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The life cycle of *Huffmanella huffmanii* Moravec, 1987 (Nematoda: Trichosomoididae), an endemic marine-relict parasite of Centrarchidae from a Central Texas spring

McLean L. D. Worsham¹, David G. Huffman¹, František Moravec² and J. Randy Gibson³

¹ Freeman Aquatic Biology, Texas State University, San Marcos, TX, USA;

² Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic;

³ U.S. Fish and Wildlife Service, Aquatic Resources Center, San Marcos, TX, USA

Abstract: The life cycle of the swim bladder nematode *Huffmanella huffmanii* Moravec, 1987 (Trichinelloidea: Trichosomoididae), an endemic parasite of centrarchid fishes in the upper spring run of the San Marcos River in Hays County, Texas, USA, was experimentally completed. The amphipods *Hyaella* cf. *azteca* (Saussure), *Hyaella* sp. and *Gammarus* sp. were successfully infected with larvated eggs of *Huffmanella huffmanii*. After ingestion of eggs of *H. huffmanii* by experimental amphipods, the first-stage larvae hatch from their eggshells and penetrate through the digestive tract to the hemocoel of the amphipod. Within about 5 days in the hemocoel of the experimental amphipods at 22 °C, the larvae presumably attained the second larval stage and were infective for the experimental centrarchid definitive hosts, *Lepomis* spp. The minimum incubation period before adult nematodes began laying eggs in the swim bladders of the definitive hosts was found to be about 7.5 months at 22 °C. This is the first experimentally completed life cycle within the Huffmanellinae.

Keywords: swim bladder nematode, Trichinelloidea, development, intermediate host, Amphipoda, *Hyaella*, *Gammarus*, fish, USA

Adult trichinelloid nematodes occur in a wide range of vertebrate hosts. The vast majority of species infect epithelial surfaces, although a few species infect spleen or liver tissues, or are found in other tissues (Anderson 2000, Anderson et al. 2009). Although the development and transmission of some species with medical importance or those parasitising economically significant groups of animal hosts have been extensively studied (for reviews see Moravec et al. 1987, Anderson 2000, Moravec 2001), the biology of trichinelloids as a whole still remains little known. Aside from well-studied trichinelloid taxa, little else is known about the morphogenesis of larvae during development or the life cycle patterns for the vast majority of trichinelloid species. This is particularly true regarding whether or not an intermediate host is required (Moravec 2001).

Most of the data available on the life cycles of trichinelloids pertains to representatives of the species-rich family Capillariidae Railliet, 1915, whereas information on the life cycles of representative of other trichinelloid families is rather scarce or absent (Anderson 2000). Within the Trichosomoididae (apparently closely related to Capillariidae), only the life cycle of *Trichosomoides crassicauda* (Bellingham, 1840) from the urinary tract of rats

has been studied in detail (e.g. Löwenstein 1910, Thomas 1924, Wahl and Chapman 1967, Hasslinger and Schwarzler 1980). The eggs of this nematode were directly infective to the definitive host. To date, very little has been reported about the life cycles of representatives of the trichosomoidid genus *Trichuroides* Ricci, 1949 (*T. chiropteri* Ricci, 1949 from the urinary bladder of bats), or of the numerous species of *Huffmanella* Moravec, 1987 parasitising fishes.

Huffmanella huffmanii Moravec, 1987 (Trichinelloidea: Trichosomoididae: Huffmanellinae) is a parasitic nematode known only from the swim bladders of centrarchid fishes from the upper spring run of the San Marcos River (SMR) in Hays County, Texas (29°53'36"N; 97°55'52"W). The first reported occurrence of *H. huffmanii* was in 1979, when Underwood and Dronen (1984) reported eggs from the swim bladders of various centrarchids in the SMR and identified the eggs as representing an unidentified species of *Capillaria* Zeder, 1800. The species was later formally described as *Huffmanella huffmanii* based on eggs alone (Moravec 1987) and the new genus *Huffmanella* was erected to accommodate it. Adults of the species were subsequently described by Huffman and Moravec (1988). The description of *H. huffmanii* preceded the reassignment of several species from *Capillaria* into the genus *Huffmanella*,

Address for correspondence: M.L.D. Worsham, Freeman Aquatic Biology, Texas State University – San Marcos, San Marcos, TX 78666-4616, USA. Phone: (406) 600-5515; Fax: (512) 245-7919; E-mail: biolyth@txstate.edu

Table 1. Akaike information criterion contrast coefficients and feeding-niche categories of centrarchid species from San Marcos Springs based on observed foraging behavior. The contrast coefficients reflect relative likelihood that fish in the category could be expected to encounter and consume benthic, spring-related, detritivorous invertebrates.

Contrast coefficient	Category definition	Example taxon
0	Non-centrarchid	<i>Herichthys cyanoguttatus</i> Baird et Girard
1	Limnetic/zooplanktivorous	<i>Lepomis macrochirus</i> Rafinesque
2	Littoral/invertivorous	<i>Lepomis miniatus</i> Jordan
3	Benthic/invertivorous	<i>Lepomis microlophus</i> Günther
4	Spring-dominant benthic/invertivorous	<i>Lepomis auritus</i> Linnaeus

as well as the erection of *Huffmaniellinae* Moravec, 2001 by Moravec (2001).

Some twenty other species of *Huffmanella* are now documented, all of which are histozoic in marine fishes from coastal and blue-water habitats of diverse geographic regions across the world's oceans (Ruiz et al. 2013, Justine and Iwaki 2014). Since all species of *Huffmanella* other than *H. huffmanii* are marine, it is likely that *H. huffmanii* is a marine relict. This assertion is consistent with the presence of numerous other marine-relict invertebrates unique to the San Marcos Springs (SMS) (Holsinger and Longley 1980). Furthermore, even though many invertebrate taxa that are not marine relicts are also restricted to just the spring-influenced Upper SMR, none of the SMR marine relicts are found outside of a subterranean or spring-confined distribution (Gibson et al. 2008). Much like the other SMR invertebrates of marine taxonomic affiliations, reports from previous surveys have indicated that *H. huffmanii* is also restricted to just the spring-influenced Upper SMR (Michel 1984, Cox et al. 2004, O'Docharty 2007). These findings are consistent with the hypothesis that *H. huffmanii* is a marine relict with a spring-associated distribution.

The majority of the species of *Huffmanella* are described from eggs alone, with adults having been described thus far from only six species (Bullard et al. 2012). Each species of *Huffmanella* deposits its eggs in a species-specific organ of its definitive host. However, aside from the location of egg deposition, little else is known about any of the life cycles of *Huffmanella* spp. The location of egg deposition by *H. huffmanii* (and many other *Huffmanella* spp. that are histozoic in internal organs of the definitive host) is remarkable because eggs cannot be released from a living definitive host to infect the next host. Previous research has been unable to get ingested eggs of *H. huffmanii* to hatch inside the definitive host (Cox 1998, Moravec 2001, Cox et al. 2004, O'Docharty 2007); therefore, an infected definitive host laden with eggs of *H. huffmanii* must be decomposed or digested in order for the eggs to become available to the presumptive intermediate host.

Eggs of *H. huffmanii* are appreciably denser than water, and once liberated from the host, they ultimately come to rest on the benthic sediments. Because eggs cannot infect fish directly and because eggs are found only among the benthos once outside the definitive host, it has been suggested that a benthic detritivore must be serving as the intermediate host (Cox et al. 2004, O'Docharty 2007). Therefore, the first step in solving the life cycle of *H. huffmanii* was to determine if there is, in fact, an intermediate host, and if

so, to identify that host and use it to experimentally complete the life cycle. Presented herein is the experimentally completed life cycle of *H. huffmanii*; the first described for any of the about 20 known species of the *Huffmaniellinae*.

MATERIALS AND METHODS

Analysis of habitat preferenda of definitive hosts to suggest intermediate hosts

Initially, centrarchids foraging around spring openings in Spring Lake (the impounded headsprings of the SMR) were periodically observed, photographed and videographed by divers during both day and night, from January through May of 2012, inclusively. After determining the preferred feeding habitats of the centrarchid species known to be susceptible to *H. huffmanii* (Moravec et al. 1987, O'Docharty 2007), each species was assigned to one of four feeding-niche categories. Because the presumptive intermediate host was suspected of being a benthic, spring-dependent, detritivorous invertebrate, we were able to use these category assignments to rank the centrarchid species by how frequently they would be expected to encounter and consume such invertebrates. These species are listed in Table 1 along with their feeding-niche categories and respective contrast coefficients.

Following the feeding preferendum analysis, centrarchids were collected from Spring Lake and necropsied in order to determine if the actual levels of parasitism for each species corresponded to the expectations that emerged from the feeding preferendum analysis. These survey fish were collected by angling with artificial lures and transported back to the laboratory in at least 10 l of spring water. Because it was not known if eggs of *H. huffmanii* are environmentally sensitive, and one of the goals of fish collections was to obtain viable eggs, transport of fish back to the laboratory was expedited in order to ensure that dissolved oxygen and temperature did not deviate substantially from optimal conditions potentially required by *H. huffmanii*. Fish were then transferred to a tank supplied with flowing, untreated artesian well water physicochemically similar to SMS water in most measurable parameters. The fish were maintained at 22 °C under a 12/12 h light/dark cycle until just before necropsy.

