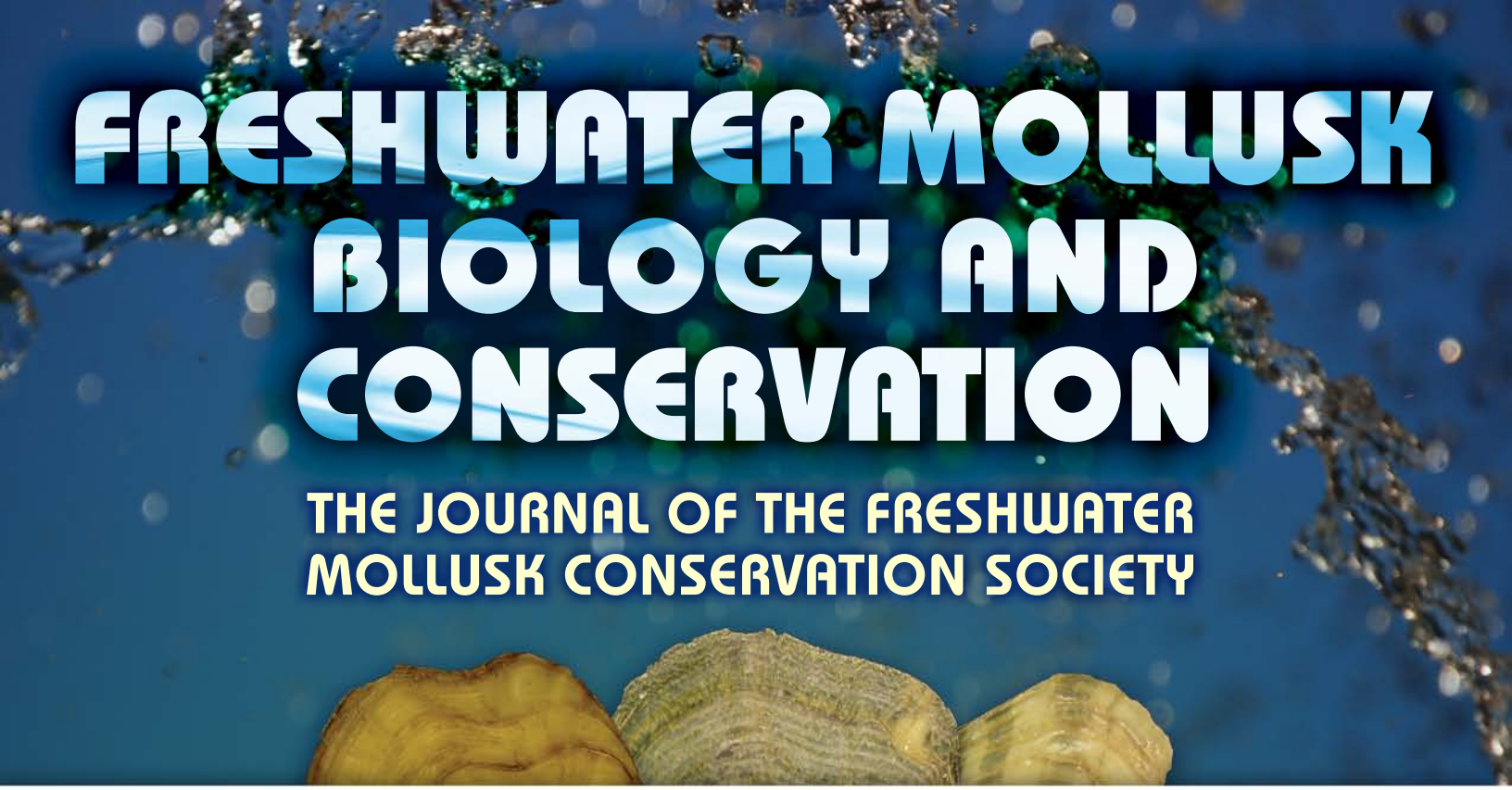

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NOTE

TRANSLOCATION AND REPRODUCTIVE BENEFITS TO A HIGHLY ENDEMIC AND ENDANGERED SPECIES, THE BANBURY SPRINGS LIMPET, *IDAHOLANX FRESTI* (MOLLUSCA: GASTROPODA)

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ABSTRACT

We have monitored four isolated populations of the endangered freshwater Banbury Springs limpet for eight or more years. One of these populations consistently exhibited low numbers and very limited recruitment. In an effort to increase its size and reproductive vigor, we translocated 19 limpets from a large, robust population to the smaller, declining one (focal population). This translocation effort was carried out along with a small-scale habitat management effort. Post-translocation monitoring has seen the focal population increase by up to 900%, with an increase in reproduction from 6% to 33–55% annually. Limpet densities in the focal population also have increased from 5.5 m^{-2} to 43 m^{-2} post-translocation, reaching densities seen in more stable populations. The augmentation of additional individuals, in addition to ongoing habitat management efforts, likely played an important role in the observed increases. The observed recruitment also suggests some level of increased genetic vigor following the translocation, but we lack the data to fully support a genetic rescue effect. Although translocation and augmentation of isolated and declining populations should be approached cautiously, our results support a growing body of literature that suggests the shortcomings associated with these techniques may have been overstated in the earlier literature. If done properly, their use can provide important conservation gains for small and isolated populations of sensitive species.

KEY WORDS: limpet, translocation, augmentation, Mollusca, freshwater, genetic rescue

INTRODUCTION

As isolated populations become smaller, they are at increased risk of extirpation due to demographic stochasticity (Lande 1988; Holsinger 2000) as well as increased inbreeding and the expression of genetic load (Frankham 1998; Keller and Waller 2002; Rowe and Beebee 2003). For vulnerable and endangered species, translocating individuals from larger, more robust populations into declining ones (population augmentation) has been proposed as an effective conservation tool (Taberlet et al. 1997; Amos and Balmford 2001; Tallman et al. 2004; Bodine et al. 2008). The use of translocation, "the human-mediated movement of living organisms from one area, with release in another" (International Union for the Conservation of Nature [IUCN] 2013), is not without risk, and the IUCN and others (Moritz 1999; Dudash and Fenster 2000; Amos and Balmford 2001) have outlined criteria and precautions to avoid or minimize these risks. Given the growing trend of small and fragmented habitats, translocation may provide an effective tool for managers dealing with species that occur in small populations and exhibit reduced genetic vigor (Moritz 1999; Tallman et al. 2004).

The Banbury Springs limpet (*Idaholanx fresti*) is a monotypic species endemic to Idaho and placed in the subfamily Lancinae, which is restricted to the Pacific Northwest, USA (Campbell et al. 2017). The Banbury Springs limpet (or limpet) has a conical shell that can measure up to 7.1 mm in length and 4.3 mm in height (Fig. 1). The species is confined to four aquifer-fed springs along the Snake River in south-central Idaho, where it prefers cobble-dominated habitat, free of fine sediments, in clear spring tributaries that maintain consistent temperatures 13–17° C year round (U.S. Fish and Wildlife Service [USFWS] 2018). They are rarely found on submerged woody debris, nor have they been associated with rooted macrophytes (e.g., *Stuckenia* spp.) as habitat. The species is presumed to feed on saxicolous periphyton, but little else of its life history (longevity, fecundity, or reproduction) is documented. These four populations are located within 10 km of one another, but they are reproductively isolated as *I. fresti* requires good water quality (Bowler and Frest 2018) and

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Figure 1. *Idaholanx fresti* is the only representative of a monotypic genus and is restricted to four spring creeks in south-central Idaho (photo credit Robert Jaeger).

