INVESTIGATIONS INTO *HUFFMANELA* (NEMATODA: TRICHOSOMOIDIDAE): NEW POPULATIONS, LIFE CYCLES, AND EGGSHELL ULTRASTRUCTURE

by

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I. INTRODUCTION

PHYLUM NEMATODA

Nematodes are bilaterally symmetrical worm-like animals. They were once included in the now obsolete phylum Aschelminthes Grobben 1910 (along with rotifers, nematomorphs, tardigrades, gastrotrichs, kinorhynchs, and priapulids), but the nematode clade was formerly erected to phylum status by Pott in 1932. However, the position of the phylum Nematoda in the tree of life is completely unresolved (Philip et al. 2005), and the fossil record is sparse.

The phylum Nematoda is one of the most speciose phyla, with over 26,000 known species and estimates of the total number of species ranging up to 1,000,000 (Hugot et al.). Nematode clades are known that have adapted to almost every aquatic, marine, and terrestrial habitat, with large clades being characterized as having free living, phytoparasitic, and zooparasitic life styles (Hyman 1951).

GENUS *HUFFMANELA*

Dr. David Huffman first discovered the dark bipolar eggs of what is now *Huffmanela huffmani* in the swim bladder of a centrarchid fish collected from the San Marcos River. Thinking they represented a new species of *Capillaria* Zeder, 1800, Huffman sent the eggs to a Czech specialist, Frank Moravec, for confirmation. Realizing these eggs represented worms of an undescribed genus, Moravec (1987) erected the genus *Huffmanela* to contain the species, described the species as *H. huffmani*, and moved two other previously described *Capillaria* spp. into the genus.

Over the subsequent decades, the subfamily Huffmanelinae Moravec 2001 was erected in the family Trichosomoididae Hall 1916 to contain the genus, which now

contains 21 nominate species, with *Huffmanela huffmani* being the only reported freshwater population (Figure 1). Recently, several other freshwater populations of *Huffmanela* have been discovered in Texas, also restricted to isolated spring systems (Figure 1).

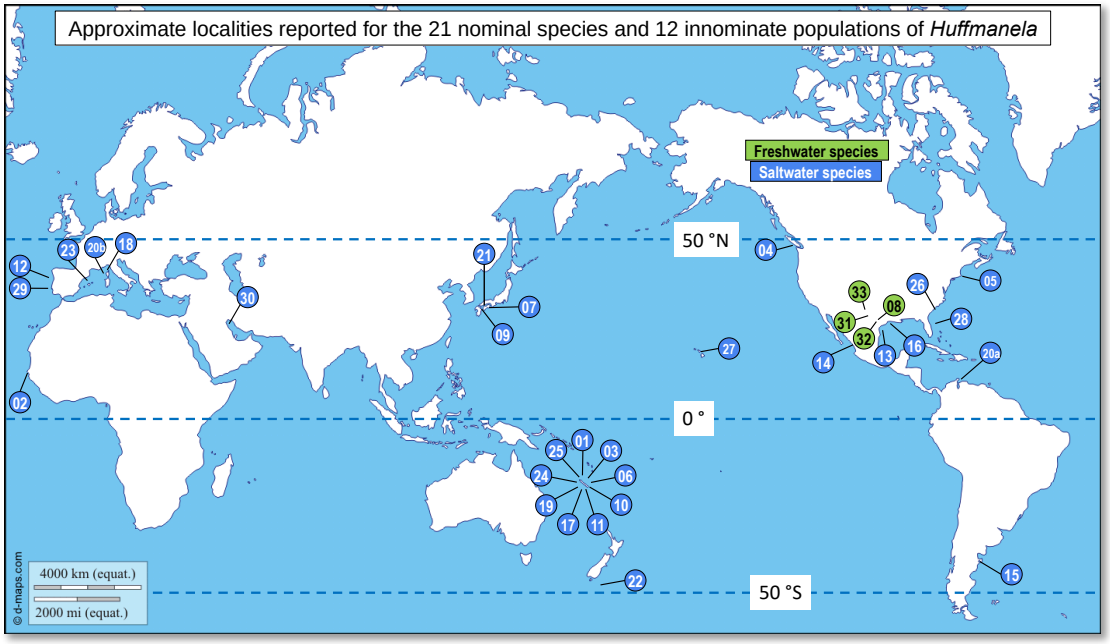

Figure 1. Map showing locations (details in Table 1) where apparently unique populations of *Huffmanela* have been reported (green circles represent Texas freshwater populations; pin #08 represents *H. huffmani*).

Table 1. Species and reported populations of *Huffmanela*: host taxonomy and locations.

Pin#	*Huffmanela* Species	Authority	Host Subclass: Order: Family	Locality
01	*Huffmanela balista*	Justine (2007)	Neopterygii: Tetraodontiformes: Balistidae	Récif Toombo, New Caledonia
02	*Huffmanela banningi*	Moravec (1987)	Neopterygii: Pleuronectiformes: Cynoglossidae	Atlantic Ocean, Senegal and Congo
03	*Huffmanela branchialis*	Justine (2004)	Neopterygii: Perciformes: Nemipteridae	Amédée Islet, Nouméa, New Caledonia
04	*Huffmanela canadensis*	Moravec et al. (2005)	Neopterygii: Scorpaeniformes: Sebastidae	Clayoquot Snd., British Columbia, Canada
05	*Huffmanela carcharhini*	MacCallum (1925) Moravec (1987)	Elasmobranchii: Carcharhiniformes: Carcharhinidae	NW Atlantic Ocean

Table 1 Continued. Species and reported populations of *Huffmanela*: host taxonomy and locations.

Pin#	*Huffmanela* Species	Authority	Host Subclass: Order Family	Locality
06	*Huffmanela filamentosa*	Justine (2004)	Neopterygii: Perciformes: Lethrinidae	Passe de Dumbéa, New Caledonia
07	*Huffmanela hamo*	Justine and Iwaki (2014)	Neopterygii: Anguilliformes: Muraenesocidae	Inland Sea of Japan
08	*Huffmanela huffmani*	Moravec (1987)	Neopterygii: Perciformes: Centrarchidae	San Marcos River headsprings; San Marcos, TX
09	*Huffmanela japonica*	Moravec et al. (1998)	Neopterygii: Perciformes: Mullidae	Inland Sea of Japan, off Shikoku Island
10	*Huffmanela lata*	Justine (2005)	Elasmobranchii: Carcharhiniformes: Carcharhinidae	Off Nouméa, New Caledonia
11	*Huffmanela longa*	Justine (2005)	Neopterygii: Perciformes: Lethrinidae	Récif Toombo, New Caledonia
12	*Huffmanela lusitana*	Ramos et al. (2019)	Neopterygii: Gadiformes: Gadidae	Near Figueira da Foz., Portugal
13	*Huffmanela markgracei*	Ruiz and Bullard (2013)	Elasmobranchii: Carcharhiniformes: Carcharhinidae	Atlantic USA & Gulf of Mexico
14	*Huffmanela mexicana*	Moravec and Fajer-Avila (2000)	Neopterygii: Tetraodontiformes: Tetraodontidae	Mazatlan, Sinaloa State, Mexico
15	*Huffmanela. moraveci*	Carballo and Navone (2007)	Neopterygii: Atheriniformes: Atherinopsidae	Nuevo & San José Gulfs, Argentinean Sea
16	*Huffmanela oleumimica*	Ruiz et al. (2013)	Neopterygii: Perciformes: Lutjanidae	Gulf of Mexico
17	*Huffmanela ossicola*	Justine (2004)	Neopterygii: Perciformes: Labridae	Amédée Islet, New Caledonia
18	*Huffmanela paronai*	Moravec and Garibaldi (2000)	Neopterygii: Perciformes: Xiphiidae	Ligurian Sea, Italy
19	*Huffmanela plectropomi*	Justine (2011)	Neopterygii: Perciformes: Serranidae	Nouméa, New Caledonia
20	*Huffmanela schouteni*	Moravec and Campbell (1991)	Neopterygii: Beloniformes: Exocoetidae	Curaçaon (20a), & Ligurian Sea, Italy (20b)
21	*Huffmanela shikokuensis*	Moravec et al. (1998)	Neopterygii: Tetraodontiformes: Monacanthidae	Inland Sea of Japan, off Shikoku Island
22	*Huffmanela* pop01	Moravec and Campbell (1991)	Neopterygii: Ophidiiformes: Ophidiidae	New Zealand, off South Island
23	*Huffmanela* pop02	Gállego et al. (1993)	Unknown	Mediterranean, off Barcelona, Spain?
24	*Huffmanela* pop03	Justine (2004)	Neopterygii: Perciformes: Nemipteridae	Passe de Boulari, Nouméa, New Caledonia

Table 1 Continued. Species and reported populations of *Huffmanela*: host taxonomy and locations.

Pin#	*Huffmanela* Species	Authority	Host Subclass: Order Family	Locality
25	*Huffmanela* pop04	Moravec and Justine (2010)	Neopterygii: Perciformes: Labridae	New Caledonia
26	*Huffmanela* pop05	Bullard et al. (2012)	Elasmobranchii: Carcharhiniformes: Carcharhinidae	Cape Fear, North Carolina
27	*Huffmanela* pop06	Bullard et al. (2012)	Elasmobranchii: Carcharhiniformes: Carcharhinidae	O'ahu, Hawai'i
28	*Huffmanela* pop07	Dill et al. (2016)	Elasmobranchii: Carcharhiniformes: Sphyrnidae	Florida
29	*Huffmanela* pop08	Esteves et al. (2016)	Neopterygii: Pleuronectiformes: Soleidae	Atlantic coast of Portugal
30	*Huffmanela* pop09	Hegazi et al. (2014)	Neopterygii: Perciformes: Sparidae	Iraqi waters of Persian Gulf
31	*Huffmanela* pop10	Worsham (2015)	Neopterygii: Perciformes: Centrarchidae	Clear Creek Springs, near Menard, TX
32	*Huffmanela* pop11	Worsham (2015)	Neopterygii: Perciformes: Centrarchidae	Comal Springs, New Braunfels, TX (presumed extinct)
33	*Huffmanela* pop12	Johnson and Negovetich (2019)	Neopterygii: Perciformes: Centrarchidae	Anson Springs, S Concho River, Christoval, TX

Adult worms of this genus are histozoic, and apparently intracellular parasites, with many species laying eggs in the host's internal organs (MacLean et al. 2006; Moravec and Garibaldi 2003). These eggs are deposited in a very early stage of development and continue their development as intracellular parasites (Justine 2007; Moravec 2001; Worsham et al. 2016). Most species are described based on the morphology of eggs alone, as the female that laid the eggs disappeared weeks prior to the eggs turning dark enough to draw attention to their presence (Justine and Iwaki 2014).

KNOWN FRESHWATER POPULATIONS OF *HUFFMANELA*

SAN MARCOS RIVER (SMR) POPULATION

The San Marcos River (SMR) issues from the Edwards (Balcones Fault Zone) Aquifer System (Jones 2019) in San Marcos, Texas (29.8939°, -97.9300°). The headsprings are impounded by Spring Lake, which inundates about 200 springs that generate an average annual discharge of 4,300 liters per second (Lps) Brune (1981). The SMR flows SE for about 121 km before joining the Guadalupe River at Gonzalez, TX, which eventually joins the Gulf of Mexico.

Prior to the work of Worsham (2015), freshwater *Huffmanela* had only been found in the thermally stable spring-fed reach of the San Marcos River, despite multiple attempts of previous students in the Huffman lab (Cox et al. 2004; Michel 1984; O'Docharty 2007) to search other karst springs across Central Texas in hopes of finding populations of *Huffmanela*.

SAN SABA RIVER (SSR) POPULATION

H. huffmani occurs in the SMR with an endemic innominate species of *Hyalella* (SMS *Hyalella*) that is distinguishable from the cosmopolitan *Hyalella* cf. *azteca* by the number of dorsal mucronations (3-4 vs. 1-2 spines) (Worsham et al. 2017). In his review of amphipod literature, Worsham noticed in that another unique spring-endemic amphipod (*Hyalella texana* Stevenson and Peden 1973) had been described from Clear Creek Springs, a tributary of the upper San Saba River (SSR), in Menard County, Texas. Worsham speculated that the SMS *Hyalella* coevolved with *Huffmanela huffmani* through the Holocene, and noted that the SMS *Hyalella* is very similar to the Clear Creek amphipod (*Hyalella texana*) in that both contain three to four dorsal spines. He

hypothesized that another population of spring dependent *Huffmanela* possibly existed in Clear Creek Spring and collected several sunfish from the spring in 2015 for examination. In the swim bladders of several of these fish he discovered the distinctive dark smudges characteristic of infection with *Huffmanela*; thus, the second known freshwater population of Huffmanela was discovered (Worsham 2015).

Clear Creek joins the SSR about 20 river kilometers from the headsprings of the SSR, which consists of several springs near Fort McKavett in eastern Schleicher County, Texas. All the SSR springs issue from the Edwards-Trinity (Plateau) Pool of the Edwards Aquifer System (Jones 2019). The SSR population of *Huffmanela* and the endemic *Hyalella texana* of Clear Creek Springs also occur in the spring run of the SSR, and all of the specimens in the current study were based on collections from the SSR headsprings and spring run as far downstream as the Toe Nail Trail crossing (30.8360°, -100.1039°).

The two largest headsprings of the SSR are at the western ends of the south (30.8259°, -100.1190°) and middle (30.8290°, -100.1320°) arms of Talbot Lake. From Talbot Lake, the river flows eastward for about 225 km before joining the Colorado River which eventually joins the Gulf of Mexico.

COMAL SPRINGS (CS) POPULATION

The Comal Springs is a series of springs located in New Braunfels, Comal Co., TX (29.7212°, -98.1277°). As with the SMS, these springs issue from the Edwards (Balcones Fault Zone) Aquifer and are very similar to those located in Spring Lake. The SMS and CS are separated by a straight-line distance of approximately 28 km.

Several workers have reported searching these springs for *Huffmanela* (Cox et al. 2004; Michel 1984; O'Docharty 2007). It seemed odd that a spring so like the SMS and such a short distance away in the same river drainage contained no trace of *Huffmanela*.

The Comal River ceased to flow for several months in 1956, and Worsham (2015) conjectured that *Huffmanela* may have existed there prior to the drought, which may have led to the extinction of the hypothetical population and its associated amphipod. Worsham was able to remove and dissect swim bladders from centrarchid museum specimens collected by Hubbs et al. (1953) at the beginning of the drought. He found that they did contain the distinctive eggs of *Huffmanela*, and thus, a third population of freshwater *Huffmanela* was discovered, though apparently extirpated by the drought of 1956, and presumed to be extinct ever since.

SOUTH CONCHO RIVER (SCR)

The third extant population of freshwater *Huffmanela* was discovered by in 2018 by Nick Negovetich of Angelo State University (Johnson and Negovetich 2019). Having been granted access to the headwaters of the South Concho River (SCR), he was able to examine centrarchids from Anson Springs in Tom Green County, Tx, and discovered that swim bladders of these fish were parasitized with *Huffmanela*.

The Concho River is also located in western-central Texas and is comprised of three major tributaries (North, South, and Middle Concho rivers), which join in the town of San Angelo and form the Concho River, which flows east for 93 km where it joins the Colorado River.

The SCR begins at Anson Springs in Tom Green County (31.1349°, -100.4925°), which issues from the Edwards-Trinity (Plateau) Aquifer System (Jones and Anaya 2019)

as do the springs of the SSR. The straight-line distance between the South Concho Springs and San Saba Springs is about 48 km. From Anson Springs, the SCR flows north for 27 km before joining the Middle and North Concho Rivers to form the Concho River, which then flows east for about 93 km before joining the Colorado River.

TAXONOMY OF NEMATODES

CLASSICAL NEMATODE TAXONOMY AND RELATION TO *HUFFMANELA*

Classical nematode taxonomy has concerned itself with morphological characteristics of adult nematodes and their mature eggs (Bird 1971). In most cases it is preferable to base descriptions on adult males, since they usually have hardened parts of the copulatory apparatus that are geometrically distinct and consistent. However, descriptions can also be based on fully developed eggs (Justine 2007; Moravec 1987). This method has been widely applied throughout the genus *Huffmanela* (Bullard et al. 2012; Esteves et al. 2016). Descriptions of new species in the genus are often based on morphological characteristics of eggs such as length and width, or any unique structures found in the vitelline membrane of the egg (Justine 2004), combined with host taxonomy, host organ, and marine province of the fish host (Bullard et al. 2012).