Fish were killed by pithing and the swim bladder was excised. A small portion of the epithelium of the ventral wall of the swim bladder was separated from the remainder of the swim bladder and was used to roughly estimate the intensity of infection using compound light microscopy at 40× to 200× magnification. Because a single swim bladder might contain a million or more eggs (Cox 1998), intensity was not determined by counts, but was estimated by the approximate percentage of the visual field where

Table 2. Descriptions of infection-intensity categories used to estimate the intensity of infection with eggs of *Huffmanella huffman* Moravec, 1987 in the ventral wall of the swim bladders of wild-caught centrarchids.

Infection-intensity category	Description
0	no eggs detected
1	trace infection; only a few eggs observed
2	up to 25% of swim bladder infected with eggs
3	greater than 25% and up to 50% of swim bladder infected
4	greater than 50% of swim bladder infected

eggs had been deposited. These intensity estimates were broken out into five infection-intensity categories as defined in Table 2.

The remaining tissue of each swim bladder was placed individually into a sealed 700 ml plastic food container filled with artesian water. These containers were maintained in a water bath of flowing artesian water to maintain a constant temperature of 22 °C (similar to the SMS water) while microbial decay was allowed to break down swim bladder tissues and free the eggs. Water in these containers was refreshed twice monthly.

Using the infection-intensity data derived from the wild-caught fish, an Akaike information criterion (AIC) analysis was performed to determine the factors that best predicted the observed intensity ratings of the wild-caught fish. This analysis used three one-way ANOVAs ($\alpha = 0.01$) employing host standard length, the contrast coefficients mentioned above (Table 1), and species as predictors of the intensity ratings observed for these fish. The factor ‘standard length’ was used as a predictor to assess whether or not there was a minimum size at which the fish could be expected to be positive for eggs of *H. huffman*. If so, it could indicate that something is preventing the fish from consuming infected invertebrates until the fish reach a certain minimum size. This, in turn, could have potentially provided useful hints about the intermediate host. The factor ‘species’ was used as a predictor to assess whether or not species-related differences in the susceptibility to *H. huffman* could explain differences in the infection-intensity ratings. The factor ‘contrast coefficients’ was used as a predictor to assess if observed differences in the prevalence and intensity rating could best be explained by feeding niche of the definitive host.

Selection of candidate intermediate hosts

At the time of this writing, approximately 200 species of macroinvertebrates have been reported from the SMS and SMR (Edwards and Arnold 1961, Bowles et al. 2007, Gibson et al. 2008, Diaz and Alexander 2010), most of which are benthic. To narrow down the most promising candidate species from this list, literature discussing the life cycles of related fish nematodes in the Trichinelloidea was reviewed (Moravec et al. 1998, Køie and Nylund 2001). Based on that review, the intermediate host for *H. huffman* was suspected of being either an annelid or an amphipod. This conclusion complicated our life-cycle investigations, as there are at least 13 species of amphipods known to occur locally (Holsinger and Longley 1980, Gibson et al. 2008) and since the literature on the annelids of the SMR was almost non-existent. Therefore, it became necessary to survey the annelids of SMS, which was found to contain at least 20 benthic species, including

at least 16 species of oligochaetes, 2 aphanoneurans and 3 leeches (Worsham et al. 2016).

Benthic invertebrates were collected from near spring openings using a Ponar grab sampler. Individual collections were transported back to the laboratory in separate containers of 22 °C spring water and maintained alive until sorted. The organisms were examined under a dissecting microscope and sorted into containers representing the lowest discernable taxon. From these collections, live cultures of candidate intermediate hosts were established and maintained in the laboratory in labelled 1200 ml plastic food containers with windows cut in the sides and covered with 200 μ m mesh. These culture containers were floated in a flow-through system of artesian water to ensure constant physicochemical conditions similar to the SMS.

Selection of candidate intermediate hosts was based on the notion that the intermediate host must be abundant enough to account for the high prevalence of infection we observed, and also the assumption that the intermediate host is a benthic species. Since the hypothetical intermediate host was also presumed to be a detritivore, only benthic species that could be maintained on a diet of detritus were used in experimental infection attempts.

Testing of candidate intermediate hosts for susceptibility

Candidate invertebrate species were tested for susceptibility to infection with *H. huffman* by placing them in a container with an aliquot of larvated and viable eggs. Controls were maintained in the same way, but without exposure to eggs of *H. huffman*. All invertebrate exposures were run for two weeks, or until all treatment specimens had died, whichever occurred first. If the rate of population decline in the treatment containers began to exceed the rate of decline in the corresponding controls by a noticeable margin, all individuals in both groups were examined immediately for larvae of *H. huffman*. At the end of the two weeks, all remaining experimental subjects were examined for larvae. When nematode larvae were discovered among the treatment replicates of a species, the associated controls were also examined to assure that they were negative. A list of the taxa tested for susceptibility to *H. huffman* is provided in Table 3.

Of all the invertebrate taxa we attempted to infect, only the talitroid amphipods of the genus *Hyalella* Smith and freshwater gammarideans of the genus *Gammarus* Linnaeus became infected. All *Hyalella* spp. tested for susceptibility showed evidence of larvae outside the gut lumen (no luminal or extraluminal nematode larvae were found in the control *Hyalella*).

Subsequent methods pertaining to the intermediate hosts of *H. huffman* employed amphipods belonging to two presumptive species of *Hyalella*. One species is a common and widely distributed species in the *Hyalella* cf. *azteca* (Saussure) eco-morph complex, while the other is an undescribed species endemic to the SMS. All amphipods used in the experiments that follow were F_1 or later generations raised in artesian water in the laboratory.

Graded exposure of candidate intermediate hosts and exposure of definitive host

Titration of amphipod exposure intensity

In an attempt to determine effective dosage regimens of eggs that would most likely produce detectable infections in amphipods without killing them, amphipods were subjected to larvated eggs

Table 3. List of invertebrate taxa exposed to eggs of *Huffmanella huffmanii* Moravec, 1987.

Higher taxon	Taxon	n
Annelida: Aphanoneura	<i>Aeolosoma</i> spp. (SMR)	10
Annelida: Clitellata	<i>Chaetogaster diastrophus</i> Baer (SMR)	10
Annelida: Clitellata	<i>Dero furcatus</i> Müller (SMR)	5
Annelida: Clitellata	<i>Dero obtusa</i> d'Udekem (SMR)	10
Annelida: Clitellata	<i>Haplotaxis</i> sp. (SMR)	2
Annelida: Clitellata	Lumbriculidae gen. sp. 1 (SMR)	5
Annelida: Clitellata	Lumbriculidae gen. sp. 2 (SMR)	5
Annelida: Clitellata	<i>Pristina</i> sp. (SMR)	10
Crustacea: Amphipoda Crangonyctidae	<i>Crangonyx</i> cf. <i>pseudogracilis</i> Bousfield (SMR)	6
Crustacea: Amphipoda Dogielinotidae	<i>Hyalella</i> cf. <i>azteca</i> (Saussure) (Comal Springs, New Braunfels, TX 29°42'54"N; 98°08'5"W)	10
Crustacea: Amphipoda Dogielinotidae	<i>Hyalella</i> cf. <i>azteca</i> (Finnegan Springs, Devils River drainage 29°53'60"N; 100°59'53"W)	10
Crustacea: Amphipoda Dogielinotidae	<i>Hyalella</i> cf. <i>azteca</i> (SMR)	10
Crustacea: Amphipoda Dogielinotidae	<i>Hyalella</i> sp. (SMS endemic)	10
Crustacea: Amphipoda Dogielinotidae	<i>Hyalella texana</i> Stevenson et Peden (Clear Creek Springs, San Saba River drainage, near Menard, TX 30°54'25"N; 99°57'39"W)	10
Crustacea: Amphipoda Gammaridae	<i>Gammarus</i> sp. (Caroline Springs, Pecos River drainage, near Sheffield, TX 30°28'09"N; 101°48'12"W)	3
Crustacea: Amphipoda Crangonyctidae	<i>Stygobromus</i> spp. (SMR)	6
Crustacea: Copepoda	Cyclopoida Burmeister (SMR)	10
Crustacea: Isopoda	<i>Cirolanides texensis</i> Benedict (SMR)	5
Crustacea: Ostracoda	<i>Cypria</i> sp. (SMR)	10
Crustacea: Ostracoda	<i>Stenocypris</i> sp. (SMR)	10
Platyhelminthes: Macrostomida	Macrostomida gen. sp. (SMR)	10
Platyhelminthes: Tricladida	<i>Dugesia</i> sp. (SMR)	10
Platyhelminthes: Turbellaria	<i>Schmidtea</i> sp. (SMR)	10

SMR – San Marcos River.

of *H. huffmanii* at various combinations of exposure intensity and incubation duration. Amphipods from each species were divided into five groups for the exposure phase, with four of the groups representing four levels of egg-exposure intensity (four durations of exposure to the same density of eggs of *H. huffmanii*), and the fifth group serving as a control (not exposed to parasite eggs). Each exposure-intensity group consisted of three replicates, with each replicate housed in a 1200 ml plastic food container.

Prior to distributing amphipods to replicate containers, the 15 marked containers were assigned random numbers, and then arranged in order of increasing random number. Each of the 15 replicate containers received 27 lab-reared amphipods at the beginning of a titration run, for a total of 405 amphipods for one entire run of the titration experiment. Disposable one-piece plastic transfer pipettes with 5 ml capacity (similar to Fisher S30467-1) were used to transfer amphipods individually from container to container. Tips of pipettes were trimmed back along the taper to a larger bore size to allow for easy passage of amphipods.

