TWO HIGHLY INVASIVE PARASITES NOW IN ECOLOGICALLY SENSITIVE TEXAS WATERS: CONSERVATION IMPLICATIONS FROM CAGED-FISH, DISTRIBUTIONAL, AND PHYSIOLOGICAL STUDIES

by

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DEDICATION

For my family, blood and not blood alike.

Dad – for always knowing I could, and for telling me "I told you so."
Mom – for your strength, grace, and patience with the black sheep.
Amber and Amanda – for inspiring me to live up to the two awesome women that came before me, and for being the goofballs you are.
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Disclaimer: The findings and conclusions in the thesis are those of the author and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

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I. INTRODUCTION

Early in the 20th Century, the exotic Asian snail *Melanoides tuberculata* (Gastropoda: Thiaridae) was introduced into the Western Hemisphere and brought along several invasive parasites that are now affecting avian, mammalian, and piscine hosts. This snail and its parasites have invaded an increasing number of sensitive aquatic ecosystems in the USA. Most of this study deals with *Haplorchis pumilio* (Looss 1899), one of two invasive heterophyid trematodes introduced into many North American springs by *Melanoides tuberculata*. However, since the parasite would not be a problem had the snail host not successfully invaded local surface waters, what appears proximally to be a parasite problem is, ultimately, a snail problem. Thus, an understanding of the invasive biology of the responsible snail, *Melanoides tuberculata* (Müller 1774), as well as the history of its introduction into North America, is apropos.

The invasive snail - *Melanoides tuberculata*

Melanoides tuberculata is a highly invasive species because of its resistance to desiccation, molluscicides, and disinfectants, and its ability to be easily spread from one location to another; once introduced, the snail out-competes almost every established and native gastropod species (Murray 1971). The snail is primarily found in freshwater, but can survive in salinity up to 30 ppt and has been reported to survive up to 8 d out of water (Mitchell et al. 2005). *M. tuberculata* primarily reproduces parthenogenetically and broods young internally; this strategy aids in the snail's ability to out-compete other species (Mitchell and Goodwin 2004), and makes it possible for a single introduced snail to potentially overwhelm an ecosystem within a few years. Because of its hardiness and

ability to establish dense populations in relatively little time, *M. tuberculata* has been introduced into novel habitats as a biological control agent for schistosomiasis in the Caribbean via competition with the schistosome's snail host *Biomphalaria* spp. (Pointier 1993).

Melanoides tuberculata is thought to have been introduced to the USA through the aquarium trade sometime prior to 1950 (Murray 1971). As of 2019, reports of *M. tuberculata* have been confirmed in Arizona, California, Colorado, Florida, Hawaii, Idaho, Louisiana, Montana, Nevada, Oregon, Puerto Rico, South Carolina, Utah, and Wyoming (Daniel et al. 2019). The first documented occurrence of *M. tuberculata* in Texas was when Murray (Murray 1964) reported the species from the effluent of the San Antonio Zoo, Bexar County. By 1971, *M. tuberculata* had spread to many other springs in Texas (Murray 1971); established populations have since been reported from spring-fed waters in over 15 counties in Texas (Karatayev et al. 2009).

The snail was first reported from the spring-fed Comal River (CR), Comal County, in 1965 (Murray and Woopschall 1965). We know of no reports of the invasive thiarids from the San Marcos River (SMR) prior to 1978, although D.G. Huffman anecdotally reported that *M. tuberculata* was abundant in the Upper SMR in the summer of 1973, with shoals in the river below Spring Lake Dam comprised almost exclusively of empty *M. tuberculata* shells. In 1978, Lindholm (1979) examined 354 *M. tuberculata* snails from the SMR for larval parasites and found no infections with any trematode species. The next documented record of *M. tuberculata* in the CR or SMR was in 1996 when a gill fluke transmitted by the snail was discovered to be causing lethal infections in

fountain darters (*Etheostoma fonticola*, Jordan & Gilbert 1886) in the CR (Mitchell et al. 2000).

Invasive parasites propagated by *Melanoides tuberculata*

Melanoides tuberculata serves as the first intermediate host for more trematode parasites than any other snail species, among these several trematodes that cause serious human diseases in the snail's native range (Pinto and de Melo 2011). At least three invasive trematodes are reported to be thriving in most *M. tuberculata* populations in Texas waters: *Philophthalmus gralli* (Mathis & Leger 1910), *Centrocestus formosanus* (Nishigori 1924), and *Haplorchis pumilio*.

Philophthalmus gralli is an eyefluke of birds that does not use fish as hosts, and so will not be discussed further in the study. *C. formosanus* and *H. pumilio* are heterophyid trematodes that exhibit broad host latitude across many Nearctic and Neotropical fish families and are thought to be spread by piscivorous birds. Several Texas fish species affected by these heterophyids are state- and federal-listed fishes of sensitive spring ecosystems, including the endangered fountain darter found only in the upper spring runs of the CR and SMR, as well as the threatened Devils River minnow (*Dionda diaboli*, Hubbs & Brown 1957) of West Texas spring systems (Huston et al. 2014).

Centrocestus formosanus

Centrocestus formosanus was originally described in Taiwan and is now distributed throughout Asia and warm-water areas of the world where *M. tuberculata* occurs. The first report we can find of *C. formosanus* in North America was in 1979, when metacercarial larvae of the worm were found encysted in the gills of black carp

imported into Mexico (López-Jiménez 1987, Amaya-Huerta and Almeyda-Artigas 1994). The parasite was confirmed in the United States in the early 1980's (Scholz and Salgado-Maldonado 2000, Johnson et al. 2012) and is now known to be widely distributed throughout most Texas populations of *M. tuberculata* (McDermott et al. 2014).

In 1990, larval stages of a "mystery fluke" were reported from *Melanoides tuberculata* in the San Antonio Zoo (Knott and Murray 1991). Although the description of the rediae and cercariae are vague and incomplete, several aspects of the report suggest that this may be the earliest report of *Centrocestus formosanus* in Texas, though this identification was not definitively confirmed by the author. Prevalence of these larvae as high as 80% was reported in snails from the Zoo (Knott and Murray 1991). The parasite was probably brought from Mexico to Texas by the migratory Green Heron, *Butorides virescens* (Linnaeus 1758), later determined to be serving as the main definitive host for *C. formosanus* in Central Texas (Kuhlman 2007).

In 1996, wild fountain darters (*Etheostoma fonticola*) routinely collected by USFWS for the refugium at the San Marcos Aquatic Resources Center (SMARC) displayed pathology suggesting potentially lethal infections of a gill parasite. D.G. Huffman examined the diseased fish and determined that the inflammation of the gills was caused by encysted metacercarial larvae of a trematode later identified (Mitchell et al. 2000) as *Centrocestus formosanus*. A retrospective study by SMARC of archived darters that had been previously collected for other purposes in 1994-95 revealed that *C. formosanus* probably arrived at the CR in 1993, since by late 1994 the prevalence among fountain darters in parts of the river had reached 100% (Mitchell et al. 2000). All wild-caught fountain darters from the CR from 1997-1998 collections (n=207) were infected

with *C. formosanus*, some with moderate to heavy infections (Mitchell et al. 2000). Comparatively, prevalence of *C. formosanus* in wild-caught fountain darters from the San Marcos River in 1997-1998 was only 3% (*n*=145). However, by 2004, 20% (*n*=5) of fountain darters from the SMR were positive for *C. formosanus* (Mitchell et al. 2005). Surveys (May 1997-May 1998) of *Melanoides tuberculata* in the CR and SMR revealed that many of the snails from the CR were shedding the infective cercarial larvae of *C. formosanus* (139 of 2,279 snails), whereas only 1 of 2,241 *M. tuberculata* examined from the SMR was shedding cercariae (Mitchell et al. 2000).

C. formosanus is the only fish parasite introduced by *M. tuberculata* that has been adequately studied in Texas, and almost all of that research on the parasite has focused on the population in the CR; thus, prevalence and intensity of *C. formosanus* in fountain darters in the SMR is understudied.

Known Geographic and Host Distribution of C. formosanus in Texas

All reports pertaining to the occurrence of *C. formosanus* in Texas through 2001 (Fleming et al. 2011) revealed that the geographic distribution of *C. formosanus* was restricted to the distribution of its snail host (*Melanoides tuberculata*). Thus, the geographic distribution of the parasite in Texas was limited to thermally stable spring runs and surface waters with winter thermal minima remaining above 17 C (Mitchell and Brandt 2005). However, in 2009, the Huffman lab discovered *M. tuberculata* thriving in surface-fed waters of the Guadalupe River several km upstream and downstream from its confluence with the Comal River. Live snails have since been discovered in the SMR as far downstream as Luling, some 50 river kilometers downstream from the headsprings, despite increasing fluctuations of water temperature and quality beyond the confluence

with the Blanco River (Groeger et al. 1997). These reports mark the first time *M. tuberculata* has been found thriving in North American waters having winter thermal minima below the reported critical thermal minimum of *M. tuberculata* (18 C) (Mitchell and Brandt 2005). This development suggests that wild fisheries in thermally ambient surface-fed waters may now be potentially subject to impact by invasive parasites transmitted by *M. tuberculata* (Mitchell and Goodwin 2004).

Caged-shiner studies conducted in the Guadalupe River in 2009 found 63% of 35 fish in six cages were infected with *C. formosanus* metacercariae (Huffman, unpubl. data). All cages had fish infected with *C. formosanu*s, including the farthest downstream cage 3.5 km downstream from the confluence with the CR. Wild-fish surveys of centrarchids, silurids, and cyprinids also detected *C. formosanus* in the Guadalupe River as far as 12 km downstream from the Comal confluence (Huffman, unpubl. data).

Life cycle

The main definitive host of *C. formosanus* in Texas is the Green Heron (*Butorides virescens*) (Kuhlman 2007). After the bird ingests an infected fish, adult worms colonize the colon; a few days later, eggs are released in the bird's feces. The first intermediate host, *Melanoides tuberculata,* is infected via direct consumption of *C. formosanus* eggs (Lo and Lee 1996). Once in the snail, the infective miracidium transforms into a sporocyst, asexually reproduces to form multiple rediae, and each redia produces multiple cercariae that are released by the snail (Mitchell and Goodwin 2004). Cercariae of *C. formosanus* will not penetrate fish skin (Lo and Lee 1996) and must be drawn into contact with gill epithelium by being passively drawn in with the respiratory current (Saunders et al. 2001). A single miracidium that has infected a snail has the potential to

shed hundreds of thousands of infective cercariae, with some snails shedding upwards of 1,500 per day (Mitchell and Goodwin 2004).

Pathology and epizootiology

The pathology of gill damage as a result of *Centrocestus formosanus* infection was first described in the 1980's (Blazer and Gratzek 1985). Inflammation in primary lamellae of the gills can lead to the cartilaginous encapsulation of metacercariae, which inhibits respiratory function through congestion and permanent deformation of gill architecture (Blazer and Gratzek 1985). The severity of a host's reaction to the presence of metacercarial cysts varies among fish species, with some fish barely reacting at all and thus experiencing little difficulty from the infection, and others (including fountain darters) reacting severely and experiencing permanent damage to gill architecture, especially in heavy infections Blazer and Gratzek (1985).

Gill damage from high intensities of *C. formosanus* metacercariae has been shown to cause mass mortalities in some fish under conditions of high-density fish culture (Mohan et al. 1999, Ortega et al. 2009, Leibowitz et al. 2019), apparently due to respiratory impairment. In fountain darters, inflammation in infected gill arches can lead to the cartilaginous encapsulation of cercariae, which permanently destroys gill architecture and inhibits respiratory function through the loss of respiratory surface area (Mitchell et al. 2000, Mitchell et al. 2002). Mitchell concluded that the presence of *C. formosanus* sometimes compromises the health of fountain darters, but that most mortalities occurred during transport of heavily infected fish, suggesting the combined effects of transport stress and infection contributed to the mortality and not solely infection by *C. formosanus*. These findings suggest that minimal stressors, potentially

including concurrent infection by other parasites, could result in the death of already heavily infected fish (Mitchell et al. 2000, Huston et al. 2014).

Haplorchis pumilio

Haplorchis pumilio is another heterophyid trematode transmitted by *M. tuberculata* (and, to a lesser extent, the related and co-invasive thiarid *Tarebia granifera*). This parasite 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. The life history of *H. pumilio* is similar to that of *C. formosanus* in that it also has specific requirements for thiarid snails for first intermediate host, but a broad host-latitude at the level of the fish second-intermediate host and the homeothermic piscivore definitive host.

Introduction to North America

The first official report we could find of *Haplorchis pumilio* in North America is that of Scholz and Salgado-Maldonado (2000), who reported intramolluscan stages in *Melanoides tuberculata* in Mexico but did not clearly specify when the infected snails were collected. The next year, metacercariae were reported for the first time from North American fish at a nearby locality in Mexico (Scholz et al. 2001). The first reported occurrence of *H. pumilio* in Texas was in 1999 when McDermott et al. (2014) found intramolluscan larvae in *Melanoides tuberculata* collected from San Felipe Creek, San Solomon Springs, and Phantom Lake Springs in western Texas. Melissa Salmon (pers. comm.) also indicated that she had seen *H. pumilio* cercariae in *M. tuberculata* collected

from the Comal River during her research on *Centrocestus formosanus* in the late 1990's (Salmon 2000).

The first reported occurrence of *H. pumilio* in fountain darter habitat was in 2001 when Tolley-Jordan and Owen (2008) noticed *H. pumilio* larvae in snails from the CR. In 2002, Cantu (2003) was studying cercarial concentrations of *C. formosanus* in the CR when he noted that *H. pumilio* cercariae were also appearing on cercariometry filters. The prevalence of *H. pumilio* in *M. tuberculata* and the density of *H. pumilio* cercariae in the CR were only sporadically monitored for the subsequent decade. To date, no extensive study focusing on the distribution or prevalence of *H. pumilio* in fishes of Texas has been reported, despite evidence of detrimental effects on fish hosts (Sommerville 1982b, Huston et al. 2014, Clements 2018). At the present time, parasitological surveys of wild fishes of Texas are likely to underestimate the intensity and prevalence of *H. pumilio* due to the unusual location of metacercarial encystment in fin insertions and splanchnocranial cartilage, as opposed to gills and organ tissues commonly examined in parasitological studies.

In 2013, Huston et al. (2014) collected and examined 10 fountain darters from both the CR and SMR; all five darters collected from each river were found to be infected with *H. pumilio*. Recent findings from the Huffman lab suggest that, while *C. formosanus* potentially poses a threat to the survival of spring-associated fishes in the wild, *H. pumilio* is also a cause for concern for the health of fountain darters and is potentially an even greater problem for more pelagic fishes such as the Devils River minnow (*Dionda diaboli*).

Life cycle

Like its *Centrocestus* cousin, *Haplorchis pumilio* also displays broad host latitude at the second intermediate and definitive host levels (Lo and Lee 1996), with several avian piscivores possibly functioning as the main definitive hosts in the Central Texas region (Kuhlman 2007, Clements 2018). While there are some indications that the Green Heron also serves as a host for *H. pumilio* (Kuhlman 2007), bird sightings reported by Clements (2018) in a habitat where snails are heavily infected suggest that the Neotropic Cormorant may also function as a definitive host in the area.

Melanoides tuberculata (and to a lesser extent *Tarebia granifera*) serves as the first intermediate host of *H. pumilio*. Once inside the snail host, the sporocysts mature into rediae, which typically contain 9-14 cercariae (Abd el-Kader Saad and Abed 1995). Infective cercariae emerge from the snail host and penetrate the skin of a second-intermediate fish host, migrating through body tissues in search of active joints and fin inserts throughout the body, with most cercariae migrating to the caudal peduncle and a high percentage encysting in the loose connective tissues of the head (Sommerville 1982a, Lo and Lee 1996).

Pathology and epizootiology

During experimental exposures to high densities of cercariae, fish exhibited melanophore responses at sites of entry and engaged in flashing and other avoidance behaviors; acute trauma resulting from migrating cercariae resulted in organ failure, swelling and blistering of encystment sites (Figure 1), and death (Sommerville 1982a, Huston et al. 2014, Clements 2018).

Figure 1. Blisters appeared (within 24 h) on the caudal peduncle of a *C. venusta* that was placed into an aquarium with snails shedding *H. pumilio*. Blisters ruptured 48 h after exposure, resulting in the death of the fish. Photo credit D. G. Huffman.

Once the cercariae reach the fin insertions or joint cartilage of the fish host, they form a metacercarial cyst composed of a thin protein wall secreted by the parasite, which is in turn surrounded by a fibrous capsule made of host reaction tissue (Sommerville 1982b). Presence of higher numbers of cysts in shiners is correlated with decreasing swimming performance and endurance (Clements 2018), suggesting an impaired ability to pursue prey and a higher likelihood of capture by a piscivore (Herting and Witt 1967).

Historic Prevalence of Heterophyids and Cercariometric Estimates

Prevalence of *C. formosanus* infection in *M. tuberculata* from the CR during 1997-1998 was 6.1% (139 of 2,279 snails) (Mitchell et al. 2000); prevalence rose minimally to 6.3% in snails collected in 2000 (Tolley-Jordan and Owen 2008). The first documentation of *H. pumilio* prevalence in *M. tuberculata* from the CR occurred in 2008,

when Tolley-Jordan and Owen (2008) reported *H. pumilio* cercariae in 0.66% (4 of 610 snails). However, EAAHCP (2014) reported prevalence of 19.2% of "other parasites" from *M. tuberculata* in the CR – perhaps a reference to *H. pumilio*. Prevalence of *H. pumilio* in snails from the CR was reported as 14% in 2014 (Harding 2016), 50% in 2015 (referred to as "other parasites"), and > 24% (n = 1,385) in 2017-18 (Clements 2018). As of this time, prevalence of *C. formosanus* in snails collected from Pecan Island is ~10%, and prevalence of *H. pumilio* in snails from the same location is > 25% (Clements 2018). Cercariometric estimates in the CR conducted by Bio-West reported estimates of 5.1 *C. formosanus* cercariae/L, and 0.6 *H. pumilio* cercariae/L (Bio-West 2014). The latter report for *H. pumilio* may be an underestimation, since Clements (2018) later determined that the protocol Bio-West was using caused many cercariae to stick to plastic containers; thus, density estimates determined through the number of cercariae caught on filters would underestimate the density of cercariae in a given volume.

Affected Spring and River Systems

There are 11 broadly defined aquatic habitat types in Texas, with springs and streams being the most common (Saunders et al. 2001). The Edwards Plateau is a region of 9.7 million hectares in central and western Texas that is home to multiple endemic biota sensitive to predation, competition, hybridization, and habitat modification through anthropogenic activities, including the introduction of exotic species (Bowles and Arsuffi 1993). The Edwards Aquifer supports about half of all Texas springs, as well as many agricultural, fishery, and tourism industries (Gibson et al. 2008). The Comal Springs and San Marcos Springs are the two largest spring systems in Texas, and both are

characterized by their superior water quality (Fahlquist 1997) and rich fish communities (Kollaus et al. 2014).

San Marcos Springs and Upper San Marcos River

San Marcos Springs arises from the Edwards Aquifer on property now owned by Texas State University. Around 200 springs issue from openings in the limestone at the bottom of Spring Lake (Guyton 1979, Groeger et al. 1997), the impounded headsprings of the Upper San Marcos River in Hays County, Texas (Brune 1981). Spring Lake Dam, which impounds the headsprings, was constructed in 1849 to power mills and gins (Saunders et al. 2001). The outflow from Spring Lake gives rise to the Upper SMR which flows through the Texas State University campus and the city of San Marcos, within two blocks of the town square.

The land surrounding the headspring reach of the SMR has been occupied by multiple indigenous cultures since 12,000 BCE, and even in modernity the springs are considered a sacred place by several tribes (Brune 1981, Shiner 1983). The SMR is a vital source of economic stability to the San Marcos region, with river-based tourism generating upwards of $30 million in 1991 (Saunders et al. 2001). Spring Lake was privately owned by a tourist attraction and resort known as Aquarena Springs from 1950 until 1996, when it was purchased by Texas State University. Glass-bottom boats, left over from the Aquarena days, are still in operation over the lake, but now serve as an ecotourism attraction.