STRUCTURE AND COMPOSITION OF NEMATODE EGGS

For nearly half a century (Bird 1971), taxonomists have described nematode eggs as being comprised of three distinct layers, a concept sometimes referred to as the trilaminar model (Figure 2).

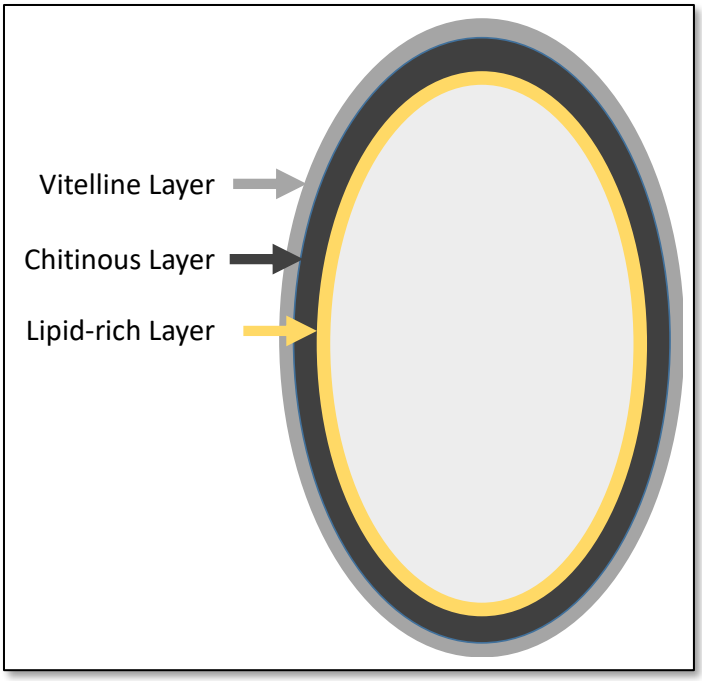

Figure 2. The classical model of a generic nematode egg recognized since early 20th Century. Gray = vitelline layer, black = middle chitinous layer, and yellow = inner lipid layer.

The outermost layer of the trilaminar eggshell was the vitelline layer which is a thin membrane surrounding the egg and which is derived from the polyspermy barrier that forms over the ovum at the moment of fertilization (Stein and Golden 2018). Next was the chitin layer which is often thick and dark in *Huffmanela* spp., and while permeable, provides physical protection and form (Stein and Golden 2018). The inner-most layer was the "lipid-rich" layer, with its lipid composition having been based upon chemical extraction and analysis done by Chitwood (1938).

A very detailed report by Olson et al. (2012) on how the various layers of the nematode eggshell are formed in *Caenorhabditis elegans* has described a total of six layers surrounding the embryo. In this new Olson model of the nematode eggshell, the outer two layers remain the same, and both the vitelline layer and the chitinous layer are still named as in the original trilaminar model. However, the third layer is where their

description of the *C. elegans* egg differs from the classical model of nematode eggs that has been in use for decades. The third layer, formerly thought to be a lipid-rich layer, was shown to contain no lipids but is composed primarily of chondroitin proteoglycans. Thus, the third layer of the Olson trilaminar model was renamed CPG Layer. The next (fourth) "layer" in the Olson model is actually a fluid- or gel-filled space containing numerous proteins, and now referred to as the Extra-Embryonic Matrix or EEM (Stein and Golden 2018). The fifth layer is composed primarily of lipids (Stein and Golden 2018) and has been dubbed the Permeability Barrier (PB), since it prevents the exchange of most molecules. The final (sixth) layer, which is known as the Peri-Embryonic "Layer," is an amorphous fluid-filled space that surrounds the embryo proper (Stein and Golden 2018).

THE POLAR PLUGS

The order Trichocephalida (sometimes Trichinellida), which includes *Huffmanela,* is unique among nematodes in that their eggs contain polar plugs (Figure 3).

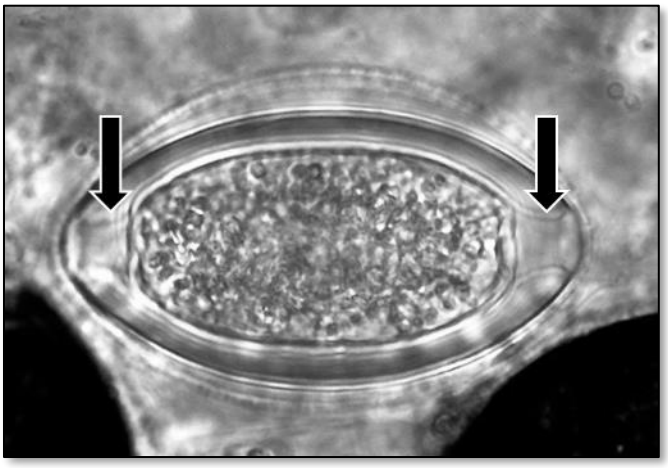

Figure 3. Early stage eggs of *H. huffmani*. Arrows point to polar plugs.

These plugs form during the development of the egg and vary from species to species. The chitinous layer forms a collar that the plug rests in, and some plugs to greatly protrude from the chitinous collar, while others hardly protrude at all.

The polar plugs of the eggs of trichocephallids were thought to conform to the same model as the trilaminar eggshell, meaning that the innermost layer was a lipid-based layer, the middle area was derived from chitin, and the outermost layer equates to the vitelline membrane (Appleton and White 1989; Preston and Jenkins 1985). The core area, derived from the chitinous layer, also appears to be subdivided into two areas, discernable as electron dense and electron lucid layers when viewed by TEM (Appleton and White 1989).

Similar to the trilaminar model of eggs, the composition of polar plugs had been considered resolved, which may be the reason that so little work has been done around polar plugs recently. In light of discoveries made by Olson et al. (2012) and the terminology proposed by Stein and Golden (2018), it is possible that the model of the polar plug should be revised as well. However, the eggs of *C. elegans* studied in their work is of a different order and lack polar plugs, and so it is not known how the eggshell layers described in the Olson model applies to trichinelloid nematodes like *Huffmanela*.

LIFE CYCLE OF *H. HUFFMANI*

Of all the populations of *Huffmanela*, only *H. huffmani* has a known life cycle, which involves one definitive and one apparently obligate intermediate host. The cycle begins when a hyalellid amphipod eats an egg of *H. huffmani*. Once ingested, the worm emerges from the egg and migrates to the hemocoel of the amphipod. It is thought that at this point the larval nematodes molt and become the L_2 stage larva within a period of 7 days (Worsham 2015). At this point they are infective to the definitive host if the amphipod is ingested (Worsham et al. 2016).

Once the amphipod is eaten, it is not known where the ingested larvae migrate, as they have never been detected until they show up as L_3 or L_4 larvae in the swim bladder at least 4.5 mo post exposure (Worsham et al. 2016). After two mature worms copulate, the female will then lay eggs in the tissues of the swim bladder (Worsham et al. 2016). Shortly thereafter, perhaps in as little as two weeks from commencement of egg deposition, the female vanishes from the swim bladder (Worsham et al. 2016).

Before the deposited eggs can be dispersed, the fish must die, either by natural causes, or from predation by a piscivore. The tissues of dead fish will deteriorate, allowing the eggs to be dispersed by natural processes, or the eggs will be dispersed in the feces of the piscivore(Cox et al. 2004). In either case, the eggs remain unharmed and are distributed among the sediment. Once the eggs are in the sediment they can be eaten by amphipods, starting the life cycle anew.

STATEMENT OF PROBLEM

NEW FRESHWATER POPULATIONS OF *HUFFMANELA*

Currently *Huffmanela huffmani* is the only nominate species from obligate freshwater fishes. Now that there are four known freshwater populations of *Huffmanela* in Texas (one extinct and three extant), and given that the two Colorado River populations (SSR & SCR; the northwestern clade) have probably been completely isolated genetically from the Guadalupe River populations (SMR & CR; the southeastern clade) for millions of years (Worsham et al. InPrep), it is probably prudent to avoid mixing the results of studies on the northwestern clade with studies on the southeastern clade, as a minimum. Since taxonomy is an established protocol for keeping the information derived from distinct gene pools separated in an efficient information storage

and retrieval system, the next question becomes, “are there taxonomically useful morphometric differences between the adult worms and eggs of the two clades sufficient to reliably separate specimens of the northwestern clade from specimens of the southeastern clade?”

Egg Morphology

A confusing mix of inadequately defined “standards” has arisen for describing differences between eggs of different *Huffmanela* species. This has led to confusion and ambiguity in the description of species and the classification of specimens. Indeed, some species descriptions were apparently based partially on artifacts induced by microtechnique, or on imaginary optical “features” caused by progressive rotation of fibers in successive layers of the highly refractive chitinous layer of the eggshell (Wharton 1978, 1980). So, the question becomes, “can the eggshell fine structure be adequately resolved such that 1) a widely applicable standard can be established for new *Huffmanela* species descriptions based on egg syntypes and 2) the discrepancies that have been discovered in the *Huffmanela* literature can be rectified retrospectively?”

Life cycle

Only the life cycle of *H. huffmani* has been completed egg to egg, and all other reported populations of *Huffmanela* are marine, and very difficult to work with due to paucity of material. Therefore, any life-cycle patterns revealed for these new freshwater populations and found to be similar to what is known for *H. huffmani* would validate the life cycle findings of Worsham et al. (2016), and any variations would also provide subsequent investigators with alternative timings and approaches in their attempts to resolve the life cycles of marine species.

GOALS AND OBJECTIVES

GOAL 1: DETERMINE IF TEXAS POPULATIONS CAN BE DIFFERENTIATED

- Objective 1.1: Identify any consistent differences between the eggs of the three freshwater *Huffmanela* populations in Texas using the suite of "classical" morphological features under light microscopy.
- Objective 1.2: Visualize the vitelline membrane and outer shell with scanning electron microscopy to determine if there are any consistent ultrastructural differences between the eggs of the populations.
- Objective 1.3: Compare the morphology of any collected adults from the two new freshwater Texas populations to those of *H. huffmani.*

GOAL 2: DETERMINE IF SOUTH CONCHO POPULATION HAS THE SAME LIFE CYCLE AS *H. HUFFMANI*

- Objective 2.1: Infect amphipod hosts with eggs of the South Concho *Huffmanela*.
- Objective 2.2: Feed experimentally infected amphipods to uninfected centrarchids.
- Objective 2.3: Dissect experimentally infected fish at various times, collecting any adults for morphometric studies
- Objective 2.4: Compare life cycle with that of *H. huffmani.*

GOAL 3: RECOMMEND STANDARDS FOR HUFFMANELA EGG DESCRIPTIONS

- Objective 3.1: Determine how the Olson model of nematode eggshells applies to *Huffmanela*.

- Objective 3.2: Using light, SEM, and transmission electron microscopy (TEM), compare local *Huffmanela* eggs with other nematode eggs described in the literature.

II. METHODS

COLLECTION SITES

SITE 1: SAN MARCOS RIVER (SMR)

Two collection sites were established for obtaining centrarchids from the SMR. The first site is the peninsula near the boat ramp of Spring Lake (29.8921, -97.9320). The second site is located at Sewell Park (29.8878, -97.9345). Both sites are on properties owned by Texas State University, and permission was granted by the Meadows Center to collect fish from Spring Lake. Fish were collected from Spring Lake location and Sewell park by angling.

SITE 2: SAN SABA RIVER (SSR)

Access is no longer granted to collect specimens from Clear Creek Springs, however, Worsham had been able to establish that there were also fish infected with *Huffmanela* near the headsprings of the SSR (about 30 km upstream from Clear Creek). Thus, collection sites were established near where a public road crosses the SSR, and we will refer to this *Huffmanela* population as the SSR population.

Three sites were chosen to sample fish from the SSR. The first is located just upstream of the crossing of Toenail Trail (30.8358, -100.1038). The second site is about 1 km farther upstream (30.8332, -100.1131). At these first two sites, collection was primarily by electroshocking and seining, with some angling with artificial lures. The third site is the impounded spring near the upstream end of the south arm of Talbot Lake (30.8259, -100.1190). Since the land surrounding the lake and springs is privately owned, travel to this site was restricted to portaging up the river from the downstream sites via

kayak. Due to the depth of the water at the headspring site, collection there was limited to angling from kayaks with artificial lures and live worms.

SITE 3: SOUTH CONCHO RIVER (SCR)

The third site, Anson Springs is located on the SCR in Tom Green Co, TX. The reach of the river that was sampled is between coordinates 31.1356, -100.4935 and 31.1411, -100.4917. Collections were accomplished with electroshocking, angling from the bank with artificial lures, and some seining.

TRANSPORT PROTOCOLS

To avoid deviating from potential thermal tolerances, centrarchids captured from the SMR were maintained in an aerated 19-liter bucket, with occasional changes of water to maintain temperature until arrival at the nearby lab. Similar methods were used for fish captured from the SSR and SCR while onsite and awaiting transport. However, due to extended transit time (~4 h), and the sometimes-hot ambient temperatures of summer, extra precautions were required to prevent them from thermally drifting away from normal spring temperature of 22 degrees. Up to three 19-liter aerated buckets containing captured fish were placed into a single 40-gallon cooler partially filled with spring water. Water temperatures in the buckets were monitored at approximately 45-minute intervals using an IR thermal sensor during transport back to the university. If the water had risen above the temperature of the spring water, ice chips were added to the water in and surrounding the bucket until the temperature returned to spring-like conditions. Once at the university, buckets were refreshed with artesian water and fish were maintained under aeration at room temperature until they could be necropsied.

FISH NECROPSY PROCEDURES

Fish were pithed in accordance with approved protocols in Texas State University IACUC#73. A longitudinal incision was cut just ventral to the spinal column and running from the operculum to a location dorsal to the insertion of the anal fin (Figure 4). This exposed the swim bladder in a way that made possible it's complete extraction in an uncontaminated condition.

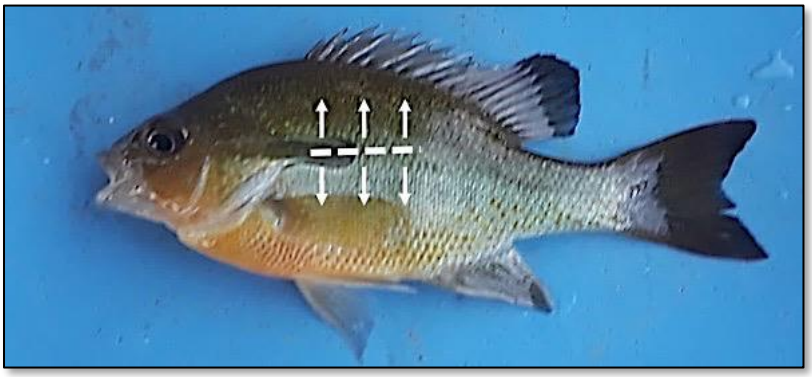

Figure 4. Method of fish dissection. Dotted line indicates area of incision; arrowed lines indicate separation of tissue and ribs to expose swim bladder.

Once the swim bladder was removed, egg-density for that individual fish was rated on a scale adapted from Worsham et al. (2016) (Table 2). To determine the rating, the swim bladder was first placed in a petri dish with enough physiological saline to cover the tissue. A dissecting scope was then used to observe five nonoverlapping views of swim bladder tissue. Each field of view through the dissecting scope was individually rated for egg density in terms of the approximate percentage of the field occupied by eggs using the rankings specified in Table 2. These five ratings were then extrapolated to the entire swim bladder to give an approximated rating of the gross infection.

Table 2. Egg density rating of infection in collected fish.

Egg-density rating	Percentage of microscope field containing eggs
0	0
1	>25
2	25-50
3	50-75
4	75-100

LIFE CYCLE PROTOCOL

EXPERIMENTAL FISH AQUARIA

Two banks of heavy-duty shelving were set up with 36 standard 10-gal (38 L) aquaria. Prior to deployment, each aquarium was fitted with a 1" drain bulkhead kit (Figure 5) by drilling a 1-1/8-in hole about 5 cm from the top of the aquarium with a diamond dust glass hole cutter while using water dripped into the work area as a lubricant during drilling. A hut consisting of split 6-in PVC about 6 in long was added later to each aquarium, and weighted with a washed rock glued to the top.

The aquaria were arranged on the shelving at a density of six per shelf without visual barriers between aquaria (Figure 6). In order to facilitate record keeping, codes were assigned to each aquarium based on Bank (L or R), Column (1-6), and Row (1-4).