The process of distributing amphipods from the main culture container to the replicate containers started with selecting one amphipod from the main culture with a transfer pipette and transferring it to the replicate container with the lowest random number. A second amphipod was then transferred to the replicate container with the next lowest random number, and so on, until all replicate containers had been supplied with 27 amphipods. This randomisation procedure was executed in order to ensure that any sequential bias introduced by the amphipod-selection procedure was randomised across treatments. The amphipods were then allowed to habituate to the replicate containers for two days prior to exposure to eggs.

Prior to distribution of eggs of *H. huffmanii* into the amphipod containers, eggs were first divided into aliquots using a two-stage randomised serial dilution protocol so as to randomise any se-

quential differences in egg concentration across the design. The eggs were first transferred into a container of artesian water. This container was then stirred gently to suspend the eggs, and the suspension was promptly divided among three intermediate dilution containers. Each of these was then stirred and its contents promptly divided among four final-dilution containers, for a total of 12 intermediate containers of relatively equal volume. These 12 containers were then randomly assigned in sets of three to one of four exposure-intensity levels (12, 24, 48 and 96 h of egg exposure), with the three containers on each level representing treatment replicates.

Following the habituation period, amphipods assigned to the four exposure-intensity levels were simultaneously exposed to eggs of *H. huffmanii* from their respective final-dilution containers. The amphipods in exposure-intensity level 1 were examined for larvae of *Huffmanella* after 12 h, exposure-intensity level 2 after 24 h; exposure-intensity level 3 after 48 h, and exposure-intensity level 4 after 96 h. The amphipods in the fifth group (controls) were not exposed to eggs. The controls were used to determine if any population declines among the corresponding treatment replicates were due to infection with *H. huffmanii* (in which case the controls were expected to show a slower decline), or to causes unrelated to egg exposure (with approximately equal decline rates expected).

Titration of minimum prepatent incubation duration

After the prescribed durations for each level of exposure-intensity had elapsed, amphipods were separated from eggs of *Huffmanella* and then incubated for varying durations. This was done to estimate the minimum prepatent time after the exposure of an amphipod to eggs before it can become infective to a centarchid definitive host.

The amphipods were first pipetted individually from their exposure containers and rinsed thoroughly in fresh artesian water

Table 4. Larval intensity ratings for amphipods exposed to eggs of *Huffmanella huffmanii* Moravec, 1987.

Larval intensity rating of exposed amphipods	Estimated number of larvae
0	no larvae observed
1	up to 3 larvae
2	3 or more larvae, but less than 25
3	25 or more larvae, but less than approximately 100
4	approximately 100 or more larvae

to ensure removal of eggs of *H. huffmanii*. The replicate exposure containers were also rinsed thoroughly. Rinsed amphipods were then placed back into their respective containers for the incubation phase.

All replicate containers at the four exposure-intensity levels showed noticeable population declines, even before rinsing in some cases, whereas the three control replicates showed no declines whatsoever, even after 96 h of incubation. However, there appeared to be no appreciable difference in amphipod survival among the four exposure-intensity levels, indicating that the maximum exposure-intensity had probably not been exceeded, even at 96 h of exposure. It was therefore decided to run all subsequent experiments using amphipods exposed to eggs for 96 h.

Examination and feeding-out of exposed amphipods

At the end of each prescribed incubation duration (following 1, 4, 7, 14, 21, 28, 35, 42 and 49 days of incubation post-exposure), 3 to 21 amphipods were sampled from each exposure-intensity group, sampling evenly from the three replicate exposure-intensity containers. Each time amphipods were sampled, one of the amphipods was necropsied and closely examined for infection with larvae of *H. huffmanii*. The remaining amphipods in each sample were fed out to experimental fish.

After incubation of amphipods for 49 days post-exposure, all infected amphipods from all exposure intensities and incubation durations had either been necropsied, fed out to experimental fish, or had died. None of the control amphipods had died at 49 days, indicating that the treatment amphipods that died had probably died from infection with larvae of *H. huffmanii*.

In most cases, exposed amphipods were so heavily infected that exact larval counts were not feasible; therefore, all amphipod infection levels were rated subjectively. The rating codes employed to represent these subjective estimates of amphipod infection intensity are described in Table 4.

Sources of experimental fish

Experimental centrarchid fish were collected via angling and seining either from the Comal River, where *H. huffmanii* currently does not occur [0% prevalence for 120 centrarchids examined by United States Fish and Wildlife Service (USFWS), and all centrarchids examined during three previous studies (Michel 1984, Cox et al. 2004, O'Docharty 2007) were negative], or from captive stock of a leucistic strain of *Lepomis cyanellus* Rafinesque raised in filtered artesian water at the San Marcos Aquatic Resource Center (SMARC) operated by the U.S. Fish and Wildlife Service. Species represented by wild-caught specimens from the Comal River were *Lepomis auritus* (Linnaeus), *L. miniatus* (Jor-

dan), *L. megalotis* (Rafinesque) and *L. macrochirus* Rafinesque, with the majority being *L. auritus* and *L. miniatus*.

Experimental fish were maintained at SMARC in cultures of one fish per 32 or 45 l aquarium depending on size of fish. Each aquarium received a constant flow-through of Edwards Aquifer well water, and the fish were maintained on commercially available fish food (TetraCichlid Fish Food Sticks), both before and after exposure to infected amphipods.

Exposure and incubation of experimental fish

For record-keeping purposes, each of the 32 experimental fish was randomly assigned a treatment number representing a unique combination of (1) the number of infected amphipods it had been fed, (2) the incubation duration of the definitive host and (3) the exposure intensity and incubation duration of the amphipods it received. Each amphipod was expected to have several viable larvae in some stage of development at the time it was fed out to fish (most of them probably had >25). Live amphipods were pipetted individually into the aquaria containing the experimental fish, whereupon the fish were observed to consume all dispensed amphipods.

Examination of experimental fish

The survey of the literature on other trichosomoidid and capillariid life cycles indicated that the prepatent period before eggs of *H. huffmanii* could be expected to appear in the swim bladders of exposed fish would probably be at least 2 months. Thus, the first three exposed fish were scheduled for examination after 3 months of incubation, with three of the remaining exposed fish to be examined each month thereafter.

After fish had been incubated for the prescribed duration, they were pithed and the swim bladder was removed for examination. The entire ventral wall of the swim bladder was cut into small pieces and each piece was mounted under a coverslip in physiological saline. Each piece was then examined for later larval stages, adults or eggs of *H. huffmanii* under a compound microscope at 40× to 100× magnification.

Completion of life cycle

F₁ eggs of *H. huffmanii* from the swim bladder tissues of experimentally infected fish were liberated via microbial decay as described above. Liberated F₁ eggs were then fed out to laboratory-reared amphipods to verify that the eggs were infective, thus completing the life cycle.

Review of the relationship between definitive host diet and organ of egg deposition for all reported species of *Huffmanella*

The organ of egg deposition in the definitive hosts for the various *Huffmanella* spp., as well as information regarding the diet and habitat preference of the host species, were gathered from the available literature. Each of the species of *Huffmanella* was then assigned to one of four cells in a 2 × 2 table (Table 5). The cells are defined by two variables, each with two categories: location of the organ in which eggs are deposited (internal vs epithelial) and the general habitat of the definitive host (benthic vs pelagic). The overwhelming majority of species of *Huffmanella* were classified as laying eggs in an internal tissue of a benthic host. This relationship was then evaluated by comparing the proportion of *Huffmanella* spp. in only two of the four cells of the table: the

Table 5. Two-by-two table for assigning known species of *Huffmanella* to a specific combination of habitat and host diet vs location of egg deposition.

		Location of egg deposition:	
		Internal	Epithelial
Habitat and diet:	Pelagic	1	1
	Benthic	12	8

proportion of all species of *Huffmanella* that deposit eggs in an internal tissue of a benthic definitive host; compared to the proportion of *Huffmanella* species that deposit eggs in a surface tissue of a pelagic definitive host.

RESULTS

Analysis of definitive host species to suggest intermediate hosts

The centrarchid *Lepomis auritus* is alien to the SMR (TISI 2015) and wild-caught individuals exhibit a high prevalence and high intensity of infection with *Huffmanella huffmanii* (Table 6). Behavioural observations indicated that individual *L. auritus* cooperated with conspecifics to ward off other centrarchid species from their preferred source of food – the benthic invertebrates around spring openings. This spring-dominating behaviour was only displayed by adult *L. auritus*, which also excluded smaller *L. auritus* from the spring openings. The adults of *L. auritus* had a prevalence of 96% and a mean rating of intensity (as defined above) of 2.8 ($n = 53$). Adults of *Micropterus salmoides* (Lacépède), despite conventional assumption of being top predators, proved to be opportunistic generalists, and were also frequently observed feeding on small benthic spring invertebrates (they were apparently too large to be excluded by *L. auritus*). *Micropterus salmoides* was also heavily infected (Table 6).

To evaluate our assumption that the intermediate host of *H. huffmanii* is a benthic detritivore, three factors were used to predict infection intensity rating and prevalence. The three factors were fish species, standard length of fish (SL) and assigned feeding-niche category. The niche model was shown to be superior to the other models (Table 7), which is consistent with the intermediate host being a benthic detritivore.

