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NOTE

VERIFICATION OF TWO CYPRINID HOST FISHES FOR THE TEXAS PIGTOE, *FUSCONAIA ASKEWI*

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ABSTRACT

We evaluated the suitability of three cyprinid fishes previously proposed as hosts for the state threatened Texas Pigtoe (*Fusconaia askewi*). We collected naturally infested fishes from the wild, held them in captivity until glochidial development and juvenile excystment occurred, and identified a subsample of juveniles to species using the mitochondrial gene ND1. The Red Shiner (*Cyprinella lutrensis*), Blacktail Shiner (*Cyprinella venusta*), and Bullhead Minnow (*Pimephales vigilax*) all carried glochidial infestations from May to August. Red Shiners and Blacktail Shiners produced large numbers of juvenile mussels (metamorphosis success = 29.4% and 46.3%, respectively), and all sequenced individuals ($N = 15$) were identified as *F. askewi*, confirming that these species serve as hosts in the wild. Bullhead Minnows carried the highest glochidial infestation but produced only two juveniles (metamorphosis success = 0.3%), neither of which could be positively identified to species.

KEY WORDS: unionid, glochidia, genotyping, freshwater mussel, conservation

INTRODUCTION

The life cycle of most freshwater mussels (family Unionidae) involves an obligate ectoparasitic stage during which the larvae (glochidia) attach to and encyst on the gills or fins of fishes where they develop into juveniles and excyst to begin a free-living existence. Many unionids are specialists whose glochidia can develop only on certain, usually closely related, fish species. Host use is known reasonably well for about one-third of North American unionids, but host information for many other species is based on unconfirmed relationships (O'Dee and Watters 2000; Haag 2012). Host information exists for only about half of the 51 unionid species reported from Texas (Howells et al. 1996; Winemiller et al. 2010; Marshall 2014).

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Two methods used to determine host fishes of unionids are laboratory-based artificial infestations and morphological or molecular identification of glochidia on the gills of wild-caught fish (e.g., Zale and Neves 1982; O'Dee and Watters 2000; Martel and Lauzon-Guay 2005; Kneeland and Rhymer 2007). Artificial infestations in the laboratory can confirm the ability of glochidia to develop on a particular fish species, but they do not incorporate all of the biotic and abiotic variables that could influence larval development in a natural setting (Neves et al. 1985; Bauer and Wächtler 2001; Gillis 2011). Identification of glochidia naturally infested on wild fishes can provide information from a more natural context, but these observations do not provide conclusive evidence of host suitability because glochidia may attach briefly to nonsuitable hosts before they are rejected by the host's immune system (Watters and O'Dee 1996; Haag 2012).

Marshall (2014) determined 17 potential host fishes for the state threatened Texas Pigtoe (*Fusconaia askewi*), with the Red Shiner (*Cyprinella lutrensis*), Blacktail Shiner (*Cyprinella venusta*), and Bullhead Minnow (*Pimephales vigilax*) showing the greatest infestations. These proposed relationships were based on observations of *F. askewi* glochidia naturally infested on wild fishes and identified by molecular markers, but production of juvenile mussels on these fish species was not confirmed. We evaluated the suitability of *C. lutrensis*, *C. venusta*, and *P. vigilax* as hosts for *F. askewi*. We collected wild individuals of the three target fish species that carried natural infestations of mussel glochidia from three eastern Texas streams, housed them in the laboratory until juvenile mussels were released, then identified the juvenile mussels with molecular methods. We also report differences in juvenile mussel production among fish species as another way to evaluate their relative suitability as hosts.