The San Marcos Springs (SMS) has the second highest spring discharge in Texas and exhibits the most constant discharge of any spring system in the southwestern United States, having flowed continuously through recorded history (Guyton 1979, Brune 1981),

even during the extreme droughts of the Mid-Holocene (Worsham et al. 2016). The SMS flows at a discharge between 125-300 CFS, with sufficient velocity to maintain relatively constant conditions for spring-dependent species. From 1956-2005, mean discharge from the SMS was 4,100 liters/second (Gibson et al. 2008). The SMS maintains an average year-round temperature of 22 C, with temperature variability increasing with distance from the springhead (Guyton 1979, Saunders et al. 2001). Beyond the confluence with the Blanco River, especially, variability in temperature and water chemistry increases as one moves from upstream to downstream, but still under spring influence compared to water temperatures in the Blanco River (Groeger et al. 1997).

The SMR is home to many environmentally sensitive and specialized endemics that occur in the spring-influenced reaches (Groeger et al. 1997, Saunders et al. 2001). According to Texas Parks and Wildlife, the main threats to the stability of the San Marcos River include reduction and cessation of flow, reduction in water quality, habitat modification, recreational impacts, and the presence of non-native species (Saunders et al. 2001). Municipal activities have resulted in channelization and the loss of some riffle and run habitats in the Upper SMR. An increase in backwater and pool habitats have reduced current velocity, increased depth, and resulted in accumulation of silt (Saunders et al. 2001). The Upper SMR continues to flow for approximately 8 km to where it receives water from the Blanco River and becomes the Lower SMR, until it joins the Guadalupe River downstream from Gonzalez, TX. Most of the SMR focus of the present study pertains to the portion of the Upper SMR between the headsprings and the IH-35 crossing, but some sampling was performed downstream from that reach to determine the downstream extent of heterophyid infections in fishes.

Comal River

Comal Springs (CS) also arises from the Edwards Aquifer in the city of New Braunfels, Comal County, TX, 28 km SW of the San Marcos Springs. CS has the highest mean discharge of any spring system in Texas (Guyton 1979, Brune 1981, Johnson et al. 2012); from 1927-2005, the mean discharge from the CS was 8,200 liters/second (Gibson et al. 2008). The headsprings were impounded by two dams in the late 1880's to form Landa Lake (Linam et al. 1993). Spring water flowing through Landa Lake is split into the New Channel and the Old Channel; these channels subsequently re-converge to form the Main Channel, which continues for ~2.5 km to the confluence of the Comal with the Guadalupe River. Flow in the Old Channel is regulated by a series of culverts, making it one of the most stable areas of the CR. Water temperatures remain around 25 C year-round, and all other physiochemical properties remain relatively constant as well (Linam et al. 1993).

Prolonged periods of low flow in the CR have been documented, though they are rare (Guyton 1979). The first recorded incidence of the CS going dry was for a period of nearly 5 months in the summer and fall of 1956; this period marked the lowest recorded flow from the SMS as well, though neither spring system completely ceased flowing (Guyton 1979). While Comal Springs were reported to cease flowing in 1956, the Comal River itself did not run dry; the primary flow during this period was from precipitation runoff, seepage, small springs, and water that was released from a local power plant (USGS 1958).

Guadalupe River

The Guadalupe River (GR), which flows from Kerr County to its mouth in San Antonio Bay, is a relatively typical Texas river system. The upper portion is formed by two main tributaries in Kerr County, the North Fork and South Fork, which converge and flow another 370 km before entering Canyon Lake. The lower portion of the GR below Canyon Lake, which is heavily used for recreation within the city of New Braunfels, joins the Comal River and flows to the south and east before it receives the water of the SMR ~3 km west of Gonzales, in Gonzales County.

The area bordering the GR has supported various indigenous tribes for several centuries, and was eventually colonized by Mexican and European settlers in the 1720's. In 1920, the Guadalupe Waterpower Company began building a series of hydroelectric dams between the cities of Seguin and New Braunfels, but the effort was largely unsuccessful due to the GR's tendency to flood. The Guadalupe-Blanco River Authority (GBRA) was established in 1933 as an entity to supervise the control and distribution of water within the Guadalupe and Blanco Rivers. Construction of Canyon Dam was completed in 1964, which provides effective flood control of downstream areas and controls flow in the lower portion of the GR. Both the upper and lower portions of the GR provide a number of utilities such as power, drinking water, and economically viable recreational opportunities to surrounding communities (Smyrl 2010).

Devils River

The Devils River (DR) watershed, in the deserts of Southwest Texas, originates in Sutton and Crockett Counties and flows intermittently southward into Val Verde County, where surface waters occur on a consistent basis (Brune 1981). The system is one of the

few remaining pristine spring systems in West Texas, though the effects of water usage, habitat degradation, and the construction of Amistad Reservoir in 1968 have resulted in substantial loss of aquatic habitats (Valdes-Cantu and Winemiller 1997, USFWS 2005). Several endemic species of fish occur in the DR, including the federally endangered Devils River minnow (*Dionda diaboli*, Hubbs & Brown 1957) and the state-threatened Conchos pupfish (*Cyprinodon eximius*, Girard 1859), Proserpine shiner (*Cyprinella proserpina*, Girard 1856), and Rio Grande darter (*Etheostoma grahami*, Girard 1859) (Valdes-Cantu and Winemiller 1997).

Affected Fishes

Most literature regarding the effects of parasitism on fish health is based on concerns for species of economic value, such as fishes produced in hatchery conditions or on ornamental fish farms, due to the potential impact of parasites on the quality of product and, therefore, the potential for economic loss (Sommerville 1982a, Yanohara and Kagei 1983, Blazer and Gratzek 1985, Tung et al. 1989, Arizmendi 1992, Mohan et al. 1999, Ortega et al. 2009, Cortés et al. 2010, Sumuduni et al. 2017, Leibowitz et al. 2019). However, the potential impacts to sensitive endemic species presents a separate issue that draws into question the stability of the entire system, and in extreme cases, the potential for local extirpation of the species.

Etheostoma fonticola (Perciformes: Percidae) is endemic to spring-influenced reaches of the SMR and CR. The population in the CR was supposedly extirpated by droughts in the 1950's, though debate still persists over the degree of extirpation; regardless, the CR was restocked in the 1970's with 457 fountain darters from the SMR

(Schenck 1975, Hubbs et al. 2008). SMARC maintains a refugium for the species and cultivates lab-reared populations of fountain darters, in the event of another population-level threat such as the 1950's drought.

Fountain darters associate with aquatic vegetation for feeding, reproduction, and refuge (Alexander and Phillips 2012), however they do not occur exclusively in areas containing vegetation and have been reported to breed in areas without vegetation (Nichols 2015). Spawning period for fountain darters lasts approximately 11 months out of the year, with peak productivity January through April (Nichols 2015). The main threats to natural populations of fountain darters are reduction in spring flow due to drought or excessive withdrawal from the Edwards Aquifer and the effects of non-native species, including invasive parasites transmitted by non-natives (McDonald et al. 2006). The species was classified as endangered in 1970 due to: (1) reduction, modification, or threatened habitat or range; (2) disease or parasitism; (3) anthropogenic factors such as hybridization, competition, or predation; and (4) a highly restricted range (Russell 1970, Jelks et al. 2008).

Dionda diaboli (Cypriniformes: Cyprinidae) is a small cyprinid originally described by Hubbs and Brown (1956). Adults are 25-53 mm in size, with a triangular caudal spot and melanophores lining the scale margins, which creates a distinctive cross-hatched diamond appearance above the lateral line (Hubbs and Brown 1956). In areas shared with its congener *Dionda argentosa* (Girard, 1856) and where both spring and stream habitats are available, the species tend to partition habitat types, with *D. diaboli* associating with stream and pool habitats and *D. argentosa* associating with spring habitats (Kollaus and Bonner 2012). Thus, *D. diaboli* is not solely reliant on stenothermal

spring systems and is therefore not strictly a “spring-dependent” species (Kollaus and Bonner 2012, Schlechte and Fleming 2015, Robertson et al. 2016).

Though no geographic distribution data was collected before 1951, the species is thought to be currently restricted to a small portion of its historic range, with remaining populations restricted to small unconnected stream reaches (Garrett et al. 2004, USFWS 2005, Schlechte and Fleming 2015). Recent distribution assessments in the US and Mexico have reported fewer individuals and reduced range compared to previous studies (Garrett et al. 1992, Contreras-Balderas et al. 2003, USFWS 2005, Schlechte and Fleming 2015). Primary threats to *D. diaboli* include loss of stream flow due to dam construction and pumping of groundwater; habitat degradation due to pollution; and impacts of non-native species (USFWS 2005), including *Melanoides tuberculata* and its associated parasites (Schlechte and Fleming 2015). The species was initially proposed as federally endangered by USFWS in 1978 due to concerns over the species’ limited habitat, and was officially listed as threatened in 1999 (Garrett et al. 2004). Since its listing, SMARC has established a refugium for the species.

Other vulnerable fishes that share habitat with the Devils River minnow include the Proserpine shiner (*Cyprinella proserpina* Girard 1856), the Conchos pupfish (*Cyprinodon eximius* Girard 1860), and the Rio Grande darter (*Etheostoma grahami*), all of which are listed as vulnerable or endangered in some capacity on a state or federal level (Valdes-Cantu and Winemiller 1997). Of these three species, only individuals of *E. grahami* were collected for this study.

General physiology of the stress response

In a biological context, stress can be broadly defined as any response reaction to a stimulus (or stressor) that alters the homeostatic balance of an individual (Barton and Iwama 1991). These responses are commonly classified into categories of primary, secondary, and tertiary responses according to the organismal level of the response (Barton and Iwama 1991). Primary stress responses occur immediately following a stimulus and are defined by the release of hormones such as catecholamines (e.g., epinephrine) and corticosteroids (e.g., cortisol). Secondary stress responses occur following the hormonal cascade during the primary stage of stress. Secondary effects can be metabolic (e.g., changes in blood glucose), hematological (e.g., changes in hematocrit or leukocyte ratios), hydromineral (e.g., plasma protein), or structural (e.g., gonadosomatic indices) (Barton and Iwama 1991). Tertiary effects, or "whole-animal" effects, involve a change in complete metabolism which can manifest as a reduction in growth, disease resistance, swimming performance, or reproduction. Beyond these three organizational levels of response, stress may also manifest on a population-level scale, when growth and reproduction are compromised due to stress, and that reduction in fitness is carried down through subsequent generations (Wedemeyer et al. 1990, Barton and Iwama 1991, Romero 2004, Britton et al. 2011, Pankhurst 2011).

The stress response on all levels has evolved to maintain homeostatic conditions within a host (Barton 2002). However, when the magnitude of a stressor exceeds the host's ability to maintain consistent physiological parameters, the host may be predisposed to lower overall fitness and premature death (Barton and Iwama 1991). Selye (1950) offered a generalized framework of an organism's response to stress, known as the

GAS, or General Adaptation Syndrome. In this model, the first stage of the stress response consists of an alarm reaction, or a rapid physiological response to a stimulus. The second stage, resistance, involves compensatory mechanisms by the host that seek to regain the homeostatic balance that was disrupted by the alarm reaction. During the resistance stage, the physiological condition may appear to have returned to normal, pre-stress conditions, or may have reached a new homeostatic balance (known as allostasis). However, the compensatory abilities of the affected individual are finite, and the added effort of maintaining homeostasis under non-ideal conditions will eventually lead to the final stage of stress, or exhaustion. Physiological exhaustion can often result in lower overall fitness, up to and including premature death (Barton and Iwama 1991).

Inflammation

Inflammation is the body's main generalized response to injury, and is intended to isolate and destroy the injurious agent and facilitate healing (Mumford et al. 2007). Inflammation can be acute or chronic in form, which differ not only in duration but also in physical processes and observable pathology.

Acute inflammation is characterized by involvement of the vascular system, tissue-based inflammatory cells, and chemical mediators; the process generally progresses over a period of 3-10 days, then resolves quickly once the invading agent is removed. A diagnostic feature of acute inflammation is exudation, or the release of fluid from the vasculature into the injured tissue. During the primary stage of stress, chemical signals trigger vasodilation and increased permeability of capillaries and post-capillary venules. As a result, protein-rich fluid containing a mixture of antibodies and other defense chemicals floods the site of injury. The composition of this edema fluid varies

with the nature, severity, and duration of the injury (Mumford et al. 2007). If the injurious agent is not removed, the number of leukocytes in the immediate area will usually increase as the inflammation progresses from acute to chronic stage. If the injurious agent is removed, macrophages destroy any necrotic tissue or remaining exudate and prepare the area for healing.

Chronic inflammation is characterized by cellular proliferation, progresses over the span of weeks to months or even years, and continues as long as the injurious agent is present. The morphology of acute and chronic lesions differs substantially (Mumford et al. 2007). Macrophages, lymphocytes, and plasma cells are dominant, and fibrosis, or the formation of scar tissue, frequently occurs. Chronic inflammatory lesions are proliferative, not exudative like acute inflammatory lesions; there is no fluid build-up at the injury site, but rather an accumulation of cells that form a lesion composed of macrophages and other leukocytes. Similar to acute inflammation, chronic inflammation will usually subside and healing will commence once the injurious agent is neutralized. However, the form of healing that can occur is limited by the type of tissue which was originally affected, and the severity of the initial injury itself (Mumford et al. 2007).

Physiological processes affected by parasitism

Tertiary effects of parasitism that have been documented in previous work include reduced growth (Lamkova et al. 2007), compromised swimming performance (Clements 2018), aberrant behavior (Sommerville 1982b, Clements 2018), impaired immune function (Barton and Iwama 1991, Lamkova et al. 2007), and increased host susceptibility to death by predation or disease (Brassard et al. 1982, Britton et al. 2011, Slavik et al. 2017).

The ability to invest enough energy to ensure healthy growth and maturation is a critical component of the fitness of an individual fish (Barton 2002). Many factors play a role in how a fish develops, including genetic and environmental factors; however, the stress of disease can detrimentally affect the process of growth (Barton 2002). In many instances, the presence of a bacterial or parasitic infection may not directly contribute to reduced growth rates but rather contribute indirectly, through decreased foraging and therefore food intake (Wu et al. 2017); alternatively, parasitized fish may increase their foraging time, as a compensatory mechanism for increased energetic demands from being infected (Slavik et al. 2017). Additionally, a fish under stressful conditions, especially disease or parasitism, will often shift energy reserves toward reproduction to ensure the maximum fitness of offspring, and away from the process of growth (Britton et al. 2011). The re-allocation of metabolic energy results in a re-distribution of resources normally reserved for the optimal function of specific systems, which may result in compromised function of those processes forced to operate with less than ideal energy reserves. In the case of cyprinids parasitized by mussel glochidia on the gills, parasitized fish expended significantly more energy on movement than uninfected fish (Slavik et al. 2017). Gill parasitism of marine copepod microparasites results in increased ventilation rates, reduced feeding, reduced growth by 66%, reduced gonad mass by 68% compared to controls, and increased mortality of infected fish by 1.8 X (Finley and Forrester 2003).

Changes in properties of the blood are valuable methods of assessing fish health (Blaxhall 1972, McLeay and Gordon 1977, Campbell and Murru 1990, Bracewell et al. 2004, Ivanc et al. 2005). Qualitative and quantitative aberrations in hematological parameters – including whole blood glucose, lactate, serum protein, hematocrit,

hemoglobin concentration, and leukocyte ratios – are commonly considered the most significant indicators of fish wellbeing (Blaxhall 1972, Silbergeld 1974, Hattingh 1975, Wedemeyer et al. 1990, Barton and Iwama 1991, Wells and Pankhurst 1999, Martins et al. 2004, Movahed et al. 2016).

In fisheries research, glucose levels in the blood are a commonly used indicator of physiological stress in fishes (Silbergeld 1974, Hattingh 1975). Elevated blood glucose is a result of increased catecholamine levels in the blood (Pottinger 1998) during the primary stage of stress, which triggers the production of glucose and provides metabolic fuel for "fight or flight" responses immediately following a stimulus (Wedemeyer et al. 1990). The glucose content of whole blood is a critical measure of health as the increase of glucose production diverts metabolic energy away from growth and reproductive processes (Wells and Pankhurst 1999). Blood glucose levels are not as precise of a quantification of stress than measuring a corticosteroid such as cortisol, since fluctuations in glucose can also vary with age, nutritional state, and season (Wedemeyer et al. 1990). Changes in glucose can, however, reliably aid in determining the severity of a stress response, and allow for the estimation of the individual's ability and time required to recover from the stressor (Pottinger 1998).

Handheld meters designed to monitor levels of glucose in human blood are commonly used in fish aquaculture and field studies, and provide results comparable to traditional colorimetric glucose assays utilized in the lab (Iwama et al. 1995, Wells and Pankhurst 1999, Eames et al. 2010, Bartoňková et al. 2016). In fact, the use of portable handheld glucometers presents advantages over traditional laboratory methods, especially regarding the minimum volume of blood required to achieve an accurate reading; while

colorimetric assays generally require a minimum of 10-20 µL of blood, handheld glucometers provide results with as little as 1-2 µL of blood. Glucometers also have the advantage of speed, since readings are generally produced in fewer than 10 s; this characteristic is advantageous when measuring multiple individuals in the shortest possible amount of time to ensure all collection events are performed as close to the same time as possible (Eames et al. 2010).

Wells (1999) compared the results of similar handheld tools to traditional laboratory procedures when measuring blood glucose, blood lactate, and plasma protein. Both the glucose and lactate monitors correlated well with laboratory methods, though the meters did report lower absolute values. This result could be due to the measurable ranges of the meters, and as such it is critical to thoroughly determine the measurable range of a glucose meter as well as the levels of glucose commonly found in the blood of the study species (Wells and Pankhurst 1999). An additional caveat to consider when measuring glucose content in fish blood is the effect of anesthetic agents. Tricaine methanesulphonate (MS-222) is a commonly used method of euthanizing fish in the lab; however, exposing fish to MS-222 results in highly variable glucose levels compared to fish anesthetized with ice-cold water (Eames et al. 2010).

Hematocrit, or packed cell volume (PCV) – the volume of erythrocytes that makes up the total volume of blood, expressed as a percentage – is a measurement commonly used in the study of parasitic infections in fish (Filipsson et al. 2017). A reduction in hematocrit reflects a lower number of red blood cells that make up the whole blood, and therefore a decrease in the oxygen-carrying capability of hemoglobin. This

reduction in oxygen-carrying ability, or anemia, is a common secondary stress effect in parasitized fish (Fox 1965, Martins et al. 2004, Panjvini et al. 2016).

Goals and Objectives of Study

Goals of this project were to estimate the time of arrival of *Haplorchis pumilio* within the Comal and San Marcos rivers; to determine the current (2018) status of *Centrocestus formosanus* and *Haplorchis pumilio* in selected Texas waters; and to estimate the impact that these parasites (especially *H. pumilio*) might be having on fish in Texas, with special emphasis on sensitive spring fishes.

Goal 1 – Determine the first occurrence of Haplorchis pumilio in fountain darters, and trends in prevalence since its introduction

Objective 1.1 – Examine archived fish specimens for H. pumilio infection

H. pumilio infections were first reported in fishes of Central Texas in 2013 (Huston et al. 2014); however, research on *C. formosanus* in the gills of fountain darters in the CR has been ongoing since 1996 (Mitchell et al. 2000). Thus, examining archived specimens preserved between 1996–2013 for signs of *H. pumilio* infection provides a timeline of the parasite's first appearance in fountain darters of the CR.

Objective 1.2 – Review published reports of H. pumilio studies in the San Marcos River and Comal River

A thorough overview of the available historical data regarding prevalence and intensity of *H. pumilio* is necessary to understand the history of the introduction and current dynamics of the species in the CR and the SMR.

