INVASIVE HETEROPHYID TREMATODES
AND THEIR NATIVE AQUATIC HOSTS
IN TEXAS
by
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1. EXOTIC SNAIL-TREMATODE SYSTEM

Historical Background of Exotic Snail Invasions

The current globalization paradigm, and mankind’s increased ease of passage across the landscape, has brought many exotic species of flora and fauna to sensitive environments where these invasives often thrive virtually unrestrained, even outcompeting native species. In the United States alone, at least 50,000 exotic species have been recorded as introduced (Pimentel, et al., 2005). Freshwater ecosystems are among the communities most adversely affected by these invasions, as they often contain endemic species, and have higher rates of extinction when compared to marine or terrestrial ecosystems (Dudgeon, et al., 2006; Ricciardi and Rasmussen, 1998). These exotic species can cause extensive alterations of community structure and ecosystem function within aquatic systems (Chapin, et al., 1997). Aquatic invaders can directly compete with, prey upon, or parasitize native species (Johnson, et al., 2009).

Among the freshwater exotics, gastropods are the most common group with high invasion success, often being introduced through the aquarium trade (Padilla and Williams, 2004). In Texas, five species of exotic gastropod are known to have reproducing populations, representing at least 50% of the known exotic freshwater invertebrates occurring in the state (Karatayev, et al., 2009). However, this figure does not take into account exotic parasites associated with these gastropods, which would greatly inflate the number of aquatic invasive invertebrates in Texas. Many mollusk species, especially snails, have a unique host-parasite relationship with a community of helminths known as the digenetic trematodes. Nearly all digenetic trematodes utilize a snail as a first intermediate host in their complex, two- or three-host lifecycles (Haseeb
and Fried, 1997). Several exotic trematode species are now known to occur in Texas (one reported herein from Texas fishes for the first time), all having been introduced following the establishment of their exotic thiarid snail hosts. Because these trematodes have a high level of host-specificity for their first intermediate snail host, one cannot examine the life history and geographical distribution of these exotic parasites without first understanding the invasion history of the snails. Two species of exotic snail introduced into Texas, *Melanoides tuberculata* and *Tarebia granifera*, are of serious concern to wildlife and human health as they are first intermediate hosts for multiple species of digenetic trematode parasites, several of which are the source of major human health problems in Asia (Pinto and de Melo, 2011).

*Melanoides tuberculata* is thought to have been introduced to the USA through the aquarium trade sometime prior to 1950 (Murray, 1971) and *Tarebia granifera* was introduced to the USA perhaps as early as 1935 (Nollen and Murray, 1978). Populations of both species of snail were first reported in Texas in 1964, when the snails were found in the San Antonio River, Bexar County (Murray, 1964). Both species were later reported in Landa Lake, New Braunfels, Texas (Murray and Woopschall, 1965). Since that time, both species of snail have been reported in multiple spring-fed systems throughout Texas (Karatayev, et al., 2009) and the USA (Benson and Neilson, 2013).

The range of thermal tolerance for *M. tuberculata* has been experimentally determined to be between 18° and 32° C (Mitchell and Brandt, 2005), and the range of thermal tolerance for *T. granifera* has been reported at being between 10° and 38° C (Karatayev, et al., 2009). As a result, these snails (especially *M. tuberculata*) are unable to establish reproducing populations in most freshwater systems of North America,
because the winter thermal minima of most of these systems often remain below 18˚C long enough to begin killing adult snails. Conversely, these tropical exotics have been thriving for years since their introduction into many perennial spring systems in several states of the USA (Brune, 1981; Hubbs, 2001; Mitchell and Brandt, 2005), because the thermal stability of these systems usually keeps the seasonal swings in water temperature well within an acceptable range for the snails.

The introduction and establishment of these snail species first became a serious concern when a species of avian eyefluke was found parasitizing waterfowl in the San Antonio zoo, subsequently identified as Philophthalmus gralli (Murray and Haines, 1969; Nollen and Murray, 1978). Later, two additional species of trematodes were discovered in Texas, the first being Centrocestus formosanus, a heterophyid trematode which parasitizes the gills of fish (Mitchell, et al., 2000; Mitchell, et al., 2005). The second trematode species identified was Haplorchis pumilio (also a heterophyid), whose larval stages were found parasitizing M. tuberculata and T. granifera (Tolley-Jordan and Owen, 2008). Although C. formosanus has received considerable attention in Texas, little is known about H. pumilio north of Mexico.

**Life Cycle of Centrocestus formosanus and Haplorchis pumilio**

*Centrocestus formosanus* (Nishigori, 1924) and *Haplorchis pumilio* (Looss, 1896) are trematodes of the family Heterophyidae. *Centrocestus formosanus* was first described from fishes and birds in Taiwan (then called Formosa). This trematode is now known to have a wide distribution through Asia (Chen, 1942; Madhavi, 1986; Premvati and Pande, 1974; Yanohara, 1985), and has since been introduced into North America, perhaps as early as 1980 (Blazer and Gratzek, 1985). *Haplorchis pumilio* was first
described from birds in Egypt. This parasite also now has a worldwide distribution as it has been reported from Africa (Sommerville, 1982a), Israel (Witenberg, 1929), India (Umadevi and Madhavi, 2006), China and East Asia (Chen, 1936; Chung, et al., 2011; Lo and Lee, 1996a; Shen, 1959), Southeast Asia (Chai, et al., 2012; Kay, et al., 2009; Pearson and Ow-Yang, 1982; Radomyos, et al., 1998), Australia (Pearson, 1964), Venezuela (Díaz, et al., 2008), Mexico (Scholz, et al., 2001), and the USA (Tolley-Jordan and Owen, 2008; snail stages only).

The definitive hosts for *Centrocestus formosanus* are typically piscivorous birds, and in Texas, the Green Heron (*Butorides virescens*) apparently serves as the functional definitive host (Kuhlman, 2007). When an infected heron defecates into the water, eggs of the adult trematodes are passed with the feces. Eggs are either consumed by first intermediate snail hosts, or the egg hatches and release free-swimming miracidia (1st larval stage) into the water column (Haseeb and Fried, 1997). These miracidia either hatch from eggs consumed by the first intermediate snail host (*Melanoides tuberculata*) and penetrate the gut wall, or become free swimming and then find and penetrate the snail epidermis. Once within the snail, the miracidium migrates to the digestive gland of the snail where subsequent larval stages (known as “parthenitae”) reproduce and multiply asexually. There, the miracidium metamorphoses into a sporocyst (2nd larval stage), which later releases multiple rediae (3rd larval stage) through asexual reproduction (Haseeb and Fried, 1997). As rediae mature, they in turn release many free-swimming cercariae (4th larval stage), which escape from the snail host through its excurrent siphon (Haseeb and Fried, 1997). These cercariae swim toward light with their whip-like tail, which keeps them suspended in the water column, and some are incidentally inhaled with
the respiratory current of a fish. Upon contact with the gills of a susceptible fish, a
cercaria sheds its tail, penetrates the gill epithelium, and migrates to the gill cartilage
where it encysts as an “immature” metacercaria (5th larval stage). In a suitable fish
species, each metacercaria then progresses through several developmental phases,
culminating several weeks later in the “mature” metacercaria that is infective to the
definitive host. When a fish that is hosting mature metacercariae is consumed by a
definitive host, the metacercariae transform into the adult stage and migrate to the rectum
where they lay eggs that are passed out with the bird’s feces (Martin, 1958).

Adult flukes of *Haplorchis pumilio* have been found inhabiting the digestive
tracts of mammals, birds and reptiles (Díaz, et al., 2008; Sommerville, 1982a; Umadevi
and Madhavi, 2006). When an infected definitive host defecates into the water, eggs are
passed with the feces. The parthenitae of *H. pumilio* within the snail hosts are similar to
those of *C. formosanus*; however, obligatory first intermediate snail hosts can be either
*Tarebia granifera* or *Melanoides tuberculata* (Tolley-Jordan and Owen, 2008; Umadevi
and Madhavi, 2006). Cercariae of *H. pumilio* are vigorous swimmers, and have a
lifespan of up to 72 hours (Díaz, et al., 2008). When cercariae come into contact with a
susceptible second intermediate fish host, the cercariae actively penetrate the host all over
the body, but primarily in the soft tissue of the fin insertions (Lo and Lee, 1996b;
Sommerville, 1982b). Once penetration is complete, the cercariae drop their tail and
encyst as an immature metacercariae (5th larval stage), which in suitable hosts mature in
15-30 days (Díaz, et al., 2008; Umadevi and Madhavi, 2006). When a fish hosting
mature metacercariae is consumed by a definitive host, the metacercariae develop into the
adult stage, migrate to the intestine and mate. The eggs are then passed with the definitive host’s feces (Haseeb and Fried, 1997).

**History of Centrocestus formosanus and Haplorchis pumilio**

The first official reports of *C. formosanus* in continental America came from a fish farm near Tezontepec de Aldama in the state of Hidalgo, Mexico (López-Jiménez, 1987), where *C. formosanus* metacercariae were reported encysted on the gills of black carp (*Mylopharyngodon piceus*) recently imported from Asia. López-Jimenez assumed that *C. formosanus* had been introduced into Mexico via these black carp in 1979; however, subsequent workers speculated (Amaya-Huerta and Almeyda-Artigas, 1994) that the trematode would not likely have become established in the wild fisheries of Mexico by importing brood-sized infected fish, even if uninfected first intermediate hosts, red-rimmed melania snails (*Melanoides tuberculata*), were already present at the farm. Their reasoning was that there would be no way for the metacercariae to enter the gut of the definitive host in Mexico, the Striated Heron (*Butorides striata*). This was because the brood fish were much larger than could be consumed by these small herons, and any snails already in the area would remain uninfected without the metacercariae entering the definitive host to develop into adults, and later defecate eggs into the water. They therefore speculated that infected *Melanoides tuberculata* must have also been imported from Asia by the farmers to culture as food for the malacophagous black carp, and it was therefore not the importation of adult carp which first introduced the parasite into the wild fisheries of the Western Hemisphere, but the importation of infected snails.

Most reports of *H. pumilio* in the USA have been incidental, with the parasite being recorded as present during studies involving the gill trematode *Centrocestus*
Cercariae of an unidentified *Haplorchis* species were reported as early as 2000 in west Texas springs (McDermott, 2000). A report from Utah also included the discovery of *Haplorchis* species cercariae from *M. tuberculata* (Harvey, et al., 2005). Furthermore, *H. pumilio* has been found in snails in the Comal River in New Braunfels, Texas (Tolley-Jordan and Owen, 2008), and cercariae have been collected from the water column using filtration techniques (Cantu, et al., 2013; Johnson, et al., 2012). *Haplorchis pumilio* is also present in the San Marcos River (Hays County, Texas), and San Felipe Springs (Val Verde County, Texas; personal obs.). It is likely that *H. pumilio* has become established in many of the aquatic systems in the United States that have reproducing populations of *M. tuberculata* and *T. granifera*. As of yet, there have been no published reports of *H. pumilio* metacercariae infecting second intermediate hosts in Texas or in the continental USA. Migratory Green Herons (*Butorides virescens*) are theorized to have brought the trematode *C. formosanus* into Texas from Mexico (Kuhlman, 2007), and this is the likely route of establishment for *H. pumilio*. However, there have not been any reports of definitive hosts of *H. pumilio* in the continental United States.

**Pathology of Centrocestus formosanus and Haplorchis pumilio**

Approximately 50% of the species in a newly exposed fish community are expected to become infected by *C. formosanus* cercariae shed from local *M. tuberculata* (Fleming, et al., 2011; Ortega, et al., 2009; Salgado-Maldonado, et al., 1995; Scholz and Salgado-Maldonado, 2000). So the presence of *M. tuberculata* in Texas spring systems is concerning because many of these spring systems contain federally and state-listed
threatened and endangered fish species (Fleming, et al., 2011; Rader, et al., 2003) whose survival prospects could be further threatened by the introduction of *C. formosanus*.

In some fish species, metacercarial cysts of *C. formosanus* may result in severe pathologies such as edema, hemorrhaging, sloughing of respiratory epithelium, lamellar fusion, and hyperplastic distortion of gill architecture through chondrocytic hyperplasia (Alcaraz, et al., 1999; Blazer and Gratzek, 1985; McDonald, et al., 2006). Gill damage from high intensities of *C. formosanus* metacercariae has been shown to cause mass mortalities in high-density fish populations, such as in fish culture (Mohan, et al., 1999; Ortega, et al., 2009). It is therefore possible that similar mortality rates might be cryptically occurring in fish populations restricted to thermally stable spring-fed systems like those found within the Edwards plateau and Trans Pecos Regions of Texas. This could be especially likely when spring discharge is low, since high metacercarial intensity in fish is known to significantly reduce oxygen uptake (Alcaraz, et al., 1999). It seems likely that progressively increasing intensity of *C. formosanus* infection in a fish would lead to the fish being progressively more vulnerable to predation (via reduced respiratory efficiency), but I have not been able to find reports that have experimentally quantified the effect of *C. formosanus* intensity on predator avoidance.

Invasion by *H. pumilio* cercariae can also have severe pathological consequences for fish hosts. Cercariae penetrate through the skin all over the body of the host, and are known to migrate to preferred locations to encyst. Hemorrhaging in the skeletal muscles has been noted in association with both the penetration and migration of cercariae (Sommerville, 1982b; Umadevi and Madhavi, 2006). Penetration by a large number of cercariae simultaneously has been shown to be lethal to both fry and adults of multiple
fish species, with hemorrhage being the likely cause of mortality (Sommerville, 1982b; Umadevi and Madhavi, 2006). Cercariae encyst primarily in the tissue of the caudal fin insertion of fish hosts, with lesser numbers encysting around the insertion tissue of other fins, the operculum and the cartilage of the head (Sommerville, 1982b). Where clusters of metacercariae occur there is often evidence of nodular formations (Sommerville, 1982b).

*Haplorchis pumilio* has been recorded in humans in Asia (Chung, et al., 2011; Radomyos, et al., 1998). Humans can ingest metacercariae when consuming raw or lightly cooked fish (Díaz, et al., 2008). Metacercariae encysted in fish can also remain viable for up to 3 days of refrigeration (Sommerville, 1982b). Currently, there is a paucity of information regarding the pathology of *H. pumilio* in humans (Kay, et al., 2009), although there have been reports attributing ulcerations and gastrointestinal disturbance to heavy intensities of *Haplorchis* spp. (Chung, et al., 2011).

