HUFFMANELA HUFFMANI: LIFE CYCLE, NATURAL HISTORY, AND BIOGEOGRAPHY

by

McLean Worsham, B.S.

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Committee Members:

David Huffman, Chair

Chris Nice

Randy Gibson

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1. THE AQUATIC ANNELID FAUNA OF THE SAN MARCOS RIVER HEADSPRINGS, HAYS COUNTY, TEXAS

Introduction

The San Marcos River, Hays County, Texas is a spring fed river supplied with physicochemically stable water from the Edwards Aquifer (Crow, 2012; Groeger et al., 1997; Musgrove & Crow, 2012). The headsprings are found on the campus of Texas State University at the bottom of a small artificial reservoir known as Spring Lake, which was impounded by a low head dam in 1849. Spring Lake and the upper 2 or 3 km of the spring run supports a rich biotic community. At present there are four species that are federally protected, threatened, or endangered, with some other endemics worthy of such recognition. Circumstances associated with the biogeographic history of the headsprings no doubt contributed to the evolution of these unique and endemic invertebrates. Therefore, endemism is high for some of the invertebrate taxa; especially taxa that are poor dispersers and that have long inhabited the San Marcos River (SMR).

The first studies of invertebrates from the SMR and nearby springs issuing from the Edwards Aquifer led to the description of several new stygobionts (Benedict, 1896; Bowman & Longley, 1976; Hershler & Longley, 1986; Holsinger, 1966; Holsinger & Longley, 1980; Ulrich, 1902) with little attention paid to epigean invertebrate species. The first study on epigean invertebrates only reported on tricopterans, and not surprisingly, this study led to the description of a new species, *Protoptila arca* (Edwards & Arnold, 1961), which was determined to be a San Marcos endemic. Thirty additional species of tricopterans were later reported from the San Marcos (Bowles et al., 2007).

More recent surveys reported additional species from the SMR and associated springs (Gibson et al., 2008; Hutchins et al.; 2013, Martin, 1951).

The study of the diet of the federally protected fountain darter, *Etheostoma fonticola*, (Jordan & Gilbert, 1886) from the SMR was the first study to report on epigean invertebrates other than tricopterans, but this diet study only reported on hard-bodied invertebrates (e.g. mollusks and arthropods) and the recovered specimens were only identified to order (Schenck & Whiteside, 1977). Despite low taxonomic resolution, the findings from this study suggest a remarkable amount of diversity, with twelve separate orders reported from gut contents. This diversity was verified by a subsequent diet study of the San Marcos salamander, *Eurycea nana* Bishop, 1941, whereby numerous taxa (also largely hard-bodied forms), were reported from the SMR for the first time (Diaz, 2010).

At the time of this writing, the only reports of free-living soft-bodied invertebrates from the SMR was mention of a stygobiontic platyhelminth and a stygobiontic hirudinean (Hershler & Longley, 1986), and the documentation of the first gastrotrich of the genus *Redudasys* (Gastrotricha: Macrodasyida) in the Northern Hemisphere (Kånneby & Wicksten, 2014). Reported herein is the first survey of annelids from the SMR, with notes on other free living vermiform fauna, including the first report and a new distribution record for a nemertean. This report adds several species to the ever-growing list of invertebrate taxa reported from the San Marcos Springs (SMS) and SMR. Several of these appear to be undescribed taxa that likely have a restricted distribution to the physicochemically stable spring run.

Materials and methods

Invertebrates were collected from January 2013 to August 2014. Several sampling methods were utilized, including a Ponar grab sampler, placement of nets over spring outflows, baited traps, dip netting of vegetation and substrate, and SCUBA diving with suction devices. All collected organisms were transported live to the Texas State University Aquatic Biology Station. Specimens were examined under a dissecting and/or compound light microscope and were identified to lowest possible taxon using the most recent literature (Brinkhurst, 1964; Brinkhurst & Jamieson, 1971; Harman, 1973; Hiltunen & Klemm, 1980; Pinder, 2010; Spencer, 1978).

Results

Taxa collected

Regarding annelids, at least 3 species of epigean Hirudinea, 2 species of Aphanoneura, 1 species of Branchiobdellida, and 16 species of clitellate Oligochaetes are present in the SMR. Additionally, at least 3 species of free-living Platyhelminthes and 1 species of Nemertea are present in the SMR. The species of Nemertea is the first record of the phylum from the SMR, though this phylum has been documented elsewhere in the Guadalupe drainage basin (Ourso & Hornig, 2000). See Table 1 for list of all vermiform taxa identified in this study.

Table 1. List of annelid and other vermiform taxa collected.

Phylum	Class	Subclass	Order	Family	Genus/species	Describer
Annelida	Clitellata	Hirudinea	Arhynchobdellida	Erpobdellidae[1]		
Annelida	Clitellata	Hirudinea	Rhynchobdellida	Piscicolidae		
Annelida	Clitellata	Hirudinea	Rhynchobdellida	Glossiphoniidae	*Placobdella parasitica*	Say
Annelida	Clitellata	Hirudinea	Branchiobdellida	Cambarincolidae		
Annelida	Clitellata	Oligochaeta	Lumbriculida	Lumbriculidae	Lumbriculidae sp_1	
Annelida	Clitellata	Oligochaeta	Lumbriculida	Lumbriculidae	Lumbriculidae sp_2	
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*Stylaria cf. lacustris*	Linnaeus
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*Chaetogaster cf. limnaei*	von Baer
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*C. cf. diastrophus*	Gruithuisen
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*C. cf. crystallinus*	Vejdovsky
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*Pristina* sp.	
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*Nais* sp.	
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*Dero* (*Dero*) *cf. obtusa*	d'Udekem
Annelida	Clitellata	Oligochaeta	Haplotaxida	Naididae	*D. (Aulophorus) cf. furcatus*	Müller
Annelida	Clitellata	Oligochaeta	Haplotaxida	Haplotaxidae	*Haplotaxis* sp.	
Annelida	Aphanoneura			Aeolosomatidae	*Aeolosoma cf. variegatum*	Vejdovský
Annelida	Aphanoneura			Aeolosomatidae	*A. cf. quarternarium*	Ehrbg
Platyhelminthes	Turbellaria		Tricladida	Dugesiidae	*Schmidtea* sp.	
Platyhelminthes	Turbellaria		Tricladida	Dugesiidae	*Dugesia* sp.	
Platyhelminthes	Rhabditophora		Seriata	Kenkiidae	*Sphalloplana mohri*[2]	Hyman
Nemertea	Enopla		Hoplonemertea	Tetrastemmatidae		Böhmig

[1] Both a stygobiotic and epigean species were collected.
[2] This species was not collected by the authors but was included for completeness.

Dichotomous key to Annelida of San Marcos Springs

1a Parasitic or commensal 2
1b Free-living.....5

2a (1a) Chaetae absent 3
2b Chaetae present (commensal on gastropods (in mantle cavity); body usually quite small, <4 mm) ..... ***Chaetogaster cf. limnaei***

3a (2a) Parasitic on exterior of vertebrates... 4
3b Parasitic on exterior of crayfish of Family Cambaridae (Figure 1) .....Order Branchiobdellida (Family **Cambarincolidae**)

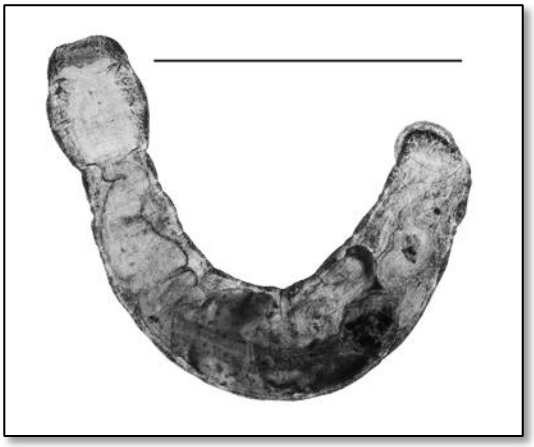

Figure 1. Branchiobdellida from Cambaridae (scale 1 mm).

4a (3a) Parasitic on fishes; anterior sucker about half the diameter of caudal sucker; body small (<2.5 cm).....Family **Piscicolidae**
4b Usually parasitic on turtles; body quite large, (>2.5 cm) ***Placobdella parasitica***

5a (1b) Chaetae present 6
5b Chaetae absent; caudal sucker well developed into pedestal .....Family **Erpobdellidae**

6a (5a) Dorsal chaetae absent (at least on 10 or more anterior segments).....7
6b Dorsal chaetae present (Figure 2) 9

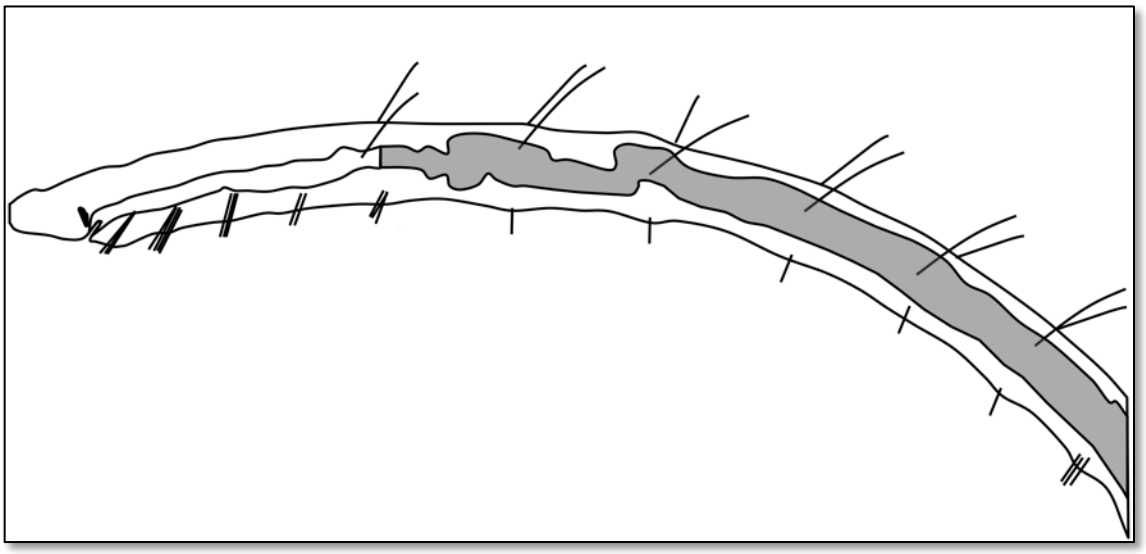

Figure 2. Drawing of generic aquatic annelid showing example positions of dorsal and ventral chaetae.

7a (6a) Ventral chaetae bifid and at least three and up to nine per bundle (Figure 3) 8

7b Ventral chaetae 1 per bundle with simple point and tip curved towards posterior of worm (Figure 4); worm elongate, up to 10 cm or more in length, but usually 4-5 cm.....***Haplotaxis*** sp.

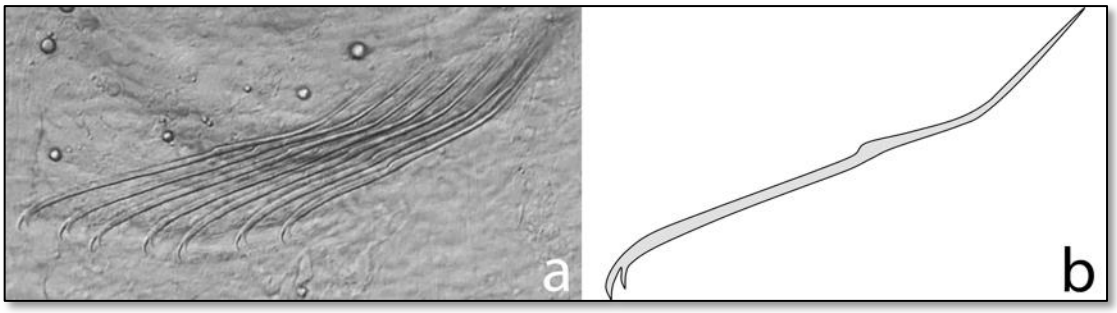

Figure 3. *Chaetogaster*: a) – photograph of typical bifid ventral chaetae bundle; b) – drawing showing shape of one chaeta.

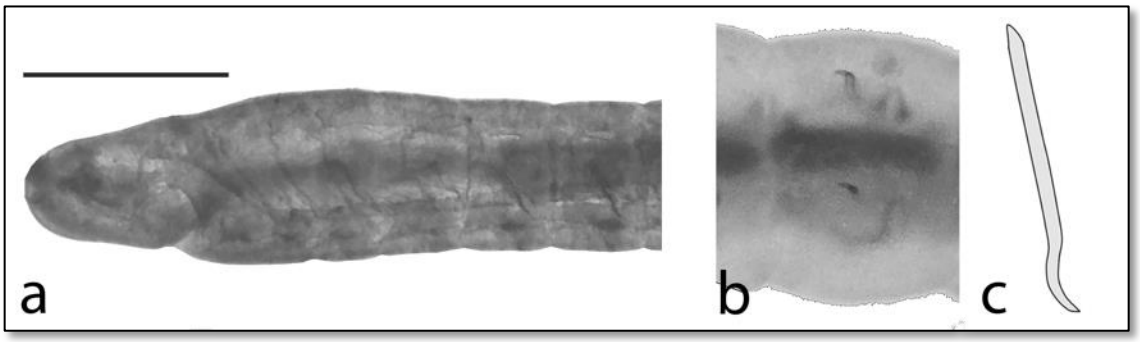

Figure 4. *Haplotaxis* sp.; a) – lateral view of anterior end showing prostomium and ventral mouth (scale 750 µm); b) – ventral view of one segment showing singlet ventral chaetae; c) – drawing of one ventral chaeta.

8a (7a) Prostomium more conspicuous than other *Chaetogaster* spp.; only ventral chaetae present; worm usually small, total length <4 mm (Figure 5) ..... ***Chaetogaster cf. diastrophus***

8b Prostomium inconspicuous with cleft (Figure 6); numerous chaetae per posterior ventral bundles; worm usually relatively large, with total length ≥ 4 mm .....***Chaetogaster cf. crystallinus***

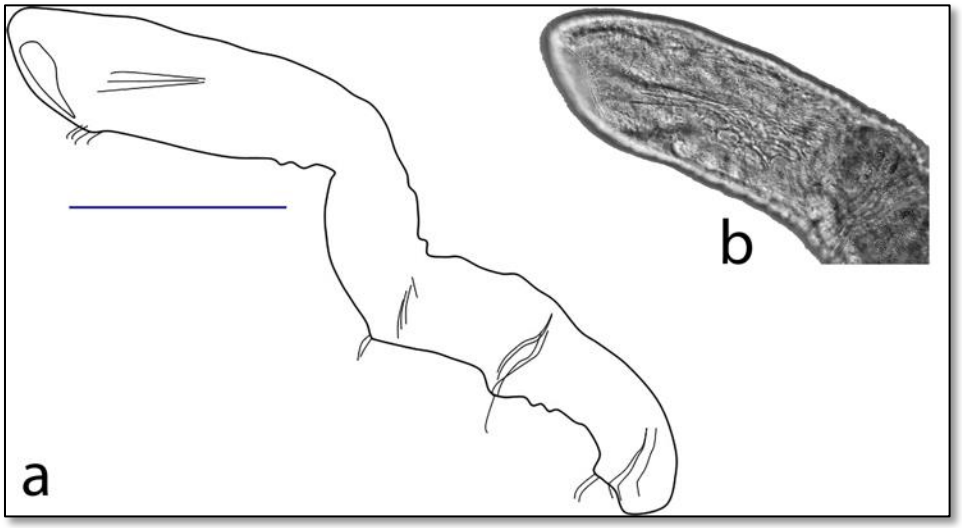

Figure 5. *Chaetogaster diastrophus*: a) – drawing of entire body (scale 250 µm); b) – photo of anterior end showing prostomium over mouth.

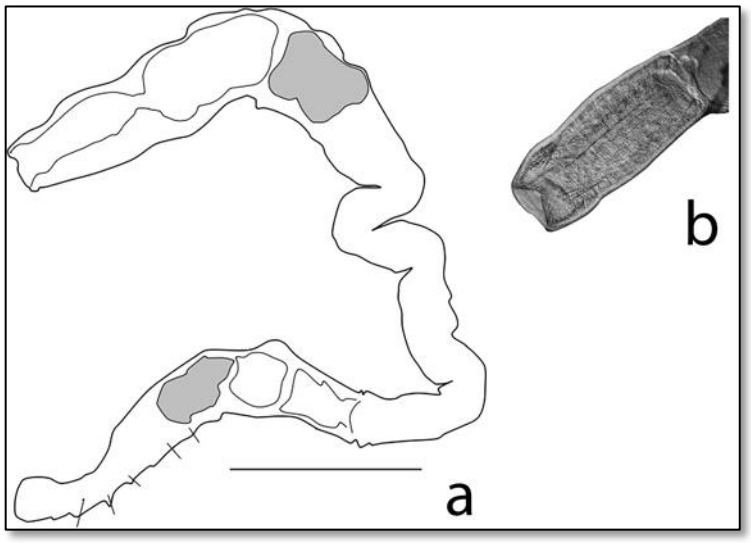

Figure 6. *Chaetogaster cf. crystallinus*: a) – outline drawing of entire body showing positions of only chaetae (scale 1 mm); b) – photo of anterior end showing cleft in prostomium.

9a (6b) Dorsal chaetae usually more than 1 per bundle and found on anterior portions of worm.....10

9b Dorsal chaetae short, only 1 per bundle, only found on posterior of worm; ventral chaetae 1 per bundle with simple point curved posteriad; worm elongate, up to 10 cm long, usually 4-5 cm.....***Haplotaxis*** sp.

10a (9a) Ventral chaetae two per bundle and with simple point (Figure 7); worm usually quite large, total length >3 cm 11

10b Ventral chaetae bifid, more than two per bundle with usually 3-9 per bundle in most species (Figure 8) 12

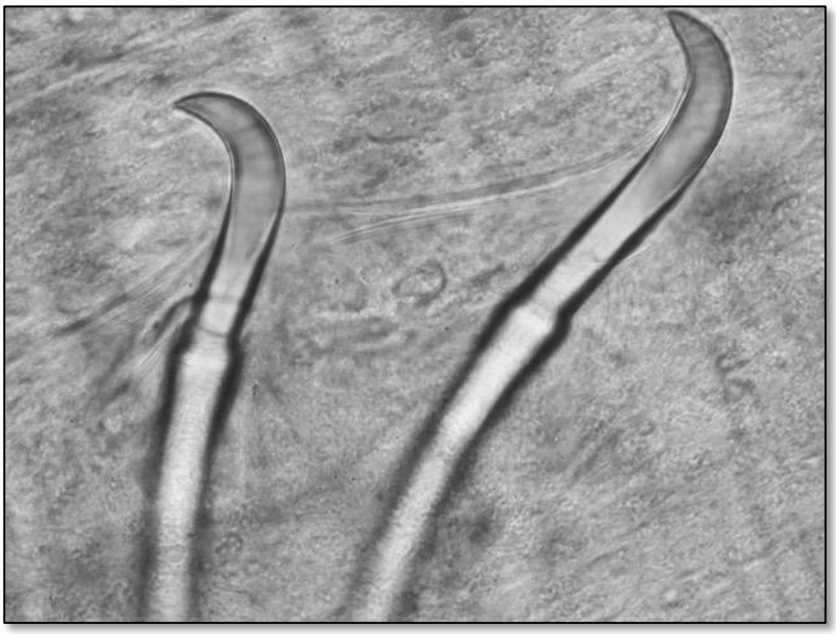

Figure 7. Paired chaetae typical of both dorsal and ventral bundles found on lumbriculids.

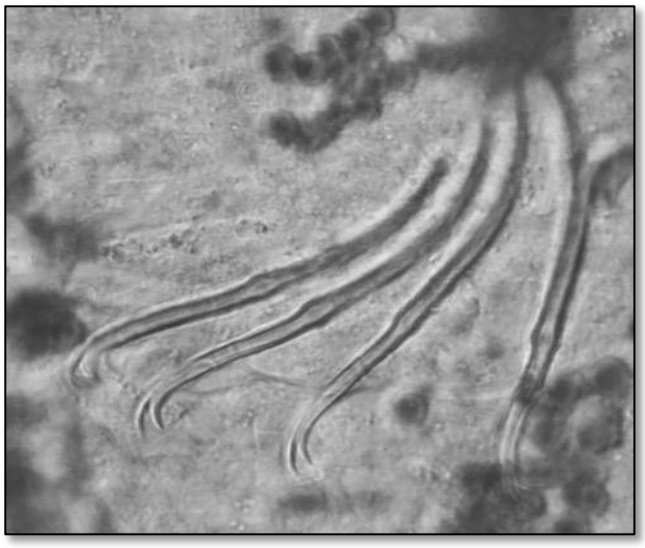

Figure 8. Multiple bifid ventral chaetae.

1a (10a) Prostomium modified into elongated proboscis (Figure 9) ..... **Lumbriculidae sp$_1$** (may be two species)

11b Prostomium inconspicuous and without proboscis (Figure 10) ..... **Lumbriculidae sp$_2$** (may be two species)

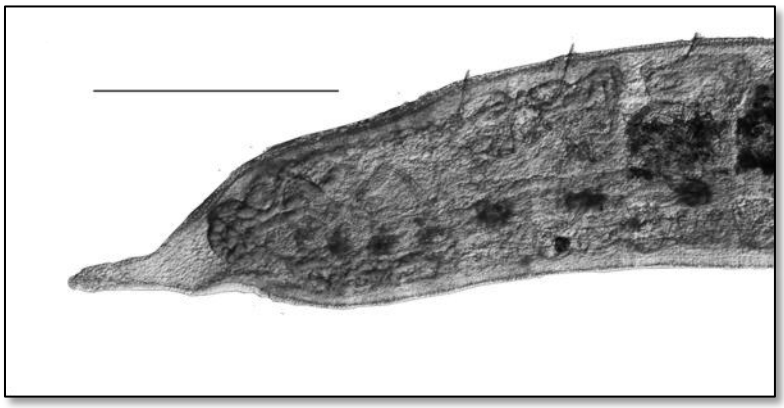

Figure 9. Lumbricullidae sp_1: lateral photo of anterior end showing prostomium with conspicuous proboscis (scale 500 µm).

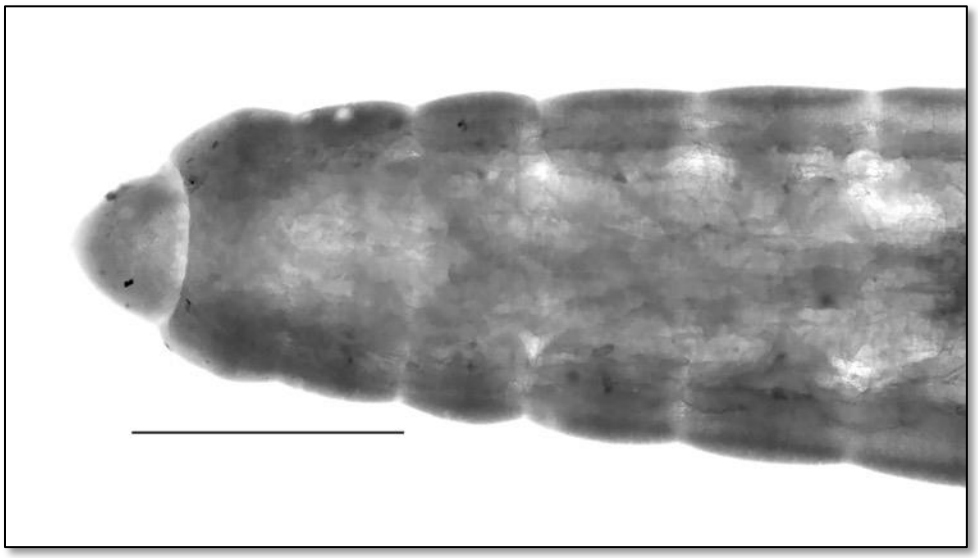

Figure 10. Lumbriculidae sp_2: photo showing inconspicuous prostomium.