Goal 2 – Determine the current infection status of both parasites in wild fishes at selected stream reaches

Infection status encompasses multiple aspects of a parasitological study – geographic distribution, prevalence estimates, and intensity estimates. To quantify the total effects that the parasites, especially *H. pumilio*, might be having on local fish, it is necessary to document each of these aspects within an area. We collected fish (cyprinids, when available) from several river systems in Texas that are home to fishes which are vulnerable in some capacity.

Objective 2.1 – Determine the geographic distribution of the parasites in wild-caught fish collected along the San Marcos River

We can reliably estimate the most downstream reach at which new infections are acquired by fish based on: (1) parasitism levels observed in wild-caught fish collected at multiple sites downstream until the heterophyids are no longer detected within fish, and (2) the presence or absence of live *M. tuberculata* in the immediate vicinity.

Objective 2.2 – Determine infection levels in naturally-occurring populations of fountain darters

Previous research has determined high densities of *Melanoides tuberculata* and both heterophyids in sections of Landa Lake at the headwaters of the CR (Clements 2018). Since infections may impact younger fish in different ways than adult fish, including compromised growth and reproductive investment (Wells and Pankhurst 1999, Lamkova et al. 2007), a complete picture of the infection dynamics in known breeding areas of fountain darters is required to understand and predict any potential effects of parasitism on both pre-reproductive and reproductive fountain darters.

Objective 2.3 – Determine prevalence and intensity estimates by collecting fish and snails from several sites along the Devils River

The Devils River is a sensitive spring system that remains relatively pristine (Harrell 1978) and is home to multiple vulnerable species (Hubbs and Garrett 1990, Valdes-Cantu and Winemiller 1997), making data on the status of heterophyids in this system a necessity for proper management of resident endemic species. Infections in native fishes such as the Devils River minnow (*Dionda diaboli*) were confirmed by Huston et al. (2014) from archived specimens collected from the DR in 2011. Little to no information has been reported on the prevalence and intensities of heterophyids (especially *H. pumilio*) affecting fish at different locations in the DR such as Blue Spring and nearby Dolan Creek.

Goal 3 – Estimate the impact that these parasites (especially H. pumilio) might be having on fish in Texas

While determining the geographic range, prevalence, and intensities of *C. formosanus* and *H. pumilio* infections in fish provides helpful baseline data, the true impact the parasites may be having on local fish within a system remains unexplored. One method of quantifying the effects of the parasites in a river system is to determine the rate at which susceptible fish are becoming infected by the parasites. Secondly, monitoring the health of infected individuals to determine any effects that may compromise reproduction or fitness is necessary to understand potential impacts that may or may not manifest on a population level (Britton 2013).

Objective 3.1 – Determine the rate of acquisition of both heterophyids in caged-fish stationed within reaches where Melanoides tuberculata occurs

Findings from previous research have revealed a discrepancy between rates of parasitism estimated through examination of wild-caught fish and rates of parasitism estimated from caged-fish studies (Cantu 2003, Fleming et al. 2011). Wild-caught fish collections do not reflect mortalities that have occurred as a direct or indirect result of parasitic infection; however, when uninfected fish are placed in stationary cages in areas where *M. tuberculata* occurs, the rates at which these fish acquire parasites can be used to estimate levels of parasite acquisition occurring in local fish (Fleming et al. 2011). This objective will be achieved by two experiments: one with caged *Cyprinella venusta* and the second with caged fountain darters.

Objective 3.3 – Determine the acute physiological effects of Haplorchis pumilio infection on Cyprinella venusta

While some research on the pathological effects of infection by *H. pumilio* has been performed (Sommerville 1982b), there are no studies currently published that address alterations in physiology as a result of stress imposed on fish exposed to *H. pumilio* cercariae. Clements (2018) and Sommerville (1982b) both observed changes in behavior (e.g., avoidance, flashing, swimming in bubble streams) when fish were exposed to high doses of cercariae, suggesting the imposition of at least enough stress to signal a fight-or-flight alarm reaction (Selye 1950). By measuring commonly used indicators of stress, some acute physiological effects of heavy *H. pumilio* infection are herein reported for the first time.

II. METHODS

Determining when *Haplorchis pumilio* was introduced into the Comal River through examination of archived fountain darters

Preserved specimens of fountain darters used in previous studies (Salmon 2000, Cantu 2003) were donated to Texas State University by the San Marcos Aquatic Resource Center (SMARC/TPWD Scientific Donation Permit SPR-0616-153) for the purposes of acquiring historical data on *Haplorchis pumilio* infections in fountain darters of the CR. In total, 56 archived fountain darters from various prior studies were dissected for definitive signs of *H. pumilio* infection. Since the earliest reports of *H. pumilio* in North American populations of *Melanoides tuberculata* was approximately 1999 (Scholz et al. 2001, McDermott et al. 2014), we chose 2002 as the starting year for the search.

Well-preserved specimens were separated into groups by site and date. Three individuals from each of these groups were then randomly selected for necropsy. Fish selected from the above groups were examined for *H. pumilio* cysts in the caudal peduncle, beginning with fish that had been placed in cages in August 2002 and working backwards from that date. Each time an infection was confirmed among any of the three fish in a group, three fish were selected from the next earlier date and examined. This process was continued until none of the three fish in a group were found to be infected.

Individual fountain darters were selected from archived specimens from Cantu's 2002 study of *C. formosanus* acquisition in caged fish in the CR (Cantu 2003). Cantu was not aware of *Haplorchis pumilio* infections in fountain darters, but based on our observations, co-infection by *C. formosanus* and *H. pumilio* is common. Therefore, fish

from four of Cantu's eight sites were selected for necropsy (*n*=36), since fish from these sites had the highest documented mean intensities of *C. formosanus*.

Twelve fountain darters collected from four sites in the CR (Old Channel, New Channel, Landa Lake, and Garden Street Bridge) for an unspecified study in September 2000 were examined. The next earliest fish available had been combined into a community jar from unspecified locations and unspecified studies spanning 1998-1999. The oldest fish available were three individuals captured from an unspecified location in Landa Lake for an unspecified study in January 1997.

Determine the current infection status of both parasites in wild fish at selected stream reaches

Fish collection sites were selected based on (1) river access, and (2) sites used in previous studies where the presence of either *Centrocestus formosanus* or *Haplorchis pumilio* infections in snails or fishes had been confirmed (Mitchell et al. 2000, Salmon 2000, Cantu 2003, Clements 2018) (Table 1).

Table 1. Sites and descriptions of each location where wild fish were collected.

Water Body	Site	Description	GPS	Study Type[1]	
San Marcos River	SMR.UP1	Spring Lake Dam E Spillway	29.889984, -97.933911	WC	CF
	SMR.UP2	Rio Vista Park	29.880031, -97.932736	WC	
	SMR.UP3	IH-35 Crossing	29.874703, -97.931594	WC	
	SMR.DS1	Old Bastrop Hwy Crossing	29.857239, -97.896806	WC	
	SMR.DS2	Scull Rd Martindale	29.849542, -97.856864	WC	
	SMR.DS4	Hwy 20 Bridge	29.752686, -97.780914	WC	
	SMR.DS5	Sherrill Rd Crossing	29.728031, -97.760786	WC	
	SMR.DS6	Prairie Lea Crossing	29.716842, -97.753125	WC	
	SMR.DS7	Stairtown Rd Crossing	29.711983, -97.737881	WC	
	SMR.DS8	Hwy 90 Crossing	29.667508, -97.699619	WC	
	SMR.DS9	Luling S Magnolia Crossing	29.665936, -97.650653	WC	
Landa Lake	LL.PEC1	Pecan Island 1	29.715463, -98.134108		CF
	LL.PEC2	Pecan Island 2	29.715117, -98.134338	WC	CF
	LL.PEC3	Pecan Island 3	29.714736, -98.134580		CF
	LL.SPISL	Spring Island	29.717957, -98.131549		CF
Comal River	COM.UP1	Old Channel	29.709332, -98.122667		CF
Guadalupe River	GUAD.UP1	Cypress Bend Park	29.713569, -98.106317	WC	
	GUAD.DS1	Faust St Bridge	29.697287, -98.107269	WC	CF
Devils River	DEV.RCH	Dolan Crossing Reach	29.887524, -100.99300	WC	
	DEV.POOLS	Upper pools above rapids	29.902174, -101.00123	WC	
	DEV.FIN	Finegan Springs	29.895805, -100.99598	WC	
	DEV.BS	Blue Spring	29.893806, -100.99468	WC	
	DEV.DOL	Dolan Creek at Yellow Bluff	29.894910, -100.98343	WC	

San Marcos River

Fish were collected from three sites upstream of the confluence with the Blanco River and seven sites downstream from the confluence (Figure 2). All fish collection was performed with straight or bag seine except for one site (SMR.UP2), where all fish (consisting solely of centrarchids) were collected by angling due to the water being too deep for seining.

[1] WC = Wild-caught fish survey; CF = Caged-fish study

In partnership with USFWS, fountain darters were collected from two locations in the SMR – the springhead at the Meadows Center and immediately downstream from the East Spillway of Spring Lake Dam (Figure 2). Fountain darters were collected by dipnet and transported back to SMARC, where they were euthanized in MS-222 and preserved in 10% formalin. All specimens were stored at SMARC until permitted transfer to Texas State University. In total, 22 individuals were collected from the Meadows Center and 15 individuals were collected immediately below the spillway.

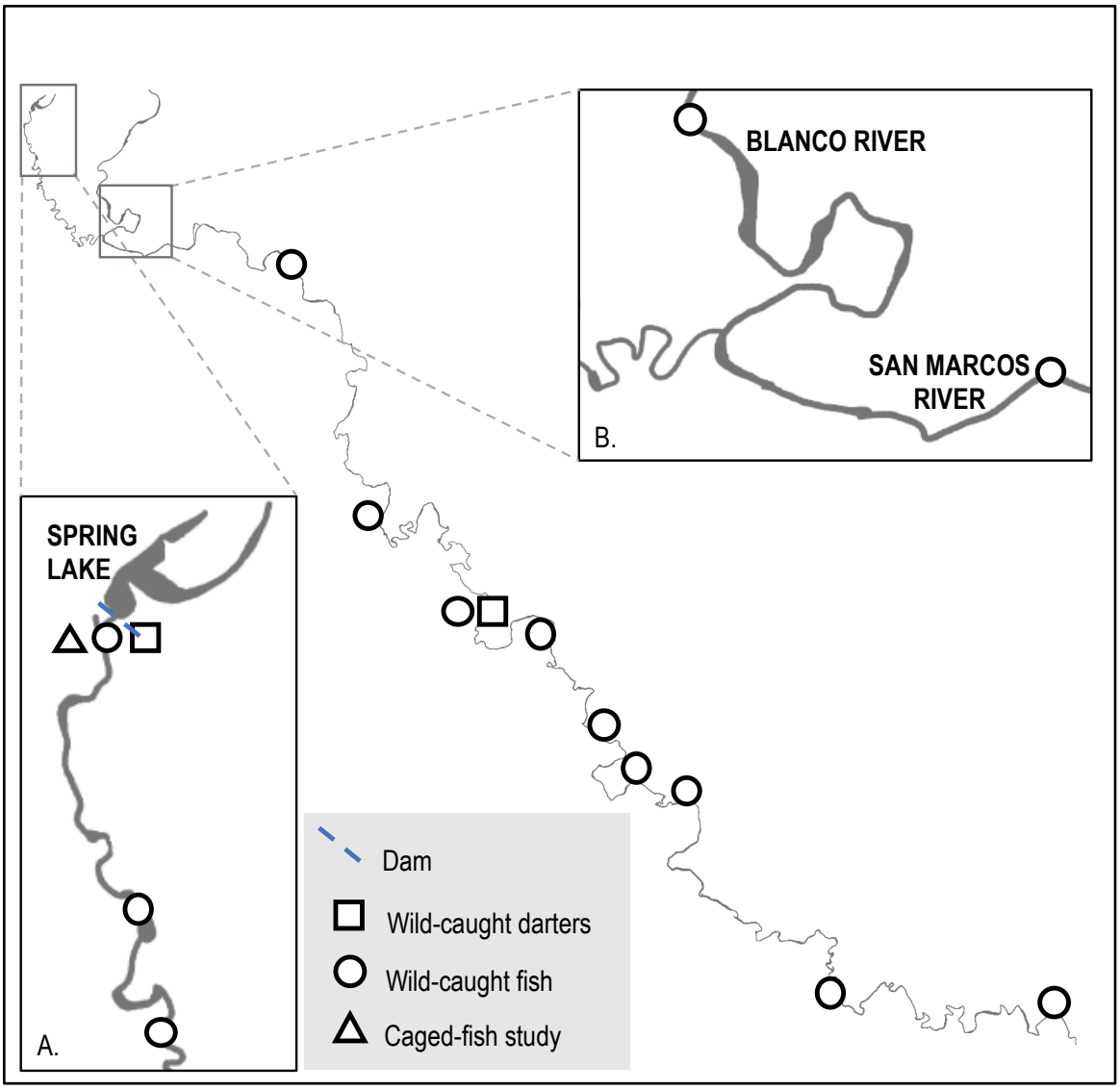

Figure 2. Sites of fish collection on the SMR. (A.) Enlarged view of Spring Lake; (B.) Enlarged view of the confluence of the BR and SMR.

Comal River

With the cooperation of USFWS, 22 fountain darters were collected via dip net along the northwest bank of Pecan Island in Landa Lake (Figure 3), the impounded headwaters of the CR, where previous studies have determined high densities of *Melanoides tuberculata* with high prevalence of heterophyid parasites (Clements 2018).

Based on total body length, we determined that all fountain darters captured were older juveniles or young adults (22-28 mm TL) (Brandt et al. 1993, McDonald et al. 2006).

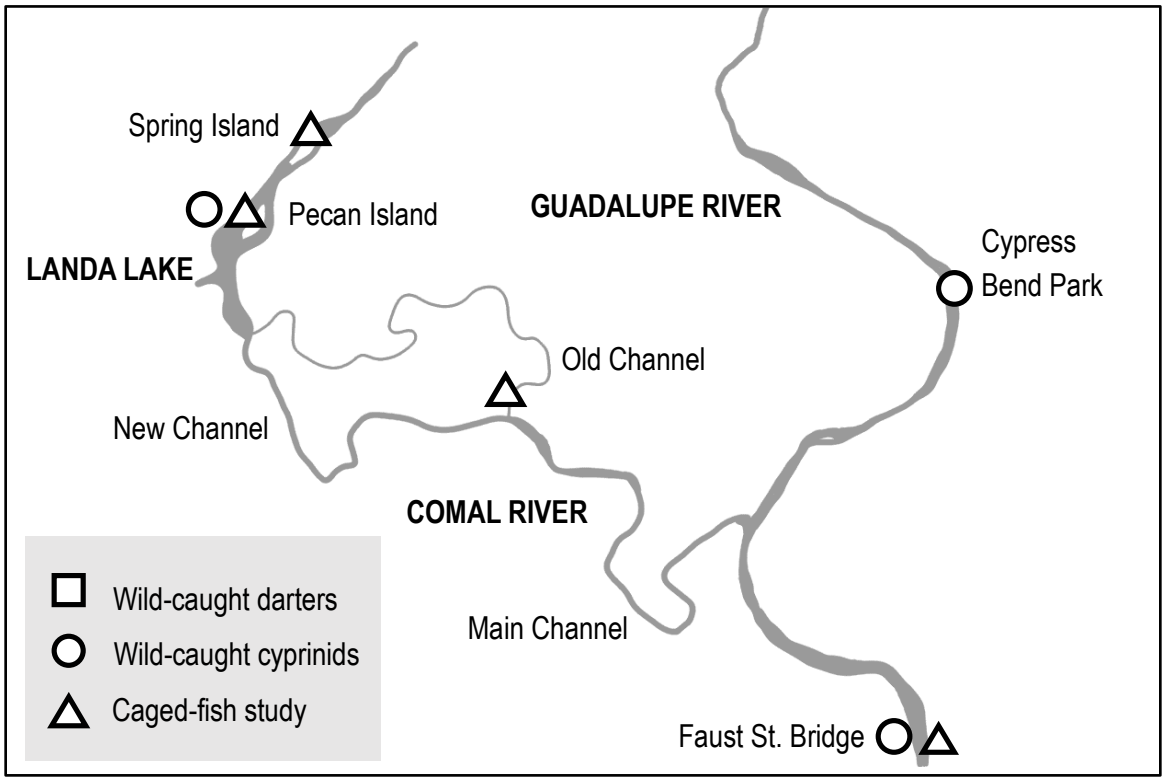

Figure 3. Fish collection sites and study types on the CR and GR.

Guadalupe River

Fish were collected from two sites in the Guadalupe River – one upstream (GUAD.UP1) and one downstream (GUAD.DS1) from the confluence with the CR (Figure 3).

Devils River

With the cooperation of The Nature Conservancy and TPWD, fish (cyprinids, Rio Grande darters, and some centrarchids) were collected via seine and angling from the Devils River State Natural Area and Dolan Creek in Del Rio, TX (Table 1). Fish were collected from four sites in the Devils River and one site below Yellow Bluff in Dolan Creek (Figure 4, Figure 5, Figure 6, Figure 8).

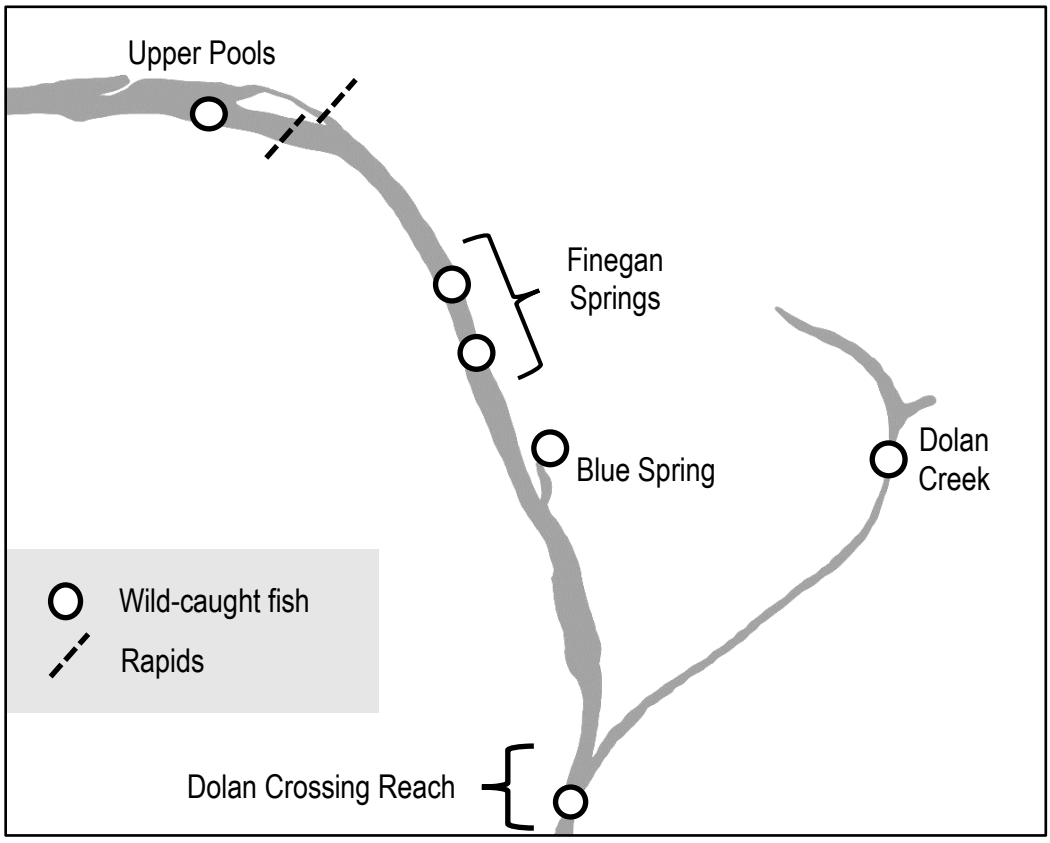

Figure 4. Fish collection sites on the DR.

