STUDIES ON THE LIFE CYCLE OF *HUFFMANELA HUFFMANI* (NEMATODA: TRICHOSOMOIDIDAE)

THESIS

Presented to the Graduate Council of
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Master of SCIENCE

by

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"If all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable, and if, as disembodied spirits, we could then investigate it, we should find its mountains, hills, vales, rivers, lakes, and oceans represented by a thin film of nematodes. The location of towns would be decipherable, since for every massing of human beings there would be a corresponding massing of certain nematodes. Trees would still stand in ghostly rows representing our streets and highways. The location of the various plants and animals would still be decipherable, and, had we sufficient knowledge, in many cases even their species could be determined by an examination of their erstwhile nematode parasites".

- N.A. Cobb

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ABSTRACT

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The histozoic nematode *Huffmanela huffmani* Moravec, 1987 (Trichosomoididae) parasitizes the gas bladders of several species of centrarchid fishes in the upper spring run of the San Marcos River, TX. Despite a previously reported prevalence of up to 90%, year-round presence of freshly laid eggs in the gas bladders, and years of research, knowledge of the parasite's life cycle is limited. The objective of this study was to gather information relevant to the life cycle of *H. huffmani*. This objective was met through prevalence studies, definitive host diet analysis, *in vitro* culture experiments, and infection challenges. The overall prevalence of *H. huffmani* was 59%. A trend of decreasing *H. huffmani* prevalence downstream from the headsprings of the San Marcos River was observed. No single diet item appeared to be more prevalent in the diet of infected definitive hosts than in that of uninfected definitive hosts. Two new techniques

successful in the extraction and induced hatching of *H. huffmani* eggs are described. No *H. huffmani* larvae were successfully cultivated. Three models depicting potential life cycles are described for *H. huffmani*. Several candidate intermediate hosts for *H. huffmani* are identified; however, use of these hosts in infection challenges did not produce infections in definitive hosts. These results suggest the life cycle of *H. huffmani* is most likely indirect, and the required intermediate host is most likely of an unknown taxon directly associated with the headsprings of the San Marcos River.

I. INTRODUCTION

The Phylum Nematoda includes an estimated 80,000 species, making it the most taxonomically rich of the animal phyla second to Phylum Arthropoda. Approximately half of the phylum is parasitic. Among the parasitic forms, approximately half infects plants and the other half infects animals. (Hugot, 2001).

Life cycles of parasitic nematodes differ in pattern and complexity among species. One species might infect only a single host, whereas another might infect a series of intermediate hosts before sexually reproducing in a final, or definitive host. Nematodes proceed through four larval stages, L1-L4, with ecdysis occurring between each successive stage. The elapsed time between hatching and infectivity is the prepatent phase (Roberts and Janovy, 2004). Intermediate and definitive hosts might be aquatic or terrestrial vertebrates or invertebrates. All members of the nematode family Trichosomoididae are histozoic parasites (Roberts and Janovy, 2004). Host specificity within this group of parasites is common, resulting in characteristic species-specific relationships with hosts.

Included in this family is the subfamily Huffmanelinae, the only trichosomoidid subfamily that is exclusively parasitic in fish, and is represented by a single genus, *Huffmanela* (Moravec, 2001).

Fourteen species of *Huffmanela* are named: *H banningi, H. branchialis, H. carcharini, H. canadensis, H. filamentosa, H. huffmani, H. japonica, H. lata, H. mexicana, H. moraveci, H. ossicola, H. paronai, H. schouteni,* and *H. shikokuensis* (Moravec, 1987; Justine, 2004; MacCallum, 1925; Moravec et al., 2005; Moravec, 1987; Moravec et al., 1998; Justine, 2005; Moravec and Fajer-Avila, 2000; Carballo, 2007; Moravec and Garibaldi, 2000; Moravec and Campbell, 1991). An additional species remains unnamed (Conboy and Speare, 2002).

The first descriptions of most *Huffmanela* species are based on eggs found deposited in host tissues. Descriptions of adults are only reported for *H. huffmani, H. canadensis*, and *H. moraveci* (Huffman and Moravec, 1988; Moravec et al., 2005; Carballo, 2007). All reported definitive hosts of *Huffmanela* are either elasmobranch (*H. carcharhini* and *H. lata*) or teleost fishes.

The genus is cosmopolitan in its marine distribution, with representatives reported from various locations in both the Atlantic and Pacific Oceans. The only freshwater species, *H. huffmani*, is limited to the Upper San Marcos River in Texas (Huffman and Moravec, 1987; Conboy and Speare, 2005). At present, life cycles have not been reported for any species of *Huffmanela* (Cox et al., 2004). Table 1 serves as a guide to the comparison and contrast of all *Huffmanela* species.

Table 1. Subfamily Huffmanelinae. Modified from Justine (2004)

Species	Marine/ Fresh	Locality	Host	Tissue	Adults	Citation
H. banningi	M	Atlantic: Senegal & Congo	*Cynoglossus browni* (tongue sole)	Muscle	No	Moravec, 1987
H branchialis	M	Pacific: New Caledonia	*Nemipterus fucosus* (threadfin bream)	Mucosa of gills	No	Justine, 2004
H. canadensis	M	Pacific: British Columbia	*Sebastes* spp. (rockfish)	Epidermis of fins	Yes	Moravec et al., 2005
H. carcharhini	M	Atlantic: North America	*Carcharhinus melanopterus* & *C. plumbeus* (Carcharhinidae)	Ligaments of gill arches	No	MacCallum, 1925
H filamentosa	M	Pacific. New Caledonia	*Gymnocranius grandoculis* (large eye bream)	Mucosa of gills	No	Justine, 2004
H huffmani	F	Texas Spring Lake, San Marcos River	Centrarchid fishes (Centrarchidae)	Epithelial layer of gas bladder	Yes	Moravec, 1987
H. japonica	M	Inland Sea of Japan. Shikoku Island	*Upensis bensasi* (goatfish)	Masses in musculature	Segments	Moravec et al., 1998
H. lata	M	Pacific: New Caledonia	*Carcharhinus amblyrhynchos* (grey reef shark)	Internal epidermis of gill slit	No	Justine. 2005
H. mexicana	M	Pacific: Mazatlan, Mexico	*Sphoeroides annulatus* (bullseye puffer)	Epithelial layer of gas bladder	No	Moravec and Fajer-Avila, 2000
H. moravec	M	Argentina: Patagonian gulfs	*Odontesthes smitti* & *O. nigricans(Atherinidae)*	Epidermis of fins and internal operculum, gill mucosa	Yes	Carballo and Navone, 2007
H. ossicola	M	Pacific. New Caledonia	*Bodianus loxozonus* (blackfin hogfish)	Bone	No	Justine, 2005
H. paronai	M	Mediterranean: Ligurian Sea	*Xiphias gladius* (swordfish)	Epidermis	No	Moravec and Garibaldi, 2000
H. schoutedi	M	Caribbean: Curacao, Mediterranean. Ligurian Sea	*Cheilopogon cyanopterus, C. heterurus, Hirundichthys affinis (Exocoetidae)* Human (spurious)	Inner layer of intestine and abdominal cavity, epithelial layer of gas bladder	No	Moravec and Campbell, 1991; Moravec and Garibaldi, 2003
H. sikokuensis	M	Inland Sea of Japan: Shikoku Island	*Stephanolepis cirrhifer* (thread sail filefish)	Evenly throughout musculature	No	Moravec et al., 1998
Huffmanela spp.	M	Pacific: British Columbia	*Sebastes* spp (rockfish)	Epidermis of fins	No	Conboy & Speare 2002

The focus of the present study is the freshwater species *H. huffmani*, which parasitizes fishes in the Family Centrarchidae.

Infections have been reported from *Ambloplites rupestis, Lepomis auritus*, *L. cyanellus*, *L. macrochirus*, *L. megalotis, L. microlophus, L. punctatus*, and *Micropterus salmoides* (Michel, 1985; Cox, 1998). Although these fishes are present throughout Central Texas, fishes with *H. huffmani* infections have only been reported from the upper spring run of the San Marcos River (Cox et al., 2004). This includes Spring Lake, the impounded headwaters of the river, which is spring-fed from the Edwards Aquifer (Young et al., 1973). *Huffmanela huffmani* is reported from the lake as well as downstream from the dam, with prevalence decreasing downstream (Cox, 1998). Prevalence of infection has been reported as high as 90%, with intensities estimated at up to 10^6 eggs per host (Michel, 1985). No infections have been reported from fish collected 6 km or farther downstream from the dam (Cox, 1998). Fish have been observed during all seasons with fresh *H. huffmani* eggs (Cox, 1998) embedded in the epithelial layer of the gas bladder (Žd'árská, 2001; Figure 1).

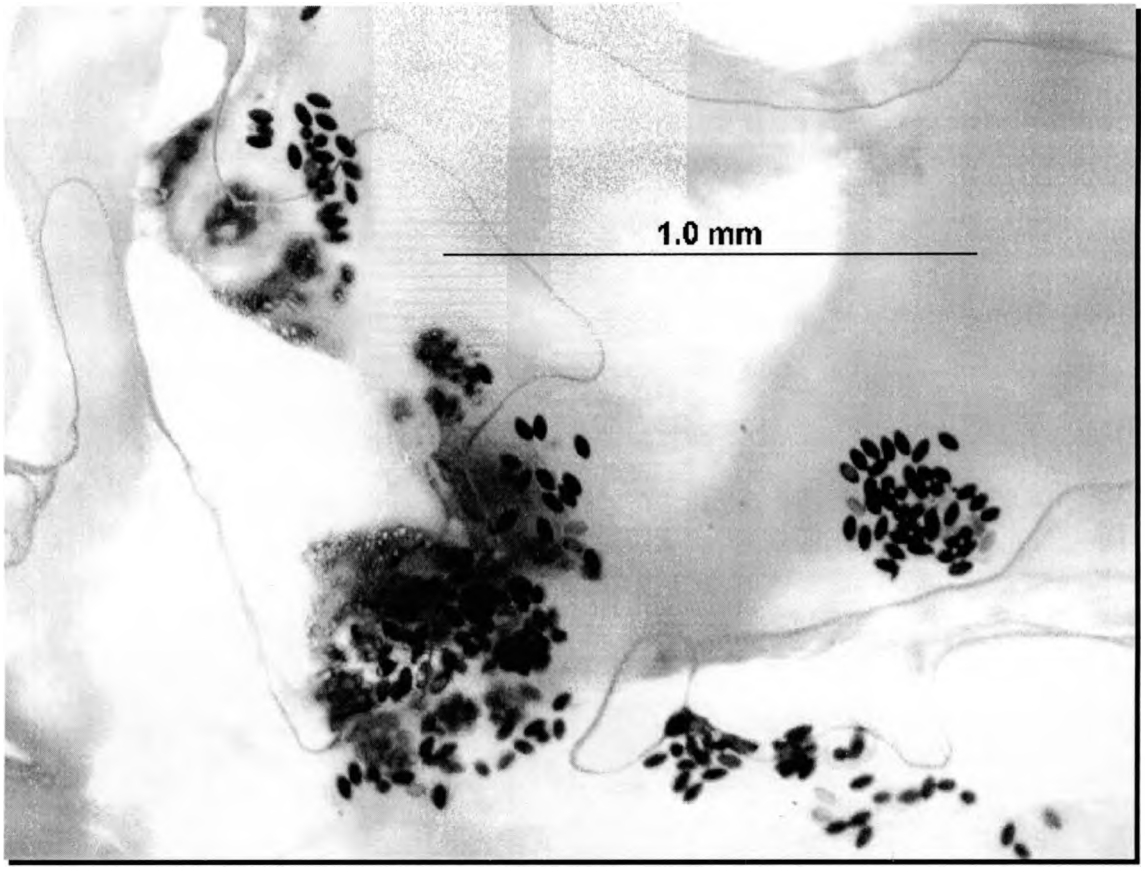

Figure 1. *H. huffmani* eggs embedded in the epithelial layer of a centrarchid gas bladder

Other trichiuroid nematodes, such as species in the genus *Capillaria*, utilize one or more invertebrate intermediate hosts (Moravec, 1999). The geographic distribution of *H. huffmani* is extremely limited; therefore, it is possible that the waters emerging directly from the aquifer might support a known or perhaps undescribed endemic invertebrate uniquely capable of serving as intermediate host for the parasite.