METHODS

Field Sites and Sampling

Cyprinella lutrensis, *C. venusta*, and *P. vigilax* were collected from three streams in eastern Texas that support

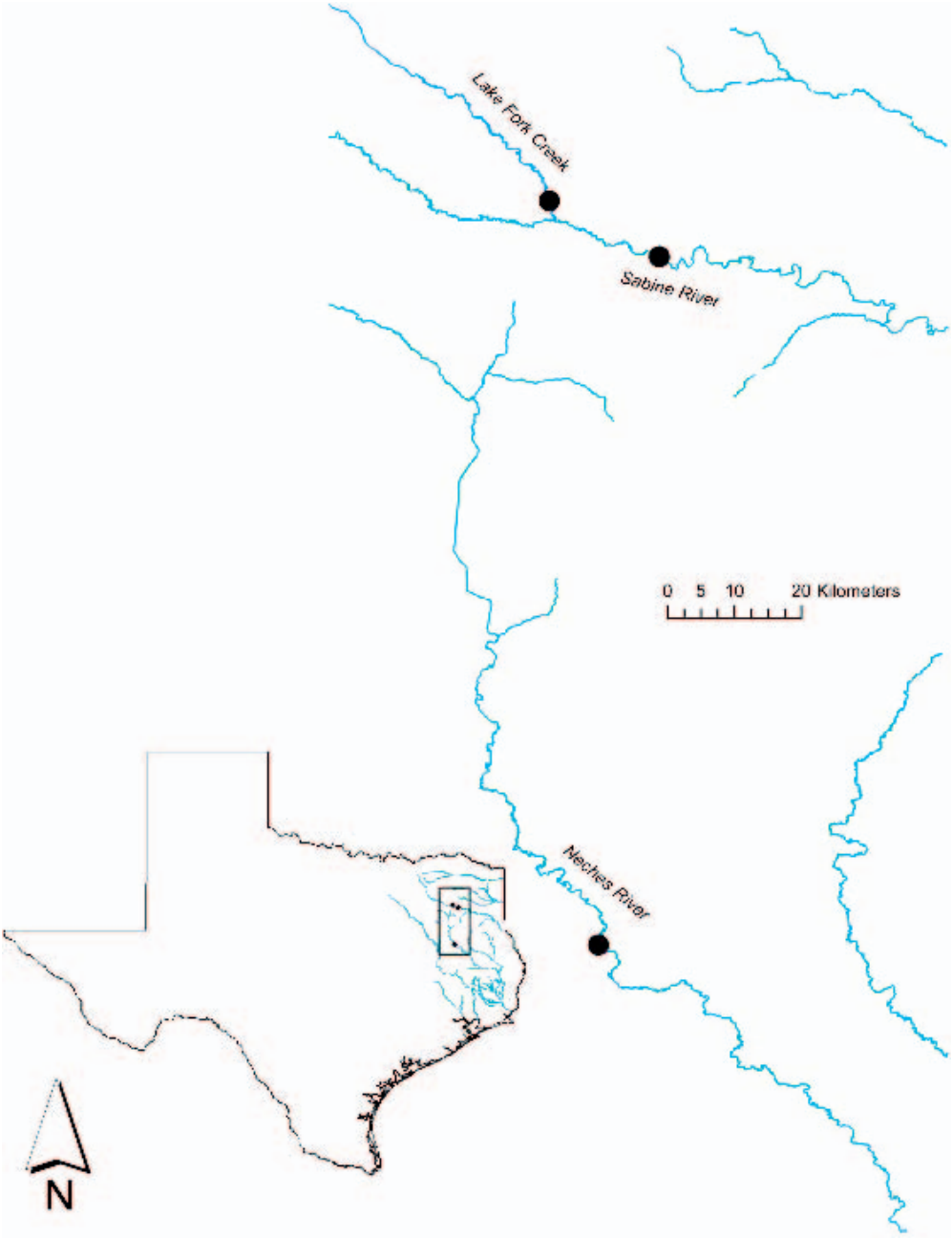

Figure 1. Texas collection locations for Red Shiners (*Cyprinella lutrensis*), Blacktail Shiners (*Cyprinella venusta*), and Bullhead Minnows (*Pimephales vigilax*).

