a few seconds from the surface film (t_0, t_2, t_4, t_6) and then start undulating the body up and down while still hanging from the tail (t_1, t_3, t_5, t_7).

The second behavior observed (7 of the 42 studied cercariae) involved the cercaria swimming across the water column in a at the same average depth while engaging in an up-and-down Zig-Zag pattern (Figure 11). This involved a directional swimming upwards for 8-10 cm, followed by directional swimming downwards for 8-10 cm, all the while moving in a circular pattern around the circular container the container. In one period of observation lasting for about 90 minutes, three cercariae were consistently moving up and down in this fashion while moving around their container in a more or less circular pattern. They seemed to be avoiding the boundary layer next to the glass, and may have swam in more of a straight line in open water.

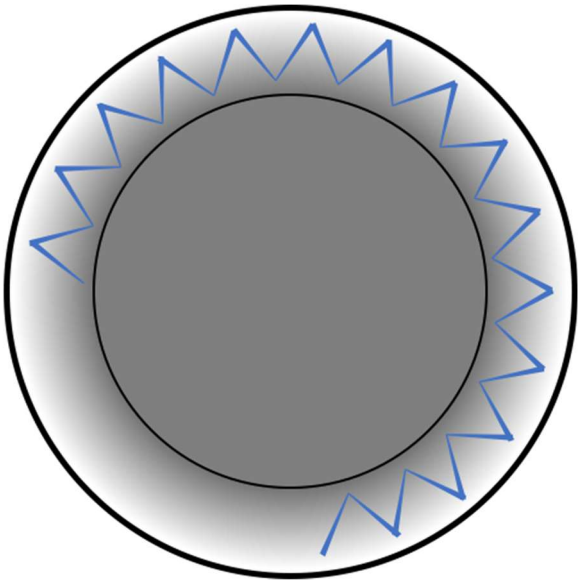

Figure 11. Schematic vertical view of circular aquarium showing Zig-Zag cercarial behavior pattern in which cercaria would move in a circle close to the wall while moving up and down in depth (vertical scale exaggerated).

Snail Co-infection Experiment

Naturally Occurring Infections

Collected Snails

A total of 396 snails (*Melanoides tuberculata*) were collected from Landa Lake between October 5th, 2018 and October 12th, 2018. Fifty-four (14%) of the snails were naturally infected with *Centrocestus formosanus* (Nishigori, 1924), 117 (30%) were naturally infected with *Haplorchis pumilio* (Looss, 1896), and 26 (7%) were naturally infected with *Philophthalmus gralli.*

Infected Waterfowl

The heads of an additional 55 waterfowl were donated by three groups of sport hunters between January 5th, 2019 and January 26th 2019. Four of these ducks were infected with a total of six adult *P. gralli* (7.2% prevalence, 1.5/eye mean intensity, 0.57 standard deviation).

Philophthalmus gralli Exposures

Exposure of Snails to P. gralli

The miracidia recovered from the six worms were sufficient to expose a total of 41 snails, but the actual exposure rates varied between 5 miracidia per snail to 15 per snail. The miracidia were used to expose 18 snails that were already infected with *C. formosanus*, 17 that were already infected with *H. pumilio*, and 16 that were uninfected. Subsequent inspection of the water in which the exposures took place resulted in the recovery of a total of three left-over miracidia, one each from three different snails, and I assumed that the remainder of the miracidia penetrated the targeted snail.

Success of Exposure

Dissection of all the treatment and control snails revealed that none (0%) of the snails exposed to *P. gralli* miracidia showed any evidence of infection with the parasite, even the snails that were uninfected when exposed.

Mortality

Of the 116 snails not exposed to *P. gralli* miracidia, 12 had died prior to the end of the 120-d shedding period. The dead snails included one (3%) in the *C. formosanus* group, two (7%) in the *H. pumilio* group, and nine (16%) in the uninfected group. Some of the 51 snails exposed to *P. gralli* miracidia had died before the end of the 120-d shedding period, including three (17%) that were previously infected with *C. formosanus*, two (12%) infected with *H. pumilio*, and five (31%) uninfected snails. A Chi-squared test for independence revealed that the factors of *P. gralli* exposure and natural infection status were independent of mortality ($p[\chi^2_{(df=5)} \geq 8.5007] = 0.1307$).