Selection of candidate intermediate hosts

Of all the invertebrate taxa exposed to eggs of *Huffmanella huffmanii*, only amphipods of the genera *Hyaella* and *Gammarus* became infected. All of the *Hyaella* that were exposed to eggs of *H. huffmanii* became infected with larvae of *H. huffmanii*, including the *Hyaella* spp. that do not co-occur with *H. huffmanii* in the SMR (Table 3). Only one of the tested species of *Hyaella* is known to be endemic to the SMS (MLDW – unpubl. data) and therefore probably coevolved with *H. huffmanii*. Only three *Gammarus*

Table 6. Prevalence of infection and mean of intensity rating categories of eggs of *Huffmanella huffmanii* Moravec, 1987 in swim bladders of wild-caught centrarchids from the San Marcos Springs.

Species	Mean of intensity rating categories ¹	Prevalence (%)	n
<i>Micropterus salmoides</i>	3.00	90	10
<i>Lepomis auritus</i> ²	2.64	89	56
<i>Lepomis miniatus</i>	1.83	67	6
<i>Lepomis microlophus</i>	1.71	57	7
<i>Lepomis macrochirus</i>	0.17	8	12

¹ categories as defined in Materials and Methods; ² calculated from 53 adults and 3 uninfected juveniles.

Table 7. Akaike information criterion (AIC) weights of competing models used to predict infection intensity ratings of *Huffmanella huffmanii* Moravec, 1987 in wild-caught centrarchids.

Model	F ratio	p	AIC weight
SL	8.23	0.00581	0.0
Species	9.33	6.65 ⁻⁰⁷	0.057
Niche	48.33	4.15 ⁻⁰⁹	0.943

individuals were exposed to eggs of *H. huffmanii*; one died, one was lightly infected and the third was uninfected. All subsequent attempts to experimentally infect centrarchids employed the SMS *Hyaella* and *Hyaella* cf. *azteca* as the experimental intermediate hosts.

Exposure of susceptible hosts

If no amphipods had died during the exposure phase, a total of 36 exposed amphipods would have been available for necropsy on the three examination days (0, 3 and 7 days of exposure). However, because infected amphipods began dying in some of the replicate containers after the examinations had been started; only 30 amphipods were necropsied for infection-intensity rating. The regression slope of infection intensity on exposure duration was not significant.

After larvae had spent 1 week in experimental amphipods, internal development was observed in the larvae (Fig. 1). Although no increase in the length of larvae was observed (larvae were always 270–350 μ m long) and no moults were observed (even after 9 weeks of incubation in an amphipod), it can be assumed that they were second-stage larvae. The internal development was not accompanied by a change in the activity level of the larvae. Of the fish that had been fed amphipods containing larvae, only those that had been fed amphipods incubated for at least 5 days post exposure became infected, suggesting the prepatent period in the intermediate host is 5 days and is likely associated with the aforementioned internal development. After this initial duration of incubation in the intermediate host, no difference in infectivity was observed for longer incubation durations.

Dead larvae of *H. huffmanii* (Fig. 2) were often observed in some of the experimental amphipods exposed to eggs of *H. huffmanii*. Surprisingly, dead larvae were only observed in individuals of the presumably co-evolved endemic SMS

Fig. 1. Naturally hatched larvae of *Huffmanella huffmanii* Moravec, 1987 after being consumed by *Hyalella* spp. **A** – a freshly hatched larva containing loose refractile bodies (reserve granules); **B** – 30 days after hatching, with none of the loose refractile bodies of freshly hatched larvae apparent, but instead, a single condensed central structure is visible throughout the posterior end of the worms.

Fig. 2. Photomicrograph of a dead and partially calcified larva of *Huffmanella huffmanii* Moravec, 1987 from San Marcos Springs *Hyalella* sp. about 5 days post exposure.

Fig. 3. Photomicrograph of numerous presumably fertilised, freshly deposited eggs of *Huffmanella huffmanii* Moravec, 1987 recovered from an experimentally infected centrarchid incubated for 7.5 months post-infection. Note the apparent lack of shells and polar plugs, and with the contents of eggs enclosed by the spinose membrane alone.

Hyalella sp. and never in any of the other exposed amphipods. In some cases, dead larvae had begun showing signs of calcification in SMS *Hyalella* sp. after as little as 4–6 days post exposure. Aside from this presumed immune response by SMS *Hyalella* sp., the life span of the larva of *H. huffmanii* in the amphipod host seems to be limited by the life span of the intermediate host, since numerous larvae were found alive and active 50 days after last exposing their amphipod host to eggs. Individuals of *Hyalella* spp. were shown to be capable of living for up to 7 months in our laboratory cultures, but it is unlikely that they live past 4 months in the wild (Othman and Pascoe 2001). No larvae of *H. huffmanii* were incubated in *Hyalella* for the duration of the life span of the *Hyalella*, and there was no evidence of progressive decline in number of live larvae at 9 weeks post exposure in *H. cf. azteca*.

Eggs of *H. huffmanii* stored for more than 21 months in a sealed plastic container outside the definitive host

were still infective to *Hyalella*, at which point the eggs still showed no sign of decreased infectivity to amphipods. This suggests that eggs released naturally into the environment could remain viable for well over two years and still be infective to amphipods.

None of the experimental fish incubated for less than 4.5 months post exposure showed any signs of infection. Of 20 fish that had been incubated for at least 4.5 months after being fed experimentally infected amphipods (that had incubated for 5+ days post exposure), 70% showed evidence of infection. Most of these fish were only lightly infected, with increasing evidence of infection as the incubation duration of the definitive host progressed. The first confirmed deposition of apparently fertilised eggs was not until after 7.5 months of incubation post exposure (Fig. 3).

Fig. 4. Eggs of *Huffmanella huffmanii* Moravec, 1987 at various stages of development recovered from experimentally infected centrarchids incubated for 11.5 months post-infection. **A** – eggs showing evidence of larvation and having fully formed shells and visible polar plugs; **B** – lower resolution photomicrographs of swim bladder tissue contrasting darker larvated eggs with lighter, more recently deposited eggs.

Fig. 5. *Huffmanella huffmanii* Moravec, 1987 recovered from the swim bladder of a fish experimentally exposed to eggs of *H. huffmanii* 6.5 months ago. **A** – gross view entire worm; **B** – view of stichosome showing alternation of dark and light stichocytes typical of *Huffmanella* spp.; **C** – view of stichosome showing visible stichocyte nuclei and alternating dark (black arrows) and light (white arrows) stichocytes.

However, it was not until after 9 months of incubation that the intensity of infection with eggs in experimentally infected fish appeared to be as heavy as infections observed in the wild; however, none of these eggs were larvated at 9 months. Partially larvated eggs were first observed after 10.5 months of incubation. Fully larvated eggs (Fig. 4) were not observed until 11.5 months post-exposure of the definitive host. Only experimental eggs from fish incubated for at least 11.5 months post infection were able to in-

fect *Hyaella* amphipods, thus experimentally completing the life cycle and overlapping one full generation with the next in captivity. Interestingly, numerous adults (Fig. 5) were observed in fish incubated for up through 9 months, but no adults could be found in infected fish examined at 10.5 months post exposure. This suggests that all the adults had naturally expired at about 10 months of age, since no additional infected amphipods had been fed to the fish after the initial exposure. Infection results are presented in

Table 8. Results of feeding amphipods experimentally infected with larvae of *Huffmanella huffman* Moravec, 1987 to centrarchids.

Amphipod taxon	Amphipods fed out (n)	Duration of incubation in fish (months)	Infected (Y/N = 1/0)	Comments
SMS <i>Hyaella</i> sp.	3	4.5	1	2 mm piece of posterior of 1 subadult female
SMS <i>Hyaella</i> sp.	4	5	1	3 worms 500 × 12 µm; probably L ₃ or L ₄
SMS <i>Hyaella</i> sp.	3	6.5	1	3 adult worms 600 × 25 µm
SMS <i>Hyaella</i> sp.	3	7.5	1	Early stage eggs lacking eggs shells found in swim bladder tissue
SMS <i>Hyaella</i> sp.	6	7.5	1	One unembryonated egg
SMS <i>Hyaella</i> sp.	8	7.5	1	Anterior section of one adult (based on morphometry)
SMS <i>Hyaella</i> sp.	5	7.5	1	One young adult male
SMS <i>Hyaella</i> sp.	9	7.5	1	10 000 of freshly laid unembryonated eggs; numerous adults
<i>Hyaella</i> cf. <i>azteca</i>	7	8.75	1	10 000 of freshly laid eggs (unshelled); 10 of active adults; <1% eggs partially larvated
<i>Hyaella</i> cf. <i>azteca</i>	9	10.5	1	10 000 of partially larvated eggs, eggs had shells indicating they were more advanced in development
SMS <i>Hyaella</i> sp.	5	12	1	10 000 of eggs; many fully larvated
SMS <i>Hyaella</i> sp.	21	12	1	10 000 of eggs; many fully larvated
<i>Hyaella</i> cf. <i>azteca</i>	21	12	1	100 000 of eggs, many fully larvated; intensity approaching that of wild-caught fish

SMS – San Marcos Springs.