populations of *F. askewi*: Sabine River near Highway 14, Smith County; Neches River near Highway 294, Anderson County; and Lake Fork Creek near Highway 80, Wood County (Fig. 1). We collected fishes from the Sabine and Neches rivers on eight different days between May and October of 2014 (Table 1) based on times of maximum glochidial infestation in these rivers reported by Marshall (2014). We collected fishes from Lake Fork Creek on a single date (August 4, 2014) to increase sample sizes of target fishes when high flow prevented sampling on the Sabine and Neches rivers. Fishes were collected from each site over a 150-m reach near mussel beds using a 7.5-m bag seine. Electrofishing was not used to avoid mortality or stress to fish that may cause release of encysted glochidia. We attempted to collect fishes of varying sizes (3–7 cm length) for each species. Water temperature, pH, and conductivity were measured using a YSI multi-probe meter (YSI Incorporated, Yellow Springs, OH, USA) for each sampling event.

Laboratory Housing of Fishes

Fishes collected from the field were brought back to the Department of Biology Aquatic Ecology Laboratory at the University of Texas at Tyler. Fish were then placed in 3-L

Table 1. Infestation of Red Shiners (*Cyprinella lutrensis*), Blacktail Shiners (*Cyprinella venusta*), and Bullhead Minnows (*Pimephales vigilax*) by mussel glochidia at three eastern Texas collection sites (Sabine = SBN, Neches = NCHS, Lake Fork Creek = LKFRC). (*n*) refers to the number of fishes examined on each date. Number of glochidia is divided into those that excysted as juveniles (Juv.), those that were sloughed prior to metamorphosis into juveniles (Gloch.), and those that remained encysted at the end of the experiment (Encysted).

	(*n*)	Date	Site	Juv.	Gloch.	Encysted	Total
Cyprinella lutrensis	46	May 29, 2014	SBN	111	205	3	319
	26	July 10, 2014	SBN	45	75	17	137
	10	July 11, 2014	NCHS	7	87	15	109
	11	August 4, 2014	LKFRC	13	172	33	218
	15	August 7, 2014	NCHS	67	45	5	117
	3	October 23, 2014	SBN	0	0	0	0
	3	October 24, 2014	NCHS	0	0	0	0
Total	114			243	584	73	900
Cyprinella venusta	14	June 3, 2014	SBN	16	0	1	17
	6	July 10, 2014	SBN	0	0	2	2
	7	July 11, 2014	NCHS	9	14	0	23
	23	August 4, 2014	LKFRC	18	57	60	135
	22	August 7, 2014	NCHS	25	8	4	37
	15	October 24, 2014	NCHS	0	0	0	0
Total	87			68	79	67	214
Pimephales vigilax	1	May 29, 2014	SBN	0	0	0	0
	2	June 3, 2014	SBN	2	251	0	253
	3	July 10, 2014	SBN	0	405	0	405
	14	October 23, 2014	SBN	0	0	0	0
	26	October 24, 2014	NCHS	0	0	0	0
Total	46			2	656	0	658

tanks in an AHAB unit (Pentair Aquatic Eco-Systems, Inc., Apopka, FL, USA) in groups of up to seven smaller individuals or two to three larger individuals and separated by species, collection date, and collection site. Multiple individuals of the same species and origin were housed together to decrease stress in these shoaling species and because our system was limited to 20 tanks. We monitored water temperature, pH, and conductivity every other day with a multi-probe YSI meter, and we adjusted these conditions to be similar to river sites where the fish were collected.

Juvenile Collection

Juvenile collection devices consisting of 3.5-cm-long polyvinyl chloride pipe segments with 118-μm mesh netting on one end were placed on each tank; these permitted water exiting the tanks to flow through while retaining glochidia and juvenile mussels (Barnhart 2006). This mesh size is smaller than the minimum size of *F. askewi* glochidia (128 μm; Howells et al. 1996). We removed and inspected juvenile collectors every other day for the first 2 wk of the trial and sporadically until termination of the trial. We examined material retained on the netting under an Olympus SZ dissection microscope (Olympus Corporation of the Americas, Center Valley, PA, USA) and counted all glochidia and juveniles. Juvenile mussels were distinguished from glochidia based on the presence of internal tissue development and movement, such as protrusion of the foot from the shell (Howells et al. 1996). We calculated overall infestation intensity ([number of juveniles + number of sloughed or encysted glochidia]/number of fish), juvenile production (number of juveniles produced/number of fish), and metamorphosis success (number of juveniles/[number of juveniles + number of sloughed glochidia]) for each potential host species across all trials. We collected subsamples of at least 10 juveniles for genetic identification from each tank on each day that tanks were inspected. Each individual was placed in a separate 1.5-mL centrifuge tube with 95% ethanol and stored at −20°C.