**Statement of Problem**

*Centrocestus formosanus* is an invasive Asian trematode that infects the gills of many species of freshwater fishes when exposed to its infective cercarial larvae. Mass fish kills caused by *C. formosanus* have been reported by some commercial fisheries practicing high-density monoculture, but despite the extensive gill damage also reported for some wild-caught fishes infected with *C. formosanus*, we can find no reports of fish kills having been attributed to the parasite in the wild. However, we speculate that wild fish with respiratory impairment will be (1) slower in their flight from pursuing predators, and/or (2) more likely to prefer the more oxygen-rich upper layers of the water column than uninfected individuals. Either effect would increase the likelihood that wild fish
gradually accumulating metacercarial cysts on the gills would experience reductions in
their fitness due to parasite-induced respiratory impairment. It reasons that infected fish
occupying the upper portions of the water column would have a greater probability of
being captured by piscivorous predators well before \( C. \text{formosanus} \) killed the host.

*Centrocestus formosanus* has been reported to infect frogs and toads in Asia,
though no reports of amphibians being parasitized by this trematode exist for North
America. It is possible that \( C. \text{formosanus} \) has been infecting native amphibian species
all along, and has gone unnoticed by previous investigators. This is of concern, as many
of the aquatic communities where \( C. \text{formosanus} \) has been reported also contain federally
threatened neotenic salamanders that could be adversely affected by this parasite.

*Haplorchis pumilio* is also an invasive trematode which infects the soft tissue
surrounding the fin insertions of a fish host. Currently, no second intermediate fish hosts
of this parasite have been reported in Texas or in the continental USA, even though first
intermediate snail hosts have long been reported as infected with the parthenitae of the
trematode. Thus, no data exists for the breadth of second intermediate host specificity for
\( H. \text{pumilio} \) in Texas. Furthermore, little data has been generated in regards to the effects
of this parasite on the viability of fish hosts, but it is likely that high intensities of
metacercarial infection in the caudal fin insertion would hinder swimming efficiency in
infected individuals. If this were the case, then fish heavily infected with \( H. \text{pumilio} \)
metacercariae would also be more likely to suffer from morbidity and subsequent
predation.
Project Goals

The goal of this study is three fold. Firstly, we intended to determine if
*C. formosanus* can infect native Texas amphibians, as well as to determine if these
amphibian species can serve as functional hosts for the trematode. The San Marcos
salamander (*Eurycea nana*) is an obligate aquatic salamander endemic to the San Marcos
River in San Marcos Texas. This federally threatened salamander could be affected by
*C. formosanus*, and we intended to determine if this salamander, as well as local leopard
frogs can become infected with the parasite. Secondly, because no data currently exists
as to the second intermediate fish hosts of *H. pumilio*, we intended to examine multiple
threatened and endangered fish specimens from Texas waters in order to begin to build a
list of hosts for future investigations.

Lastly, we intended to develop a preliminary methodology to determine the extent
to which *C. formosanus* and *H. pumilio* infection may be impacting local survival of an
important forage fish (*Cyprinella venusta*), and also a listed minnow (*Dionda diaboli*)
that is currently declining in numbers and range where *Centrocestus formosanus* (and
likely *H. pumilio* as well) now occurs. This will allow us to develop a model that will
vastly improve our ability to estimate the potential negative impact of the spread of these
trematodes into a naïve community of wild fish species. Several experimental objectives
were executed in order to obtain data and experience useful for constructing this
methodology:

1. Construct a swim tunnel that will be capable of testing the swimming endurance
   of small minnows at controlled water velocities.
2. Develop a methodology for artificially infecting minnows at various rates and intensities in order to test these fish for swimming endurance.

3. Develop a methodology for efficient necropsy of these minnows, to enumerate infection intensities.

4. Construct a working model which will be able to provide us with data regarding the following:
   
   o Determine the intensity of *C. formosanus* and *H. pumilio* infection at which the swimming endurance of the target species is reduced to 60% of that of uninfected controls, which would substantially reduce their ability to escape predation.
   
   o Determine the intensity of *C. formosanus* and *H. pumilio* infection at which the target species is affected so drastically that at least 20% can be expected to die within two weeks of exposure from direct effects of the parasite.
   
   o Determine the interaction, when the endurance of an individual is tested, between:
     
     - the rate at which *C. formosanus* and *H. pumilio* metacercariae are acquired by fish at several intensities, and
     
     - the parasite intensity that accumulates at several metacercarial acquisition rates.
   
   o Determine if the endangered *Dionda diaboli* responds differently to *C. formosanus* and *H. pumilio* metacercariae than does the much more common *Cyprinella venusta*. If so, determine:
how these differences in host response may be differentially affecting performance of the two species in the swimming-endurance test, and

- the level of parasite intensity at which the parasite begins to kill infected individuals of both species.

  o For both fish species, estimate the percentage of cercariae introduced into an aquarium (at naturally observed densities) that are able to successfully establish themselves as metacercariae, which would indicate differential susceptibility to infection.

  o Estimate the within-variance in susceptibility between cohorts of the same fish species exposed to the same cercarial density for the same length of time.
2. ARTIFICIAL EXPOSURE OF ADULT SAN MARCOS SALAMANDERS AND
LARVAL LEOPARD FROGS TO THE CERCARIAE OF *CENTROCESTUS
FORMOSANUS*

**Preamble**

At the time of this writing, this manuscript has been accepted for publication in The Journal of Parasitology. The work resulted from a collaboration between Daniel C. Huston, Valentin Cantu and David G. Huffman, and is the order of authorship on the publication. Daniel C. Huston is currently a graduate student at Texas State University as well as a Student Trainee with the United States Fish and Wildlife Service (USFWS). Valentin Cantu is also a Biologist with the USFWS. David G. Huffman is a professor of biology at Texas State University, and Daniel C. Huston’s Major Advisor.

**Abstract**

The gill parasite *Centrocestus formosanus* (Trematoda: Heterophyidae) is an exotic parasite of concern in Texas because it has been shown to infect multiple threatened and endangered fish species. The purpose of this study was to determine if *C. formosanus* could present a threat to larval anurans, as well as threatened neotenic salamanders endemic to the spring-fed systems of Texas. We exposed adults of the San Marcos salamander *Eurycea nana* (Caudata: Plethodontidae) and tadpoles of the Rio Grande leopard frog *Lithobates berlandieri* (Anura: Ranidae) to the cercariae of *C. formosanus*. The San Marcos salamander showed no signs of metacercarial infection suggesting that *E. nana* may be refractory to *C. formosanus* cercariae. *Centrocestus*
formosanus readily infects the gills of leopard frog tadpoles, but the metacercariae apparently died prior to reaching maturity in our tadpoles.

**Introduction**

The Asian trematode Centrocestus formosanus has become established in many spring-fed systems in Texas via the introduction of its obligate first intermediate snail host, the red-rimmed melania Melanoides tuberculata (Mitchell, et al., 2005).

 Centrocestus formosanus metacercariae parasitize a broad range of second intermediate fish hosts, where they are found encysted in the gills (Mitchell, et al., 2005). They also parasitize frogs and toads, where the metacercariae encyst in the stomach wall and skeletal muscles (Chen, 1948; Chen, 1942). In fish hosts, high intensities of C. formosanus metacercariae can lead to gill damage through hemorrhaging, lamellar fusion, and distortion of gill architecture (Alcaraz, et al., 1999; Blazer and Gratzek, 1985). For example, the fountain darter Etheostoma fonticola, a federally endangered fish endemic to central Texas, is particularly susceptible to C. formosanus, and can acquire metacercariae at rates that are fatal (McDonald, et al., 2006; Mitchell, et al., 2000). Previous studies pertaining to C. formosanus in Texas have predominately focused on determining spatial and temporal distribution of cercariae in aquatic systems (Cantu, et al., 2013; Johnson, et al., 2012), as well as identifying the potential negative consequences to second intermediate fish hosts (Fleming, et al., 2011; McDermott, 2000; Mitchell, et al., 2000; Mitchell, et al., 2005). However, it is equally important to examine the potential for infection in other aquatic vertebrates, such as obligate aquatic amphibians.
The upper San Marcos River (Hays County, Texas, 40°53′04″N, 74°03′28″W) is critical habitat for a number of federally endangered and threatened species including the fountain darter *Etheostoma fonticola* and the San Marcos salamander *Eurycea nana* (USFWS, 1996). *Eurycea nana* is an obligate aquatic neotenic salamander which retains its external gills throughout its life. Reduced spring discharge is considered to be the greatest threat to the salamander’s survival (USFWS, 1996). These reduced flows coupled with additional stressors such as parasitic infections, could negatively affect the population of *E. nana*. Trematode infections are known to have the potential for serious pathologies in amphibians. *Ribeiroia ondatrae* has been linked to severe deformities in multiple amphibian hosts, including frogs, newts and salamanders (Johnson, et al., 2002) *Melanoides tuberculata* and *C. formosanus* also occupy the upper reaches of the San Marcos River (Mitchell, et al., 2000) and, if spring flow decreases, exposure of the salamander to cercariae of *C. formosanus* could be intensified.

Effective management and recovery of endangered species requires the identification and reduction of factors that threaten the viability of these populations (Lawler, et al., 2002), and is a primary goal of the recovery plan for the San Marcos River (USFWS, 1996). The potential for the susceptibility of *E. nana* to local *C. formosanus* cercariae became a concern when we found a report of a neotenic *Eurycea* species in Missouri infected with unidentified metacercariae (McAllister, et al., 2006). Therefore, we sought to determine if *C. formosanus* could infect *E. nana*. We also exposed tadpoles of a local frog, *Lithobates berlandieri* to infection, to determine if amphibians with internal gills could serve as functional second intermediate hosts for the trematode.
Materials and Methods

Collections of wild caught *M. tuberculata* were obtained from Landa Lake, (Comal County, Texas, 29°42′28.08″N, 98°7′24.24″W) between January and May of 2013. Snails were placed individually in 8-dram vials filled with de-chlorinated tap water under fluorescent lighting on a 12/12 light/dark cycle. Vials were examined daily for cercariae of *C. formosanus*, and snails found to be shedding were aggregated into a 38-L aquarium. Shedding snails were maintained on commercially available fish flakes until the beginning of the infection trials.

The San Marcos Aquatic Resource Center (SMARC) in San Marcos, Texas, maintains breeding colonies of San Marcos salamanders and fountain darters. The SMARC provided 23 adult San Marcos salamanders (2.9-4.1 cm, SVL) for the experiments, and also provided ten adult fountain darters (3.7-4.9 cm, TL), that were used as controls to ensure that the cercariae used in our trials were capable of infecting susceptible hosts. Twenty tadpoles of the Rio Grande leopard frog *Lithobates berlandieri* (1.2-2.9 cm, SVL) were captured from ponds located on the SMARC grounds. All experiments were performed at the SMARC facility.

Prior to exposing the experimental hosts to cercariae, 70 *M. tuberculata* shedding *C. formosanu*s cercariae were aggregated into a 38-L aquarium filled with fresh well water, and incubated for three days to allow a large number of cercariae to accumulate in the water, as *C. formosanu*s cercariae are known to be viable for up to 50 hrs (Lo and Lee, 1996b). Three 38-L aquaria filled with four L of well water were stocked with experimental hosts. One aquarium was stocked with both ten experimental salamanders, and ten fountain darters that were used as controls to assess the viability of the
experimental cercariae. A second aquarium was stocked with ten salamanders, and the third with 20 tadpoles of *L. berlandieri*. Two L of water containing approximately 30,000 *C. formosanus* cercariae from the snail aquarium were transferred to each of the three treatment aquaria on the morning of 10 June 2013. After two hr of exposure, all ten darters and ten salamanders in the first aquarium as well as ten of the 20 tadpoles in the third aquarium were euthanized with tricaine methanesulfonate (FINQUEL MS-222®; Argent Chemical Laboratories Inc., Redmond, Washington) and fixed in 95% ethanol. The salamanders from the second aquarium and the remaining ten tadpoles from the third aquarium were transferred into separate aquaria containing fresh well water. The animals were housed for 15 and 28 days respectively, during which the tadpoles and salamanders were observed daily for condition and fed fishflakes and bloodworms three times per week.

All gill arches were excised from the fountain darters and examined for metacercariae using a dissecting microscope (5-35x). The gills and gastrointestinal tract of the euthanized tadpoles were excised and examined similarly. Likewise, the gill filaments, skin, flesh, mouth and gastrointestinal tract of the euthanized salamanders were examined for metacercariae.

Because the first ten salamanders had not become infected, an attempt was made to expose the ten experimental salamanders from the second aquarium to a higher density of cercariae at a different time of day. Therefore, in the afternoon of day 15 following the initial exposure, the ten salamanders from the second aquarium were re-exposed individually in 600-ml beakers to approximately 10,000 *C. formosanus* cercariae for one hr. The re-exposed salamanders were then returned to a cercaria-free aquarium for an
additional 12 days. Twenty-eight days following the initial exposure, a time sufficient for the metacercariae to mature (McDermott, 2000; Nishigori, 1924), the remaining salamanders and tadpoles were euthanized and preserved in 95% ethanol, and subsequently examined as above.

Since the group of ten salamanders that were examined after a second exposure to *C. formosanus* cercariae exhibited no signs of infection, a third trial was conducted to determine if water flow was necessary for *C. formosanus* to encyst. Three additional salamanders were placed in a single 1,000-ml beaker and exposed to approximately 10,000 cercariae for two hr, with an air stone to provide gentle water circulation. At the end of the two hr period, one salamander from the beaker was euthanized and preserved in 95% ethanol. The two remaining salamanders were transferred to an aquarium containing fresh well water. After four and eight days respectively, these two salamanders were euthanized and preserved in 95% ethanol, and examined as above.

**Results**

All ten fountain darters and 20 leopard frog tadpoles became infected. Metacercariae were found in the gills of the tadpoles but not in the gastrointestinal tract. Metacercarial intensity (min-max; mean) varied widely in the fountain darters (16-58; 35) and the tadpoles (3-92; 30). Metacercariae found in gills of the ten tadpoles that had been incubated for 28 days post-exposure showed signs of development beyond the initial encystment, (loss of cercarial eye spots), but all metacercariae appeared to have been killed by the host before exhibiting the classical sign of maturity (development of dark “X gland” appearance at excretory bladder) as per Yamaguti (Yamaguti, 1975). None of the 23 salamanders was found to be infected with *C. formosanus* metacercariae, including
those individuals that were exposed to cercarial pressure twice and those exposed in circulating water conditions.

**Discussion**

Our San Marcos salamanders were refractory to infection by *C. formosanus* cercariae. Conversely, both Rio Grande leopard frog tadpoles and fountain darters readily became infected, indicating that the cercariae to which the salamanders were exposed were capable of infecting susceptible hosts. Although natural infection in adult frogs and toads has been reported in Asia (Chen, 1942), and tadpoles of other species have been experimentally infected before (Chen, 1948), we could find no published reports of any North American anuran species naturally infected with *C. formosanus*. Interestingly, (Chen, 1948) reported metacercariae only in the gastrointestinal tract of an experimentally infected tadpole, whereas we found metacercariae only in the gills of our tadpoles. The metacercariae in the gills of our experimental tadpoles appeared to have begun to develop, but had died before reaching maturity. This suggests that tadpoles of *Lithobates berlandieri* are unlikely to serve as functional hosts for *C. formosanus*. Nevertheless, high intensities of infection pressure with *C. formosanus* cercariae could negatively impact tadpole survival, and in times of environmental stress, could potentially contribute to population decline in these amphibians.