Fig. 6. Diagram showing steps in the life cycle of *Huffmanella huffman* Moravec, 1987 (not to scale). **A** – adults lay eggs in definitive host; at least 7.5 months must pass between ingestion of viable larva and the appearance of fertilised eggs in the host swim bladder, but eggs do not become fully larvated and infective to amphipods until 11.5 months post infection (p.i.): **1** – centrarchid definitive host; **2** – photomicrograph of swim bladder tissue showing eggs in various stages of development; **B** – release of eggs from definitive host; **3** – definitive host dies and decomposes to release eggs; **4** – definitive host is consumed by piscivorous fish and viable eggs with sufficiently developed shells pass out in faeces; **C** – eggs free in sediments: **5a** – young eggs (<10 months p.i.) with inadequately developed shells will not survive in environment or passage through piscivore gastrointestinal (GI) tract; **5b** – unlarvated eggs old enough to have fully developed egg shells (>10 months p.i.) can survive in environment and through GI tract of piscivore, but are uninfected to amphipods until they become fully larvated while in sediments; **5c** – fully larvated eggs (≥11.5 months p.i.) are immediately infective to amphipods and are still infective to amphipods after 36+ months in sediments; **D** – fully larvated eggs ingested by amphipod: **6** – larvae hatch and migrate to hemocoel where they must remain for at least 5 days p.i. before being infective to definitive host; **7** – larva in hemocoel of amphipod and infective to centrarchid. Life cycle can be completed in a theoretical minimum of 12 months, but can probably take longer than 36 months.

Table 9. Comparison of diets, habitats, organs, and localities for reported populations of species of *Huffmanella* Moravec, 1987.

Species	Host taxon	Host diet	Host habitat	Organ infected	Classification	Locality	Reference
<i>Huffmanella balista</i> Justine, 2007	<i>Abalistes stellatus</i> (Anonymus) (Balistidae)	Invertebrates	Littoral, reefs	Outer swim bladder wall	Benthic/internal	New Caledonia	Justine 2007
<i>Huffmanella banningi</i> Moravec, 1987	<i>Cynoglossus browni</i> (Chabanaud) (Cynoglossidae)	Invertebrates	Littoral	Musculature	Benthic/internal	Atlantic Ocean, Senegal and Congo	Moravec 1987
<i>Huffmanella branchialis</i> Justine, 2004	<i>Nemipterus furcosus</i> (Valenciennes) (Nemipteridae)	Invertebrates	Littoral, neritic	Gill mucosa	Benthic/external	Nouméa, New Caledonia	Justine 2004
<i>Huffmanella canadensis</i> Moravec, Conboy et Speare, 2005	<i>Sebastes</i> spp. (Sebastidae)	Invertebrates	Sublittoral, bathyal	Skin	Benthic/external	Clayoquot Sound, British Columbia, Canada	Moravec et al. 2005
<i>Huffmanella carcharhini</i> (MacCallum, 1925)	<i>Carcharinus plumbeus</i> (Nardo) (Carcharhinidae)	Invertebrates and fishes	Littoral, sublittoral, bathyal	Skin, gill mucosa	Benthic/external	Hawaii, North Carolina	MacLean et al. 2006, Bullard et al. 2012
<i>Huffmanella filamentosa</i> Justine, 2004	<i>Gymnocranius grandoculis</i> (Valenciennes) (Lethrinidae)	Invertebrates	Littoral, neritic, reefs	Gill mucosa	Benthic/external	Nouméa, New Caledonia	Justine 2004
<i>Huffmanella hano</i> Justine et Iwaki, 2014	<i>Muraenesox cinereus</i> (Forsskal) (Muraenesocidae)	Fishes and some benthic invertebrates	Littoral	Musculature	Benthic/internal	Japan	Justine and Iwaki 2014
<i>Huffmanella huffmanii</i> Moravec, 1987	Centrarchidae Bleeker	Invertebrates	Spring-inhabiting centrarchids	Inner swim bladder wall	Benthic/internal	San Marcos River head-springs, Hays County, Texas, USA	Moravec 1987, Huffman and Moravec 1988, Cox et al. 2004, present study
<i>Huffmanella japonica</i> Moravec, Koudela, Ogawa et Nagasawa, 1998	<i>Upeneus bensasi</i> (Houttuyn) (Mullidae)	Invertebrates	Littoral, reefs	Musculature	Benthic/internal	Inland Sea of Japan, off Shikoku Island	Moravec et al. 1998
<i>Huffmanella lata</i> Justine, 2005	<i>Carcharinus amblyrhynchos</i> (Bleeker) (Carcharhinidae)	Invertebrates and fishes	Littoral, reefs, sublittoral	Outer skin between two gill openings	Benthic/external	Nouméa, New Caledonia	Justine 2005
<i>Huffmanella longa</i> Justine, 2007	<i>Gymnocranius grandoculis</i> (Lethrinidae)	Invertebrates	Littoral, sublittoral, bathyal	Mesentery, outer swim bladder wall, body walls	Benthic/internal	New Caledonia	Justine 2007
<i>Huffmanella markgracei</i> Ruiz et Bullard, 2013	<i>Rhizoprionodon terraenovae</i> (Richardson) (Carcharhinidae)	Invertebrates and fishes	Littoral, reefs, sublittoral	Outer skin, underlying musculature, gill filaments	Benthic/external	Gulf of Mexico	Ruiz and Bullard 2013
<i>Huffmanella mexicana</i> Moravec et Fajer-Avila, 2000	<i>Sphoeroides annulatus</i> (Jenyns) (Tetraodontidae)	Invertebrates	Littoral, reefs	Inner layer of swim bladder wall	Benthic/internal	Mazatlán, Sinaloa State, Mexico	Moravec and Fajer-Avila 2000
<i>Huffmanella moravecii</i> Carballo, Navone et Cremonese, 2011	<i>Odontesthes</i> spp. (Atherinopsidae)	Invertebrates and small fishes	Littoral	Gill mucosa, skin	Benthic/external	Nuevo and San José gulfs, Argentinean Sea	Carballo et al. 2011
<i>Huffmanella oleumimica</i> Ruiz, Ray, Cook, Grace et Bullard, 2013	<i>Lutjanus campechanus</i> (Poey) (Lutjanidae)	Invertebrates and fishes	Littoral, sublittoral	Skin	Benthic/external	Gulf of Mexico	Ruiz et al. 2013
<i>Huffmanella ossicola</i> Justine, 2004	<i>Bodianus</i> spp. (Labridae)	Invertebrates	Littoral, reefs	Most bones, gill arch bones	Benthic/internal	Nouméa, New Caledonia	Justine 2004, Justine 2007
<i>Huffmanella paronai</i> Moravec et Garibaldi, 2000	<i>Xiphias gladius</i> Linnaeus (Xiphiidae)	Fishes and invertebrates	Pelagic	Skin	Pelagic/external	Ligurian Sea, Italy	Moravec and Garibaldi 2000
<i>Huffmanella plectropomi</i> Justine, 2011	<i>Plectropomus leopardus</i> (Serranidae)	Invertebrates and fishes	Reefs	Mesentery near swim bladder wall	Benthic/internal	Nouméa, New Caledonia	Justine 2011
<i>Huffmanella schouteni</i> Moravec et Campbell, 1991	<i>Hirundichthys affinis</i> (Günther), <i>Cheilopogon</i> spp. (Exocoetidae)	Zooplankton and small fishes	Epipelagic	Serosa of intestine, swim bladder	Pelagic/internal	Caribbean Sea, Curaçao, and Ligurian Sea, Italy	Moravec and Campbell 1991, Moravec and Garibaldi 2003
<i>Huffmanella shikokuensis</i> Moravec, Koudela, Ogawa et Nagasawa, 1998	<i>Stephanolepis cirrhifer</i> (Temminck et Schlegel) (Monacanthidae)	Invertebrates	Reefs	Musculature	Benthic/internal	Inland Sea of Japan, off Shikoku Island	Moravec et al. 1998
<i>Huffmanella</i> sp.	<i>Pentapodus aurofasciatus</i> Russell (Nemipteridae)	Invertebrates and zooplankton	Reefs	Mucosa of mouth and gills	Benthic/external	Nouméa, New Caledonia	Justine 2004
<i>Huffmanella</i> sp.	<i>Trisopterus luscus</i> (Linnaeus) (Gadidae)	Invertebrates	Littoral, sublittoral, bathyal	Musculature	Benthic/internal	Atlantic Ocean, Portugal	Esteves et al. 2009
<i>Huffmanella</i> sp.	<i>Genypterus blacodes</i> (Forster) (Ophidiidae)	Invertebrates and small fishes	Littoral, sublittoral	Musculature	Benthic/internal	Pacific Ocean, New Zealand, South Island	Moravec 2001

greater detail in Table 8 and Fig. 6 is a summary of what is now known of the life cycle of *H. huffman*.

Relationship between definitive host diet and location of egg deposition for *Huffmanella* spp.

A survey of the literature revealed that *Huffmanella* spp. have been reported from 18 families of marine fish and one family of freshwater fishes (Table 9). The organ of egg deposition for species of *Huffmanella* (Table 9) appeared to be somewhat predictable by the habitat of the host (Table 5). Of the 20 benthic definitive host species reported in the literature, 60% were parasitised by a *Huffmanella* species that lays eggs in an internal tissue, whereas, 50% of the two reported pelagic host species were parasitised by a species of *Huffmanella* which lays eggs in a surface tissue.