Figure 5. The Upper Pools site above the rapids (DEV.POOLS).

Figure 6. Collecting snails and Rio Grande darters from the shallow channels of Finegan Springs (DEV.FIN).

Figure 7. A high density of *Melanoides tuberculata* in the swift riffles at Dolan Crossing Reach (DEV.RCH).

Figure 8. A mixture of swift riffle, dense submerged vegetation, and channel habitats at Dolan Crossing Reach (DEV.RCH).

After capture, fish were transported to the lab in covered, aerated five-gallon buckets, and euthanized in a buffered solution of 250 mg/L tricaine methanesulfonate (MS-222). In some cases, fish were euthanized in the field using MS-222 dissolved in buffered river water. Euthanized fish were preserved in 10% formalin for later necropsy.

Examination for Parasites

With free-swimming parasites, larger fish tend to have higher infection intensities due to more external surface area to encounter parasites in the water, increasing the likelihood of cercariae encountering a host (Movahed et al. 2016). Previous research has reported that the detrimental effects of parasite infection are more pronounced in smaller fish (Sommerville 1982b, Francová and Ondračková 2013); however, for fish larger than fry, this is not so much a function of body size as it is the number of cysts/mm total body length. At the same individual intensity, a 25 mm fish will exhibit greater negative effects than a 50 mm fish with the same number of cysts. Therefore, to avoid biasing mean intensity estimates toward larger fish, standardizing individual intensity by determining how many cysts occur per mm of body length allows for the comparison of parasite impact across sites that yielded differing mean lengths of fish (Clements 2018).

Centrocestus formosanus

Individual gill arches were excised using sharp-nosed forceps and placed on a slide in the order of their removal. One drop of 0.25% Bismarck Brown was applied to each gill arch to facilitate visibility of cysts, and gentle cover slip pressure was applied to a 24X50 mm glass coverslip to spread the gill filaments (Figure 9, Figure 10). The number of cysts per gill arch was recorded. A two-tailed *t*-test showed no significant

difference between the number of cysts on the right versus left side of the body (n=55, p=0.9601), thus the total number of cysts per fish was estimated by counting the cysts on the right side of the body and multiplying that number by two (Madhavi 1986). Examined gill arches were placed in a vial of 10% formalin along with the rest of that individual.

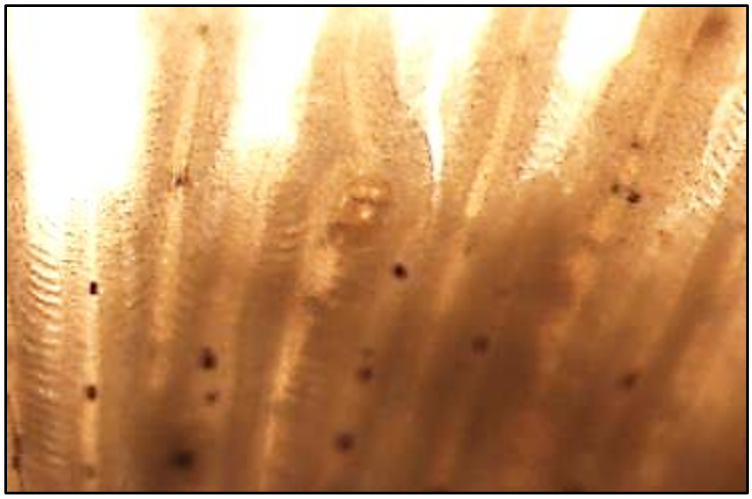

Figure 9. Metacercaria of *C. formosanus* in the gill filament of a *C. venusta*.

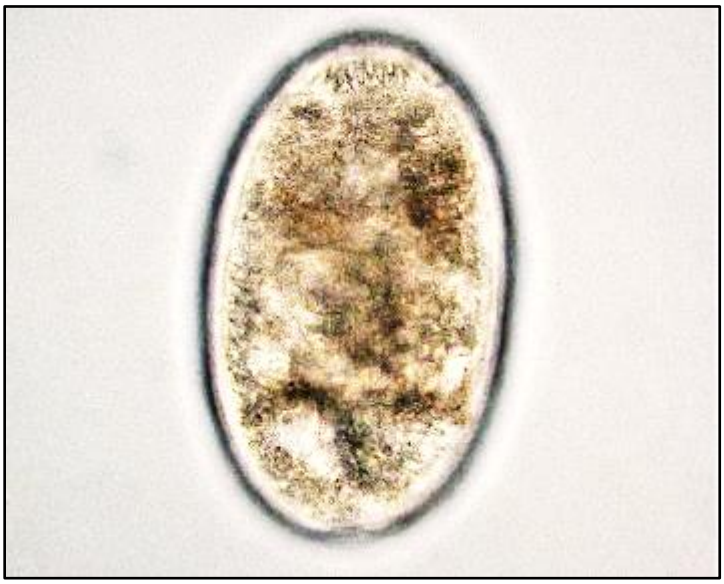

Figure 10. Excysted metacercaria of *Centrocestus formosanus* with characteristic double row of circumoral spines and dark X-shaped excretory bladder.

Haplorchis pumilio

All dissections were performed under a dissecting microscope at 4-25X magnification. The right side of the caudal peduncle (defined as the area from the last three vertebrae to the ural fan) was filleted sagittally starting at the base of the anal fin and continuing to the heads of the caudal fin rays (Videler 1993). This resulted in the separation of the right hemitrichial heads from the ural fan to more easily access embedded metacercarial cysts (Lo and Lee 1996, Clements 2018) (Figure 11).

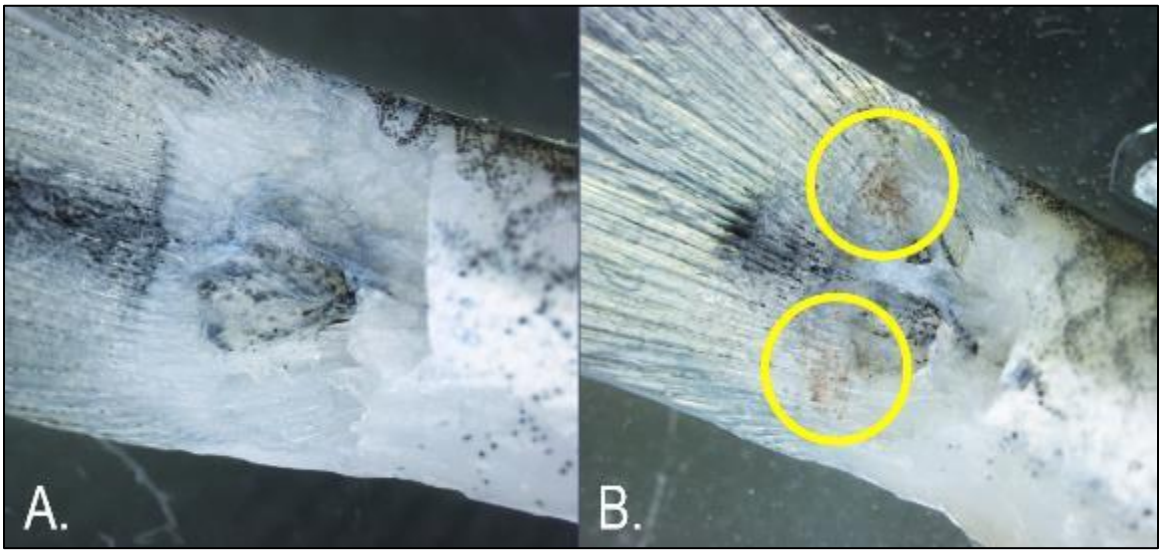

Figure 11. The dissected caudal peduncles of (A.) an uninfected *C. venusta* and (B.) a *C. venusta* with a high intensity infection of *H. pumilio* (brown cysts circled in yellow).

The exposed tissue on the right side was teased apart and individual cysts were excised from the tissue. The rest of the caudal peduncle was thoroughly teased apart to expose all cysts on both sides of the caudal peduncle. All cysts were excised, counted individually, and moved aside to eliminate the chance of double counting at the time of

removal. The total number of cysts in the caudal peduncle was recorded and all tissues including cysts were placed into the vial containing the previously examined gills.

If cysts were present in the caudal peduncle of a cyprinid, the remaining fins were examined, beginning with the anal fin and moving to the pelvic, pectoral, and dorsal fins, followed by the cartilage of the head and jaw (Figure 12). The number of cysts in each body location was recorded and expressed as a percentage of total cysts within that fish. Given that the majority of cysts occur in the caudal peduncle of cyprinids (Huston et al. 2014), a ratio was established based on previous work (Huston 2014) and our observations from 49 wild-caught *C. venusta* that allowed us to estimate the number of cysts distributed throughout the bodies of other fish based solely on the number of cysts in the caudal peduncle. Too few centrarchids were examined for *H. pumilio* to calculate a reliable ratio of cyst distribution within the body, therefore each location in the body of every centrarchid was examined for cysts. Each location in the body of fountain darters and Rio Grande darters was examined for cysts as well.

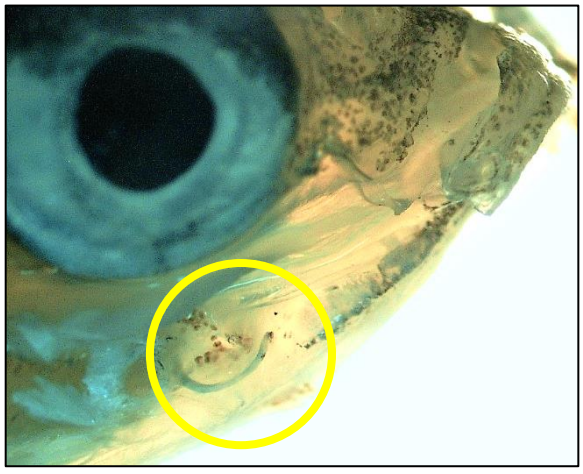

Figure 12. Metacercarial cysts (circled in yellow) of *H. pumilio* in the jaw of a *C. venusta* with the pre-operculum, operculum, and maxilla removed.

Estimating the impact of *C. formosanus* and *H. pumilio* on fishes in Texas

Rate of acquisition (caged-fish) studies

Caged-fish studies were performed using young adult *Cyprinella venusta* as a representative species, except for one study using F1-generation fountain darters bred in captivity at the SMARC facility (Table 1). One caged-fish study in the SMR was conducted using presumably uninfected fish collected from a site in the Blanco River, several km upstream of the confluence with the SMR. Ten fish from the BR site were examined before conducting the study to determine the infection status of *C. venusta* at that location and determine their appropriateness for use as experimental controls. All other caged-shiner studies used *C. venusta* collected from the hatchery ponds at the SMARC facility; 15 of these fish were examined for heterophyid infection before using them in caged-fish studies to ensure they were not infected before commencement of the study. Sites for cage placement were selected based on the previously determined geographic distributions of the parasites (Figure 2, Figure 3).

Mesh fabric minnow traps (mesh size 3 mm) purchased from Cabelas.com (Pomar Collapsible Minnow Trap (Cabelas #IK-017356; 25 X 25 X 46 cm) were modified to prevent the escape of caged fish by twisting the ends of the trap shut and securing each end with zip ties (Figure 13). Two cages were placed side by side inside of a larger turtle guard constructed of welded, 24-gauge wire, galvanized horse panel with 5X10 cm openings (Figure 13). The protective steel cages were constructed by cutting five panels of horse panel to form a rectangular frame (61 cm W, 71 cm L, and 30.5 cm H), grinding down sharp edges on the top and sides, and securing the corners together with zip ties. Vertical members of the side panels were left extended on the bottom edges to provide

secure placement for the apparatus. The top was not attached until placement at the field site, to make transport of the collapsible frame easier. The barrier was tagged with contact info, warning signs, and permit numbers and secured to a stationary object by a tether to prevent the interference of piscivorous animals with the study and to keep the cages stationary within the river.

Figure 13. Mesh minnow trap with openings zip-tied shut.

Figure 14. Field assembly of a caged-fish device, with one fish cage.

Keeping the mesh cages submerged in river water, individual fish were removed from a five-gallon bucket kept on shore and placed into the mesh cage. After placing fish in the cage, we sealed the cage by zipping it shut and secured the zipper with zip-ties. The four corners of the mesh cages were attached with braided nylon rope to the sides of the outer cage (1) to prevent the cages from collapsing when pushed by the current, and (2) to maximize the space available for fish inside the cage. The entire apparatus was secured to nearby structures, usually sturdy tree branches, with braided nylon rope as an added measure to prevent movement of the apparatus during rain events or other potential interference.

Two days after placing the cages, at least two fish were randomly selected and removed from the cage with a mesh net, then euthanized and preserved as above. The cage was then resealed and secured in place as previously described for another three days (5 d exposure), at which point 2-4 more fish would be randomly selected for removal and euthanized. The remaining fish were left in the cages for two days further (7 d exposure) before being collected, after which time the entire apparatus was removed from the site.

We conducted a study to estimate the rates of parasitism that could be expected in fountain darters living in an area of Landa Lake that is a known breeding ground for the fish and where high densities of *Melanoides tuberculata* also occur (Clements 2018). The same sites from the previously conducted caged-fish study using *C. venusta* were used (Table 1, Figure 15).

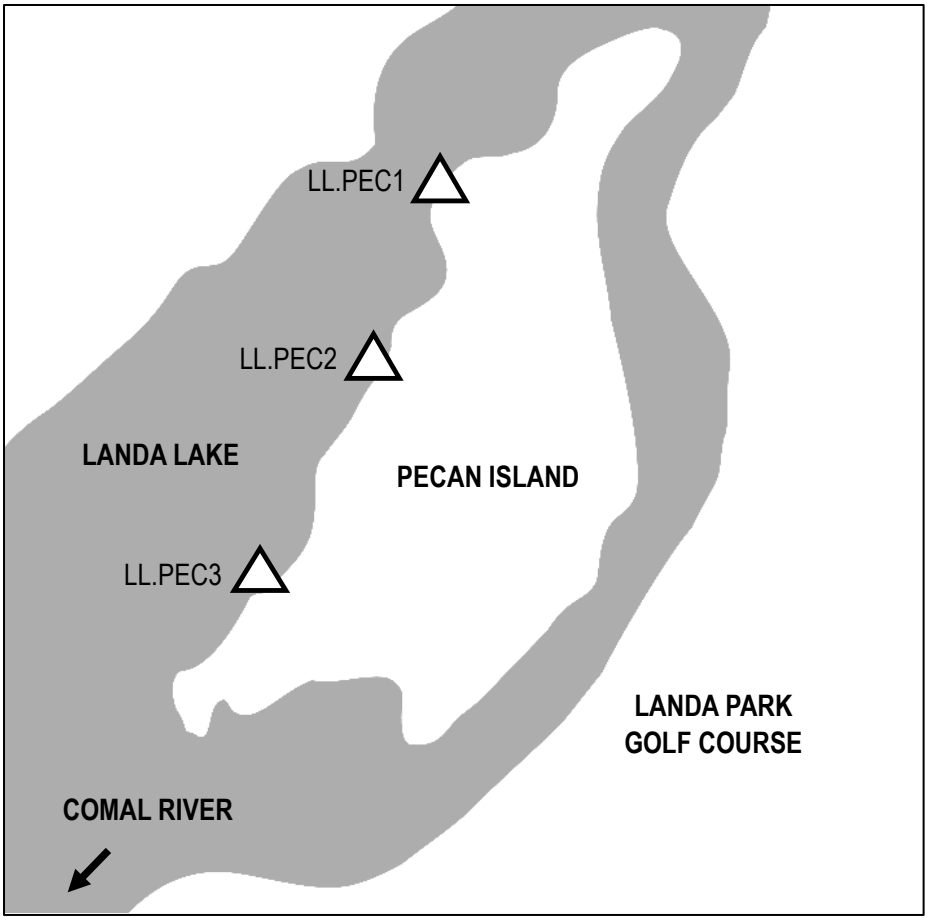

Figure 15. Stations of caged-fish studies and snail density transects at Pecan Island in Landa Lake.

The small size of fountain darters compared to *C. venusta* required cages with smaller mesh size to prevent fish from escaping. The fabric from previously used mesh minnow traps was removed and fiberglass window-screen mesh (Saint-Gobain ADFORS™ Extra Strength Black Fiberglass Mesh) was wrapped around the metal frame. The mesh was selected based on two specifications: (1) fountain darters could not fit through the openings in the mesh, and (2) the holes could not be so small that the mesh would interfere with cercariae passing through the water column. The mesh was sewn

around the frame using fishing line, then the ends were twisted and secured with zip-ties identical to cages used in previous studies.

Prior to placement in the field, each cage was tested to ensure optimum functionality by placing ten lab-reared fountain darters into a cage set inside a flow-through tank at SMARC. After 24 hours, the tank was checked to ensure no fish had escaped. If any fish had escaped, the cage was thoroughly inspected, and any escape routes were secured with fishing line or zip-ties. Two cages, each containing ten fish, were set at each station (Figure 15) and placed side by side inside a protective outer cage according to the previously described methods.

All handling of fountain darters was supervised by a USFWS lead biologist, and all specimens were stored on-site at the SMARC facility until official documented transfer of specimens to Texas State University.

Snail density transects in Landa Lake

A device to estimate the density of *Melanoides tuberculata* snails in one square meter plots was built from PVC pipe and stainless-steel mesh. The device consisted of two parts: a submergible frame of PVC filled with sand to ensure it would sink, and a floating frame of the same size that had steel mesh bolted to the frame (Figure 16). The mesh functioned as a sieve to rinse away mud and small debris but leave snails larger than ~1 cm in length sitting on top of the mesh. The density frames were placed at three stations per site along the banks of Pecan Island in Landa Lake, New Braunfels, TX. Station 1 was located closest to the bank; Station 2 was located approximately 1 m farther out from the bank, parallel to Station 1; Station 3 was located 1 m farther into the main channel than Station 2, parallel to the previous two stations (Figure 15).

Figure 16. A 1 m X 1 m snail density frame.

Using dipnets, two workers removed substrate from within the area marked by the sinking frame and dumped the contents of the net onto the mesh of the floating frame, held by a third worker (Figure 16). The substrate was carefully rinsed and sifted, and all plants and non-target invertebrates returned to the river. The workers shoveled nets of substrate until no more snails or snail shells were found, approximately 10-12 cm into the sediment. All snails from each station were sorted into individually labeled Tupperware containers and the empty shells separated from living snails. Enough water was placed in the containers to submerge the living snails, and the containers were sealed and transported back to the lab.

All living snails from each station were placed in test tubes labeled with the station number and filled with ~50 mL of artesian water. Incandescent lights were secured ~20 cm above the test tubes to induce cercarial shedding, and the tubes were left

undisturbed for 24 h. The following day, each test tube was gently inverted with the snail still inside, and approximately 10 mL of water was added to a Syracuse watch glass containing 1-2 drops of 1% Rose Bengal solution to facilitate higher visibility of cercariae. The infection status (*C. formosanus*-infected, *Haplorchis pumilio*-infected, or undetermined) was recorded for each snail, with those from the Undetermined group placed in a tube of fresh water for a further 24 h before re-inspection. Readings were completed for each Undetermined snail three times; if no cercariae were present on the third reading, the snail was recorded as Uninfected and removed from the study. Snails that were confirmed infected with either heterophyid were placed into one of two 10-gallon aquariums, designated respectively by heterophyid. Infection status of all live snails from the three stations at a site were pooled and used to determine snail density and prevalence of both heterophyids at each site.

Physiology study

On a separate occasion than when determining snail density, snails were collected from Pecan Island in Landa Lake and their infection status was determined using the above methods. Snails confirmed to be shedding *H. pumilio* cercariae were placed in an aerated 10-gallon aquarium designated for *H. pumilio*-infected snails only. Snails were fed commercial algae wafers and checked on alternating days to make sure all individuals were alive and responsive to stimuli.