Duration of each trial ranged from 3 to 6 wk. If juvenile production ceased or if fishes did not produce any glochidia or juveniles for 3 wk, we terminated the trial and euthanized all fishes in that tank. This 3-wk termination criterion was based on past observations of the authors, as well as observations that unionid glochidia tend to excyst between a few days and several weeks following encystment (e.g., Haag and Warren 1997), with this process expedited in warmer regions (Watters and O'Dee 2000). Euthanized fish were then examined for glochidial encystment on their gills and fins.

DNA Sequencing and Identification

Genomic DNA was extracted from individual juveniles using a Chelex double-stranded DNA extraction protocol

(Casquet et al. 2011). We modified the protocol of Casquet et al. (2011) by adding 50 μL of a 1:15 solution of proteinase K and 10% Chelex 100 resin instead of the recommended 150 μL; this was done to avoid diluting the small amounts of genomic DNA extracted from juvenile mussel tissue. Extracted DNA was stored at –20°C until use in PCRs. The primers Leu-uurF and LoGlyR were used to amplify mitochondrial (mtDNA) NADH dehydrogenase (ND1) gene (Serb et al. 2003). PCR reactions used for amplification of the ND1 gene consisted of 20 μL: 6.7 μL purified H_2O, 0.1 μL TopTaq, 2.0 μL PCR buffer (Qiagen Sciences Inc, Germantown, MD, USA), 0.4 μL dNTPs, 2.0 μL 10X Coral Load (Qiagen), 4.0 μL Q-Solution, 1.0 μL of each primer, 0.4 μL bovine serum albumin, and 2.4 μL of DNA (∼150 ng/μL). An extra 10% of the PCR reaction was created to provide a negative control with each PCR. An Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) with a heated lid was used to amplify the reactions. The reaction settings for amplification of double-stranded DNA were as follows: 94°C for 5 min; 30 cycles of 94°C for 45 s, 54°C for 60 s, and 72°C for 60 s; followed by a final extension of 72°C for 5 min. Gel electrophoresis was used to test the quality of amplification. The successfully amplified PCR products were purified using and E.Z.N.A. cycle pure kit (Omega Bio-tek, Norcross, GA, USA) following the protocol with an additional 30 μL of purified water for resuspension. Purified DNA was concentrated at 17–20 ng/μL with a 260/280 ratio around 1.8 to 2.0 as recommended by Eurofins MWG Operon where reactions were shipped to for sequencing using BigDye Terminator v 3.1 Cycle Sequencing kits (Applied Biosystems, Foster City, CA, USA). Sequences were edited with the Sequencher 5.2.4 program (Gene Codes Corporation, Ann Arbor, MI, USA) and then initially compared with unionid sequences available on the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). The edited sequences were also cross-referenced with an adult molecular key that provides sequences for all the 37 unionid species that occur in eastern Texas (Marshall 2014). The tissue samples from mussels used to create the molecular key included adult mussels collected from the same sampling sites we used on the Sabine River and Neches River. ClustalX2.0.11 (Conway Institute UCD, Dublin, Ireland) was used to generate an alignment file of the juvenile sequences with the adult sequences of the molecular key. The alignment file from ClustalX2.0.11 was then uploaded into Mesquite (version 2.75, Mesquite Project Team, http://mesquiteproject.org) to provide ocular observation of the alignment with the sequences of the molecular key.