Attempts to directly infect fish with *Huffmanela huffmani* eggs obtained from the gas bladders of infected *Micropterus salmoides* have been unsuccessful (Cox, 1998). This suggests the parasite does not have a direct life cycle and must develop in an

intermediate host prior to infecting centrarchids. Michel (1985) and Cox (2004) collected unspecified invertebrates species from the upper San Marcos River thought to be capable of serving as intermediate hosts. These invertebrates were fed to uninfected sunfish, which were subsequently examined for evidence of infection. Mollusks and the endemic cave shrimp *Palaemonetes antrorum* were exposed to *H. huffmani* eggs and subsequently fed to uninfected fishes. None of these attempts to infect fish were successful, nor did they provide evidence that these specific invertebrates were capable of serving as intermediate hosts for *H. huffmani*.

The aim of the current study is to provide information regarding the life cycle of *H. huffmani*. The failure of previous studies to describe the life cycle of the parasite might have been due to the choice of candidate invertebrate hosts used in feeding experiments. Invertebrates were chosen solely on the basis of their presence in the type locality, with no regard to their ecological association with the centrarchid hosts. The current study aims to identify candidate invertebrate hosts according to their presence in the diet of infected centrarchid hosts. To further investigate the life cycle of *H. huffmani*, selected invertebrates, some of which were determined to be present in the diet of infected fishes, were used as potential intermediate hosts in infection challenges.

Prevalence and intensity data for *H. huffmani* infections were collected to determine if there have been changes since the original base-line data of Michel (1985) and Cox (1998). Data were also collected from various ecologically different sites within

Spring Lake and two additional sites in the river in order to study the distribution of *H. huffmani* within the reported habitat.

The objectives of this study are to:

- Determine current prevalence and intensity of *H. huffmani* infection in centrarchids from the type locality.
- Compare the diet composition of infected fish with that of uninfected fish.
- Perform feeding experiments using candidate invertebrates wild-caught from the type locality.

II. MATERIALS AND METHODS

Prevalence Study

Fishes of the genera *Lepomis* and *Micropterus* were collected from Spring Lake and the San Marcos River immediately downstream from the dam. Samples were collected under scientific research permit number SPR-0106-011 and IACUC permit 06-0624F58C88 issued to Dr. David Huffman of Texas State University-San Marcos. Collections were accomplished using angling techniques between the dates of February 2, 2005 and December 12, 2006. Locality of capture and total length were recorded for each fish. Fish were identified to species using keys from Hubbs et al. (1991).

Within Spring Lake, three ecologically different sites were identified (Figure 2). SL Site 1 included the waters directly emerging from springs at the headwaters of the lake. SL Site 2 included the more stagnant waters in the slough area of Spring Lake. SL Site 3 included the main waters of Spring Lake downstream from the slough, but still dominated by fresh spring water, and some distance from the main source springs.

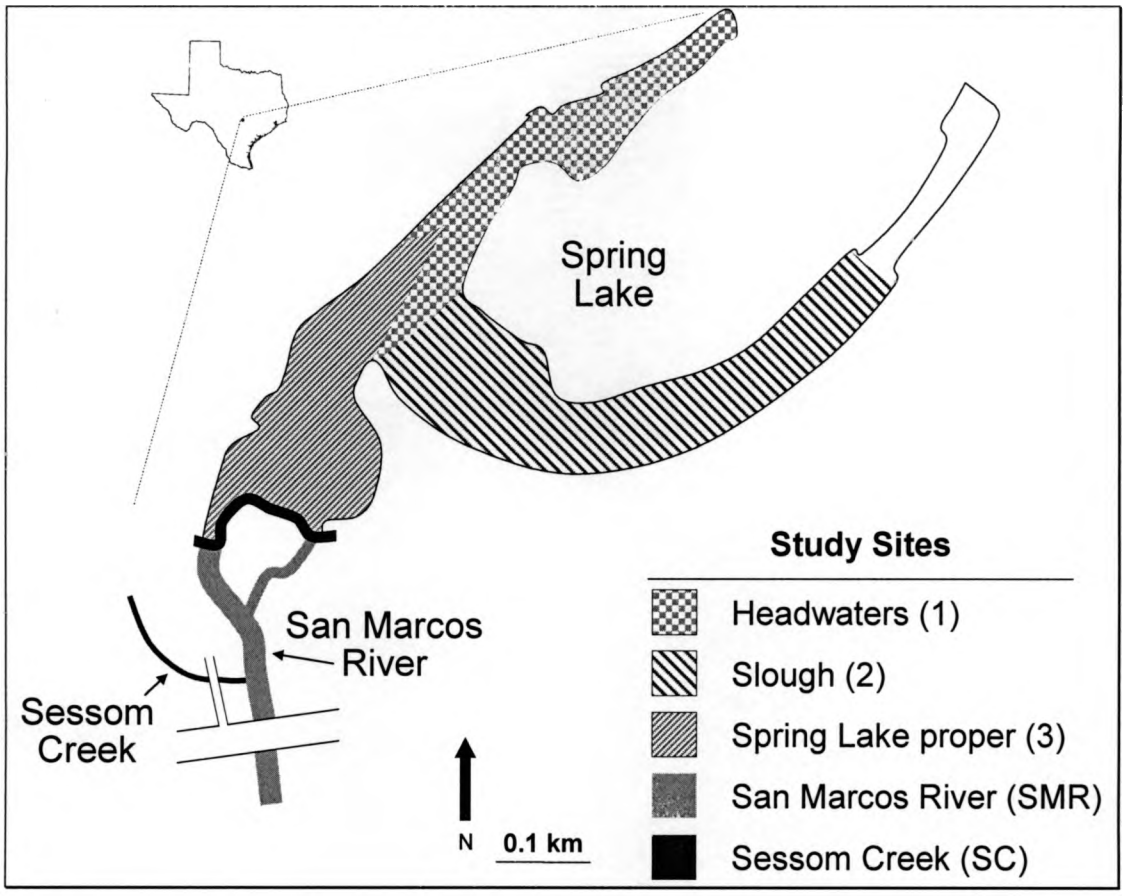

Figure 2. Map of study sites

Two ecologically different sites were established downstream from the dam. One of these (SMR) included the waters of the main stem of the San Marcos River immediately downstream from the main dam. The other (SC) included the waters of Sessom Creek just upstream from the confluence of the San Marcos River, but downstream from the Sessom Drive culvert.

All fish were examined for *H. huffmani* infections using standard necropsy techniques (Dailey, 2005; Huffman, 2005). The entire gas bladder was removed, examined for *H. huffmani* eggs, and preserved in 10% formalin.

Total prevalence of *H. huffmani* infection in all species was determined by expressing the number of fish infected (at any level) as a percentage of the total number of fish examined. Prevalence was expressed for samples collected across and within habitat sites.

The relative intensities of *H. huffmani* infections were subjectively assigned to one of the three categories using the procedure described by Cox (1998). According to this procedure, heavy infections represent greater than approximately 10^6 eggs, medium infections represent between approximately 10^3 and 10^6 eggs, and light infections represent between approximately 1 and 10^3 eggs present in the gas bladder.

The age composition of infections were subjectively described according to a modified Cox (1998) procedure. This procedure can estimate the proportion of total eggs that are freshly laid by judging the color intensity of eggs. The youngest or most recently laid eggs are clear to yellow in appearance under microscopic observation. Eggs darken progressively from clear to amber to black as they age; thus the oldest eggs are dark brown to black. In the current study, eggs that were clear to yellow in color were classified as "new eggs," and eggs that were amber to dark were classified as "old eggs."

Diet Analysis Study

Collected fish were euthanized with MS-222 and placed on ice to retard digestion. Stomach contents were removed on the day of capture and preserved in 70% ethanol. Identifiable contents were later sorted and identified to the lowest practical taxon using

keys in Thorp & Covich (2001). Fish were coded during the diet analysis to avoid potential operator bias.

After the identifiable items in the stomach contents of a fish were sorted by taxa, a subjective estimate of the number of individuals most likely represented by these items was completed for each fish. For example, if a head capsule was identified without an accompanying body, the individual taxon was counted once. However, if three head capsules were observed with enough other parts to represent five individuals, the count was recorded as five individuals, rather than eight. The number of individuals representing a taxon was then expressed as a percentage of the total number of all individuals of all taxa in that stomach. These percentages were then expressed as "mean percent" for subsequent comparisons.

Life Cycle Studies

In vivo Extraction and Induced Hatching of H. huffmani Eggs

Fishes of the genera *Lepomis* and *Micropterus* were collected from various sites within Spring Lake. Gas bladders found to be infected with eggs of *H. huffmani* were placed in the body cavity of mosquitofish (*Gambusia affinis*). The mosquitofish were then fed to *Micropterus salmoides* in a 40 gallon aquarium which was cleared of pre-existing fecal debris. Fecal masses from the bass were collected the day after the feeding and examined for *H. huffmani* eggs. Egg-laden fecal masses were maintained in 0.5 L containers of aerated artesian well water at approximately 25 °C. Water was changed at

the rate of 25% every other day. Eggs were examined frequently for evidence of hatching.

In vitro Extraction and Induced Hatching of H. huffmani Eggs

Fishes of the genera *Lepomis* and *Micropterus* were collected from Spring Lake. Gas bladders found to be infected with eggs of *H. huffmani* were placed in a 7% saline solution. Bladders were then placed in an 80/20 v/v solution of artificial digestive fluid with trypsin (Sigma) and 7% Pepsin (Sigma). The mixture was mechanically homogenized with a Seward Stomacher 80 Biomaster Lab System at normal speed for 12 minutes. The resulting slurry was sealed in a 1 L Nalgene container and incubated for 24 hours at approximately 25 °C. To extract *H. huffmani* eggs from remaining undigested host tissue, the sample was lightly centrifuged (Eppendorf 5810 R) at 4000 rcf for 5 minutes. The centrifugation suspended the extracted eggs in the supernatant. Eggs were removed from the supernatant with a plastic 1 ml pipette and maintained in a bath of 21-23 °C artesian well water with aeration for 24 hours. Eggs were then examined every 12 h for evidence of hatching.

In vitro Cultivation of H. huffmani Larvae from Eggs

Eggs were collected using the *in vitro* extraction method. Cultivation of larvae from the eggs was attempted using a modification of the methods described by Adroher et al. (2004). The only modifications to the protocol were incubation temperature (20° C rather than 13° C) and atmosphere (ambient CO_2 rather than 5% CO_2). The original culture procedure was developed for histozoic fish nematodes requiring an intermediate

host, and so the technique should also be applicable to *H. huffmani*. This technique historically supported larval nematodes through four larval stages, including ecdysis, maturation to adulthood, and sexual reproduction resulting in a second generation of nematodes *in vitro*. Extracted eggs were rinsed in a 0.9% saline solution and axenized using an antibiotic-antifungal solution of gentamicin sulfate, amphotericin B, sodium penicillin G, streptomycin sulfate, and Hanks' balanced salt solution. Eggs were then transferred to sterile 6-well polystyrene 30 ml flasks filled with a culture medium (GLIT) of Yaeger's liver infusion tryptose (YLIT) and modified Grace's insect medium. Approximately 50 eggs were placed in each well. The eggs were incubated at 20°C and the culture media was refreshed weekly. Eggs were examined daily for signs of hatching, and hatched larvae were observed for survival and molting.

Proposed Direct Life Cycle Model A

In Direct Model A (Figure 3), an infected definitive host would be ingested by an uninfected predator. This predator would become infected with larval nematodes. The larvae would mature to adults and reproduce in the gas bladder of the predator. Adults would then lay eggs while in the infected predator and the eggs would escape from the predator into the environment by an unknown means, such as death and decomposition. The life cycle would then be completed when another definitive host somehow ingests the released eggs.

