

EXPERIMENTAL TESTS OF THE EFFECTS OF EXOTIC HETEROPHYID  
METACERCARIAE ON THE SWIMMING ENDURANCE OF  
SMALL FISH HOSTS

By

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## INTRODUCTION

### 1.1 Overview

Two aquatic snail species from Asia were introduced into freshwaters of the USA sometime in the early 20<sup>th</sup> Century. The snails are enough trouble in themselves, as they are aggressive competitors and can alter the composition and dynamics of aquatic ecosystems. However, these snails also brought several parasites with them, two of which are able to infect and damage local native fishes in many families, and some of those fish are listed species of conservation concern.

#### 1.1.1 A snail problem: two invasive snails of the family Thiaridae

##### 1.1.1.1 *Original introductions into USA*

*Melanoides tuberculata* (Müller, 1774) is thought to have been introduced to the USA through the aquarium trade sometime prior to 1950 (Murray 1971), and *Tarebia granifera* (Lamarck, 1822) was introduced perhaps as early as 1935 (Nollen and Murray 1978). These snails were first reported from Texas in 1964 from the spring-fed San Antonio River (Murray 1964). Both species were later reported from the spring-fed Comal River (Murray and Woopschall 1965). Established populations of these snails have since been reported from spring-fed waters in over 15 counties in Texas (Daniel et al. 2018, Karatayev et al. 2009, Tolley-Jordan and Owen 2008).

##### 1.1.1.2 *Melanoides tuberculata*

*Melanoides tuberculata* has been reported to serve as the first intermediate host for more trematode parasites than any other snail in the world, and the list includes several trematodes that cause serious human disease (Pinto and de Melo 2011). At least four species of invasive trematodes have been introduced into Texas waters by *M. tuberculata*,

including *Centrocestus formosanus*, *Haplorchis pumilio*, *Philophthalmus gralli*, and an unidentified species of *Renicola* sp.

### **1.1.1.3 *Tarebia granifera***

*Tarebia granifera* also serves as host for many economically and medically important trematodes in its native range in Asia and is known to serve as first intermediate host for *P. gralli* and *H. pumilio* in Texas waters.

## **1.1.2 Invasive parasites propagated by invasive snails**

### **1.1.2.1 *The two heterophyids***

The metacercariae of *Centrocestus formosanus* and *Haplorchis pumilio* exhibit broad host latitude across many Nearctic and Neotropical fish families and are problematic human parasites in parts of Asia where fish are often consumed without adequate cooking heat.

### **1.1.2.2 *Centrocestus formosanus background***

The infective cercarial larvae of *Centrocestus formosanus* escape *Melanoides tuberculata* via the excurrent siphon, and swim about randomly in the water column, largely at the mercy of water currents. When inspired with the respiratory current of a susceptible fish, they attach to the respiratory membrane of the gills. Metacercariae of *Centrocestus formosanus* have been found parasitizing the gills of many fishes, including the endangered fountain darter (*Etheostoma fonticola*) (Mitchell et al. 2000, Mitchell et al. 2005) and the federally threatened Devils River Minnow (*Dionda diaboli*) (Figure 1). This trematode is now known to be widely distributed in most populations of *M. tuberculata* in Texas (McDermott et al. 2014).

### **1.1.2.3 *Haplorchis pumilio* background**

*Haplorchis pumilio* 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. While the cercariae of the species have long been known to be present in the water column of the Comal River, nothing about its distribution in fishes of North America north of Mexico had been reported until our lab discovered it in numerous local fishes, including the endangered fountain darter, the threatened Devils River minnow, and several fishes popular as game and food fish (Huston et al. 2014).

### **1.1.3 Eggs of invasive parasites brought in by migratory piscivorous birds**

The definitive host of *Centrocestus formosanus* in Texas was reported by Kuhlman (2007) to be the Green Heron (*Butorides virescens*). The definitive host of *Haplorchis pumilio* in Venezuela was reported by Díaz et al. (2008) to be the Striated Heron (*Butorides striatus*). The Striated Heron is native to South America and Panama, but the Green Heron is very closely related, and is probably serving as definitive host for both species in Texas.

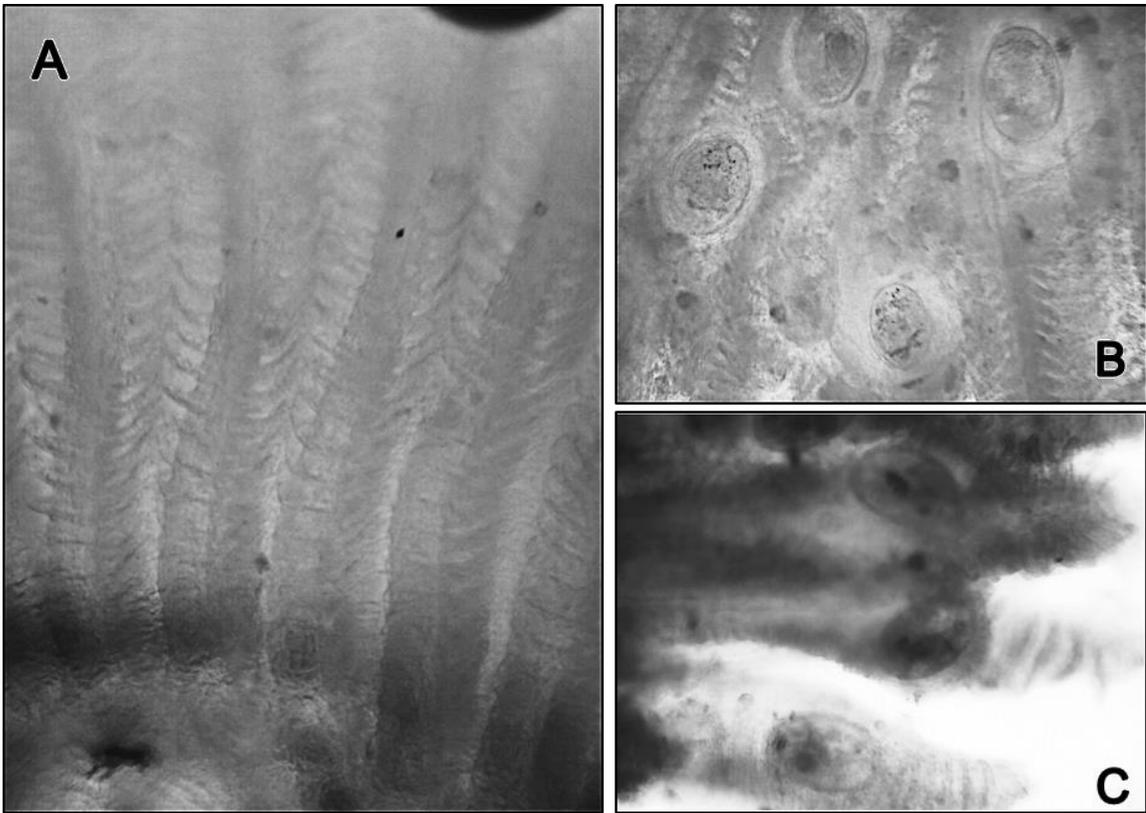


Figure 1. Gill filaments of *Dionda diaboli*: (A) normal architecture (fish raised from eggs at San Marcos Aquatic Resources Center refugium), (B) basal region of filaments of fish wild-caught from Devils River and infected with metacercariae of *Centrocestus formosanus*, (C) tips of filaments infected with *C. formosanus* (wild-caught from Devils River; photos by DGH).

The Green Heron is migratory, and spends the winter in Mexico and Central America, but returns to Texas in March laden with adult worms (Kuhlman 2007). This bird was probably responsible for bringing both parasites to Texas once they were introduced into Mexico in 1979 (Amaya-Huerta and Almeyda-Artigas 1994). So, wherever *M. tuberculata* has been introduced into North America, if the habitat is a suitably attractive feeding site for green Herons and within the range of the Green Heron, it's only a matter of time before both heterophyid parasites are introduced.

## 1.2 Pathology and Epizootiology of *Centrocestus formosanus*

### 1.2.1 In wild fisheries

After *C. formosanus* cercariae penetrate the respiratory epithelium of a susceptible fish, they burrow into the gill filaments, eventually making their way to the supporting cartilage where they can cause various levels of damage, the severity of which varies widely with the host species. The fountain darter is particularly susceptible to gill damage since it reacts much more strongly to the invasion than many other fish hosts that have been examined. According to Mitchell et al. (2000), who found up to 1,524 cysts in the gills of fountain darters wild-caught from the Comal River:

Examination of wet-mounted gills of fountain darters with flared opercular flaps revealed swollen and deformed branchial tissues. A clear layer of tissue often was noted surrounding the cyst and appeared to be a host reaction.... Infected gill filaments were shortened, thickened, and often distorted. Epithelial hyperplasia and fusion of the filaments also were noted. Affected lamellae often had several areas that were engorged with red blood cells and massive cartilage lesions and apparent proliferation resulting in displacement of the epithelial tissue.... The trematodes were encysted in chondrocytes and affixed to the side of gill filament cartilage. The proliferated cartilage caused affected filaments to expand to several times the normal diameter, and this resulted in the complete disruption of the normal gill morphology. The filaments were thick, distorted, and apparently fused. Few typical lamellae were present, and epithelial hyperplasia was extensive....

Mitchell et al. (2000) indicated that many heavily infected darters had died before he could examine them and speculated that darter deaths were probably occurring undetected in the Comal River as a result of direct and indirect factors associated with this pathology.

### 1.2.2 In fish culture

Gill damage from high intensities of *C. formosanus* metacercariae has been shown to cause mass mortalities in high-density fish culture (Blazer and Gratzek 1985, Mohan et al. 1999, Ortega et al. 2009), apparently due to respiratory impairment. Blazer and

Gratzek (1985) studied several species of tropical fishes reared in Florida ponds that had been infected with what is now thought to be *C. formosanus*. They observed the following in these natural infected fish:

... in heavily infected fish, opercula were flared and gills visible. In some cases, haemorrhage from the gills was apparent. Clotted blood was visible between gill filaments and protruding from the opercula. An increased ventilation rate as determined by opercular movement was observed. ...The amount of cartilage proliferation varied from 2 to 3 cells thick to areas in which hypertrophy and hyperplasia led to actual fusion of 2 primary lamellae.... Remnants of the original cartilage, consisting of stacked, flattened chondrocytes usually remained within the hyperplastic tissue. However, the normal structure of the gill filament and secondary lamellae were destroyed. In some cases, the capillaries were flattened along the border of the proliferating cartilage. Occasionally, the respiratory epithelium was metaplastic, with areas of squamous epithelium....

### 1.3 Pathological Effects of *Haplorchis pumilio*

Our lab has experimentally demonstrated that the encysted metacercarial larvae of *Haplorchis pumilio* also have a lethal effect on various species of fish, including the endangered fountain darter, *Etheostoma fonticola*, and the blacktail shiner, *Cyprinella venusta*, (Huston 2014, Figure 2).

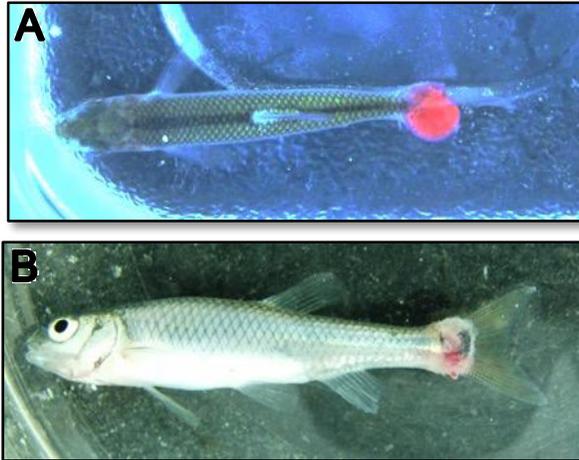


Figure 2. Blacktail shiner exposed for 30 minutes to water from a snail aquarium with *Haplorchis pumilio* cercariae. (A) Day 2 post-exposure; note serum-filled blisters on sides of caudal peduncle. (B) Day 3 post-exposure; blisters have ruptured and fish has died, probably from electrolyte loss.

*Haplorchis pumilio* penetrates the skin wherever contact is made, causing flashing and erratic swimming by the host being infected. The cercariae then migrate from the point of penetration to the fin bases or other skeletal cartilage, randomly intercepting other organs in the journey (larval migrans) (Sommerville 1982). Larval migrans would cause subsequent inflammation, micro hemorrhaging in the viscera, and in many cases, mortality in the affected host. The cercariae eventually settle at the bases of the fins between the fin rays. These fin rays are known to actively articulate relative to one another in healthy fish (Bainbridge 1962), and the presence of these metacercariae and inflammation are thought to impair the articulation of the fins during fin flaps, which would reduce swimming efficiency.

#### 1.4 Observed Inconsistencies – The *Centrocestus formosanus* Paradox

There are several frustratingly paradoxical inconsistencies in the limited information we have available to us regarding the epizootiology of *Centrocestus formosanus* and *Haplorchis pumilio* in Texas waters.

#### 1.4.1 **Low density of susceptible fishes in waters with high cercarial density**

Although anecdotal, we have observed that in some habitats where either of these parasites occur in abundance, and which appear to be ideal habitats for many centrarchids and cyprinids, there are surprisingly few individual fish of either family. This was first noted by workers in our lab in 2009 in the waters associated with the confluence of the Comal and Guadalupe Rivers. These waters receive almost no fishing pressure from anglers. This negative association could be explained by assuming that the susceptible fish in waters with high densities of heterophyid cercariae are simply killed by the cercariae – but no fish kills have been reported from these same areas.

#### 1.4.2 ***Centrocestus formosanus* intensity estimates in wild-caught shiners much lower than expected**

In a study of *Centrocestus formosanus* parasitism levels in *Cyprinella venusta* from the confluence of the Comal and Guadalupe Rivers, intensity of infection in the wild fishery was estimated through necropsy of wild-caught individuals (Fleming et al. 2011). A concurrent study assessed the acquisition rate of metacercariae in caged shiners in the same water at approximately the same time (Fleming et al. 2011). The estimates of parasite intensity in the fishery based on necropsy of wild-caught individuals dramatically underestimated (by a factor of 10X to 50X) the parasitism levels that were expected from the rate at which the caged shiners had acquired the parasite (mean=4.2 cysts/d, max=14 cysts/d; Fleming et al. 2011).

Figure 3 shows the cyst-count results based on about 50 blacktail shiners wild-caught from near the confluence of the Comal and Guadalupe Rivers (data derived from Fleming et al. 2011). The ages of the fish were determined by back-calculating from their lengths (age and growth data from Casten 2006). If the older of these wild-caught fish had been

living in the same reach for two years, the necropsy data would suggest that they would have only acquired about 200 cysts, or an average of about one cyst every 4 d. However, when uninfected shiners were placed in cages at various places near the confluence for 3-5 days on several occasions (Fleming et al. 2011), the caged fish acquired parasites at a rate many times greater than one cyst every 4 d (Figure 4). The actual rates of acquisition in the caged shiners suggests that wild-caught fish living in the water for 2 y should have acquired an average of about 3,000 cysts, and possibly as many as 10,220 cysts, instead of just 200!

#### 1.4.3 *Centrocestus formosanus* prevalence estimates in wild-caught shiners much lower than expected

Not only the intensity, but also the prevalence of *Centrocestus formosanus* in *Cyprinella venusta* was inconsistent between these two concurrent studies in the same water (Figure 4). While prevalence of *C. formosanus* in wild-caught shiners hovered around 60% for fish up to 3+ y old, almost all of the uninfected shiners held in cages at about the same time became infected within 3-5 d of exposure (to the same water from which the wild-caught shiners had been captured) with prevalence reaching 98% in less than 6 d! So, if 98% of caged shiners become infected within 5 d of exposure to river water, how could 20-40% of 1-4-year-old fish of the same species wild caught from the same water have avoided infection?

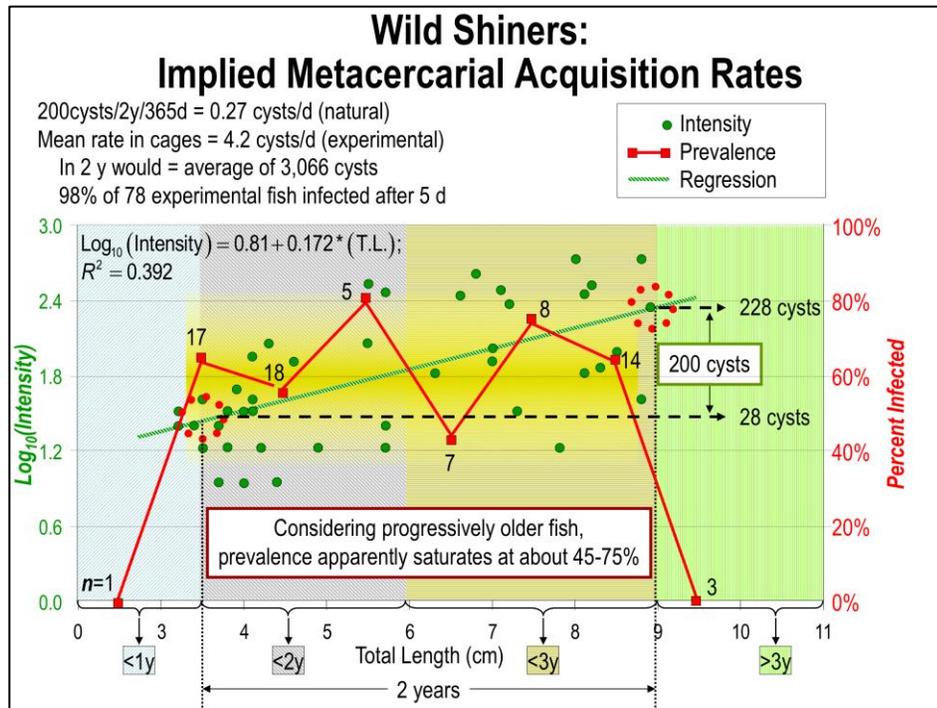


Figure 3. Apparent rate of acquisition of *Centrocestus formosanus* cysts derived from wild-caught shiners near confluence of Comal and Guadalupe Rivers. Data from Fleming et al. (2011).

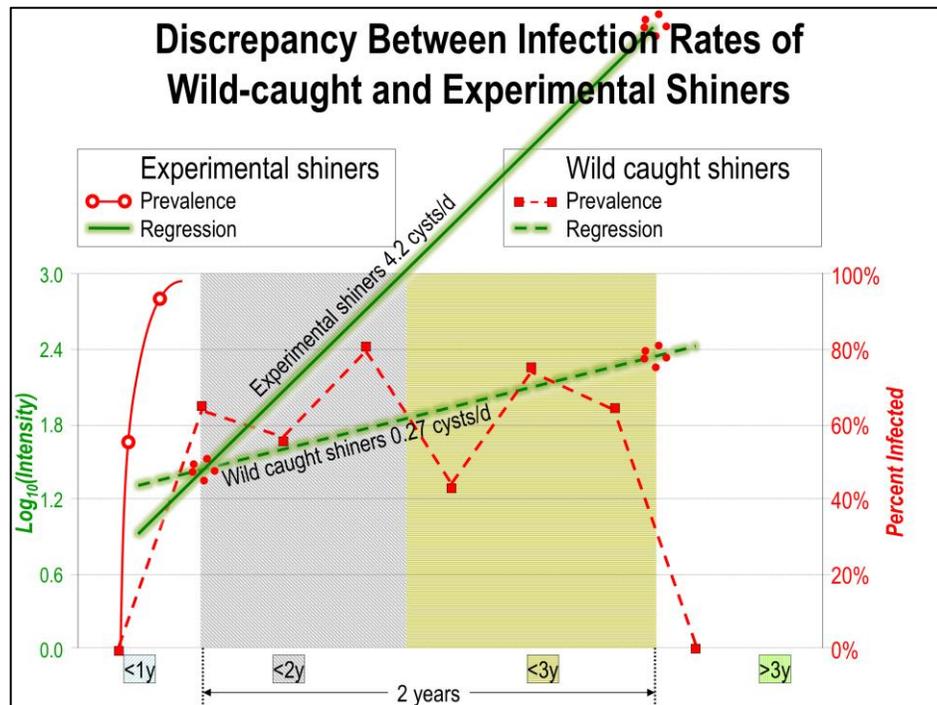


Figure 4. Discrepancy between apparent prevalence and intensity of infection based on wild-caught shiners (dashed lines) vs. realized prevalence and intensity based on experimental fish placed in cages for 3-5 days (solid lines). Data from Fleming et al. (2011).