DISCUSSION

Nine species of centrarchids from three genera were observed to be susceptible to infection with *Huffmanella huffman* (see Cox 1998). However, infection prevalence and mean intensity rating was not evenly or randomly distributed across naturally infected centrarchid species (Michel 1984, Underwood and Dronen 1984, Cox et al. 2004, O'Docharty 2007, present study). This uneven distribution of infection with *H. huffman* across wild-caught centrarchid taxa was determined not to be a function of physiological host specificity, but rather a function of the feeding niches of the various centrarchid species. Indeed, it would seem that the internal environment of all centrarchids is probably equally hospitable to *H. huffman*, as is demonstrated by the susceptibility¹ of *Ambloplites rupestris* Rafinesque, despite that species being only distantly related to *Lepomis* Rafinesque and *Micropterus* Lacepède, which are sister genera (Near et al. 2005). Despite their demonstrated susceptibility, wild-caught centrarchid species that we classified as limnetic invertivores were almost never infected in the wild-caught SMS collections, whereas wild-caught individuals of the more benthic centrarchids that feed on interstitial invertebrates were almost always infected. This is consistent with the conjecture that the intermediate host must be a benthic detritivorous invertebrate, and is further consistent with the observation that the eggs are appreciably denser than water. This conjecture was also well corroborated by the AIC contrast analysis in which the niche model explained, by far, the most variance in infection intensity (AIC weight = 0.943, Table 7).

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

Hyaella as its intermediate host, since this genus is one of the most abundant invertebrate genera in the SMR headsprings; thus, this is a more evolutionarily stable strategy than requiring a rare organism. The undescribed spring-run endemic *Hyaella* species (referred to herein as SMS *Hyaella* sp.) is restricted to the same geographic distribution as *H. huffman*, which initially led us to assume that some aspect of host specificity to this particular *Hyaella* species was responsible for limiting the downstream distribution of *H. huffman*. However, the distribution of the SMS *Hyaella* sp. does not appear to be limiting the distribution of *H. huffman*, since multiple other species of *Hyaella* were also experimentally demonstrated to be capable of serving as functional intermediate hosts. In fact, the only amphipod species which showed any sign of being less suitable was the endemic SMS *Hyaella* sp., which, presumably, coevolved with *H. huffman*, but which often killed the larvae shortly after infection. Ultimately, both species of *Hyaella* that occur in the SMR experimentally transferred heavy infections to the definitive host. However, the greatest intensity of experimental infection was observed in definitive hosts that had been fed infected *H. cf. azteca*, suggesting that the presumably coevolved SMS *Hyaella* sp. seems to have evolved immune responses to *H. huffman* that are sufficient to appreciably suppress the transmission of *H. huffman* infections to the definitive hosts.

Species of *Hyaella* are widespread in freshwaters, with a cosmopolitan distribution across the Americas, and without a single population reported from a marine environment (Serejo 2004, Witt et al. 2006, Wellborn and Broughton 2008). This observation presents an intriguing enigma, that is: “Why is it that *H. huffman* cannot be found throughout all localities where *Hyaella* spp. and centrarchids coexist, given that the former can be found co-occurring with centrarchids contiguously throughout much of North America, and *H. huffman* seems to be able to infect numerous species of *Hyaella* and centrarchids?”

This makes it difficult to explain the extremely restricted geographic distribution of *H. huffman*, which is obviously not due to the distributional limits of either its intermediate or definitive hosts. This restricted distribution has been thoroughly documented by a prior study on the distribution of *H. huffman* (Cox et al. 2004) and corroborated by the present study. Both studies determined that the farthest downstream point where *H. huffman* can be found is in the proximity of John J. Stokes San Marcos River City Park (ca 7 river km; 29°52'09"N; 97°55'43"W) which is close to the downstream extent of the physicochemically stable spring-run waters (Groeger et al. 1997). Because the remainder of the SMR has abundant *Hyaella cf. azteca* co-occurring with centrarchids, this leads us to the conclusion that the distribution of *H. huffman* is limited by factors of water physicochemistry.

It is curious that the intermediate host in the life cycle of this marine relict is an exclusively freshwater taxon (Serejo 2004), with all species of the genus *Hyaella* being found in

¹ Based on unpublished accounts from several *Ambloplites rupestris* wild-caught from the San Marcos River and examined in parasitology laboratory exercises at Texas State University.

freshwaters in the Americas. However, the family in which *Hyalella* is contained (Dogielinotidae Gurjanova) is itself contained in the superfamily Talitroidea Rafinesque, which is a highly diverse group of amphipods containing terrestrial, semi-terrestrial, freshwater and marine species (Serejo 2004). The nearest family to the Dogielinotidae appears to be Hyalidae Bulychewa, which is a talitroid amphipod family that has numerous species with a circumpolar distribution in littoral and intertidal marine environments (Serejo 2004). Therefore, it is possible that talitroids are serving as the intermediate hosts of at least some of the other species of *Huffmanella*. However, though many of the definitive hosts of marine species of *Huffmanella* have a diet consisting of benthic invertebrates, the majority of these hosts are not restricted to just littoral and intertidal habitats.

The successful infection of *Gammarus* sp., though very low in intensity, suggests that some marine *Huffmanella* may use species of Gammaridea as intermediate hosts. This suborder is extremely diverse and occurs in numerous benthic habitats across the oceans of the world (Raupach et al. 2015). However, the two species of marine *Huffmanella* with strictly pelagic blue-water definitive hosts (Moravec and Campbell 1991, Moravec and Garibaldi 2000) are paradoxical, because these fishes are unlikely to come into contact with any benthic invertebrates. With the eggs of *H. huffmanii* being appreciably denser than water (including hyper-saline water at 45 ppt), it is difficult to imagine how the eggs of these parasites of pelagic species, assuming their eggs are also denser than water, ever become consumed by a susceptible intermediate host that could then possibly be later consumed by the pelagic definitive host species. One pelagic definitive-host species, *Xiphias gladius* Linnaeus, is almost exclusively piscivorous on other pelagic fishes (Palko et al. 1981), which suggests the possibility of a second intermediate or paratenic host in the life cycle of *Huffmanella paronai* Moravec et Garibaldi, 2000.

The epipelagic host fishes in the family Exocoetidae are perhaps the most curious of all described host fishes of *Huffmanella*, as they are never found in benthic waters and feed almost exclusively on zooplankton (Casazza and Ross 2008), thus eliminating the possibility of a second intermediate host fish that transports the larvae of *Huffmanella* from the benthos to the definitive host. Aside from these two species, it is highly likely that the intermediate hosts in the life cycle of the other marine *Huffmanella* species are benthic invertebrates. If the host specificity of *Huffmanella* spp. is at all conserved at the first intermediate host level, then all *Huffmanella* spp. are probably using amphipods as intermediate hosts and most likely gammarideans.

It appears that the definitive host is crucial to the larvation of eggs of *H. huffmanii*. Freshly laid F₁ eggs that had not yet formed a shell inside the spinose membrane were placed in a sealed container filled with artesian water and were kept thermally stable in a water bath of artesian water at 22 °C. None of these eggs larvated and all soon began to decay. However, eggs which had already developed a shell successfully larvated while incubating in water. These observations suggest that eggs cannot survive or larvate out-

Fig. 7. Eggs of *Huffmanella huffmanii* Moravec, 1987 from a wild caught centrarchid showing gradient of development early in embryogenesis. Left most egg has contents contained solely inside spinose membrane and is therefore thought to have been laid recently. Egg second from left has developed egg shell and polar plugs indicating it as laid some time ago. The two right most eggs show evidence of darkening shells and embryogenesis indicating they were earliest of the four eggs to be laid.

side of the host until they have formed a shell (examples of developmental stages of eggs shown in Fig. 7).

Based on the observation that (1) eggs collected from wild-caught fish are able to survive at least 21 months without a noticeable drop in vitality and (2) eggs are not larvated and infective until approximately 4 months after being laid, it is likely that eggs of *H. huffmanii* cannot infect an intermediate host and thus advance the life cycle for at least one year after the fish first becomes infected, but can remain infective for several years after the eggs have been laid. However, it is very unlikely that the minimum life cycle time would be realised in nature, i.e. an egg would be available for consumption by an amphipod as soon as it is fully larvated, and that that amphipod would then be eaten by a centrarchid 5 days post infection.

Therefore, the typical life cycle time would probably be much longer than the one year theoretical minimum observed experimentally. Because it is not yet known how long eggs can survive, it is impossible to estimate a functional generation time; however, given our results it is almost certain that there is substantial overlap between putative generations. Therefore, the possibility for cross-generational gene flow and a lack of discrete generations is highly likely. Based on these findings it is likely that the average generation time is several years, with the majority of each individual's life span spent as an egg.

The prepatent period of *H. huffmanii* in the intermediate host appears to be very short, while quite long in the definitive host. Amphipods that had been incubated with larvae of *H. huffmanii* for only 5 days successfully transferred infection to the definitive host, even though no development was observed in the larvae until they had incubated in the intermediate host for at least 7 days. Despite the requirement of only modest developmental changes in the intermediate host before *H. huffmanii* becomes infective to the definitive host, all evidence available at this time suggests that the amphipod host is very likely an obligate intermediate host, and that the life cycle cannot be completed directly.

The prepatent period in the definitive host appears to be relatively long for a trichinelloid nematode, as most trichinelloid species for which the life cycle is known usually have a prepatent period around three months (Moravec et al. 1998, Køie and Nylund 2001). *Huffmanella huffmanii* is remarkable in that it appears that the prepatent period is at least 7.5 months at 22°C as no eggs were observed until after 7.5 months of incubation and high intensities of eggs were not observed until after nine months of incubation. After egg deposition, larvation appears to take about five months, as the first nearly larvated eggs were not recovered from experimentally infected fish until five months after the first egg deposition was observed. Because this is the first life cycle of species of *Huffmanella* to be understood, it is not known if a long prepatent period is a conserved trait across any of the other *Huffmanella* spp.