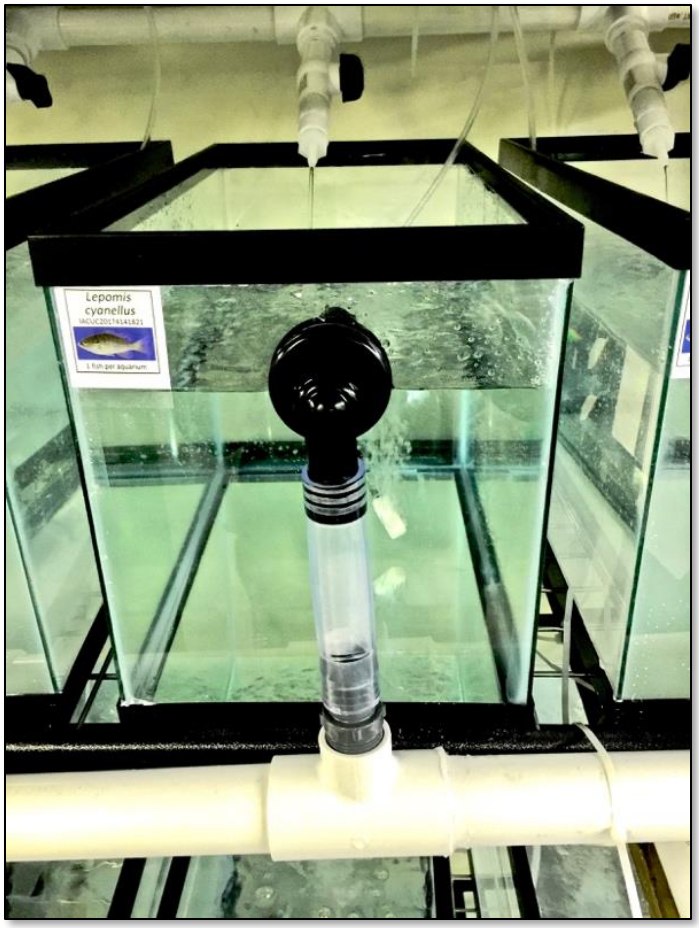

Figure 5 Example of individual aquarium setup, showing gravity-flow distribution system with metering valves, air stone connected to metered distribution system, 1-inch bulkhead drains, and common wastewater collection system.

Each 38 L aquarium was supplied by a centralized source of artesian well water from the same aquifer that feeds San Marcos Springs, which was gravity fed into the wet lab from an upstairs reservoir. The water was fed into a series of pipes, which was then distributed to the aquaria. Water flow to each aquarium was regulated by an adjustable valve attached to a nozzle. Each valve was adjusted to ensure that each aquarium was receiving adequate water, and that there was enough pressure on the nozzle to mix the water within the tank.

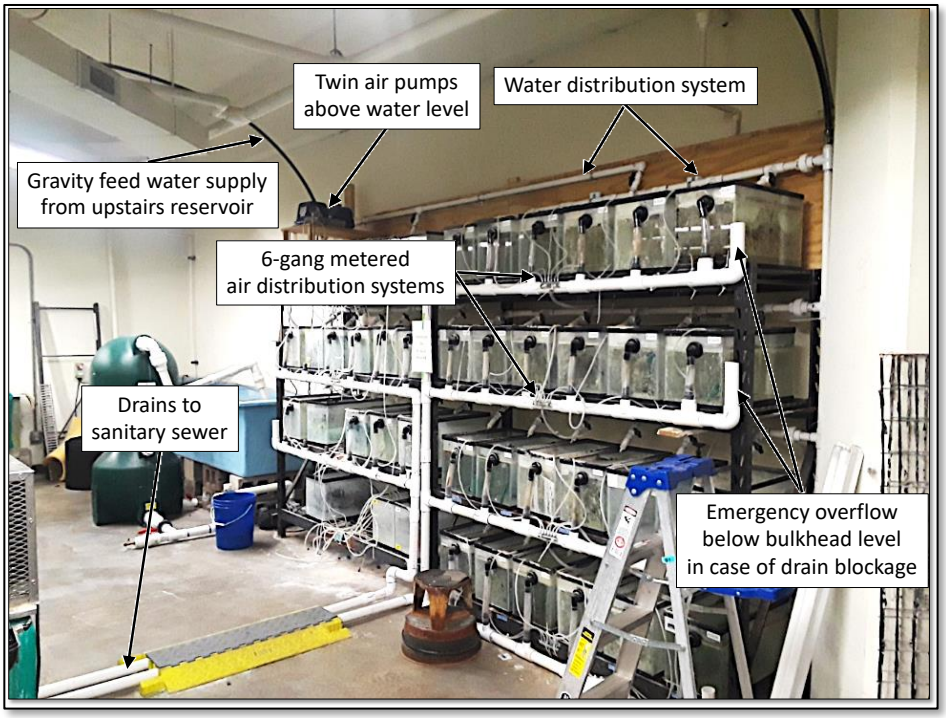

Figure 6. Flow through aquarium bank set up in FAB wet lab showing various support services.

Airflow to the aquaria was provided using two large aquarium pumps running in parallel. These pumps fed into a large pipe with six taps used to disperse compressed air across the system. A rubber hose was attached to each tap and this hose leads to a six-gang valve located at the center of each row of aquaria. The six-gang valve splits the incoming air into six separate hoses, which distribute air to a single air stone in each aquarium. Air stones were positioned at the front of the aquaria to facilitate daily monitoring of conditions.

Once the flow through system was ready, on-site reared leucistic green sunfish were collected from the USFWS San Marcos Aquatic Resource Center (SMARC). Most of these fish had been maintained for several generations in large outdoor ponds that were several miles from the San Marcos River and supplied with well water from the Edwards Aquifer. Fish traps were used to collect the fish from ponds. Other fish had been

maintained in living streams located on the facility; these fish were collected using small dip nets.

Once captured, the fish were placed in 19 L buckets with fresh water provided by SMARC. The fish were then directly transported to FAB where each fish was placed into an individual aquarium. Once the aquaria were filled, the remaining fish were euthanized and dissected to ensure they had not been previously infected with *Huffmanela*.

Fish were fed daily with flaked food and/or dried blood worms. During the feeding a daily form similar to Figure 7 was completed on paper and later transcribe into Excel. Fish-care forms were recorded daily from first fish in, to last fish out.

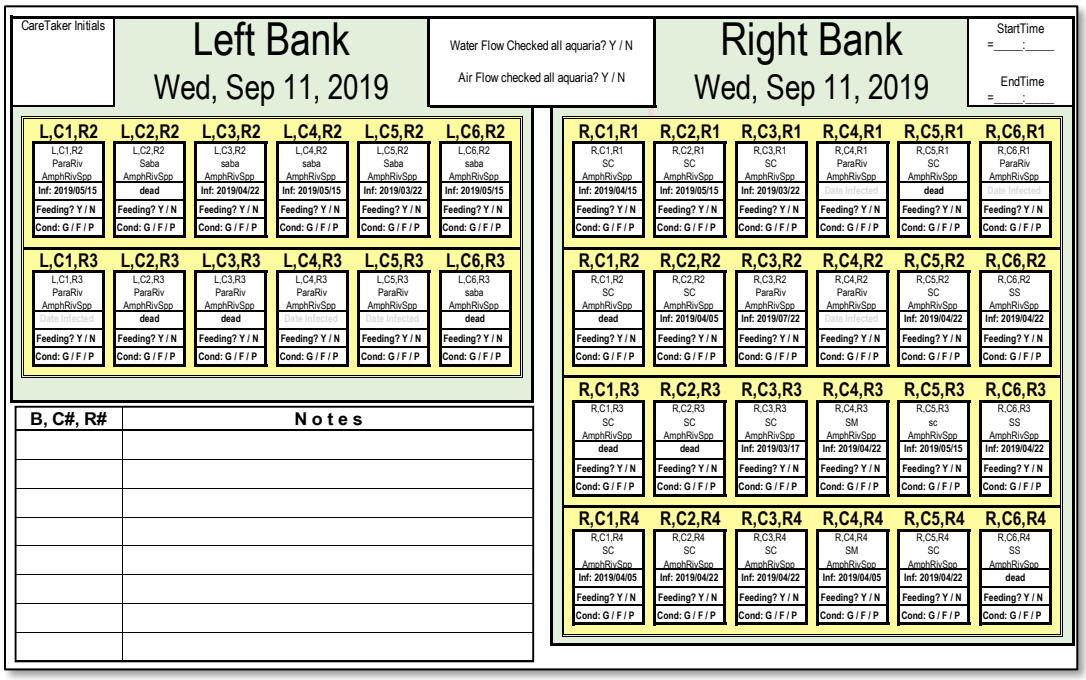

Figure 7. Excel-based daily fish-care data-collection form for life cycle experiment. Each rectangle represents descriptive data for one aquarium, and has places for keeping daily records of fish condition. Changes were noted on paper in the notes section and transcribed into a data file afterwards.

Egg and Amphipod Maintenance Protocol

All wild-caught fish were necropsied, and swim bladders graded under a dissecting microscope for likelihood of containing viable eggs. Some larger fish with obviously infected swim bladders contained only eggs from infections that were several years old, in which case all eggs would have since expired. The eggs in such swim bladders are usually surrounded by concentric layers of granulomatous tissue easily discernable during initial inspection, and these swim bladders were discarded without further testing. Other fish may have mixtures of fresh and old infections, and still others may have mostly viable eggs.

Swim bladders considered likely to contain viable eggs were transferred individually to 50 mL plastic falcon vials containing aquifer water (Egg-Incubation Vials), sealed, and maintained in a flow-through apparatus in aquifer water at 23 C (Figure 8) following the protocol of Worsham et al. (2016). Water in the vials was replaced every 14 days with fresh aquifer water.

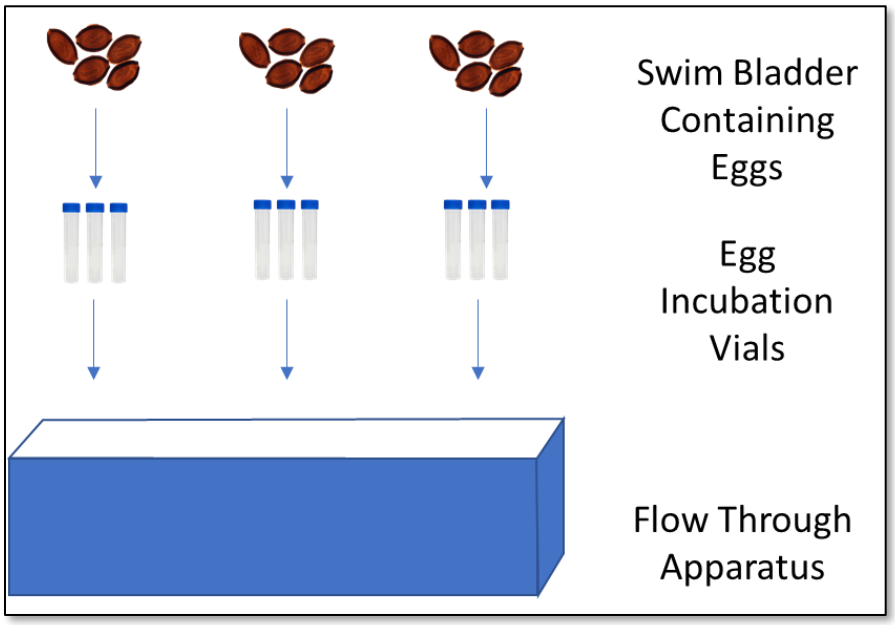

Figure 8. Egg storage protocol for life-cycle experiment

Because the concentration of viable eggs derived from such swim bladders varies substantially from fish to fish, the egg viability in each vial was determined by pipetting a random aliquot of suspended eggs into a 1-L plastic jar containing aquifer water and about 10 lab-reared amphipods. Exposed amphipods (*Hyalella* cf. *azteca)* were retrieved from their incubation containers 5 d post exposure, dissected in physiological saline, and examined for living nematode larvae. If larval nematodes were found, the eggs from that swim bladder were added to the pool of eggs being stored until needed for the infection protocol.

The contents of all vials from the SCR containing viable eggs were then combined into a 1L plastic Master Egg-Stock Container (Figure 9).

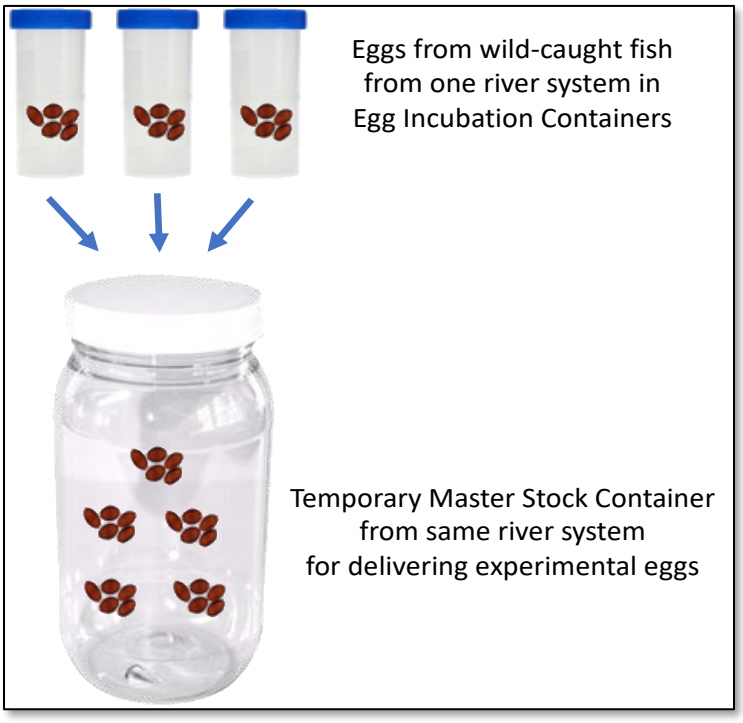

Figure 9. Creation of Master Egg-Stock Container.

Amphipod Culture Protocol

Amphipods were collected from each river system and identified to presumptive species using a dissecting scope. Amphipods from each river system were cultured in separate 10-gal amphipod-culture aquaria at room temperature (Figure 10).

Amphipods were fed an aquatic moss (presumably *Amblystegium*), that was collected from Sessom's Creek, a spring-fed tributary of the SMR that flows under the Freeman Aquatic Biology Building. Moss was rinsed thoroughly and immersed in nicotine-infused water for several hours. The nicotine water was created by soaking six Camel® snus packs in 1 L of DI water for about 6 h at room temperature, resulting in a tea-like appearance. Based on the amount of nicotine said to be delivered to a tobacco-using human per snus pack, the resulting nicotine concentration was approximately 48 mg/L. After removing the moss from the nicotine water, the moss was rinsed at least five times in fresh aquifer water over a period of about an hour to avoid exposing cultured amphipods to residual nicotine.

Establishment of lab-reared amphipod cultures

Freshly hatched juveniles of *Hyalella azteca* are reported to require 1 month at 25 C to develop to maturity (Cooper 1965). Therefore, wild-caught amphipod stocks were maintained for at least 2 months in quarantine in order to assure that juvenile amphipods later found in the cultures had been derived from in-lab mating, and were not survivors from the original field collection, which might have been exposed to *Huffmanela* eggs in the native habitat.

After the 3-mo quarantine period, about 1 L of water containing cultured amphipods was withdrawn from the amphipod culture aquarium and transferred to a

transparent glass baking dish over white paper. Young amphipods (~3mm or smaller) were individually transferred to a separate dish using a 30-mL pipette with the end trimmed to allow for easier passage of the amphipods. These amphipods were then used to establish cultures of lab-reared amphipods from each of the three river systems (Figure 10). Only these lab-reared amphipods were used for *Huffmanela* life cycle experiments.