IV. DISCUSSION

Prevalence of Native P. lacrymosus in NA Waterfowl

The occurrence of philophthalmosis in waterfowl is often described in taxonomic keys as infrequent to rare (MacDonald, 1981); thus, the finding that *Philophthalmus lacrymosus* occurs in Redhead ducks at a high prevalence (40%) was quite surprising. Indeed, while almost half of the 25 sampled Redheads were infected with *P. lacrymosus* with a mean intensity of 7.2, only 2 of 1337 ducks of other species sampled in this study were infected with *P. lacrymosus* – definitely "infrequent to rare" in these other ducks I have no reason to assume that the disparity in prevalence between Redheads and other ducks is the result of any newly discovered distributional phenomenon; however, the possibility of Redheads having a higher susceptibility than other duck species for an unknown reason cannot be discounted. At this time, I conjecture that a more reasonable explanation for this unexpectedly high prevalence would be that the eyes of Redheads have simply been overlooked in previous parasitological studies. Consequently, I suggest that Redheads may be an important and historically overlooked definitive host for *P. lacrymosus* in Texas, despite this host only being present in Texas during the winter months.

Prevalence of Invasive P. gralli in NA Waterfowl

While I only found only 2 of 1,337 examined ducks (excluding Redheads) infected with the native *P. lacrymosus*, 161 of these 1,337 ducks were infected with the exotic *P. gralli*. Notably, probably none of these 161 ducks would have been infected with *P. gralli* had *Melanoides tuberculata* not been introduced into Western Hemisphere in the 20th Century. Prior to that introduction, while *P. lacrymosus* was probably infecting

Redheads at the current rate, the likelihood of other ducks in the Central Flyway contracting philophthalmosis on their wintering grounds was almost zero. Unfortunately, the invasive snail now occurs in all major flyways of NA, and almost all ducks in NA are being exposed to increasing levels of philophthalmosis gralli as *M. tuberculata* continues to spread into novel habitats frequented by ducks.

An enlightening perspective on the disparity between the two philophthalmids in Redheads vs. the other ducks can be obtained by contrasting the habitat preferences of the two snail hosts, and also the salinity intolerance of the extramolluscan stages of *P. gralli*. *Philophthalmus lacrymosus* uses a marine gastropod, *Lampanella minima* Gmelin, 1791, as the intermediate host (Gibson et al. 2005), and the extramolluscan stages would then be in marine to brackish water. The salinity tolerance limits of the extramolluscan stages of *P. lacrymosus* have not been reported, and so I cannot speculate on whether *P. lacrymosus* cercariae could survive in freshwater long enough to complete the life cycle. However, the miracidium from a *P. lacrymosus* egg deposited into freshwater is certain to perish for lack of a compatible snail host. Thus, any North American bird infected with *P. lacrymosus* probably acquired the infections while feeding in the estuaries of the Gulf of Mexico.

While the snail host for *P. gralli* in North America *(M. tuberculata*) has been reported from marine environments (Murray et al. 2008), the extramolluscan larvae of *P. gralli* can only survive in freshwater habitats. Indeed, miracidia of the species cannot survive at salinities of 2.6%, and hatchability of the eggs decreases to 40% at a salinity as dilute as 0.9% (Nollen et al. 1979). So, any North American duck that is infected with *P. gralli* necessarily acquired the worms while feeding in freshwater, and any eggs shed

from such ducks into marine waters will not be able to infect *M. tuberculata*, even if it occurs nearby.