The discrepancy between these estimates suggests that all fish wild-caught from the habitat (some of which were 3+ years old) were recent immigrants, and that these fish could not have survived in those waters all their lives. Consequently, we have developed a hypothesis that might explain (1) the unexpectedly low intensity and prevalence of *C. formosanus* in wild-caught susceptible fishes, (2) the lack of fish kills reported where the density of the infective stages of *C. formosanus* is high, and (3) unexpectedly low densities of susceptible fishes in otherwise suitable habitats: either the infected fish are more likely to succumb to predation by various piscivores at a much higher rate than uninfected fish, or the susceptible fish that encounter high densities of *C. formosanus* cercariae emigrate preferentially to other reaches of a stream where cercarial densities are not so high. Thus, *C. formosanus* is probably causing much more damage to susceptible wild fisheries (which includes Centrarchidae, Cyprinidae, Percidae, and Ictaluridae) than previously thought, and probably also causing previously unrecognized alteration of fish community structure. However, even if intensity and prevalence estimates from caged-fish studies are more representative of the rate of metacercarial acquisition by fish, these statistics still do not provide a quantitative model that researchers can employ to explore the actual impact of the parasites upon a wild fishery.

#### 1.4.4 Fish kills in culture but not in wild

Despite the reports of severe pathologies and of mass deaths of fish in culture, we have found no reports of fish kills attributed to *C. formosanus* or *H. pumilio* metacercariae in any of the many wild fisheries where they occur naturally, or where they have been introduced into naïve ecosystems, even in waters we have studied where the cercariae of these heterophyids are overwhelmingly the most abundant cercaria in the water column.

#### 1.4.5 *M. tuberculata* distribution no longer restricted to thermally stable waters.

For over 50 years, the cold sensitivity of this tropical snail restricted its distribution in Central Texas to thermally stable spring runs, where winter thermal minima never approach the reported lethal thermal minimum of 17 °C that has been reported for *M. tuberculata* by Mitchell and Brandt 2005 (Mitchell and Brandt 2005). Just to make sure that the snail had not spread from the Comal River into the connecting waters of the Guadalupe River, Fleming et al. (2011) executed several thorough searches 2000-2001 for *M. tuberculata* in the connecting waters of the Guadalupe River near its confluence with the Comal River, which was heavily infested with the snail. Only dead shells, presumably washed out of the Comal River by high-flow events, were found in the Guadalupe River at that time, confirming that the snail was restricted to the thermally stable spring run of the Comal River. However, in 2009, our lab discovered *M. tuberculata* thriving in the ambient waters of the Guadalupe River up to several river-km upstream and downstream from its confluence with the Comal River, and since then we have discovered it thriving in the San Marcos River some 50 river-km downstream from the headsprings. So, what happened between 2001 and 2009 that has allowed *M. tuberculata* to suddenly begin thriving in ambient, surface-fed streams where winter thermal minima remain for weeks at a time well below the reported lethal thermal minimum of *M. tuberculata*?

#### 1.5 Summary of Problem

*Melanoides tuberculata* and *Tarebia granifera* are both aggressive snail species of the family Thiaridae that have successfully colonized many warm waters (>17 °C) around the world. *Centrocestus formosanus* and *Haplorchis pumilio* are invasive parasites transmitted by one or both snails. The parasites use many families of teleost fishes as 2<sup>nd</sup>

intermediate hosts, and a variety of piscivorous birds (and mammals) as definitive hosts. Any habitat in North America successfully invaded by the snails will likely be visited by a migratory piscivore that will introduce the parasite to the water via defecation, which will lead to infection of the snails. Then, it is estimated that more than 50% of the species of fish in that habitat will likely become infected by one or both of these parasites in ways that are likely to reduce the ability of the infected fish to; (1) escape predation by local piscivores, (2) pursue and capture food, (3) maintain station in flood events through oral grasping, (4) maintain its position in the dominance hierarchy of the school, (5) move between pools separated by long riffles, etc.

However, while the pathology of these parasites is well documented from intensive fish culture and laboratory settings (Blazer and Gratzek 1985, Mitchell et al. 2002), the ability to predict levels of measurable harm to wild fish based on cercariometry is nonexistent. Fish infected with either of the two heterophyids would presumably find it more difficult to perform daily activities associated with fitness than uninfected fish. So, before conservation workers can justify implementation of potentially costly and long-term mitigation efforts, it is imperative that they can specify how various densities of cercariae in the water column can affect fish in wild situations. Prior to this study, it has been difficult for managers responsible for wild fisheries to establish reasonable action thresholds based on cercarial densities because relevant information is largely unavailable for this and most fish parasite systems. We have tested fish under conditions representative of real-life demands and have linked those test results directly to cercariometry readings. We also provide relevant data regarding the effects of various levels of parasitism on the ability of fish to perform work in the form of swimming while

water speed is gradually increased. Any reduction in the ability of an infected fish to perform work in the form of swimming against increasing flow can be inferred to represent reduced fitness regarding its ability to perform the daily activities listed above.

## 1.6 Goals

We set out to determine how various levels and rates of parasitism with two invasive parasites affect the swimming ability of a native forage fish and a federally listed minnow. Our research provides experimental data from which estimates of cercarial density in the water column in affected waterways can be used to predict the levels of damage that are being caused by infection of native fishes and may also help to explain some of the paradoxical discrepancies mentioned earlier.

### 1.6.1 Effects of Parasitism

#### 1.6.1.1 *Swimming Endurance*

**Goal 1:** To be able to quantitatively predict the negative effects that *C. formosanus* and *H. pumilio* cercariae might be having on the swimming endurance of two cyprinids, one of which is a wide-spread and important forage fish, and the other of which is a threatened species that has an overlapping range with one or both of these parasites.

We hypothesized that:

- As experimental fish acquired more parasites, their swimming abilities would be negatively affected, especially after being parasitized by *Haplorchis pumilio*.
- As experimental fish acquired a given number of parasites at faster rates, they would be more dramatically affected.

### 1.6.1.2 *Lethal Level*

**Goal 2:** To determine the lethal intensity of infection (in which the host dies outright) for both parasites in both fish species, and to determine how this lethal level is affected by rate of parasite acquisition.

We hypothesized that:

- Some fish would die outright from the parasitism due to larval migrans.
- Deaths would occur at high initial exposure from sudden shock of infection.

### 1.6.2 **Maximum Swimming Speed**

The maximum swimming speed of *C. venusta* has already been estimated in a swim tunnel by Leavy and Bonner (2009), but the device used was very different. The swimming speed of *D. diaboli* has not yet been estimated experimentally.

**Goal 3:** To determine the maximum swimming speed of *Cyprinella venusta* and *Dionda diaboli* in our Swimming Endurance Apparatus using an adaptation of the speed-ramping protocol established by Leavy and Bonner (2009) and compare these estimates to the estimated maximum swimming speeds based of the formula provided by Sambilay (1990).

We hypothesized that:

- The estimated maximum swimming speed we obtain for *C. venusta* may be different from that published by Leavy and Bonner (2009), since our SEA has been demonstrated to have laminar flow in the viewing tunnel, and since their fish were tested in the field under varying conditions immediately after capture while our fish will be tested after habituation.

- *D. diabolis* will have a lower maximum swimming speed than *C. venusta* in the SEA, and the maximum swimming in the SEA will be consistent with that predicted by Sambilay (1990).

## **METHODS**

### **1.7 General Aquarium Set up**

#### **1.7.1 Aquarium components**

##### **1.7.1.1 *Aquarium dimensions***

All aquaria used in experimentation were standard 10-gallon (approximately 40 L) aquaria measuring 10 in (W) X 20 in (L) X 12 in (H).

##### **1.7.1.2 *Shelving for aquaria***

Aquaria were arrayed on two side-by-side banks of metal shelving (Gladiator® Rack Shelving Unit model # GARS774XEG), with four shelves each (Figure 5). The lower three shelves each held five fish aquaria, while the top shelf was reserved for aquaria housing the infected snails.



Figure 5. Gladiator Shelving unit with aquaria.

### **1.7.1.3 Modification of aquaria**

Each aquarium on the lower 3 shelves (fish aquaria) was divided into four quadrants separated by partitions (Figure 6). Each quadrant held a single fish, and the partitions prevented any cryptic mechanisms of social interactions, such as competition for food or hierarchical position in the aquarium, from affecting the experiment.

The partitions were fashioned from plastic fluorescent-light diffuser grating with square openings about 17 mm wide. In order to reduce likelihood of stress from social interactions between fish in neighboring quadrants, a plastic mesh with hole size of approximately 3 mm was glued to one side of the partitions using non-toxic glue (Gorilla® PVC cement). The mesh also prevented small fish from squeezing through the plastic grating. The partitions were supported in the aquaria using strips of plastic U-

channel (Gladiator® GearWall® Panel Trim) fixed to aquarium walls with silicone caulking. Even though all components were marketed as non-toxic, after installing the U-channel and partitions, aquariums were filled, soaked, and emptied several times. This ensured that any soluble chemicals potentially harmful to fish would be removed from any of the installed components. The partitions were removable, which facilitated replacement of broken parts as well as cleaning. The height of the partitions and U-channel was about 4 cm short of the top of the aquarium for convenience of installing and removing the partitions, and also allowed conservation of materials due to the original dimensions of the gratings.

Each aquarium had an air stone connected to a supply line that fit snugly into the U-channel alongside the partitioning. To reduce likelihood of fish jumping out of the aquaria or into another quadrant, every aquarium was fitted with a lid made of thin, black, plastic mesh with openings of about 17 mm. We soon discovered that most of the fish would position themselves at an angle such that one eye was looking at the bottom of the aquarium, often with the tail elevated relative to the head. We assumed that this odd behavior was a reaction to their reflection in the aquarium bottom, since they could not see other fish nearby to orient towards. Initially, black aquarium rocks were used in each aquarium to block reflection. Although effective at stopping the behavior, the rocks made the aquaria extremely difficult and time consuming to clean. So, the rocks were discarded and the outside surface of the bottom pane of glass in all aquaria was painted with white latex paint using a standard paint roller. This completely stopped the disorientation of the fish.



Figure 6. Experimental fish aquarium with partitions and U-channel installed.

## 1.8 Acquisition and Processing Fish and Snails

### 1.8.1 Fish sources

Each experiment required 60 fish of the species being tested. Fish were collected and maintained during experimentation under permits TE40343C-0 (USFWS; Exp 2022-08-31), SPR-0913-124 (TPWD; exp 2019-09-06), and IACUC20176146600 (exp 2020-09-11).

#### 1.8.1.1 *Dionda diaboli*

Experimental *D. diaboli* were acquired from the San Marcos Aquatic Resource Center (SMARC). SMARC maintains captive bred *D. diaboli* as part of the management strategy for the species. Fish are maintained on the SMARC property in an enclosed,

snail-free raceway, which ensured they had no preexisting infections with the parasites under study. SMARC provided us with their surplus production of *D. diaboli* for our experiments. Fish were transported from the SMARC facility to the Freeman lab at Texas State University (TSU) using 5-gallon, food grade buckets equipped with a portable aerator. The journey is approximately 12-16 minutes. Upon reaching the Freeman lab at TSU, fish were immediately distributed to experimental fish aquaria with small hand nets.

#### **1.8.1.2 *Cyprinella venusta***

Experimental *C. venusta* were collected from the Blanco River (29°53'40.78"N, 97°54'1.28"W) with a seine. This site on the Blanco River is approximately 7 km upstream from the confluence with the San Marcos River, and *M. tuberculata* has never been reported from any portion of the Blanco River. Because this area of the river is not influenced by any significant spring source, it is subject to ambient temperature change and far less likely to support *M. tuberculata*. Without the presence of the first intermediate host of either trematode being tested, it was very unlikely that fish in this area were already infected.

Fish were collected in groups of 70-95 individuals per sampling event. *C. venusta* with a standard length of less than 40 mm were returned to the river. Collected *C. venusta* were transferred from the seine to a 5-gallon, food grade bucket equipped with a portable aerator and transported by car to the Freeman lab at TSU. The journey is approximately 10-18 minutes. Upon reaching the Freeman lab at TSU, fish were immediately distributed to experimental fish aquaria with small hand nets.

## 1.8.2 Snail source

### 1.8.2.1 Snail collection sites

*M. tuberculata* were collected from Landa Lake in New Braunfels, TX. In order to locate a consistent source of infected snails, samples of snails were collected with dip nets from multiple sites around the lake shown in Figure 7. A population of *M. tuberculata* on the west bank of Pecan Island (29° 42' 54.17"N, 98° 8' 3.43"W) was found to have the highest prevalence of both *H. pumilio* and *C. formosanus*.

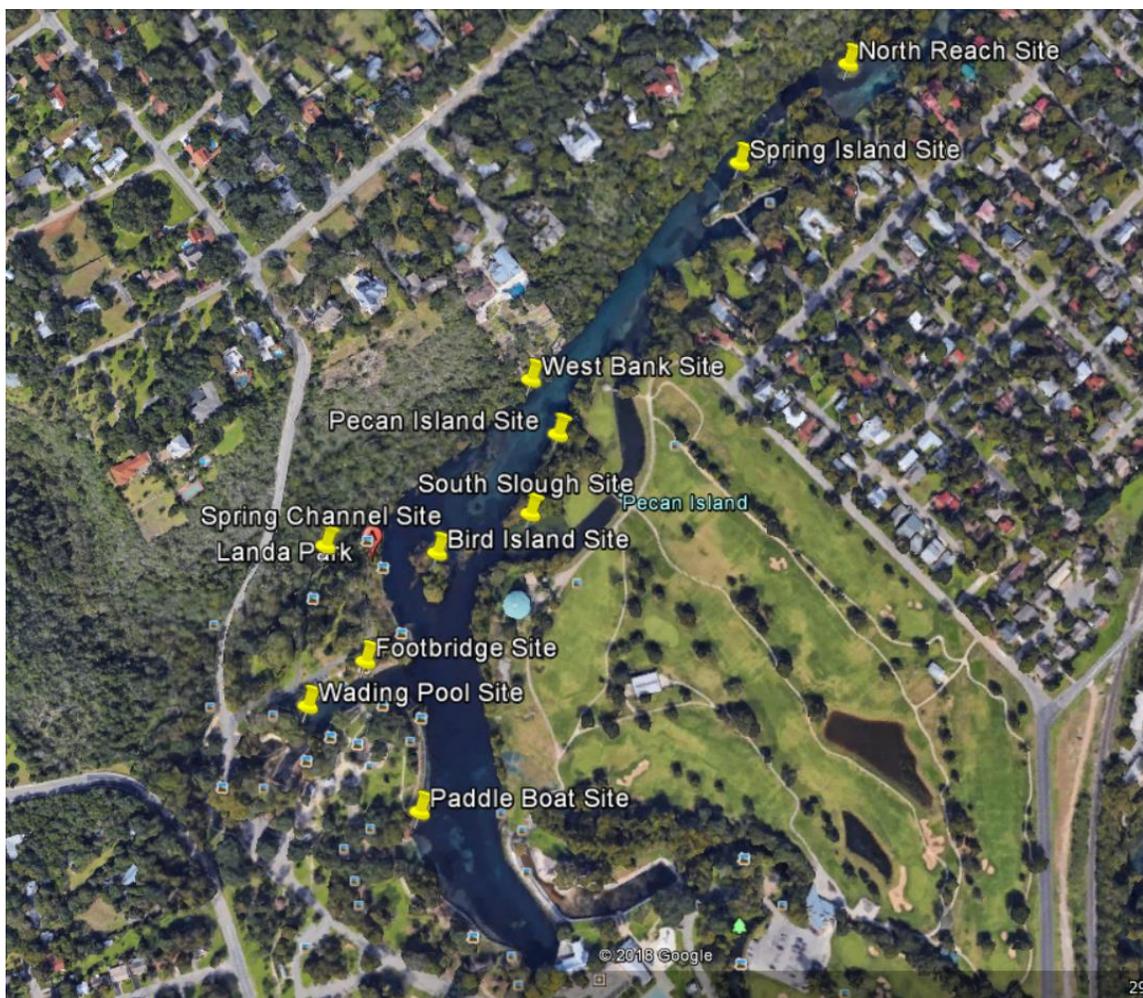


Figure 7. Map of Landa Lake showing snail sampling sites.

### 1.8.2.2 Sampling protocol

Snails were obtained from shallow water near the shore by means of standing on the shore, wading, and from kayaks, and transferred to uncovered buckets containing lake

water. Because previous workers in our lab have observed that exposure to low temperature can shock *M. tuberculata* and result in permanent reduction in cercarial production, buckets were left partially submerged in shallow lake water while sampling during colder months. This helped to maintain the temperature of the water in the collecting bucket for the duration of the sampling event.

#### *1.8.2.2.1 Frequency and duration of sampling*

Sampling events lasted for approximately 1-3 hours and were conducted over an 18-month period including winter months (November 2016-April 2018). Frequency of sampling varied and was determined by number of cercariae needed for scheduled experiments, current density of cercariae in snail aquaria, and infection prevalence of snails being collected (Section 1.9).

#### *1.8.2.2.2 Transportation issues*

Snails were transported by car in a 5-gallon, food grade bucket equipped with a portable aerator. Prior to transportation, fresh water from the lake was added to the buckets, and buckets were covered with bait-bucket lids. The ride from Landa Lake to the Freeman lab at TSU was approximately 25-30-minute. Buckets were transported in the temperature-controlled cab of the transporting vehicle during all seasons.

### **1.8.3 Snail sorting**

To separate shedding from non-shedding snails, we constructed three test-tube arrays, each with 20 rows of 5 holes suitable for holding a 1" (25 mm X 200 mm) test tube. Each array was constructed from 2X10 dimensional lumber, which was cut to 32 in, sanded, drilled with a 1-inch Forstner bit, and soaked with several coats of polyurethane. Screen door handles were added to the ends for aiding in handling.

Each test tube to be placed in the board was filled with artesian well water and received a single test snail that had been rinsed of debris. After the snails were placed into test tubes, they were incubated for approximately six hours before inspection. During inspection, the end of a test tube was placed on a fiber optic light source that was directed axially through the test tube, brightly illuminating any suspended debris or small organisms. Cercariae were detected with the unaided eye as a cloud of identical objects that were obviously animated. Initially, cercariae were identified to species with the use of a compound microscope by comparing physical features to descriptions (metrics and swimming behavior), photos, and drawings from others' published work. It soon became easy to differentiate between *H. pumilio* and *C. formosanus* cercariae by grossly visible behavior. While genomic analysis is required to know in absolute terms the identities of the cercariae, subsequent inspection of the metacercariae recovered from experimentally infected fish further confirm the identities of the parasites as *C. formosanus* and *H. pumilio*. Snails not found to be shedding were checked again the next day, and then discarded if not positive.

Snails shedding cercariae of *H. pumilio* and *C. formosanus* were placed into 2 sets of 3 aquaria with respect to species. This provided 3 aquaria of snails shedding each parasite species from which to obtain cercarial treatment water for experiments. Because cercarial production per snail can fluctuate dramatically over time, a three-aquaria design was used to maximize the chance of obtaining a consistent supply of cercariae. This also provided at least 100 L of treatment water which could be utilized if the cercarial count was low (say, less than 1 cercaria/mL) on any given treatment event. After some experimentation, we determined that a snail density of 60-95 per aquarium provided

sufficient cercarial production to carry out the daily treatment schedule. If cercarial production rate began to decrease consistently, all snails in the three snail aquaria were examined in the test tube array again. Snails found to still be shedding cercaria were distributed back into aquaria, while all others were replaced with shedding snails freshly acquired from Landa Lake.

## 1.9 Cercariometry

### 1.9.1 Cercariometer design

To filter and count cercaria (cercariometry), we designed a cercariometer modeled after Bolick (2007), later refined by Johnson et al. (2012), and recommended over other methods by Cantu et al. (2013). Our cercariometric design and protocol technique were further modified after consulting with Jube Guajardo at Bio-West, a consulting agency that has been using cercariometry to determine cercarial densities in local rivers.

The cercariometer consists of a 4-tier filter series of increasingly fine stainless-steel filter screens pressed between the mating ends of 1.5" PVC barrel unions. Each barrel union with its respective screen is referred to as a filter tier, and the tiers are connected with a short section of 1.5" PVC pipe. The filter tiers are arranged in order of descending mesh size (215 $\mu$ m, 89 $\mu$ m, 43 $\mu$ m, and 28 $\mu$ m), and are removable for independent inspection and cleaning. An example of a single tier is depicted in Figure 8.