Approximately 100 individuals of *C. venusta* were collected from a section of the Blanco River which was devoid of snails. Fish were placed into 10-gallon aquaria at a density of 4 fish per tank, and excess fish were kept in holding tanks at the same density. Water in all tanks was filtered through a Tetra Whisper 10i internal filter and changed

twice weekly. Water chemistry parameters were monitored using API 5-in-1 test strips and recorded every other day. Fish were fed commercial flake food once daily. Fish were acclimated for approximately 55 days.

Cercarial concentration was determined immediately before exposure by gently mixing the water in the *H. pumilio*-infected snail aquarium to disperse cercariae throughout the water, extracting a 200 mL water sample from the aquarium, and further dividing that sample into two 100 mL samples. The number of cercariae in each 100 mL sample was counted by distributing the 100 mL across 10 Syracuse watch glasses which each contained 1-2 drops of 1% Rose Bengal stain. We allowed the stain to penetrate live cercariae for 2-3 minutes, then added 5 mL of 70% ethanol to each watch glass to fix all cercariae. Each watch glass was placed inside a plastic petri dish marked with a 0.5 X 0.5 cm grid to facilitate systematic counting. The total number of cercariae in the 100 mL sample was calculated, and the process repeated for the remaining 100 mL sample. This provided a concentration of cercariae/mL found in the snail aquarium at the time of exposure.

Based on the calculated concentration of 4.28 cercariae/mL, the desired intensity of 200 cysts per individual fish (based on the intensity of *H. pumilio* infection acquired by *C. venusta* in one week at Pecan Island), and a 60% infection success rate – doubled from the 30% success rate observed by Clements (2018) due to the small size of the exposure chamber, since cercariae are more likely to encounter a host in smaller volumes of water and therefore more likely to infect the fish – we added 325 mL of cercarial water to 36 plastic 1-L jars. Control jars were prepared in the same manner using 325 mL of clean artesian water instead of cercarial water.

Two exposure groups were designated: the T_0 group, which was processed 15 minutes after exposure, and the T_1 group, which was processed 12 hours after exposure. All fish from both exposure groups were exposed for 15 minutes per fish. The start times for each replicate were staggered to account for 10 minutes of processing time between replicates, and to keep the total time each fish was exposed as equal as possible. After exposure, all fish from the T_1 group were returned to their aquaria for a further 12 h; all fish from the T_0 group were immediately processed. The processing station was prepared to facilitate the most rapid sampling process (Figure 17). All capillary tubes, slides (pre-cleaned with soap and 100% ethanol), Eppendorf tubes, and Falcon tubes were labeled ahead of time with the accession number of individual fish.

Fish were immobilized in a 500 mL beaker containing ice-cold water. Once a fish was unresponsive and opercular movement ceased, the individual was removed and placed on a dissection mat lined with paper towels. The fish was lightly blotted with a paper towel and the caudal peduncle was severed using a scalpel immediately posterior to the anal fin insertion. Once blood began flowing from the caudal blood vessels, a glucose test strip (Bayer ContourNext) was placed close enough to the blood to procure a satisfactory sample size (> 0.6 uL) but avoiding contact with other tissues. Glucose readings were recorded by an assistant while the experimenter inserted a microhematocrit capillary tube into the blood vessel and withdrew as much blood from the fish as possible. One drop of blood from the capillary tube was placed on a slide and a blood film immediately prepared. Concurrently, the assistant sealed the capillary tube using CritSeal and placed the capillary tube on ice until the time of centrifugation. The liver and dorsal muscles were excised and placed in separate centrifuge tubes, then

immediately frozen in liquid nitrogen and placed into a -20 C freezer. Remaining fish tissues were placed into a vial containing 10% formalin. Entire sampling event time per fish was approximately 40 seconds-2 minutes. Control fish were processed first, followed by treatment fish. Each replicate was exposed and processed separately.

After 12 h of incubation, 5 fish from the T_1 exposure group had died. Fish which had survived overnight were processed in the above methods and order, with notable pathology recorded.

Figure 17. The processing station with labeled supplies.

III. RESULTS

First occurrence of *Haplorchis pumilio* in fountain darters of Comal River

We examined a total of 56 archived fountain darter specimens collected from the CR for *Haplorchis pumilio*. Since low intensity infections with *H. pumilio* were found in fountain darters from September 2000 but not in fish from 1997-1999 (Table 2), we estimate that *H. pumilio* arrived in the CR in late 1999 and was already established in local fish by fall 2000.

Table 2. The caudal peduncles of fountain darters preserved between 1997-2002 were examined for metacercarial cysts of *H. pumilio*.

Year collected	Study type	*n*	Presence/Absence	Reference
2002	Caged-fish	30	P	Cantu 2003
2001	Caged-fish	6	P	Cantu 2003
2000	Wild-caught	12	P	Cantu 2003
1998-1999	Wild-caught	5	A	Salmon 2000
1997[1]	Wild-caught	3	A	Salmon 2000[2]

We compiled all reports we could find of either heterophyid in the CR and SMR, including peer-reviewed, institutional, and anecdotal reports. This included reports of cercariae detected by river cercariometry, examination of *M. tuberculata*, or examination of fish (Table 3). We condensed the findings from these reports into a single table,

[1] Earliest available collection.
[2] Author not explicitly stated.

resulting in a comprehensive chronological history of the status of both heterophyids in river water, snails, and fish over the years. The chronological sequence of findings in the CR reveals a steady increase in *Haplorchis pumilio*, and an apparent decrease in *Centrocestus formosanus*. The table also illustrates the paucity of heterophyid research that has been performed in the SMR in comparison to the CR.

Table 3. Historical review of presence, prevalence, and intensity data for *Haplorchis pumilio* and *Centrocestus formosanus* in the Comal and San Marcos Rivers.

		Comal River						San Marcos River						
Date	Cited	River Cercariometry		*Melanoides tuberculata*[1]		*Etheostoma fonticola*[2]		River Cercariometry		*Melanoides tuberculata*[1]		*Etheostoma fonticola*[2]		
Range	Authority	*C. formosanus*	*H. pumilio*	*C. formosanus*	*H. pumilio*	*C. formosanus*	*H. pumilio*	*C. formosanus*	*H. pumilio*	*C. formosanus*	*H. pumilio*	*C. formosanus*	*H. pumilio*	
1974-75	ref 01											0[5]	0[5]	ref 01-Davis and Huffman 1977
1977-78	ref 02									0 of 354[7]	0 of 354[7]			ref 02-Lindholm 1979
Feb-1994	ref 03					0% (of 5)								ref 03-Mitchell et al. 2000
Mar-1994	ref 03					60% (of 5)								ref 03-Mitchell et al. 2000
Aug-1994	ref 03					100% (of 5)								ref 03-Mitchell et al. 2000
Mar-1995	ref 03					100% (of 5)								ref 03-Mitchell et al. 2000
Oct-1996	ref 03					100% (of 9)								ref 03-Mitchell et al. 2000
1997-98	ref 03			6% (of 2,279)		8-1,524 cysts 100% (of 194)				0.04% (of 2,241)	0[3]	0-2 cysts 3% (of 130)	0[4]	ref 03-Mitchell et al. 2000
2000-01	ref 04					4.1/d								ref 04-Fleming et al. 2011
2002	ref 05	97% pos. (0-45 / L)	<3% pos.			0-1,662 cysts; 99% (of 232)								ref 05-Cantu 2003
2002	ref 06			6% (of 610)	0.7% (of 610)									Ref 06-Tolley-Jordan and Owen 2008
2004	ref 07	10 / L[11]												Ref 07-BIO-WEST 2005
2006	ref 08	17,563 (4.2 / L)	132											Ref 08-Bolick 2007
2006-07	ref 09	1.4 / L (L.L.); 7 / L (O.C.)	Observed, no count											Ref 09-Johnson et al. 2012
2009	Ref 19											100%; M=100[12]		Ref 19 EAAHCP 2009 Appendix D; Minimum requirements for species
2009-10	ref 09	0.08 / L (L.L.); 2.8 / L (O.C.)												Ref 09-Johnson et al. 2012
2011-12	ref 10 (wild)	4.3 / L				M=232 / fish (5%>800 / fish) 95%								Ref 10-Cantu et al. 2013
	ref 10 (caged)					M=11 / fish 55% (of 736)								Ref 10-Cantu et al. 2013
2013	ref 11	1.85 / L	0.6 / L											Ref 11-Bio-West 2014
2013	ref 12						6-34 100% (of 5)						3-26 cysts 100% (of 5)	Ref 12-Huston, Daniel C. 2014
2014	ref 11	5.1 / L		0-25% (13.7%)	?	M=16-21 / fish[6]								Ref 11-Bio-West 2014
2014	ref 11	13.3 / L		13.1%	19.2%[10]									Ref 11-Bio-West 2014
2014	ref 15			12% (of 4,767)	13% (of 4,767)					13% (of 530)	7.4% (of 530)			Ref 15- Harding 2016
2015	ref 16	3.4 / L		12.5%	50%[10]									Ref 16- Bio-West 2015
2015	ref 11									15% (of 274)	"Positive"			Ref 11- Bio-West 2014
2015	ref 15					2-52(17/fish),25								Ref 15- Harding 2016
2016	ref 17	1.9 / L												Ref 17- Blanton & Associates 2018
2017	ref 17	1.0 / L												Ref 17- Blanton & Associates 2018
2018	ref 18			>15% (of 1,385)[9]	>24% (of 1,385)[9]									Ref 18- Clements 2018
2018	ref 18	1.6 / L												Ref 20- EAAHCP 2018

[1] Number positive of Number examined

[2] Intensity range (%prevalence,*n*)

[3] We were unaware that *H. pumilio* may have been mixed in with *C. formosanus*

[4] We did not know to look for *H. pumilio* in caudal peduncle until 2014

[5] Fountain darters not examined, but gills of 1400+ suceptible gambusias were negative

[6] Fish were from historical collections; years of collection not specified.

[7] 426 specimens of *Tarebia granifera* were also negative for all trematode larvae.

[8] In all six habitats in Landa Lake where snails were positive for either heterophiid trematode, prevalence of *H. pumilio* was higher than for *C. formosanus*

[9] West bank of Pecan Island; prevalence was determined by shedding (+ or -), which substantantially underestimates actual infection prevalence

[10] Listed as "Other Parasites"! Can't find data for subsequentv years.

[11] Spring Island 4U site

Distribution of heterophyids from wild-caught fishes at study sites

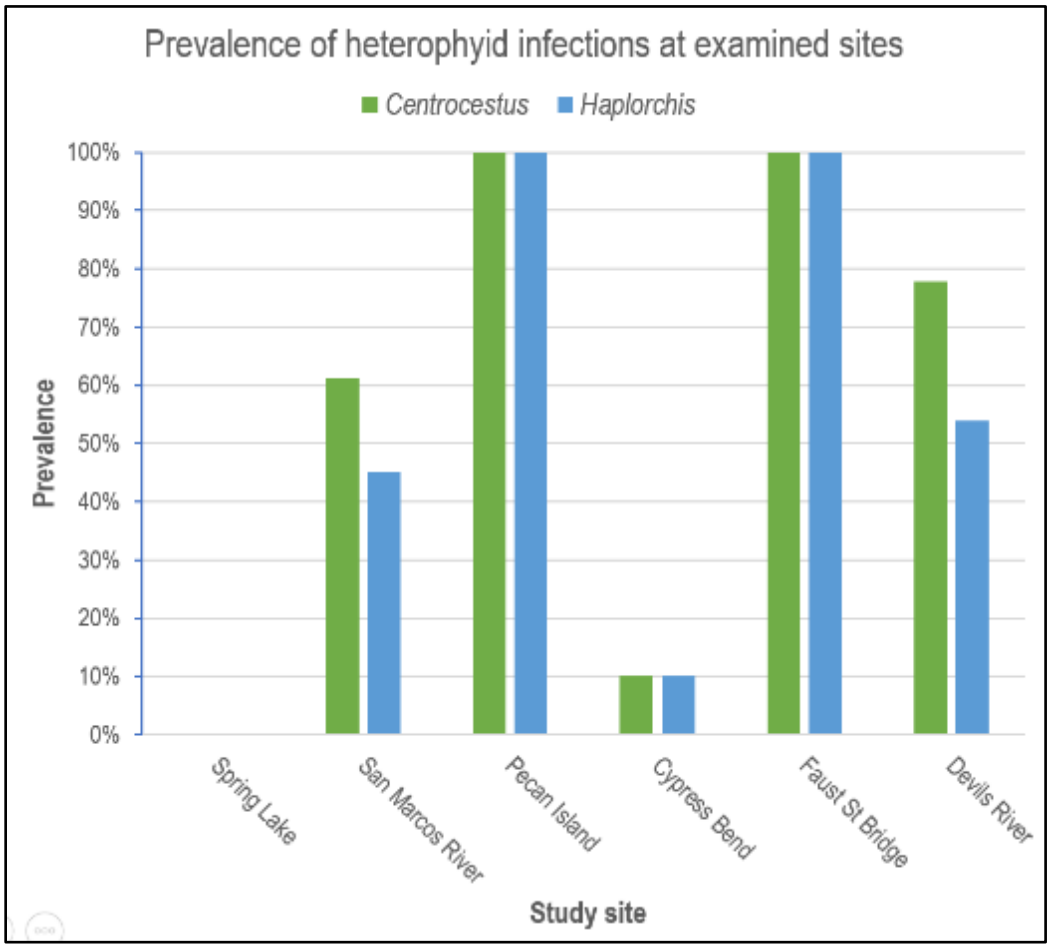

Figure 18. Prevalence of both heterophyids from all sites where wild-caught fish were collected.

San Marcos River

In collaboration with USFWS, we collected two fountain darters from the riffles directly below the spillway. Both individuals were infected with *C. formosanus* (0.59 cysts/mm) and *H. pumilio* (0.78 cysts/mm) (

Table 5). Several months later we returned with USFWS to the riffles below the spillway and collected 20 fountain darters, none of which were infected with either parasite. We also collected 20 fountain darters from the springs located just below the Meadows Center, within Spring Lake; these fish were also negative for both heterophyids. All fountain darters collected from Spring Lake and the spillway in late summer 2018 were classified as either juveniles or young adults based on growth curves reported by Brandt et al. (1993). SMARC had collected fountain darters from the SMR and CR during 2014-2015, and as early as 2015, some fish from Spring Lake were infected with *H. pumilio* and *C. formosanus.* Given that Spring Lake is a closed system, for infected fish to be present, *M. tuberculata* must be present somewhere in the lake. Based on recent surveys performed by volunteer divers and visual surveys from glass-bottom boats, however, the density of snails is so low that none were detected in all sites surveyed within Spring Lake.

At the first site downstream from the confluence with the BR (SMR.DS1) (Figure 2), all of the 18 *Cyprinella venusta* collected were infected with either *Centrocestus formosanus* or *Haplorchis pumilio* or both (Table 4,

Table 5). At the second site downstream (SMR.DS2), none of the 7 *Dionda nigrotaeniata* were infected with either parasite. The third site downstream (SMR.DS3) was inaccessible due to high water, excessive current, and heavy recreational use. At the fourth site downstream (SMR.DS4), none of the 4 *Cyprinella venusta* or the 16 *C. lutrensis* were infected with either parasite. Subsequent to the negative findings, two

additional stations were sampled downstream to Luling (SMR.DS5 and SMR.DS9), and all fish examined were negative for the heterophyids.

Table 4. Prevalence, mean intensity, and mean standardized intensity of both heterophyids in all fish from all sites.

River	Site	N	# Species	*C. formosanus*			*H. pumilio*		
				Prev.	Mn Int	Cysts/mm	Prev	Mn Int	Cysts/mm
Comal	Pecan Island	22	1	100.00%	106.55	4.29	100.00%	27.82	1.09
Devils	Blue Spring	9	3	77.78%	74.86	0.97	77.78%	17.29	0.24
	Finegan	41	2	92.68%	44.32	0.75	75.61%	15.68	0.25
	Dolan Reach	20	2	95.00%	56.53	0.86	100.00%	51.10	0.81
	Dolan Creek	27	1	81.48%	15.73	0.40	3.70%	4.00	0.10
	Upper pools	16	1	12.50%	12.00	0.16	12.50%	28.50	0.39
Guadalupe	Faust Bridge	7	1	100.00%	30.29	0.44	100.00%	15.57	0.23
	Cypress Bend	10	1	10.00%	10.00	0.21	10.00%	5.00	0.10
San Marcos	SL Dam	4	3	25.00%	20.00	0.22	25.00%	58.00	0.40
	I-35 Crossing	5	2	100.00%	9.60	0.28	100.00%	16.80	0.48
	Old Bastrop	18	1	72.22%	7.38	0.14	44.44%	2.38	0.04
	Scull Rd	8	2	0.00%	0.00	0.00	0.00%	0.00	0.00
	Hwy 20	20	2	0.00%	0.00	0.00	0.00%	0.00	0.00
	Sherrill Rd	2	1	0.00%	0.00	0.00	0.00%	0.00	0.00
	S Magnolia	4	1	0.00%	0.00	0.00	0.00%	0.00	0.00

Thus, the downstream limit of distribution of both *C. formosanus* and *H. pumilio* in the SMR in 2018 is reported herein as Old Bastrop Highway Crossing (SMR.DS1), ~9.5 km downstream from the headspring and ~1.86 km from the confluence with the BR (Figure 19). At that terminal site, *C. formosanus* was found in 13 of 18 *C. venusta*, and *H. pumilio* was found in 8 of the 18 individuals (Table 4).

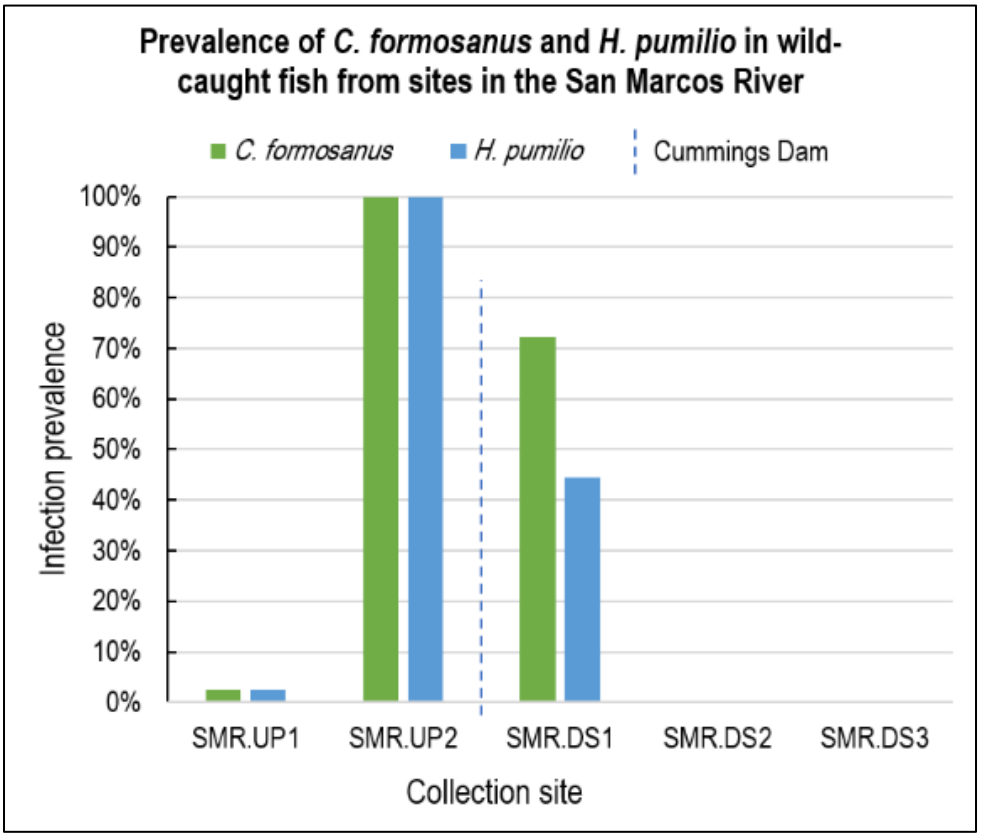

Figure 19. The highest prevalence of both heterophyids in the SMR was at the IH-35 crossing (SMR.UP2). Below the Blanco River confluence and Cummings Dam, prevalence of both parasites was lower; no infections were detected in fish downstream of SMR.DS1.