12a (10b) Gills present on posterior end (digitiform projections; in some cases inconspicuous) 13
12b Posterior end without gills 14

13a (12a) Gill fossa with two long parallel accessory palps (Figure 11) ..... ***Dero* (*Aulophorus*) *furcatus***
13b Gill fossa not prolonged, often continuous with gills (Figure 12) .....***Dero* (*Dero*) *obtuse***

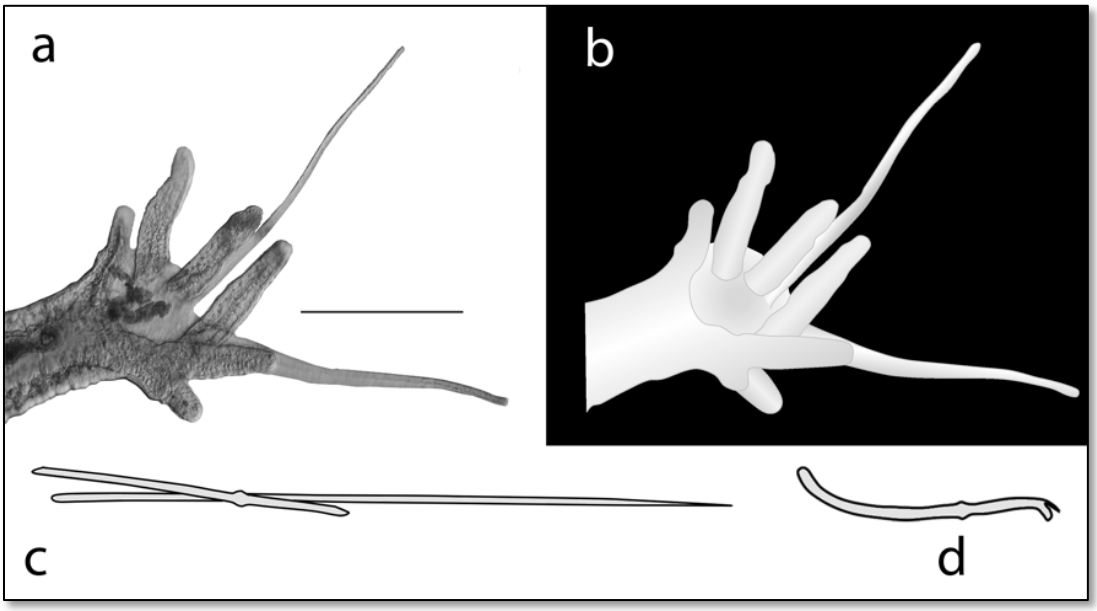

Figure 11. *Dero* (*Aulophorus*) *furcatus*: a) – photo of posterior end showing digitiform gills and elongate palps (scale 250 µm); b) – drawing of a); c) – drawing of typical chaetae bundle; d) – drawing of typical ventral chaeta.

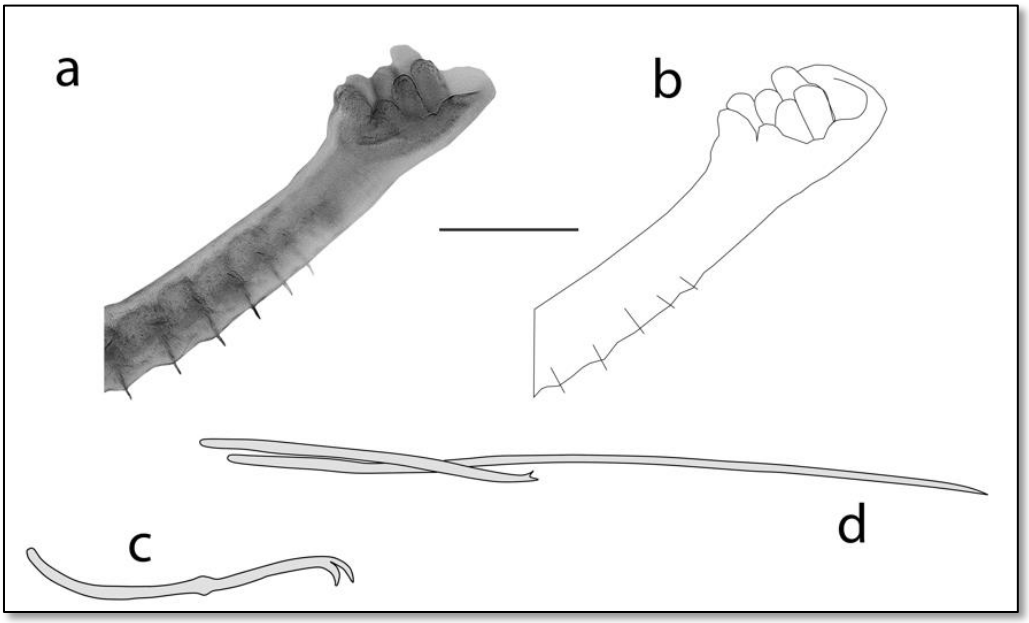

Figure 12. *Dero* (*Dero*) *obtusa*; anterior end and typical chaetae: a) – photo of posterior end showing gill fossa (scale 250 µm); b) – outline drawing of a); c) – drawing of typical dorsal chaetae bundle; d) – drawing of typical ventral chaeta.

14a (12b) Eyes present 15
14b Eyes absent.....16

15a (14a) Prostomium with elongate proboscis (Figure 13).....***Stylaria lacustris***
15b Prostomium protruding conspicuously over mouth, but without proboscis (Figure 14) ..... ***Nais*** sp.

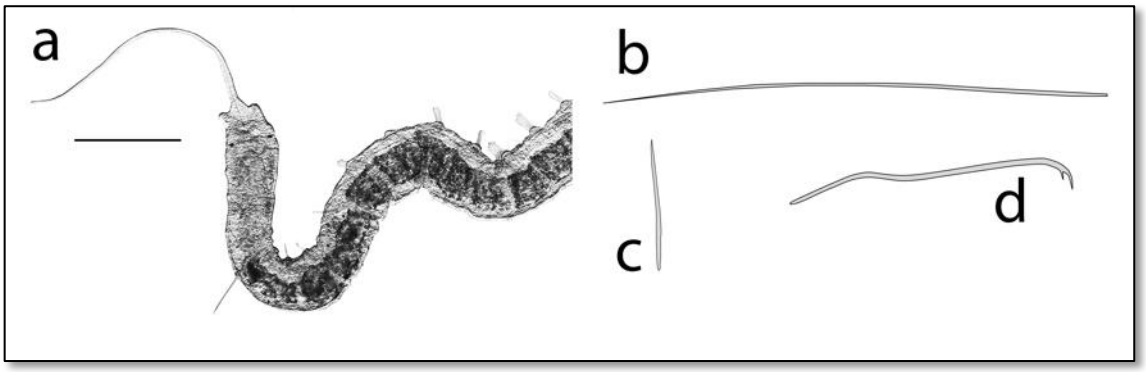

Figure 13. *Stylaria lacustris*, showing elongate prostomial proboscis, eyes, and typical chaetae: a) – photo of anterior end (scale 500 µm); b) – drawing of dorsal "hair;" c) – drawing of dorsal "needle;" d) – drawing of ventral chaeta.

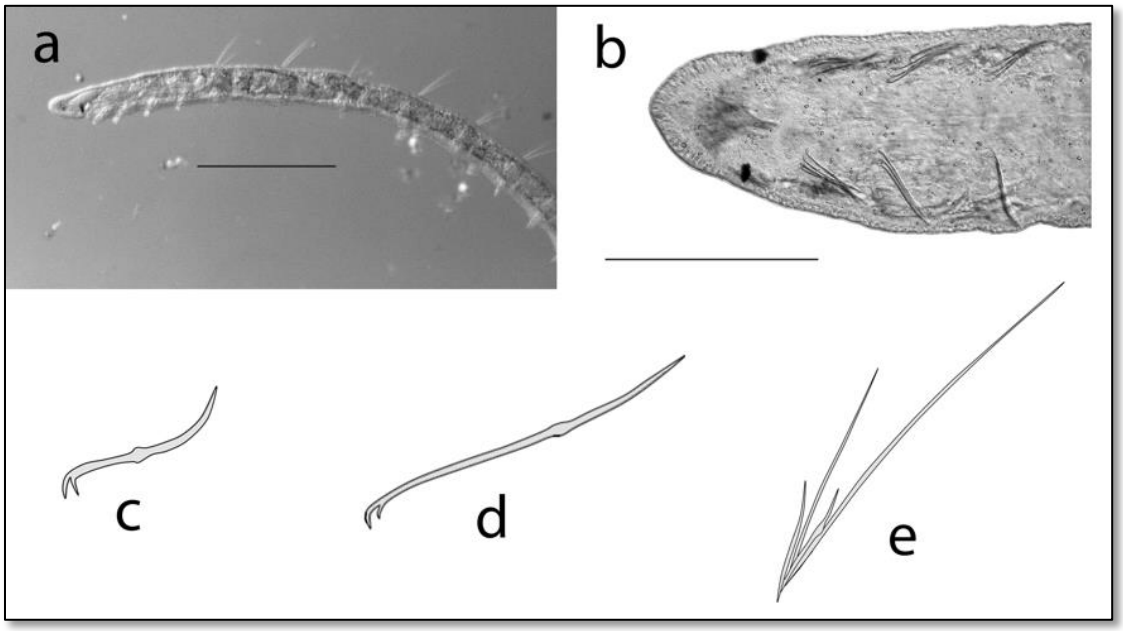

Figure 14. *Nais* sp: a) – lateral photo of anterior end showing arrangement of chaetae, eyes, and overhanging prostomium (scale 500 µm); b) – dorsal photo of anterior end (scale 250 µm); c) – drawing of typical posterior-ventral chaeta; d) – drawing of typical anterior-ventral chaeta; e) – drawing of typical bundle of dorsal chaetae.

16a (14b) Prostomium without proboscis 17
16b Prostomium with elongate proboscis (Figure 15).....***Pristina*** sp.

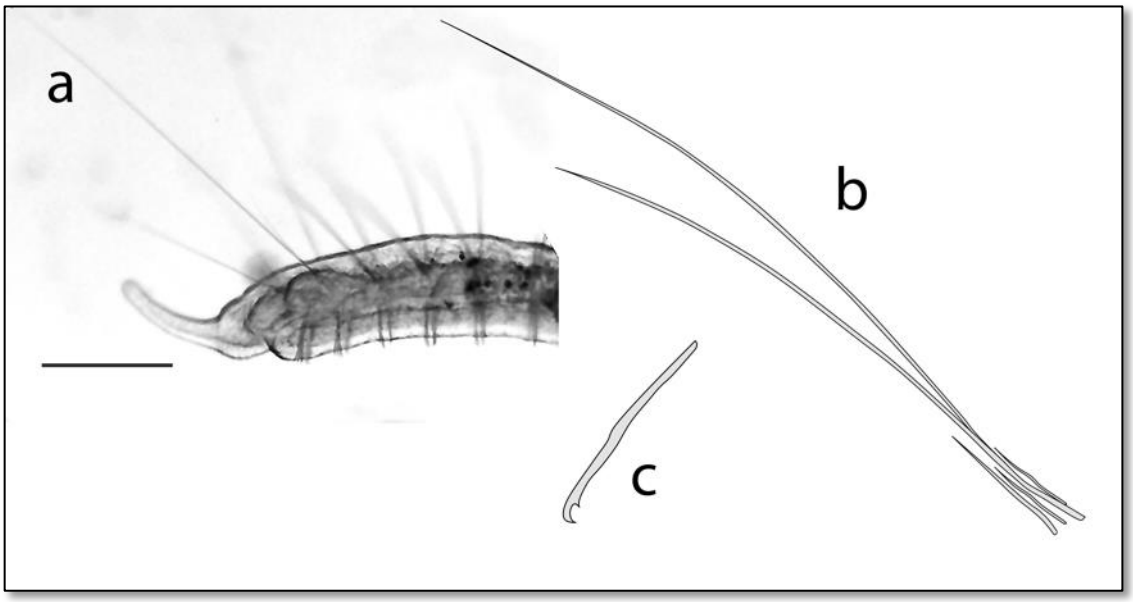

Figure 15. *Pristina* sp.: a) – lateral photo of anterior end showing elongate proboscis (scale 200 µm);b) – drawing of typical bundle of dorsal chaetae; c) – drawing of typical ventral chaeta.

17a (16a) Green epidermal glands .....***Aeolosoma cf. variegatum***
17b Red epidermal glands (Figure 16).....***Aeolosoma cf. quarternarium***

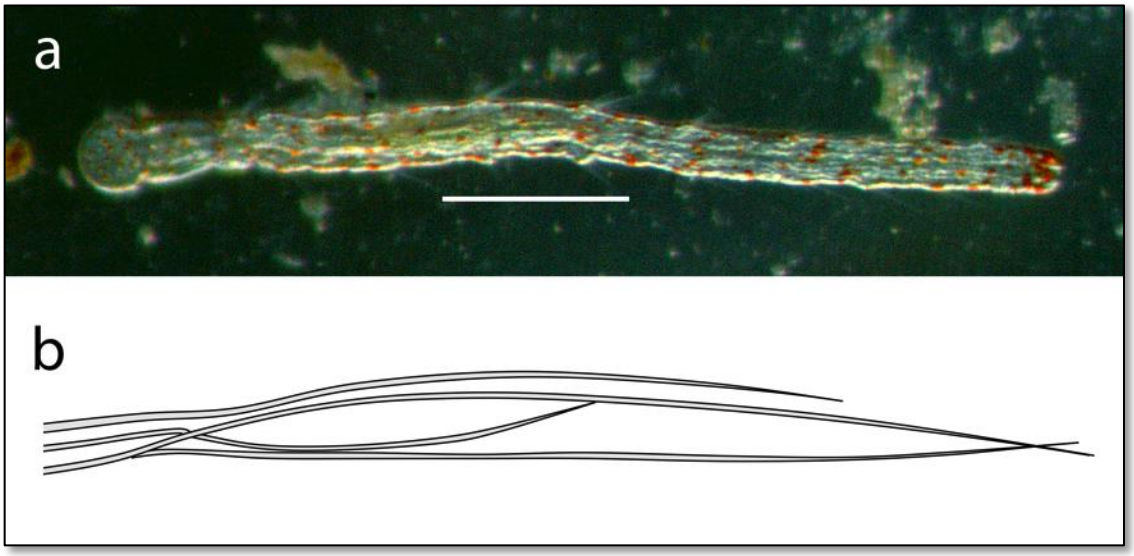

Figure 16. *Aelosoma quarternarium*: a) – photo of entire body showing red epidermal glands and disc-like anterior (scale 200 µm); b) – typical variable bundle of chaetae.

Discussion

The annelids of the SMR headwaters, not surprisingly, proved to be quite diverse. The majority of this diversity was contained within the family Naididae. The naidid annelids that were identified belong to globally common and widely distributed genera and species (Brinkhurst & Jamieson, 1971; Christoffersen, 2010; Martin et al., 2008; Park & Yeon, 2013; Pinder, 2010). Some of the naidids showed only slight morphological variation from published descriptions. However, the genera *Pristina* and *Nais* were more problematic, and none of the specimens could be assigned to any known species. Therefore, these specimens may represent two or more unique, endemic species new to science.

Haplotaxis sp. was only collected from around spring outflows. This genus is known globally to be exclusively a groundwater taxon with cryptic microdiversity (Wetzel & Taylor, 2001). Therefore, this collection not only documents a new stygobiont from the region, but might also represent a new species.

The Family Lumbriculidae may be even more speciose in the SMR than indicated in our report, as there were two collections of smaller worms that were otherwise indistinguishable from two larger worms we collected. However, we were unable to determine whether or not the smaller two forms were juvenile cohorts of the larger two forms, so only the larger two forms are reported herein. Neither of these lumbriculid species could be confidently assigned to any known genus, and it is possible that they represent undescribed endemic species. Along with the collection of two species of Aphanoneura, the occurrence of the lumbriculids is suggestive that the SMR headsprings is an ancient habitat, as the members of both of these taxa are typically collected from

ancient lakes (Martin, 1996). One of the lumbriculids (referred to herein as Lumbriculidae sp_1) was also found to contain larvae of what appeared to be a trichinelloid nematode, as suggested by the presence of a stichosome. Therefore this lumbriculid species is thought to be serving as the intermediate host in the life cycle of a potentially undescribed trichinelloid nematode.

Erpobdellid leeches are typically found free living on the benthic sediments where they hunt for small arthropods. Interestingly, a few specimens from this group were found attached to largemouth bass (*Micropterus salmoides*, Lacepede). The method of attachment was quite bizarre. Individual leeches were connected to the ventral anterior surface of the bass with a single point of attachment, and the rest of the worm was enclosed in a mesh-like sack that dangled from the point of attachment. This finding represents an interesting note of life history for this group, as it seems they can also be facultative parasites; at least locally.

Two additional types of oligochaetes were collected that are not included here because only one specimen of each type was collected and specimens were not in suitable condition for identification. A species of leech, which was only rarely collected from turtles, was also not identified.

Throughout specimen collections, numerous different forms of soil- and vegetation-dwelling nematodes were also collected. We did not attempt to identify any of these specimens. However, the variety of forms collected suggests that free-living nematodes may be the most speciose group of soft-bodied metazoans in the SMR headwaters. The study of the SMR nematode fauna would represent a great contribution to what is known of the invertebrate fauna in this habitat.

Kånneby & Wicksten(2014) noted the collection of a new gastrotrich of the enigmatic genus *Redudasys* (Gastrotricha: Macrodasyida) from the SMR headsprings. Theirs is the first report of this genus from the Northern Hemisphere. We also collected gastrotrichs from the SMR, but they were identified to the genus *Chaetonotus* (Gastrotricha: Chaetonotida).

Even from the perspective of our incomplete survey, there seems to be compelling evidence that there is much more diversity in the SMR headwaters yet to be described; particularly for the invertebrate fauna.

2. THE LIFE CYCLE OF *HUFFMANELA HUFFMANI* (NEMATODA: TRICHOSOMOIDIDAE), A MARINE RELICT PARASITE OF CENTRARCHID FISHES: THE FIRST COMPLETION OF A *HUFFMANELA* LIFE-CYCLE

Introduction

Huffmanela huffmani Moravec, 1987 (Nematoda: Trichinelloidea: Trichosomoididae: Huffmanelinae) is a parasitic nematode known only from the swimbladders of centrarchid fishes from the upper spring run of the San Marcos River (SMR) in Hays County, Texas. The first report of this worm was in 1979 (Underwood & Dronen, 1984), in which *H. huffmani* was referred to as *Capillaria* sp. *Huffmanela huffmani* was later formally described based on eggs alone (Moravec, 1987) and the new genus and the subfamily *Huffmanelinae* were also erected to contain it. Adults of the species were subsequently described following the collection of adult specimens by Moravec during a visit to Dr. David Huffman's lab at Texas State University (Huffman & Moravec, 1988). The erection of the genus *Huffmanela* was followed by the description or (reassignment from other genera) of nineteen other *Huffmanela* species, all of which are histozoic in marine fishes (Justine & Iwaki, 2014; Ruiz et al., 2013). The majority of the *Huffmanela* species are described from eggs alone, with adults having been reported thus far from only six species (Bullard et al., 2012). Each species of *Huffmanela* consistently deposits its eggs in a species-specific organ in its definitive host. Aside from the location of egg deposition, little else is known about any of the *Huffmanela* life cycles.

Since all *Huffmanela* species other than *H. huffmani* are marine, it is likely that *H. huffmani* is a marine relic. This assertion is supported by the presence of numerous other

invertebrates unique to the San Marcos Springs (SMS) that have been determined to be marine relics (Holsinger & Longley, 1980); and is further supported by previous reports that *H. huffmani* has a highly restricted geographic distribution and is found only in the upper spring run of the SMR (Cox et al., 2004; Michel, 1984; O'Docharty, 2007). Although not all centrarchid populations have been exhaustively surveyed for *Huffmanela*, at the time the present study was initiated, no freshwater populations of *Huffmanela* had been reported beyond the upper spring run of the SMR.

The location of *H. huffmani* egg deposition in the definitive host is remarkable, because there is no way for the eggs to be released from the live host to infect the next host. Previous workers have been unable to get the eggs to hatch inside the definitive host (Cox et al., 2004; O'Docharty, 2007); therefore, either the digestion of the definitive host, or its decomposition is required for the eggs to be released from the host and into the environment. *Huffmanela* eggs are appreciably denser than water, and once liberated from the host, ultimately come to rest on or in the benthic sediments. Because eggs cannot infect fish directly, and because centrarchids in contiguous populations outside the SMS are apparently uninfected, it has been suggested that a spring-obligate intermediate host endemic to the SMS is required in the life cycle (Cox et al., 2004; O'Docharty, 2007). The negative buoyancy of the eggs suggests that this intermediate host is a benthic detritivore. So, the first step in solving this life cycle was to determine if there is, in fact, an intermediate host, and if so, to identify that intermediate host. Presented herein is the life cycle of *Huffmanela huffmani*, the first described for any *Huffmanela* species.

Methods

Research was divided into several phases, some of which were executed concurrently and others, sequentially. The phases of research were as follows:

- Phase 1 – Survey of SMS invertebrate biota, and stepwise selection of candidate experimental species from among the community of SMS invertebrate species;
- Phase 2 – Attempted experimental infection of candidate invertebrate intermediates;
- Phase 3 – Graded exposure and graded incubation durations of susceptible candidate invertebrates, followed by inspection for *Huffmanela* larvae;
- Phase 4 – Attempted experimental infection of susceptible centrarchids by feeding them invertebrates experimentally infected with *H. huffmani*; and
- Phase 5 – A genus-wide literature review and functional analysis of the association between the diets of *Huffmanela* definitive hosts and the organs of egg deposition in the definitive host for all reported *Huffmanela* species worldwide.

Phase 1 Methods – Selection of candidate invertebrate species most likely to be the intermediate host of *H. huffmani*

Determination of probable ecotype of hypothetical intermediate host

At the time of this writing, close to 200 species of metazoan invertebrates have been reported from the SMS and SMR (Bowles et al., 2007; Diaz & Alexander, 2010; Edwards & Arnold, 1961; Gibson et al., 2008; Worsham et al., submitted), most of which are benthic. In order to narrow this field down to a more reasonable number of SMS

invertebrates with reasonable likelihood of being the intermediate host, it seemed prudent to do an ecological analysis of the various susceptible definitive hosts, and use this information to predict what invertebrate ecotypes would best fit the foraging preferenda of the most heavily infected centrarchid species. This required observing centrarchids *in situ* in the SMS for habitat association and foraging habits, then rating each species of fish for its intensity of infection with *H. huffmani*, and finally, examining gut contents of the most heavily infected fish to see what they were actually eating.

To begin this phase, divers observed, photographed, and videographed centrarchids foraging around Spring Lake (the impounded headsprings of the SMR), during both dark and light periods, from January through May of 2012, inclusively. Based on prior prevalence and intensity studies (Cox et al., 2004; O'Docharty, 2007) *Lepomis auritus* was expected to be the most frequently and highly infected fish species; therefore, special emphasis was assigned to studying the foraging behavior and preferred feeding sites of this species. After observing where each species of centrarchid in the SMS preferred to feed, and also analyzing gut contents to determine what they were eating, all of the SMS centrarchid species that were known to be susceptible (Moravec, 1987; O'Docharty, 2007) were categorized into four feeding-niche categories reflecting widely varying likelihoods that the fish would frequently encounter and consume benthic detritivorous invertebrates. The categories are: 1) pelagic / zooplanktivorous; 2) littoral vegetation / invertivorous; 3) benthic / invertivorous; and 4) spring-dominant benthic / invertivorous, and are described in Table 2. The category numbers will serve as contrast coefficients reflecting increasing likelihood that the species would routinely consume benthic, spring-related, detritivorous invertebrates.

Table 2. Niche categories of centrarchid species based on foraging behavior and gut analysis.

Contrast Coefficient	Category Definition	Example Taxon
0	Non-centrarchid	*Herichthys cyanoguttatus*
1	Limnetic centrarchid	*Lepomis macrochirus*
2	Littoral centrarchid	*Lepomis miniatus*
3	Benthic centrarchid	*Lepomis microlophus*
4	Spring-dominant benthic centrarchid	*Lepomis auritus*

Standard length was also used as a predictor. This predictor was employed to assess whether or not there was a minimum size at which the fish could be expected to be positive for *H. huffmani* eggs. If so, it could indicate that something is preventing the fish from consuming infected invertebrates until the fish reach a certain minimum size. This could, in turn, provide potentially useful hints about the life cycle.

A total of 54 individual fish representing five centrarchid species from the SMS were examined and categorized, and their category numbers were used as contrast coefficients to predict infection intensity ratings for the same 54 individuals. Because an infected fish might contain up to millions of eggs (Cox et al., 2004), the following intensity ratings were used to rank the perceived intensity of infection:

0) no eggs detected;

1) trace infection; only a few eggs observed;

2) up to 25% of swimbladder infected with eggs;

3) greater than 25% and up to 50% of swimbladder infected; and

4) greater than 50% of swimbladder infected.