Because none of the experimental salamanders were found to be infected, even after multiple exposures with or without water circulation, we conclude that there is some physical or physiological factor that prevents *C. formosanus* cercariae from infecting *E. nana*, despite reports of metacercariae of other trematodes infecting the gills of other species of *Eurycea* (McAllister, et al., 2006). The cercariae of trematodes express a
broad diversity of behavioral adaptations designed to bring them into contact with their preferred hosts, and rely on an equally diverse array of environmental, physical, and chemical signals to stimulate attachment to, and penetration of, these hosts (Galaktionov and Dobrovolskij, 2003). Thus, the failure of *C. formosanus* to encyst in *E. nana* could be due to a lack of proper signals exhibited by these salamanders. *Centrocestus armatus*, a species with a life history similar to *C. formosanus*, relies on its host’s inhalant respiratory current to reach the gills. Upon contact with gill epithelium, the cercariae of *C. armatus* require an interruption of water flow in order to attach successfully (Paller and Uga, 2008). An interruption in respiratory rhythm occurs in fishes at regular intervals during which the flow of current is temporarily stopped (Hughes and Morgan, 1973). Contact with a suitable substrate, coupled with a sudden cessation of flow, could stimulate *C. armatus* to initiate attachment behavior. If *C. formosanus* were to behave similarly, then it would not receive the adequate attachment stimulus from *E. nana* because the salamander breathes passively through its skin and gills, producing no rhythmic respiratory current (Duellman and Trueb, 1994). Thus, a lack of proper stimuli from *E. nana* provides a possible explanation for the failure of *C. formosanus* cercariae to encyst; however, further study will be required to confirm this hypothesis.

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here are those of the authors and do not necessarily reflect those of the U.S. Fish and Wildlife Service.
3. **HAPLORCHIS PUMILIO** (TREMATODA: HETEROPHYIDAE) INFECTING THREATENED AND ENDANGERED FISHES OF TEXAS

**Preamble**

At the Time of this writing, this manuscript has been accepted for publication in the journal BioInvasions Records. This paper was collaborated between Daniel C Huston, Mclean Worsham, David G Huffman and Kenneth G Ostrand, and is the order of authorship on the prospective publication. Daniel C Huston is currently a graduate student at Texas State University, and a Student Trainee with the United States Fish and Wildlife Service (USFWS). Mclean Worsham is also a current graduate student at Texas State University. David G Huffman is a professor of biology at Texas State University, and the Major Advisor of both Daniel C Huston and Mclean Worsham. Kenneth G Ostrand is deputy director of the USFWS San Marcos Aquatic Resource Center in San Marcos, Texas.

**Abstract**

*Haplorchis pumilio* (Trematoda: Heterophyidae) has become widely established around the world because of multiple introductions of its snail hosts, and because of its flexible host requirements at the second-intermediate and definitive levels. Although thiarid snails introduced into North American waters have been previously reported to harbor *H. pumilio*, metacercariae of *H. pumilio* have not been reported from native fishes in the continental USA. We artificially exposed several cyprinids to *H. pumilio* cercariae from infected snails and found that the trematode can be lethal when fish were exposed for only 15 minutes to high cercarial densities. We subsequently collected and examined
fountain darters *Etheostoma fonticola*, a U.S. federally endangered fish species, and examined archived specimens of largemouth bass *Micropterus salmoides*; the IUCN endangered *Dionda diaboli, Gambusia nobilis, Cyprinodon elegans*; and the IUCN vulnerable *Etheostoma grahami* for *H. pumilio* metacercarial infections. With the exception of *C. elegans*, all species examined were positive for *H. pumilio*, and these findings represent new host and locality records. Metacercariae were found encysted in the connective tissue of the head, and at fin insertions. Conversely, we found no integumental or visceral encystations, regardless of the fish species or collection locality.

We now conclude that *Haplorchis pumilio* is probably present in many aquatic systems where *Melanoides tuberculata* and *Tarebia granifera* have become established, but that the metacercariae have been missed by previous investigators because of their small size and unusual anatomical location. We recommend that subsequent investigators be on the watch for these metacercariae, and that the anatomical sites typical of the worm (fin insertions, and especially the caudal peduncle) be included in routine necropsy procedures for fishes from such habitats.

**Introduction**

*Haplorchis pumilio* (Trematoda: Heterophyidae) was first described from birds in Egypt (Looss, 1896). Various life stages of this parasite have now been reported from Africa (Sommerville, 1982a), Israel (Witenberg, 1929), India (Umadevi and Madhavi, 2006), China and East Asia (Chen, 1936; Chung, et al., 2011; Lo and Lee, 1996a; Shen, 1959), Southeast Asia (Chai, et al., 2012; Kay, et al., 2009; Pearson and Ow-Yang, 1982; Radomyos, et al., 1998), Australia (Pearson, 1964), Venezuela (Díaz, et al., 2008),
Mexico (Scholz, et al., 2001), and the USA (Tolley-Jordan and Owen, 2008) (snail stages only).

Adults of *Haplorchis pumilio* have been recovered from the digestive tracts of mammals, birds, and reptiles (Díaz, et al., 2008; Sommerville, 1982a; Umadevi and Madhavi, 2006). First intermediate hosts reported for *H. pumilio* are the thiarid snails *Melanoides tuberculata* and *Tarebia granifera* (Tolley-Jordan and Owen, 2008; Umadevi and Madhavi, 2006). *Haplorchis pumilio* metacercariae parasitize a broad range of second intermediate fish hosts, where they are found encysted in the soft tissue of the fin insertions, and the cartilage of the head (Lo and Lee, 1996b; Sommerville, 1982b). Invasion by *H. pumilio* cercariae can have severe pathological consequences for fish hosts. Cercariae penetrate through the epidermis and migrate to various parts of the body to encyst. Hemorrhaging in the skeletal muscles has been reported in association with both the penetration and migration of cercariae (Sommerville, 1982b; Umadevi and Madhavi, 2006), and simultaneous penetration by a large number of cercariae can be lethal to fry and adults of multiple fish species (Sommerville, 1982b; Umadevi and Madhavi, 2006).

*Melanoides tuberculata* is thought to have been introduced to the USA through the aquarium trade sometime prior to 1950 (Murray, 1971), and *Tarebia granifera* was introduced to the USA as early as 1935 (Nollen and Murray, 1978). Populations of both snail species were first reported in Texas (Murray, 1964), when the snails were found in the headsprings of the San Antonio River (Bexar County). Both species were later reported in the spring-fed Comal River, Comal County, Texas (Murray and Woopschall, 1965). Since that time, both snail species have been reported in multiple spring-fed
systems throughout Texas (Karatayev, et al., 2009), and the USA (Benson and Neilson, 2013; USGS, 2013).

Cercariae of an unidentified Haplorchis species were reported from snails as early as 1999 in west Texas springs (McDermott, 2000), and a report from Utah also included the discovery of Haplorchis sp cercariae from M. tuberculata (Harvey, et al., 2005). Furthermore, H. pumilio has been found in snails in the Comal River in New Braunfels, Texas (Tolley-Jordan and Owen, 2008), and cercariae have been collected from the water column using filtration techniques (Cantu, et al., 2013; Johnson, et al., 2012). It is likely that H. pumilio has become established in many of the aquatic systems in the USA that support reproducing populations of M. tuberculata and T. granifera.

While there are no reports of fishes from North America infected with H. pumilio, we do know that many native fishes are being exposed daily to cercariae of H. pumilio. Therefore, we decided to expose a common cyprinid, (Cyprinella venusta) to H. pumilio cercariae in order to check for its susceptibility to H. pumilio cercariae, and to acquire metacercariae for comparison with wild-caught fishes. After 15 minutes in a 38 L aquarium with approximately 100 infected M. tuberculata, the four experimental fish were transferred to a cercaria-free aquarium for observation. On day two post-exposure, all four fish had developed large red blisters on opposite sides of the caudal peduncle, and by day three post-exposure, the blisters had ruptured and the fish had died (Figure 1)
Figure 1. *Cyprinella venusta* showing blister on caudal peduncle (Day 2) that ruptured into a fatal ulcer (Day 3) following artificial exposure to a large number of *Haplorchis pumilio* cercariae for 15 minutes.

Because of these alarming observations, and the pathologies resulting from *Haplorchis pumilio* infection in fish hosts as reported by Sommerville (Sommerville, 1982b), it was deemed prudent to examine listed fish species from areas where *H. pumilio* cercariae had been observed in order to determine if *H. pumilio* is impacting these species. We examined fresh-caught specimens of the fountain darter (*Etheostoma fonticola*), which is listed as endangered (IUCN, 2011a), as well as archival specimens of largemouth bass (*Micropterus salmoides*) and several listed fishes from West Texas: the Rio Grande darter (*Etheostoma grahami*), which is listed as vulnerable (IUCN, 2012a); the Devils River minnow (*Dionda diaboli*), which is listed as endangered (IUCN, 2011b); the Pecos gambusia (*Gambusia nobilis*), which is listed as endangered (IUCN, 2012b);
and the Comanche Springs pupfish (Cyprinodon elegans), which is listed as endangered (IUCN, 2011c).

**Materials and Methods**

On December 9, 2013, five fountain darters (2.6-3.9 cm, TL) were collected from both the San Marcos (Hays County, 29°52′43.56″N, 97°55′57.36″W) and Comal Rivers (Comal County, 29°42′25.04″N, 98°7′20.48″W) \((n=10)\). Fish were transported to the San Marcos Aquatic Resource Center (SMARC) in San Marcos, Texas. Fountain darters were euthanized with tricaine methanesulfonate (FINQUEL MS-222®; Argent Chemical Laboratories Inc., Redmond, Washington), and preserved in 10% formalin (Hexion Specialty Chemicals INC. Springfield, Oregon). Tissues around the fin insertions, skin, flesh, mouth, head, and internal organs were examined for metacercariae.

The archival specimens that we examined from West Texas spring systems (the Devils River, Val Verde County, 29°54′04″N, 100°59′58″W; San Felipe Springs, Val Verde County, 29°22′25″N, 100°53′06″W; Phantom Lake Springs, Jeff Davis County, 30°56′06″N, 103°50′59″W; and San Solomon Springs, Reeves County, 30°56′39″N, 103°47′16″W) had been preserved and retained at the SMARC following a Centrocestus formosanus study conducted in 2011 by McDermott et al., (in press). We examined five individuals each of *D. diaboli* (3.4-4.1 cm, TL), *G. nobilis* (3.6-4.8 cm, TL), *E. grahami* (3.8-4.6 cm, TL), and *Micropterus salmoides* (6.8-9.1 cm, TL) from the Devils River; five individuals each of *D. diaboli* (3.8-5.0 cm, TL), *E. grahami* (2.6-4.7 cm, TL) and *M. salmoides* (5.8-8.6 cm, TL) from San Felipe Springs; five individuals each of *Cyprinodon elegans* (3.0-3.8 cm, TL) and *G. nobilis* (3.5-4.5 cm, TL) from Phantom Lake Springs; and five individuals of *C. elegans* (3.4-4.0 cm, TL) from San Solomon Springs.
All examinations were performed with a dissecting microscope at 5-35X. The caudal peduncle of each fish was removed from the body approximately four vertebrae from the termination of the spinal column. The caudal peduncle was then divided in half sagittally with a scalpel. Anal, pelvic, pectoral, and dorsal fins were removed intact by cutting around the tissues of the fin insertions with iridectomy scissors. The head was removed and the mandible, maxillaries, and isthmus were divided into sections. Skin and muscles were removed as a fillet with a scalpel. Internal organs were removed and examined in a petri dish. Since no *H. pumilio* metacercariae had been found in the gills of the cyprinids we had experimentally exposed to heavy cercarial concentrations, the gills of our archival specimens were not examined for *H. pumilio* infection. All of the excised tissues were then teased apart with dissecting needles, and examined for metacercariae. *Haplorchis pumilio* metacercariae were identified using a combination of descriptions provided by previous workers (Díaz, et al., 2008; Khalifa, 1977; Lo and Lee, 1996b; Scholz, et al., 2001; Shen, 1959; Umadevi and Madhavi, 2006), and by comparisons with metacercariae obtained from experimental infections resulting from exposure to cercariae shed from *M. tuberculata* that were morphologically indistinguishable from descriptions and photos of *H. Haplorchis* cercariae from the literature (Díaz, et al., 2008; Khalifa, 1977; Krailas, et al., 2011; Shen, 1959; Skov, et al., 2009; Ukong, et al., 2007).

**Results**

*Haplorchis pumilio* metacercariae were found encysted sub-dermally in the cartilage of the head, and in the tissues of the fin insertions (especially the caudal fin, Figure 2). Metacercariae often had dark oval shaped excretory bladders (Figure 3). All
fountain darters from the San Marcos River, as well as those from the Comal River, were infected with *H. pumilio* metacercariae. Metacercarial intensities of *H. pumilio* (min-max, mean) in the San Marcos and Comal Rivers were (3-26, 15.2) and (6-34, 15.4), respectively. In fountain darters, *H. pumilio* metacercariae were found primarily in the tissue surrounding the caudal fin insertion (49%) followed by the head (26%), and the pelvic (13%), dorsal (5%), pectoral (4%) and anal (3%) fin insertions. No *H. pumilio* metacercariae were found in the skin, skeletal muscles, or internal organs of the fountain darters. *Haplorchis pumilio* infection was low in the Devil’s River, where one *E. grahami* and one *D. diaboli* had a single *H. pumilio* metacercaria each. No *H. pumilio* metacercariae were found in the remaining 18 fish examined from the Devil’s River. At San Felipe Springs, *Dionda diaboli* had the highest *H. pumilio* metacercarial intensities (18-56, 27.6) followed by *M. salmoides* (0-20, 6.8) and *E. grahami* (0-10, 2.2). At Phantom Lake, *Gambusia nobilis* had low metacercarial intensities (0-16, 6), and no *H. pumilio* metacercariae were found in *Cyprinodon elegans* from this same site. At San Solomon Springs, no *H. pumilio* metacercariae were found in any of the *C. elegans* examined.
Figure 2. Lateral view of a mid-sagittal section of the caudal peduncle of a naturally infected fountain darter revealing two *Haplorchis pumilio* metacercariae (in highlighted circle at arrow).