RESULTS

Infestation on Wild-caught Fish

A total of 114 *C. lutrensis*, 87 *C. venusta*, and 46 *P. vigilax* were collected during the study (Table 1). *Pimephales vigilax* had the highest glochidial infestation intensity (average = 14.3/fish), but only two juveniles were produced in a single trial from the Sabine River (overall juvenile production = 0.04 juveniles/fish; metamorphosis success = 0.3%). No glochidia were found encysted on the gills of deceased *P. vigilax* at the end of our trials. *Cyprinella lutrensis* had a lower glochidial infestation intensity (7.9/fish), but it had the highest rate of juvenile production (2.1/fish) and moderate metamorphosis success (29.4%). In addition, 73 glochidia were encysted on the gills of deceased fish at the end of our trials. *Cyprinella venusta* had the lowest infestation intensity (2.5/fish) and the second highest juvenile production (0.8/fish), but it had the highest metamorphosis success (46.3%). Sixty-seven glochidia were found encysted on deceased fish at the end of our trials. For all three fish species, glochidial infestation was observed from late May to early June until July or early August, and no fishes were infested in October.

Molecular Identification of Glochidia and Juvenile Mussels

DNA was extracted from a total of 127 juveniles, which consisted of 86 juveniles from *C. lutrensis*, 39 juveniles from *C. venusta*, and the two juveniles from *P. vigilax*. Of these, DNA from eight juveniles from *C. lutrensis* and seven juveniles from *C. venusta* was succesfully amplified, sequenced, and identified. These juveniles included at least one individual from each fish species from all three sampling sites. We were unsuccessful in amplyfing and sequencing DNA from juveniles collected from *P. vigilax*.

Fourteen of our 15 sequences represented a single haplotype (GenBank accession number KY442832) that was 100% identical to both a National Center for Biotechnology Information sequence from *F. askewi* and one generated by Marshall (2014) for Triangle Pigtoes (*Fusconaia lananensis*) and *F. askewi*. Only one sequence represented a haplotype (GenBank accession number KY442833) not previously detected in eastern Texas, but this sequence was consistent with *F. lananensis* and *F. askewi*, and it differed from the other haplotype we detected by only a single base pair difference and was over 99% identical to that haplotype.

DISCUSSION

Our results confirm Marshall's (2014) identification of *C. lutrensis* and *C. venusta* as hosts for *F. askewi*. We show that these fishes routinely become infested by mussel glochidia in the wild, and these infestations result in production of juveniles with moderate metamophosis success (30–46%). We cannot assess the overall robustness of these host relationships because we successfully sequenced only 15 individuals, and the identity of the majority of juveniles produced by these fishes is unknown. However, we also examined the morphology of juveniles we collected and all were consistent with the distinctive shell morphology observed in *Fusconaia* from eastern Texas (Marshall 2014).

In addition to *F. askewi*, our juvenile sequences were

identical to *F. lananensis*, which also is reported from Texas (Howells et al. 1996). However, *F. lananensis* is not genetically distinguishable from *F. askewi*, and the two species are considered synonymous (Burlakova et al. 2012). Marshall (2014) also found large numbers of glochidia of the Louisiana Pigtoe (*Pleurobema riddelli*) encysted on *C. lutrensis* and *C. venusta*. However, none of the sequences we generated corresponded to this species, and shell morphology of juveniles we harvested was inconsistent with *P. riddelli* as described by Marshall (2014).

All other *Fusconaia* for which host data exist appear to be specialist on minnows, but the extent of specialization varies among species. *Fusconaia cerina*, *Fusconaia cor*, and *Fusconaia cuneolos* used a wide variety of minnow species in several genera, but *Fusconaia burkei* used only *C. venusta* (Bruenderman and Neves 1993; Haag and Warren 2003; White et al. 2008). Marshall (2014) found glochidia of *F. askewi* on a wide variety of minnow species, but we can confirm the suitability only of *C. lutrensis* and *C. venusta*. Additional laboratory studies are needed to confirm the degree of specialization in *F. askewi*.