Determination of probable taxonomic group of hypothetical intermediate host

In order to further narrow down candidate experimental species from the diverse spring-related benthic detritivores, literature summarizing what is known of the life cycles of other fish nematodes in the Order Trichinelloidea was reviewed (Køie & Nylund, 2001; Moravec et al., 1998). Based on that review, the intermediate host for *H. huffmani* was suspected to be either an annelid or an amphipod. This conclusion is complicated by there being at least 13 species of amphipods known to occur locally (Gibson et al., 2008; Holsinger & Longley, 1980), and by the paucity of literature on the annelids of SMS which have been almost entirely neglected until this study (Worsham et al., submitted). Therefore, it became necessary to survey the annelids of SMS, and the SMS was found to contain at least 17 species of annelids.

Phase 2 Methods – Attempted experimental infection of candidate invertebrates

Collection of H. huffmani *eggs*

Fish were collected by angling with artificial lures. Collected fish were transported back to the lab and maintained in at least 10 L of lake water. It is not known if *H. huffmani* is environmentally sensitive, but the restricted distribution would suggest that it might require the constant temperature of the springs. Therefore, transport of fish back to the lab was expedited in order to insure that dissolved oxygen (DO) and/or temperature did not deviate substantially from optimal conditions hypothetically required by *H. huffmani.*

Fish were euthanized by pithing. After euthanasia the fish were necropsied and the swimbladder was excised. The excised swimbladders were placed in artesian water and were either 1) left in artesian water in a sealed container to allow for microbial decay to free the eggs from the tissues; or 2) fed to a captive *Lepomis cyanellus* for digestion of the host tissues away from the eggs so the eggs could be collected from the fish feces. The digestion method produced eggs in as little as 24 hours; however, the decay method yielded a much larger number of eggs than the digestion method because the digestion method required the gathering up of eggs that had become dispersed within feces throughout an aquarium. Recovered eggs were stored in sealed 700 ml containers suspended in an artesian flow-through system to ensure thermal stability (Figure 17), and water was refreshed twice monthly. Both sources of free eggs were used in attempts to infect all candidate species of invertebrates.

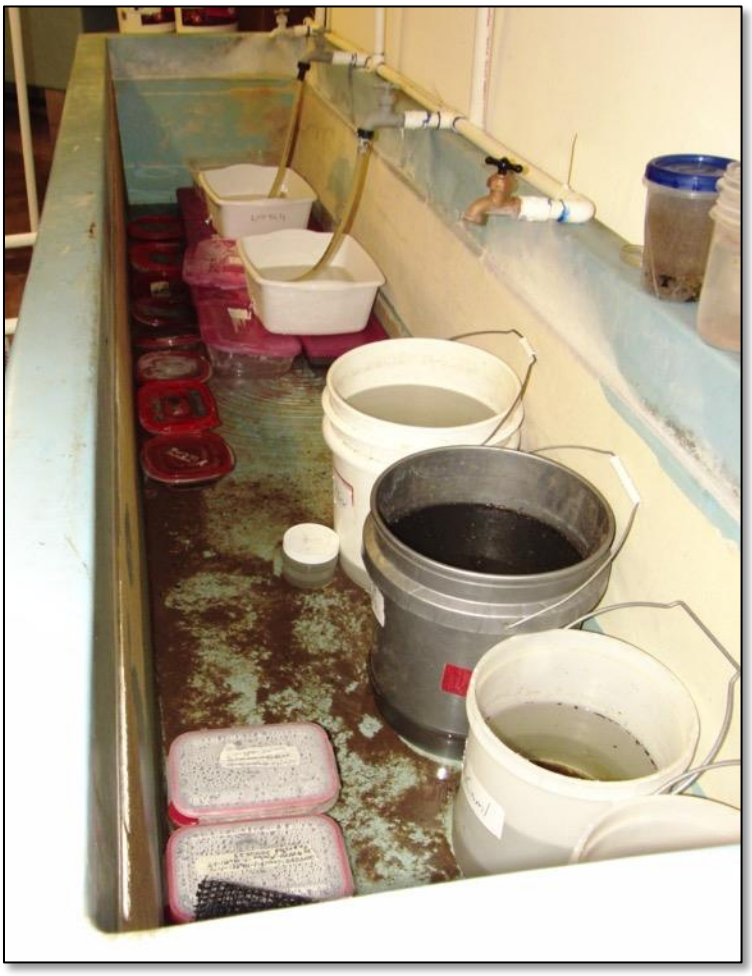

Figure 17. Water bath employed at the Freeman Aquatic Station wet lab to maintain invertebrates at constant temperatures, and on a 12/12hr Light/Dark cycle.

Invertebrate collections

Benthic sediments were collected with a Ponar grab sampler, which is a spring-loaded clamshell-like device designed to sample loose bottom sediments. The Ponar used in this project sampled a rectangular patch of surface of 17 x 24 cm in area, and had an internal volume of 2.4 L. A team of divers determined grab-sample localities real-time, positioned the Ponar, and triggered the closing of the Ponar around the sample. A crew in a small watercraft then promptly lifted the sample to the surface via a tether and

emptied the contents into a labeled five gallon bucket containing two gallons of fresh filtered artesian water maintained at or near 22 °C. Multiple samples from various localities were collected in this fashion. Immediately following a sampling session, the sediment samples were transported to the lab, and the organisms in each Ponar grab were separated and sorted under a dissecting microscope. All invertebrate specimens were identified to lowest discernable taxon. From these collections, live culture of candidate amphipods and annelids were established in the lab. Cultures were maintained in labeled 1200-ml plastic food containers with windows cut in the sides and covered with 200-µm mesh (Figure 18), and floated in a flow-through system of artesian water to insure constant physicochemical conditions. Diet varied somewhat by taxon; however, all candidate species were presumed to be detritivores, therefore only species that could be maintained on a diet of detritus were used in experimental infection attempts.

Figure 18. Photo of one of the 1,200 ml containers that were used to culture invertebrates, with 200-µm mesh window shown on one side.

Preliminary exposure experiment to test susceptibility

Candidate invertebrates were tested for susceptibility to infection with *H. huffmani* eggs by placing them in a container with an aliquot of *H. huffmani* eggs. Controls were maintained in the same way, but without exposure to *Huffmanela* eggs. All exposures were run for two weeks, or until all research specimens had died, whichever occurred first. If the population decline in the treatment containers exceeded the decline in the controls by a substantial margin, experimental and control individuals were to be examined immediately for larvae of *H. huffmani*. At the end of the two weeks, all remaining experimental subjects were examined for larvae. When nematode larvae were discovered among the treatment replicates of a species, the associated controls were also examined to assure that they were negative.

Of all the various amphipod and annelid taxa tested in the susceptibility trial, only amphipods of the genus *Hyalella* became infected when exposed to *H. huffmani* eggs; indeed, all *Hyalella* populations tested for susceptibility showed evidence of larvae (and the control *Hyalella* were never found to be infected). Thus, collections from various populations of *Hyalella* spp. were selected as experimental subjects in all subsequent methods pertaining to the intermediate hosts of *H. huffmani*.

Phase 3 Methods – Titration of optimum exposure intensity and incubation duration of *Hyalella* exposed to *H. huffmani* eggs

In this experiment, susceptible SMS *Hyalella* sp. were subjected to various exposure intensities and incubation durations with embryonated eggs of *H. huffmani* in order to titrate exposure intensities and incubation durations for subsequent experiments.

Source of H. huffmani *eggs*

H. huffmani eggs for this experiment were derived from the swim bladders of eight infected centrarchids of several species wild-caught from the SMS. The swim bladders of the eight fish were fed to one green sunfish in a clean and bare aquarium. After a 24-hour waiting period, the egg-laden fecal matter was aspirated from the bottom of the aquarium with a turkey baster. Eggs were stored in a plastic container in the artesian flow-through to maintain spring-like conditions.

Setup of amphipod exposure titration experiment

The experimental *Hyalella* were divided into five groups, with four of the groups representing four levels of treatment (four different durations of exposure to *H. huffmani* eggs), and the fifth group serving as controls that were not exposed to eggs.

Each treatment group consisted of three replicates, with the replicates housed in 1,200 ml containers, as shown in Figure 19. Each of the 15 replicate containers received 27 experimental amphipods at the beginning of the run, for a total of 405 amphipods for one entire run of the titration experiment. The amphipods were allowed to habituate to the containers for 2 days prior to exposure to eggs.

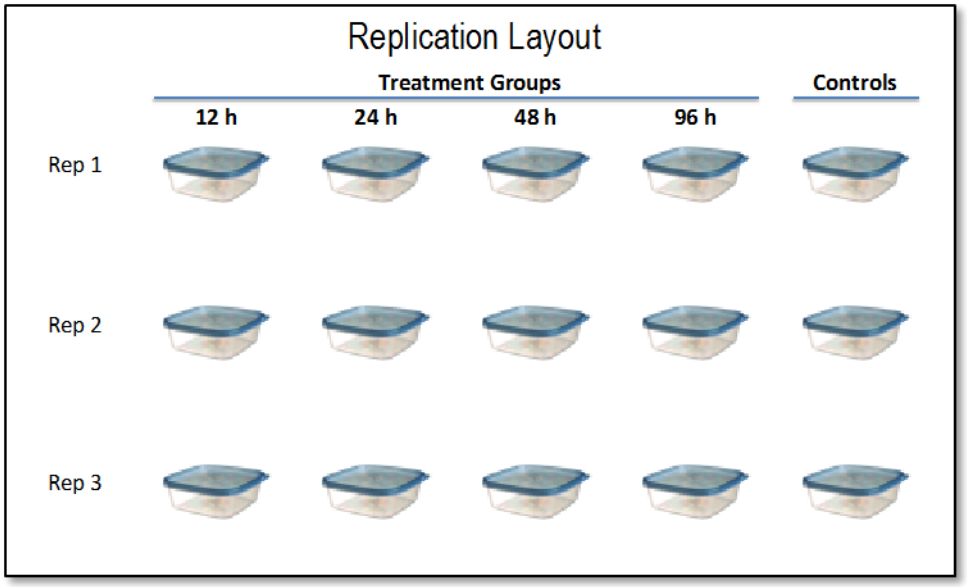

Figure 19. Layout of replication containers used in graded duration experiment, each housing 27 amphipods.

Distribution of amphipods to replicate containers

Disposable one-piece plastic transfer pipettes with 5 ml bulbs (similar to Fisher S30467-1) were used to transfer amphipods from container to container. Tips were

trimmed back to allow easy passage of amphipods. Prior to distributing amphipods to replicate containers, the containers were assigned random numbers, and then arranged in order of increasing random number. One amphipod was selected from the culture container with a plastic pipette and was transferred to the replicate container with the lowest random number. A second amphipod was selected and added to the replicate container with the next lowest random number, and so on, until all replicate containers had been supplied with 27 amphipods. This randomization procedure was executed in order to ensure that any sequential bias inherent in the amphipod-selection procedure was randomized across treatment groups.

Distribution of H. huffmani *eggs to replicate containers*

Prior to allocation of *Huffmanela* eggs into replicate containers, the source liquid containing the eggs was dispersed and divided via two serial dilution stages into 12 intermediate containers of relatively equal volumes. The first stage involved stirring the contents of the egg source and allocating the liquid equally into three intermediate dilution containers. In the second stage, the liquid in each intermediate container was then stirred and allocated to four final dilution containers of replicate 1, 2, or 3 in the order shown in Figure 20. This procedure was executed so as to randomize any sequential differences in egg dilution across the design.

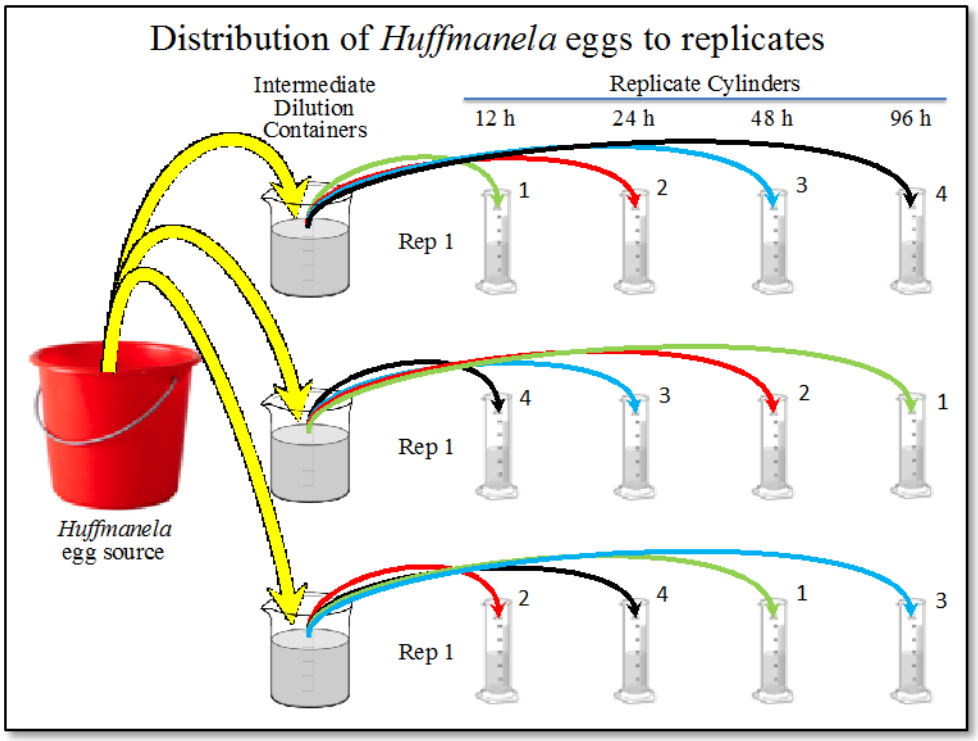

Figure 20. Illustration of the equalization and randomization of *Huffmanela* eggs prior to allocation to replicate containers.

Following the amphipod habituation period, amphipods in the four treatment groups were simultaneously exposed to approximately equal concentrations of *H. huffmani* eggs from the final dilution containers.

The titration of amphipod exposure intensity

The amphipods in Group 1 were examined for *Huffmanela* larvae after 12 hours, Group 2 after 24 hours, Group 3 after 48 hours, and Group 4 after 96 hours (Figure 21). The amphipods in the fifth experimental group (controls) were exposed to feces from a control fish that had never eaten infected swimbladders, and were used to determine if any population declines among the corresponding treatment replicates were due to *Huffmanela* infection, in which case the controls would show a slower decline, or to

causes unrelated to exposure to *Huffmanela* eggs such as fish feces, in which case controls would be expected to decline at a similar rate. There appeared to be no appreciable difference in amphipod survival among the four exposure-intensities, indicating that the maximum exposure intensity had probably not been exceeded at 96 hours of exposure. It was therefore decided to run all subsequent experiments with 96 hours of exposure.

The titration of amphipod incubation duration

After the prescribed exposure times had elapsed (at end of red lines in Figure 21), amphipods were removed from egg exposure and incubated for varying durations. The amphipods were first removed from exposure containers and rinsed thoroughly to insure removal of *H. huffmani* eggs. Replicate containers were also rinsed. After removal of eggs, amphipods were placed back into the original exposure containers for incubation of the exposed amphipods (start of green lines in Figure 21). Each exposure was allowed to incubate while the death rate of the amphipods was monitored to determine if the optimum exposure duration had been exceeded. All exposed treatment replicates showed noticeable declines even before washing in some cases, whereas controls showed no declines whatsoever.

Examination and feeding-out of exposed amphipods

Immediately following the end of each exposure duration, and once again at the end of each prescribed incubation duration (following 1, 4, and 30 days of incubation), three amphipods were taken from each exposure-intensity group (one from each replicate

container). One amphipod (represented by blue arrows in Figure 21) was necropsied and closely examined for infection with *H. huffmani*, while the other two (represented by green arrows) were fed out to one experimental centrarchid, which was then incubated without further exposure in an attempt to establish an infection from amphipods having that combination of exposure intensity and incubation duration. After 30 days of amphipod incubation, all infected amphipods from all exposure intensities and incubation durations had either been fed out to the experimental fish, or had succumbed to their infection. None of the control amphipods died, indicating that the deaths of the treatment amphipods was probably due to exposure to *H. huffmani* eggs.

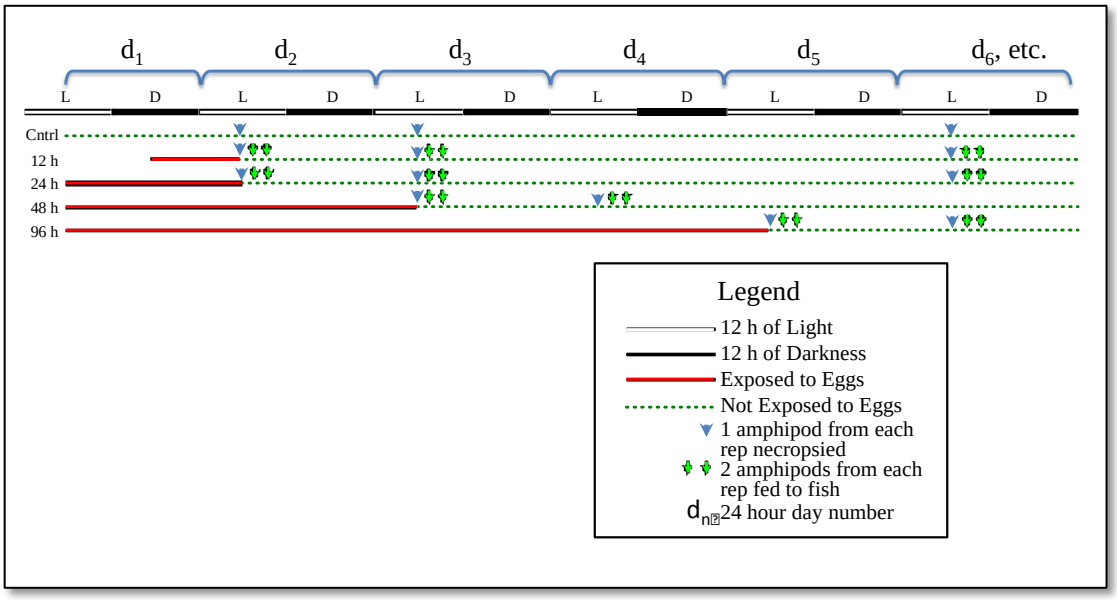

Figure 21. Timeline for graded exposure and graded incubation of amphipods.

Quantification of infection intensity in amphipods

In most cases, individual amphipods were so heavily infected that exact larval counts were not feasible. Therefore, amphipod infection level was ranked to represent relative estimates of infection intensity. The criteria for the ranks were:

0) infection not observed;

1) 1-3 larvae observed;

2) more than 3 larvae observed but less than 25;

3) more than 25 larvae observed but less than approximately 100;

4) more than approximately 100 larvae observed.

Intensity rank was used as a response variable and analyzed with a regression to determine if infection intensity was related to exposure intensity.

Phase 4 Methods – Experimental infection of fish

Sources of experimental fish

Experimental centrarchid fishes were collected via angling and seining either from the Comal River where *H. huffmani* does not occur (0% prevalence for 120 centrarchids examined by USFWS, and all centrarchids examined during three previous studies were all negative) or from captive stock at the San Marcos Aquatic Resource Center (SMARC) operated by the U.S. Fish and Wildlife Service. Captive stock at SMARC are of a leucistic strain of *Lepomis cyanellus*. Wild-caught specimens from the Comal River were *L. auritus, L. miniatus, L. megalotis,* or *L. macrochirus*; the majority were either *L. auritus* or *L. miniatus*. Collected fish were maintained in cultures of one fish per 7- or 10-gallon aquarium at SMARC. Each aquarium received a constant flow-through of conditioned well water from the SMARC water supply, and the fish were maintained on commercially available fish food, both before and after exposure to infected amphipods.

Exposure and incubation of experimental fish

Each of the 16 experimental fish was assigned a random treatment number representing the unique combination of exposure intensity and incubation duration experienced by the two amphipods it was fed prior to entering into incubation. Each amphipod was expected to have several viable larvae in some stage of development at the time it was fed out (most actually had >25). The two amphipods were simply pipetted live into the aquarium containing the fish, and the fish almost immediately ate both amphipods.

A survey of the other trichosomoidid nematodes with known life cycles indicated that the prepatent period in fish before *H. huffmani* eggs would appear in the swim bladder of experimentally infected fish would be at least 2 months, so the first three fish were scheduled for examination after 3 months of incubation, with three fish to be examined each month thereafter.

Examination of experimental fish

After fish were incubated for the prescribed duration, they were euthanized via pithing, and the swimbladder was removed. The entire epithelium of the swimbladder was cut into pieces and each piece was stretched across a wet mount and examined under a compound light microscope at 4-100x magnification for *Huffmanela* larvae, adults, or eggs.

Phase 5 Methods – Evaluation of relationship of definitive host diet and location of egg deposition for all reported *Huffmanela* species.

Data on where eggs are deposited in the definitive hosts of the various *Huffmanela* species, as well as information regarding the diet and habitat preference of the host species was gathered from the literature where available. Each of the 20 *Huffmanela* species was then assigned to one of four cells in a 2 X 2 table (Table 3). The cells are defined by two variables, each with two categories: organ in which eggs are deposited (internal vs. surface) and the habitat and source of diet of the definitive host (benthic vs. pelagic). The overwhelming majority of species were classified as using an internal-tissue in a benthic-host.

This relationship was evaluated by comparing the proportion of *Huffmanela* species in only two of the four cells of the table: the proportion of all *Huffmanela* species that deposit eggs in an internal tissue of a benthic definitive host; compared to the proportion of *Huffmanela* species that deposit eggs in a surface tissue of a pelagic definitive host.

Table 3. Two-by-two table for assigning known *Huffmanela* species to a specific combination of diet & habitat vs. location of egg deposition.

Habitat & diet:	Egg deposition location: Internal	Egg deposition location: External
Pelagic		
Benthic		

Results

Phase 1 Results – Selection of candidate invertebrate species most likely to be the intermediate host of *H. huffmani*

Clues from naturally infected fishes

Lepomis auritus, a non-native centrarchid (TISI, 2015) with high prevalence and intensity of infection with *H. huffmani* (Table 4), was observed to cooperate with conspecifics to ward off other centrarchid species from their favorite food source; the benthic invertebrates around spring openings. This spring-dominating behavior was only displayed by adult *L. auritus*. The adults of *L. auritus* had a prevalence of 96% and a mean density rating of 2.8 (n=53), whereas an increase in prevalence was not observed while considering only mature fishes of other species. *M. salmoides*, despite conventional belief of being a top predator, proved to be an opportunistic generalist and was also frequently observed feeding on small benthic spring invertebrates because it was too large to be warded off by *L. auritus*. This species was also heavily infected (Table 4).

Table 4. Mean-density rating and prevalence of infection with *H. huffmani* eggs in wild-caught fishes from San Marcos Springs.

Species	Mean-density Rating	Prevalence	*n*
M. salmoides	3.00	0.90	10
L. auritus[1]	2.64	0.89	56
L. miniatus	1.83	0.67	6
L. microlophus	1.71	0.57	7
L. macrochirus	0.17	0.08	12

Another clue was found in the observation that wild-caught centrarchids from the headsprings of the SMR and from the first 2.5 river km downstream exhibited high prevalence and mean density ratings of *H. huffmani* infection (Table 5). The abundance of *H. huffmani* declined precipitously between 2.5 to 4 river km downstream of the head springs and disappeared beyond 4 km downstream (Figure 22), suggesting that the functional intermediate host of *H. huffmani* is a true spring-run obligate invertebrate.

[1] Calculated from 53 adults and 3 uninfected juveniles.

Table 5. Mean-density rating and prevalence of infection with *H. huffmani* eggs showing decline in prevalence with distance downstream in the San Marcos River from the headsprings.