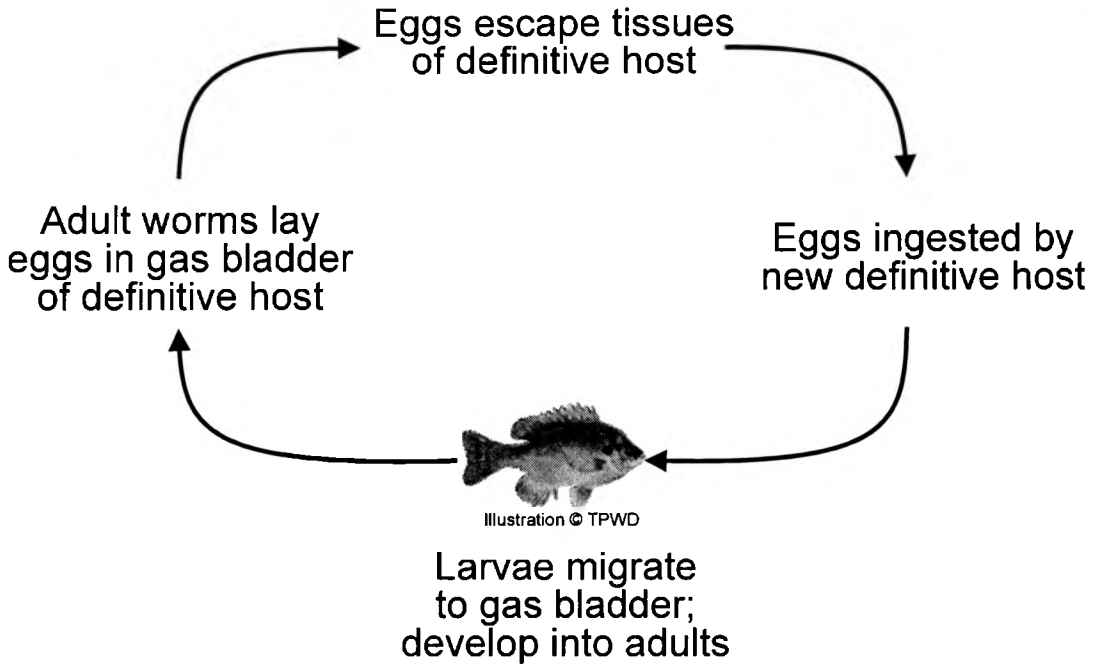

Figure 3. Proposed Direct Life Cycle Model A

Proposed Direct Life Cycle Model B

In Direct Model B (Figure 4), an uninfected predator would ingest an infected definitive host. However, the predator would not become infected, but instead would digest away the tissue of the ingested definitive host and pass the eggs with the feces. The life cycle would then be completed when these eggs are ingested by another definitive host.

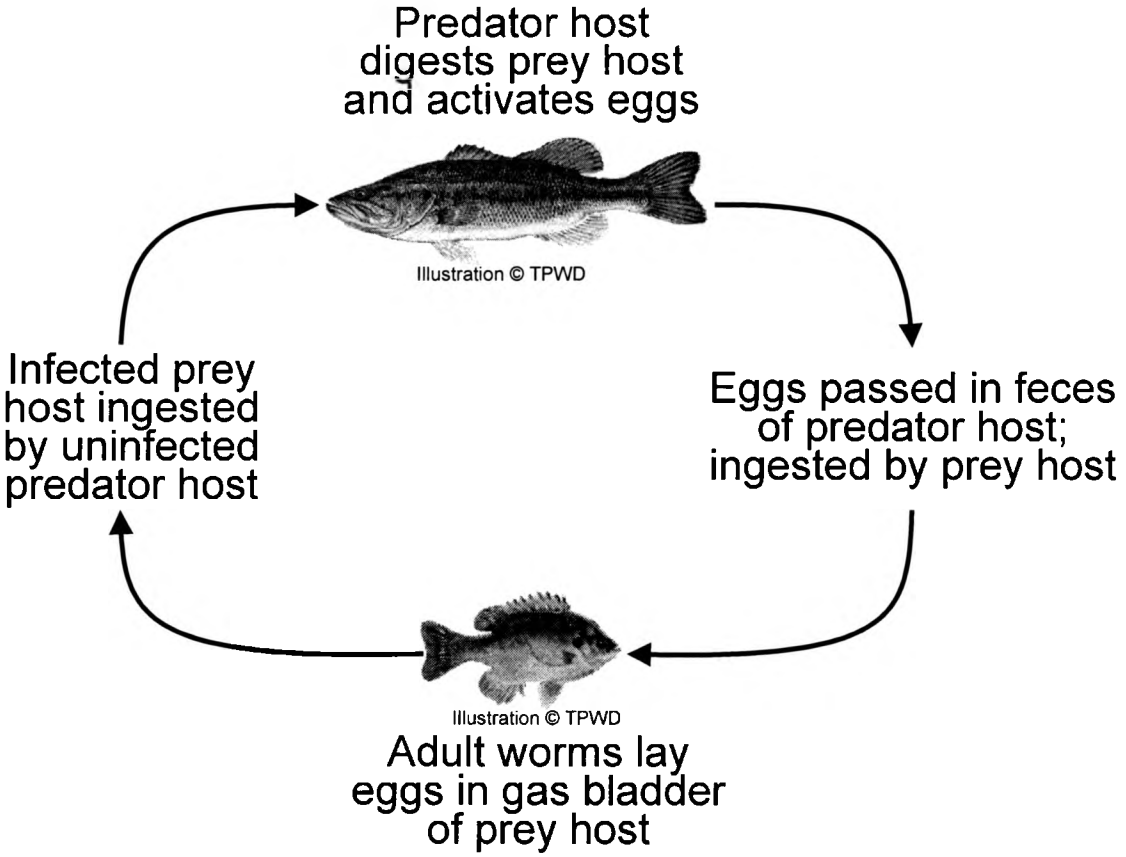

Figure 4. Proposed Direct Life Cycle Model B

Proposed Indirect Life Cycle Model

Three routes of infection are proposed for this model (Figure 5). In the first route, a definitive host with *H. huffmani* eggs in the gas bladder would be ingested by a predator. The predator would not become infected, but instead would digest away the tissue of the ingested definitive host and pass the eggs with the feces. An intermediate host, possibly a detritivore, would then ingest the released eggs. Following ingestion, the eggs would hatch, and larvae would develop into a stage infective to the definitive host. A definitive host would then ingest the intermediate host containing infective larvae. The

larvae would then migrate to the gas bladder, mature to adults, and lay eggs to complete the cycle.

In the second route, an infected definitive host would die, and through decomposition, *H. huffmani* eggs would be released as detritus. An intermediate host, possibly a detritivore, would ingest the eggs. Following ingestion, the eggs would hatch, and larvae would develop into a stage infective to the definitive host. A definitive host would then ingest the intermediate host containing infective larvae. The larvae would then migrate to the gas bladder, mature to adults, and lay eggs to complete the cycle.

The third route is an extension of either Route 1 or route 2, with an obligatory second intermediate host. The larvae that emerge from eggs ingested by the intermediate host are not infective to the definitive host. To become infective, this intermediate, now the first intermediate host, must be ingested by a carnivorous second intermediate host. In this second intermediate host, the larvae would develop into a stage infective to the definitive host. A definitive host would then ingest the second intermediate host containing infective larvae. The larvae would then migrate to the gas bladder, mature to adults, and lay eggs to complete the cycle.

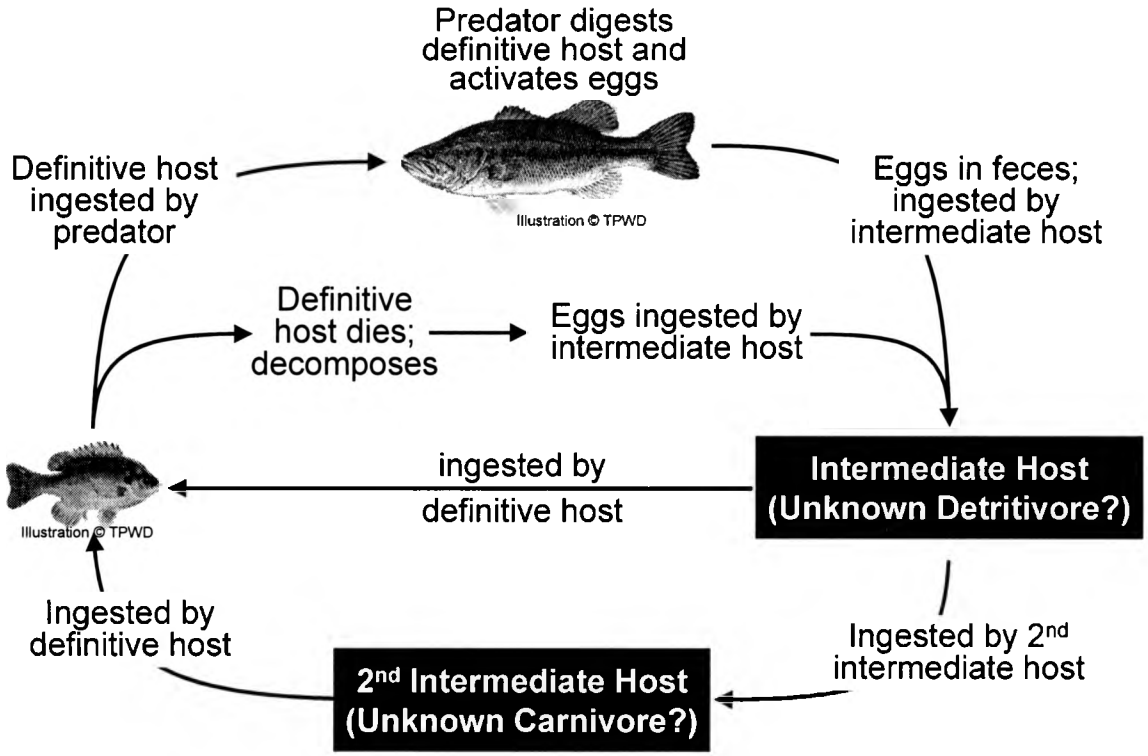

Figure 5. Proposed Indirect Life Cycle Model

Each model was investigated via a series of infection challenges, which attempted to determine the role and the identity of any intermediate host in the life cycle of *H. huffmani*. The goal of these infection challenges was to infect uninfected definitive hosts.

After the host diet had been described, invertebrate taxa determined to be common to the diet of infected hosts and hypothesized to be capable of serving as intermediate host for *H. huffmani* were selected for feeding experiments. While all aquatic invertebrates were considered, attention was focused on non-insect invertebrates due to the likelihood that the life cycle of *H. huffmani* is similar to that of other

Trichosomoidid nematodes. Attention was also focused on invertebrates that have marine counterparts, such as annelids, isopods, and decapods, since all other *Huffmanela* species are marine. Karst invertebrates from the Edwards Aquifer were collected from salamander traps previously placed on springs within Spring Lake and the artesian well. Other invertebrates were collected with D nets and kick nets. All invertebrates were maintained in 1.5 L rectangular trays filled with artesian well water and equipped with commercial aquarium gravel and aeration. Water changes (25%) were performed every other day. Invertebrates were offered the macrophyte *Hydrilla* collected from the San Marcos River and Wardley's commercial flaked fish food twice a week. Karst invertebrates were maintained in the same way in a light-free environment.

Uninfected fishes in the genus *Lepomis* were collected from the Blanco River with a bag seine. These fish were presumed to be uninfected with *H. huffmani* according to the results of a distribution study completed by Cox (1998). A total of 8 fish was maintained in each of five 40 gallon aquaria in the wet lab at the Freeman Aquatic Biology Building using 21-23 C flow-through artesian well water. Fish were fed a standard base diet of Mazuri Aquatic Gel Diet 5M70. This diet is routinely used in professional facilities such as zoos and public aquaria. This product was chosen based on the nutritional component, gel texture, and previous success with difficult-to-feed species.