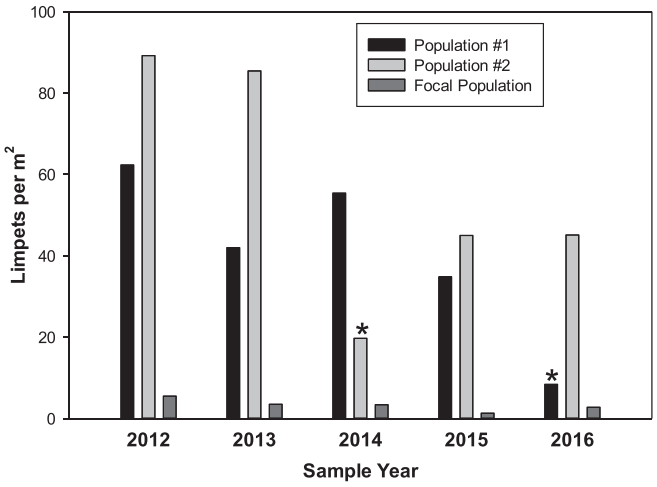

Figure 2. Estimated population densities of Banbury Springs limpets at three populations, prior to the translocation event. The focal population was consistently low throughout the study period, while the other two typically maintained larger numbers of snails found at higher densities. The two asterisks (*) denote years in which disturbance events (water diversions) caused significant declines in both numbers and densities of limpets within the denoted populations.

cannot tolerate the poor water quality found in the Snake River into which these springs feed. A recent phylogenetic review (Campbell et al. 2017) confirmed the species' distinctness from other lancines but lacked the resolution to discern differences between the four populations.

Biologists from the USFWS began annual monitoring of three of the four populations, including the population addressed in this study (focal population) of limpets, in 2012 (USFWS 2018). Frest and Johannes (1992) first documented the focal population in 1991, estimating its total numbers to range from 600 to 1,200 individuals, with densities ranging from 16 m^{-2} to 48 m^{-2}. When regular, systematic monitoring began in 2012, the number of recorded individuals was low (32 individuals, 5.5 m^{-2}), and it declined steadily with subsequent annual monitoring (Fig. 2). While other limpet populations also have encountered periodic declines due to disturbance events, they have rebounded toward predisturbance levels and typically have included a larger percentage of subadults (i.e., juveniles), exhibiting more robust recruitment. By comparison, the focal population exhibited low recruitment, with monitoring never recording more than 10% of individuals encountered being classified as subadults (Fig. 3). In addition, the area occupied by the focal population had declined from approximately 12–14 m^2 in 1991 (Frest and Johannes 1992) to no more than 2 m^2 in 2016. In comparison, limpet-occupied habitat in the other monitored populations largely remained unchanged (USFWS 2018).

In addition to the suppressed population levels at the focal population, we observed increased abundance of aquatic macrophytes (e.g., *Stuckenia pectinate*) during the spring and summer months. These macrophyte beds produce and capture fine sediments and stimulate further expansion of macrophyte growth (Mebane et al. 2014), burying and covering the limpets' preferred habitat of clean cobble substrate. Excessive macrophyte growth is regarded as a major threat to the focal population, which appeared to face possible extirpation. In 2015, the USFWS and conservation partners agreed to translocate *I. fresti* from a larger, more robust population to augment the declining focal population. Following the translocation event, periodic macrophyte

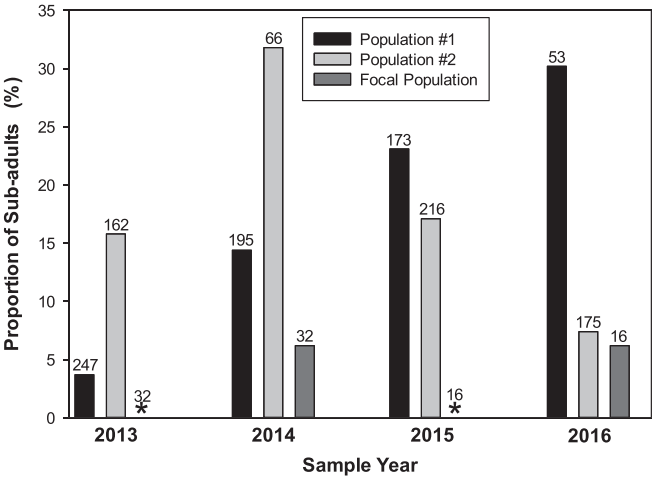

Figure 3. The proportion (%) of subadults detected in each of the three monitored populations prior to the translocation event. The asterisks (*) denote samples that lacked subadults (focal population only) and the numbers above each column show the total number of limpets recorded at each population in each of the sample years. Note that at populations 1 and 2, subadult detections made up a sizable proportion of the population even during years when low numbers of limpets were recorded.

removal was conducted at the release site to ensure sufficient habitat for the focal population. This paper provides an overview of the population augmentation effort for this federally endangered freshwater limpet; in combination with habitat management, translocation shows promise in reversing or slowing the decline of a small and isolated population.

METHODS

Monitoring

The three populations covered in this study have been monitored annually since 2012. Populations are monitored by randomly selecting the local basalt cobbles within the occupied habitat area, recording the number of individuals on each cobble, and estimating the available surface area of each cobble (Carlsson et al. 1977; McCreadie and Colbo 1991). We recorded limpets as adult (>5.0 mm in length) or subadult (≤5.0 mm) and attempted to sample a consistent number of cobbles within each population (e.g., 170–202 annually at the focal population), though the area surveyed varied based on the size of sampled cobbles. The fourth population, used as the donor population for the translocation, is not included in our analysis since the monitoring methods differed from those described above. Based on monitoring of the donor population, we estimated its size as over 1,000 individuals and regarded it as the largest population from which a limited number of limpets could be removed safely.

Translocation

On May 4, 2016, biologists from Idaho Power Company, Idaho Department of Fish and Game, and USFWS collected 19 individual Banbury Springs limpets from the large donor population approximately 8 km upriver (Snake River) from the focal population. The occupied basalt cobbles were collected from depths of 15–20 cm in riffle-glide habitats and ranged in dimension from 8 cm^{-3} to 13 cm^{-3} each. Collected limpets ranged from 3 mm to 8 mm in length with emphasis on using larger individuals (14 were ≥5 mm). Given the sensitivity of lancine gastropods, the actual translocation event was carried out as rapidly as possible (<1 h) to minimize stress to individual limpets. Cobbles containing multiple individual limpets were collected from the donor population and nontarget gastropods and other invertebrates were removed using forceps and hard-bristled toothbrushes. Cobbles and limpets were marked with nontoxic underwater markers (Sakura® Solid Marker, Sakura Corporation, Osaka, Japan), and transferred to 19-L buckets filled with local spring water. Brushing cobbles was a precaution to avoid translocation of possible invasive species between locations, although the nonnative New Zealand mudsnail (*Pomatopyrgus antipodarum*) is well established at all colonies and no other invasive species have been documented at any of the populations. In order to minimize impacts to translocated limpets, they were left on their cobbles during marking and cleaning (conducted underwater) prior to moving them to the translocation buckets. Limpets were exposed to the air less than 1 min during the entire translocation process. The buckets containing cobbles and limpets were transferred by hand to jet boats waiting on the Snake River, where they were placed in coolers and aerated. After jet-boat transport down the Snake River, the translocation buckets were transferred to biologists at the focal (recipient) population, who quickly placed the cobbles within occupied habitat (run/glide, 20–30-cm depth). The entire translocation event took place within 30 min and limpets were not exposed to any temperature shift during transport and translocation (i.e., maintained at 15.2°C).