Downstream km	Locality	Mean-density Rating	Prevalence	*n*
0	SMS	2.69	0.85	48
1.5	Upper spring run of SMR	2.60	0.93	15
2.5	Interstate Hwy-35	2.44	0.78	9
4	Stokes Park	0.25	0.23	14
4-8	Stokes Park to confluence w/Blanco R.	0.00	0.00	13

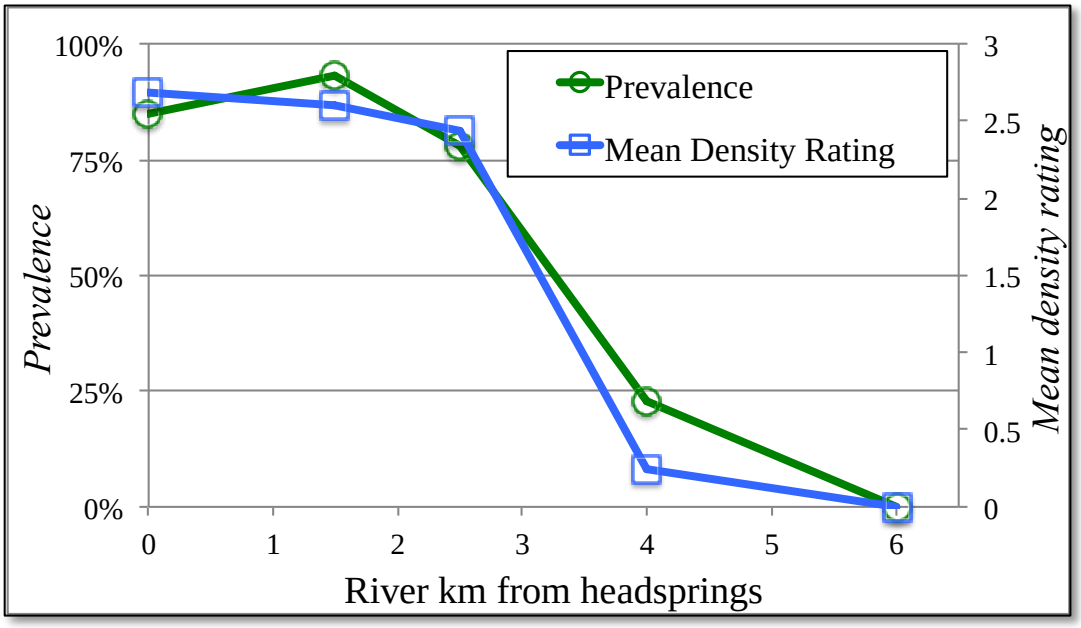

Figure 22. Rate of decline in prevalence and mean intensity rating of *Huffmanela huffmani* infection in wild-caught centrarchids with increasing distance downstream from headsprings.

To evaluate if the intermediate host of *H. huffmani* is a benthic detritivore, three factors were used to predict intensity of infected and uninfected fishes thus also predicting prevalence. The three factors compared were fish species, standard length of

fish, and assigned niche category. The niche model was shown to be superior to the other models (Table 6), which suggests that the intermediate host is a benthic invertebrate.

Table 6. AIC weights of competing models used to predict infection intensity ratings of centrarchids.

Model	*F* ratio	*p*	AIC weight
SL	8.23	0.00581	0
Species	9.33	6.65E-07	0.057
Niche	48.33	4.15E-09	0.943

Phase 2 Results – Attempted experimental infection of candidate invertebrates

Of all the invertebrate taxa exposed to *Huffmanela huffmani* eggs, only *Hyalella* became infected (including *Hyalella* spp. that do not co-occur with *Huffmanela*); 100% of the *Hyalella* that were exposed to eggs of *H. huffmani* became infected with larvae of *H. huffmani* (bolded in Table 7). However, only one of these *Hyalella* spp. is known to be endemic to the SMS and therefore to have coevolved with *H huffmani*; thus, all subsequent attempts to experimentally infect centrarchids employed SMS *Hyalella* as the experimental intermediate host.

Table 7. Infection success of invertebrates exposed to *H. huffmani* eggs.

Higher Taxon	Taxon	*n* Examined	*n* Infected
Annelida: Aphanoneura	*Aeolosoma* spp.	10	0
Annelida: Clitellata	*Chaetogaster diastrophus*	10	0
Annelida: Clitellata	*Dero furcatus*	5	0
Annelida: Clitellata	*Dero obtusa*	10	0
Annelida: Clitellata	*Haplotaxis* sp.	2	0
Annelida: Clitellata	Lumbriculidae sp_1	5	0
Annelida: Clitellata	Lumbriculidae sp_2	5	0
Annelida: Clitellata	*Pristina* sp.	10	0
Crustacea: Amphipoda	*Crangonyx cf. pseudogracilis*	6	0
Crustacea: Amphipoda	*Hyalella cf. azteca* (Comal River)	10	**10**
Crustacea: Amphipoda	*Hyalella cf. azteca* (Devils River)	10	**10**
Crustacea: Amphipoda	*Hyalella cf. azteca* (SMR)	10	**10**
Crustacea: Amphipoda	*Hyalella* sp. (SMS endemic)	10	**10**
Crustacea: Amphipoda	*Hyalella texana* (Clear Creek Springs)	10	**10**
Crustacea: Amphipoda	*Stygobromus* spp.	6	0
Crustacea: Copepoda	Cyclopoida	10	0
Crustacea: Isopoda	*Cirolanides texensis*	5	0
Crustacea: Ostracoda	*Cypria* sp.	10	0
Crustacea: Ostracoda	*Stenocypris* sp.	10	0
Platyhelminthes: Macrostomida	Macrostomida	10	0
Platyhelminthes: Tricladida	*Dugesia* sp.	10	0
Platyhelminthes: Turbellaria	*Schmidtea* sp.	10	0

Phase 3 Results – Titration of optimum exposure intensity and incubation duration of *Hyalella* exposed to *H. huffmani* eggs

If no amphipods had died during the incubation phase, a total of 36 amphipods would have been available for necropsy during the three examination days (0, 3, and 7 days of incubation). However, because infected amphipods began dying in some of the replicate containers after the examinations had been started, only 30 amphipods were

necropsied for infection intensity rating. The regression slope of infection intensity on exposure duration was not significant $\left[p\left(F_{a(1),1,29} \geq 2.3) = 0.14 \right) \right]$.

Dead *H. huffmani* larvae (Figure 23) were often observed in some of the experimental amphipods. Interestingly, dead larvae were only observed in the presumably co-evolved SMS *Hyalella* sp., and were never observed in any of the *H. cf. azteca* populations that were artificially infected.

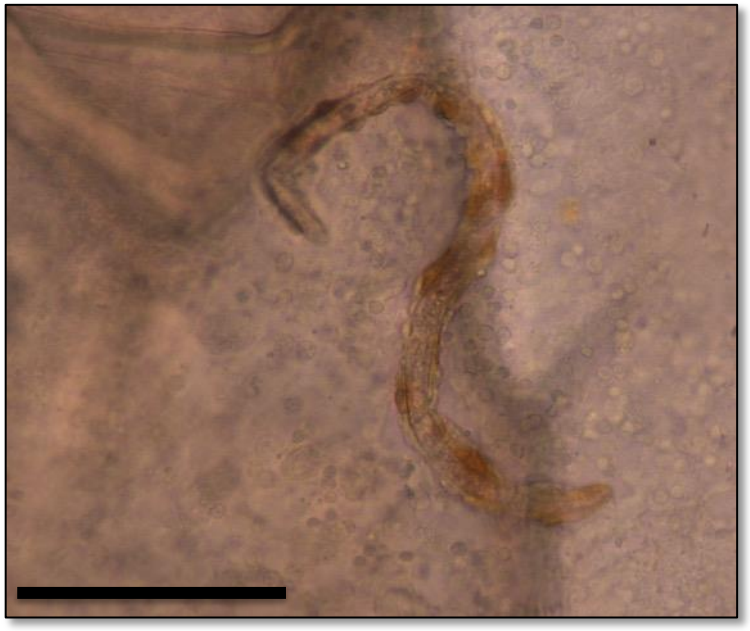

Figure 23. Photomicrograph of a dead *Huffmanela* larva from SMS *Hyalella* sp. (scale bar = 100 μm).

After 1 to 2 weeks in *Hyalella*, internal differentiation was observed in larvae, but the larvae did not increase in size and no molts were ever observed (Figure 24), even after 9 weeks of incubation in *Hyalella.* Differentiation did not appear to result in a change of activity of larvae.

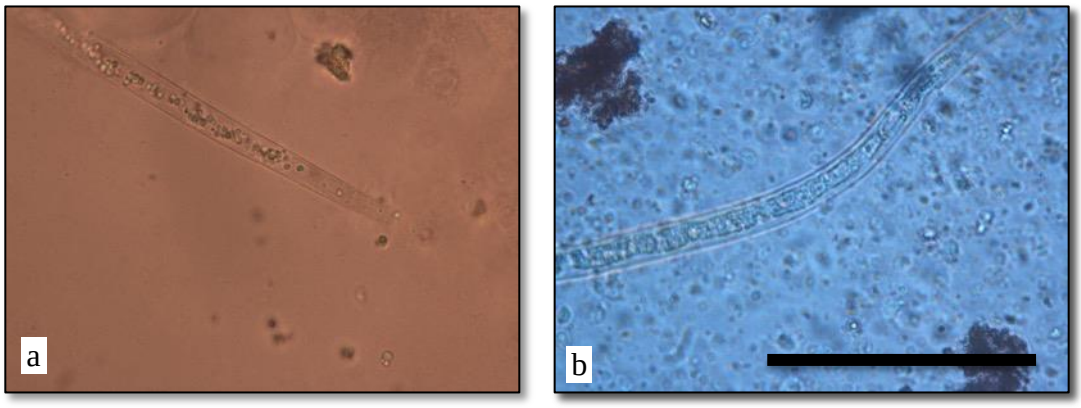

Figure 24. Naturally hatched *H. huffmani* larvae after being consumed by *Hyalella* spp.; a) freshly hatched larvae with motile spheroid internal bodies; b) 30 days after hatching; none of the motile spheroid bodies of freshly hatched larvae are apparent but instead a single condensed central structure is visible throughout the posterior of end of worms. Scale bar = 50 µm.

The life span of the *H. huffmani* larva in the amphipod seems to be limited by the life span of the intermediate host, since numerous larvae were found alive and active 9 weeks after infecting their amphipod host. *Hyalella* were shown to be capable of living for up to 7 months, but it is unlikely that they live past 4 months in situ (Othman & Pascoe, 2001). Though no *H. huffmani* larvae were incubated in *Hyalella* for the duration of the life span of the *Hyalella*, there was no evidence of progressive decline in number of live larvae after 9 weeks.

Eggs of *H. huffmani* derived from digested swimbladders were demonstrated to be still infective to *Hyalella* after 9 months of storage in a sealed plastic container outside the definitive host (Table 8), at which point the eggs still showed no sign of decreased infectivity to amphipods. This suggests that at the eggs released in the feces of piscivorous fish could withstand over a year in the environment and still be infective to amphipods.

Table 8. Infection success of eggs held in containers for various periods of time.

Days in container	Infective to amphipods? (Yes/No)
1	Yes
21	Yes
23	Yes
32	Yes
54	Yes
59	Yes
85	Yes
129	Yes
226	Yes

Phase 4 Results – Experimental infection of fish

Only the fish which had been fed *Hyalella* containing larvae that had been incubated in the amphipod for at least 5 days became infected, so the prepatent period in the amphipod seems to be as short as 5 days. None of the experimental fish incubated for less than 4.5 months showed any sign of infection. Of the fish that had been incubated for at least 4.5 months after being fed *Hyalella* infected with larvae that were incubated for at least 5 days, 50% showed evidence of infection (n=8). Of the fish that had been incubated for at least 4.5 months and fed larvae that had incubated for 30 days, 66% showed evidence of infection (n=3). None of the fish were heavily infected and could be due to the slow development of *H. huffmani*. Therefore, it is thought that the prepatent period of *H. huffmani* in the fish is at least 5.5 months and perhaps as long as 7 months or more. Infection results are presented in more detail in Table 9.

Table 9. Results of feeding infected *Hyalella* spp. to centrarchids.

Incubation Duration (months)	Prev.	*n*	Infected (Y/N)	Comments
3	0	3	N	No sign of infection in swim bladder.
4.5	0.2	5	Y	A 2-mm section of posterior end of a worm believed to be from a young adult female was recovered from the swimbladder of 1 fish (Figure 25).
5.5	0.25	4	Y	Six eggs were found in swimbladder of one fish (Figure 26); none of the eggs were completely larvated but had darkening shells (Figure 26).
6.5	0.25	4	Y	Three worms 25 x 600 µm were recovered from one fish (Figure 27). No eggs were observed in this fish and all worms appeared to be male (Figure 27).
7.5	1	1	Y	Had ovoid egg-like entities in swim bladder thought to be early stage or infertile eggs that were approximately 44 microns long (Figure 28) and had similar morphology to unfertilized eggs recovered by Esteves et al. (2009).

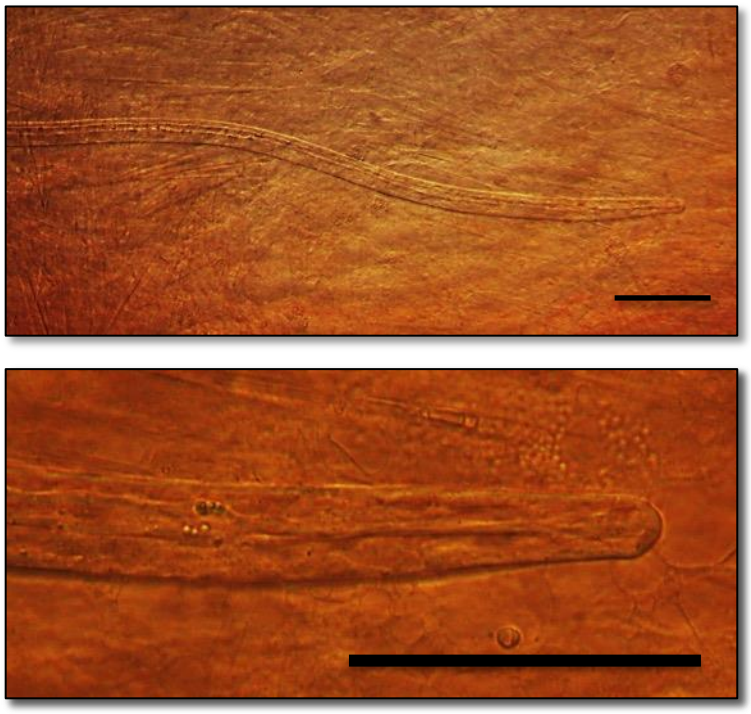

Figure 25. Posterior end of young female *H. huffmani* recovered from a fish experimentally exposed 4.5 months previously (scale bars = 100 μm).

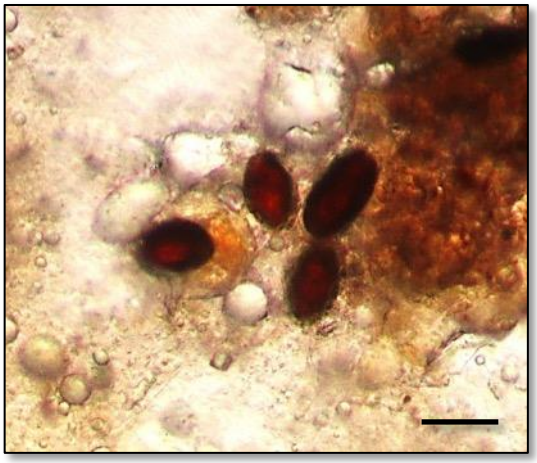

Figure 26. *H. huffmani* eggs recovered from a fish experimentally exposed 5.5 months previously (scale bars = 50 μm).

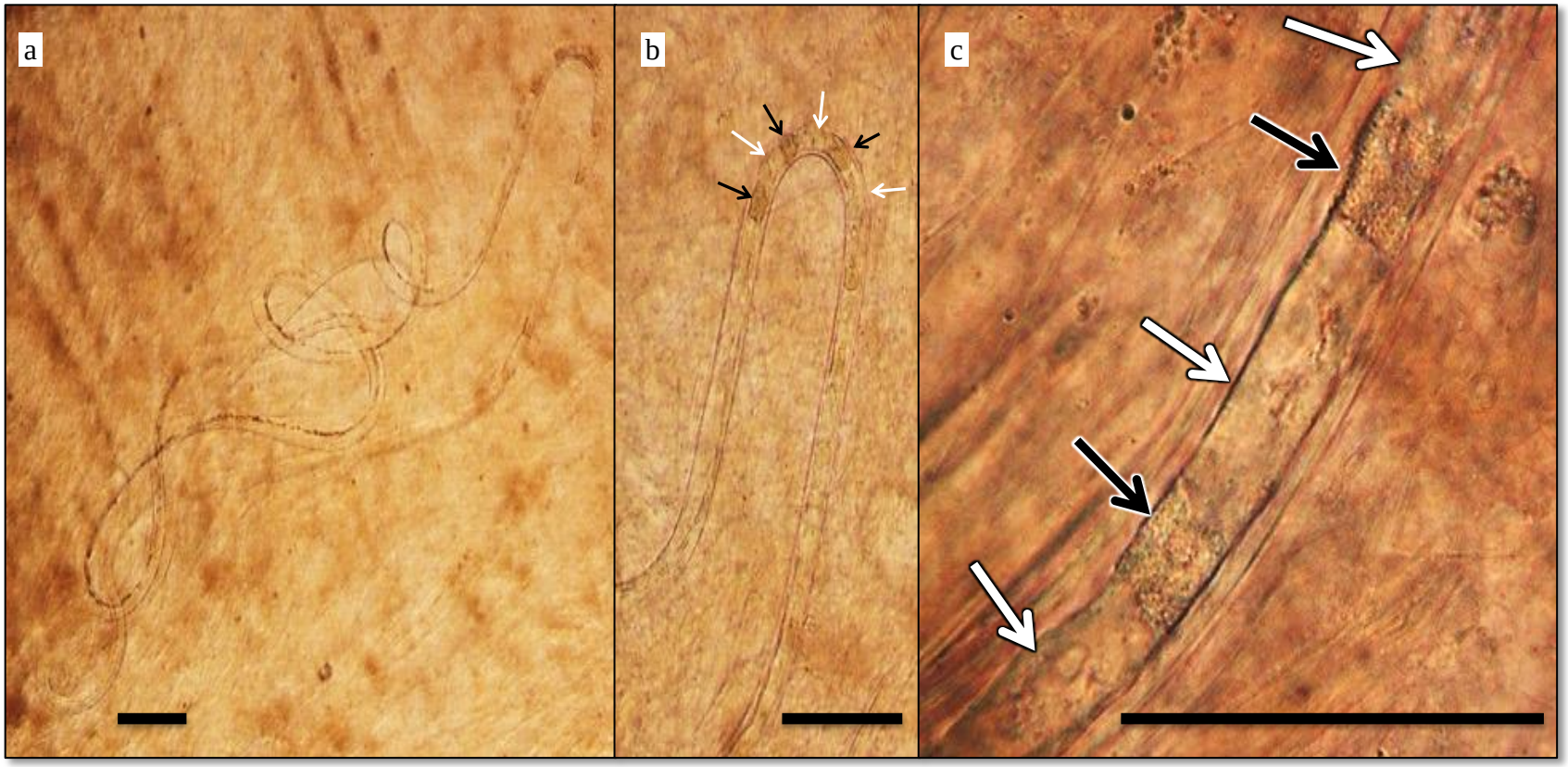

Figure 27. Worms recovered from swimbladder of a fish experimentally exposed 6.5 months previous: a) gross view of entire worm at 200 X; b) view of stichosome at 400 X showing alternation of dark and light stichocytes typical of *Huffmanela*; c) view of stichosome at 1,000 X showing visible stichocyte nuclei and alternating dark and light stichocytes (scale bars = 100 µm).

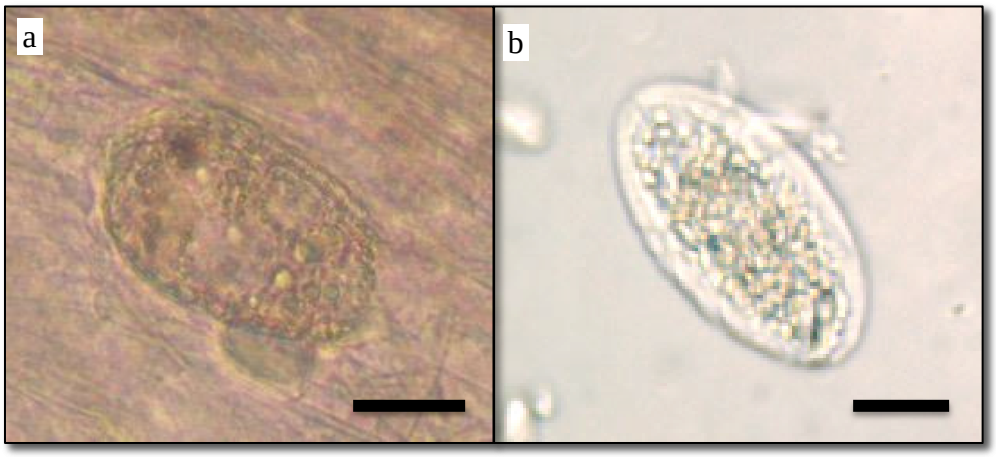

Figure 28. Presumably unfertilized egg recovered from swimbladders of fish: a) from an experimentally exposed fish in the present study; b) collected by Esteves et al. (2009) (scale bars = 20 µm).

Phase 5 Results – Evaluation of relationship of definitive host diet and location of egg deposition for all reported *Huffmanela* species

A survey of the literature revealed that *Huffmanela* species have been reported from 18 families of marine fishes and one family of freshwater fishes (Table 10). The organ of egg deposition for *Huffmanela* species (Table 10) appeared to be somewhat predictable by the habitat of the host. Of the benthic host species (n = 20), 60% were parasitized by a *Huffmanela* species that lays eggs in an internal tissue whereas, 100% of pelagic host species (n = 2) were parasitized by a *Huffmanela* species which lays eggs in a surface tissue.

Table 10. Comparison of diets, habitats, organs, and localities for known *Huffmanela* populations.

Species	#	Host taxon	Host Diet	Host Habitat	Organ	Locality	Reference
Huffmanela balista	1	*Abalistes stellatus* (Balistidae)	Benthic invertebrates	Coastal benthic; reefs	Outer swim bladder wall	New Caledonia	Justine, 2007
Huffmanela banningi	2	*Cynoglossus browni* (Cynoglossidae)	benthic invertebrates	coastal sand and mud bottoms	musculature	Atlantic Ocean, Senegal and Congo	Moravec, 1987
Huffmanela branchialis	3	*Nemipterus furcosus* (Nemipteridae)	benthic invertebrates	coastal benthic	gill mucosa	Nouméa, New Caledonia	Justine, 2004
Huffmanela canadensis	4	*Sebastes* spp. (Sebastidae)	benthic invertebrates	deep benthic	skin	Clayoquot Sound, British Columbia, Canada	Moravec et al., 2005
Huffmanela carcharhini	5	*Carcharhinus plumbeus* (Carcharhinidae)	benthic invertebrates and fishes	all ocean	skin and mucosa of gill arches	Hawaii, North Carolina	Bullard et al., 2012, MacLean et al., 2006
Huffmanela filamentosa	6	*Gymnocranius grandoculis* (Lethrinidae)	benthic invertebrates	coastal benthic, reefs	gill mucosa	Nouméa, New Caledonia	Justine, 2004
Huffmanela hamo	7	*Muraenesox cinereus (Muraenesocidae)*	benthic fishes and some benthic invertebrates	soft bottoms and estuaries	musculature	Japan	Justine & Iwaki, 2014

Table 10. Continued.

Species	#	Host taxon	Host Diet	Host Habitat	Organ	Locality	Reference
Huffmanela huffmani	8	Centrarchidae	benthic invertebrates	spring inhabiting centrarchids	inner swimbladder wall	San Marcos River headsprings, Hays County, TX, USA	Cox et al., 2004, Huffman & Moravec, 1988, Moravec, 1987 and present study
Huffmanela japonica	9	*Upeneus bensasi* (Mullidae)	benthic invertebrates	coastal benthic, reefs	musculature	Inland Sea of Japan, off Shikoku Island	Moravec et al., 1998
Huffmanela lata	10	*Carcharhinus amblyrhynchos* (Carcharhinidae)	benthic invertebrates and fishes	coastal benthic, reefs, shelves, drop-offs	outer skin between two gill openings	Nouméa, New Caledonia	Justine, 2005
Huffmanela longa	11	*Gymnocranius grandoculis (Lethrinidae)*	benthic invertebrates	continental shelves and offshore rocky bottoms	mesentery, outer swimbladder wall, and body walls	New Caledonia	Justine, 2007
Huffmanela mexicana	12	*Sphoeroides annulatus (Tetraodontidae)*	benthic invertebrates	benthic, reefs	inner layer of swimbladder wall	Mazatlan, Sinaloa State, Mexico	Moravec & Fajer-Avila, 2000

Table 10. Continued.