Table 5. Prevalence, mean intensity, and mean standardized intensity of both heterophyids by species of fish collected from the SMR.

			Centrocestus formosanus			*Haplorchis pumilio*		
Site	Species	n	Prev	Mn Int	Mn Int/mm	Prev	Mn Int	Mn Int/mm
SMR.UP.1	*E. fonticola*	39	5.1%	19	0.59	5.1%	25	0.78
SMR.UP.3	*C. venusta*	1	100%	20	0.22	0%	0	0
	M. salmoides	1	0%	0	0	100%	58	0.4
	L. auritus	2	0%	0	0	0%	0	0
SMR.DS.1	*C. venusta*	18	72.2%	7.38	0.14	44%	2.38	0.04
SMR.DS.2	*D. nigrotaeniata*	7	0%	0	0	0%	0	0
SMR.DS.4	*C. venusta*	4	0%	0	0	0%	0	0
	C. lutrensis	16	0%	0	0	0%	0	0
SMR.DS.5	*C. venusta*	2	0%	0	0	0%	0	0
SMR.DS.9	*C. venusta*	4	0%	0	0	0%	0	0

Devils River

We collected fish from multiple stations along the DR, including Blue Spring (DEV.BS), Finegan Springs (DEV.FIN), and from the reach of the DR upstream from Dolan Crossing (DEV.RCH) for ~ 2.0 km (Table 1) (Figure 4). Fish collected from these localities were all infected with one or both heterophyids. We also portaged over a class 2 rapids just upstream from Finegan Springs and collected 16 *C. venusta* from the riffles and smooth limestone pools above the rapids; only two of these fish were infected (Figure 5) (Table 4), and since there were no snails in the pool and no springs above the rapids, these two individuals may have immigrated during a flood event from the channel downstream, which was infested with *M. tuberculata*.

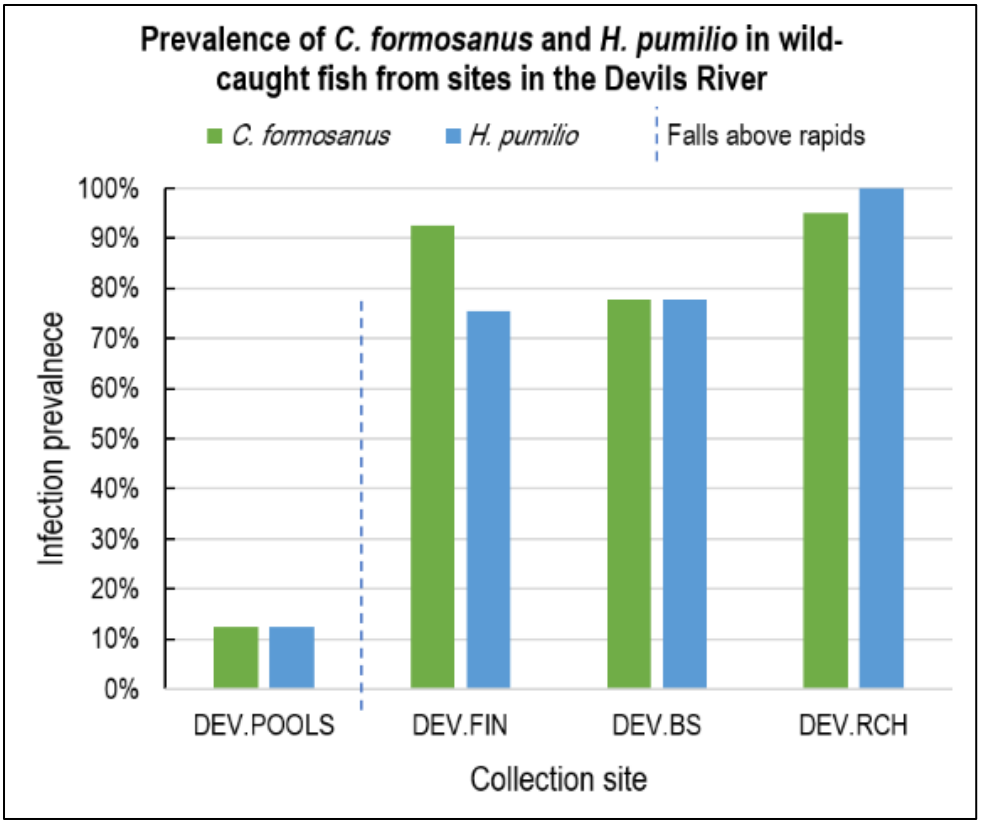

Figure 20. Prevalence of both parasites was comparable at all sites sampled in the DR except the most upstream site.

Blue Spring

Five *Cyprinella venusta* were collected, along with one *Lepomis auritus* and three juvenile *Micropterus salmoides* (Table 6). All five of the *C. venusta* were infected with both heterophyids, with an average of 0.76 *C. formosanus* cysts/mm and 0.25 *H. pumilio* cysts/mm. Two of the three *M. salmoides* were infected with both heterophyids (1.68 *C. formosanus* cysts/mm; 0.23 *H. pumilio* cysts/mm). The single *L. auritus* we collected did not show any signs of infection by either parasite (Table 6).

Table 6. Prevalence (Prev.), mean intensity (Mn. Int.), and standardized intensity of individual species of fish collected from Blue Spring in the DR.

		Centrocestus formosanus			*Haplorchis pumilio*		
Species	*n*	Prev.	Mn. Int.	Mn. Int./mm	Prev.	Mn. Int.	Mn. Int./mm
C. venusta	5	100.0%	51.2	0.76	100.0%	16.8	0.25
M. salmoides	2	66.7%	134	1.68	66.7%	18.5	0.23
L. auritus	1	0.0%	0	0	0.0%	0	0

Finegan Springs

All of the 20 *Cyprinella venusta* collected from the shallow channels at Finegan Springs (DEV.FIN) (Figure 4, Figure 6) were infected with both *Centrocestus formosanus* (0.75 cysts/mm) and *Haplorchis pumilio* (0.25 cysts/mm) (Table 7). Twenty-one Rio Grande darters were collected by dipnet from the shallow channels at Finegan Springs (Figure 4). Of these fish, 86% were infected with *C. formosanus* (0.75 cysts/mm) and 52% were infected with *H. pumilio* (0.25 cysts/mm) (Table 7).

Table 7. Prevalence, mean intensity, and mean standardized intensity of both heterophyids in both fish species collected from Finegan Springs.

		Centrocestus formosanus			*Haplorchis pumilio*		
Species	*n*	Prev.	Mn. Int.	Mn. Int./mm	Prev.	Mn. Int.	Mn. Int./mm
C. venusta	20	100.0%	73.6	1.09	100.0%	23.3	0.35
E. grahami	21	85.7%	11.8	0.38	52.4%	1.82	0.06

After observing relatively high numbers of *Melanoides tuberculata* in the channels at Finegan Springs, we collected more than 200 snails and transported them back to the lab. Examination of these snails yielded less than 5% prevalence of *C. formosanus*; no cercariae of *H. pumilio* were detected.

Dolan Crossing Reach

Habitats in this site (DEV.RCH) varied from rapidly flowing shallow riffles with cobble substrate to still backwaters in open areas with silty substrate and dense aquatic and partially submerged riparian vegetation (Figure 8). Approximately equal numbers of fish were collected from each habitat type along the reach. All three of the *Dionda argentosa* collected from the reach were infected with *C. formosanus* (0.28 cysts/mm) and *H. pumilio* (0.43 cysts/mm); 94.1% of the 17 *C. venusta* collected from the reach were infected with *C. formosanus* (0.97 cysts/mm), and all were infected with *H. pumilio* (0.90 cysts/mm) (Table 8).

Visual surveys of the reach revealed a high density of *Melanoides tuberculata*, even in the swift and shallow riffles (Figure 7).

Table 8. Prevalence, mean intensity, and mean standardized intensity of both heterophyids in both fishes collected from Dolan Crossing Reach

		Centrocestus formosanus			*Haplorchis pumilio*		
Species	*n*	Prev.	Mn. Int.	Mn. Int./mm	Prev.	Mn. Int.	Mn. Int./mm
D. argentosa	3	100.0%	11.3	0.28	100.0%	17	0.43
C. venusta	17	94.1%	65.0	0.97	100.0%	57.12	0.90

Upper Pools

All 16 *Cyprinella venusta* collected from the pools (DEV.POOLS) above the rapids (Figure 4, Figure 5) were negative for *C. formosanus* and *H. pumilio*, except for two large *C. venusta* which had likely immigrated from the infested reach downstream during a high-water event. One of the latter fish was heavily infected with *H. pumilio* (54 cysts; 0.73 cysts/mm), while the other was lightly infected (3 cysts; 0.04 cysts/mm); both fish were lightly infected with *C. formosanus* (12 cysts; 0.16 cysts/mm). All 14 other *C.*

venusta collected from above the rapids were uninfected and appeared to be in good health (Table 4). Notably, no *Melanoides tuberculata* or shells were observed in or near the upper pools.

Dolan Creek

We collected 27 *Dionda argentosa* from a large pool in Dolan Creek at Yellow Bluff (DEV.DOL) (Figure 4). Infections of *Centrocestus formosanus* were found in 81.5% of fish collected (0.40 cysts/mm), however only one of the fish was infected with *Haplorchis pumilio* (0.10 cysts/mm) (Table 4).

High densities of *Melanoides tuberculata* were observed at this site, however prevalence of both parasites among the snails was low. Cercariae of *C. formosanus* were shed by < 10% of snails, while *H. pumilio* cercariae were not shed by any of the snails collected.

Comal River - Pecan Island

All of the 22 *Etheostoma fonticola* collected from the northwestern bank of Pecan Island (LL.PEC2) were infected with both *C. formosanus* (4.29 cysts/mm) and *H. pumilio* (1.09 cysts/mm) (Table 4).

Guadalupe River

Fish (*n*=10) collected from the riffles at the upstream end of Cypress Bend Park (GUAD.UP1) had low prevalence of both *C. formosanus* and *H. pumilio*, with only one individual containing cysts of both parasites (Table 4). Considering the proximity of this site to the confluence with the CR where infection levels are particularly high, this individual fish had likely immigrated upstream from the CR instead of being naturally infected while at or upstream of Cypress Bend Park (Figure 3). No *M. tuberculata* were

observed at this site the time of collection, although small snails were found upstream of the site in previous years (Huffman, unpub. data).

All seven *C. venusta* collected from the Faust Street Bridge site downstream of the GR's confluence with the CR were infected with both *Centrocestus formosanus* (0.44 cysts/mm) and *Haplorchis pumilio* (0.23 cysts/mm) (Table 4).

Caged-fish Studies

San Marcos River – East spillway below Spring Lake Dam

Ten presumably uninfected *C. venusta* (collected from the BR where *M. tuberculata* does not occur) were placed in a cage just downstream from the east spillway of Spring Lake Dam. Fish were recovered after 2 d (*n*=2), 5 d (*n*=3), 7 d (*n*=2), and 14 d (*n*=2) of exposure to river water. One fish escaped the cage on day seven at the time of collection, bringing the total number of fish examined in the trial to nine individuals.

No signs of parasitism were observed in fish after 2, 5, or 7 d. However, both fish exposed for 14 d showed signs of parasitism. One individual contained two young *C. formosanus* cysts (not yet encysted and still possessing eyespots) and the other fish contained one young *H. pumilio* cyst (still possessing eyespots) in the caudal peduncle.

Guadalupe River – Faust Street Bridge

Ten fish were initially placed in a cage in a secluded location away from nearby recreational areas. One fish escaped the bucket at the time of placement, bringing the total number of fish used in the trial to nine individuals.

Fish were recovered after 2 d (*n*=2), 5 d (*n*=3), and 7 d (*n*=4) of exposure to river water. One of the two fish collected after 2 d of exposure was infected with *C.*

formosanus (0.05 cysts/mm); both fish were infected with *H. pumilio* (0.16 cysts/mm). Two of the three fish collected after 5 d of exposure were infected with *C. formosanus* (0.08 cysts/mm); all three were infected with *H. pumilio* (2.2 cysts/mm). All four fish collected after 7 d of exposure were infected with both *C. formosanus* (0.11 cysts/mm) and *H. pumilio* (0.12 cysts/mm) (Table 9). Overall, fish placed in a cage at this site acquired *C. formosanus* at a mean rate of 0.82 cysts/day, and *H. pumilio* at a mean rate of 2.04 cysts/day (Table 9).

Table 9. Intensity and rate of acquisition of both heterophyids in *C. venusta* placed in cages at Faust St. Bridge (GUAD.DS2).

	Centrocestus formosanus				*Haplorchis pumilio*			
Days of exposure	Individual Int.	Int./mm	Mn. Int./mm	Cysts/d	Individual Int.	Int./mm	Mn. Int./mm	Cysts/d
2	0	0	0.05	1	13	0.28	0.189	4.25
	2	0.05			4	0.09		
5	0	0	0.08	0.8	17	0.37	0.22	2.13
	6	0.11			13	0.23		
	2	0.05			2	0.05		
7	6	0.11	0.11	0.79	9	0.16	0.12	0.86
	2	0.04			6	0.13		
	8	0.16			6	0.12		
	6	0.13			3	0.06		
				0.82[1]				2.04[2]

Comal River – Old Channel

The cage at this site was placed deeper in the water column than at other sites, due to strong flow and lack of shallow locations that would also provide adequate security. Ten fish were initially placed in the cage, though two individuals escaped the cage at the time of placement, bringing the total number of fish used in the trial to eight individuals.

[1] Mean *C. formosanus* cysts acquired per day across all collection events at this site.
[2] Mean *H. pumilio* cysts acquired per day across all collection events at this site.

Fish were recovered after 2 d (*n*=3), 5 d (*n*=3), and 7 d (*n*=2) of exposure to river water. All three fish collected after 2 d of exposure were infected with *C. formosanus* and one fish was infected with a single *H. pumilio* cyst in the caudal peduncle. All three fish collected after 5 d of exposure were infected with *C. formosanus*, and one fish was infected with *H. pumilio*. Both fish collected after 7 d of exposure were infected with *C. formosanus*, and neither were infected with *H. pumilio* (Table 10). Fish placed in a cage at this site acquired *C. formosanus* at a rate of 2.1 cysts/day and *H. pumilio* at a rate of 0.35 cysts/day.

Table 10. Intensity and rate of acquisition of both heterophyids in *C. venusta* placed in a cage in the Old Channel of the CR (COM.UP.1).

	Centrocestus formosanus				*Haplorchis pumilio*			
Days of exposure	Individual Int.	Int./mm	Mn. Int./mm	Cysts/d	Individual Int.	Int./mm	Mn. Int./mm	Cysts/d
2	4	0.08	0.17	7.33	1	0.02	0.02	0.01
	2	0.05			0	0		
	16	0.37			0	0		
5	12	0.21	0.10	3.2	0	0	0.02	0.20
	2	0.04			0	0		
	2	0.05			1	0.02		
7	8	0.17	0.22	2.57	0	0	0	0
	10	0.27			0	0		
	6	0.13			3	0.06		
				4.37[1]				0.07[2]

[1] Mean *C. formosanus* cysts acquired per day across all collection events at this site.
[2] Mean *H. pumilio* cysts acquired per day across all collection events at this site.

Comal River – Landa Lake, Spring Island

Two cages, each containing five *C. venusta,* were placed at the northeastern edge of Spring Island, a privately-owned recreational island directly upstream from Pecan Island. *M. tuberculata* was present at the site; however, most living snails were < 2 cm in length, which generally do not shed heterophyid cercariae (Mitchell and Goodwin 2004). Most snail shells at this site were empty and calcified.

Fish were recovered after 2 d (n=2) and 5 d (n=4) of exposure to river water. Both fish collected after 2 d of exposure were infected with *C. formosanus*; however, neither fish was infected with *H. pumilio*. Two of the three fish collected after 5 d of exposure were infected with both *C. formosanus* and *H. pumilio* (Table 11). When we attempted to collect fish after 5 d of exposure, we discovered that one of the cages had been invaded by two centrarchids. We collected the fish remaining in the undamaged cage and abandoned the trial after 5 d of exposure instead of 7 d. Fish placed in a cage at this site acquired *C. formosanus* at a rate of 1.0 cysts/day and *H. pumilio* at a rate of 1.9 cysts/day.

Table 11. Intensity and rate of acquisition of both heterophyids in *C. venusta* placed in a cage along the northern bank of Spring Island (COM.SI.1).

Days of exposure	*Centrocestus formosanus*				*Haplorchis pumilio*			
	Individual Int.	Int./mm	Mn. Int./mm	Cysts/d	Individual Int.	Int./mm	Mn. Int./mm	Cysts/d
2	2	0.04	0.45	1	0	0	0	0
	2	0.05			0	0		
	0	0			0	0	0	
5	6	0.13	0.11	1	6	0.13	0.21	1.90
	4	0.09			13	0.29		
				1.0[1]				0.95[2]

Comal River – Landa Lake, Pecan Island

Three stations along the bank were selected based on reported snail densities (Clements 2018), with the lowest density at the easternmost station (closest to the headspring) and the highest density at the westernmost station (farthest downstream from the headspring; Figure 15). Repeated collections of snails for this study and other peripheral studies have yielded high prevalence of both parasites in *M. tuberculata* along the banks of Pecan Island, especially *H. pumilio*.

Surveys of snail-density along transects considered only live snails. Most snails from LL.PEC1 were < 2 cm in length; the second station (LL.PEC2) contained more snails of larger size, as well as higher prevalence of both parasites. The third station (LL.PEC3), farthest downstream, yielded the highest density of snails, most of which were > 2 cm in length (Table 12).

[1] Mean *C. formosanus* cysts acquired per day across all collection events at this site.
[2] Mean *H. pumilio* cysts acquired per day across all collection events at this site.

Table 12. Density of snails/m^2, live snails/m^2, and percent live snails across 3 stations per site at Pecan Island.

Site	Station	Snails/m^2	Live snails/m^2	Site – Mn Live Snails/m^2	Site – % Live Snails
Pec1	1.1	52	10	4	**7.2%**
	1.2	126	3		
	1.3	32	0		
Pec2	2.1	205	19	9	**4.5%**
	2.2	192	7		
	2.3	172	1		
Pec3	3.1	115	33	20	**17.35%**
	3.2	113	23		
	3.3	133	4		

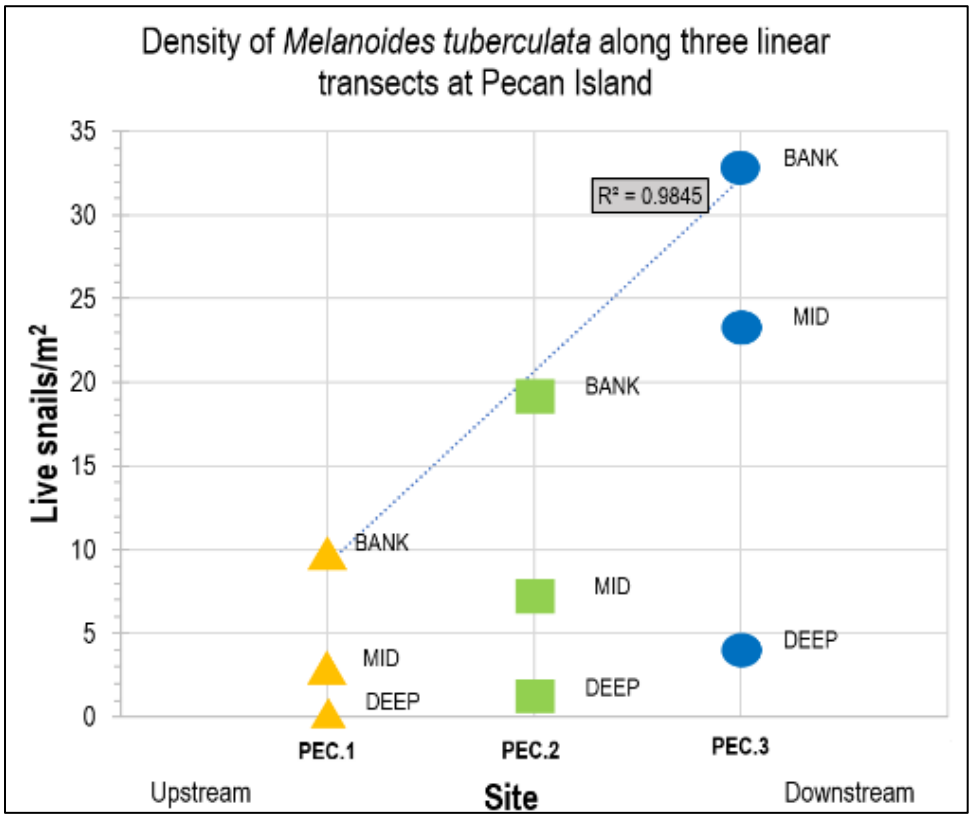

Figure 21. Density of snails was lowest at the most upstream site and highest at the most downstream site. At all sites, density of snails was highest closer to the bank and decreased with distance into the main channel.