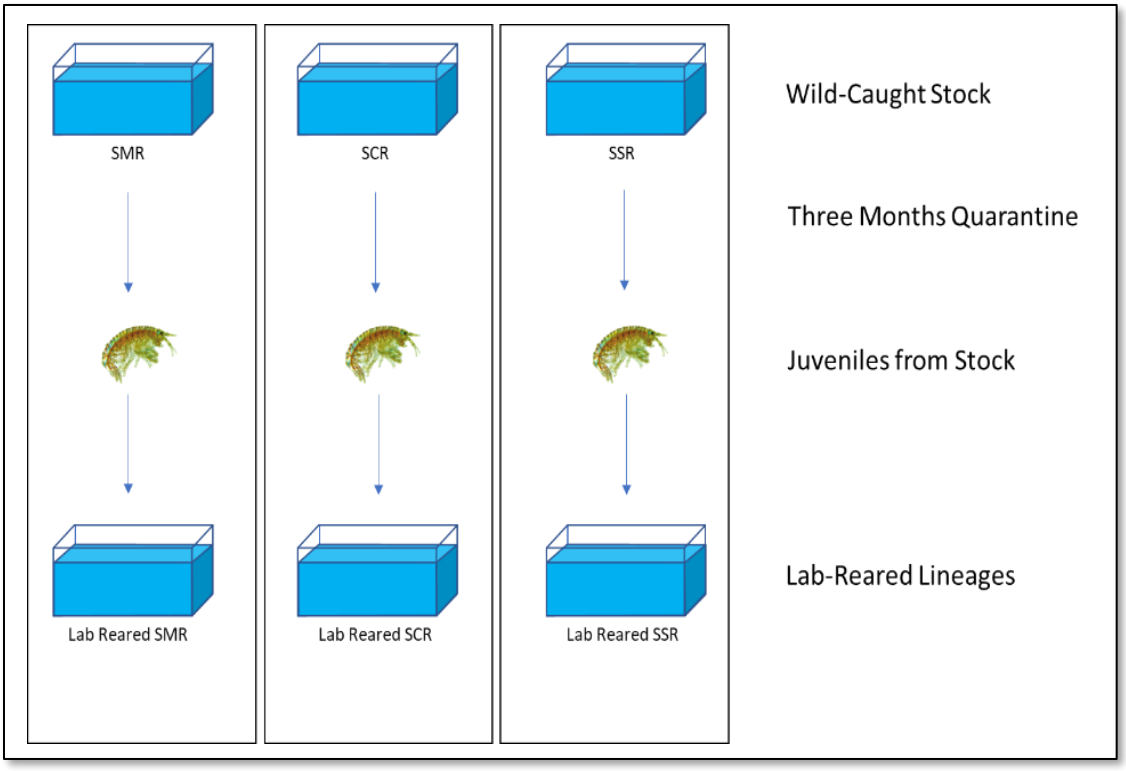

Figure 10. Creation of lab reared amphipod lineages from the three river systems.

AMPHIPOD INFECTION PROTOCOL

A clean, lidless 1-L plastic jar was inverted and submerged into the water of the amphipod culture aquarium and turned right side up. As the air and water exchanged, dozens of amphipods were drawn into the jar. The jar was then removed from the aquarium and the contents poured into a clear glass baking dish placed over a white sheet of paper. A plastic pipette with the tip trimmed back was used to collect individual

amphipods. A subset of 35 amphipods was then placed into a clean 1-L Amphipod Incubation Container and allowed to acclimate at 23 C for 2 d.

Amphipods exposed to viable *Huffmanela* eggs show an increase in mortality compared to unexposed amphipods (Worsham 2015). Because of this, it was necessary to find the balance between (1) an exposure level sufficient to ensure an adequate number of larvae per amphipod in order to effect fish infection, and (2) an amphipod survival rate sufficient to have enough infected amphipods to feed experimental fish. To determine appropriate volume of egg suspension to be delivered to experimental amphipods, the density of infective eggs per mL in the Master Egg-Stock Container was determined by titration.

Three trials were executed following the protocol described above; however, these trials exposed amphipods to different volumes of egg suspension. The protocol for the rest of the infection was derived from this trial to optimize the number of larval worms per amphipod. Three Amphipod Exposure and Incubation Containers were filled with approx. 12, 18, and 24 mL of aliquots from the Temporary Master Stock (Figure 11).

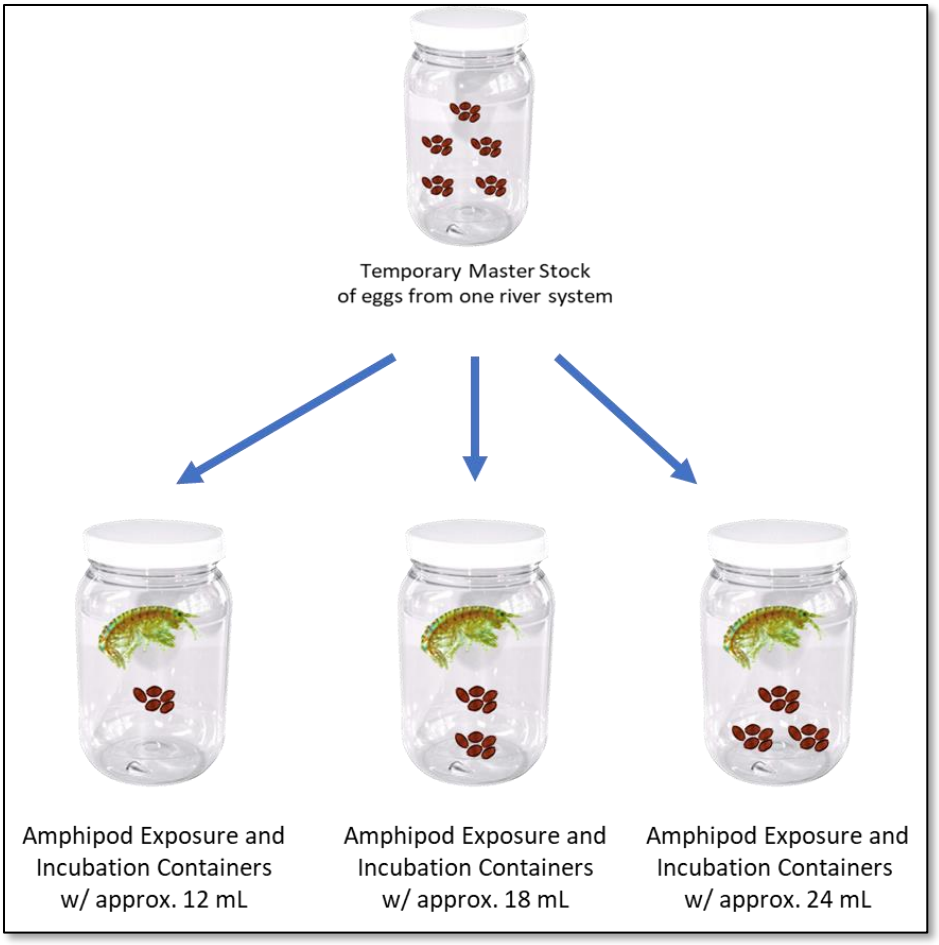

Figure 11 .Method for determining optimal exposure.

EXPOSURE OF EXPERIMENTAL AMPHIPODS TO EGGS

Prior to exposure, Master Egg-Stock Container was gently inverted several times to ensure relatively uniform dispersal in the jar, and then several aliquots (approx. 12 mL total) were withdrawn from the Master Egg-Stock Container and dispensed into the Amphipod Incubation Container with the 35 amphipods.

Amphipods were exposed to the eggs for 5 d, after which time they were removed and rinsed with fresh artesian water to remove any eggs that may have become attached. The jar was rinsed thoroughly to remove any remaining eggs, at which time the amphipods were placed back into the jar with fresh well water. Amphipods were

incubated for 5 d to ensure that any recently consumed eggs have hatched and the larva had become infective to sunfish (Worsham et al. 2016).

Before each fish-infection trial, a subsample of experimental amphipods was dissected to ensure the presence of viable larval worms. The protocol is summarized in Figure 12.

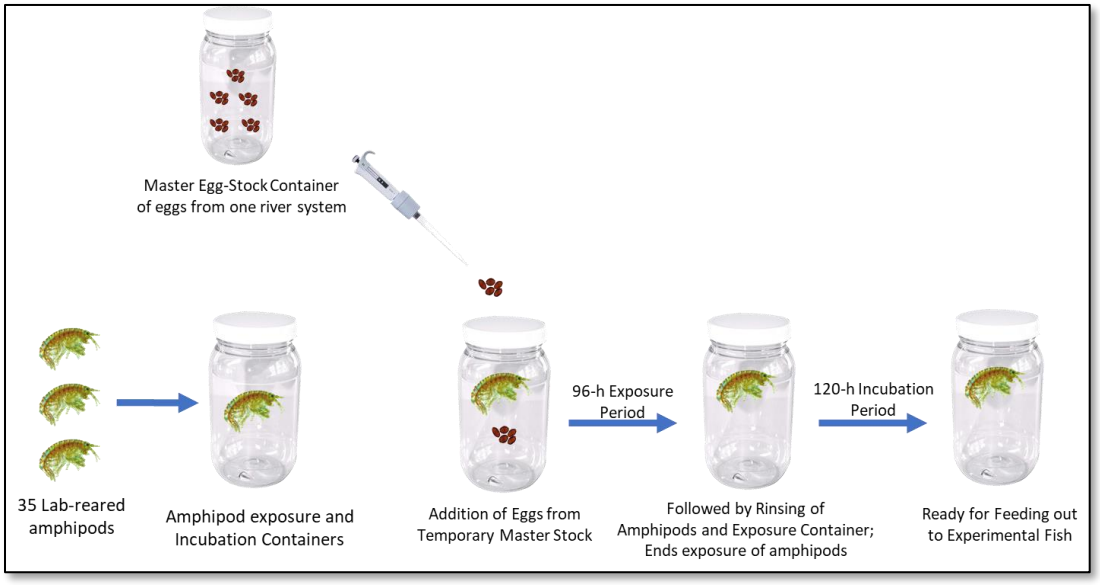

Figure 12. Amphipod infection protocol

Artificial Infection of Experimental Sunfish

During the process of collecting wild-caught fish, several times more viable eggs were collected from the SCR than from the SSR. As the number of eggs collected from the SSR was thought to be insufficient to supply the experiment, the SCR population became the basis for the lifecycle study. The eggs collected from the SSR were still used in an attempt to infect fish, however, the primary purpose of those exposures was an attempt to collect adult nematodes.

Before each infection, water flow to the fish aquaria was turned off. Several liters of water were also removed to ensure that amphipods could not escape through the drain

bulkheads. The air supply was temporarily stopped to allow for easier observation of the fish and amphipods. Most fish were fed 10 infected amphipods, however, 4 fish from each population were fed 20 infected amphipods, in an effort to increase the chances of finding adult nematodes in those fish for the planned morphometric studies. Each fish was observed until all amphipods appeared to be consumed, at which time the flow of water and air were restored to the aquaria.

Fish Dissection and Observation

For the life cycle study, it had originally been planned to dissect fish in 2-w intervals, beginning at 3 mo and ending at 12 mo post-infection, meaning that each progressive fish would have been 2 w farther along in the life cycle. However, due to unexpected fish deaths, as well as numerous exposed fish not becoming infected, this timeline was not strictly adhered to. Instead, fish were dissected in a manner as to be able to obtain as many important life cycle events as possible based on the life-cycle model developed by Worsham et al. (2016).

Fish were euthanized by pithing by IACUC approved protocol, then the swim bladder was excised with a pair of forceps as per above (Figure 4), then the rete was placed on a slide, covered with a cover slip, and inspected for the signs of nematodes and eggs. If eggs were present, their level of development was noted.

Removal and Preservation of Adult Nematodes

Adult nematodes found in experimental fish were removed from tissue using a pair of dissecting probes, killed in hot water, and transferred to formalin for morphometric comparison, or into molecular-grade EtOH for DNA studies at a later date.

Unresolvable complications with the hot-water killing protocol (ruptured worms) forced the abandonment of the protocol. Any additional nematodes that were found were placed directly into their respective fixative.

EGG STUDIES

LIGHT MICROSCOPY

Light Microscopy Specifics

Compound microscopes were used to search for discernable morphometric differences between the three populations of eggs. To qualify for measurement, each egg was required to be mature (dark brown to nearly black), oriented with long-axis approximately perpendicular to the optical viewing axis, and completely free of host tissue. Only the outer margin of the second layer of the shell (the chitinous layer) was measured since the metrics of the outermost layer of the *Huffmanela huffmani* eggs vary substantially with preparatory technique.

SEM

In preparation for imaging under the scanning electron microscope (SEM), eggs were first separated from swim bladder tissue. A swim bladder rete was placed in a petri dish and immersed in modified Hank's physiological saline. Large pieces of egg-free tissue were removed and discarded, and the remaining egg-laden tissue was shredded with dissecting probes in an attempt to free as many eggs from tissue as possible. Remaining shreds of tissue were removed, and the suspension of free eggs was pipetted into a 1.5-mL microcentrifuge tube for fixation.

The microcentrifuge tube was centrifuged at 500 rpm to condense the eggs into a pellet at the bottom of the tube. Once the pellet had formed, the supernatant was carefully pipetted off with a Pasteur pipette and discarded.

A 2% solution of glutaraldehyde was added, eggs re-suspended, and the tube capped and left to fix for 2 h. After fixation, eggs were centrifuged again, and the glutaraldehyde pipetted off.

Eggs were then re-suspended and washed twice with a 0.1M solution of cacodylate for 15 min. After each wash, the eggs were re-centrifuged.

After the wash was removed, a solution of 1% osmium tetroxide was added to the eggs, which were re-suspended and allowed to post-fix for 2 h. The osmium was then removed and neutralized by immersion in greater than twice its volume in corn oil.

Another series of cacodylate washes was performed as described above. These washes, however, were placed in corn oil, to ensure any remaining osmium was neutralized.

The eggs were then dehydrated through a series of 30, 50, 70, 80, 90, 95, and 100% ethanol. Each step in the dehydration series was given 30 min to allow for complete dehydration at that level.

To complete the drying process, a critical point dryer was used, and a porous pot with 10-µm holes was used to hold the eggs during the process. The eggs were resuspended in the microcentrifuge tube, and the eggs and ethanol were carefully pipetted drop-by-drop onto the inverted lid of the porous pot (if pipetted into the pot itself, the eggs become exceedingly difficult to remove). The base was then fitted to the cap, while keeping the entire pot in an upside-down position. and then loaded into the critical point

dryer. The chamber was then filled with acetone and run on the slow setting for approximately 2 h. Once the drying process was completed, pots were removed while maintaining them in an upside-down position. The pot was then removed from over the lid, and a piece of carbon tape was applied to the lid of the porous pot to pick up the eggs, the tape was then placed on an SEM stud.

The stud containing the carbon tape was placed in the sputter coater and coated with carbon. The studs were then stored in an indexed storage container until ready for viewing.

TEM Preparation

Square sections of egg-laden tissue measuring approximately 3x3 mm were cut from infected swim bladders and fixed in 2% glutaraldehyde for 2 h. Tissue was washed twice for 15 minutes in a 0.1M cacodylate solution. After the washes, the eggs were placed in osmium tetroxide to post-fix for 2 h. After post-fixation, another series of cacodylate washes was performed. A series of ethanol dehydrations was then performed using the same protocol as for SEM.

Once tissue was in 100% ethanol, it was placed into a TEM embedding capsule. Resin was added to the capsule at a 1 to 1 ratio of LR White Hard resin to ethanol.

Tissue in the resin/ethanol mixture was incubated for approximately 12 h at approximately 25 C. The next day the resin was pipetted out, fresh resin was added into the capsule, and the specimen was incubated for another 3 h at room temperature before a fresh batch of resin was added to the capsule. The capsules were then closed and heated in an oven at 65 C for at least 24 h to harden the resin.

Once polymerized, the resin block was shaped and placed into an ultramicrotome (Leica Reichert Ultracut S). Sections were cut to approximately 70 nm, at which time a 200 square copper TEM grid was dropped onto the section. Once dry, the section was placed in an indexed TEM grid holder until ready for viewing.

III. RESULTS

LIFE CYCLE EXPERIMENT

Of the 25 fish exposed to infected amphipods, only 5 showed signs of infection (Table 3). Two successful experimental infections were from exposure from amphipods infected with SSR larvae and three were from SCR larvae. No infections were detected in the 20 exposed fish that were examined at incubation durations shorter than 6.8 months.

Table 3. Results of feeding out experimentally infected amphipods to green sunfish.