Species	#	Host taxon	Host Diet	Host Habitat	Organ	Locality	Reference
Huffmanela moraveci	13	*Odontesthes* spp. (Atherinopsidae)	invertebrates and small fishes	coastal estuaries and lagoons	gill mucosa and skin	Nuevo and San José gulfs, Argentinean Sea	Carballo et al., 2011
Huffmanela oleumimica	14	*Lutjanus campechanus* (Lutjanidae)	benthic invertebrates and fishes	benthic; juveniles found in shallower waters	skin	Gulf of Mexico	Ruiz et al., 2013
Huffmanela ossicola	15	*Bodianus* spp. (Labridae)	benthic invertebrates	coastal benthic, reefs	most bones, gill arch bones	Nouméa, New Caledonia	Justine, 2004, Justine, 2007
Huffmanela paronai	16	*Xiphias gladius (Xiphiidae)*	pelagic fishes and invertebrates	pelagic	skin	Ligurian Sea, Italy	Moravec & Garibaldi, 2000
Huffmanela plectropomi	17	*Plectropomus leopardus (Serranidae)*	benthic invertebrates and fishes	reefs	mesentery near swimbladder wall	Noume´a, New Caledonia	Justine, 2011
Huffmanela schouteni	18	*Hirundichthys affinis, Cheilopogon spp. (Exocoetidae)*	zooplankton and small fishes	epipelagic	Serosa of intestine, swimbladder	Caribbean Sea, Curaçao, and Ligurian Sea, Italy	Moravec & Campbell, 1991, Moravec & Garibaldi, 2003

Table 10. Continued.

Species	#	Host taxon	Host Diet	Host Habitat	Organ	Locality	Reference
Huffmanela shikokuensis	19	*Stephanolepis cirrhifer* (Monacanthidae)	benthic invertebrates	benthic, reefs	musculature	Inland Sea of Japan, off Shikoku Island	Moravec et al., 1998
Huffmanela sp.	20	*Pentapodus aureofasciatus (Nemipteridae)*	benthic invertebrates and zooplankton	reefs	mucosa of mouth and gills	Nouméa, New Caledonia	Justine, 2004
Huffmanela sp.	21	*Trisopterus luscus (Gadidae)*	benthic invertebrates	continental shelves and margins	musculature	Atlantic Ocean, Portugal	Esteves et al., 2009
Huffmanela sp.	22	*Genypterus blacodes (Ophidiidae)*	benthic invertebrates and small fishes	benthic, coastal juveniles	musculature	Pacific Ocean, New Zealand, South Island	Moravec, 2001

Discussion

Nine species of centrarchids from three genera were shown to be susceptible; however infection prevalence and mean intensity rating was not evenly distributed across species. This disparity was determined to not be a function of physiological host specificity, but rather of ecological niche specificity of centrarchid species. Indeed, it would seem that the internal environment of all centrarchids is hospitable to *H. huffmani,* as demonstrated by *Ambloplites rupestris* (Rafinesque) being susceptible[1], despite the species being only distantly related to *Lepomis* and *Micropterus*, which are sister genera (Near et al., 2005). Limnetic invertivorous centrarchids were almost never infected, whereas benthic centrarchids that fed on interstitial invertebrates were almost always infected. This is consistent with the conjecture that the intermediate host must be a species of benthic detritivore and is further consistent with the observation that the eggs are appreciably denser than water. This conjecture was also well corroborated by the niche model contrast analysis explaining the most variance in infection intensity and (AIC weight = 0.943, Table 6).

Therefore, when infection trials were initiated, only benthic invertebrates thought to be detritivores were tested for susceptibility to infection with eggs of *H. huffmani*. After exposing several invertebrate taxa, only one taxonomic group proved to be susceptible, *Hyalella* amphipods. All specimens of amphipods belonging to several species of *Hyalella* (including *Hyalella* taken from localities where *H. huffmani* does not occur) became infected with larvae of *H. huffmani* when exposed to eggs. *Hyalella* is

[1] Based on unpublished accounts from several *A. rupestris* wild-caught from the SMR and examined in parasitology laboratory exercises at Texas State University.

one of the most abundant invertebrates in the SMR headsprings; thus, it is not surprising that *H. huffmani* uses this crustacean as its intermediate host, since this is certainly more advantageous than requiring a much rarer organism. Furthermore, an undescribed spring-run endemic *Hyalella* (referred to herein as SMS *Hyalella* sp.) was restricted to the same geographic distribution as was *H. huffmani*, which is suggestive that host specificity to this *Hyalella* species is responsible for limiting the distribution of *H. huffmani*. However, this common distribution does not appear to be limiting the distribution of *H. huffmani*, since it appears that multiple populations of *Hyalella. cf. azteca* can serve as suitable experimental hosts; in fact the only host which showed any sign of being less suitable was the co-occurring endemic SMS *Hyalella* sp., which often killed the larvae.

Hyalella is a widespread freshwater genus with a cosmopolitan distribution across the Americas without a single marine population (Serejo, 2004; Witt et al., 2006; Wellborn & Broughton, 2008). This observation presents an intriguing enigma, that is: "Why is it that *Huffmanela huffmani* cannot be found throughout all localities where *Hyalella* and centrarchids coexist, given that *Hyalella* can be found co-occurring with centrarchids throughout much of North America, and *H. huffmani* seems to be able to infect numerous species of *Hyalella* and centrarchids?" This makes it difficult to explain the extremely narrow distribution *H. huffmani*, which is likely not being restricted by the distribution of either its intermediate or definitive hosts. This restricted distribution was documented by a prior study on the distribution of *H. huffmani* (Cox et al., 2004), and the present study. Both studies determined that the farthest downstream point where *H. huffmani* can be found is in the proximity of John J. Stokes San Marcos River City Park which is close to the downstream extent of physicochemically stable spring run waters

(Groeger et al., 1997). This is suggestive that the distribution of *H. huffmani* is defined by factors other than the limited distribution of an obligate intermediate host, especially when considering that the remainder of the SMR has abundant *Hyalella cf. azteca* which appear to be physiologically suitable intermediate hosts.

It is curious that the intermediate host in the life cycle of this marine relict is an exclusively freshwater taxon (Serejo, 2004), with all species of the Hyalellidae being found in freshwaters in the Americas. However, the family is contained in the super-family Talitroidea, which is a highly diverse group of amphipods containing terrestrial, semi-terrestrial, freshwater, and marine species (Serejo, 2004). The nearest family to Hyalellidae appears to be Hyalidae, which is a talitroid amphipod family that has numerous species with a circumpolar distribution in littoral and intertidal coastal environments (Serejo, 2004). Therefore, it is possible that talitroids have served as the intermediate hosts in *Huffmanela* life cycles throughout the past; especially when one considers that wave action could possibly be concentrating eggs in hyper densities around coastal shore lines. However, it is difficult to imagine how requiring an intertidal species could be advantageous unless the eggs were resistant to desiccation, which appears not to be the case. Furthermore, though many of the definitive host species of marine *Huffmanela* spp. have a diet consisting of benthic invertebrates, the majority of these hosts are not restricted to just littoral and intertidal habitats. This is further complicated by the fact that two species of definitive host for marine *Huffmanela* are pelagic species (Moravec & Campbell, 1991; Moravec & Garibaldi, 2000) that are extremely unlikely to come into contact with any benthic littoral or intertidal zone invertebrates. With the eggs of *Huffmanela* being appreciably denser than water, it is difficult to imagine how the eggs

of pelagic species ever become consumed by a susceptible intermediate host that could then possibly be later consumed by the correct definitive host species. One pelagic definitive-host species, *Xiphias gladius*, is almost exclusively piscivorous on pelagic fishes (Palko et al., 1981), which suggests the possibility of a second intermediate or paratenic host in this life cycle of that *Huffmanela* species. The epipelagic host fishes in the family Exocoetidae are perhaps the most curious of all described *Huffmanela* host fishes, as they are never found in benthic waters and feed almost exclusively on zooplankton (Casazza & Ross, 2008; Casazza, 2009), thus eliminating the possibility of a second intermediate host fish that transports the *Huffmanela* larvae from the benthos to the definitive host. Aside from these two species, it is highly likely that the intermediate hosts in the life cycle of other marine *Huffmanela* species are benthic invertebrates and if host taxonomy is at all conserved at the first intermediate host level, then these hosts are probably amphipods; most likely gammarideans.

The prepatent period in the *H. huffmani* life cycle appears to be relatively long for a trichinelloid nematode, as most trichinelloid species for which the life cycle is known usually have a prepatent period around 3 months (Køie & Nylund, 2001; Moravec et al., 1998). *H. huffmani* is remarkable in that it appears to have a prepatent period of perhaps 6 months or more and, because this is the first *Huffmanela* life cycle to be understood, it is not known if a long prepatent period is a conserved trait across all *Huffmanela* spp.

To confirm the prepatent period and obtain sufficient amounts of eggs to experimentally complete the life cycle, additional exposures of centrarchids were begun in November through December, 2014. These exposures were also intended to determine if SMR *Hyalella cf. azteca* could successfully transmit infection to the definitive host.

However, as mentioned herein, the prepatent period of *Huffmanela huffmani* is quite long; therefore, these results will not be ready until summer 2015. Thus, the completed life cycle, prepatent period, and intermediate host specificity remains to be determined. Assuming the November/December 2014 exposures successfully infected fish at high intensities, eggs will be taken from these fish and fed out to *Hyalella* spp. to experimentally complete the life cycle.

3. THE BIOGEOGRAPHY AND POPULATION BIOLOGY OF *HYALELLA* AND *HUFFMANELA HUFFMANI*

Introduction

All previously reported *Huffmanela* species other than *H. huffmani* are marine (Justine, 2007; Ruiz et al., 2013) which is suggestive that *H. huffmani* is descended from a marine ancestor. There is also a suite of other invertebrates found across the Edwards Aquifer and its many springs, including the SMS, which appear to be marine derived (Gibson et al., 2008). Some relics are known only from the San Marcos Springs and an adjacent artesian well (Holsinger & Longley, 1980). Like many other spring endemic taxa, *H. huffmani* has a restricted geographic distribution to only the upper spring run of the SMR (Cox et al., 2004; Michel, 1984; O'Docharty, 2007).

It is hypothesized that *Huffmanela* were established in freshwater by the inundation of a former marine transgression and then upon regressing, isolated pockets of water contained marine organisms that eventually adapted to freshwater. It is also hypothesized that Holocene droughts extirpated most of these populations leaving only populations which survived in refugia. The hosts are obligate aquatic, which suggests that there must have been a suitable abundance of physicochemically suitable water without interruption since the introduction into the localities where they still occur. This hypothesis (referred to herein as the drought refugia hypothesis) also implies that freshwater *Huffmanela* have had all of their life cycle requirements met since their introduction into the localities they continue to occur in.

It was determined that *H. huffmani* utilizes *Hyalella* as an intermediate host, and it was determined that there is a SMS endemic *Hyalella* that is morphologically unique from other *Hyalella* (present study; referred to herein as SMS *Hyalella* sp.). Both SMS *Hyalella* sp. and *H. huffmani* are thought to be remnant populations that survived in refugia during Holocene droughts and therefore, it is thought that the *Hyalella* / *H. huffmani* host-parasite relationship could have been the result of adaption before the species became isolated in the SMS. If so, the presence of spring endemic *Hyalella* could indicate that the spring they inhabit might be a refugium for other marine relics including other populations of freshwater *Huffmanela*.

Clear Creek Springs (CCS) in Menard County, Texas is a tributary of the San Saba River (SSR) and is the only known locality of the described *Hyalella texana,* which is perhaps the most morphologically distinct of all *Hyalella* species. With this as a clue, a population of freshwater *Huffmanela* in CCS was discovered by the present study which is the only other known freshwater *Huffmanela* population in the world. The distribution of this population was later extended to include the spring run of the SSR some 23 river km to the west. At the headsprings of the SMR, CCS, and SSR, the water is relatively physicochemically stable for all parameters, but becomes increasingly more ambient with distance from the headsprings. The SSR/CCS population of *Huffmanela* is ≈800 river km from the Gulf of Mexico while the SMR population of the *Huffmanela* is ≈400 river km from the Gulf of Mexico with no known intervening populations (Cox et al., 2004; Michel, 1984; present study). Geographic distributions are shown in Figure 29.

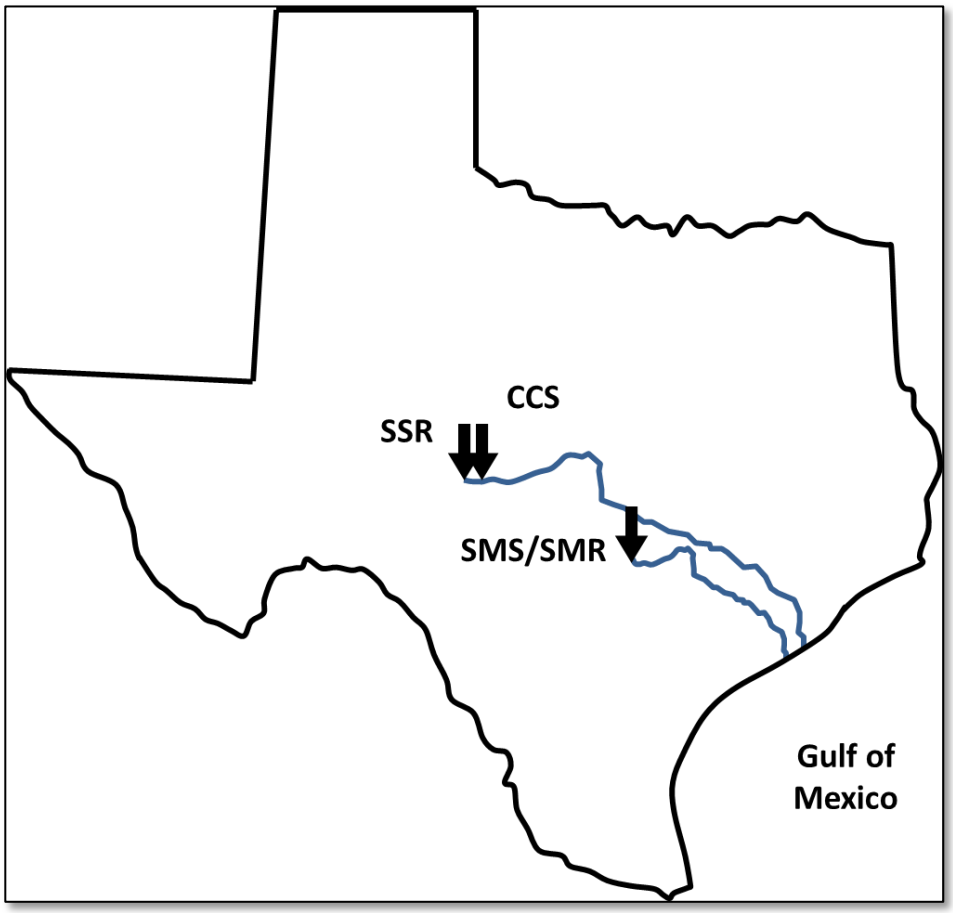

Figure 29. Known distributions of freshwater *Huffmanela*; found only at the headsprings of two rivers in drainages that ultimately empty into the Gulf of Mexico.

To try to explain the distribution of freshwater *Huffmanela* and spring endemic *Hyalella* the following questions are being asked to address the biogeography of freshwater *Huffmanela* and spring endemic *Hyalella*:

1. Does *H. huffmani* possess unique adaptations that its marine relatives do not have that allowed it to survive in freshwater?
2. Could *H. huffmani* have survived prolonged droughts which are, and have been, common features of spring environments throughout the Edwards Plateau?

3. Are the two headspring *Hyalella* species that co-occur with the two freshwater *Huffmanela* populations unique and reproductively isolated from *Hyalella* of the *H. azteca* species complex?
    - Is morphological variation between morphologically distinct populations of *Hyalella* heritable or a manifestation of environmentally induced plasticity?
    - If morphological differences prove to be heritable, are these morphological races reproductively isolated?
    - Do molecular phylogenies agree with the hypothesized phylogeography and the results of the reproductive isolation experiments?

Question #1 – Does *H. huffmani* possess unique adaptations that its marine relatives do not have that allowed it to survive in freshwater?

Assuming that *H. huffmani* originated from a formerly marine ancestor is consistent with the center of diversity hypothesis. There is ample evidence that modern-day Central Texas was inundated by an inland sea until approximately the KT boundary, approximately 65 ma (Kauffman, 1984). Subsequent to the KT boundary, the seas inundating the Central Texas area began to regress, due partly to a global decrease in sea level, and also due to regional uplifting associated with the Laramide Orogeny (Laubach & Jackson, 1990). This uplifting is known as the Llano Uplift, and is thought to have ultimately raised the strata of Central Texas perhaps thousands of meters (Barker et al., 1994; Woodruff & Abbott, 1979). The uplifting was caused by extensive igneous intrusions which contributed to the Balcones faulting during the early Miocene and produced the San Marcos Platform (Barker et al., 1994; Woodruff & Abbott, 1979), the

remnant of which is known today as the Texas Hill Country. Today, the only remaining marine deposits in central Texas were deposited by this former inland sea, and are Cretaceous or early Cenozoic in age (Hart et al., 2012; Yancey, 1995). There is no stratigraphic record of any subsequent marine transgressions over what is now central Texas. Therefore, the late Cretaceous is the earliest time that the marine relics of central Texas springs could have become isolated in freshwater. However, there is evidence that the global sea level rose high enough on multiple occasions during the Cenozoic to have submerged the areas surrounding the Llano Uplift all the way inland to what is now the Balcones Escarpment (Haq et al., 1987; Kominz et al., 2008; Kominz et al., 1998; Miller et al., 1987; Zachos et al., 2001). Therefore, there is no geologic evidence to suggest that the final event that isolated the marine relicts of Central Texas springs occurred prior to the KT boundary (c.a. 65 ma), nor later than the mid-Miocene (c.a. 6-14 ma).

The geologic history certainly seems to have allowed for the opportunity for marine organisms to diverge down freshwater lineages in Central Texas if not Central North America. However, it is still logically perplexing that freshwater taxa could be derived from marine ancestors when one considers that the osmoregulatory issues faced by a marine species are opposite of those of a freshwater species. Therefore, it is difficult to imagine how such a great osmotic leap could have occurred. However, *Huffmanela* is an endoparasite and only has to cope with the osmotic conditions of its hosts' internal environments, and the internal environments of osmoregulating marine and freshwater fishes are appreciably more similar to each other than freshwater is to salt water. Therefore, osmoregulatory adaptations would seemingly be a moot point for *Huffmanela*. However, *Huffmanela* eggs are freed from the tissues of their host when their host dies

and afterwards can be found in or on the benthic sediments of their environment (Cox et al., 2004; O'Docharty, 2007). In fact, it appears that much of the life span of at least *H. huffmani* is spent free in the environment as an egg waiting to be consumed by an intermediate host. Therefore, one would expect that the eggs of *H. huffmani* either have specific adaptations to freshwater, or they are adapted to all types of salinities and therefore salinity tolerances were not a factor in their transition to freshwater. Ultimately, the survivability of *H. huffmani* eggs in different salinities should shed light on how complicated the transition from a marine environment to freshwater or vice versa might have been for *Huffmanela* eggs.

Question #2 – Could *H. huffmani* have survived droughts which are known to affect spring environments throughout the Edwards Plateau?

Once remnant populations became established in freshwater, the question then becomes, "How did the populations that survived the intervening millions of years until present become so geographically restricted?" It is reasonable to assume that these marine relics were once much more widely distributed in Central Texas, and then their distributions became contracted by the Hypsithermal Droughts of the Holocene. Presumably, the only places where marine relicts can still be found today are those where extirpation of the relicts did not occur. The last of the Hypsithermal Droughts are thought to have relented some 2 ka, and it was at this time that the area's surface waters likely became hydrologically reconnected (Al-Rabab'ah & Williams, 2004; Bryant & Holloway, 1985; Davis & Shaw, 2001; Ellwood & Gose, 2006; Hall & Penner, 2013; Nordt et al., 1994; Russ et al., 2000).

The Hypsithermal Drought explanation suggests two additional pieces of insight. The first is that the water level of the SMS must have been maintained at or near the surface since its marine relicts first became established, and the second is that other localities that have a similar history of continuous surface water might also contain relict taxa. Therefore, if *H. huffmani* is not able to survive even short periods of desiccation, then it should not be expected to have been able to survive long periods of desiccation associated with the droughts of record as well as the Hypsithermal Droughts. If it can be shown that *H. huffmani* is not capable of tolerating desiccation, then it should be possible to speculate that *H. huffmani* formerly had a much more expansive range than presently observed, and that the present distribution is the result of survival in a drought refugium.

Question #3 – Are headspring *Hyalella* that co-occur with freshwater *Huffmanela* unique and reproductively isolated from *Hyalella* of the *H. cf. azteca* morphotype?

If *H. huffmani* survived in a drought refugium, its hosts must have also survived in the same refugium. Though all *Hyalella* populations appear to be susceptible to infection with *H. huffmani*, it is possible that extant spring populations of *Hyalella* became reproductively isolated from other *Hyalella*, and that most other populations of *Hyalella* represent a secondary introduction of *H. cf. azteca* into the region after droughts lifted; whereas spring *Hyalella* represent vestiges of an earlier introduction of the genus into the region.

Subquestion 3.1 - Is phenotypic variation between morphologically distinct populations of Hyalella *heritable or plastic?*

Spring forms of *Hyalella* have been reported to have unique phenotypes from the typical *H. cf. azteca* (Cole & Watkins, 1977; Stevenson & Peden, 1973). These differences include, larger and more numerous dorsal mucronation, more laterally compressed body, differences in the curvature of the body, different gnathopod morphology, etc. In the present study it was also noticed that spring amphipods appear to be less precocious than *H. cf. azteca*, and it was hypothesized that this was due to delayed maturation in spring forms when compared to *H. cf. azteca*. Because spring morphs share common characteristics it is possible that springs select for common morphology, or spring morphs have conserved traits retained from a common ancestor.

Subquestion 3.2 - If phenotypic differences prove to be heritable, are these morphological races also reproductively isolated?

If these spring endemic populations represent more ancient introduction to Central Texas and survived in refugia while the *H. cf. azteca* complex represents a secondary introduction of *Hyalella* to the region, then one would expect that reproduction would be successful between the different *H. cf. azteca* populations, possibly between the spring populations, but not between any of *H. cf. azteca* and either of the spring-endemic *Hyalella*.

Subquestion 3.3 – Do molecular phylogenies agree with the hypothesized phylogeography and reproductive isolation results?

The genetic structure in a group of organisms with common ancestry is thought to reflect the history of the barriers to gene flow. Therefore, it was hypothesized that the topology of a *Hyalella* phylogeny would show that SMS *Hyalella* sp. and *H. texana* are very distantly related to all other *Hyalella*, while the other *Hyalella* in this study (Devils River *H. cf. azteca*, Comal River *H. cf. azteca*, and SMR *H. cf. azteca*) would all group as close relatives to other *Hyalella* that are common across North America. It was then proposed that nodes could be attributed to historical events by using a molecular clock.

Methods

Question #1 – Salinity tolerance of eggs of *H. huffmani*

H. huffmani eggs were obtained by allowing egg-laden host tissue to decay (presumably due to microbes) in artesian water in a sealed 1,800 ml container (fish sources, $n = 8$). From this stock of eggs, a 1-ml aliquot of eggs was added to a sealed 500 ml container of 35 ppt salt water. Another 1-ml aliquot was added to 45 ppt treatment group. Eggs were placed immediately into salt water without acclimation. Vitality was assessed by determining if manipulated eggs were less infective than control eggs when fed to *Hyalella*. *Hyalella* ($n = 4$ per experimental group) were exposed to an aliquot of 50 µL of eggs in a sealed 150 ml container for 4 days and afterwards were examined for infection. All water used was artesian physicochemically stable water. *Hyalella* were fed an algae-based commercial fish food.

Question #2 – Desiccation tolerance of eggs of *H. huffmani*

Desiccation Experiment

A 100 µl aliquot of eggs was placed in a 100 ml beaker full of artesian water and was allowed to evaporate until dry. A control was established differing only in that it was not allowed to desiccate by the replenishment of water. After 1 week, the treatment group had become completely desiccated. It was allowed to sit dry for a week while the control group continued to receive water. After a week, the desiccated eggs were rehydrated and allowed to sit for a week and receive water as needed. Vitality was estimated by comparing the abilities of the control and treatment groups to infect *Hyalella*.

Comal Fish – 1951

The Comal River headsprings ceased flowing during the greatest drought of recorded history in 1957, which was an event known to have extirpated other taxa (Phillips et al., 2011). To test if the drought of the 1950's extirpated a former population of *Huffmanela* from Comal Springs, five benthic centrarchids from a museum collection that were collected in 1951 were examined for infection with *Huffmanela*.

Question #3 – Are headspring *Hyalella* that co-occur with freshwater *Huffmanela* unique and reproductively isolated from *Hyalella* of the *H. cf. azteca* morphotype?

Source of experimental Hyalella *and culture methods*

Hyalella from five populations (Table 11) were collected and placed in culture between January and August 2014. The *Hyalella* from the various collection localities

have varying degrees of morphological distinction from each other (Figure 33-Figure 32). This morphological variation occurs most noticeably in the number and length of dorsal spines. Presented in Table 11is the number of dorsal spines that have been observed in the five populations.

Table 11. Collection localities for *Hyalella* and characteristic count for each locality.