According to Anderson (2000), trichinelloids always infect the 'final' (= definitive) host in the L₁ stage even if a sojourn in an intermediate host (e.g. earthworms) is necessary for larvae to become infective. However, the larval morphogenesis in trichinelloids remains practically unknown, and data on the morphology of individual larval stages are almost absent. Generally, trichinelloid first-stage larvae (L₁) have a stylet, which may also be present in some other stages and adults (Anderson 2000), and their posterior body end is widely rounded, with a subterminal anus. Description and illustration of the first-stage larva of *H. huffmanii* expressed from the larvated egg (F.M. and D.G.H. – unpubl. data in Moravec 2001) and also its photomicrograph provided by Cox et al. (2004) show clearly that it is a typical trichinelloid first-stage larva. As in capillariids, no moulting of larvae of *H. huffmanii* was observed inside the egg capsule. On the basis of experimental studies and data from the literature, Shlikas (1978) concluded that it is a common feature of all aphasmid nematodes that their larvae do not moult within the eggshells. However, as mentioned by Moravec et al. (1987), van Banning (1980) published a photomicrograph of the larva expressed from the egg of '*Capillaria spinosa*' (= *Huffmanella banningi* Moravec, 1987), a histozoic parasite of tonguefish. This clearly indicates that the larva was undergoing its moult and, therefore, it should be considered the second-stage larva. In contrast, Køie and Nylund (2001) concluded that it is the third-stage larva that hatches from the eggs of capillariids. Obviously, more studies would be helpful in resolving this conundrum.

Based on literature data, the life cycles of some capillariid nematodes are direct (homoxenous), without participation of an intermediate host; whereas those of others are obligately heteroxenous in which the intermediate host is required. In the homoxenous life cycles, all nematode stages (L₁–L₅) occur in the definitive host during the parasite's development. Sometimes a paratenic host harbouring the L₁ may be included, but this is not obligatory for completing the life cycle. On the other hand, in capillariids with known heteroxenous cycles, the first moult of larvae occurs in the intermediate host, so that only the second-stage larvae (L₂) are infective for the definitive host (Moravec et al. 1987).

The second moult of capillariid larvae in the earthworm intermediate host was observed, e.g. by Shlikas (1967) in *Aonchotheca bursata* (Freitas et Almeida, 1934) and *A. caudinflata* (Molin, 1858), both parasites of domestic fowl and some other birds, and by Romashov (1980) in *A. erinacei* (Rudolphi, 1819), a parasite of the European hedgehog *Erinaceus europaeus* Linnaeus. According to Shlikas (1967), in contrast to the L₁ larvae, the L₂ larvae have no stylet (although a stylet was reported by Morehouse 1942 in L₂ of *A. caudinflata*) and their body is much less granulated. Taking this into account, the more fully developed larvae of *H. huffmanii* obtained from experimentally infected *Hyalomma* spp. (Fig. 1B) apparently represent second-stage L₂ larvae, even though no evidence of larval moult was observed, and we detected no increase in larval dimensions while in the intermediate host. These L₂ larvae of *H. huffmanii* have no stylet, and none of the loose refractile bodies (reserve granules) that are obvious in freshly hatched L₁ are apparent in the L₂ larvae.

Unfortunately, very little information about larval morphogenesis and the number of moults in the intermediate host has been provided by the existing life cycle studies of trichinelloids with heteroxenous cycles. Mostly an increase in size of larvae during their development in the intermediate host has been reported. For example, the body length of larval *Cystoopsis acipenseris* Wagner, 1867 (Cystoosidae Skryabin, 1923) increases in gammarids from 170 µm to about 380 µm (Janicki and Rašin 1930); larvae of *Paracapillaria philippinensis* (Chitwood, Velázquez et Salazar, 1968) in fish intermediate hosts increase from 130–150 µm to 250–300 µm (Cross 1992); larvae of *Capillaria gracilis* (Bellingham, 1840) in the fish intermediate host increase from 300 µm to 700 µm (Køie and Nylund 2001); and larvae of *Schulmanella petruschewskii* (Shulman, 1948) in oligochaetes increase from 240–350 µm to 760–1250 µm (Kutzer and Otte 1966). Because of the substantial growth reported for these larvae and considering the fact that these larvae can survive for at least several months in the intermediate host, Moravec et al. (1987) speculated that probably more than one moult is probably occurring in the intermediate host. In such a case, it could be expected that both L₂ and L₃ are infective for the definitive host. If larvae of *H. huffmanii* in amphipods become infective for the fish host 5 days p.i., as was found in this study, then the infective stage is L₂, which is also indicated by the body size of these larvae (much the same as that of L₁) much like capillariid larvae which have their first moult within about a week of hatching (see Kasparsone 1984).

Crustaceans are rarely found to be the intermediate hosts of trichinelloids. Although Markevich (1951) incidentally mentions gammarids (Amphipoda) to be the intermediate hosts of the capillariid *Piscicapillaria tuberculata* (von Linstow, 1914), no relevant source for his report was found in the literature. Most data on capillariid life cycles are on species parasitising birds and mammals. Consequently, the most frequently recorded intermediate hosts have been ascertained to be various earthworms and less often other oligochaetes, and occasionally fishes. The only trichinelloid known to utilise crustaceans as intermediate host is

the above-mentioned *Cystoopsis acipenseris* (Cystoospiidae), a parasite of the skin of sturgeons, whose intermediate hosts were found to be three species of gammarids (Amphipoda) (Janicki and Rašin 1930). Accordingly, *Huffmanella huffman* is another trichinelloid with a crustacean (amphipod) intermediate host.

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REFERENCES

- ANDERSON R.C. 2000: Nematode Parasites of Vertebrates: Their Development and Transmission. CABI Publishing, Wallingford, 650 pp.
- ANDERSON R.C., CHABAUD A.G. WILLMOTT S. 2009: Keys to the Nematode Parasites of Vertebrates: Archival Volume. CABI Publishing, Wallingford, 463 pp.
- VAN BANNING P. 1980: The occurrence of black spots in the tongue sole, *Cynoglossus browni* Chabanaud, due to nematode eggs (*Capillaria spinosa*) previously described in the shark *Carcharhinus milberti* Muller & Henle. J. Fish Biol. 17: 305–309.
- BOWLES D.E., ARSUFFI T.L. 1993: Karst aquatic ecosystems of the Edwards Plateau region of central Texas, USA: a consideration of their importance, threats to their existence, and efforts for their conservation. Aquat. Conserv. 3: 317–329.
- BULLARD S.A., RUIZ C.F., McELWAIN A., MURRAY M.J., BORUCINSKA J.D. BENZ G.W. 2012: *Huffmanella* cf. *carcharhini* (Nematoda: Trichosomoididae: Huffmanellinae) from skin of a sandbar shark, *Carcharhinus plumbeus*, in the Pacific Ocean. J. Parasitol. 98: 333–340.
- CARBALLO M.C., NAVONE G.T. CREMONTE F. 2011: Parasites of the silversides *Odontesthes smitti* and *Odontesthes nigricans* (Pisces: Atherinopsidae) from Argentinean Patagonia. Comp. Parasitol. 78: 95–103.
- CASAZZA T.L., ROSS S.W. 2008: Fishes associated with pelagic *Sargassum* and open water lacking *Sargassum* in the Gulf Stream off North Carolina. Fish. Bull. 106: 348–363.
- COX M.K. 1998: The distribution and life cycle of *Huffmanella huffman* (Nematoda: Trichosomoididae). MSc. thesis, Southwest Texas State University, San Marcos, 54 pp.
- COX M.K., HUFFMAN D.G. MORAVEC F. 2004: Observations on the distribution and biology of *Huffmanella huffman* (Nematoda: Trichosomoididae). Folia Parasitol. 51: 50–54.
- CROSS J.H. 1992: Intestinal capillariasis. Clin. Microbiol. Rev. 5: 120–129.
- DIAZ P.H., ALEXANDER M.L. 2010: Aquatic macroinvertebrates of a spring-fed ecosystem in Hays County, Texas, USA. Ent. News. 121: 478–486.
- EDWARDS S.W., ARNOLD C.R. 1961: The caddis flies of the San Marcos River. Tex. J. Sci. 13: 398–415.
- ESTEVEZ A., SEIXAS F., CARVALHO S., NAZÁRIO N., MENDES M. MARTINS C. 2009: *Huffmanella* sp. (Nematoda: Trichosomoididae) muscular parasite from *Trisopterus luscus* captured off the Portuguese coast. Dis. Aquat. Organ. 84: 251–255.
- GIBSON J.R., HARDEN S.J., FRIES J.N. 2008: Survey and distribution of invertebrates from selected springs of the Edwards Aquifer in Comal and Hays Counties, Texas. Southwest. Nat. 53: 74–84.
- GROEGER A.W., BROWN P.F., TIETJEN T.E. KELSEY T.C. 1997: Water quality of the San Marcos River. Tex. J. Sci. 49: 279–294.
- HASSLINGER M.A., SCHWARZLER C. 1980: Blasenwurm der Ratte, *Trichosomoides crassicauda*: Untersuchungen zu Entwicklung, Übertragung und Diagnose. Berl. Munch. Tierarztl. Wochenschr. 93: 132–135.
- HOLSINGER J.R., LONGLEY G. 1980: The Subterranean Amphipod Crustacean Funa of an Artesian Well in Texas. Smithsonian Institution Press, Washington D.C., 62 pp.
- HUFFMAN D.G., MORAVEC F. 1988: First description of adult *Huffmanella huffman* Moravec, 1987 (Nematoda: Trichosomoididae) from the swim bladder of centrarchid fishes of the upper San Marcos River, central Texas. Folia Parasitol. 35: 227–234.
- JANICKI K., RAŠIN K. 1930: Bemerkungen über *Cystoopsis acipenseris* des Wolga-Sterlets, sowie über die Entwicklung dieses Nematoden in Zwischenwirt. Z. Wissenschaft. Zool. 136: 1–37.
- JUSTINE J.L. 2004: Three new species of *Huffmanella* Moravec, 1987 (Nematoda: Trichosomoididae) from the gills of marine fish off New Caledonia. Syst. Parasitol. 59: 29–37.
- JUSTINE J.L. 2005: *Huffmanella lata* n. sp. (Nematoda: Trichosomoididae: Huffmanellinae) from the shark *Carcharhinus amblyrhynchos* (Elasmobranchii: Carcharhinidae) off New Caledonia. Syst. Parasitol. 61: 181–184.
- JUSTINE J.L. 2007: *Huffmanella* spp. (Nematoda, Trichosomoididae) parasites in coral reef fishes off New Caledonia, with descriptions of *H. balista* n. sp. and *H. longa* n. sp. Zootaxa 1628: 23–41.
- JUSTINE J.L. 2011: *Huffmanella plectropomi* n. sp. (Nematoda: Trichosomoididae: Huffmanellinae) from the coral grouper *Plectropomus leopardus* (Lacépède) off New Caledonia. Syst. Parasitol. 79: 139–143.
- JUSTINE J.L., IWAKI T. 2014: *Huffmanella hamo* sp. n. (Nematoda: Trichosomoididae: Huffmanellinae) from the dagger-tooth pike conger *Muraenesox cinereus* off Japan. Folia Parasitol. 61: 267–271.
- KASPARSONE Z.V. 1984: [New data on the development of the nematode *Capillaria corvorum* (Rudolphi, 1819) Travassos, 1915 in the organism of *Corvus frugilegus* L.]. Izv. AN Latv. SSR. 8: 115–117. (In Russian.)
- KØIE M., NYLUND A. 2001: The life-cycle of *Capillaria gracilis* (Capillariidae), a nematode parasite of gadoid fish. Sarsia 86: 383–387.
- KUTZER E., OTTE E. 1966: *Capillaria petruschewskii* (Schulman, 1948): Morphologie, Biologie und pathogene Bedeutung. Z. Parasitenkd. 28: 16–30.
- LÖWENSTEIN S. 1910: Epithelwucherungen und Papillombildungen der Rattenblase, verursacht durch ein *Trichosoma* (*Tr. crassicauda*?). Beitr. Klin. Chirurg. 69: 533–546.
- MACLEAN R.A., FATZINGER M.H., WOOLARD K.D. HARMS C.A. 2006: Clearance of a dermal *Huffmanella* sp. in a sandbar shark (*Carcharhinus plumbeus*) using levamisole. Dis. Aquat. Organ. 73: 83.
- MARKEVICH A.P. 1951: Parasitic Fauna of Freshwater Fish of the Ukrainian SSR. Publ. House of the USSR Acad. Sci., Kiev, 376 pp.
- MICHEL G.D. 1984: The Biology of *Capillaria* sp. (Nematoda: Capillariidae) from Swim Bladders of Sunfishes of the Upper San Marcos River. MS thesis, Southwest Texas State University, San Marcos, 59 pp.
- MORAVEC F. 1987: Revision of Capillariid Nematodes (subfamily Capillariinae) Parasitic in Fishes. Studie ČSAV No. 3. Academia, Praha, 144 pp.
- MORAVEC F. 2001: Trichinelloid Nematodes Parasitic in Cold-Blooded Vertebrates. Academia, Praha, 432 pp.