Infection Challenge 1. (Proposed Direct Life Cycle A)

Uninfected fish in four of the five aquaria were fed infected gas bladders. Infected gas bladders were placed in Mazuri gel diet cubes using forceps. The cubes were then fed to uninfected fish. The fifth aquarium was established as a control, and the control fish were maintained on pure Mazuri diet only. The incubation time was established at 30 days, because the prepatent phase of some *Capillaria* nematodes, thought to be closely related to *Huffmanela*, has been reported to be between 21 and 28 days (Roberts and Janovy, 2004). Following a 31-day incubation period, all fish in all five aquaria were examined for signs of *H. huffmani* infection. This challenge was replicated three times sequentially.

Infection Challenge 2. (Proposed Direct Life Cycle B)

Eggs were extracted and collected as described in the *in vivo* culture method. Eggs were fed to uninfected fish in four of the five aquaria using a red-rubber veterinary catheter inserted through the mouth and into the stomach. A syringe was used to collect eggs and push them through the catheter into the stomach. The fifth aquarium was established as a control, and the control fish were maintained on pure Mazuri diet only. Following a 31-day incubation period, all fish in all five aquaria were examined for signs of *H. huffmani* infection. This challenge was replicated three times sequentially.

Infection Challenge 3. (Proposed Indirect Life Cycle – Spring Lake Invertebrates)

Invertebrates were collected from Spring Lake with D nets and identified to lowest practical taxon. The invertebrates used in this infection challenge were as follows:

Arrenurus sp., *Placobdella* sp., *Chironomid* sp., *Stygobromus pecki, Hyalella azteca, Tubifex* sp. Each invertebrate species was fed to uninfected fish in separate aquaria. One aquarium was established as a control, and the control fish were maintained on pure Mazuri diet only. Following a 31-day incubation period, all fish were examined for signs of *H. huffmani* infection. This challenge was replicated three times sequentially.

Infection Challenge 4. (Proposed Indirect Life Cycle; in vivo activated eggs)

Invertebrates were exposed to *H. huffmani* eggs previously activated with the *in vivo* method. Invertebrates used in this infection challenge were as follows: *Arrenurus* sp., *Hyalella azteca, Cambarus* sp., *Palaemonetes antrorum, Chironomid* sp., *Placobdella* sp., *Cirolanides texanus, Stygobromus pecki, Dugesia tigrina, Tubifex* sp.

Fecal debris containing *H. huffmani* eggs was pipetted into the invertebrate enclosures each day for one week. During this initial exposure period, a sub-sample of invertebrates was examined daily for egg ingestion. Following the one-week exposure period, the invertebrates were incubated for two-weeks, and then fed to uninfected fish. Each invertebrate species was fed to uninfected fish in separate aquaria. One aquarium was established as a control, and the control fish were maintained on pure Mazuri diet only. Following a 31-day incubation period, all fish were examined for signs of *H. huffmani* infection. This challenge was replicated three times sequentially.

Infection Challenge 5. (Proposed Indirect Life Cycle; in vitro activated eggs)

Invertebrates were exposed to *H. huffmani* eggs previously activated with the *in vitro* method. Invertebrates used in this infection challenge were as follows: *Arrenurus*

sp., *Hyalella azteca, Cambarus* sp., *Palaemonetes antrorum, Chironomid* sp., *Placobdella* sp., *Cirolanides texanus, Stygobromus pecki, Dugesia tigrina, Tubifex* sp.

Two ml of egg slurry were placed daily into the invertebrate enclosures for one week. During this initial exposure period, a sub-sample of invertebrates was examined daily for egg ingestion. Following the one-week exposure period, the invertebrates were incubated for two-weeks, and then fed to uninfected fish. Each invertebrate species was fed to uninfected fish in separate aquaria. One aquarium was established as a control, and the control fish were maintained on pure Mazuri diet only. Following a 31-day incubation period, all fish were examined for signs of *H. huffmani* infection. This challenge was replicated three times sequentially.

III. RESULTS

Prevalence Study

A total of 147 fish representing seven species from the genera *Lepomis* and *Micropterus* were collected during the study: *L. auritus, L. gulosus, L. macrochirus, L. megalotis, L. microlophus, L. punctatus,* and *M. salmoides* (Table 2). Of these, a total of 110 fish were collected from upstream from Spring Lake dam, and 37 from downstream from the main dam.

Table 2. Number of fish collected per site

Spring Lake				Downstream from the Dam		
Study Site				Study Site		
SL 1	SL 2	SL 3	Total	SMR	SC	Total
40	32	38	110	27	10	37

Among 147 fish examined, 59% were infected with *H. huffmani*. Infections were found in 9% of *L. macrochirus*, 80% of *L. megalotis* and *M. salmoides*, 92% of *L. auritus*, 65% of *L. microlophus*, and 50% of *L. punctatus*. No *H. huffmani* infections were observed in the two *L. gulosus* examined (Figure 6A).

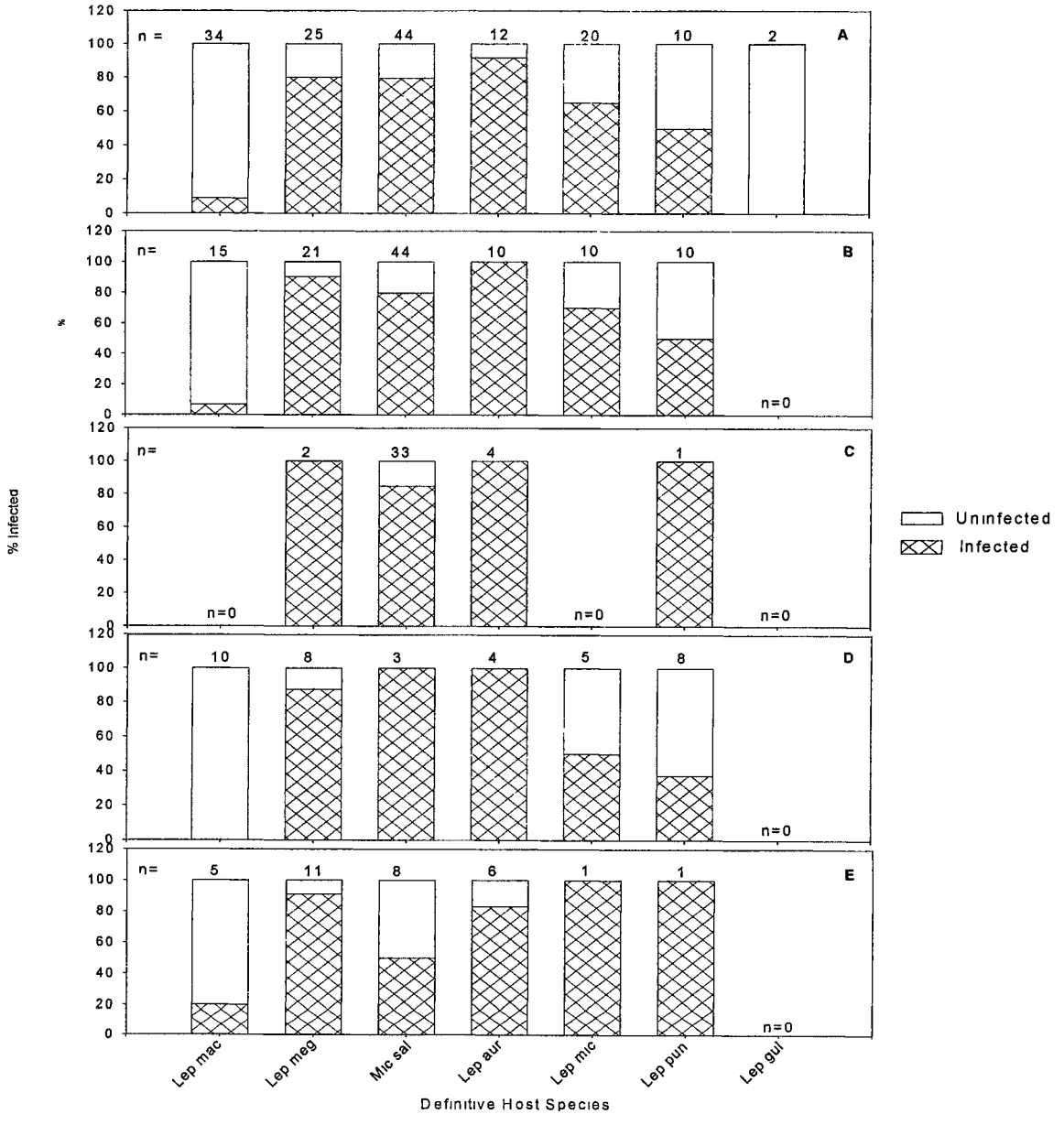

Figure 6. Overall prevalence of *H. huffmani* infections across all host species[1] and all habitats

[1] Lep mac = *L macrochirus*; Lep meg = *L megalotis*; Mic sal = *M salmoides*; Lep aur = *L auritus*; Lep mic = *L. microlophus*; Lep pun = *L punctatus*; Lep gul = *L gulosus*. A: All sites; B: All Spring Lake sites; C: Site SL1; D: Site SL2; E: Site SL3, F: All sites downstream of dam; G: Site SMR.

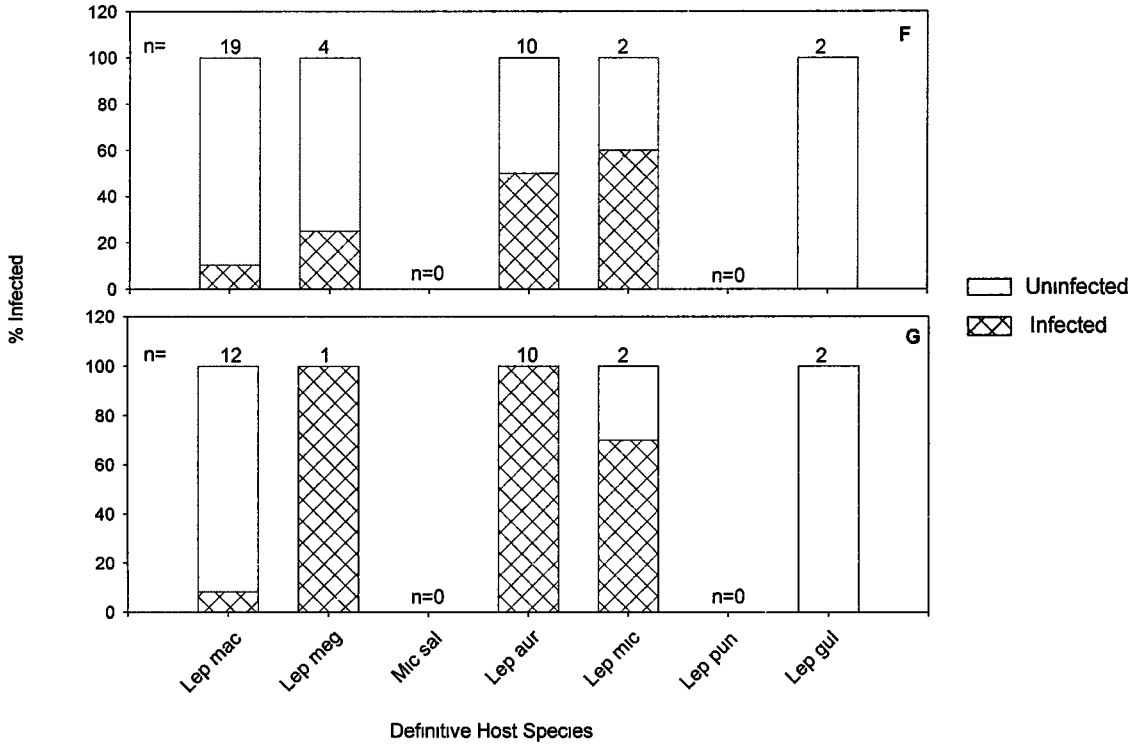

Figure 6 (cont'). Overall prevalence of *H. huffmani* infections across all host species[2] and all habitats

Among 110 fish collected from Spring Lake, 70% were infected with *H. huffmani*. Infections were found in 7% of *L. macrochirus*, 90.5% of *L. megalotis*, 79.5% of *M. salmoides*, 100% of *L. auritus*, 70% of *L. microlophus*, and 50% of *L. punctatus*. No *L. gulosus* were collected from Spring Lake (Figure 6B).