Collection Locality	Coordinates	Culture Taxon	Dorsal spine count (variants)	Figure Reference
Devils River (Finnegan Springs)	29° 54' 3.80" N 100° 59' 55.07" W	*Hyalella* sp.	2 (0-2)	Figure 30
Comal River	29° 42' 38.00" N 98° 7' 39.60" W	*Hyalella cf. azteca*	2 (0-2)	Figure 31
San Marcos River	29° 53' 27.42" N 97° 55' 56.73" W	*Hyalella cf. azteca*	2	Figure 32
San Marcos River	29° 53' 36.10" N 97° 55' 52.80" W	*Hyalella* sp. (SMS)	3 (4)	Figure 33
Clear Creek Springs	30° 54' 22.20" N 99° 57' 29.20" W	*Hyalella texana*	4	Figure 34

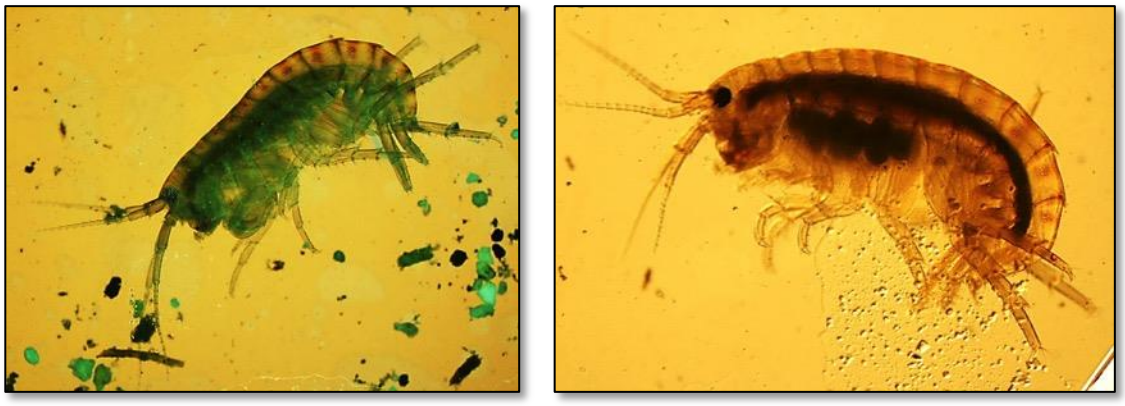

Figure 30. Devils River *Hyalella cf. azteca*: male on left; female on right.

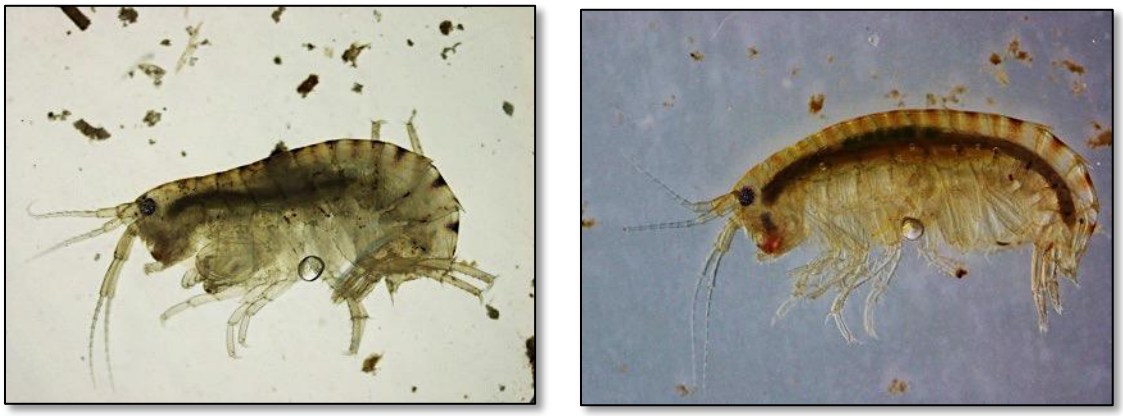

Figure 31. Comal River *Hyalella cf. azteca*: male on left; female on right.

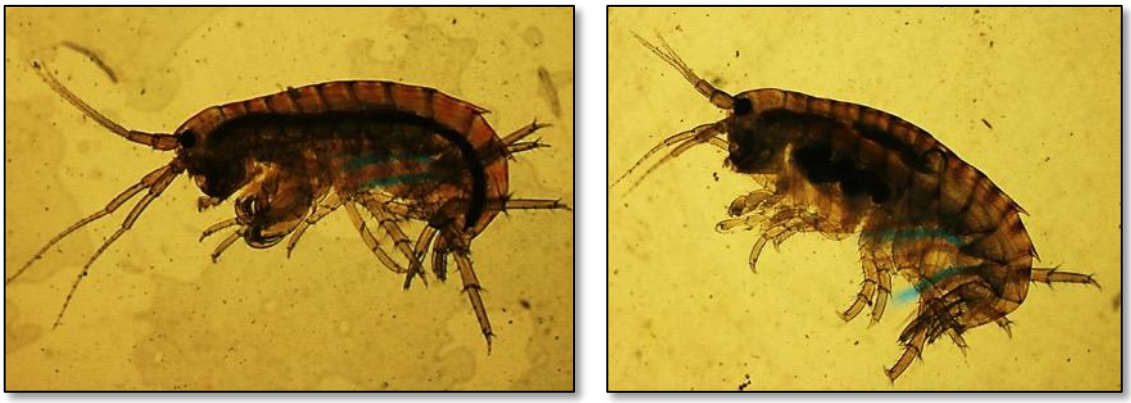

Figure 32. SMR *Hyalella cf. azteca*: male on left; female on right.

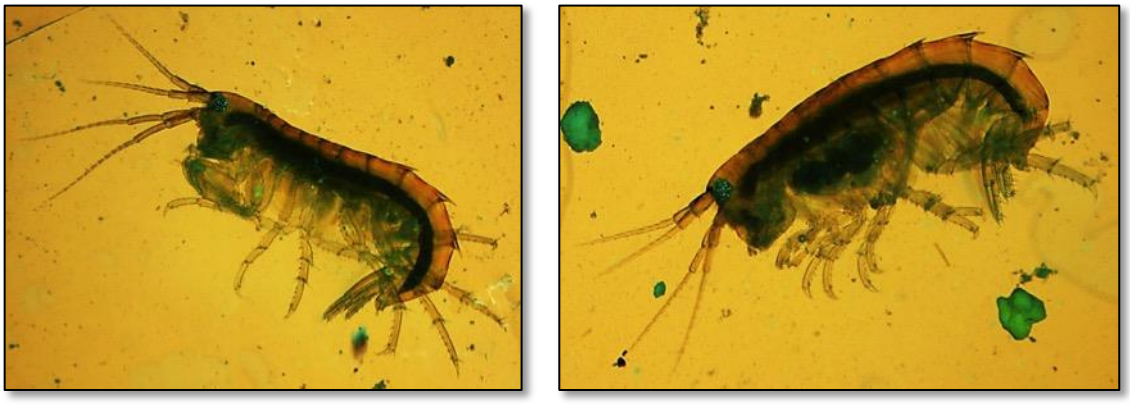

Figure 33. SMS *Hyalella* sp.: male on left; female on right.

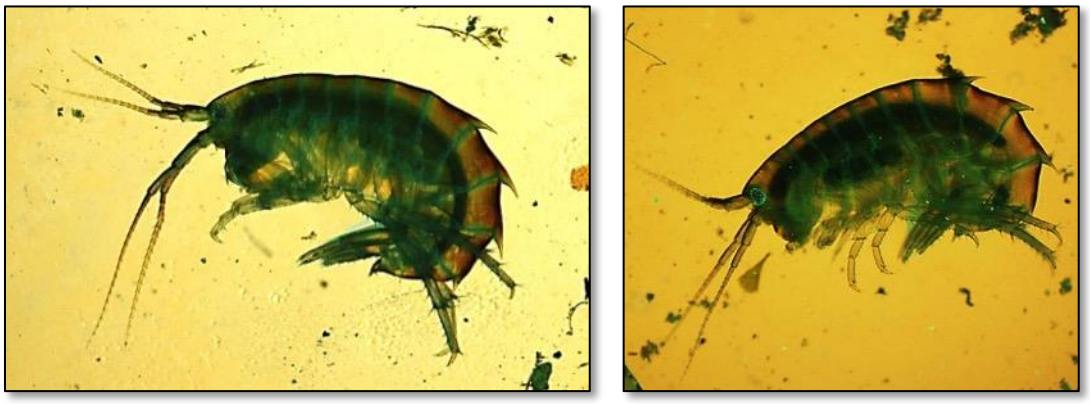

Figure 34. *Hyalella texana*: male on left; female on right.

All populations were kept in separate cultures to maintain genetically pure lines. Cultures were kept in a sealed containers suspended in a thermally stable 22 $^{\circ}$C artesian water flow through system and were kept on a 12h/12h-light/dark cycle. All populations were reared on various wild collected algae, bryophytes, and periphyton, and were supplemented with algae-based commercial fish food.

Subquestion 3.1 - Common garden methods and design

To determine if development varies between ecotypes of *Hyalella*, all taxa were reared in common-garden cultures. Cultures were started by placing one randomly selected male and one randomly selected female conspecific adults from the same population in a 150 mL sealed container. Only females that were not already brooding young were utilized. Culture containers were supplied weekly with food consisting of algae, bryophytes, and detritus. Each container was filled with artesian water, which was replaced once weekly. All other attributes were kept constant across all cultures by maintaining them in the same water bath.

If neonates were produced, adults were removed and offspring were left in the original container and were maintained under the same conditions as the parents had been. Growth rate was observed by taking length measurements once weekly. This was done by gently restraining neonates under a wet mount and then measuring with a reticle at 40x magnification. Neonates were also checked for sexual dimorphism once weekly to estimate the age and size at which each *Hyalella* population became sexually dimorphic. Growth rate was found to be almost perfectly linear with little variance for all taxa for the duration of the study (example of linear growth rate shown in Figure 35). Simple one-way ANOVAs were employed to determine if population could explain variation in growth rate, age of becoming sexually dimorphic, and size of becoming sexually dimorphic. Each common garden was replicated two to four times for each population as time and space allowed.

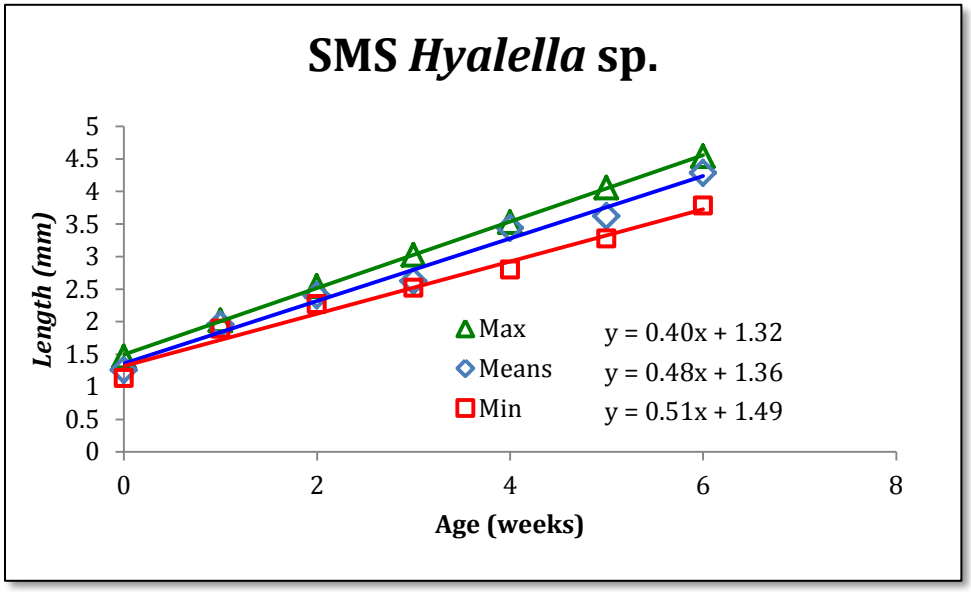

Figure 35. Age and growth curves for one brood of artificially reared *Hyalella* sp. from the San Marcos River.

Subquestion 3.2 - Attempted "no-choice" hybridization experiments with Hyalella *spp.*

This experiment was designed to see if *Hyalella* from the different populations were reproductively isolated by performing a "no-choice" attempted hybridization experiment. Neonates from the common-garden experiment were separated by sex as soon as they became sexually dimorphic to insure that they had never mated (they become sexually dimorphic before becoming sexually mature). All possible hybrid combinations of population source and sex (combinations presented in Table 12) were established in cultures and maintained the same way as in the common-garden experiment. Some combinations could not be achieved because it was difficult to find males of the various *Hyalella* types large enough to mate with female *H. texana* because *H. texana* is appreciably larger than the other *Hyalella*, and male amphipods typically must be larger than females to successfully mate (Bollache & Cézilly, 2004).

Table 12. All possible hybrid combinations of *Hyalella* population source and sex, with each count representing one pair of male and female *Hyalella*.[1,2]

	Female Type				
Male Type	SMS	SMR	Comal	Devils	*H. texana*
SMS	2*	1	1	1	1
SMR	1	1*	1	1	0
Comal	2	1	2*	1	1
Devils	2	0	0	1*	2
H. texana	1	1	1	1	2*

Cultures were checked weekly for hatched neonates. When neonates were found, the time since start of attempted hybridization was recorded. The cumulative proportions of mating success for each experimental cross was calculated each week.

Question #4 – COI phylogeny of select *Hyalella* sequences of North America

Working with Dr. Gary Wellborn from the University of Oklahoma, we generated ≈700 bp sequences of the COI locus (n = 3 for each of the five study populations). An additional 178 *Hyalella* sequences and several outgroup sequences were imported from GenBank (accession numbers included in Figure 36 on the right and listed in **Error! Reference source not found.**). The GenBank sequences were aligned with sequences generated herein using the program Geneious R6 (Kearse et al., 2012). GenBank sequences were selected on the criterion that at least some geographic information was available for the collection locality. After aligning sequences, segments with missing

[1] Conspecific controls indicated by asterisk.
[2] Red represents any of the possible *H. cf. azteca* X spring endemic *Hyalella* combinations; blue represents attempted mating of the two spring endemic *Hyalella*; yellow represents controls of conspecific *Hyalella* from same locality; and green represents any of the *H. cf. azteca* X *H. cf. azteca* from different localities.

data in the alignment were trimmed from all sequences and sequences with excessive missing data were discarded yielding a 498 bp complete alignment for all sequences included. A neighbor-joining tree (Figure 36) was built with the program MEGA6 (Tamura et al., 2013). The tree was rooted using *Gammarus* spp., *Allorchestes angusta*, *Hyale prevostii*, and *Talitrus saltator* as outgroups. Node support was estimated by bootstrapping. Divergence times since the most recent common ancestor (MRCA) were estimated based on percent substitutions and a molecular clock rate adapted from the work of Knowlton & Weigt (1998).

Results

Question #1

Eggs that were treated with salt water showed no immediate decline in infectivity. After two weeks, infectivity of treated eggs began to decline while control eggs showed no decline (Table 13).

Table 13. Differential vitality of hatched larvae after salinity trials.

Experimental Group	Treatment Duration (Weeks)			
	1	2	3	4
0 ppt	Active Larvae	Active Larvae	Active Larvae	Active Larvae
35 ppt	Active Larvae	**Inactive Larvae**	**Inactive Larvae**	**Inactive Larvae**
45 ppt	Active Larvae	**Inactive Larvae**	**Not Infected**	**Dead Larvae**

Question #2

All eggs that were desiccated experimentally were not vital while controls showed normal vitality (Table 14). This trend was evaluated in a natural environment by the fact that *Huffmanela* occurred in Comal Springs prior to being exposed to desiccation during the drought of record in 1957, but no *Huffmanela* can be found there at present (Table 15).

Table 14. Results of desiccation experiment.

Experimental Group	Duration	Vitality Test
Desiccated	1 week	Not viable
Control	1 week	Viable

Table 15. Results of necropsy of specimens from Comal Springs.

Species	*n*	Collection Date	Collection Locality	Prevalence
All Centrarchidae	60	June 23, 2014	Comal Springs	0%
All Centrarchidae	60	Jan. 8, 2015	Comal Springs	0%
Lepomis miniatus	3	Dec. 1, 1951	Comal Springs	100%
L. microlophus	2	Dec. 1, 1951	Comal Springs	50%

Question #3

Subquestion 3.1

Table 16. *Hyalella* common garden growth rate and maturation results.

Population	Reps	Brood count	Mean growth rate (mm/week)	Size of sexual dimorphism (mm)	Age of sexual dimorphism (weeks)
Devils	1	24	0.462	3.116	4
Devils	2	12	0.72	3.340	3
Devils	3	22	0.699	3.000	3
Devils	4	7	0.734	3.390	3
Comal	1	2	0.454	2.395	2
Comal	2	4	0.874	2.917	2
Comal	3	4	0.587	2.645	2
SMR	1	4	0.591	3.145	2
SMR	2	11	0.260	2.456	3
H. texana	1	8	1.110	4.709	3
H. texana	2	11	1.545	4.899	3
H. texana	3	8	0.949	4.257	4
SMS	1	7	0.503	3.437	4
SMS	2	4	0.577	3.786	4
SMS	3	5	1.039	3.498	4

Although growth rates in Table 16 appear to vary randomly within and among populations, it was found to be weakly correlated with population $p\left[\left(F_{a(1),4,10} \geq 4.05\right) = 0.03\right]$. Age of reaching sexual dimorphism proved to vary significantly between populations $p\left[\left(F_{a(1),4,10} \geq 9.15\right) = 0.002\right]$ (Table 16). Not surprisingly, with age of reaching sexual dimorphism varying between taxa, size also varied significantly $p\left[\left(F_{a(1),4,10} \geq 23.3\right) \square\ 0.001\right]$ (Table 16). Based on these findings and assuming that age of sexual dimorphism and sexual maturity are correlated, this would suggest that *H. cf. azteca* populations are maturing more rapidly when compared

to spring-endemic *Hyalella*, possibly explaining why *H. cf. azteca* populations appear to be more prolific.

Subquestion 3.2

All *Hyalella* that fit the *H. cf. azteca* morphotype successfully reproduced, though there appeared to be hesitation to reproduce with mates that were not from the same locality and morphotype (Table 17). Neither of the spring species hybridized with any other population of *Hyalella*, suggesting that they are completely reproductively isolated from all other *Hyalella*.

Table 17. Cumulative proportion of successfully reproducing pairs.

		Duration (weeks)							
Hyalella combo	*n*	1	2	3	4	5	6	7	8
SMS x *H. cf. azteca*	7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H. texana x *H. cf. azteca*	6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SMS x *H. texana*	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H. cf. azteca from different localities	4	0.00	0.00	0.00	**0.25**	**0.50**	**0.50**	**0.75**	**0.75**
Conspecifics from same locality	7	0.00	0.00	**0.29**	**0.43**	**0.86**	**0.86**	**0.86**	**1.00**

Question #4

The COI phylogeny for select *Hyalella* is presented in Figure 36. Secondary clades (Figure 36) represent groups which have less than 10% divergence from the MRCA of the clade. This criterion allows for a maximum of 20% substitutions per site between members of the same clade which is much more relaxed than most species-

designation thresholds, thus clades are probably underestimating diversity (Lefébure et al., 2006).

The molecular clock rate was developed based on the estimate by Knowlton & Weigt (1998) of 1.4% substitution per million years at the COI locus for *Alpheus* spp.; a genus of snapping shrimp. Because these shrimp have an estimated 1 generation per year whereas *Hyalella* have an estimated herein mean of 4 generations per year, we estimate that *Hyalella* probably has a mean molecular substitution rate of 5.6% substitution per million years (0.014*4 = 0.056).

Devils and Comal *H. cf. azteca* belong to the widespread and abundant Clade A. This group is quite diverse and contains fourteen subgroups for the sequences we included with an estimated MRCA around ≈1.1 ma (mid-Pleistocene), suggesting that this group was fragmented into several somewhat isolated groups during the Pleistocene. Within each of these fourteen subgroups, divergence ranged from 0.0% to 1.0% from the MRCA, with the majority ranging from 0.1% to 0.2%. This suggests that the MRCA in each subgroup lived at around 17 to 34 ka. This would suggest most diversification within the fourteen subgroups occurred since the last glacial maximum (LGM).

SMR *H. cf. azteca* belong to the widespread and abundant Clade B. Within this clade there are two main lineages for the sequences analyzed. Both subgroups are roughly 5% diverged from their MRCA, suggesting that the lineages diverged some 900 ka (a mid-Pleistocene divergence), suggesting this group was also fragmented into isolated groups during the Pleistocene. Within the subgroups within Clade B, sequence divergence from the MRCA ranged from 0.0% to 1.0% for both subgroups, with the majority of sequences ranging from 0.1% to 0.3%. This suggests that the MRCA of each

of the two subgroups lived at around 18 to 53 ka, once again indicating that these two subgroups probably diversified since the LGM.

Hyalella texana exhibited 10% divergence (≈1.78 ma, mid-Pleistocene) from its MRCA with its nearest relative (*H. muerta*, from Death Valley, CA). These two *Hyalella* populations are distant relatives to their nearest sister taxa, which are two genetically distinct populations of *H. sandra* (also from Death Valley). Sequences of the *H. texana*, *H. sandra*, and *H. meurta* group are 14% diverged (≈2.5 ma, early-Pleistocene) from their MRCA with all other *Hyalella*. Deep sequence divergence is common of many spring endemic *Hyalella* sequences we analyzed. However, this group is the most deeply diverged of all sequences analyzed for North American *Hyalella*. It is curious that *H. texana* groups with *H. sandra*, and *H. meurta* because both *H. sandra* and *H. meurta* can only be found in springs in Death Valley, CA (Baldinger, 2000). Furthermore, these three forms are morphologically dissimilar to each other; however, they are perhaps the most morphologically aberrant of all the *Hyalella*. *H. texana* is perhaps the largest and most mucronate *Hyalella* species (Stevenson & Peden, 1973) while *H. meurta* is an eyeless, amucronate, dwarf stygobiont (Baldinger, 2000).

SMS *Hyalella* sp. exhibits 10% divergence (≈1.78 ma, mid-Pleistocene) from its MRCA with its nearest relative (Clade B *Hyalella*) and 14% divergence (≈2.5 ma, early-Pleistocene) from its MRCA with all other *Hyalella*. Many other *Hyalella* collected from springs show similarly deep divergences in the tree.

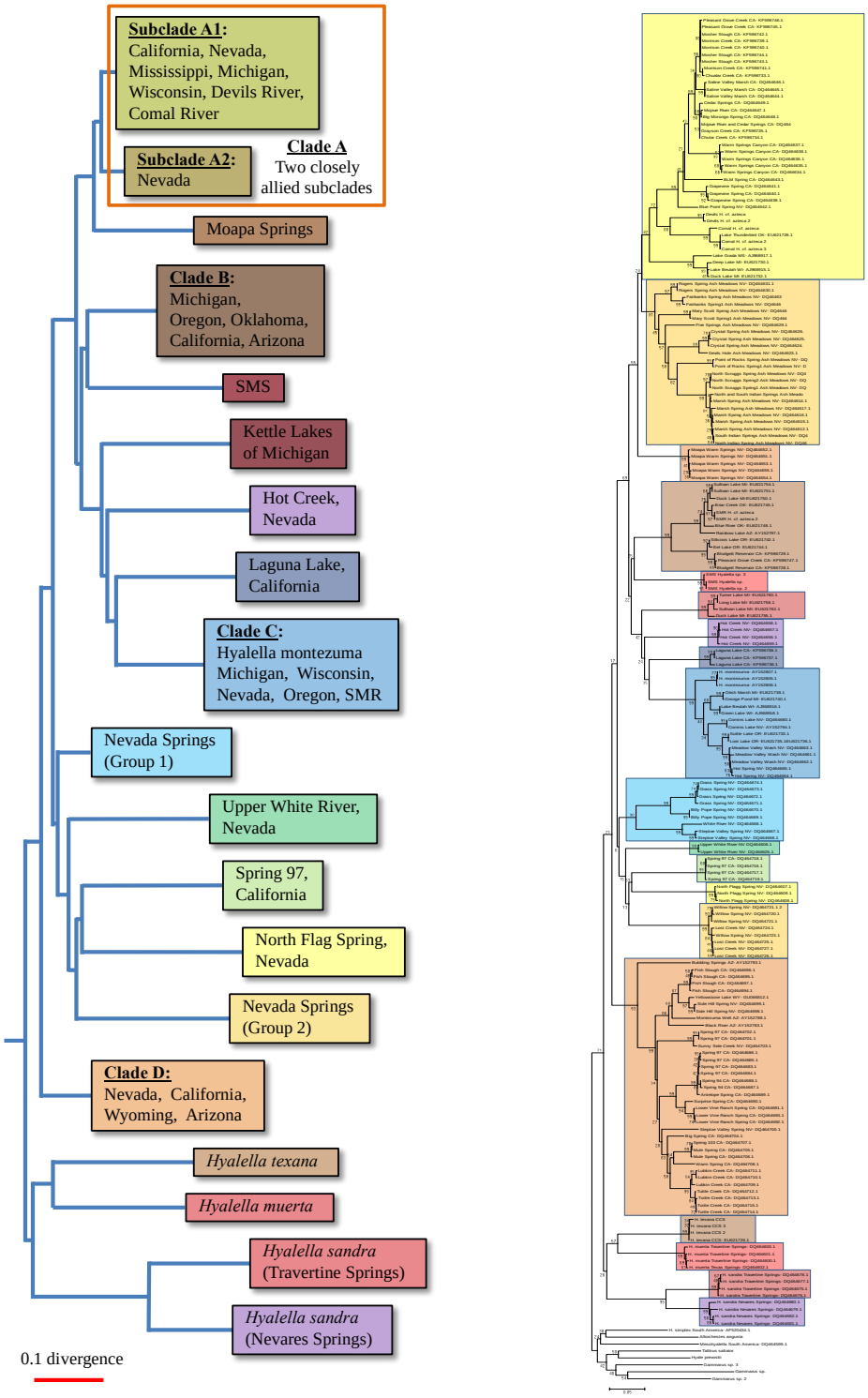

Figure 36. Phylogeny of select *Hyalella* populations based on divergence of COI gene: condensed version on left; expanded version on right.