Egg Source	Infection Evidence	Amphipods Fed Out	Mo of Incubation	Fate	Comments
C	N	10	3.0	D	Had fin rot. Treated with two cycles of API's FURAN-2 (4 doses per cycle) prior to death.
C	N	20	4.2	D	N/A
C	N	10	5.1	D	Eyes protruding out from orbital cavity, otherwise appeared healthy.
C	N	10	5.4	D	Caudal, dorsal, and pectoral fins effected by fin rot. First treated with one cycle of API's FURAN-2. Subsequently, treated with three cycles of API's Melafix and Primafix.
C	N	10	5.6	D	Prior to death had an apparent lesion on its side.
C	N	20	7.7	E	Fish immediately seized-up upon removal from water.
C	N	10	7.7	E	N/A
C	N	10	8.7	E	N/A
C	N	10	9.0	E	N/A
C	N	10	9.0	E	N/A
C	N	20	9.0	D	N/A
C	N	10	9.1	E	N/A
C	N	10	9.6	E	White fugal-like lesion dorsal. Treated with API's Melafix and Pimafix.
S	N	10	2.2	D	N/A
S	N	10	3.0	D	N/A
S	N	10	6.9	E	Immediately seized-up when removed from aquarium. Gut distended.

Table 3 Continued. Results of feeding out experimentally infected amphipods to green sunfish.

Egg Source	Infection Evidence	Amphipods Fed out	Mo of Incubation	Fate	Comments
S	N	20	8.8	E	N/A
S	N	10	8.9	E	N/A
S	N	10	9.0	E	N/A
S	N	20	9.2	E	N/A
C	Y	20	6.8	E	No eggs present. One worm found, likely an L_3 or L_4.
C	Y	10	7.1	E	Eggs clear, lacking development in chitin. Six sub-adult worms were found.
C	Y	10	8.3	E	Eggs varying in development, most have some tanning of the chitin indicating some degree of maturation. Few eggs darker and more advanced. Intensity of infection low. No worms found.
S	Y	10	9.3	E	Most eggs dark, showing maturation. Several unembryonated eggs also present. Three worms.
S	Y	10	9.7	E	Most eggs are dark showing development in chitin, most appear partially larvated. Infection intensity is very low. No worms found.

6.8 Months Post-Exposure

In one fish at 6.8 mo post-exposure, a single long worm was recovered (Figure 13). The worm lacked any discernable internal structures or eggs, suggesting that it was sub-adult.

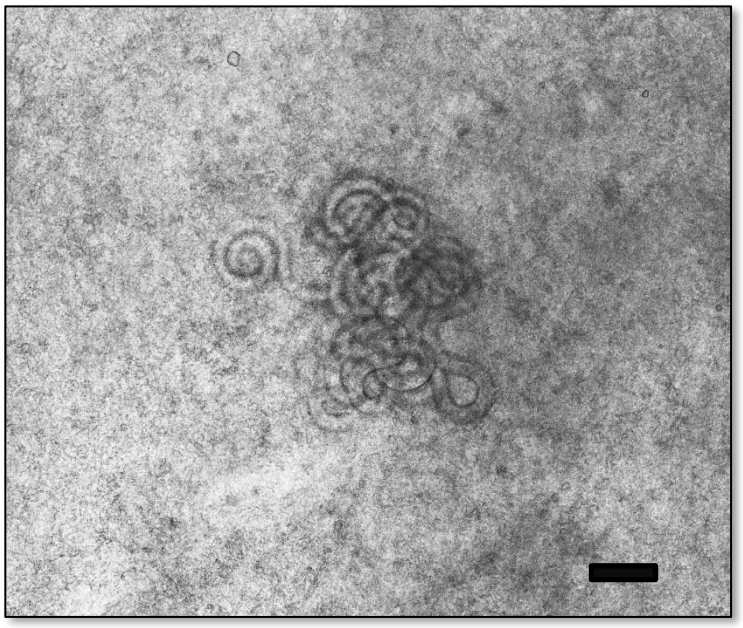

Figure 13. Sub adult nematode recovered from experimentally infected fish incubated for 6.8 mo. post-exposure; scale bar 100 µm.

In their work, Worsham et al. (2016) found that some larval worms were found prior to this, but no adult worms were seen before 6.5 mo. incubation. The lack of infection prior to 6.8 mo may be due to necropsying uninfected fish.

7.1 Months Post-Exposure

The first signs of eggs appeared several weeks later at 7.1 months (Figure 14). These eggs show signs of development of the outer shell, and polar plug development can be seen in most; however, the chitinous layer is still very light in color, indicating that the tanning process has yet to begin.

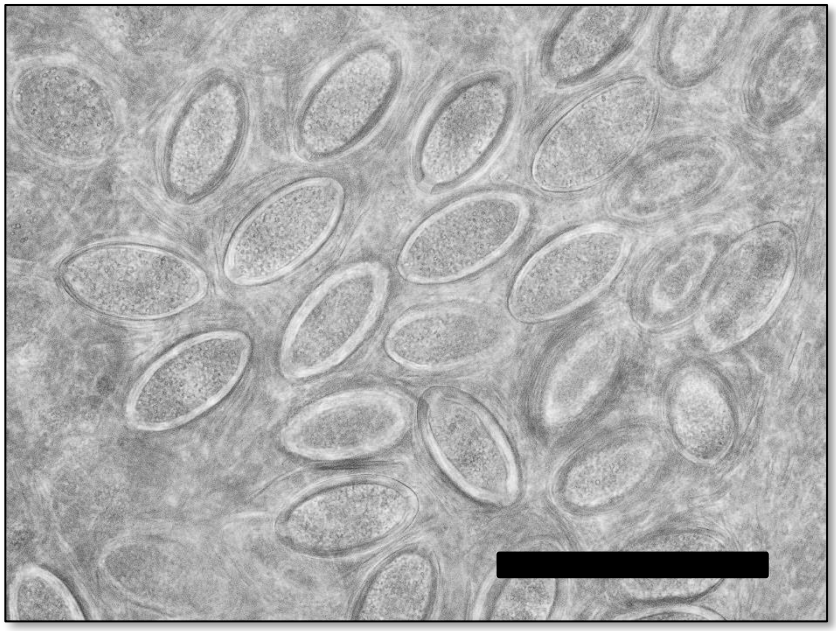

Figure 14. *Huffmanela* eggs early in development (7.1 mo post-exposure), showing formation of polar plugs; scale bar 100 µm.

The appearance of eggs at 7.1 appears to be slightly earlier than the 7.5 mo reported in Worsham (2015). However, in that study no fish were necropsied between 6.5 and 7.5 mo exposure.

8.3 Months Post-Exposure

The next confirmed infection was at 8.3 mo post-exposure (Figure 15). Most amber eggs in this fish were semitransparent; however, evidence of larval development and darkening of the chitinous layer suggests a later stage of development.

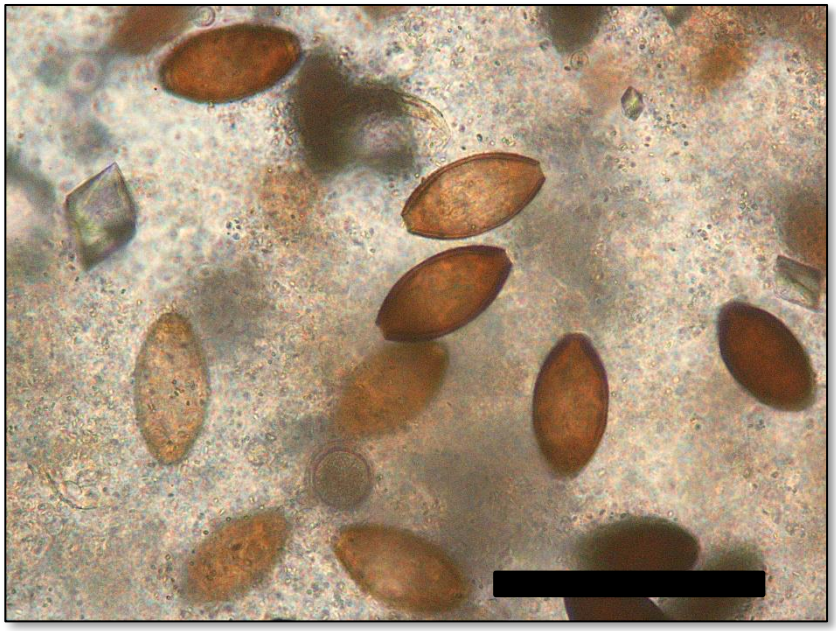

Figure 15. *Huffmanela* eggs from an experimentally infected fish 8.3 mo post-exposure showing evidence of larval development and darkening of chitinous layer; scale bar 100 µm.

Eggs of the South Concho population appear to begin to darken sometime after 8 months post-exposure. Darkening of the chitin was not reported until 8.75 months post-exposure in Worsham et al. (2016), however, no fish were inspected between 7.5 and 8.75 months post-exposure. This suggests that darkening may occur earlier, but no fish were dissected in this time frame in Worsham et al. (2016).

9.3 Months Post-Exposure

A dissection performed at 9.3 months post-exposure (Figure 16) showed evidence of at least three sessions of egg laying separated by weeks in the same area of tissue. Three worms were recovered from this fish, but all seemed to be sub adults, with poorly formed stichocytes, one of which is shown in Figure 16. As amphipods were fed to each fish in a single treatment, and eggs were found in several different stages of development,

it seems that these worms show a high degree of asynchronous development, or that they are migrating to deposit eggs at different times.

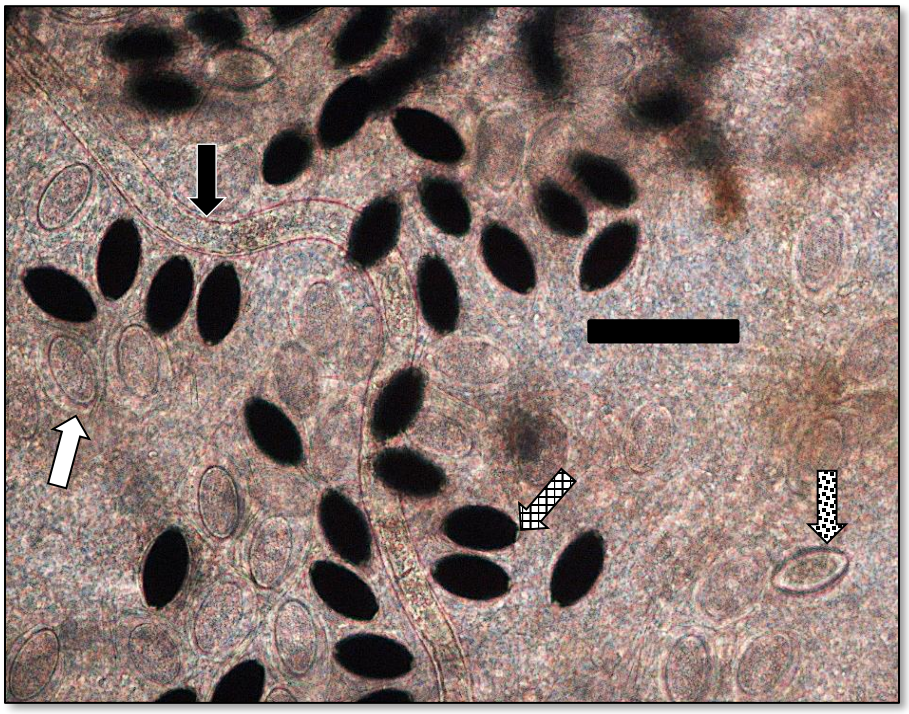

Figure 16. *Huffmanela* eggs from mixed infections in an experimentally infected fish at 9.3 mo post-infection. Arrows: **white**—ellipsoidal unembryonated egg with slight suggestion of polar development and expansive Uterine Layer (obviously zygotic); **black**—larva with diameter slightly smaller than undeveloped egg migrating through tissue; **stippled**—more fusiform intermediate-stage egg with thicker shell and obvious polar development, **cross-hatched**—late stage egg with very dark, opaque shell (note hyaline space over plugs); scale bar 100 µm.

While Worsham et al. (2016) typically found adult worms in experimental fish that had been infected for this long, eggs in numerous stages of development were noted. Even up to 11.5 mo post exposure in their study, eggs were still being observed that appeared to be early in development.

9.7 Months Post-Exposure

At 9.7 mo. post exposure, eggs in several stages of egg development still appear; however, many eggs show some indication of tanning in the chitinous layer. The darkest, and presumably oldest, eggs appear partially larvated. No worms were found in this infection. This supports the work of Worsham et al. (2016), who stated that worms stopped appearing around 10 months post-infection.

Differentiation of Freshwater *Huffmanela* Populations

Egg Morphometry of The Three Populations

Initial observation of the egg width and length measurements did not reveal obvious differences in the gross morphology between the study populations (Table 4).

Table 4. Results of measurements of eggs from each population (n = 100). Measurements in micrometers.

	Length		Width	
Population	Mean ± SE	Min, Max	Mean ± SE	Min, Max
San Marcos	55.19 ± 0.23	43.66, 60.58	28.43 ± 0.16	24.74, 34.91
San Saba	56.29 ± 0.28	39.52, 62.72	29.06 ± 0.21	26.03, 36.86
South Concho	55.60 ± 0.18	50.56, 60.40	28.76 ± 0.13	22.83, 32.65

One-way MANOVA was used to analyze the length and width measurements of eggs from the three populations, and showed significant differences between the populations [$p(F_{\alpha(1),4,594} \geq 3.77) < 0.005$]. To assess differences in length or width by population, a Bonferroni corrected one-way ANOVA was conducted on the length and width resulting in [$p(F_{\alpha(1),\,2,297} \geq 3.49) = 0.023$] and [$p(F_{\alpha(1),\,2,297} \geq 5.71) = 0.037$], respectively. Only the results of the ANOVA conducted on the lengths was significant. Tukey's HSD was then applied to the length group resulting in only the lengths of the SCR and SSR populations being significantly different [$p(q_{\alpha(2),3,297} = 3.33) < 0.003$] (Figure 17).

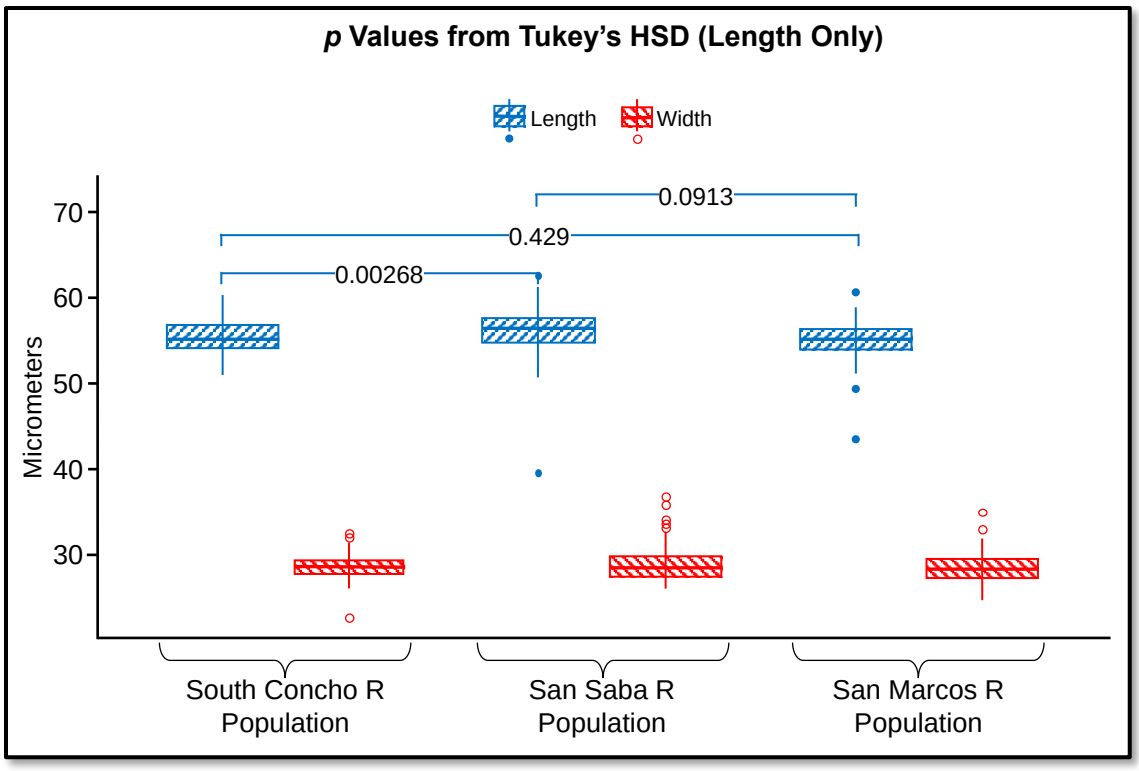

Figure 17. Egg measurements with the results of Tukey's HSD applied to the lengths of all populations. Comparison bars were not added for width measurements as they were found not to be significantly different based on the results of the ANOVA.