Among 40 fish collected from SL 1, 87.5% were infected with *H. huffmani*. Infections were found in 100% of *L. megalotis*, *L. auritus,* and *L. punctatus*, and 84.8%

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[2] Lep mac = *L. macrochirus*; Lep meg = *L. megalotis*; Mic sal = *M. salmoides*; Lep aur = *L. auritus*; Lep mic = *L microlophus*; Lep pun = *L. punctatus*; Lep gul = *L. gulosus*. A: All sites; B: All Spring Lake sites; C: Site SL1; D: Site SL2; E: Site SL3.

of *M. salmoides*. No *L. gulosus, L. macrochirus,* or *L. microlophus* were collected from SL 1 (Figure 6C).

Among 32 fish collected from SL 2, 68.7% were infected with *H. huffmani*. Infections were found in 20% of *L. macrochirus*, 90.9% of *L. megalotis*, 50% of *L. microlophus*, 83.3% of *L. auritus*, 37.5% of *L. punctatus*, and 100% of *L. auritus* and *M. salmoides*. No *L. gulosus* were collected from SL 2 (Figure 6D).

Among 38 fish collected from SL 3, 58.6% were infected with *H. huffmani*. Infections were found in 87.5% of *L. megalotis*, 50% of *L. microlophus*, 37.5% of *L. punctatus*, and 100% of *L. auritus* and *M. salmoides*. No *H. huffmani* infections were observed in any *L. macrochirus* examined from SL 3. No *L. gulosus* were collected from SL 3 (Figure 6E).

Among 37 fish collected from both sites downstream from the dam, 27% were infected with *H. huffmani*. Infections were found in 10.5% of *L. macrochirus*, 25% of *L. megalotis*, 50% of *L. auritus*, and 60% of *L. microlophus*. No *H. huffmani* infections were observed in any *L. gulosus* examined. No *M. salmoides* were collected from either site (Figure 6F).

Among 27 fish collected from SMR, 51.9% were infected with *H. huffmani*. Infections were found in 8.3% of *L. macrochirus*, 100% of *L. megalotis*, 100% of *L. auritus*, and 70% *L. microlophus*. No *H. huffmani* infections were observed in *L. gulosus*. No *M. salmoides* were collected from SMR (Figure 6G).

Among 10 fish collected from SC, no *H. huffmani* infections were observed. Fish species collected were *L. macrochirus* (n=7) and *L. megalotis* (n=3).

There appeared to be a downstream gradient of decreasing prevalence of *H. huffmani*. For example, the overall prevalence of *H. huffmani* infections was greatest at the headwaters of Spring Lake (88%), and least at the furthest downstream site (52%). When the sites are arranged in increasing distance downstream from the head springs and the host species at each site pooled, a trend of decreasing *H. huffmani* prevalence was observed (Figure 7, circles). However, larger fishes, most notably *M. salmoides*, were included in these initial prevalence calculations. Therefore, the apparent trend of decreasing prevalence downstream (Figure 7, circles) might be an artifact of host length-class effects, since the mix of length classes was not standardized across sites. The overall prevalence in *M. salmoides* was 80%; however, new infections in *M. salmoides* were primarily observed in smaller individuals. This suggests that the larger infected individuals were infected as juveniles and no longer represent active infections. Since approximately 75% of infected *Lepomis* were less than or equal to 20 cm, *M. salmoides* individuals over 20 cm were arbitrarily excluded in the next analysis. This subsequent analysis produced a very different trend (Figure 7, triangles). Instead of a trend showing decreasing *H. huffmani* prevalence with increasing distance from the springs, the highest prevalence was observed in the slough of Spring Lake, and no distinguishable downstream trend was observed.

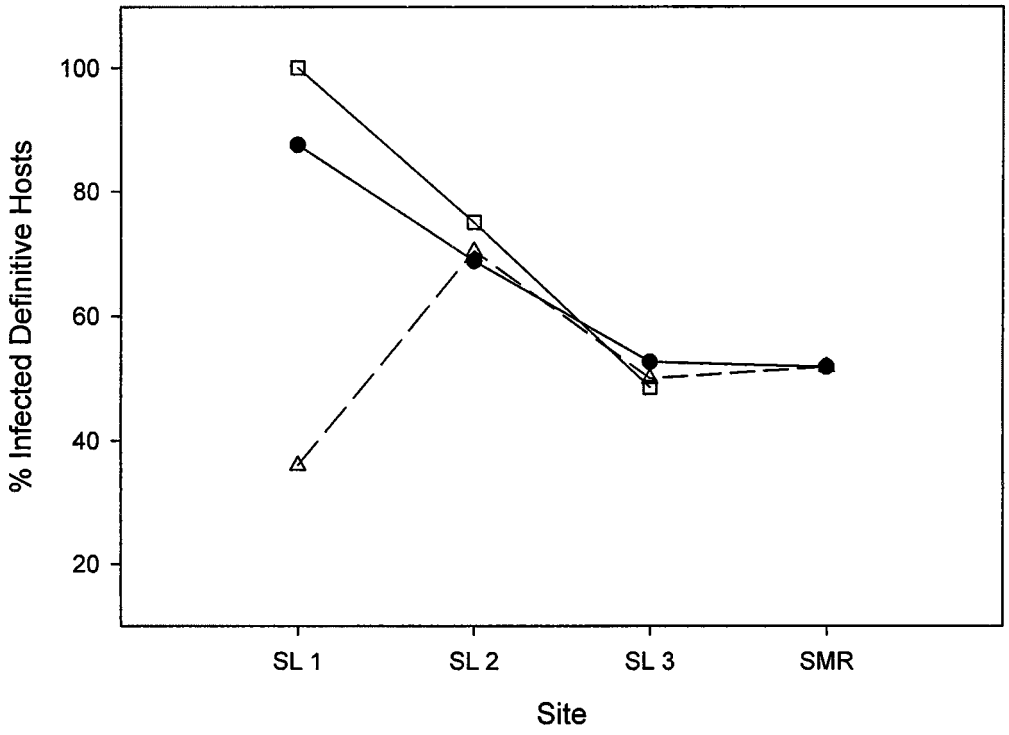

Figure 7. Distribution of *H. huffmani* prevalence across all positive sites in order of increasing distance from the source springs[3]

It is possible that any association between site and prevalence would not be accurately represented in the *M. salmoides* data because of higher mobility in *M. salmoides* as compared to *Lepomis* species. This species is more likely to move around freely in the lake than the *Lepomis* species, and the site at which a bass is captured is less likely to be the site at which the infection was acquired than it is for *Lepomis* species. In order to eliminate potentially spurious effects due to *M. salmoides*, the downstream prevalence was analyzed a third time using only *Lepomis* data. The results of this

[3] Circles: all samples; triangles: all *Lepomis* and *Micropterus* <20 cm; squares: *Lepomis* only.

analysis again show a trend consistent with a downstream reduction in prevalence (Figure 7, squares).

Among 87 definitive hosts positive for *H. huffmani*, 33.3% presented with heavy infections, 31% with medium infections, and 35.6% with light infections (Figure 8).

All of the infected *L. macrochirus* presented with light infections. Of the infected *L. megalotis*, 28% presented with heavy infections, 45% presented with medium infections, and 20% presented with light infections. Of the infected *M. salmoides*, 34.1% presented with heavy infections, 14.3% presented with medium infections, and 42.9% presented with light infections. Of the infected *L. auritus*, 15% presented with heavy infections, 46.2% presented with medium infections, and 30.8% presented with light infections. Of the infected *L. microlophus*, 25% presented with heavy infections, 45.5% presented with medium infections, and 27.3% presented with light infections. Of the infected *L. punctatus*, 10% presented with heavy infections, 50% presented with medium infections, and 40% presented with light infections.

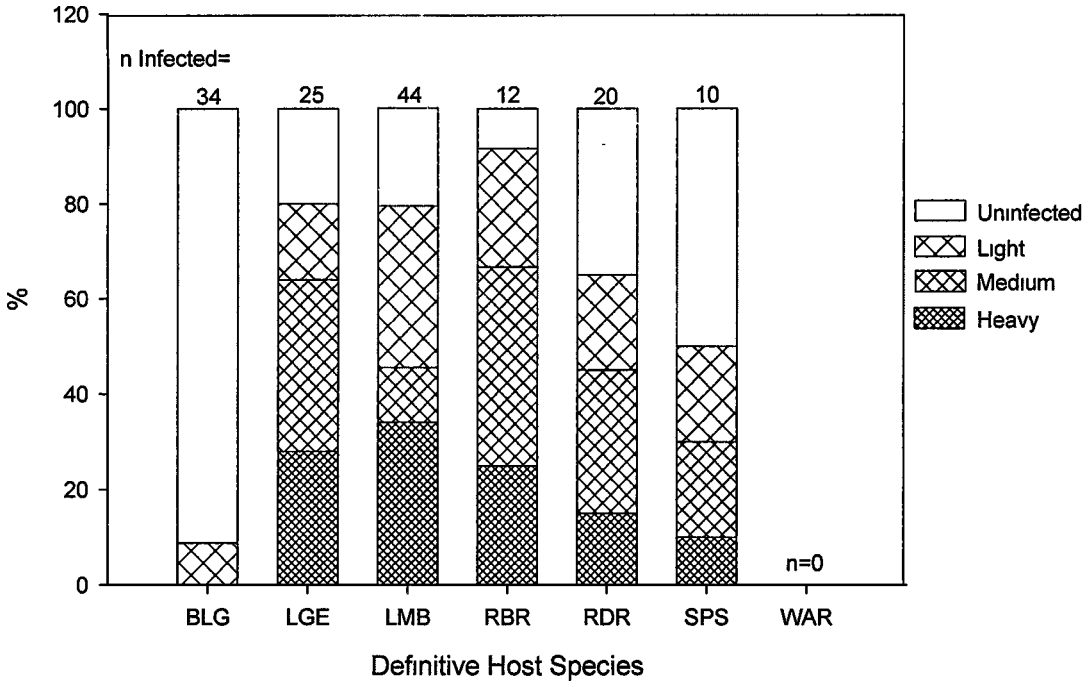

Figure 8. Relative intensity of *H. huffmani* infections across all sites by host species

Each *H. huffmani* infection was examined for eggs that appeared to be freshly laid using the criteria indicated earlier. This result was then expressed as "percent new eggs" for each infected fish. Figure 9 shows the percent new eggs in each fish species across all sites.

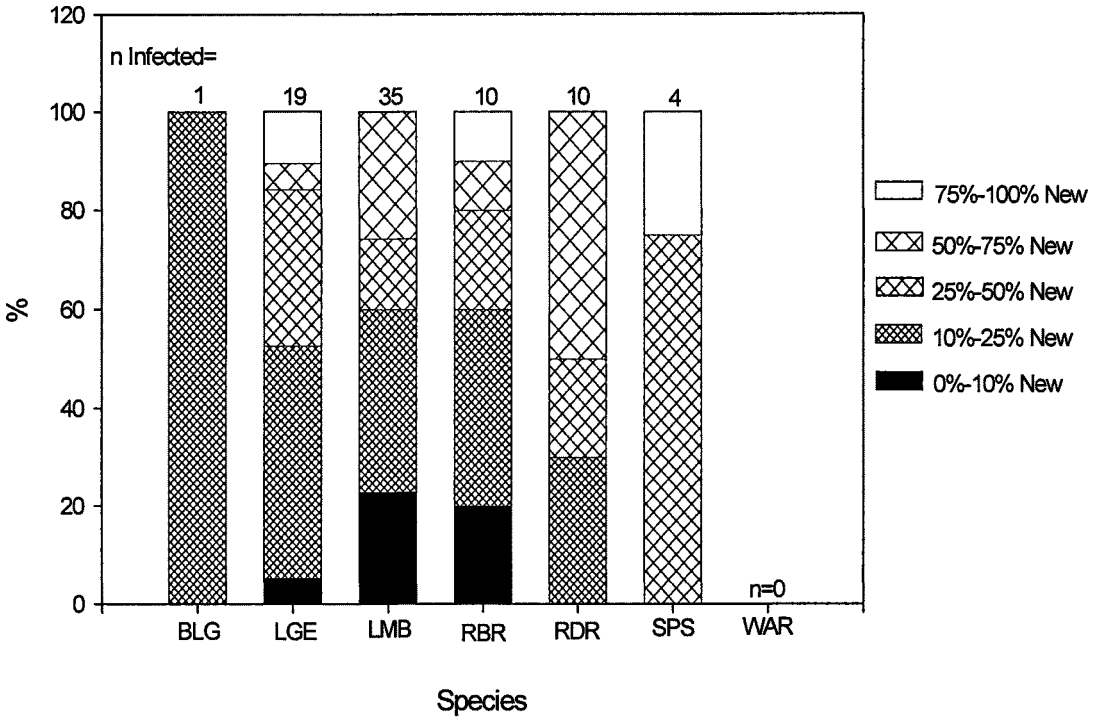

Figure 9. Percentage of new eggs found in infected definitive hosts across all sites

Infections with greater than or equal to 50% new eggs were classified as "new" infections. Infections with less than 50% new eggs were classified as "old" infections. Figure 10 shows the percentage of infected fish with either new or old infections. Mean percentages of new eggs were expressed for each site (Figure 11).