Discussion

It may never be possible to definitively determine the origins and ancestry of *Hyalella* and freshwater *Huffmanela*. However, the taxonomic affiliations of both taxa suggest that they are marine derived (Justine, 2007; Ruiz et al., 2013; Serejo, 2004). Assuming that they no longer are marine also assumes that they must be divergent from the majority of their relatives. The distribution of these two genera sheds light on the variation in successful differentiation that is possible between marine derived lineages in freshwater. *H. huffmani* and CCS *Huffmanela* appear to be relicts because they are marine derived and have highly limited distributions. *Hyalella*, however, is quite widespread across North America and therefore are not thought of as marine derived because they do not have relic distributions. However, *H. texana* and SMS *Hyalella* sp. both appear to have relic distributions due to droughts and, like other *Hyalella*, are also marine derived (Serejo, 2004). Before CCS and SMS spring endemic *Hyalella* and *Huffmanela* became relicted to just CCS and SMS, both taxa likely had a much wider distribution but were relicted by droughts of the Holocene. Therefore, it may be more appropriate to think of "marine relic" taxa of the Edwards Aquifer springs as marine derived taxa that are "drought relics."

Salinity and desiccation were both shown to affect eggs of *H. huffmani.* The gradual response of eggs to salt water (Table 13) suggests that they still have rather broad salinity tolerances for at least limited durations but the immediate response of eggs to desiccation (Table 14) suggests that drought has been a major factor in sculpting the distribution of freshwater *Huffmanela* populations. This was reinforced by evidence of *Huffmanela* in Comal Springs prior to the drought of the 1950's (

Table 15). Numerous springs in Texas have been documented to have gone dry (Brune, 2002), suggesting that these types of extirpations are not uncommon.

Both SMS *Hyalella* sp. and *H. texana* are morphologically most similar to each other (of the five types cultured in this study) with more numerous and longer dorsal spines, and laterally compressed bodies. According to the COI phylogeny, spring morphology appears to be a convergent trait as SMS *Hyalella* sp. is more closely related to all other *Hyalella* in this study than it is to *H. texana*. *H. montezuma* is also a spring endemic *Hyalella* which has a similar sort of spring morphology (Cole & Watkins, 1977) though it appears to have diverged more recently than other spring endemic *Hyalella* and does not share recent common ancestry with SMS *Hyalella* sp. or *H. texana*. The common garden experiment indicated that phenotype is heritable and not plastic suggesting that springs select for convergence on a similar phenotype. Interestingly, spring species appear to have delayed maturation when compared to populations of the *H. cf. azteca* thus suggesting that stable spring conditions may have led to the relaxation of selection on reproductive effort. The contrary explanation would be that the stable conditions of springs have conserved ancestral phenotypes in relicts while *H. cf. azteca* is a divergent, more modern phenotype which could explain the success of the phenotype. *H. cf. azteca* tend to inhabit a variety of water body types and thus appear to be generalists with widespread distributions. As multiple distinct clades apparently fit this general morphology it is likely that being a generalist selects for the *H. cf. azteca* morphotype.

All of the amphipods of the *H. cf. azteca* morphotype in this study produced offspring though it appeared to occur reluctantly when compared to controls of

conspecifics from the same locality (Table 17). This suggests that some reproductive isolation may exist between the *Hyalella* that did hybridize. Where the hybridizing taxa are positioned in the COI tree is somewhat surprising. Clade A contains the Devils River and Comal River populations while Clade B contains the SMR population. Clade B has 12% divergence from its MRCA with Clade A but has only 10% divergence from its MRCA with SMS *Hyalella*; however SMR *H. cf. azteca* proved to be reproductively isolated from SMS *Hyalella* sp. This suggests that apparently being of the *H. cf. azteca* morphotype vs. the spring morphotype does not reflect recent common ancestry and neither does the ability to hybridize. All attempted hybrid combinations were seen in amplexus whether they successfully produced offspring or not which suggests that reproductive isolation is not entirely behavioral but has a mechanistic component as well.

Degree of gene flow, genetic diversity, and population size may help to understand why reproductive isolation did not reflect common ancestry. All of the *H. cf. azteca* populations in this study represented populations of much more widespread species; i.e. Clade A and Clade B. The spring populations show no evidence of being able to hybridize with any other population including other spring populations. Spring populations also have no closely allied taxa in the COI tree and essentially represent single population species with no gene flow. The fact that Clade A and Clade B hybridized appears to verify the suggestion by Palumbi (1994) that durations without gene flow may not be as significant a factor as population size in the development of biological barriers to reproduction. Though there is no evidence of gene flow between Clade A and Clade B, there is evidence of high genetic diversity and gene flow across populations within both of these clades. If there is abundant gene flow and variation

(within and between populations), it would be difficult to retain any novel random variation that arose spontaneously in a population unless the variation was highly advantageous because it would likely be overwhelmed with introgression of more abundant alleles. However, in isolation, gene flow cannot overwhelm novel variation as readily because there is only gene flow within the population. Apparently, after enough time in isolation as a small population, variation unique to an isolated population arises that results in biological barriers to reproduction. This appears to be the case in the present study; SMS *Hyalella* would not hybridize with their closest relative, Clade B, but Clade B did hybridize with a much more distant relative, Clade A. This suggests that the exchange of genetic diversity within widely distributed and genetically diverse clades functions to reduce the probability of reproductive isolation arising within and between widely distributed and genetically diverse clades. In other words, it stands to reason that retaining the ancestral form of reproductive compatibility would be selected for in widely distributed abundant species in order to maintain reproductive compatibility within the taxon. Therefore, multiple large clades descended from a common ancestor should be expected to maintain reproductive compatibility with their common ancestor therefore maintaining reproductive compatibility between clades even with no gene flow between clades. However, if a population went through a bottleneck then the probability of becoming reproductively isolated should be increased because there less severe selection against novelty.

The biogeographic history

The COI tree largely agreed with what was predicted by the drought refugia hypothesis. The common pattern across spring *Hyalella* populations from the present study and from springs across the U.S.A. seems to be divergence into single population endemic species. The estimated divergence of most spring populations was around the beginning of the Pleistocene suggesting that spring populations are vestiges from *Hyalella* which dispersed across North America before or during the Pleistocene; the majority of which became extirpated either due to glaciation or Holocene droughts. Of course, some populations continued into the *H. cf. azteca* of today. The estimated diversification within the five major *H. cf. azteca* clades into sub-groups is estimated to have occurred during the mid-Pleistocene which suggests that these groups arose in glacial refugia. Finally, the diversification within each of these subgroups appears to have happened only very recently (since the LGM) which suggests that they diversified during the Holocene. This may indicate that the majority of the *H. cf. azteca* populations represent a second introduction of *Hyalella* to many of the drainages of Texas after the droughts of the Holocene relented.

Future research

This study was only recently initiated following the finding of *Hyalella* as the intermediate host of *H. huffmani* and the discovery of *Huffmanela* in CCS/SSR. Being relatively new research there are still many aspects that have yet to be addressed. One interesting aspect yet to be fully addressed is the host-parasite co-evolution that apparently occurred between SMS *Hyalella* sp. and *H. huffmani* in the SMS and between

H. texana and the CCS *Huffmanela* in CCS. Based on our observations, SMR *H. cf. azteca* do not have the capacity to kill off larvae of *H. huffmani* but SMS *Hyalella* sp., which presumably evolved with *H. huffmani*, seem to be able to kill off many of the larvae that infect them. Because *H. huffmani* has an estimated functional generation time of a year or more and *Hyalella* is estimated to have an average of 4 of more generations a year, it is not surprising that the presumably co-evolved SMS *Hyalella* sp. would have adapted defenses to infection. This makes for an unusual host-parasite relationship because almost never are host generations shorter than parasite generations. If SMR *H. cf. azteca* migrated into the SMR only recently as hypothesized, then they should be relatively naïve to infection with larvae of *H. huffmani* which may explain why no dead larvae have been observed in any of the *H. cf. azteca* that were experimentally infected. It is possible that aspects of host-parasite coevolution may have occurred differently in different isolated habitats. Currently there is minimal support in our study system for this idea, however, when *H. texana* were infected with CCS *Huffmanela* (whom they presumably coevolved with) only 10% succumbed to infection, but when *H. texana* was infected with eggs of *H. huffmani* (whom they did not coevolve with), 80% succumbed to infection suggesting that *H. huffmani* and CCS *Huffmanela* have evolved to affect their hosts in very different ways. This finding is only preliminary and needs to be investigated further. Building a phylogeny of *Huffmanela* would also be very useful because it would help interpret the history of cohabitation of *Huffmanela* and *Hyalella*.

The drought refugia hypothesis needs to be addressed further as well. One major area of interest would be trying to explain how it is that spring endemic *Hyalella* and *Huffmanela* have not re-dispersed out of refugia since the Holocene droughts relented.

One possible explanation is that when populations were restricted to refugia they lost most of their genetic diversity; including diversity that permits tolerance of ambient fluctuations in their physicochemical environment (at least at the organismal level if not the population). Spring runs have very minimal physicochemical fluctuation compared to the remainder of the rivers they feed into; therefore it is possible that spring endemics (because of limited genetic diversity) can only survive in physicochemically stable spring waters. This concept may explain why the distribution of *H. huffmani* is negatively correlated with an increase in variability of water temperature in the SMR (Figure 37) as a function of distance from headsprings (Figure 37, Figure 38). To further explore this, it is being proposed that the long term thermal tolerance limits and long term thermal variation tolerances of spring endemics be studied, particularly for *H. huffmani*, CCS *Huffmanlea*, SMS *Hyalella* sp., and *H. texana*. This data could then be compared to tolerances of more cosmopolitan relatives and correlated with environmental parameters to evaluate if thermal tolerances are a contemporary factor limiting the distribution of spring endemics.

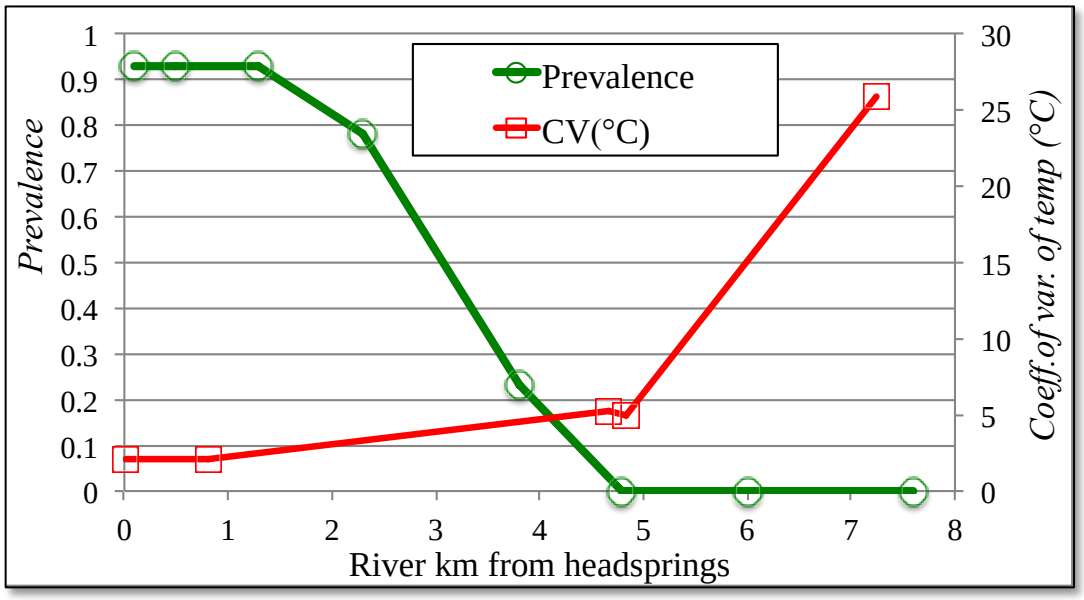

Figure 37. Prevalence for *Huffmanela huffmani* in wild-caught fish from varying distances downstream from headsprings, and associated coefficients of variation in seasonal water temperature swings at nearby stations.

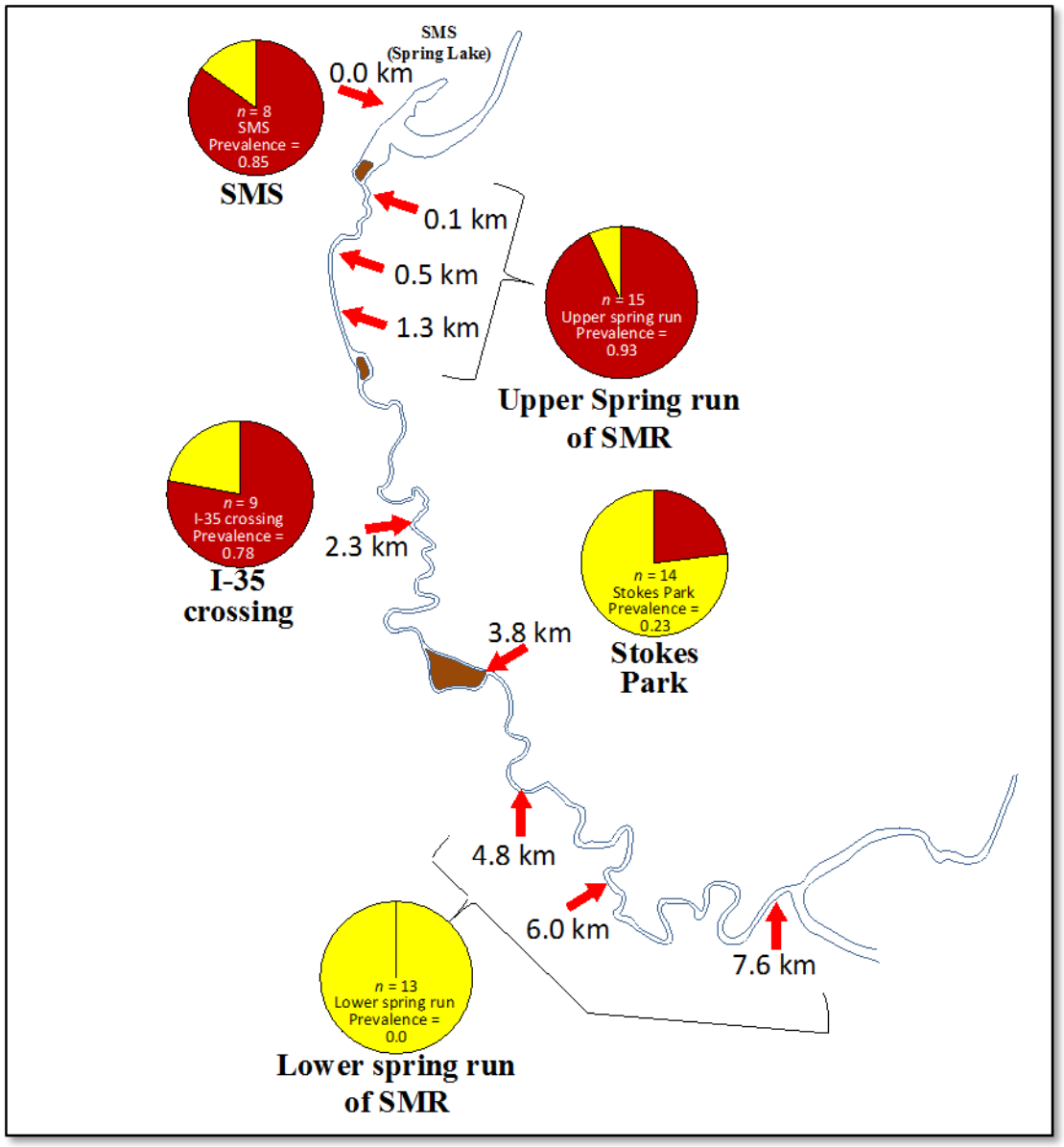

Figure 38. Prevalence of *Huffmanela huffmani* in wild-caught fish from the SMR as affected by river kilometers downstream from headsprings.

Reproductive isolation results of *Hyalella* presented herein are only preliminary and need to be verified further with more replicates of the no-choice mate experiments (already commenced research) as well as mate choice experiments. Mate choice experiments would involve allowing males and females to choose mates from all of the possible types of experimental *Hyalella* including their own. The frequency with which

mates are chosen within and between the same and different morphotypes and clades would help address behavioral aspects of prezygotic isolation.

Another aspect which needs to be properly addressed before too strong of inference can be made about the heritability of phenotype is more extensive characterization of the morphology of each of the five types of experimental *Hyalella*. This research is planned for summer 2015 and will also likely include the formal description of SMS *Hyalella* sp. as a species.

Finally, this study used only one mitochondrial (COI) locus to construct the *Hyalella* phylogeny; therefore, many aspects of the phylogeny could be suspect. The single locus approach was used because it was accessible. A multi loci analysis would be much more informative but this type of data was not generated herein nor was it available for most populations of *Hyalella* across the Americas.

4. METACERCARIAE OF *MACRODEROIDES* FROM CRANIAL BONES OF *DIONDA DIABOLI* OF SPRING-FED SYSTEMS IN WEST TEXAS

Introduction and Background

The Devils River minnow (*Dionda diaboli*) is a federally protected species with a geographic distribution limited to only a few watersheds in west Texas and Mexico (Garrett et al., 2004). A captive refugium population is maintained at the San Marcos Aquatic Resource Center (SMARC) operated by the US Fish and Wildlife Service (USFWS). Periodically, wild minnows would be collected by the SMARC staff from locations where the minnow has been known to occur and brought back to the facility for use as breeders. On several occasions during the past few years, one or more fish collected from the Pinto Creek spring run would abruptly exhibit erratic darting and spinning behaviors, go off feed, and later die of exhaustion and emaciation. In 2013, a fresh-dead "spinner" was necropsied for parasites. Because the symptoms seemed neurotic, the cranium and brain of the fish were examined first, and numerous metacercarial cysts of a trematode later identified as *Macroderoides* sp were found encysted in the neurocranial and splanchnocranial bones (Figure 39; Figure 40).

Figure 39. Photomicrograph (incident light with dissecting microscope) of several metacercariae of *Macroderoides* from cranial bones of a Devil's River minnow (*Dionda diaboli*) from Pinto Creek.

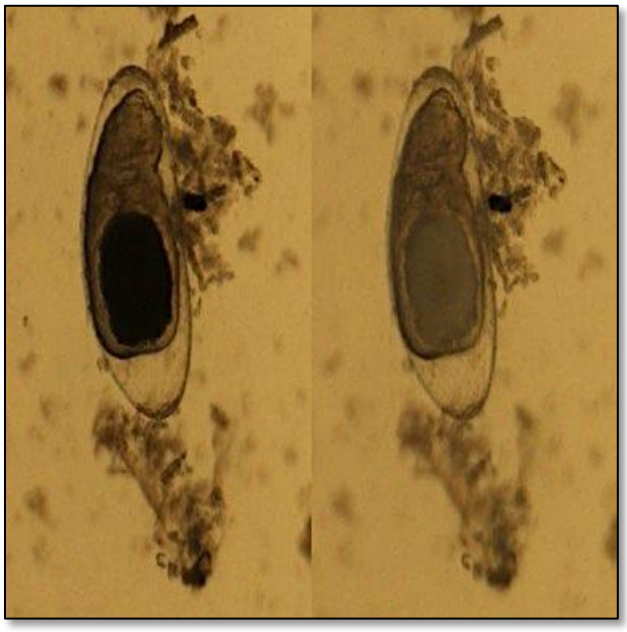

Figure 40. *Macroderoides* cyst in transmitted and incident light.

Preliminary Results with Discussion

Following this discovery, archived specimens of other wild-caught spinners that had died earlier were obtained and examined, and cysts from the same trematode species were found in the cranial bones of all fish examined. While these findings are highly suggestive that the metacercariae were the cause of this fatal neurosis, it should be noted that no control fish were examined (i.e., wild-caught *D. diaboli* from Pinto Creek not exhibiting spinning behavior), so the cause of the spinning neurosis cannot be definitively attributed directly to the metacercariae. A few asymptomatic fish wild-caught from Pinto Creek were still available at SMARC, but a decision was made to avoid sacrificing them for examination due to the low abundance of the fish at Pinto Creek, and also low numbers of wild-caught minnows from that site remaining alive at the USFWS refugium. Nonetheless, in the absence of results from control fish, it is assumed that these metacercariae were probably responsible for the aberrant spinning behaviors and deaths of the spinners.

It should be noted that, while the refugium has collected parental stock from other sites, including the Devils River, and San Felipe Springs, all infected spinners we know of from the refugium had been wild-caught from Pinto Creek. Additionally, we were later given three very small F_1 *D. diaboli* spinners to examine. These F_1 minnows were produced at the refugium by breeding wild-caught parental stock of unknown parentage. Curiously, all three F_1 spinners were negative for *Macroderoides* cysts.

The fact that the F_1 spinners were negative for *Macroderoides* cysts complicates the cause/effect picture. A further complication underlying the cause/effect picture is that the neurosis exhibited by the spinners does not seem to have been caused directly by

pressure on or physical displacement of the central nervous system (CNS). Fish affected by pressure on the CNS often become listless, broody, and develop outwardly visible pigmentation mosaics due to interference with peripheral nerves, and also usually show some degree of exophthalmia ("pop eyes"). But the spinners, in contrast, become more active than their unaffected cohorts and wear themselves out in bursts of very fast darting and spinning behaviors, and do not show outward signs of skeletal deformation or exophthalmia. It is possible that the metacercariae of *Macroderoides* is a carrier of a rickettsial organism that is transmitted to the host fish, which in turn causes the neurosis. Although *Macroderoides* has never been shown to transmit a microbe of any sort to fish, several other trematodes have been shown to harbor and transmit microbial disease to some of their definitive hosts. The presence of a potentially pathogenic microbe in *Macroderoides* would help explain how the three F_1 spinners became spinners without having been exposed to cercariae of *Macroderoides*. If the mother of these F_1 spinners was infected with *Macroderoides* metacercariae that had transmitted a *Neorickettsia* to her prior to spawning, the resulting offspring may have become spinners due to transovarial transmission of the *Neorickettsia*. However, this is mere speculation until there is a search for neorickettsial DNA in affected minnows. It should also be noted that we could find no reports of fish, the second intermediate hosts, being adversely affected by neorickettsial diseases transmitted by trematodes.

The USFWS is charged with the responsibility of successfully maintaining a breeding population of *D. diaboli* in the refugium, and maintaining disease-free stock is crucial to this goal. Therefore, it became imperative to determine that these metacercarial infections were not being contracted at the refugium. To determine this, information

about the life cycle of the parasite was crucial in order to know how it might be transmitted. This required identification of the parasite to at least genus. Analysis of both the mitochondrial and nuclear genomes by Dr. Dittmar Hahn's lab at Texas State University indicated that the encysted parasites belong to the genus *Macroderoides*. A 1,000 bp sequence of the 18S rRNA was 100% congruent with that *M. typicus*, while a 1,000 bp segment of the ITS region was 100% congruent with that *M. spiniferus*.