After translocation, the recipient team of biologists observed the translocated limpets for 30 min to determine if there was any immediate mortality associated with the translocation event. The focal population was observed the following day, the following week, and monthly through August in an effort to track translocated individual limpets and assess survivorship.

Macrophyte Removal

During subsequent visits to the focal population after translocation, we observed the encroachment of rooted macrophytes, which reduced the availability of suitable habitat for the limpets. To help ensure long-term success of the translocation, we began a periodic small-scale effort to carefully remove macrophytes by hand to ensure preferred cobble habitat would not become overgrown and sediment-embedded (Fig. 4). Prior experimental studies conducted by the USFWS and others, where plots were cleared of macrophytes and fine sediments, documented *I. fresti*'s ability to recolonize these habitats in as little as 5 mo (G. Burak, personal observation). We continued to remove macrophytes during periodic monitoring visits throughout the summer months through 2019, ensuring a relatively macrophyte-free area of 3–4 m^2 within the occupied area.

RESULTS

Prior to the translocation, we carried out annual monitoring of the focal population on April 20, 2016, and found 16 individual limpets on 10 of the 201 cobbles inspected. From 2012 through 2015, the focal population fluctuated between a high of 32 (2012, 2013, and 2014) to a low of 15 in 2015 (G. Burak, personal observation). This monitoring data indicates a population that continued to function and reproduce at very low levels prior to the translocation.

As stated above, the translocation of 19 individual limpets from the donor population to the focal population occurred on May 4, 2016. One week subsequent to population augmentation, we were able to relocate 68% (13 of 19 limpets) of the translocated individuals utilizing colored markings on their shells. It is possible that the unrecovered 32% could represent mortality, poor retention of shell markings, or lack of visual

Figure 4. Pre– and post–macrophyte removal at the focal population. Prior to these management efforts, the majority of limpets had been concentrated in the lower left portion of macrophyte-free cobbles.

detection. All of the relocated individuals appeared healthy and we did not find any sign of mortality of marked limpets.

We continued periodic monitoring of the focal population through the summer of 2016, visiting on three additional occasions. By July 19, we found only two marked limpets with faded marking, one of which moved approximately 30–40 cm from the translocated cobbles. One month later, we found zero marked limpets at the focal population. While there may have been mortality of translocated limpets, the extremely faded markings on the two limpets recovered in July leads us to believe that marking retention was poor and not indicative of actual survivorship.

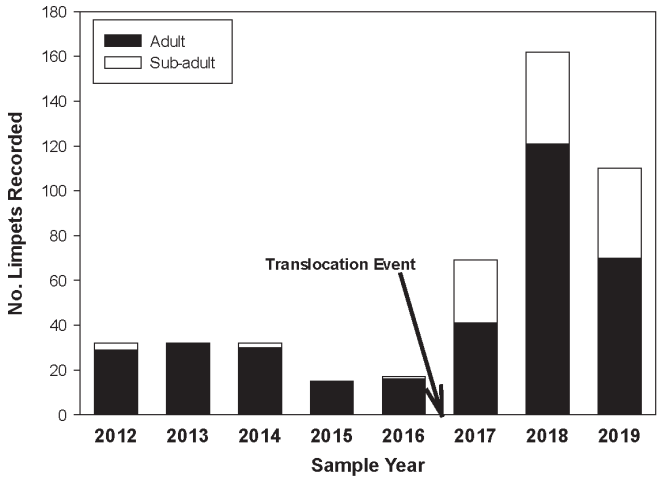

Figure 5. Recorded limpets, adult and subadult, detected at the focal population before and after the translocation event. Density of limpets showed a similar increase, ranging from 1.3–5.5 m^{-2} to 23–43 m^{-2} (before and after the translocation event, respectively).

Prior to the 2016 translocation, results of annual monitoring of the focal population had been flat or in decline over the previous 5 yr (Fig. 5). However, following translocation, the number of detected individuals increased substantially over the following 3 yr (Fig. 5). The number of individuals observed also corresponded to an increase in density at the focal population, with pretranslocation densities ranging from 1.3 limpets m^{-2} to 5.5 limpets m^{-2}, increasing to 22.9 limpets m^{-2}, 42.9 limpets m^{-2}, and 40.0 limpets m^{-2} for 2017 through 2019, respectively. Furthermore, posttranslocation densities at the focal population were comparable to those of the other monitored populations during normal years (years without disturbance events), which typically ranged from 27 limpets m^{-2} to 85 limpets m^{-2}, with an average of 50.2 limpets m^{-2} (G. Burak, personal observation) (Fig. 2).

The translocation of limpets also coincided with an observed increase in the number of subadults detected at the focal population (Fig. 5). The number of recorded subadults went from a high of three individuals in 2012 (10.3% of limpets encountered) to 23 in 2017 (54.8%), 41 in 2018 (33.3%), and 40 in 2019 (36%) (Fig. 5). While we did not attempt to make direct comparisons of changes in the donor population before and after the translocation, our continuing monitoring has shown that population to be as consistent in size and variation as it was historically (G. Burak, personal observation).

DISCUSSION

The history of isolation between the four populations is unknown but could date from prehistoric events such as the Bonneville Flood (14.5 thousand years ago), when Lake

Bonneville drained from Utah through the Snake River of Idaho, or it could predate that event, dating to when Lake Idaho underwent its last contraction (est. 1.7 million years ago). While these events likely played roles in the species' current distribution and isolation, the more recent environmental changes brought on through anthropogenic activities and modifications (agriculture, dams, flood control, irrigation diversion) to the Snake River in south-central Idaho and springs that feed it, have maintained, if not amplified, the observed isolation. We believe recent changes in habitat condition, primarily driven by changes in water quality from the aquifer springs, have led to reduced population size. While we lack the detailed genetic data, the small and declining numbers of limpets at the focal population size, coupled with the very low juvenile recruitment prior to the translocation, have all the hallmarks of a population with low reproductive vigor (Dudash and Fenster 2000) and suggests that genetic factors could be at play in addition to compromised habitat condition.

A number of studies have documented increasing nitrate concentrations over time in this aquifer system and its associated springs (Clark et al. 1998; Schorzman et al. 2009; G. Burak, personal observation), and Mebane et al. (2014) identified total nitrogen as the most important contributor to macrophyte growth in these spring systems. The aquatic macrophytes that seasonally encroach into occupied limpet habitat are native species, but we believe their increasing dominance is due to anthropogenic changes in water chemistry. Seasonal macrophyte encroachment poses the same threat to at least one other limpet population in the study area, and without consistent removal efforts, it will reduce or eliminate suitable habitat available to the species at these locations.

Other population augmentations undertaken to increase genetic diversity in declining populations have provided compelling successes (Hogg et al. 2006; Bossuyt 2007; Finger et al. 2011; Miller et al. 2012; Weeks et al. 2017), and this may have played an important role in the current conservation effort. Nonetheless, the habitat management actions (macrophyte removal) conducted during our visits helped ensure that ample suitable habitat remained available and provided resources necessary to support the observed population growth. Previous habitat manipulations carried out at the donor population site resulted in rapid colonization of limpets from adjacent habitats into areas cleared of macrophytes and fine sediments (G. Burak, personal observation), so we know the species can respond rapidly to habitat availability. However, that earlier colonization event did not result in the rapid population response observed in the current study, with mean densities at the donor population dropping from 24.7 limpets m^{-2} to 14.9–16.0 limpets m^{-2} in the 2 yr following the habitat management event (G. Burak, personal observation). Given this, we feel some of the observed reproductive vigor was driven by some level of genetic rescue as well as increased habitat availability.