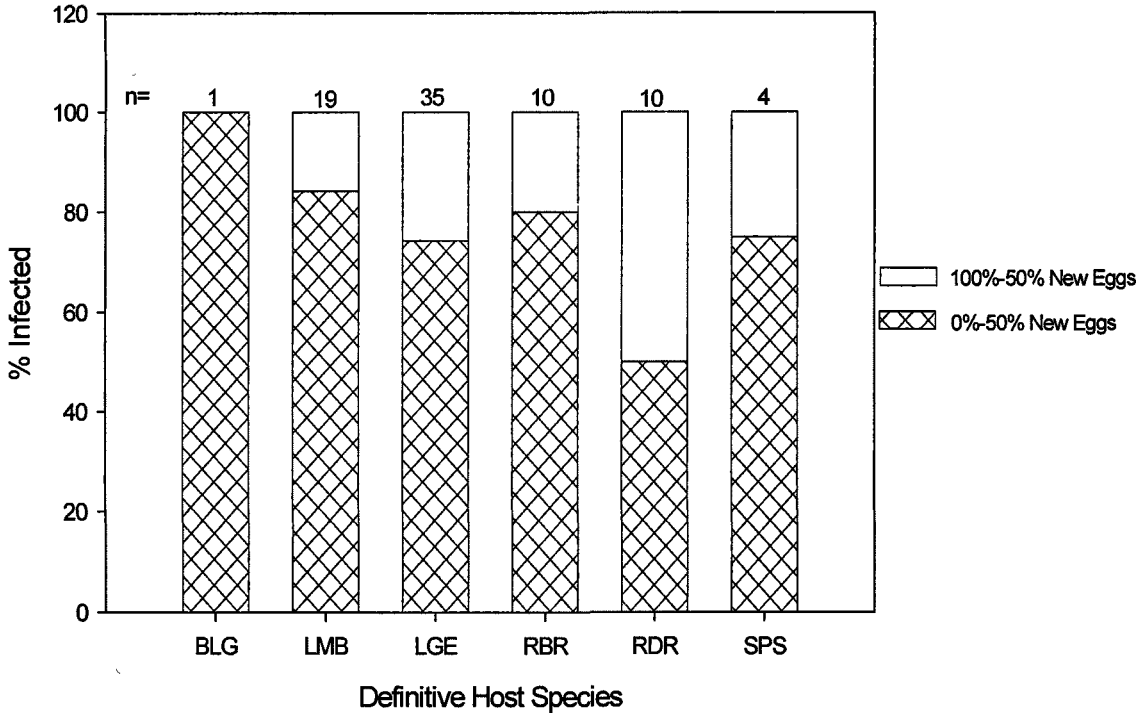

Figure 10. Percentage of new and old infections found in definitive hosts across all sites

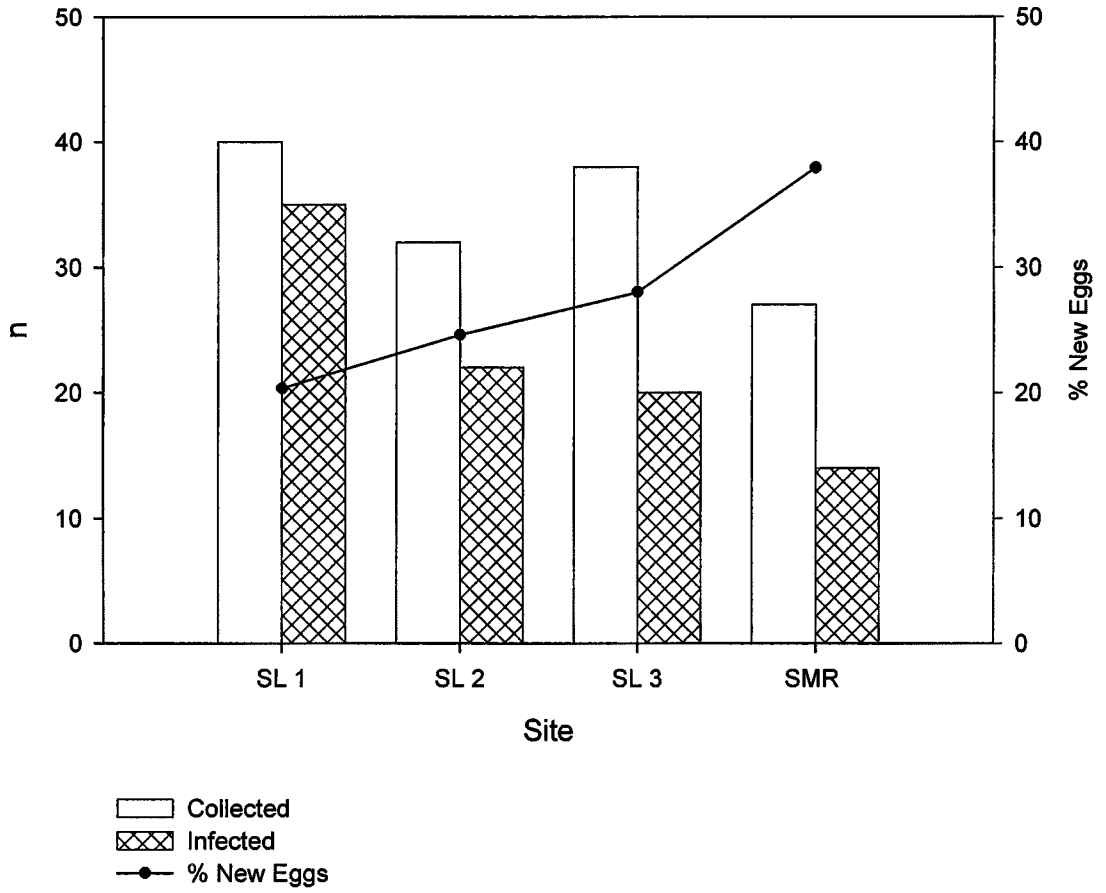

Figure 11. Distribution of new eggs across all host species[4] by site

Diet Analysis Study

Items common to all species of infected definitive hosts included *Hyalella azteca* and vegetation. The most abundant diet items present in both infected and uninfected definitive hosts were *H. azteca* and chironomids (Figure 12). Items present in infected definitive hosts but not in uninfected definitive hosts were mesoveliids (0.15%), leuctrids

[4] *Micropterus salmoides* greater than 20 cm were excluded to remove any potential length effects.

(0.15%), perlodids (0.15%), and fish (1.5%). Items that were more prevalent in infected hosts than in uninfected hosts were simuliids (1.6% vs. 1.3%), chironomids (23.2% vs. 15.1%), isonychiids (0.75% vs. 0.5%), cladocerans (0.9% vs. 0.16%), hydracarinids (0.3% vs. .0.31%), and vegetation (3.4% vs. 0.23%).

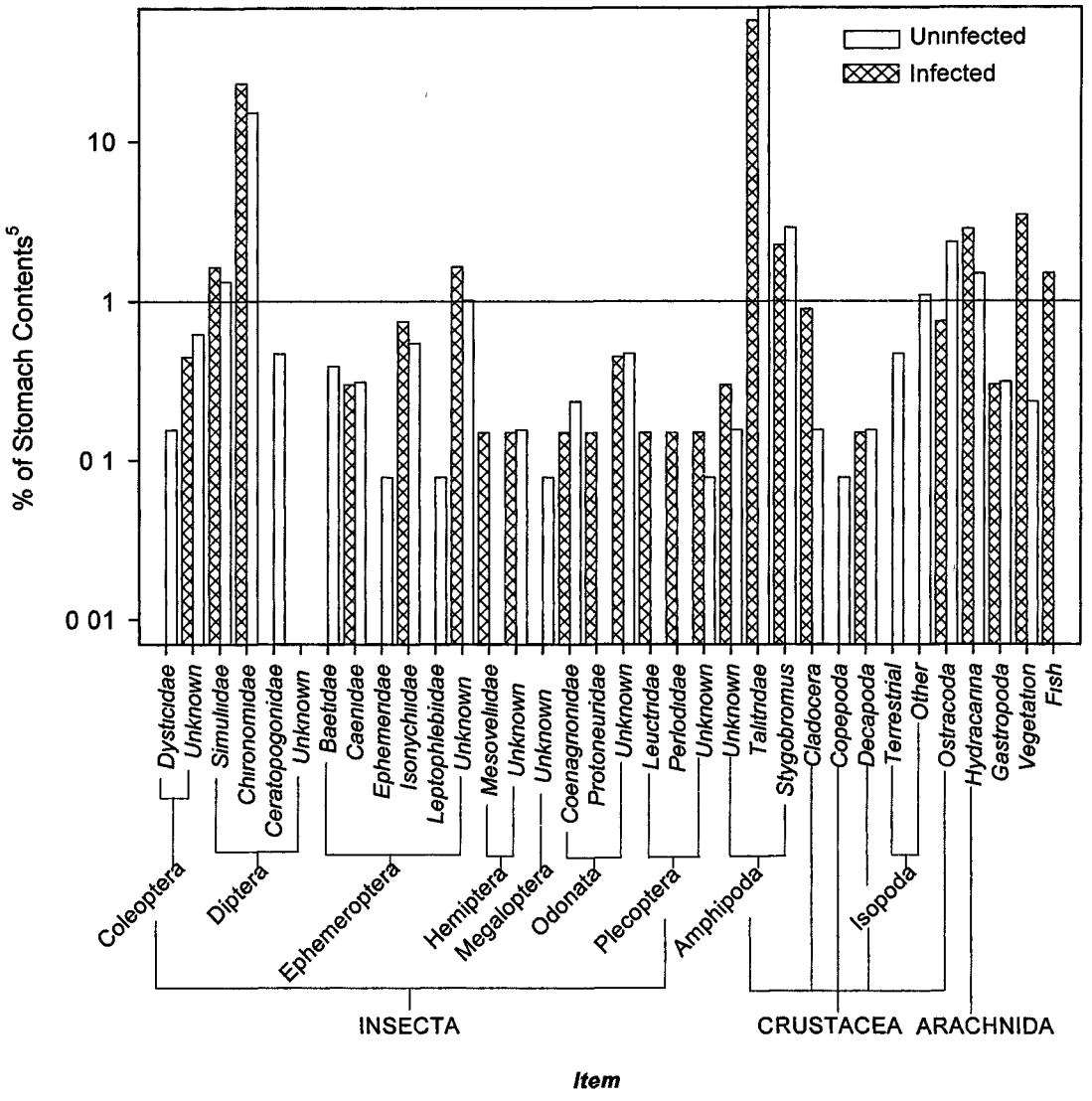

Figure 12. Mean percent of stomach contents for each dietary taxon in infected versus uninfected fishes over all sites[5]

[5] Log transformed.

Life Cycle Studies

In vivo Extraction and Induced Hatching of H. huffmani Eggs

Eggs fed to *M. salmoides* were collected from the resulting fecal debris. This *in vivo* digestive process freed the eggs from host tissue (Figure 13). Eggs hatched readily during examination (Figure 14). Eggs processed through this technique are herein referred to as "activated" eggs.