Presumed Consequences of P. gralli Introduction

Impact on Overwintering Success

I derived these data from birds that had recently arrived in Texas during their southward migration, and most of them probably did not have philophthalmosis upon arrival in Texas (Table 5). In general, birds that had younger worms tended to have lower pathology ratings than birds with older worms, and this trend progressed up through worms as old as 50 d. However, there was a notable drop in the mean pathology ratings of birds hosting worms greater than 51 d old (age category 7), and this was accompanied by a drop in mean intensity. The apparent drop in mean intensity with age of oldest worm was unexpected, because birds with older worms should have acquired the worm early in the season and should have been acquiring additional worms for several months; thus, they should have even higher mean intensity or at the very least a similar mean intensity. One possible explanation for this departure from expectation is that many of the worms die before reaching an age greater than 51 days. This does not seem likely because many experimental exposures of birds to the parasite report no difference in survivability between 14 and 34 d old worms (Nollen 1989). Those experimental results would suggest that once the worm successfully migrates to the conjunctival sac, it has a good probability completing its expected life span. Another explanation for the reversal of the trend could be that birds with older worms and the expected higher mean intensity predicted from the trend, were so compromised by the pathology that they did not survive to be harvested late in the season. This seems more reasonable, since studies of other host/parasite

systems have indicated that harvesting hosts from the wild provides an estimate of parasite impact that is at least an order of magnitude lower than estimates of impact based on caged host studies or estimates based on an assessment of infection pressure (Fleming et al. 2011, Cantu et al. 2013).

Impact on Northward Migration

The majority of the birds examined in our study were harvested well before the beginning of the northward migration; so, our pathology findings probably underestimate the magnitude of the impact that *P. gralli* has on these birds with inflamed eyes when they begin dealing with the demands of northward migration. Since the likelihood of successful migration relies heavily on the eyes, and since the impact estimates from this study are based on birds that would have been acquiring parasites for another month or two before heading north, one can assume that the introduction of *P. gralli* is most likely having nontrivial effects upon the success of the northward migration, and that the total impact of *P. gralli* on NA waterfowl is likely to increase as the parasite continues to spread.

Overall Impact

While some of the findings pertaining to impacts on individual infected birds may seem alarming, it is important to remember that the parasite was found at a low prevalence waterfowl at most sampling locations. Therefore, it would be unreasonable to argue from these data that the parasite is having major population-level or flyway-level impacts at this time. However, if the prevalence of *P. gralli* in North American waterfowl increases, which usually happens with invasive parasites, one could expect the toll taken by this parasite to become a management issue. This would be especially be true if the

cold-water tolerant morph of *Melanoides tuberculata* continues to expand into additional waterways frequented by waterfowl. If that particular clone continues to spread, high prevalences of *P. gralli*, such as I found in ducks from Choke Canyon Reservoir (55.9%), may become more common. Unfortunately, the dynamics of this host-parasite system are not well known, and inferences in this regard remain speculative at best.

The Dynamics of the Invasion

Multiple Introductions

Based on published reports and the phylogenetic analyses in this study, the spread of *P. gralli* in North America most likely occurred through multiple introductions to the U.S. and the Western Hemisphere. If the worms introduced into Texas in 1968 and subsequently transplanted to Landa Park Lake in 1974 had been responsible for the worms at the veld exhibit in a Florida zoo 6 y later, why had the worms not traveled 30 km north from Landa Lake to the San Marcos River in 4 y?

Genetic Diversity of the Texas Population

Evidence of multiple introductions, beyond the inconsistencies with the geographical spread, can also be found in the unexpectedly high genetic diversity of the Texas populations. If all Texas *P. gralli* worms had been derived from worms brought once to the San Antonio Zoo in a few birds from Hawaii, one would expect such a bottleneck event just 40 y ago to still be suppressing genetic diversity in the descendant populations. Quite the contrary, the diversity of haplotypes recovered from Texas worms (7 COI haplotypes among 15 worms) suggests that *P. gralli* has found its way to the U.S. more than once.