Figure 3. Encysted metacercaria of *Haplorchis pumilio* from a fountain darter.
**Discussion**

Though the parthenitae of *H. pumilio* have been known to occur in Texas waters for over a decade, the unusual location of metacercarial encystment is likely the reason fish have yet to be reported as infected. This is concerning because fish populations have likely hosted *H. pumilio* for many years while the implications for the health of wild fisheries and humans have been ignored in North America. Natural infection intensities of *Haplorchis pumilio* observed in our study were low; however, the exotic gill trematode *Centrocestus formosanus* co-occurs in all the locations where we found *H. pumilio* (McDermott et al., in press). Indeed, we were able to match accession numbers of our archival specimens to the data from McDermott et al. (in press) and approximately 50% of the fish infected with *H. pumilio* had previously been found to have gills infected with *C. formosanus*. Episodes of mortality attributed to either *H. pumilio* or *C. formosanus* have been reported in high-density fish farms (Blazer and Gratzek, 1985; Ortega, et al., 2009; Sommerville, 1982a; 1982b), and concurrent metacercarial infections may have larger impacts on individual host fitness than single-species infections. However, there is still a paucity of data regarding the effects of these exotic trematodes on native fish species in natural systems, though it has been suggested that *C. formosanus* can be lethal to fountain darters at metacercarial intensities above 800 in the wild (Mitchell, et al., 2000). Moderate to heavy concurrent infection with *H. pumilio* could exacerbate the degenerative effects of *C. formosanus* infections, increasing the likelihood of parasite-induced morbidity, heightened predation risk, and mortality.

Infection with these parasites could intensify in periods of reduced spring discharge. For example, reduced spring discharge is considered to be the greatest threat
to the endemic spring-adapted species of the San Marcos and Comal rivers (USFWS, 1996), as a drought in the 1950’s resulted in a cessation of spring discharge in the Comal River. Fountain darter populations in the Comal River are believed to have been extirpated during this time, and were subsequently restocked from the San Marcos River (Schenck and Whiteside, 1976). The combination of these exotic trematodes would likely present a serious threat to fountain darters, as well as the other species in this study, if similar environmental conditions were to occur again in any of the spring fed systems in Texas.

Commercially important sport fish species such as bass and catfish could also be adversely affected by this trematode, if not directly, then by the reduction of forage density. The finding of *H. pumilio* in largemouth bass is also concerning, because the location of encysted metacercariae in fish hosts provides this parasite with a more likely route of transmission to humans than does *C. formosanus* which encysts almost exclusively in the gills. *Haplorchis pumilio* has been recorded in humans in Asia (Chung, et al., 2011; Radomyos, et al., 1998), as humans can ingest these tiny flesh-colored metacercariae when consuming raw or lightly cooked fish (Díaz, et al., 2008). Metacercariae encysted in fish can also remain viable when refrigerated for 5-8 days at 4°C (Sommerville, 1982b). Currently, there is little information regarding the pathology of *H. pumilio* in humans (Kay, et al., 2009), although there have been reports attributing ulcerations and gastrointestinal disturbance to heavy intensities of *Haplorchis* spp. (Chung, et al., 2011).

Our study is the first report of *Haplorchis pumilio* in fishes of North America north of Mexico, and lists five new spring-influenced localities in Texas for the
metacercariae. We also list five native North American fishes as new host records for
*H. pumilio*: *Dionda diaboli, Etheostoma fonticola, E. grahami, Gambusia nobilis,* and
*Micropterus salmoides*. As a result, we suggest that future efforts not only include
monitoring these populations but also their infection rates, particularly during periods of
altered spring discharge as well as other variables critical to their survival and
reproductive success. This is particularly important, given the inclusion of State of Texas
and federally listed species as acceptable parasite hosts.

**Acknowledgments**

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of earlier versions of this manuscript. Use of trade names or mention of specific
companies does not imply endorsement of these companies or their products. The views
presented are those of the authors and do not necessarily reflect those of the United States
Fish and Wildlife Service.
4. DEVELOPMENT OF A METHODOLOGY FOR ESTIMATING THE EFFECTS OF EXOTIC TREMATODES ON THE SWIMMING ENDURANCE OF TEXAS MINNOWS

Abstract

Little is currently known as to the effects of the exotic heterophyid trematodes *Centrocestus formosanus* and *Haplorchis pumilio* on native fish species in Texas. A model for quantifying the potential effects that may occur as these trematodes invade new communities is needed for adequate assessment of the impact, and for the development of management plans. I describe the development of methodology, and associated specialized equipment, designed to test the swimming endurance of small fishes artificially infected at various rates and intensities with metacercarial parasites. My study used *Cyprinella venusta* and *Dionda diaboli* experimentally infected with either *Centrocestus formosanus* or *Haplorchis pumilio* as test systems. Although time constraints prevented me from bringing metacercarial intensities in my experimental fish to a level sufficient to estimate an infection intensity impact, this study provides a framework upon which future research can develop a reliable model of the effects these two parasites (and others) are having on native fishes.

Introduction

While mass mortalities have been attributed to the trematodes *Centrocestus formosanus* and *Haplorchis pumilio* in high-density monoculture fish farms (Mohan, et al., 1999; Ortega, et al., 2009; Sommerville, 1982a), I have found no reports of mass mortalities due to these trematodes in the wild. But the actual effects these parasites are
having on native fishes cannot be restricted to mass mortality events. In the absence of such mortality data, it reasons that wild fish with respiratory impairment (due to metacercariae in the gills) and/or large numbers of metacercariae encysted within the caudal peduncle will be (1) slower in their flight from pursuing predators, (2) have lower endurance, (3) be in need of greater recovery times, and/or (4) alter their behavior so that they reside in the upper portions of the water column (where oxygen content tends to be greater) than would uninfected individuals. Either effect would increase the likelihood that wild fish gradually accumulating metacercarial cysts on the gills or in the fin insertions would experience gradual reductions in their fitness.

Herein, I document the development and refinement of a successful methodology through the design and testing of specialized experimental equipment, as well as the design and testing of repeatable methods for the exposure of small fish to varying intensities and acquisition rates of metacercariae. The intent was to determine at what levels of trematode metacercarial infection fish would exhibit reduced swimming performance.

The specialized experimental equipment was constructed from parts available at local hardware stores, and is herein referred to as the “Swimming Endurance Apparatus” or “SEA.” This apparatus was designed to test the swimming endurance of small fish infected with various intensities of trematode metacercariae at constant flow velocities near the maximum swimming speed of the species. *Cyprinella venusta* (a common forage minnow on the Gulf coastal plain), and the Devils River minnow (*Dionda diaboli*), which is native to west Texas and listed as endangered (IUCN, 2011b), were tested in my experiment.
**Biology of *Cyprinella venusta***

*Cyprinella venusta* is widely distributed along the coastal drainage of the Gulf of Mexico from central Texas to Florida. It is found in small to large streams and the clear waters of reservoirs, and prefers riffles and the upstream end of pools and runs with gravel or bedrock substrates (Page and Burr, 1991). The species is a strong swimmer with a maximum swimming speed of 61 cm/s (Leavy and Bonner, 2009). It also seems to be relatively hardy, as it persists or even increases in some stressed habitats where other fish in the local community have been reported to be declining due to habitat degradation (Casten and Johnston, 2008).

*Cyprinella venusta* is highly susceptible to *Centrocestus formosanus* cercariae, but does not exhibit a strong reaction to the presence of *C. formosanus* metacercariae (personal obs.), such as has been documented for the fountain darter *Etheostoma fonticola*, and several other fishes (Alcaraz, et al., 1999; McDonald, et al., 2006). There are currently no data regarding the susceptibility of *Cyprinella venusta* to the cercariae of *H. pumilio*.

**Biology of *Dionda diaboli***

*Dionda diaboli* is very limited in geographic and physicochemical distribution, being known only from a limited number of spring-influenced sites in Mexico and West Texas. *Dionda diaboli* has apparently become locally extinct in several habitats where it was formerly abundant (Contreras-Balderas, et al., 2003; Edwards, 1999; Garrett, et al., 2002; Scharpf, 2005; TOES, 1995; USFWS, 1999; Williams, et al., 1989), and has now been declared endangered (IUCN, 2011b). Rather than seeking open waters and runs like *C. venusta*, *D. diaboli* prefers quieter, deeper waters associated with riffles or channels.
where it is often found associated with aquatic macrophytes (Edwards, 1999; Garrett, et al., 2002). Although I could not find any studies reporting the maximum swimming speed of *Dionda diaboli*, a congener in central Texas (*Dionda episcopa*) has a reported maximum swimming speed of 18 cm/s (Leavy and Bonner, 2009).

The most important of the differences between the two species from the perspective of our study is that *D. diaboli* is not only highly susceptible to *Centrocestus formosanus* cercariae, but it also hyper-reacts to the encystment of metacercariae. The host reacts with extensive chondrocytic hyperplasia, which in turn results in destructive and permanent rearrangement of gill architecture similar to that observed in the fountain darter (personal obs.). However, there are no reports regarding the susceptibility of *D. diaboli* to the cercariae of *Haplorchis pumilio*.

**Project Goals**

The goal of this study was not only to develop the necessary methodology, but also determine the extent to which *C. formosanus* and *H. pumilio* infection may be impacting swimming performance of *Cyprinella venusta* and *Dionda diaboli*. Several experimental objectives were executed:

1. Determine the intensity of *C. formosanus* and *H. pumilio* infection at which the swimming endurance of the target species is reduced to 60% of that of uninfected controls, which would substantially reduce their ability to escape predation.
2. Determine the intensity of *C. formosanus* and *H. pumilio* infection at which the target species is affected so drastically that at least 20% can be expected to die within two weeks of exposure from direct effects of the parasites.
3. Determine the interaction, when the endurance of an individual is tested, between:
o the rate at which *C. formosanus* and *H. pumilio* metacercariae were acquired by fish at various intensities, and

o the parasite intensity that has accumulated at various cercarial exposure rates.

4. For both fish species, estimate the percentage of cercariae introduced into an aquarium (at densities observed in the wild) that are able to successfully establish themselves as metacercariae, which would indicate differential susceptibility of the two fishes to infection.

**Materials and Methods**

The experiment consisted of experimentally exposing *C. venusta* and *D. diaboli* to the cercariae of *Centrocestus formosanus* (Trials 1 and 2) or *H. pumilio* (Trial 3). The artificial exposures were performed at various densities of cercarial pressure, and various durations of cercarial exposure (which determines rates of metacercarial acquisition). Then the swimming endurance of the infected fish and their respective controls were tested in the SEA. Lastly, the experimental fish were necropsied, and numbers of metacercariae counted. The metacercarial intensity data coupled with associated swim times in the SEA allowed me to estimate the effects of metacercarial infection upon swimming endurance. I was also able to estimate percentages of cercariae acquired versus cercariae applied at various densities.

*Cyprinella venusta* and *Dionda diaboli* were obtained from the U.S. Fish and Wildlife San Marcos Aquatic Resource Center (SMARC) and acclimated for at least two weeks prior to experimentation. The fish were fed commercially available flaked aquarium food ad libitum daily.
In order to provide cercariae for artificially exposures, it was necessary to collect naturally infected snails from the wild. All snails were collected from the Comal River, (Comal County, Texas, 29°42′28.08″N, 98°7′24.24″W) between October 2012 and October 2013. Approximately 5,000 *Melanoides tuberculata* were collected and transported to the Freeman Aquatic Biology building, Texas State University, San Marcos, Texas. All animals used for and during experimentation were maintained at the SMARC at a water temperature between 20 and 23 °C and a 12/12 Light/Dark cycle.

Detection and Isolation of Infected Snails

Snails were distributed individually into 30 ml clear glass vials nearly filled with de-chlorinated tap water. Fluorescent lighting was placed above these vials on a 12/12 L/D cycle. Two hundred snails were incubated simultaneously for up to three days. Snails infected with *C. formosanus* or *H. pumilio* were isolated by candling the incubation vials and visual observing movement of trematode cercariae. Trematode cercariae were identified to species through morphological examination and observation of cercarial swimming behaviors. Three species of digenean were found infecting *M. tuberculata* from the Comal River, and identification typically required the use of a dissecting microscope. However, a size differences among the three species and multiple diagnostic behaviors allowed identification to species after some experience (Appendix E).

I housed uninfected snails, snails infected with *C. formosanus* and snails infected with *H. pumilio* in separate aquaria. Snails were fed commercially available fish flakes twice weekly. Snails initially classified as non-shedding were subjected to the above examination procedure two additional times, once on the second week and again on the
third week post-capture, to ensure that the snails were indeed uninfected. For endurance trials, uninfected snails were divided equally among 3 non-shedding snail aquaria and infected snails were divided equally among 3 shedding-snail aquaria

**Physical Set-up for Experiment**

**Snail Aquaria**

Each endurance Trial utilized only one species of trematode. non-shedding snails were used to control for potential snail-effects. These two groups of aquaria (three shedding and three non-shedding) served as the source for Treatment Water and Placebo Water (Appendix A), respectively, for the next phase of the experiment. Trial 1 utilized 60 non-shedding snails and 60 shedding snails infected with *C. formosanus*; Trial 2 utilized 75 non-shedding snails and 75 shedding snails infected with *C. formosanus*; and Trial 3 utilized 50 non-shedding snails and 50 shedding snails infected with *H. pumilio*.

**Fish Aquaria**

Sixty *Cyprinella venusta* and 60 *Dionda diaboli* were utilized in each trial. Experimental fish were housed in 30 10-gallon (38 L) aquaria arranged on heavy duty shelving units in two tandem groups of 15 (Bank A and Bank B). The 15 fish aquaria in each Bank were arranged on the shelves in an array of five vertical columns of three horizontal rows each (Figure 4).
At the beginning of each Trial, four *C. venusta* (mean = 55mm ± FL) were stocked into each of 15 aquaria, and four *D. diaboli* (mean = 49mm ± FL) were stocked into each of the 15 remaining aquaria. Fish were then allowed to acclimate for four days. During the acclimation period water was exchanged daily at a rate as prescribed by the treatment (Appendix A). Water exchanges and the number of days it was exchanged differed among the aquaria in order to produce fish with low to progressively higher parasite loads, as well as to produce fish with similar parasite loads at different rates of metacercarial acquisition. Fish were exposed to *C. formosanus* (Trials 1 and 2) or *H. pumilo* (Trial 3) cercariae at controlled densities by taking equal aliquots of Treatment Water drawn from each of three aquaria that contained shedding snails.

The aliquots of water were then combined into a plastic bucket. Each day of treatment, a one L sample was taken from the pooled Treatment Water and used to estimate cercarial density/L using the method described by Cantu et al., (2013). The remaining treatment water was then exchanged into the treatment aquaria (N = 24) daily in specific volumes intended to deliver various total numbers of cercariae to an individual
aquarium. Thus, daily numbers of cercariae applied to each aquarium was dependent upon the designated volume of water applied to each aquarium. Volumes of treatment water exchanged were (Trial 1, range = 60-800 ml, mean = 450 ml; Trial 2, range = 180-2400 ml, mean = 1356 ml; Trial 3, range = 100-5000 ml, mean = 2800 ml). *Centrocestus formosanus* cercariae densities per liter were (Trial 1, range = 1200-3525, mean = 2310; Trial 2, range = 3200-4800, mean = 3703) during water exchanges. *Haplorchis pumilio* cercariae densities were (Trial 3, range = 0-800, mean = 239). Control aquaria (N = 6) received Placebo Water drawn from each of three non-shedding snail aquaria. Equal aliquots of water (1L) were combined into a plastic bucket and exchanged into the control aquaria at the same volume and rate as the defined treatments and in treatment aquaria when water containing cercariae was not exchanged.