While we regret not having better genetic information on the four limpet populations to assess their divergence from one another or their unique genotypic characteristics, these resources were not available to us and we regarded the focal population as too small to support the sacrifice of individual limpets for this purpose. Further, we considered the low observed population numbers and low recruitment of the focal population as a sign of impending collapse, and we implemented the translocation effort as a needed emergency action to help ensure the population did not become extirpated in the immediate future (Moritz 1999).

When designing this translocation effort, our intent was to augment a declining population with conspecifics from a more reproductively vigorous population. The subsequent and ongoing habitat management that began after the translocation event may have been as or more beneficial than the augmentation of conspecifics, but we lack the genetic data to assess this and did not design the study to address these factors independently. The merits and hazards of translocations and population augmentations have been well discussed (Moritz 1999; IUCN 2013), and precaution is warranted before using these actions as management tools. However, there is a growing literature that supports population augmentation as a means to prevent local extinctions and achieve conservation successes (Frankham 2015; Waller 2015; Whiteley et al. 2015; Weeks et al. 2017). While the benefits and risks of translocations and population augmentations require careful consideration, they can be used as important conservation tools in the recovery of vulnerable species and populations.

ACKNOWLEDGMENTS

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REGULAR ARTICLE

A SURVEY OF THE FRESHWATER MUSSELS (MOLLUSCA: BIVALVIA: UNIONIDA) OF THE UPPER BARREN RIVER SYSTEM, TENNESSEE

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ABSTRACT

The freshwater mussel fauna of the Barren River system in Kentucky is well documented, but information on mussel occurrence in the Tennessee portion of the system was lacking. We conducted mussel surveys at 56 sites in 22 streams in the Barren River system in Tennessee. We found six species at 14 sites: *Alasmidonta viridis* (Slippershell), *Fusconaia flava* (Wabash Pigtoe), *Lampsilis cardium* (Plain Pocketbook), *Lampsilis siliquoidea* (Fatmucket), *Pyganodon grandis* (Giant Floater), and *Villosa ortmanni* (Kentucky Creekshell). Our records of *V. ortmanni* are the first reports of this species from Tennessee, and our records of *L. siliquoidea* considerably expand the known range of that species in the state. We found live or freshly dead *V. ortmanni* at five sites, and at least two sites supported relatively large populations with evidence of recent recruitment. These observations represent important information for the conservation of this imperiled species. Overall, mussel populations in the Barren River system of Tennessee were small and scattered, which may be due, in part, to the lower mussel abundance typical of headwater streams. However, the occurrence of widespread mussel declines in this region suggests that human factors may have further reduced mussel abundance.

KEY WORDS: Barren River, mussel records, Tennessee, headwaters, *Villosa ortmanni*, *Alasmidonta viridis*, *Lampsilis siliquoidea*

INTRODUCTION

The Barren River drains approximately 4,302 km^2 and is the largest tributary of the Green River (Fig. 1). The Green River joins the Ohio River south of Evansville, Indiana, and drains a greater percentage of Kentucky's land area than any other river system in the state (Burr and Warren 1986). The upper Barren River system is the only portion of the Green River drainage in Tennessee and drains 1,119 km^2 in that state. The Green River drainage supports high fish and mussel species richness, including eight endemic fishes and, potentially, one endemic mussel species (*Villosa ortmanni*; Haag and Cicerello 2016). The fish fauna of the Barren River system, including the Tennessee portion, is well known (Burr and Warren 1986; Etnier and Starnes 1993; Ceas and Page 1997). The mussel fauna of the Kentucky portion of the Barren River system is similarly well documented (Haag and Cicerello 2016), but the fauna of the Tennessee portion is largely unknown. No mussel records exist in the databases of the Tennessee Department of Environment and Conservation (TDEC) (D. Withers, TDEC, personal communication), Tennessee Wildlife Resources Agency (D. Hubbs, Tennessee Wildlife Resources Agency, personal communication), and Tennessee Valley Authority (T. Amacker, Tennessee Valley Authority, personal communication). Parmalee and Bogan (1998) provided no Tennessee mussel records from the Barren River system and did not mention it in their discussion of river systems of the state even though it appeared on two state

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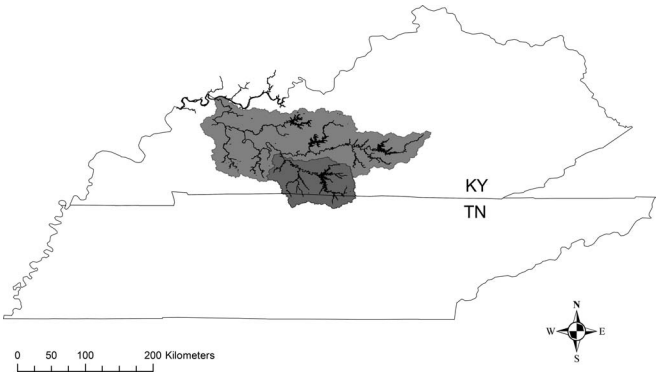


Figure 1. Location of the Barren River system within the Green River drainage, Kentucky and Tennessee, USA (Barren River system in dark shade, Green River system in light shade).

drainage maps. Finally, there are no specimens from the Tennessee portion of the Barren River system in any of the mollusk collections we contacted (North Carolina Museum of Natural Sciences, Carnegie Museum of Natural History, University of Michigan Museum of Zoology, Florida Museum of Natural History, University of Tennessee McClung Museum of Natural History and Culture [MMNHC], The Ohio State University Museum of Biological Diversity, and Harvard University Museum of Comparative Zoology). Several Barren River tributaries in Tennessee are of substantial size, suggesting that the lack of mussel records is due to lack of sampling.

We conducted a comprehensive mussel survey of the Tennessee portion of the Barren River system from December 2016 to May 2019. We discuss how our results expand our knowledge of mussel distribution in this region and contribute to conservation efforts for the Green River drainage mussel fauna.

METHODS

Study Area

The Barren River system in Tennessee lies in Sumner, Macon, and Clay counties, and includes 906 km of streams and 18 ha of impoundments (TDEC 2007; Fig. 1). On the western side of the system in Tennessee, the largest tributaries are West Fork Drakes Creek, Middle Fork Drakes Creek, and Trammel Creek, which converge in Kentucky to form Drakes Creek, the largest tributary of the Barren River. On the eastern side of the system in Tennessee the largest tributaries are Long Creek, Salt Lick Creek, Long Fork, and Line Creek, all of which ultimately flow into the upper Barren River. Streams in the Barren River system in Tennessee are on the Eastern Highland Rim or Western Pennyroyal Karst subunits of the Interior Low Plateaus physiographic province. Streams in this area are upland in character and flow over sand, gravel, and bedrock substrates.