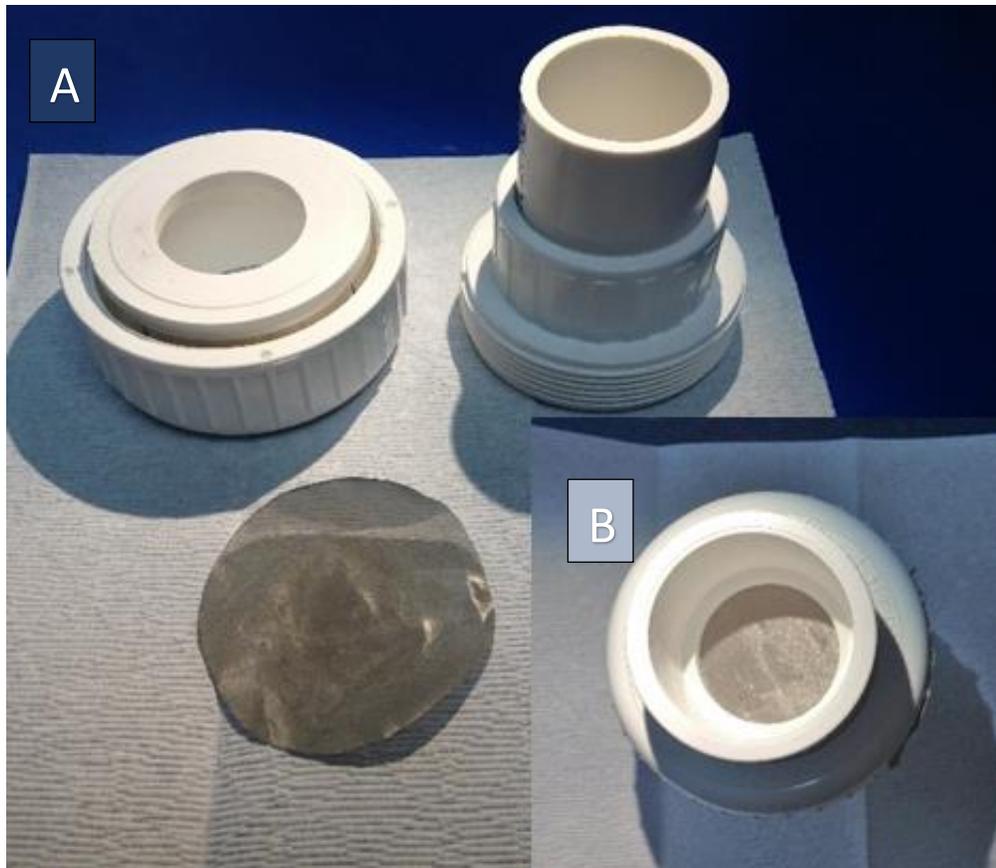


Figure 8. A.) Barrel union and screen separate. B.) Axial view of assembled filter tier.

### 1.9.2 Cercariometry protocol

Before each daily treatment event, a cercariometry reading was taken to determine the volume of cercarial water that would be required to treat each fish aquarium with its respective number of cercariae per day. This ensured that each aquarium consistently received its designated number of cercaria per day, regardless of cercarial density in the snail aquaria. Using a 1500 mL beaker, 18 L of cercarial water was accumulated into a 5-gal bucket by drawing 6 L of water from each of the three snail aquaria housing the cercarial species being tested. Before drawing the 6 L of water from an aquarium, the aquarium water was stirred thoroughly but gently to ensure uniform distribution of cercariae within the aquarium. Exceptional care was taken when depositing the samples taken from snail aquaria into the collecting bucket to avoid creating unnecessary

turbulence, decreasing the chance of potential damage to the cercariae. Once the water from all three snail aquaria had been combined into the bucket, the water was gently stirred and a 200 mL cercariometric sample was drawn by dipping a beaker into the bucket.

The 200 mL sample was fixed by adding 22 mL of 100% formalin (bringing the formalin concentration to approximately 10%). The sample was allowed to fix for 10 minutes before filtration. *Note: 10 minutes after the introduction of 10% formalin was found to be the minimum amount of fixation required for successful filtration. Shorter fixation times or lower formalin concentrations resulted in cercariae losing their tails, which decreased the confidence of identification and reduced count accuracy.* After fixation, the sample was poured through a filter tier containing the 28 $\mu$ m filter screen. Coarser pre-filters were deemed unnecessary when filtering samples from the snail aquaria, as they did not contain substantial amounts of debris more typical of samples retrieved from rivers or lakes. Water samples collected in the field generally involve much more volume and are usually expected to contain more debris and fewer cercariae. When filtering field samples, pre-filters are used to reduce the amount of debris on the target filter (28- $\mu$ m).

After filtration, the 28- $\mu$ m filter screen was then removed from the barrel union with fine point tweezers and transferred to a petri dish where it was stained with 1% rose Bengal. The 1% rose Bengal was applied drop-wise with a 1-mL pipet until the entire filter surface was covered with stain. The filter was then allowed to soak in the stain for a minimum 5 min. *Note: shorter staining intervals resulted in specimens that were partially stained or unstained.*

After 5 min, the excess rose Bengal was rinsed from the screen with 10% formalin using a 1-mL pipet. The resulting collection of fluid (residual) was transferred to a separate petri dish and examined under a dissecting microscope at 35X magnification to count the number of cercariae that had been rinsed off the screen. The rinsed filter screen was then examined as above, and the two counts added together to determine the number of cercariae in the 200 mL sampled. To aid the counting of cercariae, petri dishes were set on a transparent 1 mm X 1 mm grid, and all cells in the grid were examined for cercariae. The grid facilitated a more systematic procedure for counting.

The combined cercarial count of the filter screen and residual rinse fluid was divided by the volume of water filtered to determine the number of cercaria per mL, which will be henceforth referred to as “cercarial density” [Ex: (200 cercaria counted) ÷ (200mL filtered) = cercarial density of 1.0/mL]. Using the cercarial density, the volume of cercarial water to be administered to fish aquaria was adjusted such that each fish aquarium consistently received its assigned number of cercariae per day.

A benefit of using stainless steel filter screens is that they are reusable. After completing daily cercariometry, filters were rinsed with deionized water and placed in a 20% solution of potassium hydroxide (w/v) for cleaning. Filters were allowed to soak in 20% KOH for approximately 48 hours before being used again to dissolve any organic material from a previous filtration event. This ensured that no contamination from previous filtration events would affect daily counts. Before being used, filters were taken from the 20% KOH and placed in acetic acid, then rinsed with deionized water.

## 1.10 Procedures for all Experiments Testing Effects of *Haplorchis pumilio* and *Centrocestus formosanus*

### 1.10.1 Generalized treatment schedule for all experiments

Unless otherwise stated, experiments testing parasite effects on swimming endurance began with a 3-day habituation phase to allow fish to habituate to the aquarium, feeding on flake food, and the presence of people. During the habituation phase, 80% of the water in the fish aquaria was exchanged daily with artesian well water to prevent the accumulation of harmful metabolic products and to keep aquaria clean. The habituation phase was followed by a 14-d treatment phase in which a daily treatment of cercarial water was given to the experimental aquaria. The treatment phase was followed by a 3-d incubation phase, during which the fish were fed and 80% of water in each aquarium was exchanged. The incubation phase was followed by the swimming endurance assessment in the SEA. After being assessed for swimming endurance, fish were euthanized in MS-222 and fixed in 10% formalin for later examination.

### 1.10.2 *Haplorchis pumilio*

#### 1.10.2.1 *Cyprinella venusta*

##### 1.10.2.1.1 *Lethality experiment*

While exposure to *H. pumilio* has been demonstrated to cause mortality in various fish species (including *C. venusta*), the minimum exposure level sufficient to do so had yet to be quantified and lacked cercariometric estimates of the water to which the fish were exposed. In order to establish a reasonable treatment range, we conducted a preliminary experiment to determine, not only the exposure of cercariae that would kill the fish outright, but also the maximum exposure a fish could endure and survive for the duration of an experimental trial. Because there is limited data on the effects of *H. pumilio*, a treatment range was established based on caged fish recovered by Fleming et

al. (2011) from the confluence of the Comal and Guadalupe Rivers, where the highest acquisition rate by an individual fish was 13 metacercariae per day. Although Fleming was measuring the incidence of *C. formosanus*, the data he collected provided a realistic basis for establishing a treatment range for *H. pumilio*.

Lo and Lee (1996) have demonstrated that *H. pumilio* has an estimated infection success of 45% in ideal conditions; therefore, for a fish to acquire 13 metacercaria per day, it would need to encounter approximately 29 cercariae. To replicate Fleming et al. (2011) caged fish study, we simply multiplied 29 cercariae by four fish that would inhabit a single aquarium, resulting in a treatment per fish aquarium of 116 cercariae per day. The proposed rate was not noted to cause mortality in the caged fish study by Fleming, therefore, we set the minimum treatment to 250 cercariae per day to ensure an observable effect. The maximum treatment was set to 600 cercariae per day. We then used an Excel spread sheet to evenly distribute 10 treatments between 250 and 600 cercariae per day resulting in 12 treatments rates. The spreadsheet also randomly assigned the treatments to the aquaria in a single bank. The leftmost column in the bank was reserved for control aquaria.

The treatments were administered daily for one week. The highest treatment level was expected to cause mortality in a matter of days, however fish showed few to no symptoms even at the highest treatment level. Because of this, we doubled the number of cercariae per treatment in all treatment aquaria. We continued to increase the treatment every seven days until mortality occurred, which extended the duration of the experiment to four weeks. The final treatment for the highest exposure was 7,200 cercaria/d per aquarium.

At the end of the 4-week testing period, swimming endurance of surviving fish was assessed using the SEA. This swimming endurance testing of the surviving fish from the lethality experiment was not intended to be used in the assessment of the effects of the parasite, but rather, to work out the logistics of processing fish through the SEA, as well as streamlining the SEA testing protocol to elicit a consistent response from tested fish.

*1.10.2.1.2 Experiment 1: Testing effects of acquisition rate of the cercariae of Haplorchis pumilio*

After reviewing the results of the lethality experiment, we were able to calculate the total number of cercariae that had been administered to each aquarium over the course of the experiment (cumulative exposure); however, the cumulative exposure causing mortalities occurred over a 4-week period instead of a 2-week period as originally designed. To achieve the same cumulative exposure over a 2-week period, exposure rate per day would have had to have been doubled.

We predicted mortalities likely would have occurred sooner at this higher rate due to an increased number of successful cercarial penetration and subsequent larval migrans in a shorter period of time. This would result in fish dying sooner, not receiving the expected total number of cercariae. To test the hypothesis that mortalities can occur at lower cumulative exposure if applied over a shorter time interval, we chose six cumulative exposures from the lethality experiment that caused noticeable symptoms but few mortalities. These six cumulative exposures were tested in six aquaria over a 5-day treatment (high rate of exposure) and six aquaria over 14-day treatment (lower rate of exposure). The daily treatment range for the 5-day experiment was 9,000-24,000 cercariae per day and the treatment range for the 14-day experiment was 3,500-8,500 cercariae per day. The treatments were randomly assigned to aquaria. Testing the same

cumulative exposure using different time intervals would elucidate any effects associated with a high rate of acquisition. The surviving fish were tested in the SEA to assess the parasite's effects on endurance.

#### *1.10.2.1.3 Experiment 2: Effects of *Haplorchis pumilio* on swimming endurance*

As a result of high mortality rates observed in experiment 1, and the seemingly random pattern of mortality likely caused by larval migrans, few fish survived to testing in the SEA. We decided that further testing of *H. pumilio* should be done at lower rates of exposure with replication to increase the number fish that would survive the treatment phase. After reviewing the results of experiment 1, we decided that the tested treatment range should be approximately half of the original treatment proposed for experiment 1. This determination was based on the condition of the fish that survived to the end of experiment 1.

The range of treatment for experiment 2 was 1,000 - 4,000 cercariae per day which was divided among two sets of six aquaria in the right bank. This provided replication for six levels of treatment. The treatment range was randomly assigned to aquaria. Endurance of fish was assessed with the SEA at the end of the treatment phase.

#### **1.10.2.2 *Dionda diaboli***

Due to the limited number of *D. diaboli* available for experimentation, a single experiment was conducted to determine the exposure of *H. pumilio* that would cause mortality as well as the effects of the parasite on swimming endurance. To accomplish this, a wide treatment range was established based on the data collected in testing *C. venusta*. Because *D. diaboli* is a spring associated fish with a small distribution, it was expected to be more sensitive to treatment than *C. venusta*, which is found in a wide

variety of habitats and is often able to tolerate pollution and poor water quality. The treatment maximum for *D. diaboli* was decreased to approximately half the maximum treatment administered to *C. venusta*. This made the treatment range for *D. diaboli* 1,500-13,000 cercariae per day. The treatments were randomly assigned to aquaria 12 aquaria in a single bank.

Although one bank of aquaria was intended to test 60 fish, limitations in availability of mature *D. diaboli* reduced the number of tested fish to 40, and so the number of fish per aquarium was reduced from four to three. This allowed us to test the proposed range of treatment at higher resolution than would result from reducing the number of treatment aquaria from 12 to 8 in order to accommodate the limited fish availability. As a result, all aquarium treatments were reduced by 25% to maintain the assigned number of cercariae per fish per day. In addition, there were only two control aquaria, which housed two fish each.

Another problem that arose with *Dionda diaboli* was that the fish seemed intent on squeezing around the partitions, apparently in an attempt to join their cohorts, sometimes injuring themselves or becoming stuck. Consequently, partitions were permanently removed in this part of the study. The fish were monitored closely to ensure they were not attacking or injuring one another, and no aggressive behavior was observed.

During the 3-d habituation phase, all fish in all aquaria were almost always scattered around the aquaria and resting quietly very near the bottom. After treatment was initiated, the three fish in each treatment aquarium were almost always seen resting together near the bottom, radially arranged in a corner with the heads of all three fish almost touching. This behavior seemed most pronounced in the aquaria with the highest

treatment levels. The fish in the control aquaria were never observed engaging in this support-group behavior. However, we cannot be sure they never did this, because the aquaria with the highest treatment levels were usually checked first at the beginning of daily rounds, and the activities of the worker may have aroused the other fish from their group before they were observed. While this was indeed a curious behavior, it seemed benign, and was not considered to be a threat to the experiment.

Fish endurance was assessed using the SEA at the end of the treatment phase.

### 1.10.3 **Determining experimental range of cercarial exposure for *Centrocestus formosanus***

We arbitrarily chose to test fish at metacercarial intensities equivalent to the total number of metacercariae a resident fish in the Comal River would have acquired in 1 y, given the highest recorded natural acquisition rate. However, the time allocated for cercarial exposure in our experiments was compressed to 14 d. Therefore, we had to devise a cercarial treatment rate that would result in the experimental accumulation (in 14 d) of a year's worth of metacercariae. We first considered Fleming et al. (2011) caged fish study, which showed that shiners in the Comal River could acquire as many as 14 metacercariae per day. We calculated that after one year, such a fish would have acquired roughly 5,000 metacercariae.

Based on studies by (Lo and Lee 1996) the rate at which *C. formosanus* cercariae successfully infect a fish and develop into detectable metacercariae (infection success) is 45%. Given this success rate, a hypothetical shiner in the Comal River that had acquired 5,000 metacercariae in 1 y would have encountered approximately 13,000 cercariae. However, in our initial investigations, we measured infection successes as low as 14%, meaning that a fish with 5,000 metacercariae would have had to encounter 40,000

cercariae over 1 y. Therefore, we established our highest level of cercarial exposure (one aquarium with four fish) at 4,500 cercariae per day, with the goal of causing these fish to accumulate in 14 d of treatment the maximum number of metacercariae that a resident shiner in the Comal River might acquire in 1 y. To establish a gradient of lesser exposures that would likely result in a gradient of detectable effects in the other treatment aquaria, we set the lowest treatment level to 500 cercariae per day.

The range of 500-4,500 was divided among 12 aquaria within a bank (rounded to the nearest 100 cercariae) and randomly assigned to aquaria. Swimming endurance of surviving fish was assessed with the SEA at the end of the treatment phase. Regrettably, testing the effects of *C. formosanus* on *D. diaboli* was not possible due to lack of available stock at SMARC.

#### 1.10.4 Assigning treatments

The following is an account of the method by which treatments for each experiment were assigned to fish aquaria. After establishing the treatment range for an experiment, an Excel spreadsheet was used to randomly assign the desired treatment range to the fish aquaria within a bank. This was done to avoid a progression of increasing or decreasing treatment assignments by column or row, and randomized any extraneous effects on fish due to location of aquaria in the bank such as; proximity to lights or higher traffic areas, or seeing human faces (top shelf) vs. feet and legs (bottom shelf), etc.

Three control aquaria were assigned to the left most column of each bank to decrease the chance of accidental exposure of control fish to cercaria-laden water during the treatments, such as by accidental splashing and spills, which might have occurred if control aquaria had been under treatment aquaria.

### 1.11 General Treatment Schedule Details

Daily treatments followed the same procedure for all previously described experiments. During the treatment phase, daily cercariometry was performed to adjust the volume of cercarial water required to ensure each treatment aquarium received its assigned number of cercaria each day. After estimating the cercarial density in the snail aquaria, the treatment volume for all fish aquaria was adjusted with an Excel spreadsheet (rounded to the nearest 100 mL) and a treatment sheet was printed (Figure 9). The excel spread sheet also randomized the order in which the aquaria received treatments to randomize effects due to any bias introduced by the technique of the attendant.

<b>Venusta Trial Water Exchange Schedule: RightBank</b>					Ch, R#, S\$	Notes
Day 5: Cercarial Density of Treatment Water = 1.0/ml <b>Right (venusta)</b> Wed, Mar 14, 2018 CareTaker Initials: _____ StartTime = _____ EndTime = _____						
<b>R,C0,R1</b>	<b>R,C1,R1</b>	<b>R,C2,R1</b>	<b>R,C3,R1</b>	<b>R,C4,R1</b>		
<b>13</b>	<b>7</b>	<b>14</b>	<b>11</b>	<b>5</b>		
4,000 ml Placebo	2,400 ml Treatment	1,400 ml Treatment	1,000 ml Treatment	1,700 ml Treatment		
LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF		
<b>R,C0,R2</b>	<b>R,C1,R2</b>	<b>R,C2,R2</b>	<b>R,C3,R2</b>	<b>R,C4,R2</b>		
<b>12</b>	<b>1</b>	<b>3</b>	<b>15</b>	<b>9</b>		
1,000 ml Placebo	2,100 ml Treatment	4,200 ml Treatment	3,100 ml Treatment	4,500 ml Treatment		
LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF		
<b>R,C0,R3</b>	<b>R,C1,R3</b>	<b>R,C2,R3</b>	<b>R,C3,R3</b>	<b>R,C4,R3</b>		
<b>8</b>	<b>10</b>	<b>6</b>	<b>2</b>	<b>4</b>		
2,500 ml Placebo	500 ml Treatment	3,800 ml Treatment	3,500 ml Treatment	2,800 ml Treatment		
LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF	LR   RR ---- LF   RF		

Figure 9. Treatment sheet showing the volume of cercarial water (assuming 1 cercaria/mL) required for each treatment aquarium, in treatment order randomized for each day.

Before treatment began, plastic top screens were removed from all aquaria and all fish were inspected in the aquaria to assess condition and to remove any fish that had died. Fish in the rear quadrants of aquaria were inspected with a mirror. Fish that had died

were removed with a dip net and placed in 250 mL plastic bottles of 10% formalin. After all fish were inspected, the aquaria were drained with a syphon until about 5 cm of water remained in the aquaria using the randomized order of treatment established for that day. This established room for adding the treatment water. During the draining procedure, feces and uneaten food were also removed from each quadrant of the aquarium. The syphon hose used was 1" reinforced plastic tubing fitted with a ball valve which could be closed to maintain the syphon when switching between aquaria. The suction end of the syphon hose was covered by plastic mesh to prevent fish from being pulled into the syphon tube.