A single daily water exchange for each aquarium consisted of: 1) removing and discarding the defined volume of old water from an aquarium, 2) stirring the pooled water type to homogenize the contents, 3) removing the defined volume of the desired water type from the pooled water, 4) adding the water type to the aquarium at the defined volume and rate. To avoid cross contamination water was removed and added to aquaria utilizing three different turkey basters and three 600 ml plastic beakers assigned to Treatment, or Placebo water.

On day 21 of each trial, two-thirds of the water was replaced in all aquaria with well water that contained no cercariae. This prevented additional infections, and allowed encysted metacercariae to mature during the next 12 days. Trials were terminated on day 33, and fish were tested on day 34 and 35. The next trial was begun on day 36.
Before conducting the third Trial, which was to utilize *H. pumilio*, I ran two preliminary range-finding experiments. The first experiment involved placing *Cyprinella venusta* directly into aquaria containing snails infected with *H. pumilio*, and was employed to determine numbers of metacercariae needed to induce direct mortality. Two aerated 38-L aquaria were filled, and 18 *M. tuberculata* infected with *H. pumilio* were added to the first aquarium, and 38 infected snails were placed in the second aquarium. Four *C. venusta* were placed into each of these aquaria, and checked at 24 and 48 hour intervals for mortality. The second experiment was intended to determine effects of long term exposures to cercariae, as well as to determine if a single body section could be used as a model to estimate numbers of metacercariae. During this second preliminary trial, 15 *C. venusta* were distributed individually into Bank A, and 15 *D. diaboli* were distributed individually into Bank B. Fifty *M. tuberculata* infected with *H. pumilio* were placed into a separate, gently aerated 38-L aquarium. Cercarial densities/L were not estimated, however range of water volumes exchanged during the titration were 0-2500 ml per day. These fish experienced a 3-day habituation period, 14-day treatment period and a 14-day maturation period. Several individuals from the preliminary trial were tested in the Swimming Endurance Apparatus. For the Third Trial I utilized the same 50 infected snails from the preliminary trial.

**Swimming Endurance Apparatus**

A semi-portable apparatus was constructed from hardware available at local stores to test the swimming endurance of small fish at various current velocities (Figure 5). Water velocity was calibrated by injecting 5-mm calibration balls into the tunnel and filming them with a high-speed camera. Velocities were calculated by determining the
distance traveled per frame of the film. The spreadsheet produced a measure of average velocity for each Diverter/Flow valve combination of the tunnel, as well as respective coefficients of variation for the calibration balls. Coefficients of variation produced during all calibrations were between 4-7%. (see Appendix B for components, calibration, and operation details of the SEA).
Figure 5. Diagram of the Swimming Endurance Apparatus viewed from the front of the device. Individual components (coded by letter) are described in detail in Appendix B.
The Swimming Endurance Tests

The swimming performance challenges were conducted in the SEA (Appendix B) on day 34 ending on day 35. The swim chamber was continuously supplied with aerated water at 20±3 °C and operated on a recirculating basis during the swimming challenges. Testing protocols were based upon preliminary work (Appendix C). Fish were acclimated at a water flow rate of 0.0 cm/sec for 2 min. Following acclimation, C. venusta and D. diaboli were subjected to a series of stepwise velocity increments at 10 second intervals until water flow rates reached 30 cm/sec. Once this water flow rate was reached fish swam for two minutes. After this two minute warm-up period water flows were increased in 10 second intervals to 54 cm/sec or 45 cm/sec (about 76 and 77% of maximum swimming speed of C. venusta and D. diaboli, respectively), until the fish was fatigued. Fatigue was defined as the point at which the fish became impinged on the rear blocking screen and refused to swim for 5 continuous seconds. When a fish reached exhaustion, time was stopped and the total swim time recorded. The exhausted fish was removed, euthanized with MS-222 (FINQUEL MS-222®; Argent Chemical Laboratories Inc., Redmond, Washington) and preserved in a labeled vial of 10% formalin.

Swim times were recorded in minutes and seconds. Due to scheduling restraints, fish in the first two trials were removed from the SEA if they swam longer than 10 minutes. In the third Trial, the maximum swim time was capped at 15 minutes. These maximum swim caps were necessary because of the large sample sizes utilized, as each swimming endurance phase of a Trial needed to remain under 48 hours to avoid confounding time influences. Furthermore, some of the fish were seen to swim up to an
hour in preliminary work, and the amount of time needed to swim all fish in a trial without a maximum time cap would be astronomical.

Necropsy Protocol

All preserved fish were necropsied and examined for metacercariae. All gill arches were removed from fish from the first two trials and examined for *C. formosanus*. Each gill arch was examined under a dissecting microscope at 5-45X and numbers of *C. formosanus* metacercariae were counted for each arch. These counts were summed across all eight arches to determine *C. formosanus* intensity for each fish. We examined the caudal peduncle of each fish from the third trial for *H. pumilio* metacercariae. *H. pumilio* metacercariae can be found in the fin insertions, mouth and operculum of fish however, preliminary work (Appendix C) indicated that we could consistently expect approximately 50% of the metacercariae to be found in the caudal peduncle region of the fish. Therefore, we removed tails from the fish at approximately four vertebrae from the termination of the caudal peduncle and examined the caudal region for metacercariae under a dissecting microscope at 5-45X.

Data Analyses

We used regression analyses to determine the relationship between the metacercarial intensities induced in *C. venusta* and *D. diaboli* and their swimming performance relative to the uninfected controls. We compared total swim times to metacercarial intensities, using an adjusted metacercarial intensity value to standardize data for length. The adjusted metacercariae values for each fish were calculated by dividing the raw intensity by the length of the fish squared. We also calculated the number of cercariae that were able to encyst in fish relative to the cercarial density that
the fish were exposed to. We used Welch’s one-way t-tests to determine significant differences between numbers of metacercariae acquired by *C. venusta* and *D. diaboli*.

Several fish died during the course of the experiments, but their mortality did not appear to be directly caused by the trematodes due to the low infection intensities observed. In the *C. formosanus* trials two *D. diaboli* died prior to testing, and had 18 and 34 metacercariae respectively. In the *H. pumilio* trial, four *D. diaboli* died prior to testing (range= 5-49 metacercariae, mean = 20.75 metacercariae). These 6 fish were not included in the analysis. An additional 29 *D. diaboli* and 13 *C. venusta* were small enough to move through the viewing screens (O and Q, Figure 5). As a result, these fish were not included in analysis. Because many of the fish in our Trials were able to swim for extremely long periods of time (some for over an hour) This led to a large number of data points that were, in effect, not real swim-to-exhaustion times. As a result an additional 40 *C. venusta* and 34 *D. diaboli* were not included in analysis. We were able to use 63 *C. venusta* and 31 *D. diaboli* from the first two trials for analysis. From the third trial we were able to use 43 *C. venusta* and 49 *D. diaboli* for analysis.

**Results**

**Swimming Endurance Apparatus**

Though the Swimming Endurance Apparatus (SEA) was a prototype machine, the performance of the device was deemed highly successful. Calibration trials of the SEA between all possible water velocity settings, except for the very lowest, yielded consistently low (3-7%) coefficients of variation between calibration balls during all calibration events. Dissolved oxygen in the SEA reservoir was kept above 90% while the
SEA was powered on due to the splashing return of the water from the SEA into the reservoir after passing through the viewing tube. The high level of dissolved oxygen prevented challenges of dissolved oxygen limitations affecting results.

**Susceptibility of C. venusta and D. diaboli to metacercariae**

*Cyprinella venusta* appears more susceptible than *Dionda diaboli* to both *Centrocestus formosanus* and *Haplorchis pumilio* cercariae, hosting 60% more ($p<0.001$) *C. formosanus* metacercariae and 37% more ($p<0.01$) *H. pumilio* metacercariae on average than *D. diaboli*. Total metacercarial intensities induced during Trial 1 were (0-697, 140) for *Cyprinella venusta* and (0-229, 25) for *D. diaboli*. Mean success rate of cercarial establishment based upon estimated cercarial exposure was 10% for *C. venusta* and 2% for *D. diaboli*. Total metacercarial intensities induced during Trial 2 were (0-290, 71) for *C. venusta*, (0-189, 18) for *D. diaboli*, and mean success rate of cercarial establishment was estimated at less than 1% for both fish species. Metacercariae intensities induced during Trial 3 were (0-72, 16.3) for *C. venusta*, (0-61, 10.7) for *D. diaboli* and success rate of cercarial establishment was estimated at less than 1% for both fish species.

**Haplorchis pumilio Preliminary Trials**

All eight *C. venusta* stocked directly into aquaria with infected snails died within 48 hours. The four *C. venusta* stocked in the aquarium with 38 infected snails died within 24 hours, as did one of the four fish placed in the aquarium containing 15 infected snails. The other three *C. venusta* stocked in the aquarium containing 15 infected snails died within 48 hours. There was evidence of hemorrhaging in the caudal peduncle, as well as in the tissues surrounding the other fin insertions of all eight fish. Metacercarial
intensity for the eight fish was relatively high (range = 816-3520, mean = 1470). During the necropsy of these fish a large number of unencysted cercarial bodies were observed throughout the skeletal muscles and skin.

I performed a thorough necropsy on 14 C. venusta and 12 D. diaboli that were used in the second preliminary H. pumilio trial. Metacercarial intensity induced during the second preliminary trial was (range = 14-1105, mean = 277) for C. venusta and (range = 5-519, mean = 110) for D. diaboli. I obtained a single mortality during the titration; however, the metacercarial intensity for this fish was quite low (48), so it is unlikely that H. pumilio infection was directly responsible. A consistent pattern of H. pumilio metacercarial loads was found for specific anatomical locations of encystment within both host species (n=26). Approximately 52.5% of H. pumilio metacercariae were found in the tissue of the caudal fin insertion, with lesser numbers being found in the head and fin insertion tissues of the pectoral, dorsal, anal and pelvic fins (Table 1). A two-week post-exposure period seems sufficient for all cercariae to either migrate and encyst, or to be cleared by the fish, as no cercarial bodies or encysting metacercariae were found in parts of the body other than the head and fin insertions.
Table 1. Percentages of *Haplorchis pumilio* metacercariae found encysted per body section in 26 minnows from the second preliminary trial.

<table>
<thead>
<tr>
<th>Body Section</th>
<th><em>Cyprinella venusta</em> 1</th>
<th></th>
<th><em>Dionda diaboli</em> 2</th>
<th></th>
<th><em>Total</em> 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudal</td>
<td>52.87</td>
<td></td>
<td>Caudal</td>
<td>52.1</td>
<td>Caudal</td>
</tr>
<tr>
<td>Anal</td>
<td>6.70</td>
<td></td>
<td>Anal</td>
<td>4.67</td>
<td>Anal</td>
</tr>
<tr>
<td>Pelvic</td>
<td>4.87</td>
<td></td>
<td>Pelvic</td>
<td>5.82</td>
<td>Pelvic</td>
</tr>
<tr>
<td>Dorsal</td>
<td>6.54</td>
<td></td>
<td>Dorsal</td>
<td>7.65</td>
<td>Dorsal</td>
</tr>
<tr>
<td>Pectoral</td>
<td>15.53</td>
<td></td>
<td>Pectoral</td>
<td>8.65</td>
<td>Pectoral</td>
</tr>
<tr>
<td>Head</td>
<td>13.50</td>
<td></td>
<td>Head</td>
<td>21.12</td>
<td>Head</td>
</tr>
</tbody>
</table>

1 $n=14$ fish  
2 $n=12$ fish  
3 All 26 fish combined
Cyprinella venusta had \(p(F_{5,78} \geq 67.63) < 0.001\) more metacercariae located in the caudal peduncle fin insertion than all other anatomical locations (Table 2). Although the head and pectoral fin insertions had similar numbers of metacercariae, \((p’s < 0.02)\) more metacercariae were located in the head and pectoral fin insertions when compared to the anal, dorsal or pelvic fin insertions, which did not differ from each other.

Dionda diaboli, had \(p(F_{5,68} \geq 34.03) < 0.001\) more H. pumilio metacercariae at the caudal peduncle fin insertion than all other anatomical locations (Table 3). More metacercariae \((p’s < 0.01)\) were observed in the soft tissue of the head than the pectoral, anal, pelvic and dorsal sections that did not differ from each other.

Collectively, C. venusta and D. diaboli had more \(p(F_{5,150} \geq 87.99) < 0.001\) H. pumilio metacercariae in the caudal peduncle insertions than any other anatomical location (Table 4). The head and pectoral fin insertions contained more \((p’s < 0.05)\) metacercariae than the anal, pelvic, or dorsal fin insertions that did not differ from each other.
Table 2. ANOVA table (a) for ARCSIN SQRT transformed percentages of encysted metacercariae per body section for *C. venusta*, and graphical representation of Tukey’s Test results (b).

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<thead>
<tr>
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<th>MS</th>
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<tr>
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<tr>
<td>Total</td>
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(b) Results of Tukey’s Test$^{1,2}$

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<th>Mean</th>
<th>0.204</th>
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<td>Anal</td>
<td>Pectoral</td>
<td>Head</td>
<td>Caudal</td>
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</tbody>
</table>

$^{1}$ Bold lines unite body sections hosting similar mean percentages of total metacercariae.  
$^{2}$ Alpha (2) set at 0.05.
Table 3. ANOVA table (a) for ARCSIN SQRT transformed percentages of encysted metacercariae per body section for *D. diaboli*, and graphical representation of Tukey Test results (b).

(a) ANOVA table

<table>
<thead>
<tr>
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<tr>
<td>Total</td>
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(b) Results of Tukey’s Test$^{1,2}$

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<td>Dorsal</td>
<td>Pectoral</td>
<td>Head</td>
<td>Caudal</td>
</tr>
</tbody>
</table>

$^1$ Bold lines unite body sections hosting similar mean percentages of total metacercariae.

$^2$ Alpha (2) set at 0.05.
Table 4. ANOVA table (a) for ARCSIN SQRT transformed percentages of encysted metacercariae per body section for both fish species, and graphical representation of Tukey’s Test (b).