In vitro Extraction and Induced Hatching of H. huffmani Eggs

Infected gas bladders were placed in simulated gastric fluid and processed via the *in vitro* technique. The homogenized slurry of eggs, host tissue, and digestive fluid resulted in a supernatant in which the eggs were suspended. This simulated *in vitro* digestive process also freed the eggs from host tissue and these eggs also hatched readily during examination. Eggs processed through this technique are also referred to as activated eggs.

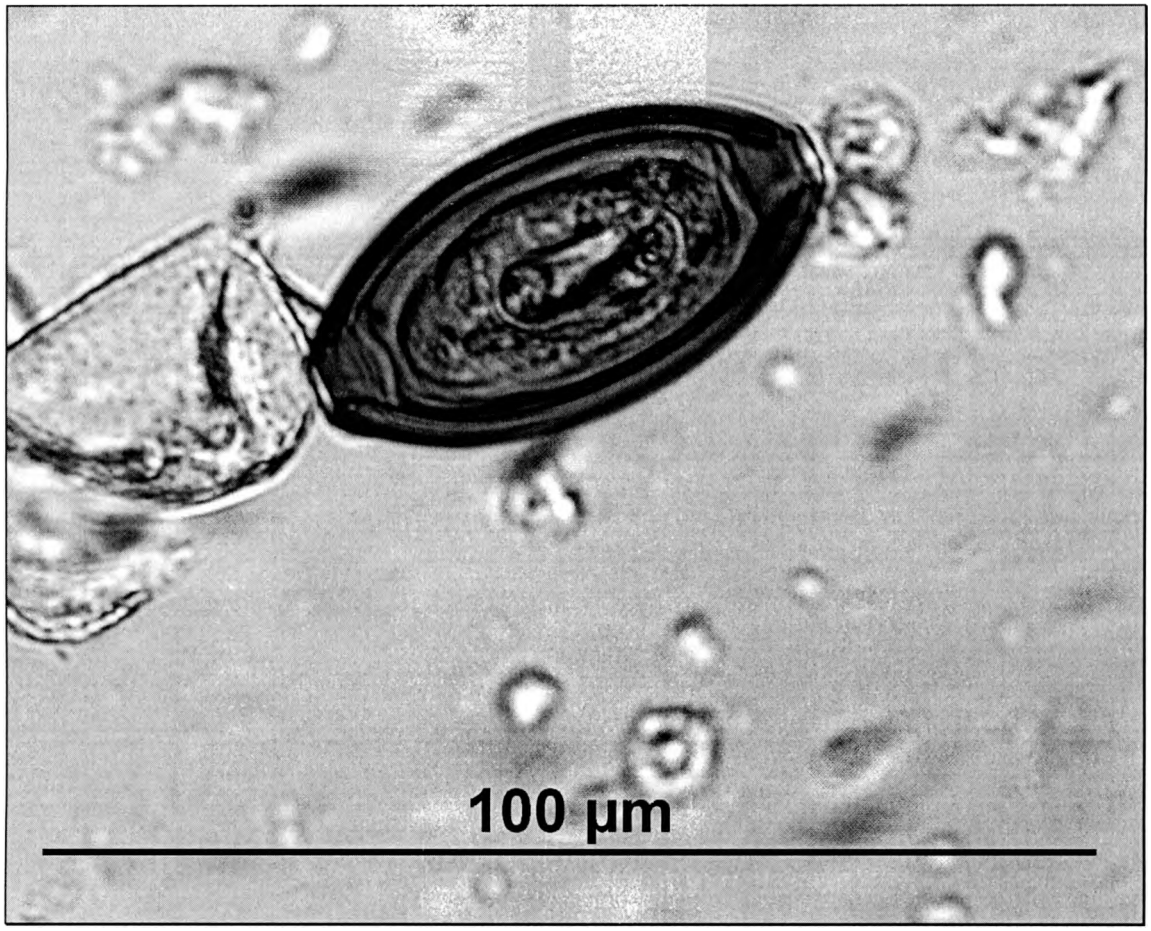

Figure 13. Egg with larva inside extracted from host tissue

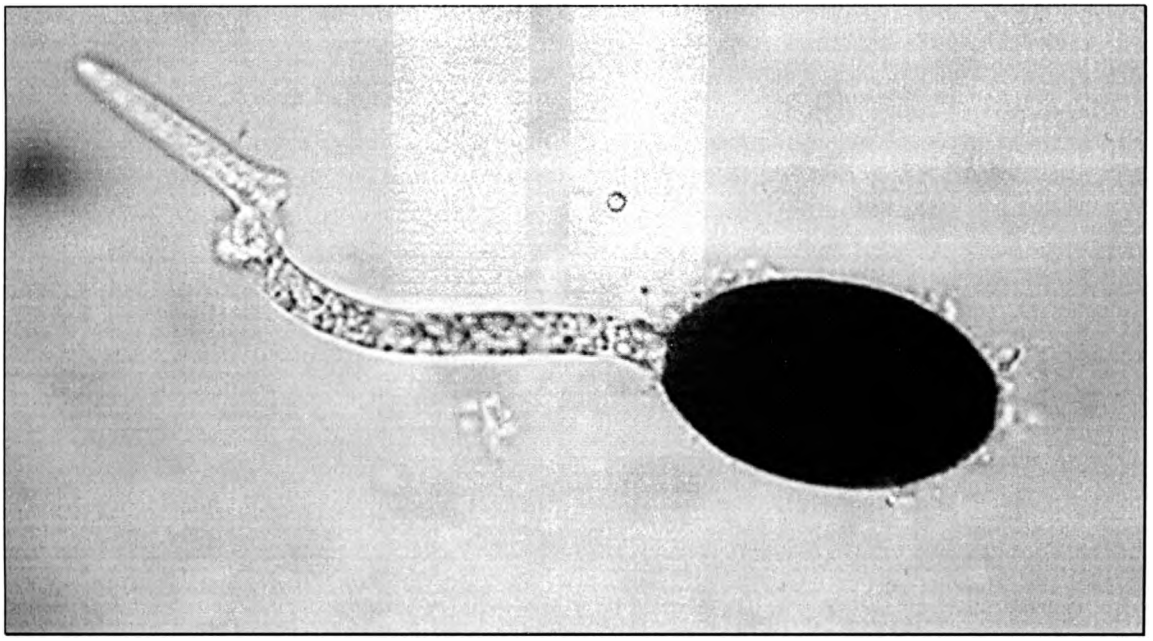

Figure 14. *H. huffmani* larva hatching from extracted egg

In vitro Cultivation of H. huffmani Larvae from Eggs

Hatching of *H. huffmani* eggs was observed immediately following exposure to culture media. Mortality of all hatched larvae occurred between 30 minutes to an hour post hatch. No molting was observed.

Infection Challenge 1. (Proposed Direct Life Cycle A)

Gas bladders infected with *H. huffmani* eggs were fed to uninfected fish. The fish were examined following a 31-day incubation period. No *H. huffmani* infections were found.

Infection Challenge 2. (Proposed Direct Life Cycle B)

Eggs activated by the *in vitro* technique were tube fed to uninfected fish. These fish were examined following a 31-day incubation period. No *H. huffmani* infections were found.

Infection Challenge 3. (Proposed Indirect Life Cycle; Spring Lake)

Invertebrates were collected from Spring Lake and fed to uninfected fish, which were examined following a 31-day incubation period. No *H. huffmani* infections were found in either the invertebrates or the fish.

Infection Challenge 4. (Proposed Indirect Life Cycle; in vivo activated eggs)

Eggs activated by the *in vitro* technique were exposed to candidate intermediate hosts. There was no evidence that any of the exposed candidate intermediate hosts had ingested *H. huffmani* eggs. No *H. huffmani* eggs or larvae were observed in the stomachs of any invertebrates during or following the 1-week exposure period. Even though there was no evidence that the exposed invertebrates had ingested eggs, all were fed out to uninfected definitive host fish. No *H. huffmani* infections were found in any of these definitive hosts following the 31-day incubation period.

Infection Challenge 5. (Proposed Indirect Life Cycle; in vitro activated eggs)

Eggs activated by the *in vitro* technique were exposed to candidate intermediate hosts. There was no evidence that any of the candidate intermediate host invertebrates exposed to the egg slurry had ingested *H. huffmani* eggs. No *H. huffmani* eggs or larvae

were observed in the stomachs of any invertebrates during or following the 1-week exposure period. Even though there was no evidence that the exposed invertebrates had ingested eggs, all were fed out to uninfected definitive host fish. No *H. huffmani* infections were found in any of these definitive hosts following the 31-day incubation period.

IV. DISCUSSION

Prevalence

The prevalence study serves as a guide to the ecological distribution of the parasite and, consequently, its intermediate host. One of the most striking observations resulting from the prevalence study is that the overall prevalence of *H. huffmani* in its habitat is much lower than previously reported. According to such reports, *H. huffmani* infects approximately 90% of definitive hosts in the upper San Marcos River (Cox, 1998). According to the results of the present study, however, the prevalence of *H. huffmani* in the same locality is 59%. Similarly, prevalence in the current study was lower than in previous studies for two of the three sites (Table 3).

Table 3. Prevalence of *H. huffmani* as reported by Cox (1998) compared to the results of the current study

Slough		Spring Lake		San Marcos River	
1998	2007	1998	2007	1998	2007
25% (n=4)	69% (n=32)	100% (n=4)	59% (n=38)	100% (n=9)	52% (n=27)

These differences in prevalence reported by Cox (1998) and the current study might be the result of much smaller sample size in the previous study. Other potential explanations for the differences are more complex and are not orthogonal. These might

include such factors as species mix, seasonality, and host length, none of which were controlled for in either study.

Apparent associations between prevalence and host species were noted in the results of the current study (Figure 6), but Cox (1998) reported that he found no evidence that prevalence of *H. huffmani* was affected by host species. This absence of apparent species effects on prevalence in the 1998 study might be due to sample sizes that were inadequate to reveal any existing species effects.

It is also possible that the differences in prevalence between the two studies are due to seasonality. Cox (1998) noted the potential for seasonal effects in the life cycle of *H. huffmani*, but found no trends consistent with this idea. Similarly, the current study did not observe any seasonal trends in prevalence. Fish were collected throughout the year in both studies, and new *H. huffmani* eggs were observed at all seasons.

The downstream gradient in prevalence (Figure 7, squares) suggests that infected definitive hosts are most prevalent near the springs at the headwaters of Spring Lake. However, this apparent trend might still be an artifact of species effects, since species mix of definitive hosts was not standardized across sites. While every attempt was made to collect an accurate representation of the ichthyofauna present throughout the entire study habitat, not all host species were collected at all sites.

If the observed trend of decreasing prevalence in the definitive hosts with increasing distance from the head springs is valid, then this would suggest that the availability of infected intermediate hosts also decreases with distance from the head

springs. This conclusion is also consistent with the observation that the prevalence of infection continues to decline towards near zero at approximately 2 km downstream (Cox 1998). The unknown intermediate host is, therefore, most likely associated with the immediate vicinity of the springs at the headwaters of Spring Lake. This claim is not only consistent with the downstream distribution of prevalence in the definitive hosts, but also with the fact that the distinctive habitat at the sites where the springs actually emerge is not replicated anywhere else in the ecosystem. The sediment surrounding the springs is characteristically different from the sediments throughout the rest of Spring Lake and the San Marcos River. This sediment resembles very fine sand but is composed of microcrystalline quartz that is released from the limestone when the calcite is dissolved. This sediment type is restricted to the immediate vicinity of actively flowing springs. In contrast, Del Rio clay lines the areas of the lake without actively flowing springs (Chappell, pers. comm.). Thus, it is possible that the intermediate host might be endemic to the quartz sediments surrounding the spring openings.

It is unlikely the intermediate host of *H. huffmani* is endemic to the Edwards Aquifer itself. While parasitic life cycles might be complex, it is illogical to surmise that an organism would begin its life cycle in the aquifer, mature outside of the aquifer, and then, through its definitive host, complete its life cycle by returning to the aquifer. There is no known portal through which infected definitive hosts might travel back and forth between the aquifer and the lake to advance the life cycle of a parasite.