Post-introduction Spread

Many other species of bird hosts can contribute to the spread of *P. gralli*, as evidenced by the extensive list of known definitive hosts. Thus, the parasite does not need waterfowl for its continued spread. This means that introductions to areas in the Western Hemisphere outside of Texas will likely result in the species being brought to Texas during migration events. Texas serves as a major nesting area for many bird species, thus conditions here are suitable for year-round maintenance of its life cycle.

Pathological Responses Among the Study Birds

An inspection of Table 4 reveals what seems to be a pattern of differences between the sampled host species regarding their pathological responses to *P. gralli*, even at the generic level (*Anas* vs. *Dendrocygna*). However, our pathology data were based on samples of naturally occurring infections rather than experimentally controlled infections. Thus, the various other potential interactions, such as host species and intensity and age of infection, could not be accounted for, and so the independent impacts of any one of these factors could not be reliably teased out independently from the others. Thus, it is virtually impossible to draw definitive conclusions regarding host-specific differences in pathological responses from these data.

Substrate Selection by Cercariae

The Role of Diet in the Spread from Point of Introduction

Evidence of selection for water stargrass as encystment substrate by *P. gralli* cercariae does suggest that anatids are major contributors to the spread of the parasite, relative to other more carnivorous birds feeding in waterways. However, the observation of naturally occurring *P. gralli* metacercariae on crayfish, dragonfly larva, mayfly larva,

and gastropods means that many insectivorous aquatic birds are also contributing to sustaining and spreading the parasite. This evidence also suggests that the parasite may be impacting many other migratory and non-migratory birds in Texas. It is also possible that the non-migratory birds are being impacted more by the parasite and it would be prudent to investigate the impact *P. gralli* is having on those populations.

Why Do Cysts Occur on Invertebrates?

Selection of *P. gralli* cercariae for water stargrass is interesting because that brings the cercaria into a position in the water column that increases the likelihood of it encountering an invertebrate. Emergent plants, such as water stargrass, serve as habitat for many invertebrates, and since the cercariae of *P. gralli* usually seek the surface of the water column and will seemingly encyst on the first suitable substrate they encounter, selecting for such emergent vegetation as encystment substrate serves to increase the likelihood of encountering an invertebrate on which the cercariae can encyst. This would also serve to increase the diversity of animals that can serve as definitive hosts; thus, increasing the impacts of the parasite on the native avian fauna.

Cercarial Behavior

The most commonly observed pattern of cercarial behavior was the radially undulating pattern (Figure 10), which keeps a cercaria continually at the surface of the water. This behavior places a cercaria at the position in the water column where it would be most likely to encounter vegetation at the water surface where many waterfowl species will be feeding. Wriggling at the surface of the water would also increase the likelihood of being ingested by a potential host prior to encystment. Many waterfowl and other aquatic birds will feed by pecking invertebrates at the surface of the water or sieving the

water at the surface and could be ingesting cercariae before they have an opportunity to encyst on a suitable substrate. Since the cercariae do not develop any further after forming a cyst (Alicata and Noda 1960, Díaz et al. 2002) and yet are still infective, it is likely they are also infective as free-swimming cercariae. If so, the radially undulating behavior would also explain the reports of *P. gralli* infecting birds that are not known to feed in the water because the parasite would be able to infect a definitive host if it is ingested while a suitable host is drinking.

Interestingly, despite hours of observation, not a single cercaria was observed switching from one of these behaviors to the other. Thus, it appears that the unexpectedly high genetic diversity observed in *P. gralli* from Texas may indicate that the two behavioral patterns are behavioral polymorphisms associated with distinct strains of *P. gralli* from different introduction events.

Also interesting is the fact that, while numerous workers have reported incidentally noticed patterns of cercarial swimming in *Philophthalmus*, neither of these patterns, that were so obvious in my study, has been found in the literature I have seen. However, most people observing cercarial behavior are observing them in a shallow dish under a microscope, and the cercariae under these conditions and at such low Reynold's numbers are constrained by the viscosity of the surface and boundary layer, while the cercariae in our study that exhibited the zig-zag pattern seemed to be avoiding the boundary layer, and swam in a circular pattern near the periphery of the circular aquaria.