After all aquaria had been drained, each aquarium received its assigned treatment of cercarial water in the randomized order established by the treatment sheet for that date. Treatments were drawn from the 18 L of water previously set aside from the snail aquaria and administered to fish aquaria with a 1,500 mL beaker. Treatment water was distributed evenly among the quadrants of an aquarium to ensure that each of the four fish in the aquarium received approximately the same amount of cercarial water. After all treatments had been administered, the fish remained at low water levels for 30 minutes, which was the amount of time required for all viable cercaria in the Lo and Lee 1996 (Lo and Lee 1996) study to penetrate their experimental fish. The aquaria were then topped off (leaving treatment water in the aquaria) in the order of treatment using a hose that supplied untreated water from the Edwards Aquifer. Fish were then fed with standard flake food (TetraMin<sup>®</sup> Tropical Flakes). When fish in an aquarium were fed, fish in the adjacent aquaria in that row attempted to get the floating food in the other aquarium, resulting in collisions with the glass and potential injury. Therefore, food was

administered to aquaria in row-by-row order to minimize the time between when a fish saw food in adjacent aquaria and when it had food available. Fish were observed during and after each treatment and any behavioral changes in response to contact with cercariae were recorded.

Aquaria top screens were then replaced and the snail aquaria were topped off with artesian water after each treatment was concluded.

### **1.12 Effects of Aquarium Habituation on Swimming Behavior**

A potential threat to the validity of any conclusions that might be drawn by comparing the swimming endurance of parasitized fish to the swimming endurance of their unparasitized cohorts might be the possibility that the swimming endurance of the controls themselves might have been altered in some way by their habituation to the aquaria. Such effects would confound attempts to exclusively assign reduced swimming endurance to parasitism alone. In order to assess the magnitude of this threat, we compared the swimming ability of *C. venusta* freshly caught from the river (unhabituated) with the control fish pooled from all experiments testing the swimming endurance of parasitism on *C. venusta*. All of these controls had been in aquaria for at least 3 w. Endurance assessments were conducted in the SEA.

### **1.13 Swimming Endurance Apparatus Operation, Calibration, and Consistency**

#### **1.13.1 Design and operation**

The components on the apparatus have been assigned alpha-codes that we will use as reference points in the associated figures. 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 10). When the pump (D) is powered on, water is pumped at full volume out of the

reservoir (B) and through the mounting board to the front side of the apparatus (at E). The water from the pump (D) enters the front side (Figure 11) at (J), and immediately enters a tee fitting, which splits the flow. The relative flow rates of the two pathways exiting this tee are 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 permit the operator to control the flow velocity of the water passing through the viewing tube (P) in which the swimming endurance of experimental fish will be tested.

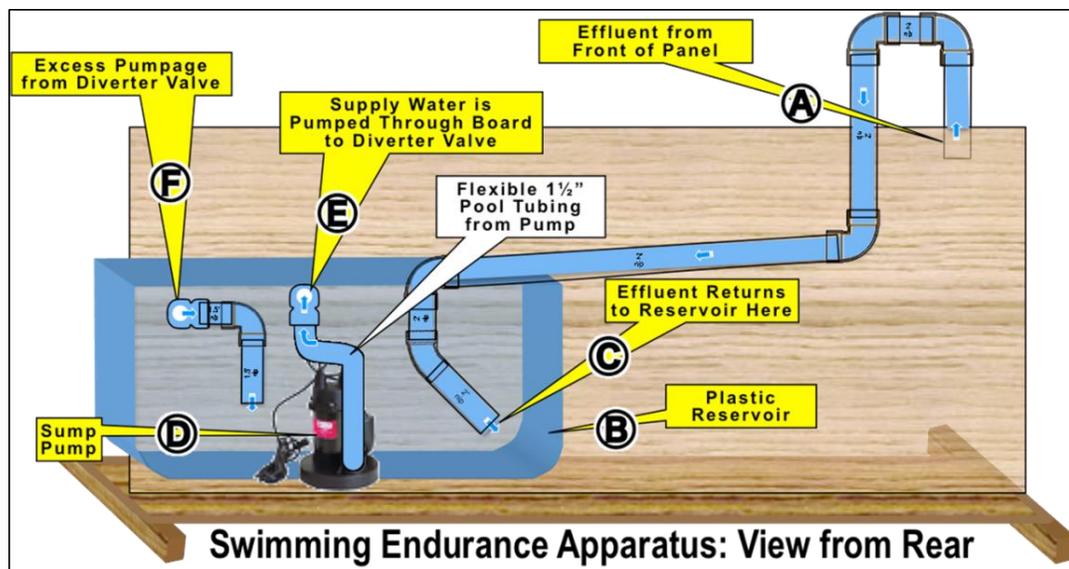


Figure 10. Swimming Endurance Apparatus (SEA); rear view.

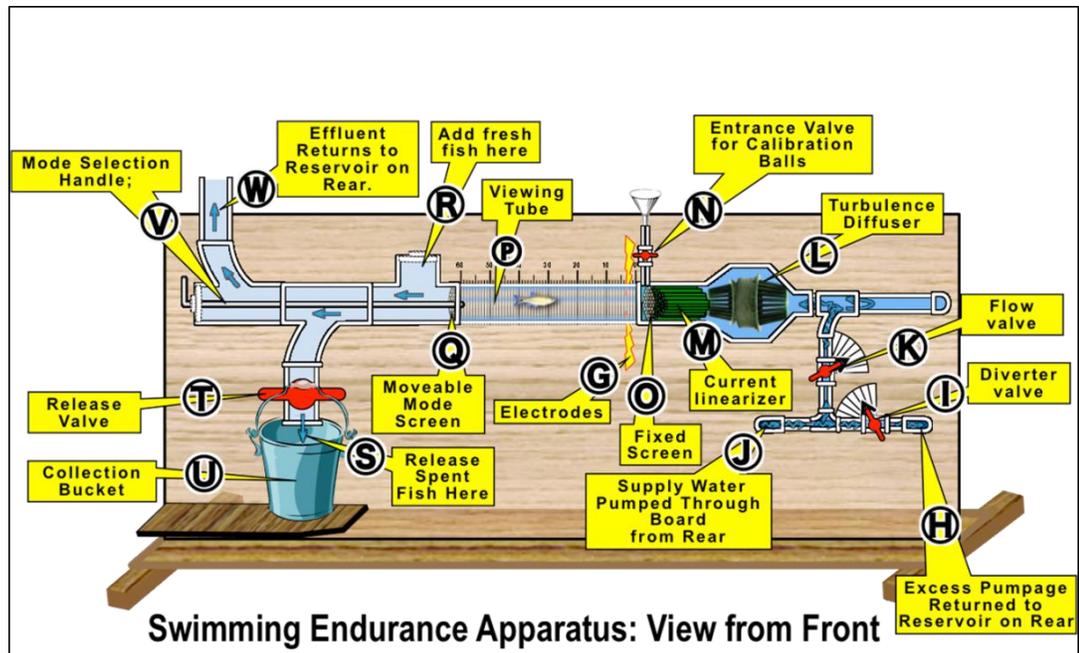


Figure 11. Swimming Endurance Apparatus (SEA); front view.

The water exiting the Flow Valve is under considerable pressure (depending on valve settings) and this initially caused unacceptable turbulence in the viewing tube and prevented the establishment of laminar flow for the testing of experimental fish. Therefore, a diffuser/linearizer subassembly (L and M) was developed through several trial iterations and installed between the Flow Valve (K) and the Viewing Tube (P). This dramatically reduced turbulence and linearized the flow through the viewing tube and achieved near laminar flow (Figure 12). A fixed screen (O) was installed at the upstream end of the viewing tube in order to prevent fish from moving out of the viewing tube and into the linearizer (L).

The Diffuser Subassembly (Figure 12, L) consists of two floor drains of different sizes (NDS #75 polyolefin Atrium gate floor drains, Figure 13) arranged in series, with the smaller drain upstream and with its base inserted inside the base of the larger drain. These serve to break up large-scale currents into small-scale turbulence.

The Linearizer Subassembly (Figure 13, M) contains numerous 5" long,  $\frac{1}{2}$  in diameter plastic tubes designed and marketed for use as guttering ferrules (Amerimax Home Products #21060 Home 21060 Plastic Gutter Ferrule, UPC: 0 49821 21060 1). These tubes are very thin-walled, which helps minimize backpressure, and the length to diameter ratio of 12:1 breaks up any remaining large torsional currents. Each of the tubes also has six straight internal fins, which were designed for reinforcement, but also serve to linearize any remaining small torsional currents from the diffuser and provide reasonably laminar flow with no rotational component into the viewing tube.

### 1.13.2 Calibration of water velocity in the viewing tube

The velocity of water through the viewing tube is determined by setting the Flow and Diverter valves to various combinations of settings, powering on the pump, and then injecting Calibration Balls (5-mm multicolored pom-poms, Tree House Studio®, <http://shop.hobbylobby.com/products/5mm-bright-pastel-multi-pom-poms-105171/>) into the tunnel (N; Figure 11). Near neutral buoyancy is achieved for these pom-poms by soaking them in soapy water and then rinsing before use. Behind the viewing tube, there is a ruler calibrated in cm. As the pom-poms move through the viewing tube at a particular valve-setting combination, they are filmed with a high-speed video camera (Casio® EX-FH25) that can shoot sequences of video up to 1000 fps.

By viewing the resulting video frame-by-frame, we are able to record the frame number when a pom-pom passed across the 0 cm mark and the frame number when the same pom-pom passed across the 50 cm mark on the ruler behind the viewing tube. The difference in frame number from 0 cm to 50 cm divided by the frames per second the video was recorded in gives the total seconds required to travel 50 cm. When the distance the pom-pom traveled was divided by the time seconds, the product was the velocity the

water was traveling in cm/s. An Excel spreadsheet was developed to analyze the videos and determine the mean flow velocity (in cm/s) of all the pom-poms recorded in a calibration session, given (1) the camera speed in fps, (2) the starting frame number when a pom-pom's position crossed the 0-cm mark, (3) the ending frame number when the pom-pom crossed the 50 cm mark, and (4) the coefficient of variation for the pom-poms in a calibration session. By filming these calibration trials across all possible Flow and Diverter valve combinations, the range of water-velocity capabilities of the tunnel was determined.

All valve combinations yielding coefficients of variation  $>10\%$  were judged to be unusable because the high variance of pom-pom velocities indicated that there was excess turbulence in the viewing tube, which would make it impossible to know what water velocity the experimental fish were actually experiencing. Some of the lower velocities utilized early in the ramping protocol had higher CVs, but these velocities were only used to gradually acclimate the fish to swimming and did not demand consequential effort from the fish.

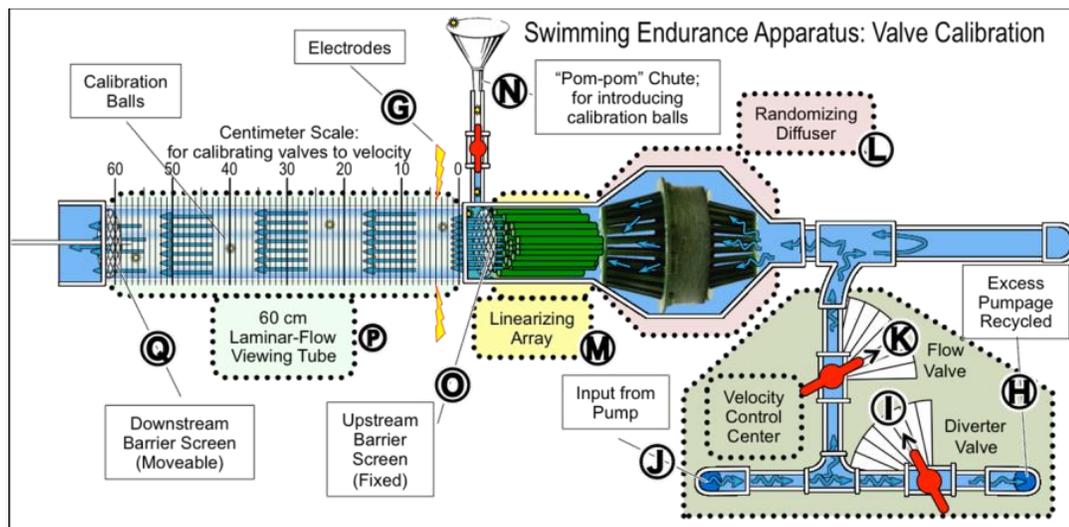


Figure 12. Swimming Endurance Apparatus (SEA); valve-calibration diagram.

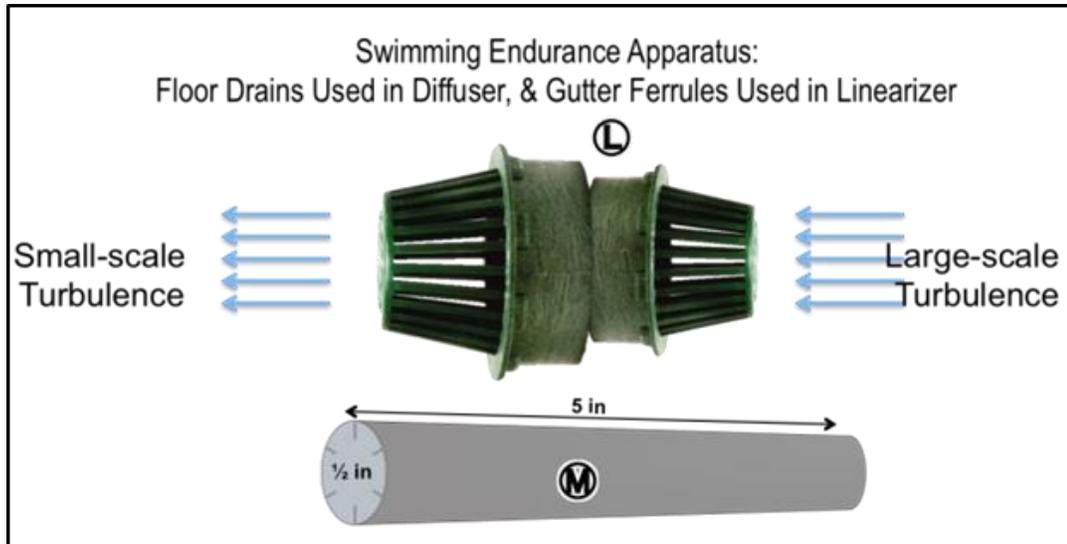


Figure 13. Swimming Endurance Apparatus (SEA); floor drains and gutter ferrules used in diffuser/linearizer subassembly.

### 1.13.3 Swimming endurance tests

The assessment of swimming endurance of individual fish consists of challenging the fish to swim continuously in the viewing tube at a systematically increased water velocity until the fish can no longer avoid being pinned against the downstream exclusion screen by the current (exhaustion). When assessing the swimming endurance of a fish, it was first retrieved from the aquarium and placed in a bucket containing artesian water with a hand net and then transferred from the bucket to the SEA.

The SEA is equipped with a movable downstream screen attached to a rod (V; Figure 11), allowing us to introduce a fish through the Access Port (R), and then force the fish into the viewing tube (Figure 15).

After fish entered the viewing tube, the test began by introducing a low-velocity flow (10 cm/s) for 3 min. This allowed the fish to acclimate to the SEA and orient to the current. After the 3 min acclimation, the speed was increased every minute on the minute by approximately 8 cm/s until the fish could no longer maintain itself in the current and became pinned against the downstream exclusion screen.

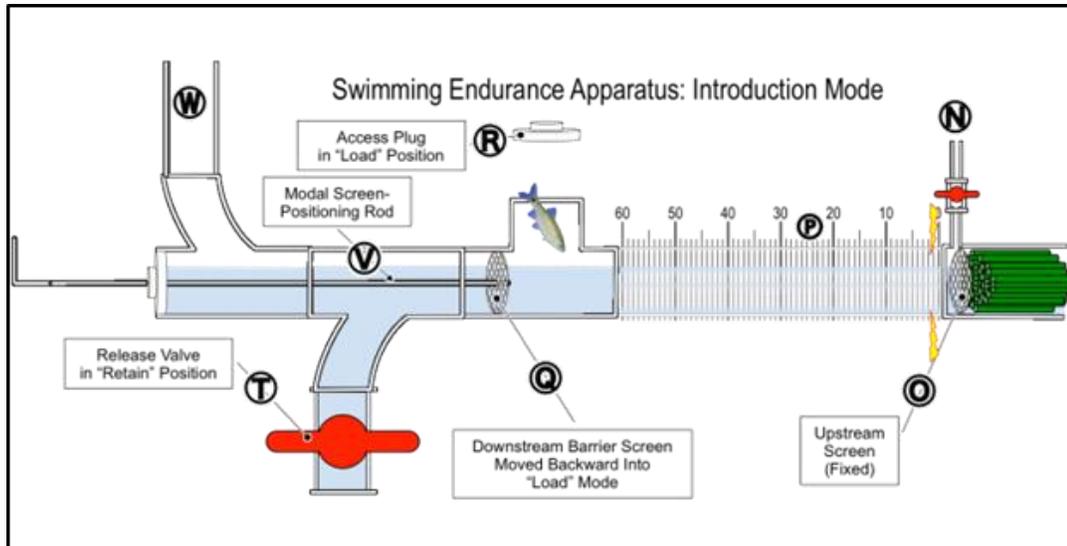


Figure 14. Swimming Endurance Apparatus (SEA); testing subassembly in fish-introduction mode.

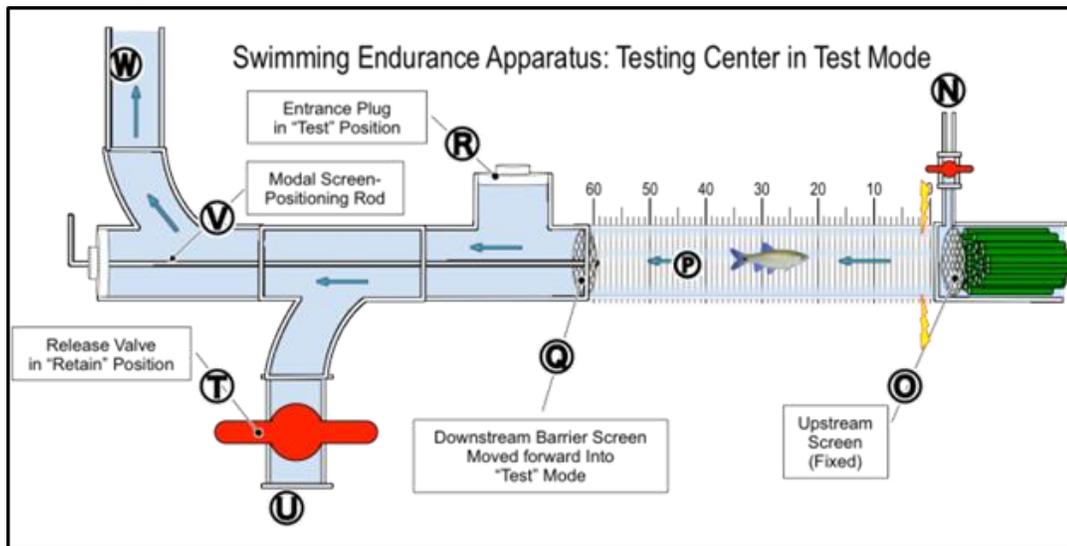


Figure 15. Swimming Endurance Apparatus (SEA); testing subassembly in test mode

After a fish became pinned, the flow was allowed to continue for 10 s, as some fish recovered shortly after being pinned the first time and continued swimming. After the fish had been unable to free itself from the screen for 10 s, the flow was stopped, and fish were transferred from the SEA into a bucket by pulling the movable screen (V) back and opening the release valve (T; Figure 16). Each swimming endurance test was filmed by a high-speed camera (120 fps) for later analysis.

Exhausted fish were transferred from the bucket to a beaker containing MS-222 (250 mg/L) where they were euthanized (cessation opercular movement for at least 5 m). Then the standard and total lengths were measured, and the fish were preserved in 10% formalin to be necropsied at a later date.