The Barren River system in Tennessee is largely rural and undeveloped. The largest municipality (Portland) has fewer than 12,000 people (US Census Bureau 2020). Land use in the Tennessee portion of the system is 50.2% forest (deciduous, evergreen, and mixed), 23.8% developed and barren land, 21.1% hay pasture and herbaceous, 1.9% cultivated crops,

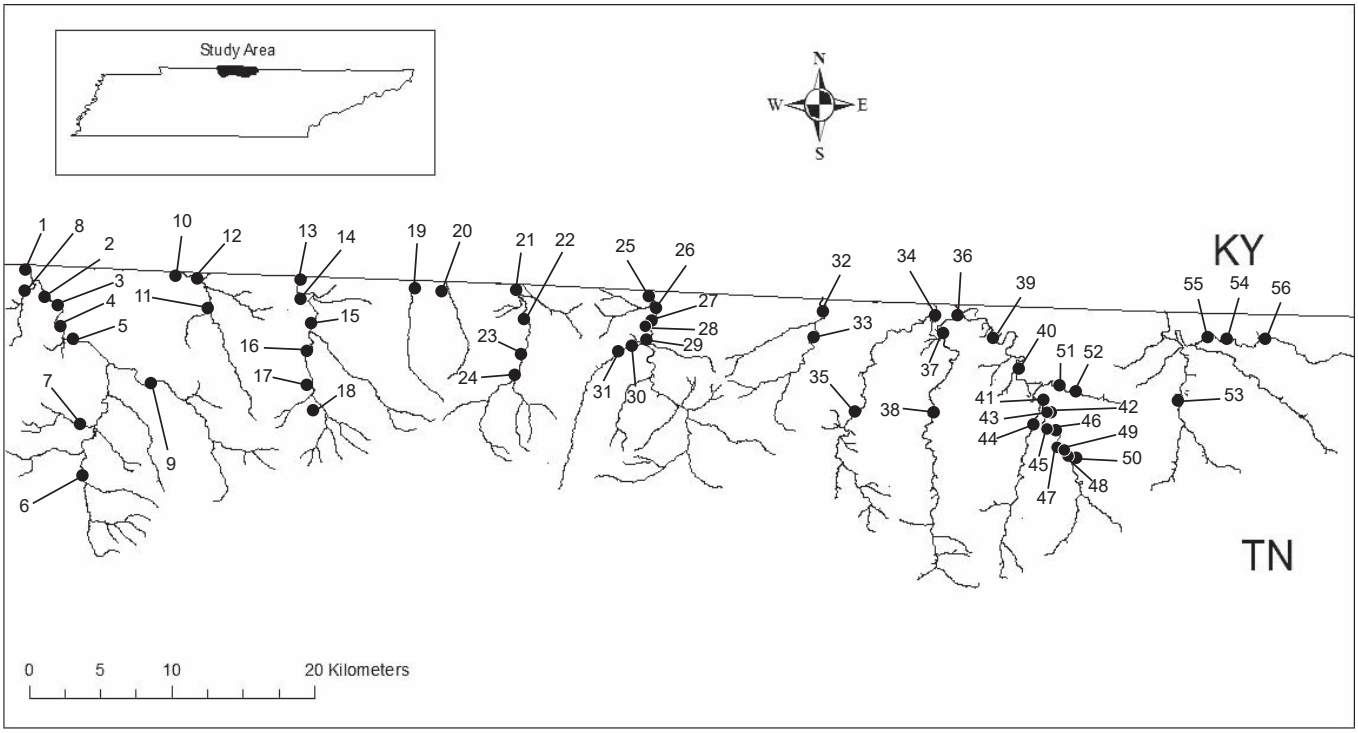


Figure 2. Mussel sampling sites in the Barren River system, Tennessee. Inset map shows the Barren River system in Tennessee (shaded).

Table 1. Mussel survey sites in the Barren River system, Tennessee.

Site	Stream	Location	County	Coordinates	Date	Stream Order
1	West Fork Drakes Creek	Coker Ford Road	Sumner	36.64760, 86.50632	December 13, 2016	3
2	West Fork Drakes Creek	Rapids Road	Sumner	36.63122, 86.49150	December 13, 2016	3
3	West Fork Drakes Creek	State Route 259	Sumner	36.62369, 86.48972	December 13, 2016	3
4	West Fork Drakes Creek	Below Denning Ford Road	Sumner	36.60750, 86.47914	December 13, 2016 December 1, 2017	3
5	West Fork Drakes Creek	Above Denning Ford Road	Sumner	36.60673, 86.46865	December 1, 2017	3
6	West Fork Drakes Creek	Railroad Lane	Sumner	36.50152, 86.46963	December 13, 2016	3
7	West Fork Drakes Creek	Butler Bridge Road	Sumner	36.55553, 86.46758	December 16, 2016	3
8	Grace Creek	500 m upstream of mouth	Sumner	36.64297, 86.50388	December 13, 2016	1
9	Caney Fork Creek	State Route 52	Sumner	36.58240, 86.41974	December 16, 2016	3
10	Sulfur Fork	North Jones Road	Sumner	36.64780, 86.40761	May 17, 2019	2
11	Sulfur Fork	Gregory Road	Sumner	36.62643, 86.39260	December 14, 2016	2
12	Sulfur Fork	Absher Branch Road	Sumner	36.63798, 86.39447	December 14, 2016	2
13	Middle Fork Drakes Creek	State line	Sumner	36.64781, 86.33271	May 16, 2019	3
14	Middle Fork Drakes Creek	Hershel Lyles Road	Sumner	36.63608, 86.32859	December 14, 2016	3
15	Middle Fork Drakes Creek	Confluence w/Dutch Creek	Sumner	36.61704, 86.33000	December 14, 2016	3
16	Middle Fork Drakes Creek	Keen Hollow Road	Sumner	36.60292, 86.32947	December 14, 2016	3
17	Middle Fork Drakes Creek	Haskell Akin Road	Sumner	36.58310, 86.32889	December 14, 2016	3
18	Middle Fork Drakes Creek	State Route 52	Sumner	36.56636, 86.32060	December 14, 2016	3
19	Little Trammel Creek	Old U.S. 31E (State Route 174)	Sumner	36.63788, 86.26626	December 16, 2016	3
20	Garrett Creek	John Beasely Road	Sumner	36.63686, 86.24210	May 16, 2019	2
21	Trammel Creek	State line	Macon	36.63861, 86.19751	May 16, 2019	3
22	Trammel Creek	Buck Haynes Road	Macon	36.62149, 86.19234	December 15, 2016	3
23	Trammel Creek	Hawkins Road	Macon	36.60054, 86.19722	December 15, 2016	3
24	Trammel Creek	Sister Hollow Road	Macon	36.58633, 86.20043	December 16, 2016	3
25	Long Creek	State Line	Macon	36.63479, 86.11480	May 16, 2019	3
26	Long Creek	Hanestown Road	Macon	36.62362, 86.11152	December 15, 2016	3
27	Unnamed tributary to Long Creek	Negro Hollow	Macon	36.62118, 86.10926	May 16, 2019	1
28	Long Creek	Adjacent to Clifty Road	Macon	36.61881, 86.11500	December 15, 2016	3
29	Long Creek	Adjacent to Clifty Road	Macon	36.61131, 86.11568	December 15, 2016	3
30	Long Creek	Shiloh Road	Macon	36.60717, 86.11895	December 15, 2016	3
31	West Fork Long Creek	Shiloh Road	Macon	36.60681, 86.12221	December 15, 2016	2
32	Puncheon Creek	Green Valley Road	Macon	36.62828, 86.00595	May 16, 2017	3
33	Puncheon Creek	Puncheon Creek Road	Macon	36.61174, 86.01224	November 30, 2017	3
34	White Oak Creek	Cook Road	Macon	36.62254, 85.93653	November 30, 2017	3
35	White Oak Creek	Coley Road	Macon	36.56656, 85.98312	November 30, 2017	3
36	Long Fork	Hagan Circle	Macon	36.62209, 85.92411	May 17, 2019	3
37	Long Fork	Wilson Road	Macon	36.61519, 85.92942	November 30, 2017	3

Table 1, continued.