<table>
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(b) Results of Tukey Test\(^1,2\)

<table>
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<tr>
<th>Mean Body Section</th>
<th>Pelvic</th>
<th>Anal</th>
<th>Dorsal</th>
<th>Pectoral</th>
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<tbody>
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<td>0.235</td>
<td>0.33</td>
<td>0.411</td>
<td>0.813</td>
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</table>

\(^1\) Bold lines unite body sections hosting similar mean percentages of total metacercariae.
\(^2\) Alpha (2) set at 0.05.
Swimming Performance

I was able to test 43 *C. venusta* and 25 *D. diaboli* in the SEA during Trial 1, with 46 *C. venusta* and 40 *D. diaboli* being tested in Trial 2. Swimming performance was not influenced by *Centrocestus formosanus* infection for either *Cyprinella venusta* \( p(F_{1,61} \geq 0.56) = 0.45 \) or *D. diaboli* \( p(F_{1,29} \geq 0.12) = 0.73 \) (Figure 6). Swim times for *C. venusta* (mean = 281 ± 99 sec) were similar regardless of metacercarial intensities (0-697). Likewise, *D. diaboli* swim times (mean = 315 ± 103 sec) were similar regardless of metacercarial intensities (0-229).
Figure 6. Scatterplots of swim times vs adjusted C. formosanus metacercarial intensities for *Cyprinella venusta* (top) and *Dionda diaboli* (bottom). Data was pooled for both species from Trial 1 and Trial 2.
Seven of the *C. venusta* from the second *H. pumilio* preliminary were run in the SEA. These fish had various metacercarial intensities that ranged from 70 to 406 encysted *H. pumilio* in the caudal peduncle insertion (mean = 193). Metacercarial intensity was significant \([p(F_{1, 5} \geq 7.76) = 0.038]\) and negatively related to swimming performance (Figure 7, \(R^2 = 0.608\)). However, the sample size used was far too low to provide a reliable model.

Figure 7. Linear regression for the second *H. pumilio* preliminary trial.

**Trial 3 Main**

I was able to test 57 *C. venusta* and 53 *D. diaboli* in the SEA during Trial 3. *Haplorchis pumilio* cercarial densities per liter of treatment water was (0-800, 238.5). In the evening of the second day of the treatment phase, an unknown issue caused the heating unit for the lab space to fail. The following morning, water temperatures in the fish aquaria were found to be 14 °C; therefore I must assume that temperatures remained near this low for at least the previous 12 hours. The heating unit was fixed the same
morning, returning water temperatures to 21 °C within 6 hours, and all fish and snails survived the sudden drop of temperatures. Due to time constraints on the project, I was unable to start over with fresh snails and fresh fish, so I continued with the trial as is. However, no cercariae were found in the 1-liter water sample drawn from the Treatment Water on the third and fourth days of treatment and, although cercariae were again present on the 5th and subsequent days of the Treatment Phase, the cercarial density remained substantially lower than before the cold event (Figure 8). Linear regression for adjusted metacercarial intensity was not significantly related to the swimming performance of C. venusta \[ p(F_{1,41} ≥ 0.0005) = 0.98 \] or D. diaboli \[ p(F_{1,47} ≥ 0.62) = 0.43 \] (Figure 9). Swim times for C. venusta (mean: 245 ± 127sec) were similar regardless of metacercarial intensity (0-72). Likewise, swim times for D. diaboli (mean: 358 ± 151sec) were similar regardless of metacercarial intensity (0-61).
Figure 8. Trial 3: Reduction in cercarial density following unexpected cold event.
Figure 9. Scatterplots comparing swim times vs. adjusted *H. pumilio* metacercarial intensities for *C. venusta* (top) and *D. diaboli* (bottom).
Discussion

Susceptibility of Fish to Cercariae

Though I predicted that *Centrocestus formosanus* and *Haplorchis pumilio* would adversely affect *Dionda diaboli* more than *Cyprinella venusta*, my results are inconsistent with this prediction. *Cyprinella venusta* hosted higher *Centrocestus formosanus* and *H. pumilio* metacercarial intensities across all 3 Trials compared to *D. diaboli*. It seems that *D. diaboli* is more resistant to these exotic trematodes than *Cyprinella venusta*. The nature of this difference in susceptibility is not known, however it seems that host size is not a significant factor. Immune response and an inherited resistance to these trematodes may be responsible for these observed differences in susceptibility. The *D. diaboli* used in the Trials were first generation descendants of wild fish collected from San Felipe Springs (Val Verde County, Texas, 29°22′25″N, 100°53′06″W). San Felipe Springs has historically had a high *Centrocestus formosanus* density (McDermott et al., in press). The *D. diaboli* used in my study may have inherited a level of resistance to *C. formosanusi* from the parent fish brought to the SMARC from San Felipe Springs. Conversely, the *Cyprinella venusta* used in my study came from a pond located on the SMARC grounds. These fish have been isolated at the facility for many years, and have not had previous exposure to *Centrocestus formosanusi*. Thus, there is a potential for the *D. diaboli* used in the experiment to have had a level of genetic resistance that *Cyprinella venusta* did not. This may also be the case for differences in susceptibility to *H. pumilio*, as the trematode has been present in San Felipe Springs as well since at least 1999 (McDermott, 2000). The *C. venusta* from the SMARC may indeed prove to be a superior model host for future work with these exotic trematodes, as they probably represent
genetically naïve hosts, allowing modeling of the effects of new invasions of these parasites.

Swimming Performance

The swimming endurance tests utilizing Centrocestus formosanus did not provide sufficiently high metacercarial intensities to break through a threshold at which Cyprinella venusta and D. diaboli would begin to show reduced swimming performance. I conclude that cercarial densities during the first two trials were not high enough to induce sufficient morbidity to provide a detectable effect. However, from the data obtained it seems that as long as metacercarial intensities remain at or below those induced in the Trials (~700 for C. venusta, ~300 for D. diaboli) that Centrocestus formosanus alone would not impact the swimming abilities of our experimental fishes.

The third Trial, utilizing H. pumilio, also did not provide sufficient data for determining a threshold at which our fish would exhibit reduced swimming performance. I conclude that I was again unable to induce sufficiently high H. pumilio metacercarial intensities in my fish to produce a detectable impact on swimming performance during the third Trial. Thus, so long as H. pumilio metacercarial intensities remain below those induced in the third Trial (~80 for Cyprinella venusta, ~60 for D. diaboli), H. pumilio alone is unlikely to cause significant damage to either C. venusta or D. diaboli.

However, the preliminary H. pumilio experiments performed prior to the third trial provide an indication of serious consequences for fish hosting much higher H. pumilio metacercarial intensities than those induced in the third Trial. Exposure to a large amount of H. pumilio cercariae within a short period of time was shown to be lethal to C. venusta, with mortality being induced by penetration of as few as 800 cercariae.
Furthermore, though I was only able to test seven *C. venusta* from the long term *H. pumilio* titration experiment, a significant effect on swimming performance was observed, as *C. venusta* hosting over 300 *H. pumilio* metacercariae could be expected to exhibit a reduction in swimming performance.

**Threats to the Validity of Conclusions**

**Limitations of the Swimming Endurance Apparatus**

Coefficients of variation in calibration across the entirety of the experiment, including preliminary work, did not vary, indicating durability and consistency of the SEA’s performance. The SEA also required very little maintenance and repair across all trials, though there were several leaks along the seals of the viewing tube as well as the downstream screen plunger rod. However, these leaks were minor and did not adversely affect the performance of the SEA. The sump pump performed consistently throughout over 100 hours of use, though future models should have a spare in case of pump failure during the critically time-sensitive endurance testing. The SEA was a prototype machine, and with every man-made device there are inherent problems. The flow-through nature of the diffuser/linearizer device, as well as the return of the water to a static 100 gallon reservoir kept the dissolved oxygen well above 95% while the SEA was powered on. However, the static reservoir suffered from a slow increase in temperature during extended testing because of heat generated by the sump pump. Because of this, the water in the reservoir needed to be exchanged after testing 5-10 fish, in order to keep the water temperatures within the 20-23°C range that the fish from each Trial had been maintained. Though the water used for these exchanges all came from the same source, fish testing in the SEA experienced subtle fluctuations in temperature depending on the order in which
they were tested between water exchanges. These differences in water temperatures could have adversely affected some of the fish more than others during the shock of transfer between their holding aquaria and the SEA. Furthermore, the 2 minutes habituation period allowed inside the SEA may have not been sufficient for proper acclimation of the fish to the SEA environment. Future models should include a heater/chiller unit plumbed into the reservoir to maintain temperature, or the reservoir should be converted into a flow-through design.

Another potential problem that may have occurred during testing was the presumed laminar flow and consistent velocity of the water in the SEA viewing tube. In calibration, coefficients of variation of the calibration balls were low (3-7%), but this level of consistency may have wavered between tests, with each fish in turn experiencing slightly different experimental conditions across a Trial. I calibrated the SEA before and after each Trial, and no large amount of variation was observed between the beginning and end calibrations, or between Trials. However, there were likely non-detectible differences in the flow conditions within the SEA that could have affected swimming performance. Though great care was taken in the design of the diffuser/linearizer section of the SEA, we observed what appeared to be an area within the viewing tube of slightly slower flow near the bottom of the downstream screen. Though this area was available to all fish that were run in the SEA, not all of the fish tested chose to utilize it. Further non-detectible variation could have occurred within the plumbing of the SEA where I could not observe obstructions.

Though I attempted to standardize size of fish in selection for a Trial, it was extremely difficult to do so because of the availability of fish. The *C. venusta* used came
from a pond located on the SMARC, were then maintained indoors in a living stream system for over 3 months, and fed daily. Even with these conditions there was still a level of size variation within these fish. Selecting *D. diaboli* of similar sizes large enough to be run in the SEA proved even more difficult, as adult *D. diaboli* do not reach a suitable size for the SEA until they are at least a year of age, and even so there is a large amount of variation in size between cohorts. Furthermore I was unable to make fine enough adjustments to the water velocity in the SEA in order to account for variations in the size of a fish. Though I did account for size in our statistical analyses, the differences in size between individuals may have confounded a portion of our swimming performance results. A larger fish may be able to accommodate more metacercariae than a smaller fish before exhibiting similar reductions in fitness.

Behavioral issues of individual fish may have lent variation at a level that could have confounded my results. In each Trial, multiple fish essentially refused to swim in the tunnel, becoming pinned against the downstream screen at the slightest increase in water velocity. Multiple Control fish (fish without metacercariae) in each Trial also exhibited this behavior, so it was not an effect brought on by parasitism. Furthermore, some fish seemed to “choose” to quit swimming after a short period. It was fairly obvious when a fish swam to exhaustion, as these fish would touch tails with the downstream screen and dart forward, and then be pushed back against the screen for many iterations. The fish that “chose” to cease swimming typically quit before they were even experiencing their maximum testing velocities, and would not struggle once they became pinned against the downstream screen. The reason behind these behaviors are
not known, however it may be a response that would be intended to direct the fish into a slower pool of water, without effort, in the wild.

Because many of the fish in our Trials were able to swim for extremely long periods of time (some for over an hour) we were forced to end each swimming test if the fish swam for longer than 10 minutes (Trials 1 and 2) or 15 minutes (Trial 3). This became necessary because of the intensity of effort and time restrictions required to test the large sample sizes used in our Trials. Unfortunately, this led to a large number of data points that were, in effect, not real swim-to-exhaustion times. These capped swim times, as well as the strange behaviors that resulted in extremely short swim times, may have served as a significant confounding factor in our data analysis.

The SEA is a remarkable device, and has many potential applications. However, the SEA suffers from some limitations that manifested physically as well as in synergy with the experimental design. Modifications to the SEA itself, as well as adjustments to the experimental design employed during a Trial could improve the utility and efficiency of future work. A major issue with the SEA was the effort required in order to test the large sample sizes used in our Trials. The test of a single fish required 5 minutes at a minimum, with the majority of fish requiring from 10 to 15 minutes to test. Time had to be taken in between each test to process and archive specimens, take water quality measurements and exchange water from the reservoir. Thus, a single Trial took a total of 36-48 hours of constant work. Modifying the experimental design in such a way as to allow smaller groups of fish to be tested at a time would reduce the level of labor intensity experienced by the workers involved in each Trial. However, the cost of reduced degrees of freedom would probably jeopardize a trial in other ways.
Metacercarial Intensity and Acquisition

The very low numbers of metacercariae recovered in my fish hosts versus estimated cercarial exposure is puzzling. Lo and Lee estimated (Lo and Lee, 1996b) that approximately 45% of Centrocestus formosanus and 42% of H. pumilio cercariae were able to establish themselves as metacercariae in experimentally exposed Cyprinus carpio. In our study, mean percentages of Centrocestus formosanus metacercariae recovered based upon estimated cercarial exposure was 10% for Cyprinella venusta and 2% for D. diaboli in Trial 1. In Trial 2, these values dropped significantly, with C. venusta and D. diaboli acquiring less than 1% of metacercariae based upon estimated cercarial exposure. I did not estimate cercarial densities per liter during the H. pumilio titrations, however percentages of metacercariae acquired versus estimated exposures are assumed to be much higher than in Trial 3, in which the percent of metacercariae acquired by my host fish was less than 1% for both species.

The causes of the very low presumed infectivity of the cercariae used in my study are unknown. Immune response in my fish exposed to cercariae may explain some of the variation in transmission success on an individual basis. It is well known that previous trematode infections may provide a fish with some level of protection from future infections through stimulation of the immune system, in a way similar to vaccination (Karvonen, et al., 2004; Karvonen, et al., 2009; Voutilainen, et al., 2009). However, specific antibodies for fighting a specific species of trematode begin to be detectible between 10 and 20 days post exposure, but do not reach their peak density until 3 to 6 weeks post exposure (Evans and Gratzek, 1989; Whyte, et al., 1987). Therefore, it is unlikely that the fish in my study were able to mount specific immune response to
Centrocestus formosanus and H. pumilio as all the fish used in the study were naïve hosts, having not been previously exposed to the cercariae of either species of trematode. However, non-specific immune response such as inflammation and production of immune cells likely did occur. Macrophages, neutrophils, eosinophil, and lymphocytes have all been reported in fish in response to trematode infection, though these immune cells likely need to be activated by a long term antibody response attained through previous exposure (Secombes and Chappell, 1996). Inflammation has been frequently reported in fish hosting trematode parasites, which can lead to the production of encapsulating granulomas and the subsequent death of metacercariae (Alvarez-Pellitero, 2008). Though these immune responses may kill some metacercariae, the encapsulation process typically affords the metacercariae some level of protection from the host’s immune system, as once encapsulated the parasites are protected from antibodies (Karvonen, et al., 2012). If however, encapsulated metacercariae were protected, circulating antibodies may be available to attack cercariae penetrating the host at a later date, killing many cercariae before they could establish themselves. If this were the case, then my host fish would have acquired the majority of their metacercariae early on in the Trial, with subsequent exposures having less of an effect, leading to a reduction in compounding infection intensities.

Though it is unknown if this were occurring, a level of immune response probably did occur in our Trials, and may explain why I was unable to achieve high metacercarial numbers in Trial 1, but it does not explain the subsequent drop in metacercarial acquisition observed in Trial 2. Furthermore, my fish hosts in the H. pumilio titration were exposed over the course of 14 days of treatment and 14 days of maturation, and
were still found to host large numbers of metacercariae at the end of that period. Therefore, I do not think it likely that my fish were able to clear the majority of the metacercariae in the timeframe used in the Trials.