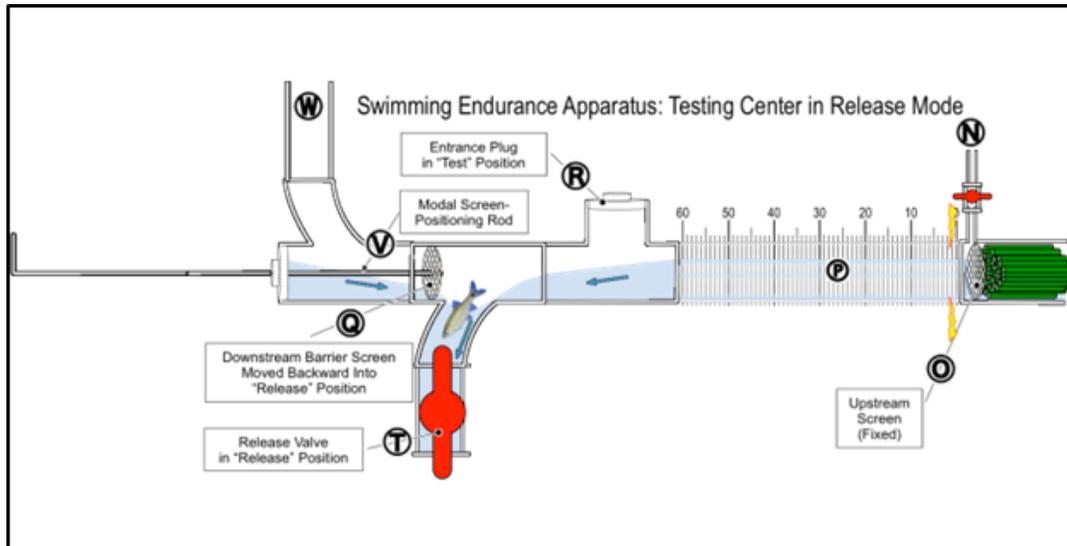


Figure 16. Swimming Endurance Apparatus (SEA); testing subassembly in spent-fish release mode

#### 1.14 Necropsy

To count the number of metacercariae accumulated by fish over the course of experimentation, fish were dissected and inspected under a dissecting microscope.

Techniques varied by parasite species. Before being dissected, fish were remeasured to confirm label accuracy, and to detect any errors in the cataloging process.

##### 1.14.1 *Haplorchis pumilio*

*H. pumilio* can be found in the body anywhere cartilage exists; however, only the caudal fin was inspected, as this location contains 23% of *H. pumilio* metacercariae present in the body on average (Lo and Lee 1996). So, total infections were estimated by multiplying the number of metacercariae found in the caudal fin by 4. Inspection of the caudal fin can take between 30 and 60 m, depending on infection intensity.

To inspect the caudal fin, all muscle was fileted with a scalpel from both sides of the body posterior to the anal fin and placed in a petri dish. The tissue was teased apart with foreceps under 25X magnification and recovered metacercariae were counted. Then the entire caudal fin was severed from the body approximately two vertebrae anterior to the termination of the vertebral column and placed in a petri dish. The fin was teased apart using foreceps under 25X magnification and number of recovered metacercariae was recorded. No stain was required to distinguish *H. pumilio* metacercariae from host tissue. Metacercariae appeared dark brown while host tissue was transparent or white with black melanophores, that were readily distinguishable from cysts after some practice. After dissection and counting were complete, all cysts and fish remains were placed in a vial containing 10% formalin and sealed.

#### 1.14.2 **Centrocestus formosanus**

*C. formosanus* encysts in the gills of fish and is found to be equally distributed in the left and right gill arches (Lo and Lee 1996). Conveniently, this allowed us to estimate total infection by inspecting the left four gill arches of each fish and multiplying the metacercarial count obtained by two.

To remove the gills, the operculum was cut away along with most of the lower jaw. Gill arches were removed one by one from the fish and placed in a petri dish using forceps. Metacercarial cysts of *C. formosanus*, unlike *H. pumilio*, are nearly transparent and difficult to distinguish from host tissue, therefore, the gills were placed into 1% Bismarck brown where they were allowed to stain for 5-8 min. Several other stains were evaluated for this purpose, but Bismarck brown was superior. Loose stain was then rinsed away by placing the gill arches in 1% acidulated alcohol for 1 m. The arches were then laid side by side on a microscope slide for study. The gill arches were flattened with

moderate pressure by placing another microscope slide on top of the gills arches and pressing it down gently with gloved fingers. Flattening the gill arches forced all the filaments into one plane where they could be examined easily. After all metacercariae were counted, all cysts and fish remains were placed in vials containing 10% formalin and sealed.

## 1.15 Analysis

### 1.15.1 Effects of parasitism on swimming endurance

To assess the effects of *Haplorchis pumilio* and *Centrocestus formosanus* on the swimming endurance of *Cyprinella venusta*, we performed a simple linear regression of total time spent swimming in the SEA plotted against the infection intensity (total number of metacercariae). Although intuitive, this model fails to account for the variation in swimming ability of fish purely due to fish length. Sambilay (1990) demonstrated that there are two main factors dictating the maximum potential speed of a fish: the aspect ratio of its caudal fin (varies between species) and its standard length (varies between individuals of a species). This means, given the same caudal-fin aspect ratio, larger fish are able to achieve a higher maximum speed than smaller fish.

This phenomenon is driven by the physics of a body moving through a viscous fluid. The resistance encountered by a fish as it moves through water is dictated by many factors including viscosity and velocity of the water, the shape of the fish, the smoothness of its body, the surface area of its body, and especially its length. All the forces acting to resist the movement of a fish (or any object) through water can be reduced to a single number known as the total drag which is measured in Newtons ((kg m)/s<sup>2</sup>). The total drag encountered by a fish moving through the water at a given velocity increases approximately linearly with increasing length of fish (larger fish must overcome more

drag than smaller fish). However, since the volume of a fish increases with the cube of its length, and volume equates roughly to muscle mass, smaller fish have less muscle per unit drag and therefore must expend more energy than larger fish to maintain station at the same water velocity. Additionally, as water velocity increases linearly, drag relative to any length increases with a quadratic power function. This means that the amount of effort that a fish must exert in order to overcome drag (maintain station) does not increase linearly with increasing velocity. Therefore, fish that swim to a higher speed before exhaustion expend exponentially more energy than fish that become exhausted at lower speeds.

The amount of effort that a fish must exert in order to maintain station in a given water velocity can be estimated by calculating the drag in Newtons that a fish must overcome at that velocity, relative to the length of the fish. The calculated drag can then be multiplied by the total distance the fish traveled at each velocity ( $V \cdot T$ ). The product of drag  $\times$  distance gives the amount of work the fish performed before exhaustion during its endurance assessment. Once all fish in an experiment have been tested and necropsied, a model of the effects of the test parasite on the swimming performance of the fish can be represented by regressing work performed against metacercarial intensity. This would allow us to determine if increasing parasite load decreased the amount of work the fish could perform, which may give us insight on how the parasite may affect the daily life of the fish.

Additionally, we wanted to determine if the maximum speed is affected by parasite load, which may help us understand how the parasite affects ability of fish to avoid predators or swim upstream through riffles. Sambilay (1990) studied the maximum

swimming speed of 126 fish species in a custom swim test apparatus and developed a mathematical expression that accurately represented the observed sustained maximum speeds (Equation 1, hereinafter referred to as “expected Sambilay maximum speed”). This expression is a purely descriptive model, and does not require any assessments of forces exerted or work performed. The only variables in the expression are the standard length of a given fish and the aspect ratio of the caudal fin for that species. In order to account for differences in swimming ability of fish due to length, we used this expression to calculate the expected Sambilay maximum swimming speed of each experimental fish, and compared that to the observed maximum speed that fish actually attained in the SEA. We then performed a simple linear regression of the difference between observed and expected swimming speed as a function of intensity of infection per unit length in mm (corrected intensity), since severity of effects of a given number of metacercariae diminishes with increasing length of fish.

Equation 1. Equation for determining the maximum sustained swimming speed of a fish (Sambilay 1990)

$$\log_{10}(\text{km/h}) = -0.828 - 0.6196 \log_{10}(\text{SL}) - 0.3478 \log_{10}(\text{AR})$$

Where: SL = Standard Length of fish in cm,  
AR = Aspect Ratio of caudal fin (height<sup>2</sup>/total surface area; both sides)

The observed maximum swimming speed was adjusted based on the time spent swimming at the observed maximum speed. The SEA testing protocol tests fish at a habituation speed for 180 secs followed by an increase in speed every 60 secs until the fish can no longer maintain station. This creates a stepwise increase in velocity as opposed to a smooth linear progression of increasing speed. When considering only the maximum speed the fish reached, there would be no difference between a fish that swam for 10 s and a fish that swam for 40 s at the same speed even though the fish that swam

for 40 secs likely expended much more energy and probably would have attained a higher maximum speed. To account for the time a fish spent in the final speed, we calculated the percentage of time the fish completed for that step in the protocol. We then multiplied that percentage by the difference between the cm/s of the final speed class the fish reached and the cm/s of the next speed class and added that to the water velocity at which the fish became exhausted. An example of this is shown in Figure 17.

Aquarium	Fish ID	10 cm/s	18 cm/s	27 cm/s	34 cm/s	46 cm/s	54 cm/s	60 cm/s	Total Time	Top Speed	Corrected TS
C3, R1	RF	180	60	14	0	0	0	0	254	27	28.63

$$\left[ \left( \frac{\text{Time (sec) in final speed}}{\text{Time (sec) required for completion}} \right) * (\text{Next Speed} - \text{Final Speed}) \right] + \text{Final Speed} = \text{Corrected}$$

$$\left[ \left( \frac{14}{60} \right) * (34 - 27) \right] + 27 = 28.63$$

Figure 17. Maximum speed reached was 27 cm/s, however, after accounting for the 14 seconds spent in the stage, the final speed is 28.63 cm/s.

#### 1.15.2 Effects of aquarium habituation on swimming behavior

After recording maximum swimming speed of unhabituated and habituated fish, each fish was compared to its respective expected Sambilay maximum speed. Observed and expected swim times were compared using a simple linear regression of maximum speed as a function of total length. This allowed us to compare the habituated and unhabituated groups to determine if habituation to aquaria affected swimming endurance. Comparing the slopes of expected swimming speed and observed swimming speed of habituated fish also helped us to determine if the equation used to calculate the expected Sambilay maximum speed was reliable.

## RESULTS

### 1.16 Prevalence of Parasites in *Melanooides tuberculata*

The mean combined prevalence of all three trematodes across all sampling sites was 30% ( $n=2606$ ), and the highest prevalence recorded among the sampling sites was from the Pecan Island West Bank site, with a combined prevalence of 41% ( $n=1385$ ). Spring Channel was the only site studied that had no collected snails parasitized ( $n=35$ ). The number snails collected from each site is shown in Figure 18. The prevalence of each species of parasite recovered is shown in Table 1 broken down by collecting site. *H. pumilio* had the highest prevalence across all sampling sites except for the Bird Island and Foot Bridge sites, where *Philophthalmus gralli* was the only parasite present with a prevalence of 1-2% ( $n=163$ ).

These prevalence data include only the collected snails that were incubated individually in test tubes and examined for shed cercariae up to three times over periods of 6-24 h. The non-shedding snails were discarded without checking for cryptic (non-shedding) infections. Based on our experience in previous studies (Huston 2014), the actual prevalence is potentially much higher than recorded above.

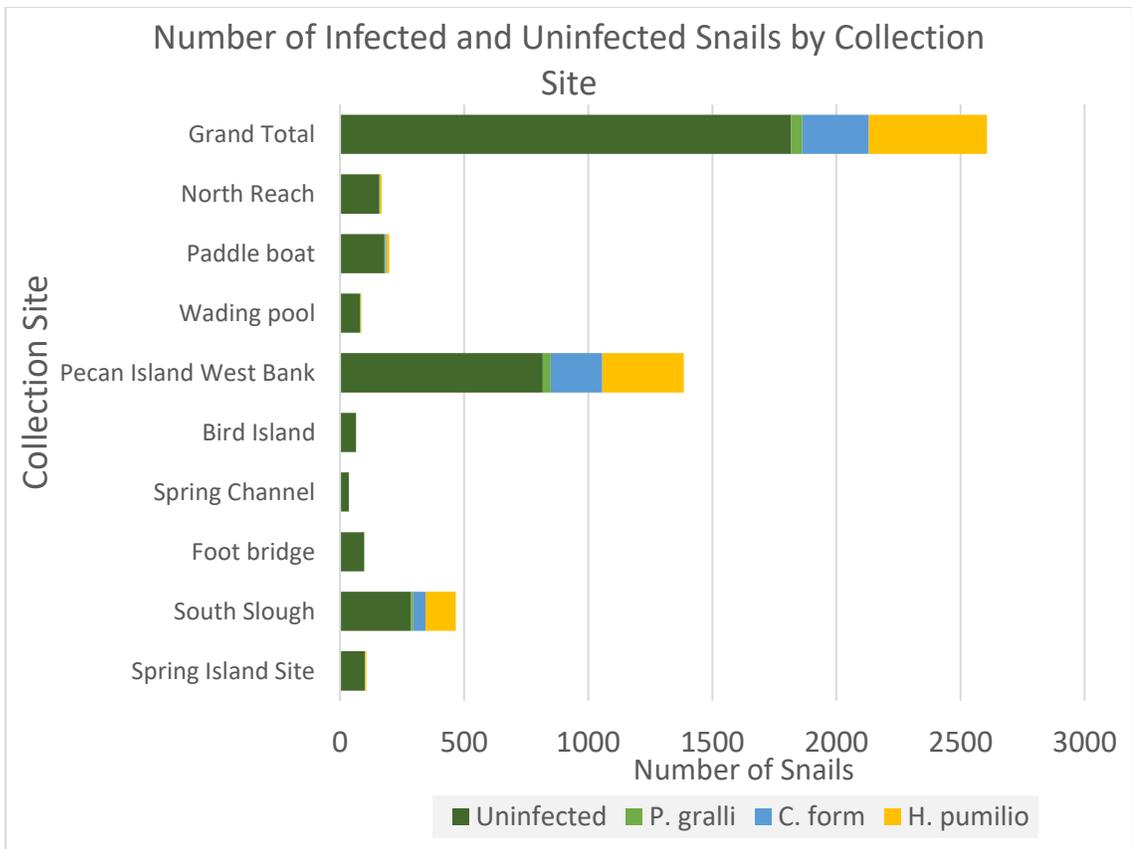


Figure 18. Number of infected and uninfected snails collected by collection site

Table 1 Prevalence of infection by species and collection site.

Site	<i>P. gralli</i>	<i>C. form</i>	<i>H. pumilio</i>	Uninfected	Total	Prevalence
Spring Island	0%	2%	5%	93%	106	<b>7%</b>
South Slough	2%	11%	26%	61%	467	<b>39%</b>
Foot bridge	1%	0%	0%	99%	98	<b>1%</b>
Spring Channel	0%	0%	0%	100%	35	<b>0%</b>
Bird Island	2%	0%	0%	98%	65	<b>2%</b>
Pecan Island WB	2%	15%	24%	59%	1385	<b>41%</b>
Wading pool	1%	0%	2%	96%	85	<b>4%</b>
Paddle boat	1%	3%	6%	90%	198	<b>10%</b>
North Reach	0%	1%	4%	95%	167	<b>5%</b>
<b>Grand Total</b>	<b>2%</b>	<b>10%</b>	<b>18%</b>	<b>70%</b>	<b>2606</b>	<b>30%</b>

### 1.17 Effects of Cercarial Exposure Rate on Fish Mortality

#### 1.17.1 Mortalities of *Cyprinella venusta* associated with *Haplorchis pumilio* infection

Cercarial exposure and fish mortality rates were pooled for analysis by considering all *C. venusta* that had been used in all experiments testing the effects of *H. pumilio* on this host. Mortalities occurred earlier for fish that were exposed to higher densities of cercariae, and fish that were exposed to lower densities of cercariae survived longer (Figure 19,  $p \ll 0.0005$ ,  $r^2=0.68$ ). The highest experimental exposure rate of *H. pumilio* cercariae in a single aquarium was 6,000/fish, and all four fish died within 5-24 h of the first treatment.

Even though some fish could withstand high numbers of cercariae given a low exposure rate, there were other effects caused by the accumulation of metacercariae from exposure that would likely cause mortality if damage from initial infection did not. For instance, fish that experienced high cumulative exposures at lower rates would generally have a latent response to food or ignore food altogether, exhibit brooding behavior, or have reduced response time to the presence of people. In some cases, the jaws of a few

fish became stuck open due to infection. While mortality may not occur due to infection itself, fish with locked jaws would die from the secondary issue of starvation.

Accumulation of metacercariae was also observed to cause significant fin deterioration. This is possibly due to the loss of circulation to the fins caused by the swelling of the fin insertion. Many fish show an inflammatory response to metacercarial encystment, which can cause large blisters or nodules to develop at the caudal fin.

Mortalities among treatment fish were excluded from analysis if they died without any obvious association with pathology (such as poor aquarium water quality, injury, etc.). Some mortalities also occurred among control fish in the tests of *H. pumilio* on *C. venusta*; five control fish died due to injury, and all four control fish in one aquarium died within hours of each other due to what we concluded was a fungal outbreak that was contained to one aquarium.

#### 1.17.2 **Mortalities of *Cyprinella venusta* associated with *Centrocestus formosanus* infection**

While some mortalities were recorded during the test of the effects of *Centrocestus formosanus* on *Cyprinella venusta*, none of the dead fish exhibited any pathology consistent with *Centrocestus formosanus* infections, confounding our ability to confidently assign cause of death to infection. We therefore concluded that no mortalities occurred as a direct result of infections with *C. formosanus*.

#### 1.17.3 **Mortalities of *Dionda diaboli* associated with *Haplorchis pumilio* infection**

As with *Cyprinella venusta*, *Dionda diaboli* mortalities occurred earlier for fish that were exposed to higher densities of cercariae, and fish that were exposed to lower densities of cercariae survived longer (Figure 19,  $p < .0002$ ,  $R^2=0.83$ ). The highest

exposure rate tested was 10,100 cercariae in a single treatment, which caused mortality of all three fish inhabiting that aquaria within 24 h. The lethal exposure necessary to kill all experimental *D. diaboli* in an aquarium within 24 h of treatment (10,100) was less than half the rate that killed all *C. venusta* in an aquarium (24,000). As with *C. venusta*, any mortalities among *D. diaboli* that coincided with poor water quality readings or apparent pre-existing conditions were not considered during analysis. No control fish died over the course of the experiment testing the effects of *H. pumilio* infections on *D. diaboli*.

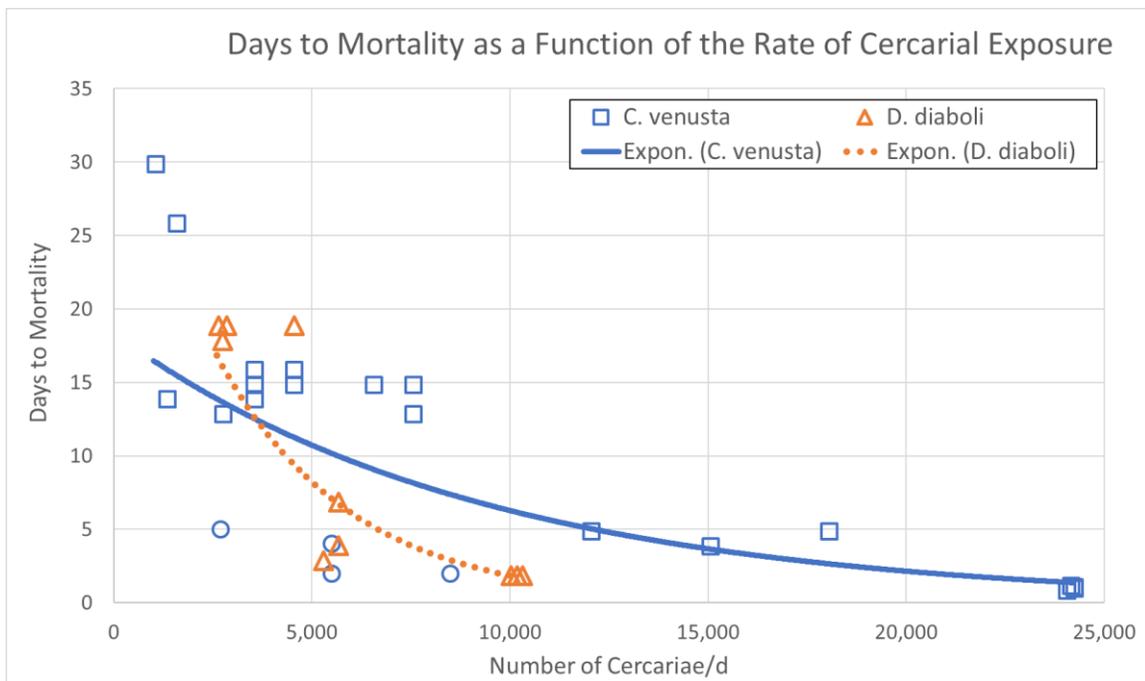


Figure 19. Days till mortality of *Cyprinella venusta* and *Dionda diaboli* as a function of the treatment rate (number of cercariae/d). Open circles represent *C. venusta* that are included in the regression but may have died as a result of chance migration through a vital organ by a single larva instead of simultaneous damage incurred by numerous larvae migrating through nonvital organs.