No *H. huffmani* infections were observed in definitive hosts collected at the Sessom Creek site. When samples were collected, the water at the Sessom Creek site was separated from the water of the river by a gravel bar. If samples had been collected from this site while Sessom Creek was flowing, infected fish might have migrated into the mouth of the creek from the river. This observation is consistent with the claim that *H. huffmani* is geographically restricted to the main stem of the upper San Marcos River.

The results of neither the relative intensity study nor the percent new eggs study provided any apparent trends that would advance the knowledge of *H. huffmani*.

Diet Analysis Study

The overall purpose of the diet analysis was to identify taxa present in the diet of infected definitive hosts that might serve as intermediate hosts for *H. huffmani*. The diet of infected definitive hosts was compared with that of uninfected definitive hosts in an attempt to reveal the identity of potential intermediate hosts. The soft body parts of most identifiable taxa observed in the stomach contents of definitive hosts, such as larval insect gills, had already been destroyed by digestion. Because the bodies of some organisms expected to be in the diet are entirely soft, they are likely to digest quickly, and might not persist in the stomach in any identifiable form. Therefore, the dietary taxa found in the study cannot serve as an exhaustive catalog of candidate intermediate hosts, and many other organisms should be considered.

In general, the taxa found in the diet analysis in this study are consistent with other analyses reported for the examined species (Werner and Mittelbach, 1981; García-

Berthou and Moreno, 2000). Taxa found to be present in the diet of infected definitive hosts but not present in that of uninfected hosts were only present in percentages less than 0.2%. Each of these three records represents a single fish with one of the indicated food items. All three fish were collected from the slough rather than from the headwaters site. Therefore, these data are insufficient to explain the observed prevalence patterns of *H. huffmani*.

Eight taxa were found to be more prevalent in the diet of infected definitive hosts than in uninfected hosts. However, three of these were present at levels less than 1%, and among those present at greater than 1%, the differential between infected and uninfected was usually small. The only dietary taxon with a biologically interesting differential between infected and uninfected fish was Chironomidae, which was used unsuccessfully in the infection challenges.

The presence of vegetation in the stomachs of infected hosts might be due to incidental ingestion when the definitive host is preying upon invertebrates that inhabit the vegetation. This vegetation often occurred as a bolus, and upon further examination, several larval insect taxa were often observed to be entangled in the vegetation. These taxa included those already represented in the diet analysis.

Life Cycle Studies

Both the *in vivo* and *in vitro* extraction and induced-hatching techniques were successful in removing *H. huffmani* eggs from host tissue and activating the eggs to hatch. In the *in vivo* method, it is likely that the digestive processes of the definitive host

freed the histozoic eggs by digesting away the surrounding host tissue. The chemicals in the digestive process also appear to remove or digest away the thin outer vitelline layer of the egg. This layer remains intact when the eggs are mechanically extracted from host tissue. The absence of the outer vitelline layer appears to be the only physical difference between activated eggs and inactivated eggs. It is also possible that the digestive process might include a biochemical reaction that activates the eggs by altering the integrity of the outer egg layers or the polar plugs, allowing the larvae to escape through a polar plug.

In contrast to the "Press Method" utilized by Cox (2004), these new techniques might be applied to thousands of eggs at once rather than a few eggs at a time. Furthermore, the techniques simulate natural hatching conditions. This technique permits the subsequent use of larvae for other purposes, such as infection challenges. Larvae that are hatched unnaturally via pure physical force might not be viable for further studies, and could lead to false negatives in life-cycle studies. These new techniques might also be valuable in studies pertaining to other *Huffmanela* species, as well as any other trichosomoidid nematodes.

Despite the failed attempts to culture *H. huffmani* larvae from eggs, the concept of larval cultivation still has potential. It is possible that minor modifications to the existing technique will render it successful.

Two Direct Life cycle Models, A & B, were proposed for *H. huffmani* (pages 14 and 15), and both models were tested by infection challenges. In these models, transmission of *H. huffmani* would not rely on any intermediate host.

To test Direct Model A, uninfected fish were fed infected gas bladders. The results of this infection challenge were inconsistent with predictions based on the proposed Direct Model A. None of the infection challenges resulted in successful infections of previously uninfected definitive hosts. However, this model was not expected to be a solution to the life cycle question because of difficulties in surmising possible routes the parasite might take between leaving one host and entering the next. Despite the arguments against this model, it was important to test every general type of nematode life cycle.

One Indirect Life cycle Model was proposed for *H. huffmani* (page 17), and this model was also tested by infection challenges. In this model, transmission of *H. huffmani* would rely on one or more intermediate hosts.

The results of this infection challenge were inconsistent with predictions based on the proposed model. None of the infection challenges resulted in successful infections of previously uninfected definitive hosts. While the results are not consistent with prediction, there are several lines of argument suggesting that this model should be investigated further.

One such argument is incubation and development time. The actual prepatent phases for the various stages of *H. huffmani* development are unknown; therefore, it is difficult to foresee appropriate experimental incubation times for both the intermediate host and the definitive host. The incubation time chosen for the infection challenges was based on the generally accepted prepatent phase of 21-28 days for *Capillaria*, a

trichiuroid genus with well-understood life cycles. However, it is possible the prepatent phase of *H. huffmani* is much longer than that of *Capillaria*, and that the timing chosen for the phases of these experiments was insufficient for one or more stages to develop fully.

Another argument is the invertebrate species chosen as candidates for the intermediate host. Candidate intermediate hosts used in the infection challenges were chosen according to several criteria. Some species were chosen because they were found in the diet of some infected definitive hosts, such as *Arrenurus* sp., *Hyalella azteca* and chironomids. Some soft-bodied forms that might have been missed in the dietary analysis and those with marine counterparts, such as *Dugesia tigrina*, *Placobdella* sp. and *Tubifex* sp. were also chosen. Because all other representatives of Huffmanelinae are marine, it was proposed that the intermediate host of *H. huffmani* is a marine relict. Several marine invertebrates, most notably annelids, are restricted to similar sediment types. Other invertebrates selected as candidate intermediate hosts were chosen due to their association with the Edwards Aquifer. *Cirolanides texanus*, *Palaemonetes antrorum,* and *Stygobromus pecki* are endemic to the aquifer, and have a geographic distribution similar to that of *H. huffmani*. While ten candidate species were chosen for experiments using these criteria, there are many other candidate species, some of which might even be undescribed, that were not investigated in this study.

A third argument to consider is the unknown larval development of *H. huffmani*. It is unknown if *H. huffmani* does proceed through four typical larval stages like most

nematodes. It is also unknown which larval stage is infective to either an intermediate host or definitive host. For most nematodes, the third or fourth larval stage is infective (Moravec, 1999, 2001). Information regarding the larval development of the parasite could dramatically affect the experimental protocols.

Potential Host-parasite Interactions

The physiological effect of *H. huffmani* on its definitive host has yet to be studied. Because the parasite infects the host gas bladder, it is conceivable that an infection might interfere with gas bladder functions, such as depth regulation and gas exchange. In definitive hosts with particularly heavy infections, the functions of adjacent organ systems, like the kidneys, might also be affected. Circulatory and respiratory functions might also be impeded due to the presence of parasite eggs in the rete mirable. All of these potential impediments might compromise other more general functions such as feeding. If this is true, an infected fish species might be easier to capture via normal angling techniques. This might also explain the observed higher prevalence in certain species of definitive hosts over others. These changes might influence the response of a species under capture stress, thus influencing the collection assemblage when normal angling techniques are utilized.

Suggestions for Future Research

While the presented results certainly advance knowledge regarding the prevalence and distribution of *H. huffmani*, several questions remain.

The results of the prevalence study would be more compelling if the centrarchid species were uniformly represented across all of the sites. Consistent species mixes across sites would allow for separate analyses of downstream effects independently of species effects.

Acquiring a consistent species mix will demand uniformly stratified sampling with consistent numbers of individuals from each species collected at each site. While electrofishing seems the best option for such sampling, it is unlikely this method would be feasible in such a sensitive habitat. Another option that would allow such sampling would be the combined use of SCUBA and spearfishing. Again, this option might not be feasible given the regulated nature of the habitat.

The failure of the intensity studies to provide any information that might advance knowledge regarding *H. huffmani* might mean that previously recommended methods of estimating the intensity of *H. huffmani* infection might need further work. Instead of subjective rankings based on gross appearance, better estimates would be provided by counting a standard subset of all eggs in a gas bladder. In this study, various mixtures of pepsin and trypsin failed to free all eggs, even after days of incubation. Although this method did not work in this study, further experimentation with digestive enzyme mixtures might yield a protocol that would free all eggs. Another possible method, which could supplement enzyme digestion, might be high-speed shredding of partially digested tissue with a blender. After all eggs are freed from gas bladder tissue, the eggs would be

suspended in a standard volume, divided into several small sub sample, counted in a Palmer counting chamber, and expressed as eggs/g fish.

A method that would not be feasible is the comparison of infected and uninfected gas bladder masses. Even if compared within the same length classes or species, this method would be biased depending on the age of the eggs in the infection. For example, infected definitive hosts with infections consisting of primarily older eggs most likely have excess tissue from immune responses. The masses of such gas bladders would be greater than that of a gas bladder infected with primarily new eggs and no scar tissue.

Based on the results of the prevalence and intensity studies, it might be advantageous to compare the diets of infected and uninfected definitive hosts according to length class. The current analysis does not consider ontogenetic diet shifts. Perhaps doing so will identify potential intermediate hosts present in the diet of definitive hosts during the age at which they become infected.

The *in vivo* and *in vitro* extraction and induced hatching techniques provide a reliable method of supplying *H. huffmani* eggs for further life cycle studies. The techniques might also be useful for other nematodes. It would be worthwhile to investigate any physical or biochemical effects sustained by the egg during the digestive process, particularly regarding the vitelline layer of the egg.

In vitro cultivation of any organism provides a working, observable model from which development might be studied. Successful *in vitro* cultivation of *H. huffmani* might provide information regarding the time required for prepatent phases, the ecdysis

schedule, and the growth rates of the various stages of the nematode. Such information would be helpful in the design of future infection challenges. Certain aspects of Adroher's protocol were adapted to the cultivation of *H. huffmani*. The *H. huffmani* eggs were incubated at 20°C rather than at 13°C, and ambient air was used rather than an atmosphere regulated at 5% CO_2. The temperature change was made to simulate the normal temperature at which *H. huffmani* exists. Adroher's CO_2 protocol was not followed because suitable equipment was not available for the study. Perhaps the higher temperature used in this study altered the medium in some way such that it could not support nematode development, but this is not as likely a cause of failure as the altered CO_2 levels. In future attempts to cultivate *H. huffmani* using Adroher's protocol should probably restrict modifications temperature only.

The proposed Indirect Life Cycle Model is a good basis for future infection challenges. The results of the extraction and induced hatching techniques support the idea that the infected tissues of an infected intermediate host must be digested by a predator to release *H. huffmani* eggs. In future studies, new candidate intermediate hosts should be used along with those used in this study, but with more time allowed for prepatent phases. Given the observed downstream trend in the distribution of *H. huffmani*, it would be advisable to investigate the sediments immediately surrounding the springs at the headwaters in the search for new candidate intermediate host species. These sediments might harbor an unknown endemic invertebrate capable of serving as intermediate host for *H. huffmani*. Any new invertebrates found there should also be

examined for existing larval *H. huffmani* infections, and used in subsequent infection challenges.

It would be worthwhile to investigate the physiological effects of *H. huffmani* on its definitive host. Histological studies of infected gas bladders might reveal physiological changes that could result in decreased function of the organ. For instance, because the gas bladder is known to be involved in many functions, complications arising from heavy *H. huffmani* infections might affect buoyancy, circulation, and perhaps sensory perception. Another technique that might be useful when investigating physiological changes is nuclear magnetic resonance imaging (NMR). This tool would provide detailed images of infected gas bladders *in vivo*, resulting in a more comprehensive understanding of the infection. For example, NMR might help understand how the eggs are deposited in the gas bladder, and might even reveal otherwise cryptic adults in other parts of the host.

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VITA

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