The presence of two distinctly different cercarial behaviors of *P. gralli* in Texas, is consistent with the argument for multiple introductions. There are reports of distinctly different cercarial behaviors within *Schistosoma mansoni* Sambon, 1907 in response to

differences in the dominant definitive host (Theron 1984). If multiple introductions have occurred for *P. gralli*, then it is possible that the behavioral differences I observed in these cercariae are a result of behavioral differences in the populations of origin. The two different behaviors might also increase the diversity of available definitive hosts.

Issues With the Snail Co-infection Study

The finding that none of the snails that had been exposed to *P. gralli* miracidia in our co-infection experiment resulted in successful infection of the snails was unexpected and initially disappointing. However, a systematic review of the experimental design and the constraints that had been imposed on the experiment by availability of infected snails and ducks revealed an interesting conjecture that should be experimentally investigated.

The exposure protocol used in the co-infection experiment had tested before the experiment was launched in order to make sure the protocol would produce viable infections in uninfected snails. A student, who was examining the conjunctival sac of a fresh, locally harvested Wood Duck (*Aix sponsa* Linnaeus, 1758), had discovered a single specimen of *P. gralli*. The specimen was shedding eggs, which allowed us to expose six uninfected *Melanoides tuberculata* to about 10 to 15 miracidia each from the student's specimen. The snails were incubated 4 months and then dissected for evidence of infection. Three of the snails died before the 4 months had passed, and of the remaining three, two were infected with *P. gralli*. Based on these exploratory results, and assuming that some of the dead snails may have died from over exposure, I expected that an adequate number of the co-infection snails exposed to *P. gralli* miracidia would also become infected with *P. gralli*. To recap our experimental procedure used in the co-

infection experiment, I used an exposure protocol reported to be successful in the literature (Alicata and Noda 1960, Alicata 1962)

(Alicata and Noda 1960, Alicata 1962, Díaz et al. 2002), and which I had successfully tested in our lab, to expose 51 snails to at least five miracidia from multiple ducks collected at three different times; yet, not even one of the snails became infected.

One possible explanation for the failure to infect is that all the philophthalmids that produced the miracidia might not have been *P. gralli* but another *Philophthalmus* species that can't infect *M. tuberculata*. This is not likely, however, because before experimental miracidia were transferred to the containers holding the snails to be infected, the adult worm was examined as wet mounts and the morphology was observed to be consistent with descriptions of *P. gralli*. No doubt one could find several other explanations as to why the exposed snails failed to become infected, but the success of the exploratory tests of this protocol would most likely discredit any explanation involving problems with the protocol. Indeed, a thorough re-examination of the experiment, coupled with findings from other aspects of this study, led to a potential explanation that may have management implications.

The Source of Experimental Miracidia

In order to generate enough miracidia to infect the treatment snails I had to acquire enough heads of infected ducks before the hunting season was over. Since the highest waterfowl prevalence in the distributional survey had been Choke Canyon Reservoir, I decided to make the 2 hr drive down there and beg ducks from hunters and return to the lab and infect snails the same day. The prevalence had dropped off since the

previous year (in response to a dramatic decline in snail density for unknown reasons) and although 55 ducks were returned and examined, only 4 were infected. However, these four ducks yielded six worms which provided enough miracidia to expose 41 snails to at least 5 swimming miracidia each.

The Source of Experimental Snails

Since the density of *Melanoides tuberculata* at Choke Canyon Reservoir had declined so much and the water level had recently risen by about 4 m, we could not find enough snails there to populate the cells of the experiment. Thus, snails for the experiment were obtained

Melanoides tuberculata in Texas

Recent phylogenetic work on *M. tuberculata* from Central Texas has revealed three distinct 16S haplotypes, and phylogenetic analysis suggests that the Texas populations of the snail originated from different regions of Asia and Africa (Harding et al. 2019). One of the haplotypes is of special interest because this haplotype of *M. tuberculat*a has been found thriving in waterways with winter thermal minima that will, for weeks at a time, drop well below the reported critical thermal minimum of *M. tuberculata*. Indeed, our lab seldom finds the H2 haplotype in the thermally stable spring flows—the only habitat where the other two haplotypes can be found, often in exceptionally high densities.