- MORAVEC F., CAMPBELL B.G. 1991: A new *Huffmanella* species, *H. schouteni* sp. n. (Nematoda: Trichosomoididae) from flying fishes in Curaçao. *Folia Parasitol.* 38: 29–32.
- MORAVEC F., CONBOY G.A., SPEARE D.J. 2005: A new trichosomoidid from the skin of *Sebastes* spp. (Pisces) from British Columbia, Canada. *J. Parasitol.* 91: 411–414.
- MORAVEC F., FAJER-AVILA E. 2000: *Huffmanella mexicana* n. sp. (Nematoda: Trichosomoididae) from the marine fish *Sphoeroides annulatus* in Mexico. *J. Parasitol.* 86: 1229–1231.
- MORAVEC F., GARIBALDI F. 2000: *Huffmanella paronai* sp. n. (Nematoda: Trichosomoididae), a new parasite from the skin of swordfish *Xiphias gladius* in the Ligurian Sea (Western Mediterranean). *Folia Parasitol.* 47: 309–314.
- MORAVEC F., GARIBALDI F. 2003: First record of *Huffmanella schouteni* (Nematoda: Trichosomoididae), a histozoic parasite of flying-fishes, in Europe. *Dis. of Aquat. Organ.* 57: 173–175.
- MORAVEC F., KOUDELA B., OGAWA K., NAGASAWA K. 1998: Two new *Huffmanella* species, *H. japonica* n. sp. and *H. shikokuensis* n. sp. (Nematoda: Trichosomoididae), from marine fishes in Japan. *J. Parasitol.* 84: 589–593.
- MORAVEC F., PROKOPIĆ J., SHLIKAS A.V. 1987: The biology of nematodes of the family Capillariidae Neveu-Lemaire, 1936. *Folia Parasitol.* 34: 39–56.
- MOREHOUSE N.F. 1942: Life cycle of *Capillaria caudinflata*, a nematode parasite of the common fowl. Iowa State College, Ames, Iowa, 133 pp.
- NEAR T.J., BOLNICK D.I., WAINWRIGHT P.C. 2005: Fossil calibrations and molecular divergence time estimates in centrarchid fishes (Teleostei: Centrarchidae). *Evolution.* 59: 1768–1782.
- O'DOCHARTY E.M. 2007: Studies on the Life Cycle of *Huffmanella huffman* (Nematoda: Trichosomoididae). MS thesis, Texas State University, San Marcos, Texas, 55 pp.
- OTHMAN M.S., PASCOE D. 2001: Growth, development and reproduction of *Hyalomma azteca* (Saussure, 1858) in laboratory culture. *Crustaceana* 74: 171–181.
- PALCO B.J., BEARDSLEY G.L., RICHARDS W.J. 1981: Synopsis of the biology of the swordfish, *Xiphias gladius* Linnaeus. NOAA Tech. Rep. NMFS Circ., 441 pp.
- RAUPACH M.J., BARCO A., STEINKE D., BEERMANN J., LAAKMANN S., MOHRBECK I., NEUMANN H., KIHARA T.C., POINTNER K., RADULOVIC A. 2015: The application of DNA barcodes for the identification of marine crustaceans from the North Sea and adjacent regions. *PLoS ONE* 10: e0139421.
- ROMASHOV B.V. 1980: Life cycle of *Capillaria erinacei* (Nematoda, Capillariidae). *Helminthologia* 17: 181–189.
- RUIZ C.F., BULLARD S.A. 2013: *Huffmanella markgracei* sp. n. (Nematoda: Trichosomoididae) from buccal cavity of Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Carcharhiniformes: Carcharhinidae), in the northwestern Gulf of Mexico off Texas. *Folia Parasitol.* 60: 353–358.
- RUIZ C.F., RAY C.L., COOK M., GRACE M.A., BULLARD S.A. 2013: A new species of Trichosomoididae (Nematoda) from skin of red snapper (Perciformes: Lutjanidae), on the Texas-Louisiana Shelf, Northern Gulf of Mexico. *J. Parasitol.* 99: 318–326.
- SEREJO C.S. 2004: Cladistic revision of talitroidean amphipods (Crustacea, Gammaridea), with a proposal of a new classification. *Zool. Scr.* 33: 551–586.
- SHLIKAS A.V. 1967: [Ontogenesis of the nematodes *Capillaria bur-sata* Freitas et Almeida, 1934 and *Capillaria caudinflata* (Molin, 1858) Travassos, 1915]. *Acta Parasitol. Lithuan.* 7: 119–130. (In Russian.)
- SHLIKAS A.V. 1978: [Studies on the development of the nematode *Trichocephalus leporis* Froelich, 1789 in the organism of the definitive host.]. *Acta Parasitol. Lithuan.* 16: 81–87. (In Russian.)
- THOMAS L.J. 1924: Studies on the life history of *Trichosomoides crassicauda* (Bellingham). *J. Parasitol.* 10: 105–136.
- TISI 2015: Redbreast Sunfish (*Lepomis auritus*). Texas Invasive Species Institute, Huntsville, Texas.
- UNDERWOOD H.T., DRONEN N.O. 1984: Endohelminths of fishes from the upper San Marcos River, Texas. *Southwest. Nat.* 29: 377–385.
- WAHL D.V., CHAPMAN W.H. 1967: The application of data on the survival of eggs of *Trichosomoides crassicauda* (Bellingham) to the control of this bladder parasite in laboratory rat colonies. *Lab. Anim. Care* 17: 386–390.
- WELLBORN G.A., BROUGHTON R.E. 2008: Diversification on an ecologically constrained adaptive landscape. *Mol. Ecol.* 17: 2927–2936.
- WITT J.S., THRELOFF D.L., HEBERT P.N. 2006: DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Mol. Ecol.* 15: 3073–3082.
- WORSHAM M.L.D., GIBSON R., HUFFMAN D.G. 2016: The aquatic annelid fauna of the San Marcos River headsprings, Hays County, Texas. *ZooKeys*: (in press).

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