Site	Stream	Location	County	Coordinates	Date	Stream Order
38	Long Fork	Spring Hollow Road	Macon	36.56791, 85.93932	November 30, 2017	2
39	Saltlick Creek	Pitcock Road	Macon	36.61074, 85.89944	November 29, 2017	3
40	Saltlick Creek	Parkhurst Road	Macon	36.59100, 85.88032	November 29, 2017	3
41	Saltlick Creek	1.1 km W of Bethany Cemetery	Macon	36.56933, 85.86982	May 17, 2019	3
42	Unnamed tributary to Saltlick Creek	1.1 km WSW of Bethany Cemetery	Macon	36.56657, 85.86944	May 17, 2019	1
43	Saltlick Creek	Above confluence with Long Hungry Creek	Macon	36.56492, 85.86948	May 17, 2019	2
44	Long Hungry Creek	Above confluence with Saltlick Creek	Macon	36.56477, 85.87075	May 17, 2019	2
45	Saltlick Creek	3.1 km SE of Sunrise	Macon	36.55566, 85.86714	May 17, 2019	2
46	Saltlick Creek	Maxie Bluff Road	Macon	36.55170, 85.85699	November 29, 2017	2
47	Saltlick Creek	Red Boiling Springs	Macon	36.54040, 85.85042	November 29, 2017	2
48	Saltlick Creek	Below old dam site	Macon	36.54431, 85.85877	November 29, 2017	2
49	Saltlick Creek	Old Lake Road to old dam site	Macon	36.54205, 85.85469	November 30, 2017	2
50	Unnamed spring	Red Boiling Springs	Macon	36.53888, 85.84972	November 29, 2017	1
51	Little Saltlick Creek	Sutton Road	Macon	36.58147, 85.85700	November 29, 2017	1
52	Unnamed tributary to Little Saltlick Creek	Sutton Road	Macon	36.58219, 85.85346	November 29, 2017	1
53	Trace Creek	State Route 52	Clay	36.57592, 85.78019	November 30, 2017	2
54	Line Creek	Copars-York Road	Clay	36.60908, 85.75448	November 28, 2017	2
55	Line Creek	Homer-Bray Road	Clay	36.60930, 85.76675	November 28, 2017	2
56	Line Creek	Adj. to Line Creek Road	Clay	36.60880, 85.73158	November 28, 2017	2

Table 2. Mussels found in the Barren River system, Tennessee, 2016–19. Cell entries are the combined number of live and freshly dead mussels or the number of relic shells (in parentheses). Totals do not include unidentifiable shell fragments or *Corbicula fluminea*, which is reported only as present (P) or not present (NP). See Table 1 for site specifications.

	Site																							
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Alasmidonta viridis																								
Fusconaia flava		(1)																						
Lampsilis cardium		1																						
Lampsilis siliquoidea	(1)	1 (3)	(1)				(1)						24	1										
Pyganodon grandis																								
Villosa ortmanni				1 (1)																				
Unidentifiable unionid shell fragments		(1)					(1)																	
Corbicula fluminea	P	P	P	P	P	NP	P	NP	NP	NP	NP	NP	P	NP	NP	NP	NP	NP	NP	NP	P	NP	NP	NP
Total no. of species	1	3	1	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Total no. of individuals	(1)	1 (5)	(1)	1 (1)	0	0	(1)	0	0	0	0	0	24	1	0	0	0	0	0	0	0	0	0	0

1.9% shrub/scrub, and 0.8% wetlands (open water, woody, and herbaceous) (Yang et al. 2018). Despite the undeveloped nature of the system, a number of major tributaries of the Barren River system in Tennessee are considered impaired due to siltation, habitat degradation, or poor water quality associated with point and nonpoint discharges (TDEC 2007). These include Big Trammel Creek, Little Trammel Creek, Long Creek, Long Fork, West Fork Drakes Creek, Middle Fork Drakes Creek, Salt Lick Creek, Trace Creek, Town Creek, and West Fork Long Creek. A widespread problem in the Barren River system of Tennessee is illegal gravel dredging, which is widespread because of the area's relative remoteness and the abundance of gravel substrate in the larger streams (TDEC 2007).

Mussel Surveys

We conducted mussel surveys at 56 sites on 22 streams from December 2016 to May 2019 (Fig. 2 and Table 1). Sample sites were selected based on access, stream position, distance to other sample sites, and presence of suitable mussel habitat, such as shallow riffles and runs with gravel and cobble substrates. At each site, we conducted qualitative visual and tactile searches for live mussels, and we searched shorelines, gravel bars, and submerged vegetation for stranded live mussels and shells. We spent at least 1 person-h at each site except at sites where the habitat was extremely degraded or the water quality was obviously compromised. Longer search times were used at sites where live mussels or freshly dead shells were found. At some sites, we used a rake to disturb the top few centimeters of substrate. We sampled upstream of bridges, fords, and culverts to examine reaches unaffected by those structures. Live mussels were identified to species, measured (anterior to posterior length, nearest 1 mm), counted, and reinserted into the substrate. Freshly dead shells (tissue remaining, shiny nacre) and relic shells (chalky nacre, weathered periostracum) were identified, counted, and cataloged at MMNHC. At each site, we also recorded presence or absence of the invasive species *Corbicula fluminea* (Asian Clam). Our nomenclature follows Williams et al. (2017).

RESULTS

We found live mussels, freshly dead shells, or relic shells at 14 sites; only relic shells were found at four sites (Table 2). Mussels were found in nine third-order streams and in five second-order streams; no first-order streams yielded evidence of mussel occurrence. We found a total of six mussel species, but only five were represented by live individuals or freshly dead shells, and only one to three species were observed at each site. Live mussels were generally uncommon and represented by only one to three individuals at most sites. Exceptions were site 13 (Middle Fork Drakes Creek), where 24 individuals of *Lampsilis siliquoidea* were found, and sites 45 and 46 (Saltlick Creek), where *V. ortmanni* was represented by nine individuals at each site. *Corbicula fluminea* was present at 25 sites, including all but two of the sites with mussels (Table 2). Live *Corbicula* were uncommon at all sites.

Alasmidonta viridis, *L. siliquoidea*, and *V. ortmanni* were the most widespread species in the system, each present at five to six sites (Table 2). *Alasmidonta viridis* was represented by adults only; no juvenile individuals were found (Fig. 3). *Lampsilis siliquoidea* was represented by a range of sizes, but no juveniles were found. *Villosa ortmanni* was represented by a range of sizes and included small individuals indicative of recent recruitment (Fig. 3). Three species were found at a single site and represented by single individuals: *Fusconaia flava*, *Lampsilis cardium*, and *Pyganodon grandis*; *F. flava* was represented only by a single relic shell at site 2 (West Fork Drakes Creek). In addition, we found two freshly dead *L. fasciola* at one site in Middle Fork Drakes Creek a few hundred meters downstream of the Kentucky state line, but we

Table 2, extended.

Site																																								
25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	53	54	55	56								
																(1)				1	3 (1)		1							(1)	2										
	1															2 (2)				9	9		2																	
NP	NP	NP	NP	NP	NP	NP	NP	NP	P	P	P	NP	P	P	P	P	P	P	P	P	P	NP	P	P	NP	NP	NP	NP	NP	P	P	P								
0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	2	0	2	0	0	0	0	0	0	1	1	0								
0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2 (3)	0	0	0	10	12 (1)	0	3	0	0	0	0	0	0	(1)	2	0								

could not confirm its occurrence in the Tennessee portion of the Barren River system.