One of the puzzling findings from the distributional survey of *P. gralli* in waterfowl of Texas was that the highest prevalence of the parasite was found at Choke Canyon Reservoir. I expected the highest prevalence in waterfowl to occur in or near

waterways that support dense populations of the intermediate host, *M. tuberculata*; that is, thermally stable spring runs.

The H2 haplotype was discovered by our lab in the summer of 2017 in Choke Canyon Reservoir after noticing an unexpectedly high prevalence of *P. gralli* in waterfowl harvested there. The H2 haplotypes were found to originate from parts of Africa, while the other haplotypes were found to originate from Southeast Asia. Given that the snails recovered from Texas waters originated from different geographic regions separated by such great distances, it is quite possible that there are also differences in susceptibility to the trematodes that can be found in the invasive snails collected in Texas.

Philophthalmus gralli in Texas

As with the snail intermediate host, the *P. gralli* population in Texas is also genetically diverse. The genetic analysis of trematodes I recovered from waterfowl in Texas revealed seven unique haplotypes from the 15 specimens sequenced. It is not possible to determine the origins of these haplotypes, since there is very little genetic information on *P. gralli* from its native range. However, it is know from other reports of *P. gralli* infections that the geographic range of *P. gralli* largely overlaps with the range of one of its snail intermediate hosts, *M. tuberculata*, and it is likely that it shares a degree of genetic variation similar to its snail host (Webster et al. 2004)). It is clear though that much genetic variation in both the parasite and the intermediate host has made it to Texas. Additionally, there is most likely great deal more genetic diversity of *P. gralli* in Texas than was detected in the 15 worms sequenced simply because I found a ratio of haplotypes to specimens of almost 1:2.

Host/parasite Compatibility in Texas

There is empirical support for the co-evolution of host-parasite systems at small scales (Webster et al. 2004). This would suggest that parasite-host systems, such as the *M. tuberculata-P. gralli* system studied here, have adapted to one another in the areas they originated from. Since there is such great genetic diversity of both the snail and the parasite in Texas, it is likely that the genetic diversity I have detected in Texas is a combination of adaptation evolved from the populations of origin.. As such, it is possible that the failure of our miracidia to infect the snails they were exposed to is a result of incompatibility between the strain of *P. gralli* and our experimental snails.

It turns out that the snail morph supporting the high prevalence of *Philophthalmus* in birds from Choke Canyon Reservoir is the H2 haplotype, and the snails I used in the coinfection experiment were all the H1 haplotype. When the findings from our co-infection experiment are considered in light of the findings by Harding et al. (2019), it seems possible that the two different snail haplotypes may not even be able to support the same trematode communities. The snails in the co-infection experiment came from Comal River, Comal County and were assigned the designation H1 by Harding et al. (2019). The adult worms used as a source of miracidia from the study came from Choke Canyon Reservoir, where the only morph of *M. tuberculata* recovered were morphologically assigned to the H2 haplotype. I conclude, then, that the cause of failure in our experiment was an unexpected incompatibility between the strains of *P. gralli* recovered from Choke Canyon Reservoir with the snails of the H1 haplotype.

The question still remains as to why the exploratory experiment that encouraged us to proceed work so well. A feasible answer is that the worm used to infect snails

during the exploratory test of our infection protocol came from a definitive host harvested in Caldwell County, where both H1 and H2 haplotypes can be found. It could very well be that the miracidia used during the exploratory investigation of our protocol had developed from a cercaria that emerged from a H1 snail, and thus, was compatible with the H1 snails used in our exploratory experiment.