Cyprinella venusta

Cyprinella venusta were obtained from SMARC and placed in mesh cages at a density of no more than four fish per cage. Two cages were placed side by side within the larger steel cage (Figure 13), for a total of 6-8 fish per station. Cages were secured to trees and fully submerged in ~1 m of water for the duration of the trial. Fish were recovered after 2 d (*n*=6), 5 d (*n*=6), and 7 d (*n*=9) of exposure to river water (Table 13). This study ran from 17-22 June 2018.

All fish collected from all three stations after 2 d of exposure were infected with *C. formosanus* and *H. pumilio*. All fish collected from stations two and three after 5 d and 7 d of exposure were also infected with both heterophyids. One fish was found dead at the second station after 5 d of exposure, and another was found dead after 7 d exposure. Two fish were found dead at the third station after 7 d of exposure (Table 13).

Fish acquired cysts of both parasites at increasing rates in conjunction with increasing snail densities (Figure 21, Table 12, Table 13). Fish placed in cages at LL.PEC1 with the lowest snail density acquired cysts at a lower rate (*C. formosanus* – 1.8 cysts/d; *H. pumilio* – 3.4 cysts/d) than fish placed in cages at LL.PEC3 with the highest snail density (*C. formosanus* – 12.7 cysts/d; *H. pumilio* – 59.9 cysts/d). Fish placed in cages at LL.PEC2 acquired cysts at a rate (*C. formosanus* – 5.5 cysts/d; *H. pumilio* – 22.6 cysts/d) consistent with its positioning between LL.PEC1 and LL.PEC3 and their respective snail densities (Tables 12, 13).

Table 13. Rates of *C. formosanus* and *H. pumilio* cyst acquisition in *C. venusta* over one week of exposure.

	Centrocestus formosanus												
	2 d of exposure				**5 d of exposure**				**7 d of exposure**				
Station	n	Mn.Int.	SE	Mn.Int./mm	n	Mn.Int.	SE	Mn.Int./mm	n	Mn.Int.	SE	Mn.Int./mm	Cysts/d
PEC1	2	7.00	1.00	0.15	2	7.00	3.00	0.15	4	7.50	0.50	0.16	1.76
PEC2	2	16.0	0.00	0.36	2	18.0	6.00	0.42	2	34.0	16.0	0.86	5.49
PEC3	4	31.0	1.00	0.67	2	86.0	18.0	1.62	3	55.3	9.26	1.30	12.7

	Haplorchis pumilio												
	2 d of exposure				**5 d of exposure**				**7 d of exposure**				
Station	n	Mn.Int.	SE	Mn.Int./mm	n	Mn.Int.	SE	Mn.Int./mm	n	Mn.Int.	SE	Mn.Int./mm	Cysts/d
PEC1	2	4.50	0.50	0.05	2	28.0	3.00	0.59	4	24.00	5.69	0.51	3.43
PEC2	2	57.0	2.00	0.92	2	109.5	24.5	2.56	2	175.5	70.5	4.43	22.6
PEC3	4	142	35.0	3.01	2	310.5	20.5	5.80	3	357.7	128.9	8.21	59.9

Fountain darters

Captive-reared fountain darters were obtained from SMARC and placed in mesh cages at a density of no more than 11 fish per cage. Two cages were placed side by side within the larger steel cage, for a total of 17-21 fish per station. Fish were recovered after 2 d (*n*=30) and 7 d (*n*=26) of exposure to river water. Mean intensity, mean standardized intensity, and cysts/d were calculated for each heterophyid at the three stations (Table 14).

Table 14. Rates of *C. formosanus* and *H. pumilio* cyst acquisition in *E. fonticola* over 1 w of exposure.

	Centrocestus formosanus								
	2 d of exposure				**7 d of exposure**				
Station	*n*	Mn. Int.	SE	Mn. Int./mm	*n*	Mn. Int.	SE	Mn.Int. /mm	Cysts/day
PEC1	11	1.27	0.53	0.05	9	5.33	1.69	0.19	0.69
PEC2	10	2.80	0.70	0.10	9	4.44	1.25	0.16	1.04
PEC3	9	2.44	0.75	0.09	8	7.50	1.10	0.27	1.15

	Haplorchis pumilio								
	2 d of exposure				**7 d of exposure**				
Station	*n*	Mn. Int.	SE	Mn. Int./mm	*n*	Mn. Int.	SE	Mn.Int. /mm	Cysts/day
PEC1	11	2.64	0.30	0.09	9	5.78	1.42	0.20	1.10
PEC2	10	2.33	0.38	0.09	9	7.67	1.13	0.28	1.12
PEC3	9	3.89	0.55	0.14	8	29.9	3.30	1.11	3.04

Alteration of physiological parameters in *C. venusta* exposed to *H. pumilio*

During exposure, treatment fish showed signs of avoidance behavior when placed into 325 mL of cercarial water. Flashing, attempts to escape, and rapid twitching were observed for most of the exposure period of 15 minutes, then stopped abruptly when fish were placed into clean water. A rapid melanophoric response was noted in every treatment individual within 1-6 minutes of initial exposure; within 10 minutes of their first appearance, all of the melanophoric blotches had faded and the fish was uniform in color once again (Figure 23).

All fish that had died overnight (< 12 h post exposure) showed obvious pathology such as hemorrhaging in the tissues of the head and substantial inflammation at the base of the pectoral and caudal fins (Figure 24, Figure 25). Blood glucose levels of fish in the T_0 and T_1 group were normalized using a logarithmic transformation and subjected to a Welch's *t*-test. The means of the T_1 control and treatment groups showed significant

difference ($p < 0.001$), with glucose significantly elevated in fish exposed to cercariae (Figure 22).

Hematocrit readings were not performed for all fish due to the difficulty in acquiring sufficient blood volume for reliable analysis. Thus, hematocrit readings were anecdotally reported for some fish in the T_1 group but were not subjected to analysis (Table 15).

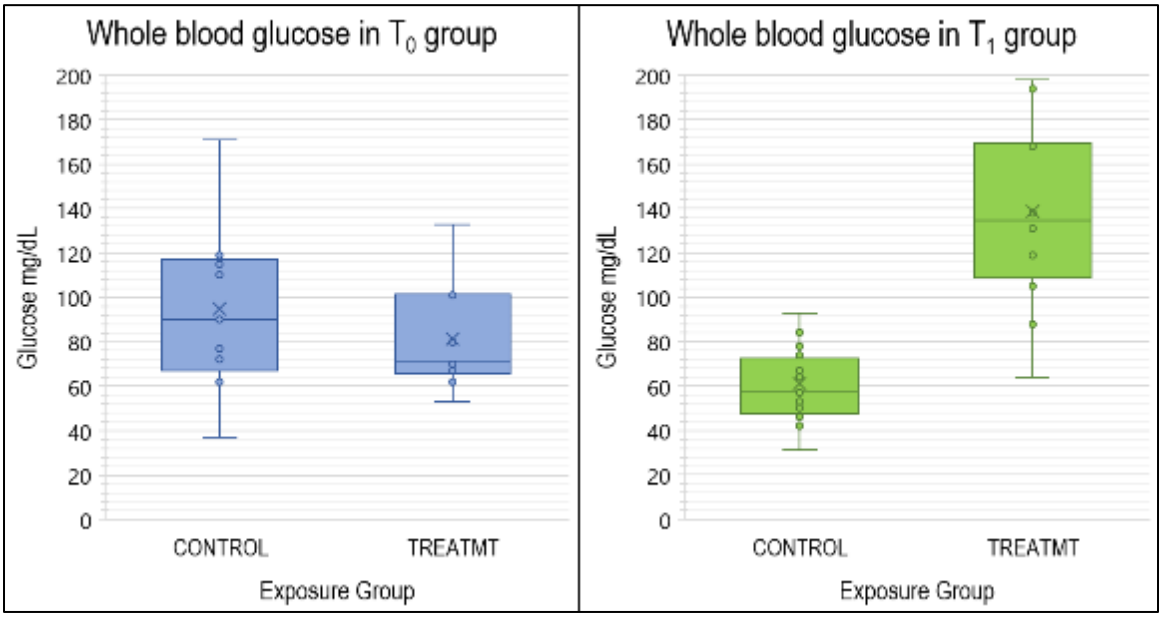

Figure 22. Whole blood glucose was significantly ($p < 0.001$) elevated in treatment fish 12 h after exposure.

Figure 23. Melanophoric response on the epidermis of *C. venusta* exposed to *H. pumilio*.

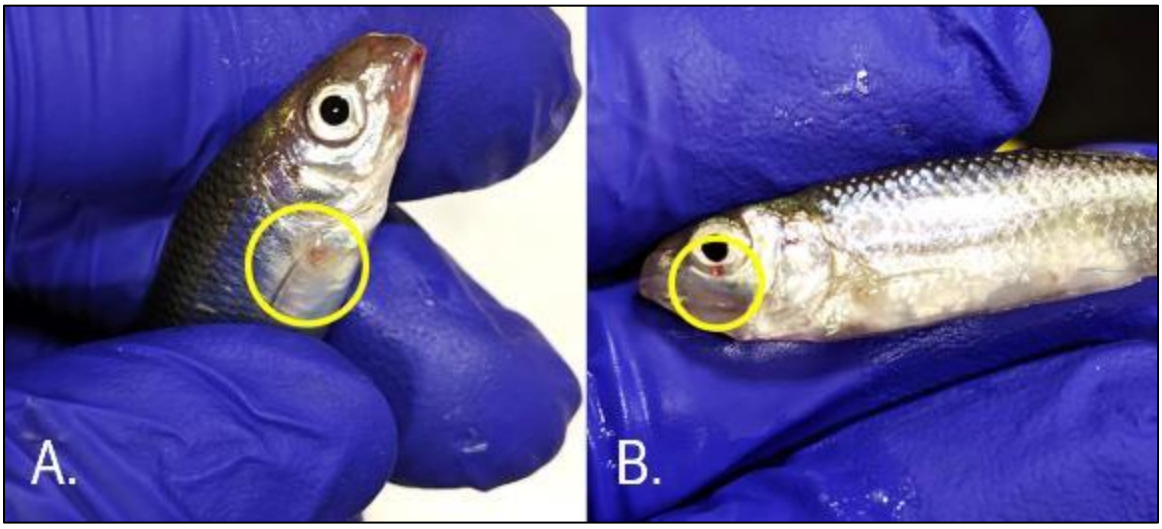

Figure 24. Two individuals from the T_1 treatment group showing acute pathology. (A.) Swelling and visible cysts at the base of the pectoral fin, irritation and hemorrhaging on the face; (B.) hemorrhaging in the eye, swelling around the jaw.

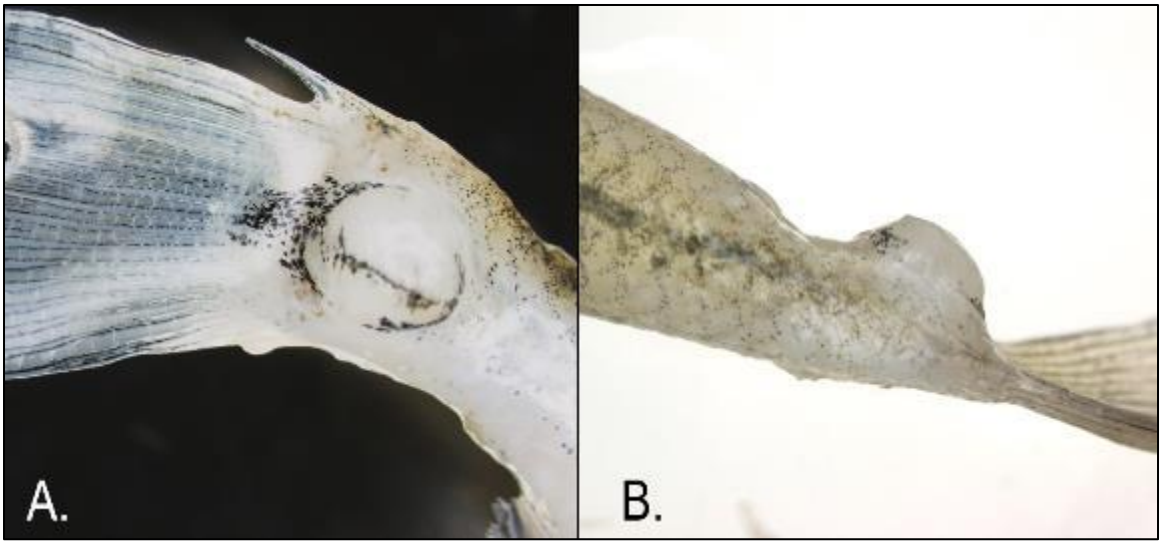

Figure 25. Blister on the caudal peduncle (A., sagittal view; B., dorsal view) of a *C. venusta* from the T_1 treatment group; formed within 12 h of exposure.

Table 15. Blood glucose levels of all fish from both T_0 and T_1 groups.

Time	Group	Rep	Accession	TL mm	Glucose mg/dL[1]	Hct%	Pathology Notes
0	**Control**	**1**	**T0.C1.1**	**55**	**77**		
	Control		**T0.C1.2**	**55.5**	**62**		
	Control		**T0.C1.3**	**58.5**	**90**		
	Treatmt		T0.T1.1	63	67		
	Treatmt		T0.T1.2	40.5	133		
	Treatmt		T0.T1.3	39.5	103		
	Control	**2**	**T0.C2.1**	**56**	**37**		
	Control		**T0.C2.2**	**49.5**	**72**		
	Control		**T0.C2.3**	**37**	**171**		
	Control		**T0.C2.4**	**42**	**110**		
	Treatmt		T0.T2.1	49	70		
	Treatmt		T0.T2.2	62	71		
	Treatmt		T0.T2.3	64	72		
	Control	**3**	**T0.C3.1**	**37**	**115**		
	Control		**T0.C3.2**	**45**	**119**		
	Treatmt		T0.T3.1	67	53		
	Treatmt		T0.T3.2	58.5	101		
	Treatmt		T0.T3.3	51.5	80		
	Treatmt		T0.T3.4	67	62		
1	**Control**	**1**	**T1.C1.1**	**61**	**64**	**20.0%**	
	Control		**T1.C1.2**	**69.5**	**84**	**23.5%**	
	Control		**T1.C1.3**	**55**	**53**	**27.3%**	
	Control		**T1.C1.4**	**48**	**57**		
	Treatmt		T1.T1.1	53.5	170		Blood in eye; CP, PV swollen
	Treatmt		T1.T1.2	63	119	60.6%	Bloody face; jaw/inserts swollen
	Treatmt		T1.T1.3	52	168	72.7%	Bloody jaw; CP swollen
	Control	**2**	**T1.C2.1**	**51.5**	**47**	**35.7%**	
	Control		**T1.C2.2**	**53.5**	**46**		
	Control		**T1.C2.3**	**60**	**53**		
	Control		**T1.C2.4**	**71.5**	**42**	**20.0%**	
	Treatmt		T1.T2.1	56	138		CP, PC swollen; bloody abdomen
	Treatmt		T1.T2.2	78.5	194	66.2%	
	Treatmt		T1.T2.3	59.5	64	5.00%	CP, PC swollen; CP reddened
	Treatmt		T1.T2.4	49	131		
	Control	**3**	**T1.C3.1**	**42**	**31**		
	Control		**T1.C3.2**	**45**	**57**		
	Control		**T1.C3.3**	**44**	**74**		
	Control		**T1.C3.4**	**69**	**84**	**16.9%**	
	Treatmt		T1.T3.1	51.5	121		
	Treatmt		T1.T3.2	56.5	198	35.1%	CP, PC, PV swollen
	Treatmt		T1.T3.3	64	88	11.1%	Bloody isthmus; jaw/inserts swollen
	Treatmt		T1.T3.4	51.5	168		CP, PC swollen; dermatitis
	Control	**4**	**T1.C4.1**	**42**	**67**		
	Control		**T1.C4.2**	**48.5**	**50**		
	Control		**T1.C4.3**	**62**	**59**		
	Control		**T1.C4.4**	**47.5**	**68**		
	Treatmt		T1.T4.1	61	105		
	Control	**5**	**T1.C5.1**	**51.5**	**93**		
	Control		**T1.C5.2**	**60**	**78**		
	Control		**T1.C5.3**	**48.5**	**67**		
	Control		**T1.C5.4**	**88**	**47**		

[1] Mean glucose levels in T_0 control fish = 94.8 mg/dL; mean glucose levels in T_0 treatment fish = 81.2 mg/dL; mean glucose levels in T_1 control fish = 61.1 mg/dL; mean glucose levels in T_1 treatment fish = 138.7 mg/dL.

IV. DISCUSSION

Estimating impacts of Centrocestus formosanus and Haplorchis pumilio

In locations where *Centrocestus formosanus* infections have been confirmed in snails or fish, infections by *Haplorchis pumilio* almost always soon follow. This phenomenon is observable in the historical documentation of the heterophyids in the SMR and CR (Table 3). Historical prevalence data reveal a tendency of *C. formosanus* and *H. pumilio* to shift dominance within a system over time. In Landa Lake, *C. formosanus* was previously more prevalent in snails than *H. pumilio*; however, current surveys show that prevalence in snails has shifted in favor of *H. pumilio* (Clements 2018). In the SMR, current prevalence of *C. formosanus* and *H. pumilio* in fountain darters are comparable (USFWS 2016). Regardless of which species arrives first, the decrease in host fitness that results from co-infection of the host individual benefits both parasites equally – both heterophyids rely on the same first- and second-intermediate hosts and rely on piscivores to spread. Therefore, when conditions are ideal for one parasite, they are ideal for the other as well. Indeed, any time *C. formosanus* is attributed with being the cause of a mass fish kill in high-density aquaculture facilities (Mohan et al. 1999, Ortega et al. 2009, Leibowitz et al. 2019), it would be prudent for managers to also look for *H. pumilio*, since both heterophyids rely on *M. tuberculata* as a first-intermediate host and since co-infection is frequently observed in wild-caught fish.

Despite the lethal effects on some fish following experimental exposure to cercariae of *H. pumilio* or *C. formosanus*, and the documented occurrence of mass mortalities in high-density fish farms, mass fish kills have not been reported in wild fisheries, even in areas where the density of cercariae in the water column are moderate

to high. However, collecting fish from natural habitats is not necessarily reflective of mortalities occurring within the population, as the collection contains only those fish that have survived thus far, and does not allow estimation of mortality that may be occurring as a direct or indirect result of parasitism (Brassard et al. 1982, Cantu et al. 2013). Fleming et al. (2011) estimated *C. formosanus* parasitism levels in *Cyprinella venusta* from the confluence of the Comal and Guadalupe Rivers through necropsy of wild-caught individuals and found that this method underestimates – as much as 16 X – the rate at which caged *C. venusta* acquired *C. formosanus* at the same time at the same locality. This discrepancy is consistent with the findings of Cantu et al. (2013), who found that estimates of cercarial density determined through cercariometry were consistent with caged-fish estimates, but that infection pressures estimated from wild-caught fish surveys underestimated the rates at which fish were acquiring parasites (Cantu et al. 2013).