Another potential host for *F. askewi* identified by Marshall (2014) was *P. vigilax*, but this species did not appear to be a suitable host in our study despite having the highest infestation intensity. We were unable to sequence and identify the two juveniles produced from *P. vigilax*, but their shell morphology was inconsistent with *F. askewi* juveniles identified from *C. lutrensis* and *C. venusta* (see Marshall 2014). *Pimephales vigilax* was an unsuitable host for *Fusconaia cerina*, and *Pimephales notatus* was a marginal host that produced inconsistent and low numbers of juveniles (Haag and Warren 2003). Mussel host infection strategies are thought to be highly evolved mechanisms to reduce glochidial mortality from encystment on unsuitable fishes (Haag 2012). The few studies that identified naturally encysted glochidia on fishes or juveniles produced from natural infestations generally show a low incidence of glochidial encystment on unsuitable fish species (Neves and Widlak 1988; Boyer et al. 2011; Hove et al. 2012). The high incidence of glochidial parasitism but low metamorphosis success on *P. vigilax* is unusual and seems maladaptive. Glochidia can be rejected from otherwise suitable hosts prior to metamorphosis due to stress of the fish, acquired immune responses, or the presence of scar tissue from multiple prior encystments (Meyers et al. 1980; Neves et al. 1985). We do not know if our unusual results for *P. vigilax* are due to one of these or other factors or if they simply show that host attraction strategies for some mussel species are relatively inefficient and nonspecific.

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LITERATURE CITED

Barnhart, M. C. 2006. Buckets of muckets: A compact system for rearing juvenile freshwater mussels. Aquaculture 254:227–233.

Bauer, G., and K. Wächtler. 2001. Ecology and Evolution of the Freshwater Mussels Unionoida. Springer-Verlag, Berlin and Heidelberg, Germany. 396 pp.

Boyer S. L., A. A. Howe, N. W. Juergens, and M. C. Hove. 2011. A DNA-barcoding approach to identifying juvenile freshwater mussels (Bivalvia:Unionidae) recovered from naturally infested fishes. Journal of the North American Benthological Society 30:182–194.

Bruenderman, S. A., and R. J. Neves. 1993. Life history of the endangered fine-rayed pigtoe, *Fusconaia cuneolus* (Bivalvia: Unionidae) in the Clinch River, Virginia. American Malacological Bulletin 10:83–91.

Burlakova, L. E., D. Campbell, A. Y. Karatayev, and D. Barclay. 2012. Distribution, genetic analysis and conservation priorities for rare Texas freshwater molluscs in the genera *Fusconaia* and *Pleurobema* (Bivalvia: Unionidae). Aquatic Biosystems 8:12.

Casquet, J., C. Thebaud, and R. G. Gillespie. 2011. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Molecular Ecology Resources 12:136–141.

Gillis, P. L. 2011. Assessing the toxicity of sodium chloride to the glochidia of freshwater mussels: Implications for salinization of surface waters. Environmental Pollution 159:1701–1708.

Haag, W. R. 2012. North American Freshwater Mussels: Natural History, Ecology, and Conservation. Cambridge University Press, New York, New York. 538 pp.

Haag, W. R., and M. L. Warren, Jr. 1997. Host fishes and reproductive biology of 6 freshwater mussel species from the Mobile Basin, USA. Journal of the North American Benthological Society 16:576–585.

Haag, W. R., and M. L. Warren. 2003. Host fishes and infection strategies of freshwater mussels in large Mobile Basin streams, USA. Journal of the North American Benthological Society 22:78–91.

Hove, M. C., M. T. Steingraeber, T. J. Newton, D. J. Heath, C. L. Nelson, J. A. Bury, J. E. Kurth, M. R. Bartsch, W. S. Thorpe, M. R. McGill, and D. J. Hornbach. 2012. Early life history of the winged mapleleaf mussel (*Quadrula fragosa*). American Malacological Bulletin 30:47–57.