While the results of the post hoc test did show significant differences between the lengths, the actual difference between the means was small (1.1 µm). In addition, great overlap was seen between the measurements for both the length and width variables.

Outer Layer Features as Revealed by SEM

Results of scanning electron micrographs from the three populations reveal that the two new populations have spinous membranes similar to that of *H. huffmani* (Figure 18, Figure 19, Figure 20).

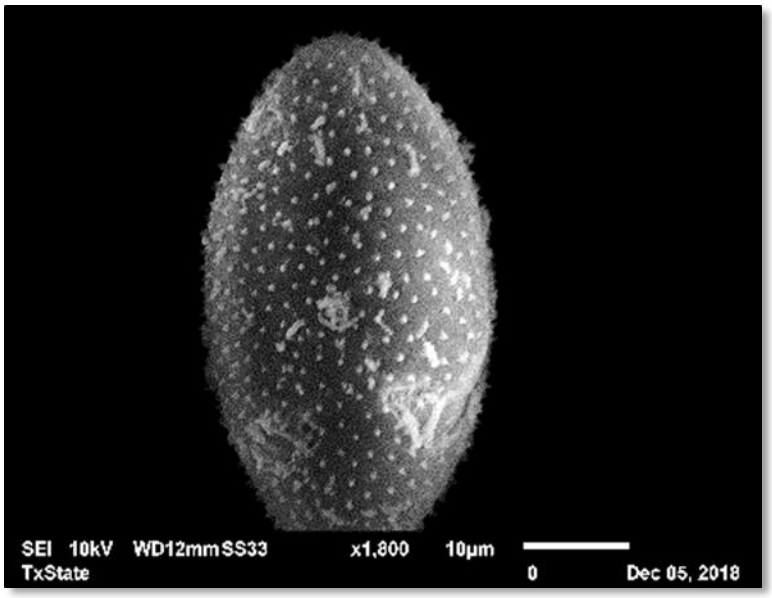

Figure 18. Scanning electron micrographs of the San Marcos population of *Huffmanela*.

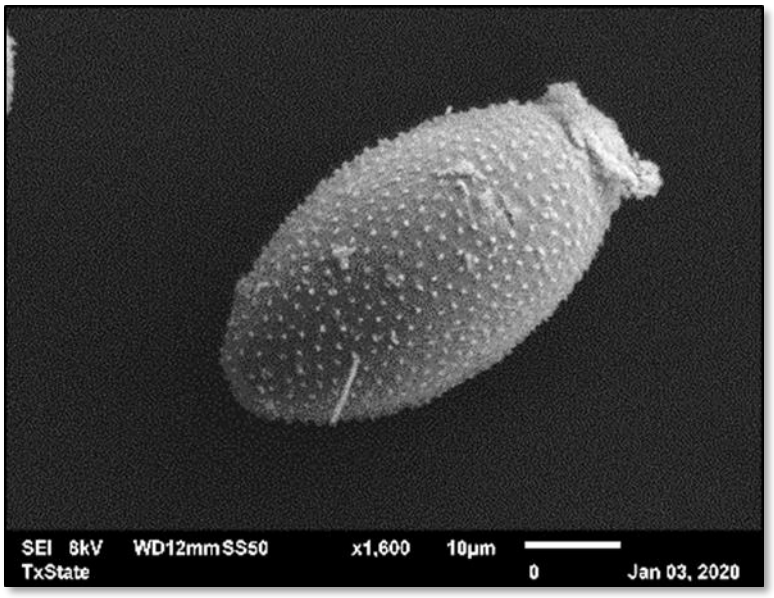

Figure 19. Scanning electron micrograph of an egg from the South Concho population of *Huffmanela*.

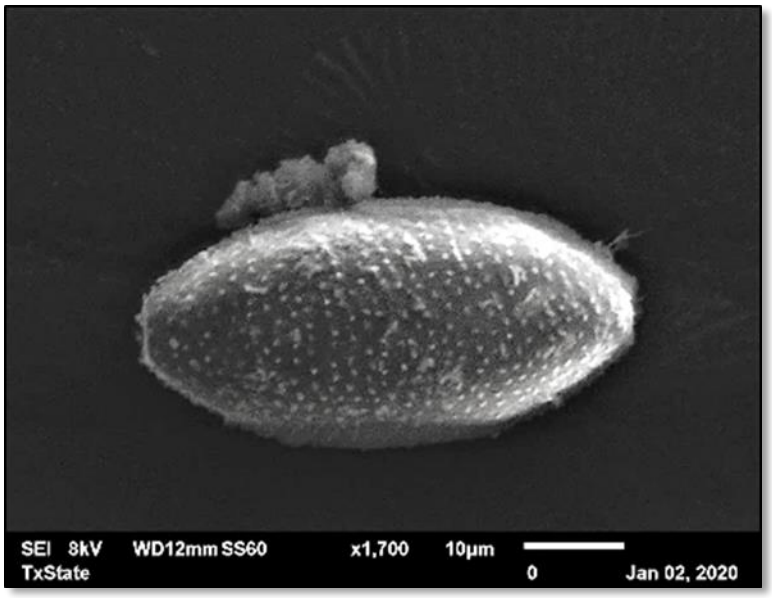

Figure 20. Scanning electron micrograph of an egg from the San Saba *Huffmanela* population.

Comparison of Late Larval and Adult Worms

All successfully collected intact nematodes were from the SCR population. Unfortunately, these worms were all late larval stages and lacked the defining features that are typically used to differentiate nematode species. Several fragments of late larvae were also collected from the SSR population, but the only discernable features were the stichocytes (Figure 21). The stichocytes were of two types, darker and shorter (25 to 33 µm long) vs. lighter and more elongate (50 to 62 µm long). The pattern of alternation between dark and light stichocytes was irregular within a worm: sometimes with 1 dark, 1 light, 1 dark; but other times with 1 dark, 2 light, 1 dark. In no examples were there two dark stichocytes in sequence or more than two light stichocytes in sequence.

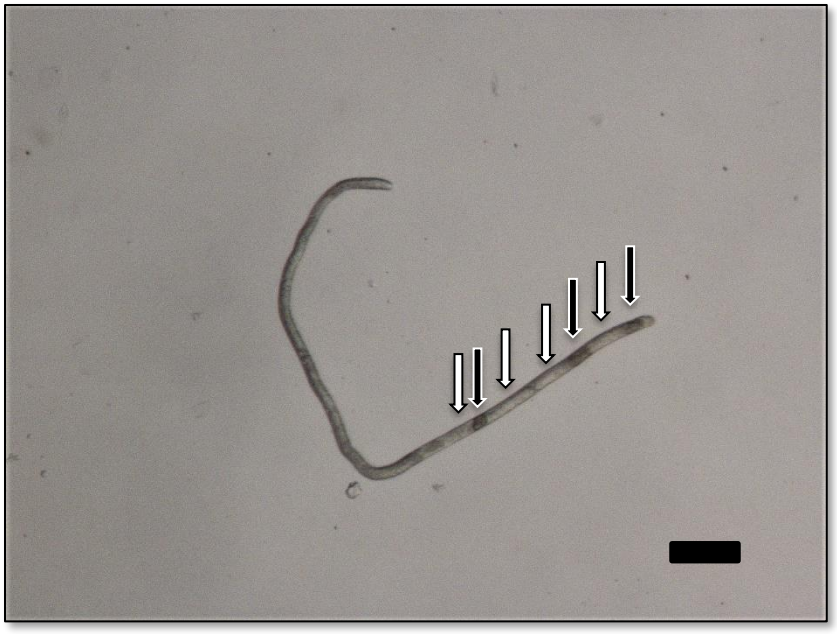

Figure 21. Worm fragment collected from an experimentally infected fish 9.3 mo post-exposure, with arrows showing dark and light stichocytes. Scale bar represents 100 µm.

STRUCTURE OF THE *H. HUFFMANI* EGGSHELL

TEM imaging performed in this study revealed peculiarities in *H. huffmani*, that may indicate the discovery of several structures not previously reported in *Huffmanela* literature. The names given to these structures are based on reviewed literature and the characteristics of the structures. As many of the structures noted in Stein and Golden (2018) appear to be more comparable to a space rather than a layer, such labels will be changed to reflect what was observed in this study.

A NEW MODEL FOR EGGS OF *HUFFMANELA* SPP

Observations in this study using light microscopy, scanning electron microscopy and transmission electron microscopy, when compared to the findings of Olson et al. (2012) for the eggs of *Caenorhabditis elegans*, indicate that many misinterpretations of egg anatomy have been made in the descriptions of *Huffmanela* eggs, as well as in

classical literature regarding the eggs of other trichurid nematodes. This study aims to correct as many of those errors as possible, and to propose a new model for the *Huffmanela* egg (Figure 22) that can be applied to many other genera of trichurid nematodes with polar plugs. All subsequent discussion of egg anatomy in this work will use the terminology developed in this proposed model.

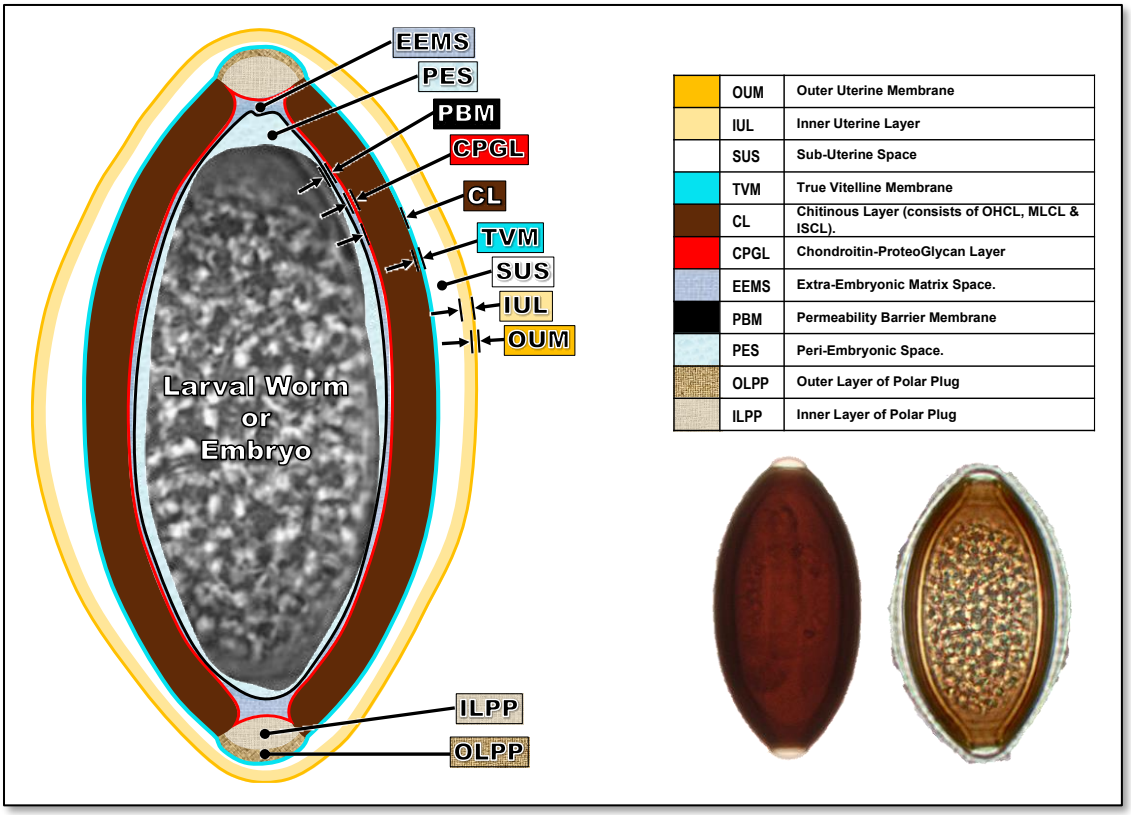

Figure 22. Proposed new model for describing eggs of *Huffmanela* spp. based on then new model proposed for *Caenorhabditis elegans* eggs by (Olson et al. 2012), and the results of SEM and TEM observations in this study.

Outer Membranes

Visualization under light microscopy has shown the outermost layer to be variable, with a fluid or gel filled inner section (Figure 23). TEM micrographs show that this area is formed by an electron dense outer layer and an inner electron lucid inner layer (Figure 24). As these characteristics are more similar to a Uterine Layer (UL), the two

sections have been termed the Electron Dense Uterine Layer (EDUL) and the Electron Lucid Uterine Layer (ELUL).

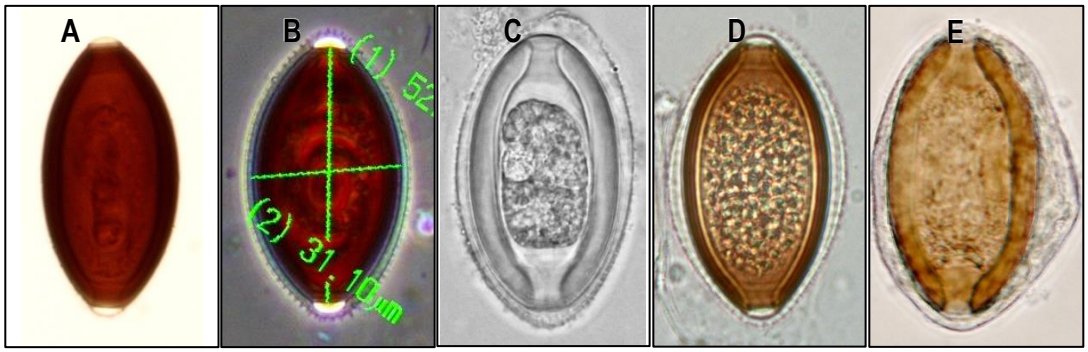

Figure 23. Variation in the appearance and dimensions of *Huffmanela huffmani* eggs, depending on status of outer uterine layer and age. A) Larvated egg missing uterine layer entirely; B) larvated egg with outer membrane of uterine layer tightly and uniformly appressed to vitelline layer; C) early pre-larvation egg with outer membrane of uterine layer spaced out fairly uniformly away from vitelline layer; D) later pre-larvation egg with much thicker chitinous layer and outer membrane of uterine layer spaced irregularly but farther from vitelline layer; E) dead egg with remnant of outer membrane of uterine layer bulging away from shell proper.

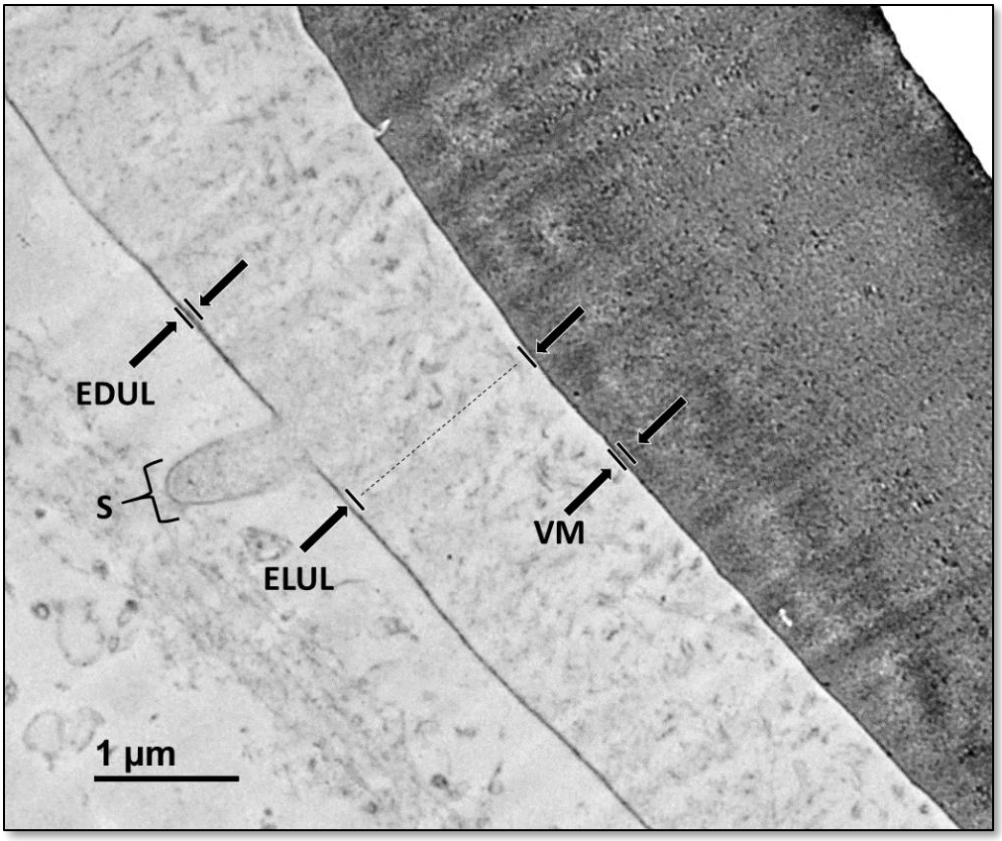

Figure 24. TEM micrograph of eggs of *H. huffmani* showing a protruding spine (SP), the Electron Dense Uterine Layer (EDUL), the Electrron Lucid Uterin Layer (ELUL), and what is presumed to be the true vitelline membrane (VM).