Prevalence and intensity estimates from wild-caught fish collected during our study reflect this same disparity. Rates of parasite acquisition determined by the caged-fish study conducted at Faust Street Bridge Crossing (Figure 3) suggest that the wild-caught cyprinids we collected had not lived in those waters all their lives. Fish the size of the ones we collected would have acquired substantially more intense infections if they were consistently exposed to the same infection pressures as the caged-fish placed in the same area; while wild-caught adult fish had a mean standardized intensity of 0.44 *C. formosanus* and 0.23 *H. pumilio* cysts, caged fish had a mean standardized intensity of 0.09 *C. formosanus* and 0.17 *H. pumilio* cysts after just one week of exposure. Given constant rates of acquisition over a one-year period – despite the unlikelihood of perfectly constant cercarial pressures – a 40 cm fish living in the area would acquire approximately

299 *C. formosanus* cysts and 745 *H. pumilio* cysts (7.48 and 18.63 cysts/mm, respectively). Experimental infections conducted by Clements (2018) suggest that swimming performance is significantly impaired as intensity of infection increases, and that a steep increase in mortality occurs in *C. venusta* with intensities higher than 15-20 cysts/mm (Clements 2018). If infection levels approached these intensities in nature, these fish would likely die either by infection effects or predation (Brassard et al. 1982, Clements 2018) before being collected, biasing not only prevalence but also mean intensities of infection within a system.

It should be noted, however, that the above scenarios – projecting from, for instance, what happens to a caged fish with restricted mobility versus what happens to a fish with unrestrained mobility – do not reflect realistic scenarios, and that infection dynamics are more complicated in natural environments. Parameters such as flow conditions, precipitation, flooding events, disturbance, and various species-specific attributes of fish hosts should be considered when estimating effects of parasitism on wild fish. The source of caged fish and the degree of stress (e.g., novel pathogens, temperature variability, and foraging ability) which those fish are subjected to by being placed in the river should also be considered. These factors may influence the rate and severity of infection, given the significant effects of infection by *H. pumilio* on blood glucose (Figure 22) and the general physiology of stress, especially the tendency of physiological stress to escalate (from secondary to tertiary to quaternary effects), where fitness may be compromised and population effects may ultimately manifest if the magnitude of the imposed stress is not mitigated (Romero 2004, Pankhurst 2011, Britton 2013).

Experimental infections have confirmed that exposing fish to high densities of heterophyid cercariae can cause rapid-onset mortality (Sommerville 1982b, McDonald et al. 2006, Huston et al. 2014, Clements 2018). Infection with *H. pumilio* results in significantly impaired swimming performance and endurance in *Cyprinella venusta* and *Dionda diaboli* (Clements 2018), and while high intensity infections had significant detrimental effects on *C. venusta*, the effects were more pronounced on the smaller, endemic *D. diaboli* (Clements 2018). Clements' (2018) experimentally infected test fish had acquired their infections at substantially faster rates than would be encountered even in natural waters with high densities of cercariae (McDonald et al. 2006, Clements 2018). The total metacercarial intensities in the tested fish were based on estimated accumulation of metacercariae by a fish remaining in water where infected snails occur over the span of 1 y; however, it should be noted that this study did not take into account the effects of acute trauma or physiological shock from rapid cercarial overdose (Farrell and Lloyd 1962).

Farrell and Lloyd (1962) suggest that fish in wild systems are capable of withstanding daily exposure to low concentrations of parasites without causing acute trauma to the host; low but constant doses of cercariae can lead to the fish host accumulating large numbers of metacercarial cysts over time, making the host reaction more chronic than acute. By comparison, fish which are exposed to the same number of cercariae all at once instead of over time may not be able to tolerate such a substantial acute reaction (Farrell and Lloyd 1962). Therefore, experimental studies that reflect natural infection pressures are the most accurate and realistic method of exploring the

effects that infection by *C. formosanus* and *H. pumilio* may be having on wild fish (Baldwin et al. 1967).

Previous research has examined the time to death of three age classes of fountain darters exposed to *C. formosanus* (McDonald et al. 2006). However, the cercarial concentrations used were higher than any naturally occurring concentrations – about 550,000 cercariae/L. In comparison, Cantu et al. (2003) detected a concentration of 4.3 cercariae/L, with a maximum concentration of 45 cercariae/L, in water from the SMR, as determined through cercariometry. It is unlikely that fish living in a natural system would be exposed to concentrations as high as those used by McDonald et al. (2006), at least not under normal conditions. Historical records confirm that drought conditions have the potential to affect the water volume and flow conditions occurring in the San Marcos and Comal Springs (USGS 1958, Guyton 1979). The ability of *Melanoides tuberculata* to withstand harsh environmental conditions suggests that drought conditions would likely not immediately affect their survivorship or population sizes. For fish sharing habitat with infected snails, though, drought conditions could exacerbate infection pressures and effects, especially for more pelagic fish such as the Devils River minnow, which has already experienced severe fragmentation of its habitat (Garrett et al. 2004, Robertson et al. 2016). Reduction in water volume (while the number of snails remains constant) would mean increased cercarial concentration, resulting in increased likelihood of infection, and potentially increased infection intensities.

Life history and infection effects

The life history of a fish host affects how parasites impact the host's ability to swim and respire. *C. venusta* swims near constantly, meaning the fish has a large amount of oxygenated water moving across its gills at any given time, and could passively respire while swimming or maintaining station in an area of high flow (Clements 2018). Fountain darters, comparatively, spend most of their time resting on the bottom and must actively pump oxygenated water – which may also contain cercariae – across their gills via opercular movement. The process of respiration may require more energy in darters than in cyprinids, and this energetic demand almost certainly increases if the gills are clogged with cysts and the surface area where gas exchange occurs is reduced (Mitchell and Goodwin 2004). Clements (2018) assessed the swimming performance of *C. venusta* infected with *C. formosanus* and found that swimming performance, a reliable indicator of stress effects at the tertiary level, was not noticeably compromised. However, other research has demonstrated that infecting the gills of rainbow darters (*Etheostoma caeruleum*, Storer 1845) with bivalve glochidia resulted in increased respiration rates, reduced foraging activity, reduced growth rate, and increased susceptibility to predation (Crane et al. 2011). Histological examination of darter gills infected with *C. formosanus* has revealed cartilaginous cell proliferation around the metacercarial cysts as well as hyperplasia, hypertrophy, and fusion of lamellae on infected gill arches (McDonald et al. 2006), and consequent distortion of the gill architecture and reduction in surface areas where gas exchange occurs (Mitchell and Goodwin 2004).

Based on rates of parasite acquisition occurring at Pecan Island, fountain darters are not as susceptible to rapid infection by *C. formosanus* or *H. pumilio* as are cyprinids

(Figure 26). At the same stations, cyprinids acquired *C. formosanus* cysts at a rate 5-11 X the rate which fountain darters acquired them, and *H. pumilio* cysts as high as nearly 20 X the rate they were acquired by fountain darters (Figure 26). Cercariae of *C. formosanus* are positively phototactic (Cantu 2003), and though phototactic behavior has not been confirmed in cercariae of *H. pumilio*, Veerappan and Achuthan (1992) documented phototaxis in cercariae of an unidentified species belonging to the genus *Haplorchis*. This documentation, coupled with the tendency we have observed of *H. pumilio* cercariae to aggregate near the top of the water column within a test tube, suggests that phototaxis may play a role in the life history strategies of both *C. formosanus* and *H. pumilio*. Further, the disparity in infection rates in fountain darters versus cyprinids when placed in cages at Pecan Island suggests that there is a natural mechanism protecting fountain darters from such high rates of infection. Most likely, this disparity is due to the bottom-dwelling nature of fountain darters versus the pelagic nature of *C. venusta*. If cercariae of both parasites are positively phototactic, then fish occupying higher levels of the water column would be exposed to greater cercarial densities than fish dwelling on the river bottom, and therefore would be predisposed to greater infection pressures than bottom-dwelling fish such as fountain darters or other percids.

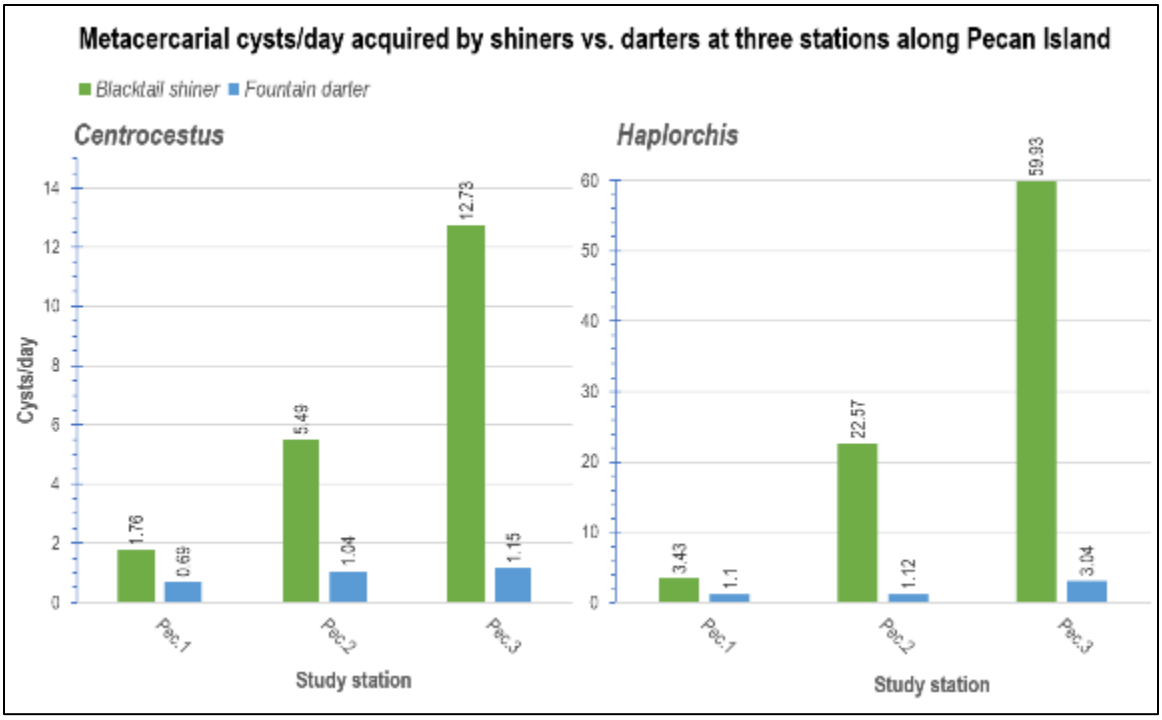

Figure 26. Cyprinids acquired metacercarial cysts of both heterophyids at rates higher than those observed in fountain darters placed in cages at the same stations.

The disparity in standardized intensities between cyprinids and Rio Grande darters collected from Finegan Springs (Figure 27) suggests that Rio Grande darters are not as predisposed to high intensity infections as cyprinids living in the same area. This is likely due to the tendency of Rio Grande darters to inhabit shallow, fast-flowing riffles directly below spring outflows, where they are less exposed to downstream effects of snails shedding cercariae; comparatively, the cyprinids we collected from this area were obtained from deeper channels and pools that either contained *Melanoides tuberculata* or were located downstream of snails. Thus, Rio Grande darters living directly below spring outflow are not as subject to the effects of *M. tuberculata* as more pelagic fish within the DR system, including *Dionda diaboli* and *Cyprinella proserpina*.

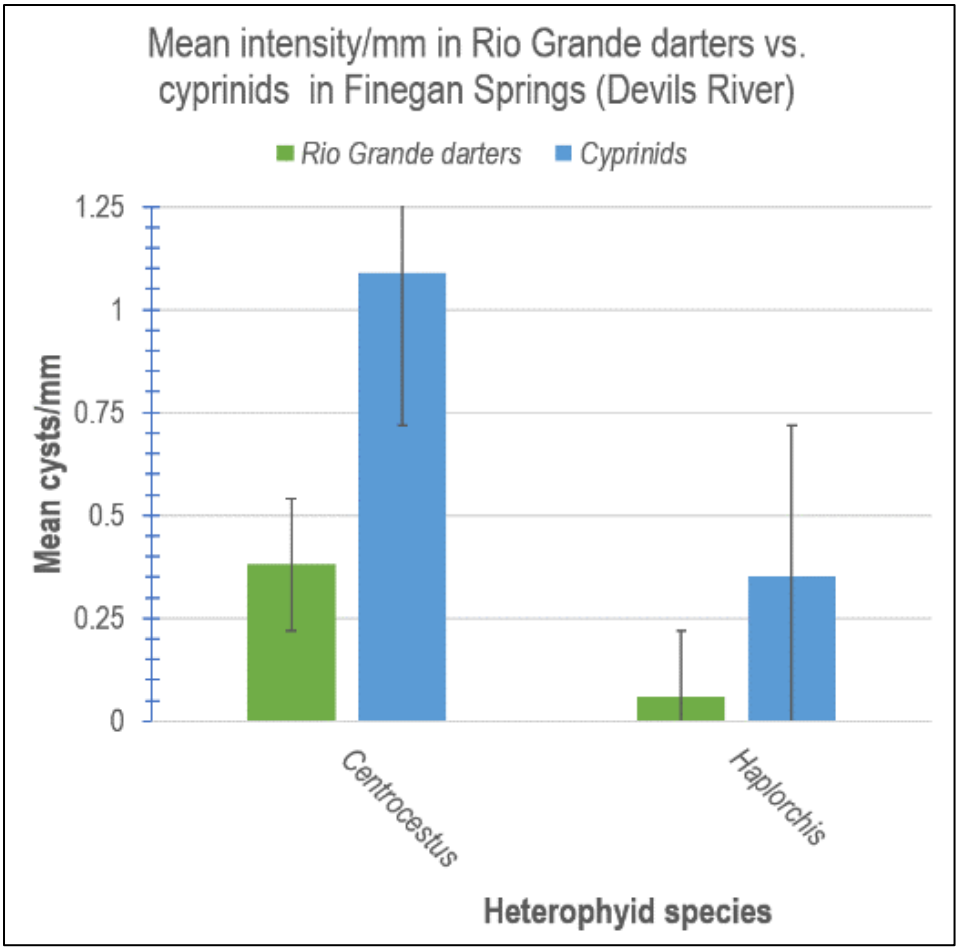

Figure 27. Standardized intensities of heterophyid infection were higher in cyprinids than in Rio Grande darters collected from Finegan Springs.

Physiological consequences of infection

Significantly elevated blood glucose levels (Figure 22) in fish exposed to *H. pumilio* cercariae indicate that infected fish (*Cyprinella venusta*) are placed under physiological stress following exposure to cercariae of *H. pumilio* (Table 15). This metabolic shift may destabilize the energetics of infected fish and divert energy away from processes such as growth or reproduction (Lamkova et al. 2007, Britton et al. 2011, Slavik et al. 2017). Given the high incidence of co-infection by both parasites, and the reported effects of each parasite separately, fish hosts which are concurrently exposed to

the pressures of both *C. formosanus* and *H. pumilio* may exhibit even greater disruptions to physiological homeostasis and, potentially, a compounded reduction in fitness. Future physiological research should analyze the effects of infection and co-infection in imperiled species such as the fountain darter and Devils River minnow. Such an experiment would provide a foundational understanding of the species-specific effects of each parasite, as well as tease out any additive or multiplicative effects that may occur as a result of co-infection, as often occurs in naturally-infected fish.

Management and conservation implications

Increased density of live snails is correlated with increased rates of cercarial acquisition and increased intensities of infection in fish (Figure 21, Figure 26). Therefore, mechanical snail removal programs may be the simplest, most cost-effective means of mitigating infection by both parasites in a river system. It is unfeasible that all snails could be removed from an entire system by mechanical removal surveys alone; however, reducing the number of larger individuals may produce measurable decreases in the rates of parasitism in fish, since snails > 2 cm shed cercariae at appreciably higher rates than smaller individuals (Mitchell and Goodwin 2004).

Cercariometry of river water provides a valuable snapshot of cercarial concentrations at the time of sampling. However, because of the dynamic interaction between factors affecting cercarial concentrations, all cercariometry must be corrected for discharge and expressed in the form of total cercariae produced per hour. At the time of this writing, the Edwards Aquifer Authority has recently expanded a cercariometry-based monitoring program for *C. formosanus* to also include monitoring for *H. pumilio* in the

CR (EAAHCP 2019a), at little extra cost since both heterophyids use the same snail host and almost always occur together within a system. Additionally, other entities have been monitoring the presence of cysts of *H. pumilio* in wild-caught fountain darters and made their data available to us for the purposes of this study (USFWS 2016).

In addition to the monitoring programs already in place, we propose that the most accurate and cost-effective method of monitoring the effects of cercarial levels in river systems that are home to sensitive fishes is caged-fish studies using captive-bred individuals of the target species. Specifically, we recommend that caged-fish studies be conducted concurrently with cercariometry surveys to thoroughly assess the infection pressures on fish in local systems and to evaluate any correlations between cercarial density determined through cercariometry and infection rates occurring in fish placed in cages immediately adjacent to the cercariometry collection site. Caged-fish estimates are not perfect reflections of natural conditions; the caged fish are unable to move far and may be prone to stress effects due to the change in environment from hatchery conditions to a natural system. However, estimates from caged-fish studies provide the most direct path to quantifying the relative health of fish living in the area where the cage is placed, and could be conducted by monitoring agencies at little extra cost to the costs of cercariometry- and fish-collection-based programs already in place.

The Devils River system is one of the last remaining relatively pristine spring systems in Texas; despite this, we observed high prevalence of heterophyid infections in multiple fish species collected from multiple sites (Figure 4). McDermott et al. (2014) reported increasing populations of *M. tuberculata* (from 1999 to 2011) and high prevalence of *C. formosanus* infections in fish in West Texas spring systems, including

the Devils River. Given the high prevalence and intensities of *H. pumilio* infection we discovered in the DR, historical rates of infection would provide valuable data on the status and dynamics of parasitism in the DR and allow for better management of the sensitive fishes occupying the system. Caged-fish studies should also be performed at several sites in the DR, with focus on the rates of parasite acquisition occurring in ideal habitats of endangered or otherwise vulnerable fishes living in the system.

In the case of fountain darters, no alarming declines in natural population numbers have been reported as of the time of this writing (EAAHCP 2019b). Given that we have documented *H. pumilio* in fountain darters as far back as September 2000, the parasite is not a novel threat, and fountain darters apparently reproduce at high enough rates to compensate for mortalities occurring directly or indirectly from parasitism by *C. formosanus* and *H. pumilio,* even in relatively low flow conditions (Mora et al. 2013, Nichols 2015, Olsen et al. 2016). Monitoring the status of infections in fountain darters in Spring Lake is critical to maintaining a healthy source population, especially if an influx of *M. tuberculata* occurs and establishes snails in the lake. *Dionda diaboli,* however, has declined in abundance due to factors such as habitat fragmentation and decreased stream flow (Anderson et al. 1995, Garrett et al. 2004), and certain populations already isolated enough to be considered genetically distinct may be threatened with extinction due to groundwater pumping and drought conditions (Schlechte and Fleming 2015, Robertson et al. 2016). Combined with these effects, the added stress of increasing snail densities and high infection prevalence may impose an added burden on the wellbeing of the individuals making up the already-threatened populations, thus potentially jeopardizing

the continued health of the sub-populations that make up the remainder of the species in Texas (Romero 2004, Pankhurst 2011, Britton 2013, Schlechte and Fleming 2015).

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