Mechanical damage to cercariae during transfer from the snail aquaria to the treatment aquaria was a potential issue considered to explain the low infection intensities induced. However, I think that it is unlikely that the cercariae were mechanically damaged during their transfer from the snail aquaria to the treatment aquaria, as cercariae of both species had previously been tested for potential mechanical damage by sealing large numbers of cercariae in a glass jar with water, and then shaking the jar vigorously. None of the cercariae of either species showed signs of mechanical damage such as loss of their tails (Personal obs.). Although I did not test these shaken cercariae for infectivity, they still showed high levels of swimming activity. Therefore I do not believe that mechanical damage during transfer could have resulted in a significant decrease in transmission success.

Another potential cause of the presumed low transmission success could have been human error in cercarial density estimations. If the cercarial densities had been grossly overestimated, then perhaps the actual percentages of cercariae able to establish themselves as metacercariae would have been closer to the observations of previous workers (Lo and Lee, 1996b). However, the same technique for estimation of cercariae density (Cantu, et al., 2013) was employed across all three Trials, and this method is thought to actually underestimate cercarial density as opposed to overestimation. During the first Trial, the snail aquaria were kept at full capacity (38 L) throughout the entire Trial, whereas in the second Trial water volumes in the snail aquaria were kept at the
minimum volume necessary for daily water exchanges. This was intended to increase the
density of cercariae per liter in order to increase metacercarial intensity in our
experimental fish hosts. Estimations of cercariae per liter increased during the second
Trial, but final metacercarial intensity decreased. Though I did not estimate cercariae per
liter in the *H. pumilio* titrations, I also observed a significant drop in metacercarial
intensities between the titration and Trial 3. It seems that the low level of infectivity of
my cercariae may have originated from the snails themselves, as opposed to immune
responses in our fish hosts or human error through mechanical damage and/or poor
estimation of cercarial densities.

**Loss of Parthenitae**

Loss of parthenitae stages and the subsequent reduction in quantity and/or quality
of cercariae shed from my snails could explain the low levels of transmission success
observed in the Trials. Infection by trematode parthenitae can have severe consequences
such as high mortality rates and parasitic castration of molluscan hosts (Lafferty and
Kuris, 2009). In the ongoing evolutionary arms race between parasites and their hosts it
would seem reasonable that snails may be able to clear or at least suppress trematode
parthenitae. Suppression of trematode parthenitae stages by molluscan hosts does occur,
but is not widely reported (Thompson, 1997). Several individual *Planorbella trivolvis*
were reported to have completely lost their trematode parthenitae as well as subsequent
production of snail eggs (Sears and Rohr, 2013).

Though only 5 of the snails infected with *C. formosanus* used in the first Trial
died during the course of the Trial, mortality of infected snails in the holding tanks during
collections was relatively constant. Indeed, while collecting *M. tuberculata* infected with
C. formosanus, snails infected with H. pumilio were also aggregated into their own aquaria. Of the 144 snails infected with H. pumilio collected between October 2012 and May 2013, 98 were still alive in September 2013. Of these 98 M. tuberculata, only 32 were still found to be shedding H. pumilio cercariae. I dissected several of the snails found to be non-shedding, and found a few that were still infected with H. pumilio parthenitae and a few that showed no signs of infection. This indicates that M. tuberculata may be able to clear H. pumilio (and likely C. formosanus as well), or at the very least, suppress the parthenitae.

If the snails used in our trials were indeed fighting their trematode infections, then it would seem likely that the surviving snails brought in for Trial 1 would have already begun to suppress their trematode infections. At the beginning of Trial 1, they would probably not have yet cleared their infection, as I was observing large numbers of cercariae in the daily density estimates. Even though I estimated higher cercarial densities per liter of treatment water in Trial 2 than in Trial 1, I found lower metacercarial intensities in my experimental fish in Trial 2. Fifty five of the 75 snails used in Trial 1 were utilized again over a month later in Trial 2, and this may account for the drop in metacercarial intensities induced. The higher cercarial densities estimated in Trial 2 could perhaps be accounted for by the reduction in snail-aquarium water volumes utilized in Trial 2. Though I did not estimate cercarial densities in the H. pumilio titration, I again saw a drop in metacercarial intensities between the titration and Trial 3, with Trial 3 utilizing the same snails as the titration over a month later. It seems that the drop in infection intensities between Trial 1 and 2, and the H. pumilio titration and Trial 3, correlates with utilization of the same snails over a month apart. Even if this theory can
explain the drops in metacercarial acquisition between trials it does not adequately explain the extremely low transmission success of the cercariae applied to the treatment aquaria, which was below 10% on average throughout the entirety of the experiment.

If *M. tuberculata* was suppressing trematode infections in our Trials, and quantity of cercariae did not suffer, then perhaps the quality of the cercariae suffered. Infectivity of cercariae can be affected by the particular characteristics of the parasite, the host, and environmental factors (Seppälä, et al., 2007). Snail hosts supplied with lower quality diets are known to produce fewer cercariae, with shorter lifespans and lower levels of infectivity than snails fed high quality diets (Seppälä, et al., 2008). This may be due to a limitation of resources available to the parasites for reproduction and subsequent cercarial output. Some snails may also be resistant to trematode parthenitae, and this resistance may reduce the quality of the cercariae produced (Seppälä, et al., 2007). Furthermore, cercarial quality may decline if the parasite invests more heavily in asexual multiplication than cercarial output (Louhi, et al., 2013). Age of infection in the snail host could also affect the quality of cercariae as host resources are used up by the parasite. The subsequent drop in cercarial infectivity between Trials 1 and 2, as well as between the *H. pumilio* titration and Trial 3, could be explained by this reduction in cercarial quality. The long infection history in my experimental snails could suggest that the trematodes had used the majority of the resources available to them, and the cercariae produced were low in quality and infectivity. Little is currently known about this phenomenon, and further study will be required to confirm these hypotheses.
Conclusions

The present preliminary investigation yielded a large amount of data useful for future work in determining the effects of *C. formosanus* and *H. pumilio* on native fish hosts. Though the results of my swim trials did not provide sufficient data to make strong predictions as to these effects, this work will pave the way to future investigations. I have shown that the Swimming Endurance Apparatus is an effective tool for testing the endurance of small fish, and that a large sample size can be processed using my methods. Further refinement of the SEA will be necessary, though these modifications will be simple to accomplish. I have also shown that artificial infection of fish with naturally infected snails is extremely labor intensive and suffers from complications occurring at the snail-trematode interaction level. The acquisition of experimentally infected cohorts of *M. tuberculata* would greatly improve the efficiency of future work. Interactions of larval trematodes and their hosts is an extremely complicated and dynamic system. Understanding of these systems will only be elucidated with future carefully controlled laboratory experiments. The present study has provided an effective methodology for such experiments, as well as the groundwork for future investigations.
APPENDIX SECTION

Appendix A: Water Exchange Theory, Establishing the Range of Treatment-Level Combinations and Determination of Daily Water Exchange Rates

Theory of Water Exchange

There was a need to control the rates and densities of cercarial exposure in order to test the endurance of fish over a broad spectrum of metacercarial acquisition rates and final intensities. However, infected *Melanoides tuberculata* do not all shed *C. formosanus* or *H. pumilio* cercariae at the same rate, and nor does an individual snail shed cercariae at a constant daily rate (Lo and Lee, 1996a). For example, an infected snail may shed 100 cercariae on day 1, none on days 2 and 3, and 500 on day 4. Thus, snails cannot be directly placed into treatment aquaria in order to infect fish, as this would prevent any assessment of the number of cercariae to which each fish was exposed. Therefore, metered allocation of cercariae to each fish aquarium required the exchange of a specified water volume from the aquaria housing Shedding snails. This method is far more labor intensive, but affords the advantages of averaging out the shedding rates of many snails, and also allows an investigator to monitor the numbers of cercariae applied to treatment aquaria each day.

Although all Experimental Water was drawn from the same source (water from a well in the Edwards Aquifer at the SMARC facility), it was divided into three functionally different types: Supply Water, Treatment Water, and Placebo Water. At the beginning of each day of a Trial, the required volume of each water type was drawn into plastic buckets from which the various water types would be later exchanged into the fish aquaria at specified rates.
Supply Water

Supply Water was drawn fresh each day and was used for three purposes: 1) to top off all snail aquaria after each Daily Water Exchange was drawn out, 2) it was exchanged into all fish aquaria at their specified Daily Exchange Rates during the three-day Habitation Phase (pre-treatment), and 3) it was exchanged into all fish aquaria at their specified Daily Exchange Rates during the 14-day Maturation Phase (post-treatment).

Treatment Water

Treatment Water was the water used to deliver infective cercariae from the Shedding-Snail Aquaria to the 24 Treatment Aquaria of each Trial. Treatment Water consisted of equal aliquots of water drawn from each of three Shedding-Snail Aquaria, and then pooled into a plastic bucket from which it was exchanged into the Treatment Aquaria each day of the Treatment Phase.

Placebo Snail-Water

Placebo Snail-Water consisted of equal aliquots of water drawn from each of three Non-Shedding Snail Aquaria, and then pooled into a plastic bucket from which it was exchanged into the three fish aquaria of each bank designated as Control Aquaria for each Run. Placebo water was also exchanged into Treatment Aquaria during the Treatment Phase of the Trial on the days during which those particular aquaria were not scheduled for cercarial exposure. Placebo Snail Water was exchanged into the Control Aquaria at various rates to determine if there were potentially meaningful snail only-effects on the swimming endurance of the fish. Such effects were not expected, but could have confounded analysis if they were to exist, unless controlled.
Overview of the Water Exchange Schedule

Each Trial of the experiment consisted of three sequential phases over the course of 33 days.

Habituation Phase

The Habituation Phase of each Trial began with the Initiation Event (the distribution of 120 fish to be tested in a Trial into the 30 fish aquaria) and lasted for four days. During the Habituation Phase, all 30 fish aquaria were exchanged with Supply Water at their prescribed Daily Exchange Volumes (Appendix A). The purpose of the Habituation Phase was to get the experimental fish accustomed to the daily human activities associated with the water exchange and feeding procedures described above.

Treatment Phase

Days 5 through 19 of each Trial constituted the 16-day Treatment Phase, during which the fish in the aquaria were exposed to their prescribed levels of Treatment or Placebo Water. Each day of the Treatment Phase, a 1-liter sample was removed from the water pooled from the three Shedding-Snail Aquaria using the baster/beaker pair assigned to Treatment Water. This water sample was used to estimate the number of cercaria per liter of the Treatment Water on that day. These estimates were accomplished utilizing the same procedure employed by a previous worker (Cantu, et al., 2013). This involves filtering the cercariae out of the water onto a fine meshed screen and then staining with Rose Bengal. The screen was then placed in a counting tray and the number of cercariae counted.
Control Aquaria

During the Treatment Phase, each Control Aquarium received either Supply Water or Placebo Water (as determined by spreadsheet calculations) at its prescribed Daily Exchange Volume.

Treatment Aquaria

Some Treatment Aquaria were treated with Treatment Water for the entire Treatment Phase (16 days), while some others only received Treatment Water during the last two days of the Treatment Phase. The remainder of the Treatment Aquaria received Treatment Water for varying Durations between 3 and 15 days inclusively. The Treatment Phase of each Trial ended with the water exchange on the 16th day of treatment (Day 20 of the experiment), and was followed by a 14-day Maturation Phase.

Maturation Phase

On Day 21 of each Trial (the first day of the Maturation Phase), about ⅔ of the water was drawn out of all aquaria and replaced with Supply Water, and the procedure repeated once per aquarium. This was intended to purge the vast majority of the remaining cercariae or Placebo Water out of the experimental aquaria, and was meant to prevent the challenges of Placebo Water, and more importantly the cercaria-laden Treatment Water from lingering on into the early days of the Maturation Phase. The purpose of this phase is to allow all metacercariae that had encysted in experimental fish by the last day of treatment to mature. Once the Maturation Phase was complete, the swimming endurance of each fish in the Trial was tested in the SEA in a random aquarium-by-aquarium sequence.
Determination of MinDEV and MaxDEV:

The first step in setting up a trial was to determine the minimum volume of Treatment Water that would be exchanged into an aquarium for the last two days of the Treatment Phase of the experiment (i.e. 25 ml/d for 2 days). This value was the Minimum Daily Exchange Volume (MinDEV). MinDEV established the minimum final intensities of metacercariae for the Trial as well as the minimum rate of metacercarial acquisition. Maximum Daily Exchange Volume was determined next (MaxDEV), which was the maximum volume of Treatment Water that would be exchanged into an aquarium for the last two days of the Treatment Phase of the Trial (i.e. 5,000 ml/d for 2 days). MaxDEV established the maximum final intensities of metacercariae for the Trial as well as the maximum rate of metacercarial acquisition.

After MinDEV and MaxDEV were determined as above for a planned Trial, the Aquarium-Set-Up-File assigned to that Trial would be opened and the blanks completed on the “Start Here” Sheet (Figure 10). Then, MinDEV and MaxDEV would be entered into the highlighted cells of the Balancer/Randomizer Sheet (Figure 11). The Balance/Randomizer Sheet performs a series of calculations that allocate intensity and rate loads into the aquarium array such that gradients in various positional factors such as light intensity, human activity, temperature, etc. will be randomized across the array, both horizontally and vertically (Figure 12). The worksheet has been tuned to accommodate most combinations of MinDEV and MaxDEV. The overall fit to a perfectly randomized array is measured by the Overall Index of Deviation in the yellow box near the center of the array, with lower values indicating better randomization. Values lower than 50 are likely sufficiently randomized to avoid confounding bias from environmental gradients.
Success is also indicated when neither the extreme Intensity Ranks nor Rate Ranks (dark blue and dark red) are concentrated in one region of the treatment array (Top right diagrams), and also when the circles representing various combinations are more or less uniformly distributed into the boundary spaces of Charts 1-4 (top center).

Once satisfied with the load balance, the Program-Summary tab calculates the Daily Exchange Volume and Duration for every aquarium in the 5 X 3 array of aquaria. The Program Details Tab illustrates the entire run laid out in detail (Figure 13), based entirely upon the Daily Exchange Volumes and Durations determined in the Balancer/Randomizer Sheet.

Figure 10. “Start Here” worksheet that establishes labels for other sheets.
Figure 11. Example values for MinDEV and MaxDEV properly entered into the Balancer Randomizer sheet.