## 1.18 Effects of Cercarial Exposure on Swimming Ability

### 1.18.1 *Haplorchis pumilio*

#### 1.18.1.1 *Cyprinella venusta*

##### 1.18.1.1.1 *Effects on maximum swimming speed*

Since the severity of effects of a given number of metacercariae diminishes with increasing fish length, the max swimming speed of fish was regressed against caudal metacercarial intensity per mm total length (corrected metacercarial intensity). The maximum swimming speed of *C. venusta* was negatively correlated with the corrected metacercarial intensity (Figure 20,  $p < 0.001$ ,  $r^2 = 0.74$ ). Deviation from expected maximum speed grew as corrected metacercarial intensity increased (Figure 21 **Error! Reference source not found.**,  $p < 0.001$ ,  $r^2 = 0.76$ ). Thus, when corrected metacercarial intensity of a fish increases by 1.0, the observed maximum swimming speed can be expected to decrease by 0.72 cm/s from the expected maximum swimming speed based on that fish's total length.

##### 1.18.1.1.2 *Effects on total work performed*

Work performed prior to exhaustion was also negatively correlated with corrected metacercarial intensity (Figure 22,  $p < 0.0001$ ,  $r^2 = 0.57$ ), with exponential decay.

#### 1.18.1.2 *Dionda Diaboli*

##### 1.18.1.2.1 *Effects on maximum swimming speed*

The maximum swimming speed of *D. diaboli* was negatively correlated with the corrected metacercarial intensity (Figure 20,  $p < 0.001$ ,  $r^2 = 0.86$ ). Deviation from expected maximum swimming speed became larger as corrected metacercarial intensity increased. Thus, when corrected metacercarial intensity of a fish increases by 1.0, the observed maximum swimming speed can be expected to decrease by 1.06 cm/s from the

expected maximum swimming speed based on that fish's total length (Figure 21,  $p < 0.001$ ,  $r^2 = 0.76$ ).

1.18.1.2.2 Effects on total work performed

Work performed prior to exhaustion was also negatively correlated with corrected metacercarial intensity (Figure 22,  $p < 0.001$ ,  $r^2 = 0.71$ ), with exponential decay.

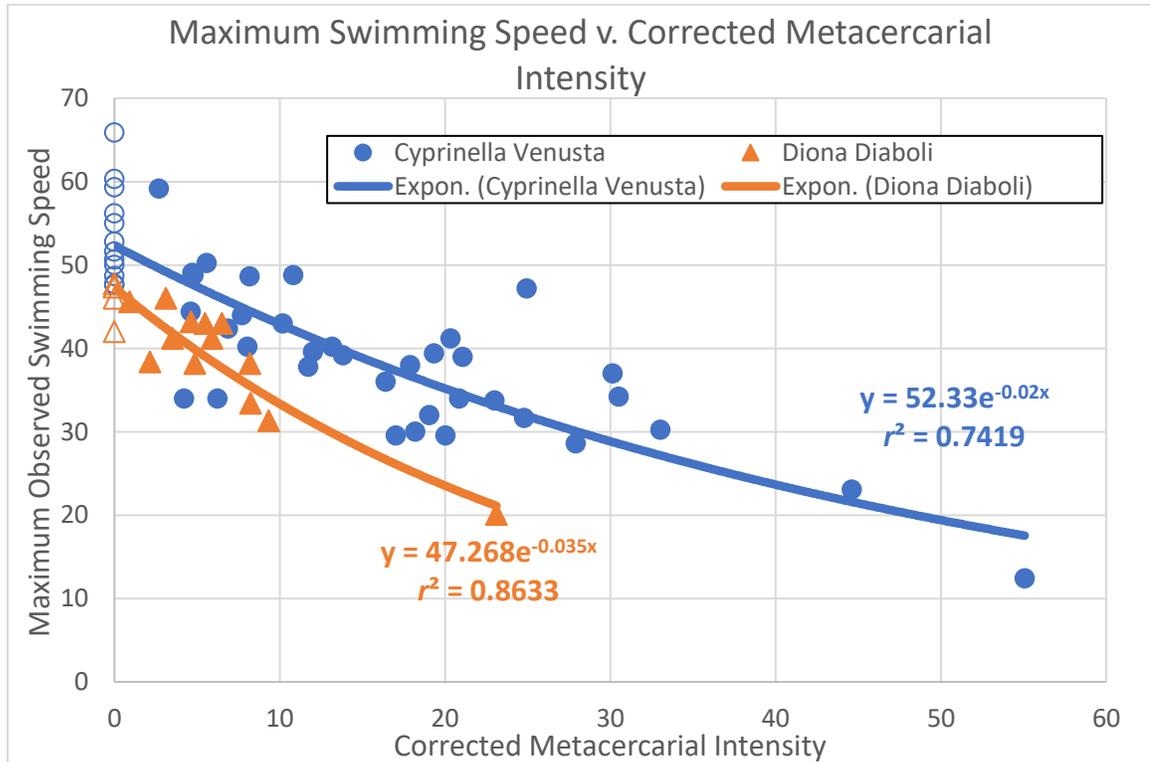


Figure 20. Swimming speed of individual *Cyprinella venusta* and *Dionda diaboli* as a function of corrected metacercarial intensity.

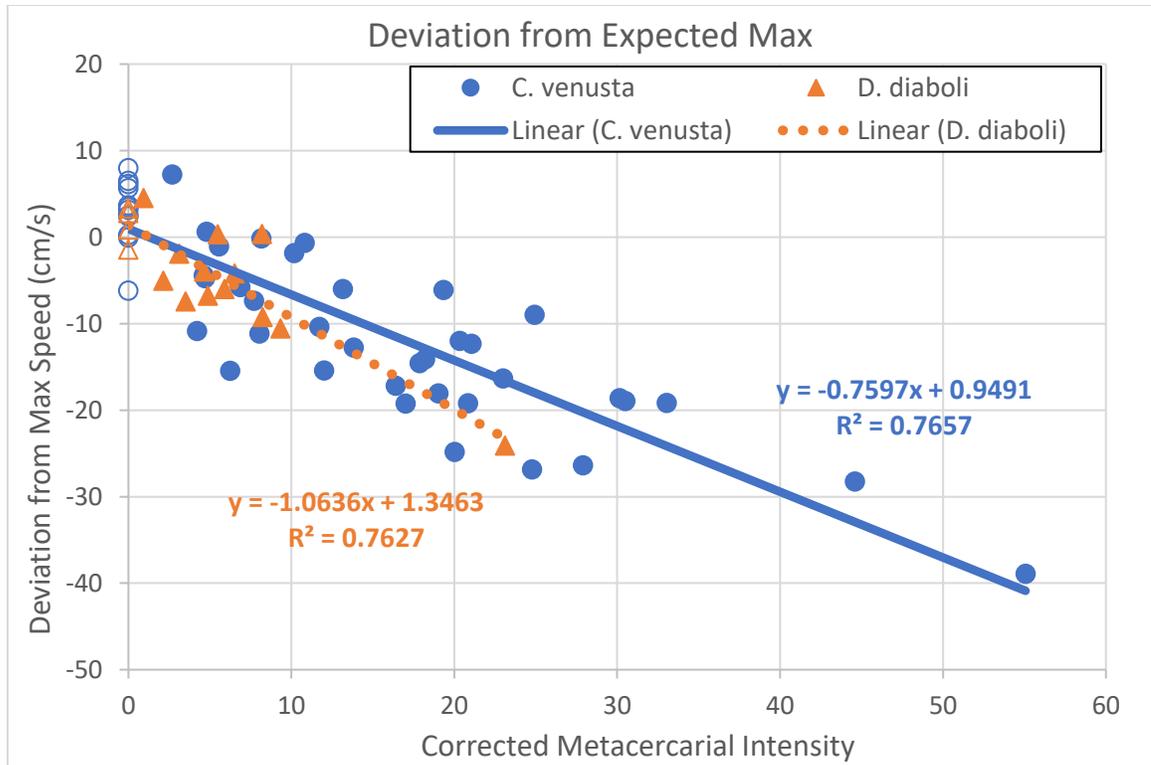


Figure 21 The deviation of individual *Cyprinella venusta* and *Dionda diaboli* from expect maximum swimming velocity as a function of the corrected metacercarial intensity of that fish. Deviation is express as observed maximum speed minus expected maximum speed. Open triangles represent control fish.

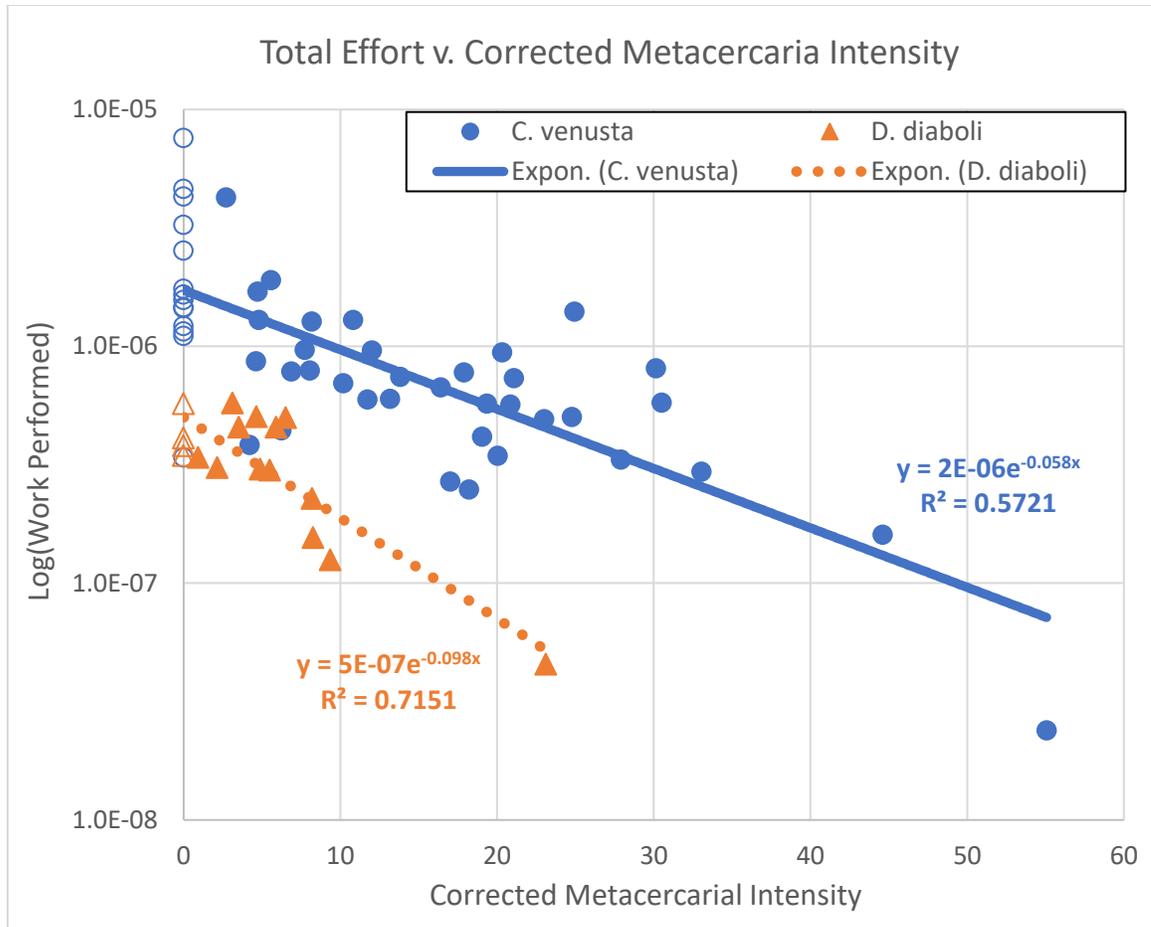


Figure 22. The log of work done by individual *Cyprinella venusta* and *Dionda diaboli* as a function of corrected metacercarial intensity of the fish. Open triangles represent control fish.

### 1.18.2 *Centrocestus formosanus*

No correlation was found between the maximum swimming speed of *Cyprinella venusta* and the corrected metacercarial intensity of *Centrocestus formosanus* in the gills ( $p=0.412$ ,  $r^2=0.01$ ). The work performed by fish prior to exhaustion was not correlated with number of corrected metacercarial intensity ( $p=0.514$ ,  $r^2=0.018$ ).

## 1.19 Successful Establishment of Metacercariae in Host

### 1.19.1 *Haplorchis pumilio*

When *C. venusta* was exposed to cercariae of *H. pumilio* (all experiments combined), the mean rate at which the cercariae successfully established themselves as metacercariae (infection success) was estimated to be 39%. The maximum infection success recorded

for a single fish was 62%, while the lowest infection success recorded for a single fish was 14%. There was no apparent correlation between cercarial exposure density and infection success ( $p=0.88$ ,  $r^2=0.028$ ), however there was a positive correlation between total length of experimental fish and infection success (Figure 23,  $p=0.0015$ ,  $r^2=0.24$ )

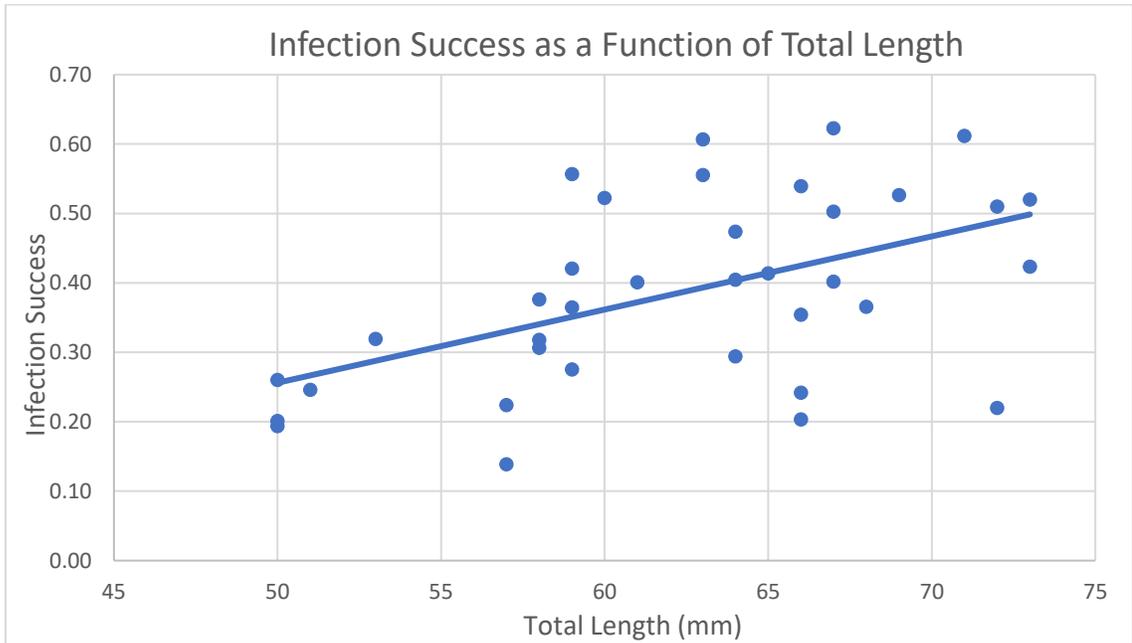


Figure 23. Infection success from an individual fish as a function of total length.

### 1.19.2 *Centrocestus formosanus*

Mean infection success of *Centrocestus formosanus* in *Cyprinella venusta* was  $16\% \pm 6.5\%$ . The maximum infection success was 33% while the minimum was 6%. There was a detectable but insignificant negative correlation between infection success and treatment level ( $p=0.086$ ,  $r^2=0.08$ ). A slight correlation was detected between infection success and total length (Figure 24,  $p=0.048$ ,  $r^2=0.12$ ).

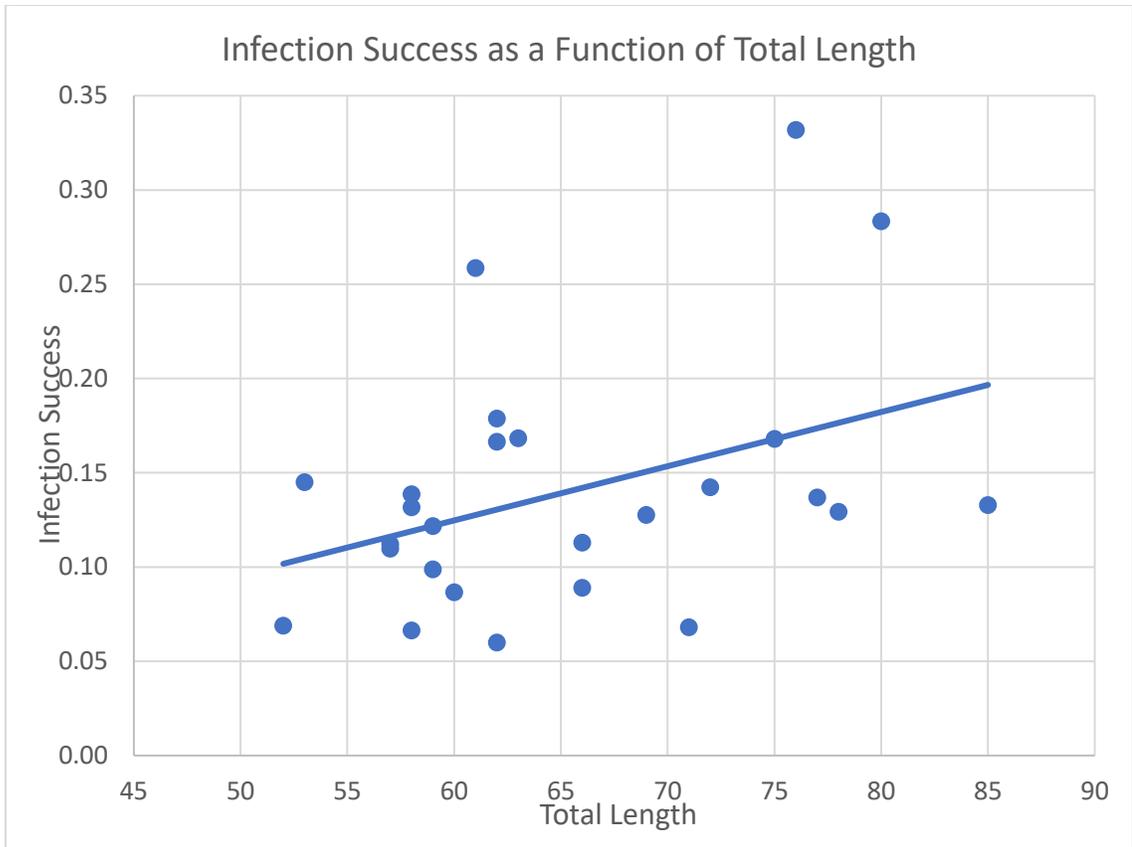


Figure 24. Infection success on an individual fish as a function of total length.

### 1.20 Effects of Habituation to Aquaria on Swimming Behavior

No positive correlation was detected between maximum swimming speed and standard length of fish that had not been habituated to aquaria (Figure 25;  $p=0.99$ ,  $r^2=0.002$ ). Habituated fish, on the other hand, showed a positive correlation between maximum speed and standard length (Figure 25;  $p=0.001$ ,  $r^2=0.48$ ).