While viewing the UL, another layer was observed. This layer appears as a dark membrane surrounding the chitinous layer (Figure 24). As this layer appears to tightly bind to the chitin and is of a width consistent with other reported vitelline membranes, this layer is thought to be the True Vitelline Membrane (TVM).

The UL also appears to be delicate, during viewing under SEM, the majority of the viewed eggs lacked this membrane, and some images appear to show a layer of fuzzy fibers radiating from the TVM (Figure 25). These fibers are thought be remnants of the ELUL.

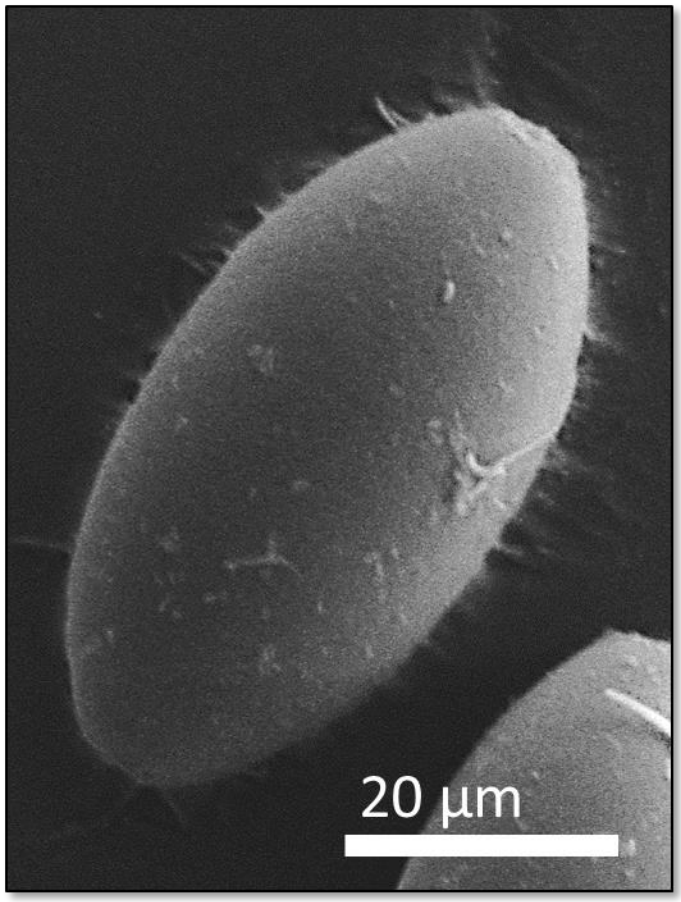

Figure 25. Micrograph of an egg from the SSR, that lack the Uterine Layer showing apparent fibers emitting from the chitinous layer.

In addition, eggs from all populations, both with and without the outer layer, showed that the polar plugs were no longer protruding, but in fact shrunken into the collar of the chitinous layer (Figure 25).

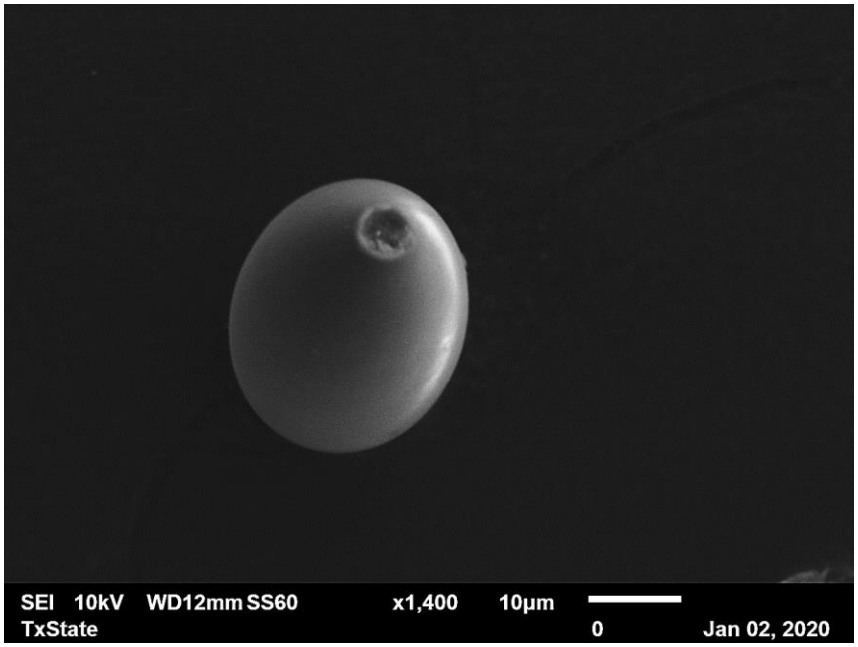

Figure 26. An egg from the SSR, lacking a Uterine Layer. The polar plug appears to be sunken within the collar of the chitinous layer.

CHITINOUS LAYER

The chitinous layer appears to be similar to that reported by Žďárská et al. (2001), consisting of three apparent layers (Figure 27). The first layer appears to consist of a single band of homogenous, electron dense material; thus, it was named the Outer Homogenous Chitin Layer (OHCL). The second layer appears to be laminar, consisting of 25 + alternating bands of electron dense and electron lucid material, deemed the Middle Laminated Chitinous Layer (MLCL) (Figure 27Figure 28). The final layer consists of an extremely electron dense area that was described as a spongy by Žďárská et al. (2001), who was able to obtain better resolution micrographs of the layer; thus, the layer was deemed the Inner Spongy Chitinous Layer (ISCL) (Figure 27). In addition, in Žďárská et al. (2001) noted that the Lipid-Rich Layer appeared to be dispersed throughout the pores of this layer, however, according to the Olson et al. (2012) model, this is likely the CPG Layer.

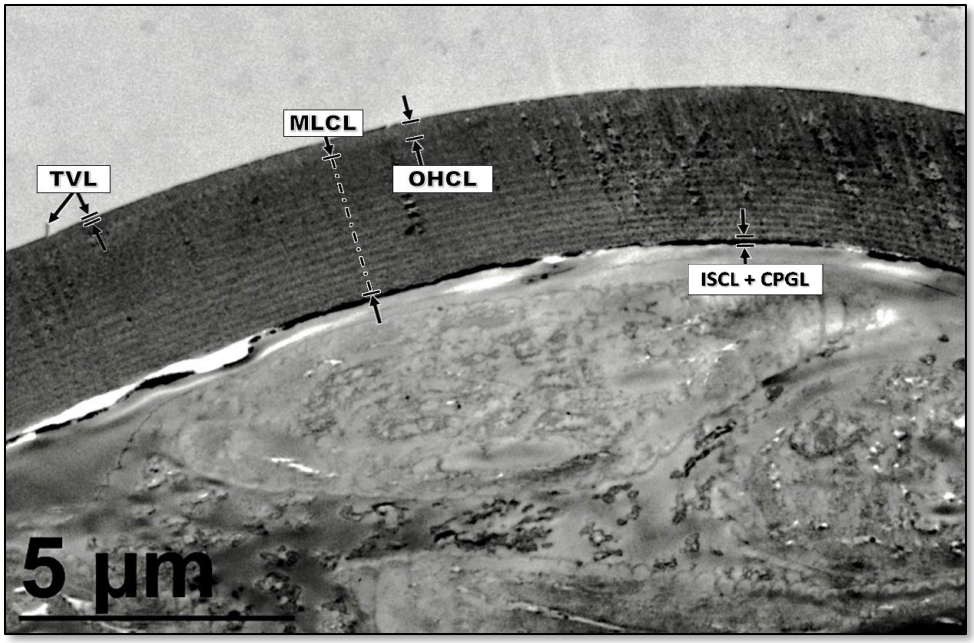

Figure 27. Section showing the Inner Spongy Chitinous Layer (ISCL) and CPG Layer (CPGL) having apparently broken away from the Middle Laminated Chitinous Layer (MLCL) in places. Also present is the True Vitelline Layer (TVL) appressed to the Outer Homogeneous Chitinous Layer (OHCL), except where pulled away from it by the microtomy knife, which was cutting from bottom to top of the imaged section.

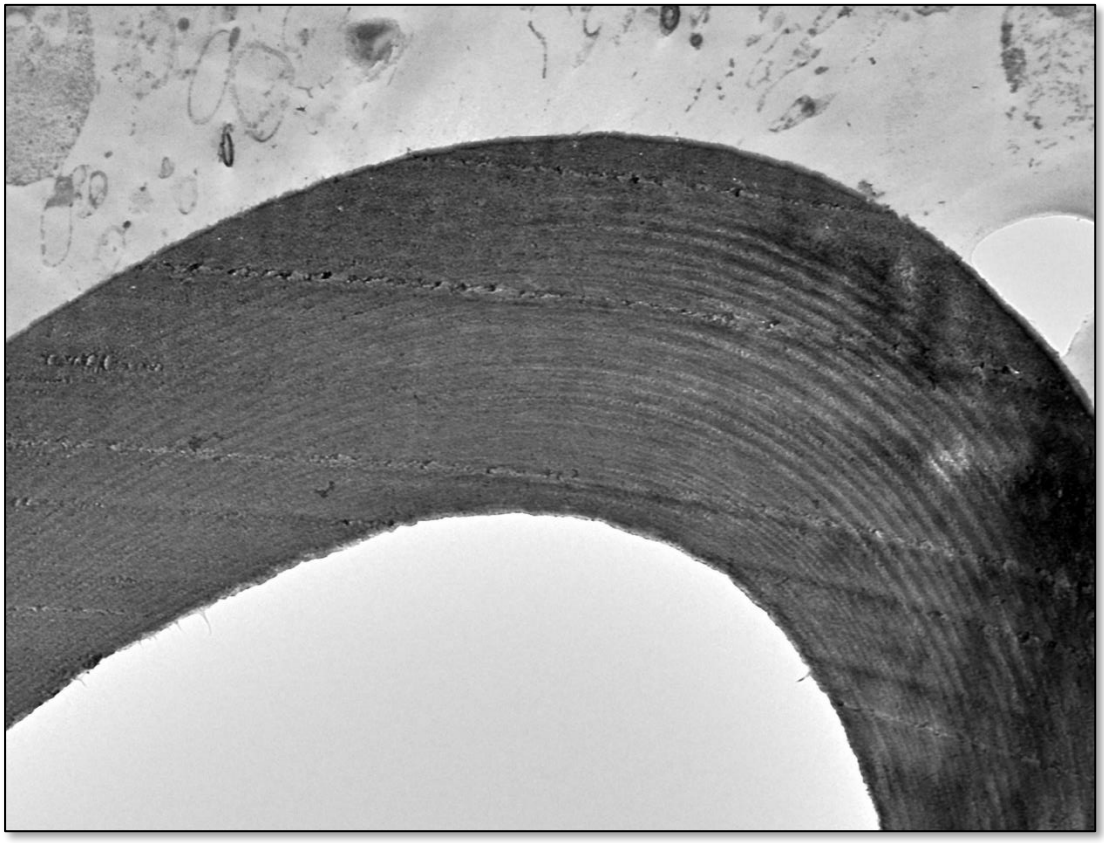

Figure 28. Chitinous layer of a *Huffmanela huffmani* eggshell showing alternating light and dark layers of chitin.

INNER LAYERS

The first layer, which forms a boundary between the chitin and the rest of the inner egg is the CPG Layer (CPGL). This layer can be seen in Figure 29 and to a lesser extent Figure 30.

The Extra-Embryonic Matrix Space (EEMS) is purported to be the space in the egg underneath the CPGL. If this is the case, and the CPGL was diagnosed correctly, the EEMS is likely to be the large structure observed in Figure 29 and Figure 30.

The Permeability Barrier Layer (PBL) is noted as a small electron dense layer that creates the inner boundary for the EEMS. Again, if previous diagnoses are correct,

that would imply that the electron dense area noted in Figure 29 and Figure 30 are likely the PBL.

Finally, the Peri-Embryonic Space has been described as the final space before the embryo or larval worm. It seems likely that this layer has been correctly diagnosed in both Figure 29 and Figure 30 as the remaining space surrounding the larvae.

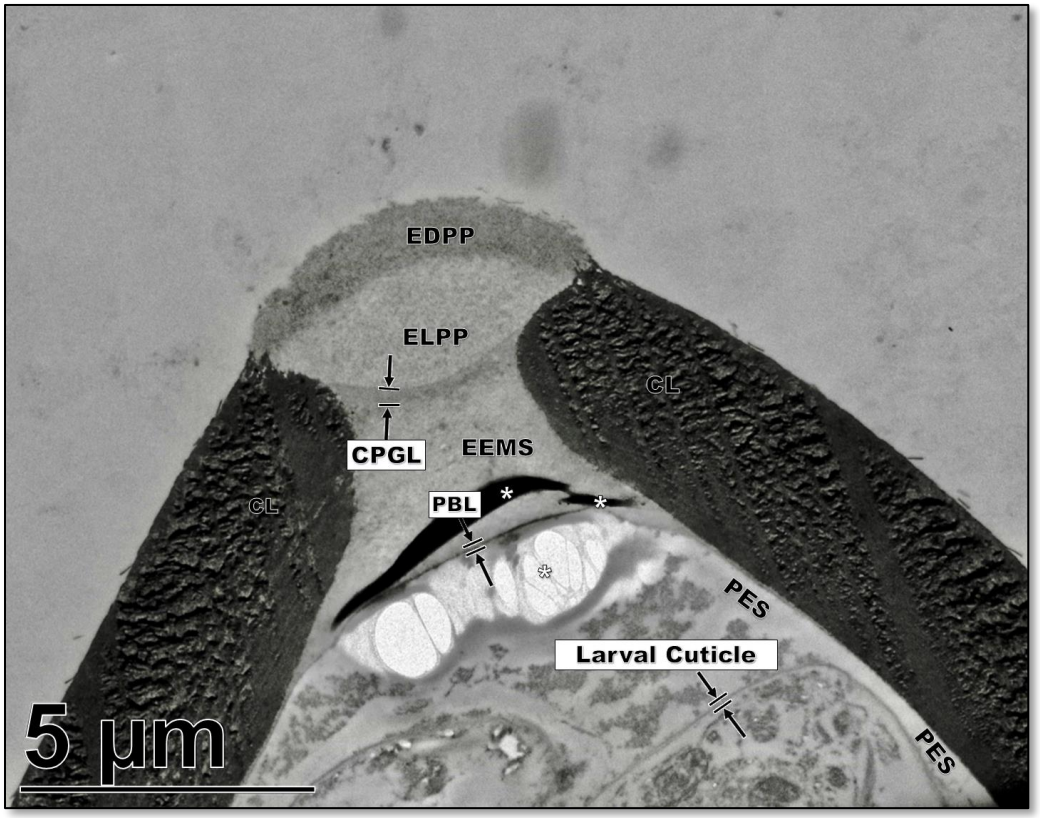

Figure 29. Section through the polar plug of an *H. huffmani* egg lacking the Uterine Membrane. CL - the Chitinous Layer. CPGL – possible Chondroitin Proteoglycan Layer. EDPP – Electron Dense Area of the Polar Plug. ELPP – Electron Lucid Area of the Polar Plug. EEMS – possible Extra-Embryonic Matrix Space. PBL – possible Permeability Boundary Layer. PES – possible Peri-Embryonic Space. * - technical artifacts.