DISCUSSION

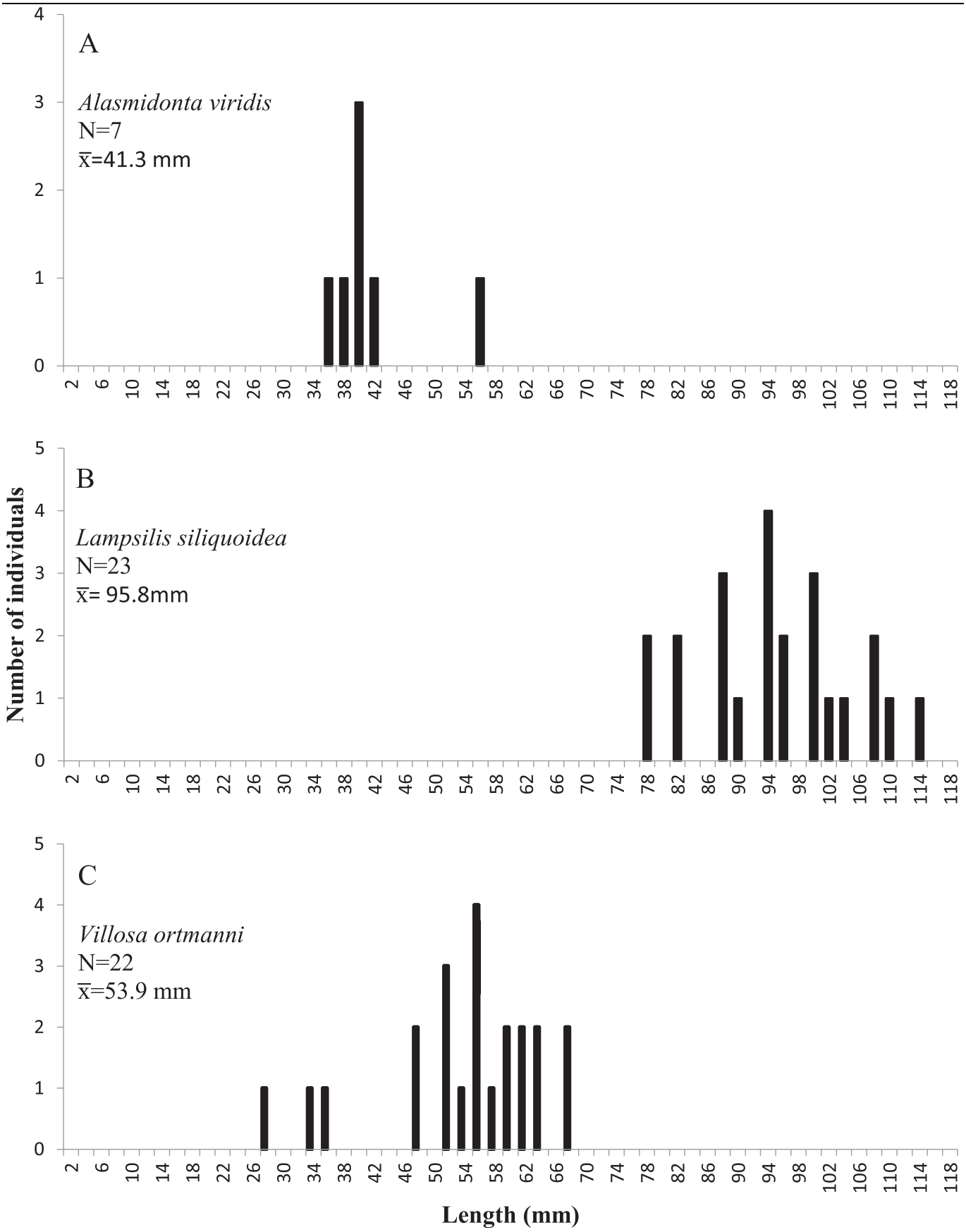
The Barren River system in Tennessee supports a limited mussel fauna typical of headwater streams in the Green River system and elsewhere in the Ohio River basin. *Alasmidonta viridis* and *L. siliquoidea* are characteristic headwater species throughout much of this region, and *P. grandis* is a stream-size generalist that is often common in small streams (Parmalee and Bogan 1998; Watters et al. 2009; Haag and Cicerello 2016). *Lampsilis cardium* and *F. flava* also occur in a wide variety of habitats, but neither species typically occurs far into the headwaters (Haag and Cicerello 2016). We found both of these species only in a larger stream (West Fork Drakes Creek), about 3 stream km upstream of the Kentucky state line. *Lampsilis fasciola* is widely distributed in the Barren River system in Kentucky and also may occur in the lower reaches of Barren River tributaries in Tennessee, but we could not confirm its presence.

Villosa ortmanni traditionally is considered endemic to the upper Green River drainage, but there is uncertainty about its relationship to *Villosa vanuxemensis* in the adjacent Red River system (Cumberland River drainage; Kuehnl 2009; Haag and Cicerello 2016); until that issue is resolved, we follow the traditional view of this species as endemic to the Green River drainage. *Villosa ortmanni* occurs in a wide variety of stream habitats from the mainstem Green River to small streams, but it is a characteristic species of headwaters, particularly spring runs, where it may be the only species present (Haag and Cicerello 2016). Along with *A. viridis*, *V. ortmanni* was the only species we found in second-order streams. Prior to our study, *V. ortmanni* was considered endemic to Kentucky (Haag and Cicerello 2016).

Tennessee has the second-highest number of mussel species in the USA, behind Alabama (Parmalee and Bogan 1998, Williams et al. 2008). Our discovery of *V. ortmanni* in the Barren River system of Tennessee brings the total number of recognized species in the state to 140 (G. Dinkins, personal observation). This is the first new record of a previously recognized species from Tennessee since reports of *L. siliquoidea* in 1985 and 1994 (MMNHC; Kesler and Manning 1996). Prior to our study, *L. siliquoidea* was reported in Tennessee only from direct tributaries of the Mississippi River (Reelfoot Lake and Wolf River), and our records of that species are the first from the Ohio River basin in Tennessee. In addition, our study provides the first mussel records of any species from the Barren River system in Tennessee.

In part, the scarcity of mussels in the upper Barren River system may be a natural feature of these headwater streams, where mussel abundance typically is lower than it is in larger streams (Haag 2012). However, the extremely low abundance we observed may be a result of human factors that have further reduced mussel populations. Entire mussel assemblages have nearly disappeared from much of the Barren River system and from many other upland streams in the southeastern USA, but the reasons for these disappearances are unknown (Irwin 2018; Reed et al. 2019; Haag 2019). The upper Barren River system is now isolated by Barren River Reservoir in Kentucky, which hinders mussel dispersal and gene flow. We observed several sources of stream degradation including illegal gravel mining (West Fork Drakes Creek, Middle Fork Drakes Creek, Long Creek, Trace Creek), channelization (Salt Lick Creek, Line Creek), and brine discharge from abandoned gas wells (Little Salt Lick Creek, Middle Fork Drakes Creek), but we have no information about the extent of these impacts or their effects on water quality or mussels in the Barren River system.

Our discovery of additional populations of *V. ortmanni* is important from a conservation perspective, regardless of this species' taxonomic status. The species was once widespread and common in the Green River drainage, but it has declined


A
Alasmidonta viridis
N=7
x̄=41.3 mm
B
Lampsilis siliquoidea
N=23
x̄= 95.8mm
C
Villosa ortmanni
N=22
x̄=53.9 mm
Number of individuals
Length (mm)

dramatically in the last 30 yr and now survives in only a few small populations; populations of *V. vanuxemensis* in the adjacent Red River system have declined similarly (Haag and Cicerello 2016; M. Compton, Office of Kentucky Nature Preserves, personal communication). In 2010, the Center for Biological Diversity petitioned the U.S. Fish and Wildlife Service to include *V. ortmanni* on the federal list of endangered species. Our findings considerably expand the known range of this species, and at least two of the sites we surveyed supported relatively large populations with evidence of recent recruitment.