Howells, R. G., R. W. Neck, and H. D. Murray. 1996. Freshwater Mussels of Texas. Texas Parks and Wildlife Press, Austin, Texas. 224 pp.

Kneeland, S. C., and J. M. Rhymer. 2007. A molecular identification key for freshwater mussel glochidia encysted on naturally parasitized fish hosts in Maine, USA. Journal of Molluscan Studies 73:279–282.

Marshall, N. 2014. Identification of potential fish hosts from wild populations of state-threatened East Texas freshwater mussels using a molecular identification dataset. Master's thesis, University of Texas at Tyler.

Martel, A. L., and J. S. Lauzon-Guay. 2005. Distribution and density of glochidia of the freshwater mussel *Anodonta kennerlyi* on fish hosts in lakes of the temperate rain forests of Vancouver Island. Canadian Journal of Zoology 79:419–431.

Meyers, T. R., R. E. Millemann, and C. A. Fustish. 1980. Glochidiosis of salmonid fishes. IV. Humoral and tissue responses of Coho and Chinook Salmon to experimental infection with *Margaritifera margaritifera* (L.) (Pelecypoda: *Margaritanidae*). Journal of Parasitology 66:274–281.

Neves, R. J., L. R. Weaver, and A. V. Zale. 1985. An evaluation of host fish suitability for glochidia of *Villosa vanuxemi* and *V. nebulosa* (Pelecypoda:Unionidae). American Midland Naturalist 119:111–120.

Neves, R. J., and J. C. Widlak. 1988. Occurrence of glochidia in stream drift

and on fishes of the Upper North Fork Holston River, Virginia. American Midland Naturalist 119:111–120.

O'Dee, S. H., and G. T. Watters. 2000. New or confirmed host identifications for ten freshwater mussels. Pages 77–82 *in* R. A. Tankersley, D. I. Warmolts, G. T. Watters, B. J. Armitage, P. D. Johnson, and R. S. Butler, editors. Freshwater mollusk symposia proceedings. Part I. Proceedings of the conservation, captive care and propagation of freshwater mussels symposium. Ohio Biological Survey Special Publication, Columbus.

Serb, J. M., J. E. Buhay, and C. Lydeard. 2003. Molecular systematics of the North American freshwater bivalve genus *Quadrula* (Unionidae: Ambleminae) based on mitochondrial ND1 sequences. Molecular Phylogenetics and Evolution 28:1–11.

Watters, G. T., and S. H. O'Dee. 1996. Shedding of untransformed glochidia by fishes parasitized by *Lampsilis fasciola* Rafinesque, 1820 (Mollusca: Bivalvia: Unionidae): Evidence of acquired immunity in the field? Journal of Freshwater Ecology 11:383–388.

Watters, G. T., and S. H. O'Dee. 2000. Glochidial release as a function of water temperature: beyond bradyticty and tachyticty. Pages 135–140 *in* R. A. Tankersley, D. I. Warmolts, G. T. Watters, B. J. Armitage, P. D. Johnson, and R. S. Butler, editors. Freshwater mollusk symposia proceedings. Part I. Proceedings of the conservation, captive care and propagation of freshwater mussels symposium. Ohio Biological Survey Special Publication, Columbus.

White, M.P., Blalock-Herod, H.N., and Stewart, P.M. 2008. Life history and host fish identification for *Fusconaia burkei* and *Pleurobema strodeanum* (Bivalvia: Unionidae). American Malacological Bulletin 24:121–125.

Winemiller, K., N. K. Lujan, R. N. Wilkins, R. T. Snelgrove, A. M. Dube, K. L. Scow, and A. G. Snelgrove. 2010. Status of freshwater mussels in Texas. Texas A&M Department of Wildlife and Fisheries Sciences and Texas A&M Institute of Renewable Natural Resources, College Station, Texas.

Zale, A. V., and R. J. Neves. 1982. Fish hosts of four species of lampsiline mussels (Mollusca: Unionidae) in Big Moccasin Creek, Virginia. Canadian Journal of Zoology 60:2535–2542.