The genus *Macroderoides* Pearse, 1924 (Digenea: Macroderoididae) includes nine species of plagiorchoid digeneans parasitic in fishes, including the seven North American species *M. spiniferus* Pearse, 1924; *M. typicus* (Winfield, 1929); *M. parvus* (Hunter, 1932); *M. flavus* (Van Cleave & Mueller, 1932); *M. trilobatus* (Taylor, 1978); *M. texanus* (Tkach et al., 2008); and *M. minutus* (Tkach & Kinsella, 2011); and the two Indian species *M. seenghali* (Gupta & Agrawal, 1968); and *M. raychaudhurii* (Agarwal & Kumar, 1983).

Following identification to genus, it became possible to speculate about the life cycle and therefore, the feasibility of fish-to-fish transmission at the *Dionda diaboli* refugium. The species of *Macroderoides* for which life cycles have been reported use planorbid snails as first intermediate host, then a small fish as second intermediate host, and usually a gar or bowfin for the definitive host.

Upon further investigation at SMARC, it was discovered that *Helisoma* sp. (a planorbid snail that serves as first intermediate host for *M. spiniferus*) is used in the *D. diaboli* refugium tanks as a cleaning agent. However, all of these snails are cultured in captivity and should not be expected to have ever encountered a miracidium of *Macroderoides* because this would require that the snails had been exposed to water that

infected gar had contaminated with *Macroderoides* eggs. Neither gar nor bowfins occur on station at SMARC but gar does occur in abundance in Pinto Creek (Mike Fonteyn, pers. Comm.), therefore it was deemed highly unlikely that any of the refugium minnows had become infected after arrival at SMARC. To verify this, fifteen *Helisoma* were taken from the tanks that had housed infected fish and examined for larval trematodes. None of the snails were positive for any trematode larvae. These two observations are consistent with all infected wild-caught spinners having been already infected before they arrived at the SMARC facility.

The next concern was to determine if *Macroderoides* occurred in the other habitats where *Dionda diaboli* is endemic. Archived specimens of six species of small fishes that had been collected during the years 2011, 2012, and 2014 were examined for infection with *Macroderoides* from four different drainages: Pinto Creek, Dolan Creek, Devils River, and San Felipe Springs. No fishes examined from Dolan Creek or San Felipe Springs were found to be infected with *Macroderoides*; however, at least some individuals of all species examined from Pinto Creek and the Devils River were found to be infected with *Macroderoides* metacercariae. Prevalence, mean intensity, and sample size is presented in Table 18 and Table 19.

Table 18. Prevalence, mean intensity, and mean density of *Macroderoides* in pooled species examined by drainage.

Locality	Prevalence	Mean Intensity	Mean Density	*n*
Devils River	30%	5.67	2.13	10
Dolan Creek	0%	0.00	0.00	10
Pinto Creek*	36%	1.40	0.50	14
San Felipe Springs	0%	0.00	0.00	31

*Does not include data for spinners examined from refugium stock discussed earlier.

Table 19. Prevalence, mean intensity, and mean density of *Macroderoides* by host in infected drainages.

Locality	Species	Prevalence	Mean Intensity	Mean Density	*n*
Devils River	*Dionda argentosa*	17%	3.00	0.50	6
Devils River	*Cyprinella venusta*	50%	7.00	3.50	4
Pinto Creek	*Gambusia affinis*	14%	1.00	0.14	7
Pinto Creek	*Notropis amabilis*	75%	1.67	1.25	4
Pinto Creek	*Poecilia latipinna*	33%	1.00	0.33	3
Pinto Creek*	*Dionda diaboli*	100%	14.10	14.10	10

*These are the wild-caught spinners from refugium stock mentioned earlier.

From the available data it can be determined that:

1. Infected fishes from the refugium wild stock became infected before they were brought into captivity.
2. This parasite only occurs in the Devils River and Pinto Creek, or at least is appreciably lower in abundances at San Felipe and Dolan Creek, if it occurs there at all.
3. Probably many small fishes belonging to the Cypriniformes are susceptible to infection and can serve as reservoir hosts.
4. Observed morbidity was likely due to infection with *Macroderoides*.

Future direction of research

1. Obtain reliable estimates of the prevalence and mean intensities for the various species of small fishes in the infected drainages because existing sample sizes are too small. Therefore, more robust sample sizes are needed, preferably at least 30 fish of each affected species from each drainage. We plan to start these collections in May.
2. Neurosis was not conclusively determined to be directly caused by *Macroderoides* infection; therefore, it is suggested that captive healthy fish are experimentally infected at SMARC using wild caught *Helisoma* which are shedding *Macroderoides* cercariae.
3. If morbidity is conclusively linked to infection with metacercariae of *Macroderoides*, it is still possible that the metacercariae themselves are not the cause of morbidity, but rather an intracellular bacterium, *Neorickettsia*. This microbe is potentially being transmitted to host fishes by the metacercariae and could be the true cause of

morbidity. To approach this, we must determine if the *Macroderoides* are in fact harboring a *Neorickettsia* and if this *Neorickettsia* is being transmitted to the host. The best way to do this would be to attempt to amplify *Neorickettsia* DNA from encysted metacercariae, morbid hosts, and healthy hosts uninfected with *Macroderoides.* Because it is possible that *Neorickettsia* can be transmitted vertically through host generations, care needs to be taken to insure that uninfected fish are not descended from an infected ancestor. However, to confirm vertical transmission, it should also be attempted to amplify *Neorickettsia* DNA from fish reared at SMARC that are known to be direct descendants of *Macroderoides* infected wild-stock *D. diaboli* from Pinto Creek

4. Determine if *Macroderoides* has contributed to the decline of the Pinto Creek population of *D. diaboli*, archival specimens should be necropsied to try to determine if prevalence and intensity of infection has increased in recent years. The recent droughts are thought to have severely affected the flow in Pinto Creek thus possibly enhancing the infection rate of *D. diaboli* with *Macroderoides*. This is because, with a lower stand of water fish should have less volume to move about in thus increasing the probability that a cercariae could find a host. To test this, flow data should be analyzed if available for correlation with past infection rates of *D. diaboli* with *Macroderoides.*
5. Both 18S rRNA and the ITS region identified the trematode as belonging to the genus *Macroderoides*, however, the two loci identified the worm as two different species. Additionally, since *Macroderoides* metacercariae have never been reported from cranial bones of fishes, it appears that this may be a new species of *Macroderoides.*

Because immature trematodes rarely have taxonomically identifiable characteristics and also poorly studied, adult specimens must be obtained in order to determine if this is a known species or to describe the species if it proves to be undescribed.

APPENDIX SECTION

APPENDIX 1

Table 20. Invertebrates identified from the SMS by various authors.

Phylum	Subphylum	Class	Subclass	Order	Family	*Genus (Subgenus)*	*species*
Annelida		Aphanoneura			Aeolosomatidae	*Aeolosoma*	*tenuidorsum*
Annelida		Aphanoneura			Aeolosomatidae	*Aeolosoma*	*quarternarium*
Annelida		Clitellata		Branchiodellida			
Annelida		Clitellata	Hirudinea	Arhynchobdellida	Erpobdellidae		
Annelida		Clitellata	Hirudinea	Rhynchobdellida	Piscicolidae		
Annelida		Clitellata	Hirudinea	Rhynchobdellida	Glossiphoniidae	*Placobdella*	*parasitica*
Annelida		Clitellata	Oligochaeta	Lumbriculida	Lumbriculidae		
Annelida		Clitellata	Oligochaeta	Haplotaxida	Haplotaxidae	*Haplotaxis*	sp.
Annelida		Clitellata	Oligochaeta	Haplotaxida	Naididae	*Stylaria*	*lacustris*
Annelida		Clitellata	Oligochaeta	Haplotaxida	Naididae	*Chaetogaster*	*limaei*
Annelida		Clitellata	Oligochaeta	Haplotaxida	Naididae	*Chaetogaster*	diastrophus
Annelida		Clitellata	Oligochaeta	Haplotaxida	Naididae	*Chaetogaster*	crystallinus
Annelida		Clitellata	Oligochaeta	Haplotaxida	Naididae	*Pristina*	sp.
Annelida		Clitellata	Oligochaeta	Haplotaxida	Naididae	*Nais*	sp.
Annelida		Clitellata	Oligochaeta	Haplotaxida	Naididae	*Dero (Dero)*	*obtusa*
Arthropoda	Chelicerata	Arachnida		Halacarida			
Arthropoda	Chelicerata	Arachnida		Hydracarina	Arrenuridae		
Arthropoda	Chelicerata	Arachnida		Hydracarina	Aturidae		
Arthropoda	Chelicerata	Arachnida		Hydracarina	Hydryphantidae		
Arthropoda	Chelicerata	Arachnida		Hydracarina	Lebertidae		

Table 20. Continued.

Phylum	Subphylum	Class	Subclass	Order	Family	*Genus (Subgenus)*	*species*
Arthropoda	Chelicerata	Arachnida		Hydracarina	Limnesiidae		
Arthropoda	Chelicerata	Arachnida		Hydracarina	Limnocharidae		
Arthropoda	Chelicerata	Arachnida		Hydracarina	Pionidae		
Arthropoda	Chelicerata	Arachnida		Hydracarina	Torrenticolidae		
Arthropoda	Chelicerata	Arachnida		Orbatida			
Arthropoda	Crustacea	Branchiopoda		Cladocera	Chydoridae	*Eurycercus*	sp.
Arthropoda	Crustacea	Branchiopoda		Cladocera	Daphniidae	*Daphnia*	sp.
Arthropoda	Crustacea	Maxillopoda	Copepoda	Cyclopoida			
Arthropoda	Crustacea	Maxillopoda	Copepoda	Harpacticoida	Canthocamptidae		
Arthropoda	Crustacea	Maxillopoda	Copepoda	Harpacticoida	Laophontidae		
Arthropoda	Crustacea	Malacostraca	Eucarida	Decapoda	Cambaridae		
Arthropoda	Crustacea	Malacostraca	Eucarida	Decapoda	Palaemonidae	*Palaemonetes*	*antrorum*
Arthropoda	Crustacea	Malacostraca	Eucarida	Decapoda	Palaemonidae	*Palaemonetes*	sp.
Arthropoda	Crustacea	Malacostraca	Eucarida	Decapoda	Palaemonidae	*Macrobrachium*	*carcinus*
Arthropoda	Crustacea	Malacostraca	Peracarida	Amphipoda	Crangonyctidae	*Crangonyx*	*pseudogracilis*
Arthropoda	Crustacea	Malacostraca	Peracarida	Amphipoda	Crangonyctidae	*Stygobromus*	spp.
Arthropoda	Crustacea	Malacostraca	Peracarida	Amphipoda	Hyalellidae	*Hyalella*	*azteca*
Arthropoda	Crustacea	Malacostraca	Peracarida	Amphipoda	Hyalellidae	*Hyalella*	sp. (Spring Endemics)
Arthropoda	Crustacea	Malacostraca	Peracarida	Isopoda	Asellidae	*Lirceolus*	spp.
Arthropoda	Crustacea	Malacostraca	Peracarida	Isopoda	Cirolanidae	*Cirolanides*	*texensis*
Arthropoda	Crustacea	Ostracoda				*Bradleycypris*	*oblique*
Arthropoda	Crustacea	Ostracoda				*Candona*	cf. *neglecta*
Arthropoda	Crustacea	Ostracoda				*Chlamydotheca*	*texasiensis*

Table 20. Continued.

Phylum	Subphylum	Class	Subclass	Order	Family	*Genus (Subgenus)*	*species*
Arthropoda	Crustacea	Ostracoda				*Cypria*	*ophthalmica*
Arthropoda	Crustacea	Ostracoda				*Darwinula*	*stevensoni*
Arthropoda	Crustacea	Ostracoda				*Eucypris*	cf. *pigra*
Arthropoda	Crustacea	Ostracoda				*Physocypria*	*kreapelini*
Arthropoda	Crustacea	Ostracoda				*Pseudocandona*	cf. *stagnalis*
Arthropoda	Crustacea	Ostracoda				*Stenocypris*	cf. *major*
Arthropoda	Crustacea	Ostracoda				*Typhlocypris*	sp.
Arthropoda	Hexapoda	Insecta		Coleoptera	Elmidae		
Arthropoda	Hexapoda	Insecta		Coleoptera	Dryopidae		
Arthropoda	Hexapoda	Insecta		Coleoptera	Dytiscidae		
Arthropoda	Hexapoda	Insecta		Coleoptera	Hydrophilidae		
Arthropoda	Hexapoda	Insecta		Coleoptera	Psephenidae		
Arthropoda	Hexapoda	Insecta		Diptera	Athericidae		
Arthropoda	Hexapoda	Insecta		Diptera	Ceratopogonidae		
Arthropoda	Hexapoda	Insecta		Diptera	Chironomidae		
Arthropoda	Hexapoda	Insecta		Diptera	Culicidae		
Arthropoda	Hexapoda	Insecta		Diptera	Empididae		
Arthropoda	Hexapoda	Insecta		Diptera	Rhagionidae		
Arthropoda	Hexapoda	Insecta		Diptera	Simuliidae		
Arthropoda	Hexapoda	Insecta		Diptera	Stratiomyidae		
Arthropoda	Hexapoda	Insecta		Diptera	Tabanidae		
Arthropoda	Hexapoda	Insecta		Diptera	Tipulidae		
Arthropoda	Hexapoda	Insecta		Ephemeroptera	Baetidae		
Arthropoda	Hexapoda	Insecta		Ephemeroptera	Ephemeridae		

Table 20. Continued.

Phylum	Subphylum	Class	Subclass	Order	Family	*Genus (Subgenus)*	*species*
Arthropoda	Hexapoda	Insecta		Ephemeroptera	Isonychiidae		
Arthropoda	Hexapoda	Insecta		Ephemeroptera	Leptohyphidae		
Arthropoda	Hexapoda	Insecta		Ephemeroptera	Leptophlebiidae		
Arthropoda	Hexapoda	Insecta		Hemiptera	Gerridae		
Arthropoda	Hexapoda	Insecta		Hemiptera	Naucoridae		
Arthropoda	Hexapoda	Insecta		Hemiptera	Veliidae		
Arthropoda	Hexapoda	Insecta		Lepidoptera	Crambidae		
Arthropoda	Hexapoda	Insecta		Odonata	Aeshnidae		
Arthropoda	Hexapoda	Insecta		Odonata	Cordulidae		
Arthropoda	Hexapoda	Insecta		Odonata	Gomphidae		
Arthropoda	Hexapoda	Insecta		Odonata	Libelullidae		
Arthropoda	Hexapoda	Insecta		Odonata	Calopterygidae		
Arthropoda	Hexapoda	Insecta		Odonata	Coenagrionidae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Glossosomatidae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Heliocopsychidae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Hydrobiosidae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Hydropsychidae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Hydroptilidae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Leptoceridae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Philopotamidae		
Arthropoda	Hexapoda	Insecta		Trichoptera	Polycentropodidae		
Cnidaria					Hydradae	*Hydra*	sp.
Gastrotricha				Macrodasyida		*Redudasys*	sp.
Gastrotricha				Chaetonotida		*Chaetonotus*	sp.

Table 20. Continued.

Phylum	Subphylum	Class	Subclass	Order	Family	*Genus (Subgenus)*	*species*
Mollusca		Bivalvia		Veneroida	Corbiculidae		
Mollusca		Bivalvia		Veneroida	Sphaeriidae		
Mollusca		Gastropoda		Caenogastropoda	Ampullariidae	*Marisa*	*cornuarietis*
Mollusca		Gastropoda		Cerithioidea	Pleuroceridae	*Elimia*	*comalensis*
Mollusca		Gastropoda		Lymnaeoidea	Lymnaeidae	*Pseudosuccinea*	*columella*
Mollusca		Gastropoda		Lymnaeoidea	Lymnaeidae	*Fossaria*	*dalli*
Mollusca		Gastropoda		Mesogastropoda	Thiaridae	*Melanoides*	*tuberculata*
Mollusca		Gastropoda		Mesogastropoda	Thiaridae	*Tarebia*	*granifera*
Mollusca		Gastropoda		Planorboidea	Ancylidae	*Hebetancylus*	*sp.*
Mollusca		Gastropoda		Planorboidea	Ancylidae	*Laevapex*	*fuscus*
Mollusca		Gastropoda		Planorboidea	Ancylidae	*Gundlachia*	*radiata*
Mollusca		Gastropoda		Planorboidea	Physidae	*Physa*	sp.
Mollusca		Gastropoda		Planorboidea	Planorbidae	*Gyraulus*	*parvus*
Mollusca		Gastropoda		Planorboidea	Planorbidae	*Helisoma*	*anceps*
Mollusca		Gastropoda		Planorboidea	Planorbidae	*Biomphalaria*	*obstructa*
Mollusca		Gastropoda		Rissooidea	Hydrobiidae	*Amnicola*	*limosa*
Mollusca		Gastropoda		Rissooidea	Hydrobiidae	*Cincinnatia*	*comalensis*
Mollusca		Gastropoda		Rissooidea	Hydrobiidae	*Pyrgophorus*	*spinosus*
Nemertea		Enopla		Hoplonemertea	Tetrastemmatidae	*Prostoma*	cf. *graecense*
Platyhelminthes		Rhabditophora		Seriata	Kenkiidae	*Sphalloplana*	*mohri*
Platyhelminthes		Turbellaria		Macrostomida			
Platyhelminthes		Turbellaria		Triclada	Dugesiidae	*Schmidtea*	sp.
Platyhelminthes		Turbellaria		Triclada	Dugesiidae	*Dugesia*	sp.

Table 20. Continued.

Phylum	Subphylum	Class	Subclass	Order	Family	*Genus (Subgenus)*	*species*
Porifera		Demospongiae		Haplosclerida	Spongillidae		
Tardigrada				Heterotardigrada	Coronarctidae		

Identifications by David Bowles, Pete Diaz, Jay Lyndon (masters thesis), and McLean Worsham.

APPENDIX 2

Table 21. Accession information for amphipod DNA sequences used to generate phylogenetic trees.

Accession	Geographic Location	Clade
AJ968915.1	Lake Beulah WI	Clade A1
AJ968917.1	Lake Grada MS	Clade A1
DQ464634.1 - DQ464638.1	Warm Springs Canyon CA	Clade A1
DQ464639.1 - DQ464641.1	Grapevine Spring CA	Clade A1
DQ464642.1	Blue Point Spring NV	Clade A1
DQ464643.1	BLM Spring CA	Clade A1
DQ464644.1 - DQ464646.1	Saline Valley Marsh CA	Clade A1
DQ464647.1	Mojave River CA	Clade A1
DQ464648.1	Big Morongo Spring CA	Clade A1
DQ464649.1	Cedar Springs CA	Clade A1
DQ464650.1	Mojave River and Cedar Springs CA	Clade A1
EU621728.1	Lake Thunderbird OK	Clade A1
EU621730.1	Deep Lake MI	Clade A1
EU621732.1	Duck Lake MI	Clade A1
KF596733.1 & KF596734.1	Chualar Creek CA	Clade A1
KF596735.1	Grayson Creek CA	Clade A1
KF596739.1 & KF596740.1	Morrison Creek CA	Clade A1
KF596742.1 - KF596744.1	Mosher Slough CA	Clade A1
KF596745.1 & KF596746.1	Pleasant Grove Creek CA	Clade A1
DQ464610.1	North Indian Spring, Ash Meadows NV	Clade A2
DQ464611.1	South Indian Springs, Ash Meadows NV	Clade A2
DQ464612.1 & DQ464614.1 - DQ464617.1	Marsh Spring, Ash Meadows NV	Clade A2
DQ464613.1	North and South Indian Springs, Ash Meadows NV	Clade A2
DQ464618.1 - DQ464620.1	North Scruggs Spring, Ash Meadows NV	Clade A2
DQ464621.1 & DQ464621.1	Point of Rocks Spring, Ash Meadows NV	Clade A2
DQ464623.1	Devils Hole, Ash Meadows NV	Clade A2
DQ464624.1 - DQ464626.1	Crystal Spring, Ash Meadows NV	Clade A2

Table 21. Continued.

Accession	Geographic Location	Clade
DQ464627.1 & DQ464628.1	Mary Scott Spring, Ash Meadows NV	Clade A2
DQ464629.1	Five Springs, Ash Meadows NV	Clade A2
DQ464630.1 & DQ464631.1	Rogers Spring, Ash Meadows NV	Clade A2
DQ464632.1 &DQ464633.1	Fairbanks Spring, Ash Meadows NV	Clade A2
AY152797.1	Rainbow Lake AZ	Clade B
EU621742.1	Siltcoos Lake OR	Clade B
EU621744.1	Eel Lake OR	Clade B
EU621745.1	Briar Creek OK	Clade B
EU621748.1	Blue River OK	Clade B
EU621750.1	Duck Lake MI	Clade B
EU621751.1 & EU621754.1	Sullivan Lake MI	Clade B
KF596728.1	Blodgett Reservoir CA	Clade B
KF596729.1	Blodgett Reservoir CA	Clade B
KF596747.1	Pleasant Grove Creek CA	Clade B
EU621733.1	Suttle Lake OR	Clade C
AJ968916.1	Lake Beulah WI	Clade C
AJ968918.1	Green Lake WI	Clade C
AY152794.1	Comins Lake NV	Clade C
DQ464660.1	Comins Lake NV	Clade C
DQ464661.1 - DQ464663.1	Meadow Valley Wash NV	Clade C
DQ464664.1 & DQ464665.1	Hot Spring NV	Clade C
EU621735.1 & EU621736.1	Lost Lake OR	Clade C
EU621738.1	Otis Marsh MI	Clade C
EU621740.1	George Pond MI	Clade C
AY152789.1	Montezuma Well AZ	Clade D
DQ464690.1	Surprise Spring CA	Clade D
AY152783.1	Black River AZ	Clade D
AY152793.1	Bubbling Springs AZ	Clade D
DQ464683.1 - DQ464688.1	Spring 97 CA	Clade D
DQ464689.1	Antelope Spring CA	Clade D
DQ464691.1 - DQ464693.1	Lower Vine Ranch Spring CA	Clade D
DQ464694.1 - DQ464697.1	Fish Slough CA	Clade D
DQ464698.1 & DQ464699.1	Side Hill Spring NV	Clade D
DQ464700.1	Steptoe Valley Spring NV	Clade D
DQ464701.1 & DQ464702.1	Spring 97 CA	Clade D

Table 21. Continued.

Accession	Geographic Location	Clade
DQ464703.1	Sunny Side Creek NV	Clade D
DQ464704.1	Big Spring CA	Clade D
DQ464705.1 & DQ464706.1	Mule Spring CA	Clade D
DQ464707.1	Spring 103 CA	Clade D
DQ464708.1	Warm Spring CA	Clade D
DQ464709.1 & DQ464711.1	Lubkin Creek CA	Clade D
DQ464712.1 - DQ464715.1	Tuttle Creek CA	Clade D
GU066812.1	Yellowstone Lake WY	Clade D
AY152805.1 - AY152807.1	Montezuma Well AZ	*H. montezuma* (Clade C)
DQ464600.1 - DQ464603.1	Travertine Springs & Texas Springs, Death Valley, CA	*H. muerta*
DQ464679.1 - DQ464682.1	Nevares Springs CA	*H. sandra* Nevares Springs
DQ464675.1 - DQ464678.1	Travertine Springs, Death Valley, CA	*H. sandra* Travertine Springs
AF520434.1	Punta Arenas, Chile, South America	*H. simplex*
EU621726.1	Clear Creek Springs (CCS)	*H. texana*
DQ464656.1 - DQ464659.1	Hot Creek NV	Hot Creek
KF596736.1 - KF596738.1	Laguna Lake CA	Laguna Lake
EU621755.1	Duck Lake MI	Michigan Kettle Lakes
EU621758.1	Long Lake MI	Michigan Kettle Lakes
EU621760.1	Turner Lake MI	Michigan Kettle Lakes
EU621762.1	Sullivan Lake MI	Michigan Kettle Lakes
DQ464651.1 - DQ464655.1	Moapa Warm Springs NV	Moapa Warm Springs
DQ464666.1 & DQ464667.1	Steptoe Valley Spring NV	Nevada Springs 1
DQ464668.1	White River NV	Nevada Springs 1
DQ464669.1 & DQ464670.1	Billy Pope Spring NV	Nevada Springs 1
DQ464671.1 - DQ464674.1	Grass Spring NV	Nevada Springs 1
DQ464720.1 & DQ464721.1	Willow Spring NV	Nevada Springs 2
DQ464723.1	Willow Spring NV	Nevada Springs 2
DQ464724.1 - DQ464727.1	Lost Creek NV	Nevada Springs 2

Table 21. Continued.

Accession	Geographic Location	Clade
DQ464607.1 - DQ464609.1	North Flagg Spring NV	North Flagg Spring
DQ464716.1 - DQ464719.1	Spring 97 CA	Spring 97
DQ464605.1 & DQ464606.1	Upper White River NV	Upper White River

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