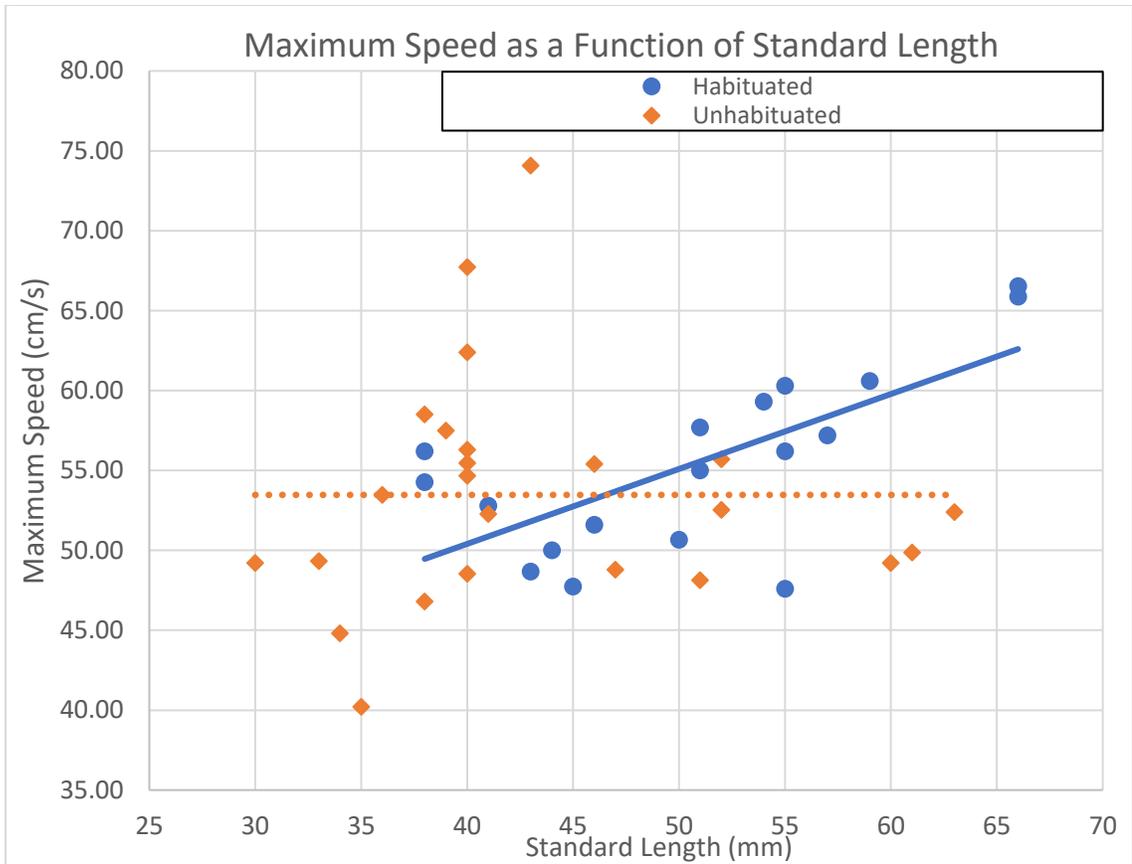


Figure 25. Maximum swimming speed attained by an individual fish plotted as a function of its standard length.

### 1.20.1 Determining the validity of the expected maximum speed formula

The validity of the formula used to calculate expected maximum speed was determined by comparing the slopes of two regression lines based on the observed and expected maximum swimming speeds of habituated control fish from all experiments. The first regression was performed by regressing the *observed* maximum speed of the fish against their standard length. The second was performed by regressing the *expected* maximum swimming speed against their standard lengths. The slopes of the two regressions were not significantly different [Figure 26,  $p(t_{a(2),32} \geq -|1.294|) = 0.204$ ], indicating that Equation 1 was performing as expected.

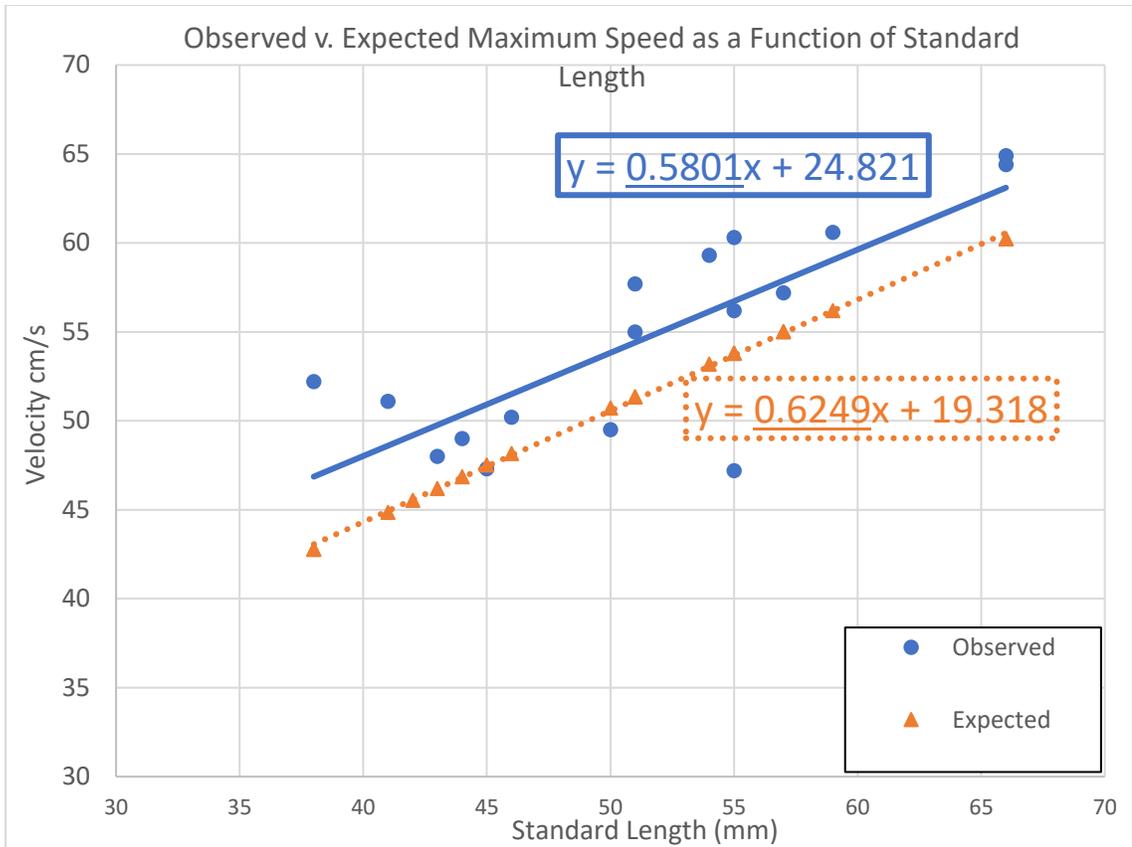


Figure 26. Slope of the regressions of observed and expected Sambilay maximum swimming speed are underlined.

### 1.21 Swimming Endurance Apparatus Calibrations and Consistency

Achieving consistently lamellar flow is paramount in retrieving dependable, repeatable results from the SEA. Without the assurance that the SEA produces lamellar flow, there is no way to know that each fish encountered the same velocities of water at each set valve settings. Turbulence and eddies within the current of water in the viewing tube can provide an escape from faster moving water, introducing unmeasurable variation to the experiment.

Lamellar flow of water at each ramp of SEA protocol is demonstrated by the low coefficient of variation of pom-poms that passed through the viewing tube (Table 2). Low CV in all speed classes indicate that the flow through the viewing tube has no significant turbulence or eddies, providing consistent, lamellar flow.

Table 2. Consistency is displayed in terms of the coefficient of variation and standard deviation associated with each water velocity. The number of pom-poms recorded during calibration is n.

Ramp	n	cm/s	Std Dv	C V
Habituation	7	10	1.04	10.7%
1	11	18	1.45	8.2%
2	10	27	2.94	11.0%
3	10	34	2.58	7.6%
4	14	46	3.02	6.5%
5	11	54	2.22	4.1%
6	11	60	3.91	6.5%
7	11	68	3.27	4.8%

## DISCUSSION

### 1.22 Prevalence of Heterophyid Parasites in *Melanoides tuberculata*

#### 1.22.1 Sampling-site factors associated with prevalence

When compared to historical data, prevalence of *H. pumilio* appears to have increased substantially over the past 17 yrs. Other workers have reported *H. pumilio* prevalences no higher than 1%-1.6% in Landa Lake (Bio-West 2014, Tolley-Jordan and Owen 2008). However, our data for *H. pumilio*, pooled across all sample sites (n=2606), revealed a prevalence of 18%. It is important to note that, while no previous study has reported prevalences as high as what we have observed, most of our snails came from Pecan Island, where we consistently found the highest prevalence, and where the previous workers did not sample.

The high prevalence of exotic heterophyids in snails we collected from Pecan Island West Bank (PIWB) relative to other sites that we sampled was likely due to the high number of piscivorous birds that frequented the area. Although the Green Heron (*Butorides virescens*) is thought to be the main definitive host for *C. formosanus*, and probably also hosts *H. pumilio*, only a single Green Heron was seen over the course of numerous visits to PIWB. The Double-crested Cormorant (*Phalacrocorax auritus*),

however, was seen roosting by the dozen at the PIWB collection site during every sampling event. While it would appear that the Double-crested Cormorants were serving as the definitive host, previous studies assessing the prevalence of *C. formosanus* in Double-crested Cormorants at Landa Lake did not detect any infections. The paradox of seeing numerous cormorants at PIWB on every visit and seeing only a single Green Heron suggests that the Double-crested Cormorant should be reassessed to determine if they in fact can serve as a definitive host for *C. formosanus*.

#### 1.22.2 **Incomplete detection of cercariae**

Accurate determination of *C. formosanus* and *H. pumilio* prevalence in *M. tuberculata* in our experiments was complicated by the fact that infected snails had to be kept alive in order to provide a source of cercariae to conduct experiments on fish. In other studies whose objective was to solely determine prevalence of infection in *M. tuberculata*, snails were simply crushed and examined for the presence of sporocysts or generations of rediae in the digestive gland. This is a relatively quick and efficient method allowing the detection of infection even when the snail has recently been infected and is still incubating or is experiencing a senescence in the rate of shedding of cercariae. The incubation period between becoming infected and shedding cercariae for all three trematodes commonly infecting *M. tuberculata* in Texas varies between 83-89 d (Pinto et al. 2018, Díaz et al. 2002). In order to detect infections using presence or absence of cercariae in our test tube array, collected snails would have had to have become infected three months prior to being sampled. Otherwise, infections in snails that were still incubating would not be detected. Snails that were examined over a 24 h period and found not to be shedding any cercariae were discarded, some of which were likely harboring rediae with immature cercariae. Dissection of every discarded snail was not

attempted because this was beyond the scope of the study. Thus, our reports of prevalence are likely lower than actual prevalence in the wild population around Landa Lake.

### **1.23 Effects of Cercarial Exposure Rate on Fish Mortality Rate**

#### **1.23.1 General relationships**

Fish mortality resulting from infection with *H. pumilio* is likely caused by four main mechanisms; (1) physiological shock and trauma incurred when a fish is exposed to a large number of cercariae in a short time, (2) accumulated histopathological damage when a fish is exposed to cercariae at a lower rate per day, resulting in the surviving fish encountering perhaps a much larger total number of cercariae, (3) chance migration of a cercaria (perhaps independently of exposure rate) that just happens to intercept a vital organ causing catastrophic damage, and (4) excessive electrolyte loss through wounds created when cercariae aggregate in sufficient numbers to cause flaring of scales or open sores at the fin insertions.

The number of days that infected *C. venusta* survived while being treated with cercariae of *H. pumilio* decreased at an exponential rate as cercarial exposure rate increased (Figure). Mortality in fish was observed after a single exposure of 6,000 cercariae in one day, but fish exposed to three times as many total cercariae (18,750) survived for as long as 15 d when the exposure rate was reduced to 1,850/d. This would indicate that mortality due to high exposure rate is not caused by the total amount of damage done by cercariae, but rather, is caused by physiological shock to all systems of the body in a very short period of time. Conversely, mortality in fish receiving a lower daily cercarial exposure rate that survive much longer is possibly caused by the accumulated damage to tissues resulting from numerous cercarial migrations over time.

While the rate of cercarial exposure is likely the main factor driving the relationships in Figure 19, some of the fish in aquaria receiving low rates of exposures (1,200 cercariae/d) died much earlier than their cohorts. Such fish usually showed grossly obvious signs of catastrophic damage to vital organs as revealed by bright red, green, and yellow stains showing through the abdomen, but lacked other symptoms of infection. The probable cause of death in these instances was larval migration by cercariae that caused catastrophic damage to, and ultimate failure of, a vital organ. Sommerville (1982) has shown that migrating cercariae of *H. pumilio* were found in every organ of the body in experimentally infected fry of three species tilapia. These observations suggest that an element of chance is associated with the path taken by a cercaria migrating through host tissue. It follows that fish exposed to higher numbers of cercariae are more likely to experience at least one of these catastrophic organ failures. The chance occurrence of migration causing significant damage to a vital organ is likely the result of cercariae that have gotten lost in their journey to a suitable place to encyst and began migrating through tissue aimlessly.

Roughly a quarter of cercariae that successfully develop into metacercariae in fish are distributed in the caudal fin (Lo and Lee 1996). When metacercariae accumulate in large numbers, the caudal peduncle can swell into a large blister that can rupture (Figure 2), causing loss of blood and electrolytes. Mortality due to blood and electrolyte loss was presumed to have occurred in fish with sudden development of an open sore near the caudal fin and subsequent death.

### 1.23.2 Differences in mortality rates between *Cyprinella venusta* and *Dionda diaboli*

The slope of the regression of mortality on exposure rate for *Dionda diaboli* was much steeper than the slope for *Cyprinella venusta*, and the exposure rate sufficient to kill all *D. diaboli* in an aquarium after one treatment was less than half the exposure rate killing all *C. venusta* in an aquarium after one treatment (Figure 19). Also, *D. diaboli* died sooner than *C. venusta* at similar exposure rates on average, meaning that a given exposure rate is more likely to cause mortality in *D. diaboli* than in *C. venusta*. This result was consistent with our expectation that *D. diaboli* would be much more sensitive than *C. venusta* to the effects of infection by *H. pumilio*.

### 1.23.3 Interpretations and other observations

Mortality caused by high cercarial exposure is probably not a biologically significant problem for fish in local river systems. The continuous flow of water from upstream will sweep emergent cercariae downstream, preventing density of cercariae from accumulating in one spot. Therefore, it is unlikely that fish inhabiting local rivers will encounter cercariae of *H. pumilio* at sufficient densities to cause sudden mass mortality. It is worth noting, however, that as flow rate from springs decrease, the density of cercariae will increase, which would also increase the risk of parasite induced mortality. While there has not been any assessment of acquisition rates of *H. pumilio* in wild fish, caged fish have been shown to acquire as many as 14 metacercariae of *Centrocestus formosanus* per day in the Comal River (Fleming et al. 2011). Because the prevalence of *H. pumilio* is higher than the prevalence of *C. formosanus* in the snail host at Landa Lake, we estimate the rate of acquisition to be similar for *H. pumilio*. Based on our lowest observed infection success rate for *H. pumilio* cercariae, the caged fish would have had to have encountered at least 100 cercariae/d to acquire 14 metacercariae. This estimate is

approximately 10% of the cercarial exposure that caused mortality in experimentally infected fish over the course of 30 d. Even considering the highest estimates of cercarial rates in the river, fish mortalities due to infection is more likely to occur from secondary causes resulting from decreased fitness associated with metacercarial accumulation over longer periods of time, than mortality due to high cercarial exposure.

Should wild fish actually encounter water with cercarial densities similar to those that were lethal in our experimental studies, the observed behaviors of experimentally exposed fish in our study and those of other workers (Sommerville 1982) suggest that immigrating fish would quickly seek other waters with lower cercarial densities. fish experimentally exposed to *H. pumilio* cercariae displayed signs of agitation including flashing and spastic swimming. In addition, some fish in high exposures even attempted to jump from the aquaria, leaving the water several times after initial treatment. It has also been observed that seemingly pristine habitat in rivers where the parasite is known to exist are void of fish. This would indicate that fish selectively avoid areas where cercarial density is high in spite of suitable habitat. Avoidance of areas with high cercarial density is expected for highly mobile fish in rivers such as *C. venusta*, however, fish that are confined in a reservoir or fish that have low mobility within the river due to habitat requirements (such as *D. diaboli*) or territorial behavior, may be more affected by high cercarial densities. The low mobility of *D. diaboli*, combined with its apparent sensitivity to effects of *H. pumilio* infection, would put the already threatened species at even greater risk given its limited range and strong association with calm, spring influenced water and thick vegetation.

## 1.24 Effects of *Haplorchis pumilio* and *Centrocestus formosanus* on the Swimming Ability of *Cyprinella venusta*

### 1.24.1 Effects of *Haplorchis pumilio*

#### 1.24.1.1 *Observed effects on Cyprinella venusta*

*Cyprinella venusta* that encounter non-lethal exposures of cercariae of *H. pumilio* will experience decreased fitness in terms of swimming ability due to infection. Measures expressing such decreases in swimming ability include reduced maximum swimming speed and reduced total work the fish is able to perform in terms of maintaining station before becoming exhausted. Infected *C. venusta* experienced a consistent decrease in maximum swimming speed as infection intensity increased. An increase of one metacercariae per each mm of total length of the fish reduced maximum swimming speed by 1.2-1.6% (depending on fish length). Given a fish with a total length of 62 mm (the mean total length of the experimental fish), and a total accumulation of 442 metacercariae (7.1 metacercariae/mm), its maximum swimming speed would be reduced from an expected 51 cm/s to 45.9 cm/s. This amounts to a 10% decrease in maximum swimming speed relative to an uninfected control of the same length. Considering the mean recorded rate at which fish acquire metacercariae in the Comal River (Fleming et al. 2011), some resident fish would acquire a metacercarial load sufficient to reduce its maximum swimming speed by 10% in about 1 y. A 10% decrease in the maximum swimming speed would likely be biologically relevant when evading predators and navigating riffles in order to find new food sources and breeding areas. Considering that *C. venusta* commonly reach 3 yrs of age, *C. venusta* surviving this length of time would experience a 30% decrease in maximum swimming speed given the same rate of metacercarial acquisition. A resident fish acquiring the highest measured rate of metacercarial acquisition (14 metacercariae per day) would accumulate about 5,000

metacercariae in a single year which would surely cause death long before it reached 1 year of age. It is also important to note that because swimming ability decreases with metacercariae per mm total length of the fish, as total length decreases, fewer total metacercariae cause a decrease in swimming ability. As stated before, 7.1 metacercariae per mm causes an approximate 10% decrease in maximum speed of *C. venusta*, meaning the number of metacercariae that would cause this change in a 40 mm fish (284 metacercariae) is much less than would be required in a 70 mm fish (479 metacercariae). Consequently, the 40 mm fish would experience this decrease in fitness much sooner (21d) than the 70 mm fish (35d), given an acquisition rate of 14 metacercariae per day. This suggests that smaller fish are at potentially higher risk than larger fish and may experience a decrease in fitness sooner than larger fish at the same acquisition rate. This could mean that younger, smaller fish are at higher risk than older larger fish, which may alter the demographics of fish populations where the parasite exists.

Fish also showed a decrease in the amount of total work they could perform before becoming exhausted as corrected metacercarial intensity increased. Total work performed is equivalent to the amount of force a fish can exert over a given distance. For fish, this translates to the amount of time a fish can maintain station at a given water velocity, which is obviously an important aspect of fitness. Unlike the linear decrease observed in maximum swimming speed due to infection, the total work a fish can perform before becoming exhausted declines with an exponential decay as metacercarial intensity increases (Figure 20). This means that if a fish accumulates metacercariae at a constant rate, the time required for the fish to become exhausted (given a fixed water velocity) decays exponentially. As mentioned before, an infection of 7.1

metacercariae/mm causes a 10% decrease in maximum swimming speed but causes a 23% decrease in the amount of work the shiner is able to perform before becoming exhausted. A shiner with a 23% reduction in ability to maintain station in flowing water will surely be outcompeted when contesting for optimal feeding positions and spawning sites, both activities of which generally occur in flowing water.

#### **1.24.1.2 Observed effects on *Dionda diaboli***

Much like *Cyprinella venusta*, *D. diaboli* exhibited reduced maximum swimming speed and reduced total work performed when infected with *H. pumilio* (Figures 21 and 22). However, the effects of *H. pumilio* infection are more dramatic in *D. diaboli* than in *C. venusta*.

Maximum swimming speed of *D. diaboli* can be expected to decrease by 2.1 to 2.8% for each unit increase in corrected metacercarial intensity (depending on fish length), which is much greater than the decrease observed in *C. venusta* (1.2 to 1.6%). So, given the number of metacercariae that causes a 10% decrease in max swimming speed of *C. venusta*, *D. diaboli* would experience a 17% decrease. A decrease in swimming ability of this magnitude would, undoubtedly, be biologically relevant, as it would surely compromise the ability of *D. diaboli* to escape predators or compete with uninfected fish. As with *C. venusta*, shorter *D. diaboli* will experience negative effects sooner than longer fish at the same metacercarial acquisition rate.