<table>
<thead>
<tr>
<th>Minimum Daily Exchange Vol. MinDEV ==&gt;</th>
<th>Minimum Final Mean Intensity MinFinInt ==&gt;</th>
<th>Minimum Rate of Metacercarial Acquisition MinRateAcq ==&gt;</th>
<th>25 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ml</td>
<td>50 ml</td>
<td>25 ml</td>
<td></td>
</tr>
<tr>
<td>Maximum Daily Exchange Volume MaxDEV ==&gt;</td>
<td>Maximum Final Mean Intensity MaxFinInt ==&gt;</td>
<td>Maximum Rate of Metacercarial Acquisition MaxRateAcq ==&gt;</td>
<td>400 ml</td>
</tr>
<tr>
<td>400 ml</td>
<td>6,400 ml</td>
<td>400 ml</td>
<td></td>
</tr>
</tbody>
</table>

Target Species: Cyprinella venusta ;
Run #1;
Run Date: 2013-6-2

Target Species: Cyprinella venusta ;
Run #1;
Run Date: 2013-6-2
Figure 12. Example of Balancer Randomizer sheet showing intensity and rate loads balanced across the aquarium array so as to avoid systematic bias due to horizontal and vertical gradients of exposure to such incidental factors as light, human activity, temperature, etc.
Figure 13. Example Program-Details chart showing all water exchanges on a day-by-day basis throughout the experiment.
A semi-portable apparatus was constructed from hardware available at local stores to test the swimming endurance of small fish at varying speeds. The apparatus is mounted on a sheet of 8 feet X 4 feet X ¾ inch exterior-glue plywood mounted on a wheeled frame constructed from ¾ inch exterior-glue plywood on pressure-treated 2-inch dimensional lumber. Components of the apparatus are explained in the text that follows by referring to alpha-codes assigned to reference points in the associated figures.

**Construction and Theory of Operation**

Water flow through the swim tunnel is provided by a fixed-speed sump pump (Utilitech®, 1/3 hp, #0079356) (D), submersed in a 100 gallon plastic reservoir tank (B) mounted on the backside of the apparatus (Figure 15). When the pump (D) is powered on, water is pumped at full volume out of the reservoir (B) and into the front side of the apparatus (at E). The water from the pump (D) enters the front side (Figure 16) at (J),
and immediately enters a tee fitting, which splits the flow. The relative flow rate of the two pathways coming out of this tee is dependent upon the combination of settings of two adjustable ball cocks, the “Flow” Valve (K) and the “Diverter” Valve (I), both of which have been equipped with pointers and radial dials marked with 11 calibration settings from 0 (fully closed) to 10 (wide open). Combinations of valve settings are selected (based on prior calibration trials) to adjust velocity during swimming endurance tests. The Flow Valve (K) restricts the amount of the incoming water that will pass through the experimental pathway of the apparatus, while the Diverter Valve (I) shunts the excess volume of the constant-speed pump (D) back through the mounting board (at H) and returns it to the reservoir (F) & (B). Various combinations of adjustments of these two valves relative to each other permit the operator to control the flow velocity of the water passing through the viewing tube in which the swimming endurance of experimental fish will be tested.

The tests consist of challenging an individual experimental fish to swim continuously in the viewing tube infused with a mass flow of water at a constant velocity close to the maximum swimming speed of the species until the fish can no longer avoid being pinned against the downstream screen by the current.
Figure 15. Swimming Endurance Apparatus: Rear View.
Figure 16. Swimming Endurance Apparatus: Front View.
The water exiting the Flow Valve is under considerable pressure (depending on valve settings) and this would cause unacceptable turbulence in the viewing tube, and prevent uniform testing of experimental fish. Therefore, a diffuser/linearizer subassembly was designed and installed between the Flow Valve and the Viewing Tube to reduce turbulence and linearize the flow through the viewing tube to as near laminar as possible (Figure 17).

The Diffuser Subassembly (Figure 17L) consists of two floor drains of different sizes (NDS #75 polyolefin Atrium gate floor drains, Figure 18) arranged in series, with the smaller drain upstream and with its base inserted inside the base of the larger. These serve to break up large-scale currents into small-scale turbulence.

The Linearizer Subassembly (Figure 18) contains numerous 5” long, ½ in diameter plastic tubes (Figure 18M) designed and marketed for use as guttering ferrules (Amerimax Home Products #21060 Home 21060 Plastic Gutter Ferrule, UPC: 0 49821 21060 1). The tubes themselves are very thin-walled, which helps minimize backpressure, and the length to diameter ratio of 12:1 breaks up any remaining large torsional currents. The tubes also have six straight internal fins, which serve to linearize any small torsional currents and provide reasonably laminar flow into the viewing tube.
Figure 17. Swimming Endurance Apparatus: Valve Calibration Diagram.
Figure 18. Swimming Endurance Apparatus: Floor drains and gutter ferrules used in diffuser/linearizer subassembly.

Figure 19. Swimming Endurance Apparatus: Testing Center in Introduction Mode.

Figure 20. Swimming Endurance Apparatus: Testing Center in Test Mode.

Figure 21. Swimming Endurance Apparatus: Testing Center in Release Mode.
Calibration of Velocity Control Valves

The flow rates are determined by setting the Flow and Diverter valves to various settings relative to one another, powering the pump on, and injecting Calibration Balls (5 mm Pom-Poms) into the tunnel. As the Pom-Poms move through the viewing tube, they are filmed with a high fps video camera that can shoot sequences of video up to 1000 fps. Behind the viewing tube, there is a measuring tape calibrated in cm. By analyzing the resulting video, we are able to determine the velocity of each Pom-Pom by counting the number of frames required to travel 50 cm at the current recording speed of the camera.

Gathering Calibration Data

To begin calibration of a particular combination of valve settings the high-speed video camera is positioned on a tripod such that the entire viewing tube fills the frame, and the pump is powered on. After the air has been expelled from the viewing tube, the camera is activated, the valve settings and camera speed are recorded in a journal with the start time, the valve on the calibration subassembly (N) is slowly opened, and Pom-Poms are introduced individually into the funnel such that consecutive Pom-Poms are always dyed with differing colors, in case they overlap in a frame. After about 15 Pom-Poms have been captured on the video, the pump is powered off, and the camera is deactivated, and the stop time recorded with the journal entry. The setting of one of the valves is then incremented and the protocol repeated. When the adjusted valve has been calibrated at its furthest setting, it is set back to its lowest setting, and the other valve is incremented.
and the entire series is repeated. Downstream screen clearance, pump filter clearance, and water level in the reservoir are adjusted to normal between calibrations.

**Analysis of Calibration Data**

An Excel 2011 spreadsheet has been developed to analyze the videos and determine the mean flow velocity (in cm) of the Pom-Poms recorded in a video, given the camera speed in fps, the starting frame number when a Pom-Pom’s position can be determined after it enters the right side of the video frame, the reading in cm behind the Pom-Pom in that frame (from the cm ruler behind the viewing tube), and the ending frame number and cm reading behind the Pom-Pom before it disappears out of the video frame to the left.

After videos of the Pom-Poms have been recorded for a series of valve-setting combinations, the videos are transferred into a Macintosh iMac computer and opened with QuickTime 7.X (the ability to count frames has been removed from later versions of QuickTime). The above data for the 15 Pom-Poms in one video are then entered into an array in the spreadsheet that has been dedicated to one of the many combinations of valve settings in which the Flow valve is partially or fully open.

When all the Pom-Pom data for one combination of valve settings has been entered, the spreadsheet returns the number of Pom-Poms with entered data, the mean velocity (in cm) of the Pom-Poms entered, and the coefficient of variation of the velocities behind the mean. All valve combinations yielding unacceptably high coefficients of variation (>10%) are rejected as unusable for reliable experimentation.
Appendix C: Preliminary Swim Tunnel Studies

A number of preliminary swim trials were necessary to determine the limits of the SEA as well as to determine the maximum swim speeds for both *Cyprinella venusta* and *Dionda diaboli*. The necessity of the acclimation and warm-up phases were elucidated during this preliminary work. The testing phase of each swimming endurance test was the phase in which water velocities were raised to 54 cm/sec (77% of maximum swimming speed) for *C. venusta* and 45 cm/sec (76% of maximum swimming speed) for *D. diaboli*.

**Behavioral Issues of Experimental Fish**

A distinctive behavior reported for *C. venusta* is oral grasping (Adams et al., 2003). This is a station maintenance behavior that needed to be controlled as this could potentially confound swim times. Early versions of the SEA had an upstream screen composed on 1/3 inch diagonal hardware cloth. The divisions in this screen were a size and shape such that appropriately sized *C. venusta* could bite and hold onto the screen. Modification of the upstream and downstream screens of the SEA to ¼ inch square hardware cloth prevented this behavior. Reducing the size of the screens also prevented the challenges of very small minnows attempting to pass through the upstream or downstream screens. Oral grasping has not been reported for *D. diaboli* and was not noted during the preliminary trials or in the 3 Experimental Trials.

**Development of the Acclimation, Warm up, and Testing Phases**

It became clear during the preliminary studies that we could not introduce a fish into the SEA, increase speed to maximum and expect the fish to swim normally to exhaustion. Fish tended to refuse to swim when treated in this manner. Thus, methods
for encouraging fish of both species to swim properly in the tunnel needed to be developed.

**Acclimation Phase**

The acclimation phase begins by setting the SEA diverter and flow valves to 5. This would fill the viewing tube with water. After the swim tunnel had been primed, the flow valve would be reduced to 0, representing a water velocity of 0 cm/sec. Next a fish would be removed from an aquarium and introduced into the SEA. This fish would then be gently encouraged and locked into the viewing tube of the SEA with the downstream screen plunger. It was found that allowing the fish a 2-minute acclimation period without water flow was sufficient to prevent behavioral issues and swim refusals in both species of fish, so long as the next phase (Warm-up) was also included.

**Warm-up Phase**

Fish often refused to swim if not given a proper acclimation time. Fish also tended to refuse to swim at their testing speeds (70-80% of maximum swimming speed) if not given a proper swim Warm-Up. The Warm-Up phase of the swimming endurance test was designed to encourage the fish to begin to swim in the SEA at low velocities. It should be noted that both *C. venusta* and *D. diaboli* required this Warm-Up. At the 2-minute mark of the Acclimation phase the flow valve would be increased from 0 to 1 (velocities at both these settings were 0 cm/sec). At 10-second intervals the flow valve (and subsequent water velocity) would be increased to 2 (12 cm/sec), 3 (21 cm/sec) until the flow valve was set at 4 (30 cm/sec). The duration between increasing the Flow valve from 0 to 4 took 30 seconds. A fish would be allowed to swim at this speed for 2 minutes, which represented a 2-minute, 30-second Warm-Up.
Testing Phase

The testing phase was to be the phase of the endurance test in which the velocity of water flowing through the SEA would be increased to represent approximately 70% of the maximum swimming speed of the respective species being tested. Determination of SEA valve setting for maximum swim speed of both *C. venusta* and *D. diaboli* required the use of calibration techniques (Appendix B), as well as uninfected experimental fish. Twenty fish of each species were tested in the SEA to determine maximum swimming speeds. All fish received an acclimation and warm-up time as explained above. At the 2 minute 30 second mark the Flow valve was increased from 4 to 5, and subsequently increased one valve setting every 10 seconds until the fish was unable to overcome the velocity of water in the viewing tube. Average max speed for *C. venusta* was (70 cm/sec) which is slightly higher than 61 cm/sec as reported by previous workers (Leavy and Bonner, 2009). Average maximum swimming speed for *D. diaboli* was 60 cm/sec. Though there have been no reports of the maximum swimming speed of *D. diaboli* it was surprising to find it to be much higher than 18 cm/sec as was reported for a closely allied species *Dionda episcopa* (Leavy and Bonner, 2009). It was decided that *C. venusta* would be tested with the SEA flow valve set at 7 and the diverter valve set at 5. This velocity was calculated as approximately 54 cm/sec which represented 77% of the calculated maximum swimming speed of *C. venusta*. *Dionda diaboli* was to be tested with the SEA flow valve set at 6 and the diverter valve set at 5. This velocity was calculated as approximately 45 cm/sec, which represented 76% of the calculated maximum swimming speed of *D. diaboli*. 
**Determination of Maximum Swimming Speeds**

Twenty fish of each species were tested in the SEA to determine maximum swimming speeds. All fish received an acclimation and warm-up time as explained above. At the 2 minute 30 second mark the Flow valve was increased from 4 to 5, and subsequently increased one valve setting every 10 seconds until the fish was unable to overcome the velocity of water in the viewing tube. Average max speed for *C. venusta* was (70cm/sec) which is slightly higher than 61cm/sec as reported by Leavy and Bonner (2009). Average maximum swimming speed for *D. diaboli* was 60cm/sec. Though there have been no reports of the maximum swimming speed of *D. diaboli* it was surprising to find it to be much higher than 18cm/sec as was reported for a closely allied species *Dionda episcopa* (Leavy and Bonner 2009). It was decided that *C. venusta* would be tested with the SEA flow valve set at 7 and the diverter valve set at 5. This velocity was calculated as approximately 54 cm/sec which represented 77% of the calculated maximum swimming speed of *C. venusta*. *Dionda diaboli* was to be tested with the SEA flow valve set at 6 and the diverter valve set at 5. This velocity was calculated as approximately 45 cm/sec which represented 76% of the calculated maximum swimming speed of *D. diaboli*. 
Appendix E: Differentiation of cercariae

Three exotic species of digenetic trematode were found utilizing *M. tuberculata* as a first intermediate host in the Comal River, *Philophthalmus gralli*, *C. formosanus* and *H. pumilio*. In most cases, accurate identification of cercariae to species requires the use of a dissecting microscope; however, size differences among these three species, as well as multiple uniquely diagnostic cercarial behaviors allowed easy diagnosis without the aid of a microscope after some experience.

**Detection of Centrocestus formosanus Cercariae**

Snails infected with *C. formosanus* tended to shed a large number of cercariae, which often appears to the naked eye as a cloudy suspension in the vial. *C. formosanus* cercariae are small, with two distinct eyespots and have an inverted heart-shaped body when at rest. These cercariae swim in tight circles, largely staying in the same location, with short periods of rest in between frantic swimming sessions.

**Detection of Haplorchis pumilio Cercariae**

*Haplorchis pumilio* cercariae also have two distinct eyespots, but are much longer than those of *C. formosanus*, and have a larger oval body. The tail of *H. pumilio* is also proportionately much longer, at approximately twice the length of the body. *H. pumilio* cercariae also swim in short bursts, but cover a greater linear distance during swimming sessions than those of *C. formosanus*. Snails infected with *H. pumilio* tend to shed fewer numbers of cercariae than those infected with *C. formosanus*; however, the large size of these cercariae and their determined linear movement can be easily detected with the naked eye.
Detection of *Philophthalmus gralli* Cercariae

*Philophthalmus gralli* cercariae are the largest of the three species. This larval trematode has an elongated, narrowly oval body, with a tail roughly the same length. The cercariae have a clearly visible disc shaped sucker at the center of the body. Swimming behavior is a slow undulating whip like motion of the body and tail. Cercariae of *P. gralli* were rarely encountered in suspension, since they tend to encyst on the bottom of the vial after a short swim. However, when encountered, snails infected with *P. gralli* were discarded. No snail was observed to be shedding more than one species of cercaria.
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