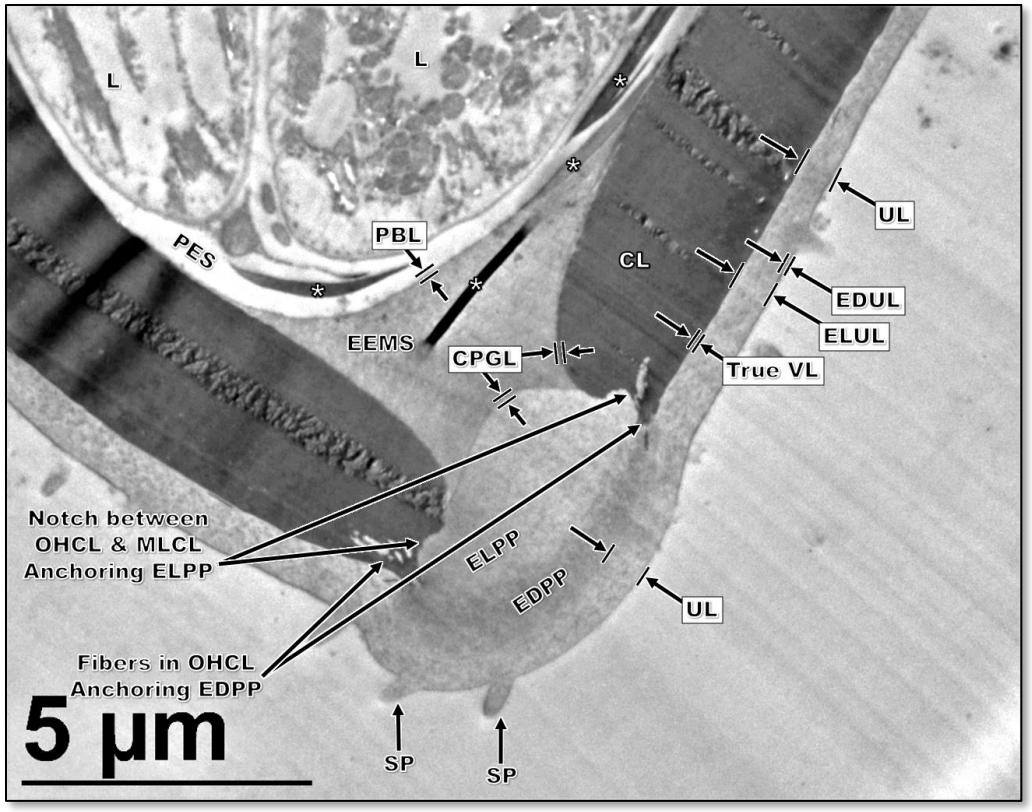

Figure 30. Mid-sagittal section of the polar region of an egg of *H. huffmani*. See Table 5 for symbol explanation.

Table 5. Symbol interpretation for Figure 29.

***:**	Presumptive artifactual folds in section caused when knife encountered chitin broadside.
CL:	Chitinous Layer (consists of OHCL, MLCL & ISCL).
CPGL:	Chondroitin-ProteoGlycan Layer.
EDPP:	Electron Dense layer of Polar Plug.
EDUL:	Electron Dense Uterine Layer.
EEMS:	Extra-Embryonic Matrix Space.
ELPP:	Electron Lucid layer of Polar Plug.
ELUL:	Electron Lucid Uterine Layer.
L:	Larval body.
OHCL:	Outer Homogeneous Chitinous Layer.
PBL:	Permeability Barrier Layer.
PES:	Peri-Embryonic Space.
SP:	Superficial Projections of uterine layer.
UL:	Uterine Layer, formerly “vitelline” layer (consists of EDUL & ELUL).
TVL:	True Vitelline Layer (previously undocumented layer on surface of OHCL).

POLAR PLUG

The plug itself appears to be comprised of two distinct layers, the Electron Dense Layer of the Polar Plug (EDPP) and the Electron Lucid layer of the Polar Plug (ELPP) (Figure 29 andFigure 30). In addition, this study also found that these structures appear to originate from the OHCL and MLCL, respectively (Figure 30). It was also noted that even with the removal of the OUL, the polar plug still maintained its shape (Figure 29).

IV. DISCUSSION

LIFE CYCLE

Due to the amount of uninfected fish, as well as fish that died prior to their proposed necropsy date, this study is not as complete as the study in Worsham et al. (2016). However, the few infected fish necropsied provide some insight into their life cycle, as well as the ability to compare the life cycle of *H. huffmani* with the San Saba and South Concho populations.

The lifecycle of *H. huffmani* appears to be similar to that of the San Saba and South Concho populations (Worsham et al. 2016). While timing events of the necropsy of fish in the study do not precisely overlap, we can infer that they show similar timelines. If the life cycles are the same, we can also make some inference into some of the gaps in the *H. huffmani* life cycle, such as narrowing down when adult worms begin to lay eggs, or when chitin synthesis begins in the shell.

One of the major limitations of this study was the inability to infect all experimental fish. This greatly reduced the ability to compare the life cycle with that of *H. huffmani*. One possible explanation for this could be the number of larval worms attained per amphipod.

Unlike Worsham et al. (2016), who typically encountered greater than 25 larvae in each amphipod, during this lifecycle study, most amphipods harbored substantially less worms. Generally, 1-5 larval worms were found per exposed amphipod prior to feeding the rest out to experimental fish. This does have some interesting implications for the number of larvae required to infect the definitive hose.

Worsham et al. (2016), states that it required the feeding out of 21 infective amphipods to approach an intensity similar to that of wild caught fish. This suggest that a many larval worms are required in order to infect the definitive host. Similarly, they found that feeding of 9 amphipods infected with several hundred larval nematodes collectively, resulted in only 10's of adult nematodes in the fish. It is not known what causes this reduction of nematodes; if the worms are lost during consumption by the centrarchid, immune response by the centrarchid, or the worm simply migrating at different times or failing to migrate to the correct organ.

This leads to an interesting question regarding the biology of this parasite, how are these populations stable in the wild? Due to the perceived narrow window for these worms to mate, and the number of adult nematodes required to produce infections consistent with those in the wild, it would be speculated that these nematodes should be commonly found within the intermediate host. This, however, does not appear to be the case as no infections have been detected in wild amphipods, even after numerous amphipods have been examined in the Huffman lab.

Future studies concerned with the life cycle of these new populations, as well as *H. huffmani* can focus on some of the issues brought up in this study, such as how many nematodes are required in order for the life cycle to be completed. Other studies can try and narrow some of the gaps with the timing of different portions of the lifecycle, such as when eggs are first deposited, or when exactly do worms begin to disappear.

COMPARISON OF TEXAS POPULATIONS

Results of the egg morphometrics from each population shows great overlap in sizes, both in length and in width. In addition, while the SMR and SSR populations did

significantly differ in length, the actual difference in means (1.1µm) would not be useful as a taxonomically delimiting characteristic.

In their redescription of *Huffmanela* eggs, Huffman and Moravec (1988) reported finding width measurements of the chitin from 30 to 33 µm, which is in contrast to this study, which found the majority of all population's eggs were < 30 µm wide. In addition, this study found several eggs from the SMR and SSR populations that were larger than reported in the redescription. While the upper bounds of their redescription for length (60 µm) seemed accurate for all populations except the SSR, eggs from all populations were found that were much smaller than the lower bounds of the length (54 µm) measurement in the redescription. This shows that there is more variability in the size of the eggs than previously thought.

Comparison of the Uterine Layer also showed similarities between the populations. Eggs from all populations showed a membrane dotted with spines in a non-uniform manner, which is consistent with observations made by Žďárská et al. (2001) of *H. huffmani*. As most eggs had only partially intact membranes, statistical comparison on the number of spines was not conducted. However, after viewing numerous specimens from each population, the number of spines does not appear to vary greatly between the three.

Comparison of the length and organization of stichocytes from the segmented worm of the SSR population also appears to fall within the range of *H. huffmani* as reported by Huffman and Moravec (1988). While their study found some stichocytes that were longer than were found in this worm, this may be due to this worm being sub adult, and the stichocytes may not have reached their full length.

Due to the overlap of egg measurements between populations, large variation in size among the eggs, and the similarities in the Uterine Layer; declaring them independent species based on egg characteristics is difficult. A more reliable method for differentiation would be to compare intact adult nematodes, as they have more morphometrics available for comparison.

Even with comparisons of adults, what constitutes a morphologically significant difference to delimit a species is often debatable and can prove a hard hypothesis to test (Abebe et al. 2011). Perhaps, an even more definitive method for trying to differentiate these nematodes would be on the genetic level. Genetic analysis could help show how these freshwater populations compare with each other, as well as confirm the genus' placement within higher taxonomic classifications.

STRUCTURE OF THE *H. HUFFMANI* EGGSHELL

The discovery of the UL membrane not being the true vitelline membrane resolves several inconsistencies within the *Huffmanela* literature. This explains why different populations have be described as lacking a "vitelline membrane". In addition, it also explains why some populations are described as having a filamentous appearance.

Similarly, the model proposed in this study will help to resolve many of the issues described in the literature regarding the composition of *Huffmanela* eggs. Knowledge of the true structure of these eggs will likely inform descriptions of new populations, as well as help identify technical artifacts previously described in the literature.

According to Žďárská et al. (2001), in their TEM study of the eggshell of *H. huffmani*, the eggshell consists of,

"*…three main layers: an outer vitelline layer, a middle chitinous layer, and an inner lipid layer. The vitelline layer, forming the superficial projections of the egg shell, comprises two parts: an outer electron-dense, and an inner electron-lucid part. The chitinous layer is differentiated into three parts: an outer homogenous electron-dense part, a lamellated part, and an inner electron-dense net-like part. The lipid layer comprises an outer net-like electron-lucid part, and an inner homogenous electron-lucid part. The polar plugs are formed by electron-lucid material with fine electron-dense fibrils.*"

This is apparently the first time that, in a peer-reviewed paper, the term "vitelline layer" was applied to the delicate outer two layers of a *Huffmanela* egg. Earlier egg studies of other trichinelloid species that did not have this delicate outer layer had used the term "vitelline layer" to refer to the thin, uniform, electron-dense layer tightly appressed to the outer surface of the much thicker chitinous layer (Appleton and White 1989; Wharton 1980). However, this layer was overlooked by Žďárská et al. (2001), perhaps due to the fact that the chitinous layer of the older and dark *H. huffmani* eggs they studied were almost as electron dense as the true vitelline layer. Thus, the compound external layer of the *H. huffmani* eggs that Žďárská et al. (2001) referred to as the vitelline layer is likely not homologous to the layer that forms over the ovum at the time of fertilization, which is what Wharton (1980), Appleton and White (1989), Bleve-zacheo et al. (1993), Olson et al. (2012), Stein and Golden (2018), and others likely referred to as the vitelline layer.

Upon careful examination of the micrographs in Žďárská et al. (2001) it was observed that there was a dark membrane closely adhered to the chitin, similar to the membrane found in this study. This also appears to agree with the description of the vitelline membrane in several other related nematodes (Appleton and White 1989; Bird and McClure 1976; Wharton and Jenkins 1978).

If the membrane Žďárská et al. (2001) deemed the vitelline layer is incorrect, then what is this new layer? In addition to a vitelline layer, some genera also possess a coating or a layer external to the vitelline layer. Bleve-zacheo et al. (1993), in a study of the eggshell of the phytoparasitic nematode *Xiphinema diversicaudatum*, stated: "*The vitelline layer, derived from the vitelline membrane* [formed at fertilization], *retains a single membrane-like structure but becomes thickened by adhering particulate material to its outer surface…. This coating material in X. diversicaudatum clearly originates from secretory products of the oviduct cells*." Wharton (1980) stated: "*The outer layers of the egg-shells of some species of nematodes consist of material secreted by the cells of the uterus.*" The layer these authors are referring to, which is external to the true vitelline layer and is secreted by the lining of the uterus, is often termed the "uterine layer." Indeed, in the schematic drawings provided by Wharton (1980) of the various types of uterine layers known at that time, are drawn with a thin electron-dense outer layer and a thick, electron-lucid inner layer similar to the outer layer of *H. huffmani*. Thus, it appears that the "delicate outer membrane" documented in eggs of many *Huffmanela* species, may in fact be a uterine membrane. Indeed, consider the "spines" described for the vitelline layer of *Huffmanela* and dubbed the Superficial Projections by Žďárská et al. (2001). (Appleton and White 1989) says this of the Uterine Layer: "*trichurid egg-shells*

lack a 4th layer, the uterine layer, which forms the proturberances and spines that decorate eggs such as those of Ascaris *spp.*"

What has not yet been made clear prior to this study is that, while the outer sub-layer (EDUL) of the uterine layer *H. huffmani* is a thin membrane with some tensile strength, the inner sublayer (ELUL) is apparently more of a fluid- or gel-filled space in the natural state than it is a "layer," and may be vulnerable to osmotically induced alterations. Upon fixation, however, this layer congeals or coagulates and adheres to the EDUL much more strongly than to the True Vitelline Layer, so that the entire Uterine Layer may separate from the eggshell by mechanical or chemical processes. Both layers also easily separate from the vitelline layer and the resulting space fills with fluid sometimes resulting in a fibrous appearance from the remnants of the ELUL. Unfortunately, misunderstandings of the phenomena generating such artifactual features now serve as misleading diagnostic characters in the formal descriptions of some *Huffmanela* species.

Synthesis of the rigid chitinous shell starts immediately after fertilization (Stein and Golden 2018). The first layer is laid down against the inside of the vitelline layer, and additional layers are deposited inwardly as the egg matures (Olson et al. 2012). Counting from inside out in Figure 28 there are 25+ dark layers, with the oldest layers on the outside of the bend and the youngest layers nearest the inside of the bend. The thickness of the deposition decreases with increasing maturity as chitin deposition is waning toward completion, and is eventually completed as the Inner Spongy Chitinous Layer. When the chitin synthesis stops, the Chondroitin Proteoglycan Layer is deposited against the inside

of the chitin layer and apparently also into the pores of the ISCL (Olson et al. 2012; Žďárská et al. 2001).

Žďárská et al. (2001) referred to the innermost layer of chitin (ISCL) as the net-like chitin, and claimed that the "lipid layer" is applied against it and partly intermingles with it in the pores of the spongy spaces. However, Olson et al. (2012) has determined that the "lipid layer", as it had been called for decades preceding 2012, does not contain lipids, but proteoglycans. Thus the first layer inside the innermost layer of chitin is now the CPG Layer (CPGL), which is what Žďárská et al. (2001) observed mingling with the net-like chitin. Figure 27 shows the Inner Spongy Chitin Layer (ISCL) as an interrupted (because of microtomy problems) dark line just inside the chitin, similar to that found by Wharton (1980).

While no references have been found in the literature comparing the proposed new models to other species of nematodes besides *Caenorhabditis elegans*, numerous studies show layers similar to those described in Stein and Golden (2018). Possibly the best example of these layers being present, but unacknowledged in a study is Appleton and White (1989). While they categorize everything below the chitin as the lipid layer, their micrographs showed several easily distinguishable features such as an electron dense layer below the chitin, thought to be the boundary between CPGL and the CL as seen in this study and Žďárská et al. (2001). Below the boundary, a large space is observable, which could equate to the EEMS as noted by this study. An electron dense boundary can be seen dividing what is thought to be the EEMS and another space surrounding the embryo. If previous assumptions are correct, this likely means that this boundary is the PBL and that the space surround the embryo is the PES.

The polar plug of *H. huffmani* appears similar to other nematode eggs that contain polar plugs, as similar structures were observed originating from similar locations. However, unlike most other eggs was the addition of the UL surrounding the plug. It was noted, however, that even if the UL was removed, the polar plug retained its shape. This may be due to the true vitelline membrane still surrounding the plug, or it could be due to the nature of the EDPP, as it is thought to be formed from ridged chitinous fibers (Appleton and White 1989; Žďárská et al. 2001).

CONCLUSIONS

Results of population comparisons done in this thesis, including egg shell morphometrics, visualization of the uterine membrane, and worm morphometrics show similarities between all populations. In addition, similarities in life cycles makes it difficult to delimit these three populations as individual species.

Realization that the outer membrane of *H. huffmani* is not the vitelline membrane as noted by Žďárská et al. (2001), and is likely a uterine membrane, will cause the need to redescribe many of the species within *Huffmanela*. This does, however, solve many outstanding issues regarding this membrane in the literature.

Finally, this is the first attempt that has been seen to attempt to describe eggs other than *C. elegans* using the Olson et al. (2012) model. Again, this helps resolve issues regarding erroneously described egg structures in *Huffmanela* literature. In addition, this model will likely become more commonplace for describing and redescribing numerous species of nematodes.

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