As I was considering the design issues for the co-infection experiment, I was encouraged to proceed by the success of our exploratory proof-of-concept experiment. However, I was unaware of a cryptic but critical issue regarding the selection criteria for the experimental subjects. While our exploratory experiment involved extracting eggs from adult trematodes from the eyes of a locally collected bird and using them to infect locally collected snails, our coinfection experiment was going to require many more hunter-donated birds than I could obtain locally. So, I chose Choke Canyon Reservoir for a source of infected birds, because I knew it had a high prevalence of birds infected with *Philophthalmus*, and chose Landa Lake for a source of heterophyid-infected snails. I was aware that the only *M. tuberculata* morph that populated Choke Canyon Reservoir where I was getting the adult worms was the cold-water tolerant H2 morph, and that the dominant snail morph in Landa Lake, where I collected the snails for the coinfection study was the cold-water sensitive H1 morph. However, I never considered that the birds at Choke Canyon Reservoir might be harboring a different strain of *Philophthalmus* that was adapted to the cold-water tolerant H2 snail morph but also incompatible with the cold-water sensitive H1 morph I had collected for co-infection.

If it is the case that the two haplotypes are not susceptible to the same parasites, then snails obtained from Choke Canyon Reservoir would not have been suitable for the

experiment because none would have been naturally infected with either of the two heterophyids. Indeed, in our examinations of thousands of *M. tuberculata* from many Texas habitats, I have found no natural infections of either *H. pumilio* or *C. formosanus* in the H2 haplotype, even though I have found about 2% of the H2 snails in some locations infected with philophthalmids. Another observation that corroborates our conjecture that the H1 and H2 haplotypes differ in their susceptibilities to the heterophyids is that none of the 20 fish that were necropsied from Choke Canyon Reservoir had even a single cyst of either *Haplorchis* or *Centrocestus* even though there had been a very high density of snails and numerous herons in the sampled area of the lake.

Future work

In order for any future attempts at investigating the relationship between the intramolluscan stages of *P. gralli* with those of the two heterophyids to be successful in the future, some additional steps must be taken. The first step would be to develop in vitro strains of at least *P. gralli* but ideally all three of the parasites investigated during the experiment and a development of laboratory strains of *M. tuberculata*. Development of a laboratory strain of *M. tuberculata* would be straight-forward because the snail is parthenogenetic and can thrive in conditions common in household aquaria. In vitro laboratory strain of the trematodes would be much more difficult, but possible. While I am not aware of any attempts to develop laboratory strains of *P. gralli*, *C. formosanus*, or *H. pumilio*, there are examples of other trematodes grown in vitro (Basch et al. 1973, Chaithong et al. 2001, Fredensborg and Poulin 2005, Pung et al. 2009). Additionally, increased and updated techniques for culturing trematodes in vitro would make it possible

for a greater number of institutions to conduct parasitological experiments especially if the institution lacks the necessary facilities to maintain live vertebrate hosts. Culturing a trematode in vitro will certainly have, at the very least, genus specific challenges. None the less, producing such cultures will be necessary for better understanding host-parasite interactions in wildlife, and specifically understanding the interactions examined in this study.

In order for the experimental interactions of the two heterophyids and *P. gralli* to be meaningful when applied to the natural settings found in Texas, cross exposures of the different wild strains of both host and parasite will also be necessary. This would mean exposing H2 snails to H2 derived miracidia and H1 derived miracidia and exposing H1 snails to H1 derived miracidia and to H2 derived miracidia for all three trematode species if possible. A cross exposure experiment would also aid in better understanding the host-parasite system that may lead to better management plans for the snail and its associated trematodes.

V. CONCLUSIONS

The invasive trematode *Philophthalmus gralli* has been able to invade most of Texas and has been spread to the state more than once. The survey revealed the parasite can occur at a high prevalence and has the potential to impact the health of waterfowl populations that winter in Texas. The selection by cercariae, survey of naturally occurring cysts, and observed behavior of the cercariae reveals that many other species of aquatic birds are also at risk from the parasite. Additionally, the evidence for multiple introductions and multiple strains of the parasite could mean that any attempts at creating a management plan for the parasite may have to be done at small scale, local levels based on the dominant strain of the parasite.

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