The total amount of work that an individual *D. diaboli* was able to perform was also negatively impacted by increasing metacercarial intensity (Figure 22). The amount of work an individual was able to perform decayed exponential as corrected metacercarial intensity increased. As stated before, because this relationship is not linear, the number

of metacercariae sufficient to cause only a 10% decrease in maximum swimming speed for an individual *D. diaboli* would result in about a 23% decrease in the amount of work that fish is able to perform. A decrease in swimming ability of this magnitude is certainly biologically relevant, as infected fish will be out-competed when contesting for optimal feeding areas and breeding grounds.

#### **1.24.1.3 Ecological ramifications of *Haplorchis pumilio***

Another way of looking at these effects is to consider populations of *C. venusta* and *D. diaboli* coexisting in a community of small fishes and currently existing in a cercaria-free ecosystem. If *H. pumilio* is introduced into such a naïve system, *D. diaboli* will experience fitness consequences earlier than *C. venusta*, and the other species will probably also be affected differentially. Also, the next generation of young for each susceptible species will be affected much more dramatically than their parents, leading to an increase in mean age of the population. Perhaps more importantly, species not susceptible to *Haplorchis pumilio* will benefit from the invasion, due to decreased competition. Thus, the demographics, composition, distribution, etc., of the community will all change post introduction. Indeed, because of the more intense effects of infection on smaller fish, recruitment of offspring into the reproducing population of some species may be suppressed sufficiently as to bring about local extirpation.

#### **1.24.2 Mechanisms causing decreased swimming ability**

The decrease in swimming ability observed in *C. venusta* and *D. diaboli* as a result of *H. pumilio* infection is probably caused by two main factors; (1) overall damage in the muscle tissue from larval migration (Sommerville 1982) and (2) metacercariae encystment between the fin rays in all fins, stifling the ability for fish to articulate their fin rays for efficient swimming. To swim efficiently, fish articulate the rays of the caudal

fin to form a cup, which they use to create thrust as the fish beats its tail (Bainbridge 1963). The concavity of the caudal fin cup inverts on every tail beat and is led by the rays on the dorsal and ventral margin of the caudal fin, indicating that the formation of the cup is an active process. Formation of the cup requires muscles to work in moving fin rays against resistance. An increase in metacercarial cysts between the articulation points of the rays likely hinders this process, resulting in reduced swimming efficiency. In addition to reduced articulation of the fin itself, damage to muscle tissue from hemorrhaging caused by cercarial migration may reduce the amount of thrust fish are able to generate per tail beat.

It is important to note that all experiments testing swimming ability of *C. venusta* and *D. diaboli* infected with *H. pumilio* were conducted over a relatively short time (3 weeks) at densities much higher than the rates observed in local rivers. While it is not inconceivable that fish in local rivers could acquire enough metacercariae to experience the decrease in total fitness observed in experimentally infected fish, the acquisition of a considerable number of metacercariae over the course 1yr as opposed to 3 w may produce different effects on fitness.

#### 1.24.3 **Centrocestus formosanus**

Counter to prediction, the range of metacercarial load tested in *C. venusta* was not associated with a negative impact on swimming ability. Inspection of the gills during necropsy revealed that metacercariae caused minor damage and minimal swelling to gill filaments of *C. venusta*. This is inconsistent with specimens of different species that have been experimentally infected in the past. Unlike *Cyprinella venusta*, fountain darters (*Etheostoma fonticola*) infected with *C. formosanus* exhibited a copious amount of

swelling in the gills in response to infection, as well as severe damage to the gill tissue that often results in death (Mitchell et al. 2000). This indicates that pathological effects of *C. formosanus* infection is heavily dependent upon the host species' immune response to infection. While *C. formosanus* may have some effect on the fitness of small cyprinids, our data suggest that *H. pumilio* should be of far greater concern to fish conservation workers.

### 1.25 Success of Metacercarial Establishment by *Haplorchis pumilio* and *Centrocestus formosanus*

There are many variables in rivers and lakes that potentially affect the rate at which cercariae successfully infect a host, which are not testable using aquaria. As such, it is important to note that any data retrieved from experiments using aquaria to determine the infection success of parasites describe an ideal scenario for cercariae given that the host cannot escape, there is no water current for cercariae with which to contend, and the cercariae are with the host at all times in a relatively confined space. Conversely, cercariae in a river or lake exist in a much larger volume of water where the chance of encountering a suitable host is arguably lower; furthermore, if a suitable host is encountered, cercariae must also successfully penetrate the host, migrate through the host tissue, and successfully develop into a metacercaria. Additionally, cercariae are largely subject to the velocity of the water, only able to travel at approximately 0.5-2.5 mm/s (Lo and Lee 1996) and can be damaged by turbulence or riffles which lowers the viability of cercariae. Consequently, predicting the infection success rate of cercariae of either trematode species under study is arduous.

Regardless of the accuracy of the infection success rate reported from experimentally infected fish, it appears from the results in section 1.19 that density of cercariae does not

affect infection success rate in either species of trematode. Lack of density dependence represented in our data may be a result of the extreme high densities of cercariae tested, resulting in a saturation effect. We expect that as cercarial densities decrease to levels found in the wild, infection success will decrease. While no density dependency was observed, infection success rate was positively correlated with total length of fish in both species of trematode. While the positive correlations could be spurious, the increased success rate of cercariae on larger fish could be explained by larger fish having larger respiratory currents compared to smaller fish. Larger respiratory currents increase the chance cercariae contact unscaled skin and membranes of the fish, making it easier for cercariae to successfully penetrate the fish. Additionally, larger fish push more water with every fin movement than compared to small fish, even when maintaining orientation within an aquarium. This creates a larger continuous current of water close to the body near the fin insertions, possibly increasing the number of cercariae the fish encounters.

## **1.26 Effects of Aquarium Habituation on Swimming Behavior**

### **1.26.1 Effects of habituation to aquaria on swimming ability of fish**

Behavior in the SEA was markedly different between habituated and unhabituated fish. It was originally hypothesized that unhabituated fish may perform better in the SEA than habituated fish, considering that habituated fish are in aquaria for up to 3 weeks without any swimming exercise from exposure to flowing water. Inconsistent with prediction, habituated fish attained higher swimming speeds on average and responded more consistently to the SEA speed-ramping protocol. Unhabituated fish behaved erratically when first put into the SEA, but also frequently tried to move laterally to escape the current. Upon encountering the wall of the glass tube, unhabituated fish began darting unpredictably. Fish also displayed a fright response when the SEA operator made

adjustments to the speed, resulting in similar erratic darting behavior. This made it difficult to obtain consistent, reliable data with unhabituated fish. As a result, recorded maximum swimming speed was not correlated with the standard length of unhabituated fish, which was inconsistent with our expectations. Conversely, maximum swimming speed of habituated fish was positively correlated with standard length, as expected. The consistent, predictable behavior of habituated fish was likely affected by the fact that habituated fish were accustomed to living in the confines of a glass container and had become acclimated to the presence of people.

#### 1.26.2 **Validity of Sambilay's speed equation**

Sambilay (1990) equation for predicting the maximum swimming speed was determined by fitting a regression across observed maximum swimming speed of over 126 species of fish using the aspect ratio of the caudal fin and standard length of the fish tested. Given the high degree of variation among fish in terms of morphology and natural history, a test was conducted to ensure the proposed equation accurately and reliably predicted the expected Sambilay maximum swimming speed of *Cyprinella venusta*. To determine if the equation was an acceptable way of calculating the expected Sambilay swimming speed of *C. venusta*, the slope from the regression of observed maximum speed on standard length was compared to the slope of the regression formed by expected Sambilay maximum swimming speed on Standard Length (Figure 26). As reported in section 1.20.1, a *t*-test determined that the slopes from the two regressions were not different, indicating not only that Sambilay's equation was a reliable estimation of expected maximum swimming speed of *C. venusta*, but also gave us confidence that the SEA produced consistent, repeatable results.

### **1.27 Swimming Endurance Apparatus Calibration and Consistency**

Previous attempts to estimate the maximum swimming speed of fish used devices the measurement of total volume sent through the speed assessment apparatus to estimate the velocity of the water in the apparatus and did little to ensure that each fish tested experience lamellar flow that is free of eddies and turbulence. Without the assurance of lamellar flow, each fish that enters the swimming assessment apparatus could potentially experience a current velocity that is not accurately reflected in the measurement of the volume output of said apparatus. Other measurements of maximum speed employed the use of a current meter to estimate the velocity of water fish are experiencing. However, without the assurance of lamellar flow, there is no way of knowing the current velocity measured is uniform throughout the testing apparatus.

To ensure lamellar flow within the viewing tube of the SEA, meticulous steps were taken in the design (section 1.13.1) and calibration of velocity (section 1.13.2) to ensure the uniformity of flow. Low coefficients of variation at each speed generated indicates the SEA operates with a high degree of consistency and repeatability (Table 2). This is likely the lowest coefficient of variation ever retrieved from a device designed to test the swimming ability of fish, giving us confidence in the results it has produced.

The variance present in calibration is partially due to the inevitable adherence of water to the surface of the pipe through which the water is flowing. The attraction of water to the inside surface of the pipe creates a layer of water next to the pipe surface which moves slower than the water in the center of the pipe. This phenomenon is referred to as the boundary layer and creates a parabolic velocity profile in the water flowing through the pipe. This simply means that as you move from the center of the pipe to near

the wall of the pipe, the velocity of water will approach 0 cm/s. The decrease in velocity close to the wall of the pipe is partially accounted for since the velocities measured for each flow and diverter setting during calibration are produced from the mean of pom-poms that were filmed moving through the SEA, many of which encountered the boundary layer for more than half the journey through the viewing tube. The effect of the boundary layer was not a significant concern, as the majority of fish swam in the boundary layer, experiencing the same decrease in velocity of water at every tested speed. The only potential consequence of the boundary layer is that the estimated maximum swimming speed of fish is slightly higher than what fish actually experienced during testing; meaning that the actual speed the fish achieved is slightly lower than the observed speed. This is potentially explained in (Figure 26) when comparing expected Sambilay maximum and actual maximum swimming speeds of habituated fish. While the slopes of the two regressions were not different from each other, the intercepts were marginally different [ $p(t_{a(2),32} \geq 1.992) = 0.055$ ], indicating that the observed maximum speeds are slightly overestimated. We would attribute this consistent overestimation of maximum speed to the uniform decrease in velocity of water caused by the boundary layer. However, because the intercepts were not significantly different, and the effect was consistent across all fish tested, no correction was made to observed maximum swimming speed.

Further improvements to design can still be made to increase the utility of the SEA. A variable speed pump would allow us to increase the velocity of water in a continuous fashion over time as opposed to the step-wise increase in velocity used in this experiment. Smooth, linear increase of velocity would allow us to more accurately assess

the actual maximum swimming speed of fish given that the testing protocol would not be limited by low resolution of water velocities that are able to be reliably generated. A variable speed pump would also reduce the amount of materials required to build the SEA and also simplify the operation protocol.

## **1.28 Recommendations for Future Research**

### **1.28.1 Re-assessment of the Double-crested Cormorant as a definitive host for *Centrocestus formosanus* and *Haplorchis pumilio***

To investigate the hypothesis that Double-crested Cormorants serve as a definitive host for *C. formosanus* and *H. pumilio*, Double-crested Cormorants could be shot and dissected to determine the prevalence, if any, of the two trematodes. This may be difficult and time consuming due to the relative difficulty of obtaining the appropriate permits to euthanize non-game, water dwelling birds. Alternatively, fresh fecal samples could be obtained from Double-crested Cormorant roosts around Landa Lake and examined for the presence of eggs of either *C. formosanus* or *H. pumilio*. This method presents its own set of challenges as there is no way to ensure that fecal samples obtained from roosts did indeed come from Double-crested Cormorants unless the birds were physically restrained, and fecal samples were taken directly from the bird.

### **1.28.2 Estimating infection success in the wild**

To better understand infection success rates or trematode cercaria present in Landa Lake and surrounding rivers, current our lab is currently conducting research into the relationship between cercarial levels measured in the river and metacercarial intensity acquired by concurrent caged-fish studies. The caged fish will be retrieved, euthanized and dissected to estimate acquisition rates, which can be used to infer the relative success

rates of cercariae present in the wild. These data will allow us to relate river cercariometry readings to actual fitness consequences.

### 1.28.3 Exploring the effects of long-term, low exposure rate of *Haplorchis pumilio* on *Cyprinella venusta*

To explore the effects of low cercarial exposure rate of *H. pumilio* on *C. venusta* over longer periods, we intend to conduct a similar experiment using cercarial exposure rates more consistent with cercariometric estimates of local rivers and lakes where the trematodes exist. Current research is being conducted to estimate the cercarial density in local rivers and lakes which will be paired with caged fish studies from the same areas. Testing effects of *H. pumilio* on *C. venusta* over longer periods of time will also be useful in assessing the ability of *C. venusta* to abort metacercariae embedded in fin insertions

## LITERATURE CITED

- Amaya-Huerta D, Almeyda-Artigas RJ (1994) Confirmation of *Centrocestus formosanus* (Nishigori, 1924) Price, 1932 (Trematoda: Heterophyidae) in Mexico. *Research and Reviews in Parasitology* 54:99-103
- Bainbridge R (1963) Caudal fin and body movement in the propulsion of some fish. *J Exp Biol* 40:23-56
- Bainbridge R (1962) Training, speed and stamina in trout. *J Exp Biol* 39:537-555
- Bio-West I (2014) Comprehensive final report for gill parasite (*Centrocestus formosanus*) activities in the Comal River. Bio-West, Inc., Round Rock, TX
- Blazer VS, Gratzek JB (1985) Cartilage proliferation in response to metacercarial infections of fish gills. *Journal of Comparative Pathology* 95:273-280. doi: DOI: 10.1016/0021-9975(85)90013-1
- Bolick AE (2007) The effects of spring flow on the abundance of heterophyid cercariae in the Comal River, New Braunfels, TX. MS thesis, Texas State University–San Marcos,
- Cantu NV, Brandt TM, Arsuffi TL (2013) An evaluation of three sampling methods to monitor a digenetic trematode *Centrocestus formosanus* in a spring-fed ecosystem. *Parasitology* 2013:1-7
- Casten LR (2006) Life history plasticity of the blacktail shiner (*Cyprinella venusta*) across disturbance gradients in Alabama streams. , Auburn University, Auburn, Alabama
- Daniel WM, Benson AJ, Neilson ME (2018) *Melanoides tuberculata* (Muller, 1774): U.S. Geological Survey, Nonindigenous Aquatic Species Database. USGS. <https://nas.er.usgs.gov/queries/FactSheet.aspx?SpeciesID=1037> 2018
- Díaz MT, Hernandez LE, Bashirullah AK (2008) Studies on the life cycle of *Haplorchis pumilio* (Looss, 1896) (Trematoda: Heterophyidae) in Venezuela. *Revista Científica* 18:35-42
- Díaz MT, Hernández LE, Bashirullah AK (2002) Experimental life cycle of *Philophthalmus gralli* (Trematoda: Philophthalmidae) in Venezuela. *International Journal of Tropical Biology and Conservation* 50:629-641
- Fleming BP, Huffman DG, Bonner TH, Brandt TM (2011) Metacercarial distribution of *Centrocestus formosanus* among fish hosts in the Guadalupe River drainage of Texas. *Journal of Aquatic Animal Health* 23:117-124
- Huston DC (2014) Invasive heterophyid trematodes and their native aquatic hosts in Texas. MS thesis, Texas State University, San Marcos, Texas

- Huston DC, Worsham MLD, Huffman DG, Ostrand KG (2014) Infection of fishes, including threatened and endangered species by the trematode parasite *Haplorchis pumilio* (Looss, 1896) (Trematoda: Heterophyidae). *BioInvasions Records* 3:189-194
- Johnson MS, Bolick AE, Alexander ML, Huffman DG, Oborny EL, Monroe A (2012) Fluctuations in densities of the invasive gill parasite *Centrocestus formosanus* (Trematoda: Heterophyidae) in the Comal River, Comal County, Texas, USA. *J Parasitol* 98:111-116
- Karatayev AY, Burlakova LE, Karatayev VA, Padilla DK (2009) Introduction, distribution, spread, and impacts of exotic freshwater gastropods in Texas. *Hydrobiologia* 619:181-194. doi: 10.1007/s10750-008-9639-y
- Kuhlman TA (2007) The predominant definitive avian hosts of the invasive Asian trematode, *Centrocestus formosanus*, in the headwaters of the Comal, San Antonio, and San Marcos rivers of Central Texas. MS thesis, Texas State University, San Marcos, TX
- Leavy TR, Bonner TH (2009) Relationships among swimming ability, current velocity association, and morphology for freshwater lotic fishes. *North American Journal of Fisheries Management* 29:72-83
- Lo CT, Lee KM (1996) Infectivity of the cercariae of *Centrocestus formosanus* and *Haplorchis pumilio* (Digenea: Heterophyidae) in *Cyprinus carpio*. *Zoological Studies* 35:305-309
- McDermott KS, Arsuffi TL, Brandt TM, Huston DC, Ostrand KG (2014) Distribution and occurrence of the exotic digenetic trematode (*Centrocestus formosanus*), its exotic snail intermediate host (*Melanooides tuberculatus*), and rates of infection of fish in springs systems in western Texas. *Southwest Nat* 59:212-220
- Mitchell AJ, Brandt TM (2005) Temperature tolerance of red-rim melania *Melanooides tuberculatus*, an exotic aquatic snail established in the United State. *T Am Fish Soc* 134:126-131
- Mitchell AJ, Goodwin AE, Salmon MJ, Brandt TM (2002) Experimental infection of an exotic heterophyid trematode, *Centrocestus formosanus*, in four aquaculture fishes. *North American Journal of Aquaculture* 64:55-59
- Mitchell AJ, Overstreet RM, Goodwin AE, Brandt TM (2005) Spread of an exotic fish-gill trematode: A far-reaching and complex problem. *Fisheries* 30:11-16
- Mitchell AJ, Salmon MJ, Huffman DG, Goodwin AE, Brandt TM (2000) Prevalence and pathogenicity of a heterophyid trematode infecting the gills of an endangered fish, the fountain darter, in two central Texas spring-fed rivers. *Journal of Aquatic Animal Health*:283-289
- Mohan CV, Shankar KM, Ramesh KS (1999) Mortalities of juvenile common carp, *Cyprinus carpio* associated with larval trematode infection - a case study. *Journal of Aquaculture in the Tropics* 14:137-142

Murray HD (1971) The introduction and spread of thiarids in the United States. *Biologist* 53:133-135

Murray HD (1964) *Tarebia granifera* and *Melanooides tuberculata* in Texas. Annual Report to the American Malacological Union 53:15-16

Murray HD, Woopschall LJ (1965) Ecology of *Melanooides tuberculata* (Müller) and *Tarebia granifera* (Lamarck) in South Texas. *B Am Malacol Union* 32:25-26

Nollen PM, Murray HD (1978) *Philophthalmus gralli*: Identification, growth characteristics, and treatment of an oriental eyefluke of birds introduced into the continental United States. *J Parasitol* 64:178-180

Ortega C, Fajardo R, Enríquez R (2009) Trematode *Centrocestus formosanus* infection and distribution in ornamental fishes in Mexico. *Journal of Aquatic Animal Health* 21:18-22

Pinto HA, Gonçalves NQ, López-Hernandez D, Pulido-Murillo EA, Melo AL (2018) The life cycle of a zoonotic parasite reassessed: Experimental infection of *Melanooides tuberculata* (Mollusca: Thiaridae) with *Centrocestus formosanus* (Trematoda: Heterophyidae). *PloS one* 13:e0194161-13

Pinto HA, de Melo AL (2011) A checklist of trematodes (Platyhelminthes) transmitted by *Melanooides tuberculata* (Mollusca: Thiaridae). *Zootaxa* 2799:15-28

Sambalay VC (1990) Interrelationships between swimming speed, caudal fin aspect ratio and body length of fishes. *Fishbyte* 8:16-20

Sommerville C (1982) The pathology of *Haplorchis pumilio* (Looss, 1896) infections in cultured tilapias. *Journal of Fish Diseases* 5:243-250

Tolley-Jordan LR, Owen JM (2008) Habitat influences snail community structure and trematode infection levels in a spring-fed river, Texas, USA. *Hydrobiologia* 600:29-40