With the exception of *V. ortmanni*, all of the mussel species we observed remain widespread and common in at least some parts of their ranges, but enigmatic mussel declines in the Barren River system and elsewhere threaten the survival of even widespread species. Headwater streams provide unique aquatic habitats but are vulnerable to a wide range of human impacts (Downing et al. 2012; Wohl 2017). Approximately 12,000 m of a tributary to Line Creek is being restored as part of the Tennessee Stream Mitigation Program (T. Dinkins, Stantec Consulting Services, Inc., personal communication). Efforts such as this are necessary to improve and ensure the health of headwater streams and the mussel assemblages they support.

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Figure 3. Length frequency distributions of live and freshly dead (a) *Alasmidonta viridis* in Salt Lick and Line creeks, (b) *Lampsilis siliquoidea* in West Fork Drakes and Middle Fork Drakes creeks, and (c) *Villosa ortmanni* in Salt Lick and West Fork Drakes creeks, Barren River system, Tennessee. Sample size (N) and mean length ($\bar{x}$) is provided for each species.

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REGULAR ARTICLE

ASSESSMENT OF BURROWING BEHAVIOR OF FRESHWATER JUVENILE MUSSELS IN SEDIMENT

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ABSTRACT

Standard laboratory sediment toxicity methods have been adapted for conducting toxicity tests with juvenile freshwater mussels. However, studies looking at juvenile mussel burrowing behavior at the water-sediment interface are limited. Juvenile mussels burrow in sediment for the first 0 to 4 yr of life but also may inhabit the sediment-water interface. The objective of this study was to evaluate burrowing behavior of various species and ages of juvenile freshwater mussels in three control sediments: West Bearskin Lake, Spring River, and coarse commercial sand. Species tested included (1) Fatmucket (*Lampsilis siliquoidea*), (2) Notched Rainbow (*Villosa constricta*), (3) Washboard (*Megalonaias nervosa*), (4) Rainbow (*Villosa iris)*, (5) Arkansas Fatmucket (*Lampsilis powellii*), and (6) Oregon Floater (*Anodonta oregonensis*). Greater than 95% of the mussels burrowed into test sediment within 15 min. Across species, life stage, and substrate type, most mussels were recovered from the upper layers of sediment (91% at a sediment depth of 3.4 mm or less), and only 2% of the mussels were recovered at a depth >5.1 mm. No mussels were recovered from a depth >6.8 mm. There was no difference in mussel burrowing depth at 4 h versus 24 h across species, age, and sediment type. Two ages of Fatmucket burrowed to a significantly greater depth in the West Bearskin Lake sediment compared to the Spring River sediment or Coarse Sand. However, there was no significant difference in mean depth across sediment type with the other five species of mussels tested. Based on species and age of mussels tested, juvenile mussels up to an age of at least 20 wk and a length of at least 5 mm readily burrow into sediment and likely would be exposed to contaminants in whole sediment and associated pore water throughout a laboratory sediment toxicity test.

KEY WORDS: freshwater mussel, Unionidae, behavior, benthic ecology, Fatmucket, Notched Rainbow, Washboard, Oregon Floater, Rainbow, Arkansas Fatmucket, control sediments

INTRODUCTION

Freshwater mussels of the family Unionidae are widely distributed throughout North America. Mussels have been reported from lakes and streams, on substrata varying from mud and clay to sand and coarse gravel, and they are often associated with vegetation (Clarke 1973). Freshwater mussels are among the most imperiled groups of fauna in North America (Ricciardi and Rasmussen 1999; Lydeard et al. 2004; Strayer et al. 2004). North America has the world's most diverse freshwater mussel fauna, with more than 300 taxa, but over 70% of species are considered extinct, endangered, threatened, or of special concern (Williams et al. 1993). The decline in the U.S. mussel fauna has been attributed to a variety of factors, including habitat modification, introduction of exotic species, over-utilization, and contaminants (Watters 1999; Wang et al. 2007a, 2007b, 2013; Bringolf et al. 2007; Okay and Karacik 2008; Cope et al. 2008; Downing et al. 2010; Besser et al. 2015).

The three main life stages of freshwater mussels are glochidia, juveniles and adults, and each life stage uses a different habitat. Glochidia are primarily found in the water column while in the free-living stage (Cope et al. 2008). Juvenile mussels reportedly burrow in sediment for the first 0 to 4 yr of life after transformation (Strayer et al. 2004; Schwalb

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and Pusch 2007; Cope et al. 2008). Numerous studies have documented the burrowing behavior of older juvenile and adult mussels (Lewis and Riebel 1984; Hull et al. 1998; Watters et al. 2001; Archambault et al. 2014; Block et al. 2013; Hazelton et al. 2014); they have been observed using their shell and foot to burrow into sediment. Though mussels generally are considered to be sessile, several studies have documented both vertical and horizontal movements (Kat 1982; Amyot and Downing 1991; Downing et al. 1993; Balfour and Smock 1995; Amyot and Downing 1997; Schwalb and Pusch 2007; Allen and Vaughn 2009). Populations of Eastern Elliptio (*Elliptio complanate*) were found to move up to 3 m/yr, and this movement was nondirectional (Balfour and Smock 1995). Horizontal movement of up to 15 cm/wk has been documented for Painter Mussels (*Unio pictorum*) and Duck Mussels (*Anodonta anatina*) in a river setting (Schwalb and Pusch 2007). Mussels may burrow completely or partially in sediment throughout the year, depending on water temperature (seasonal migration) and reproductive activity (Amyot and Downing 1991, 1997; Watters et al. 2001; Cope et al. 2008; Block et al. 2013).

Mussels spend much of their lives at or just below the sediment/water interface. This interface is a particularly important factor when assessing the environmental effects of chemical contaminants such as metals and persistent hydrophobic or nonpolar organic chemicals (e.g., oil and polychlorinated biphenyls). The sediment surface is the bioactive zone, where organisms interact with sediment and can receive the greatest exposure, whereas organisms may not be exposed to contaminants that are present in deeper sediments (National Research Council 2003). This upper layer of sediment is a microbially active layer and can have important redox properties that affect metal speciation and subsequent uptake and toxicity. Here mussels can be exposed to contaminants resulting in adverse effects on mussel recruitment, reproduction, or survival (Thorsen 2004; Cope et al. 2008; Hazelton et al. 2014).

Sediment toxicity bioassay methods, used to determine the bioavailability and toxicity of chemicals in sediment, rely on organisms that come in direct contact with the sediment. Standard laboratory organisms commonly used to conduct sediment toxicity tests are either epibenthic (e.g., amphipods) or create irrigated tubes on the sediment surface (e.g., midges) or into surficial sediments (e.g., mayflies). Juvenile freshwater mussels are an ideal candidate for use in toxicity bioassays because they are in direct contact with sediment. Standard bioassay methods using freshwater mussels in water-only bioassays have been developed (ASTM 2019a) and were modified in the current study to evaluate toxicity of field-collected sediments to mussels (Wang et al. 2013; Besser et al. 2011, 2015; Ingersoll et al. 2015; Schein et al. 2015). However, because freshwater mussels have a complex life history, they require specialized methods for laboratory culture (Neves 2004; Barnhart 2006), and there has been uncertainty about contaminant exposure and the role of bioavailability in laboratory toxicity tests with juvenile freshwater mussels. The objective of this study was to improve our understanding of the burrowing behavior of juvenile mussels (3- to 20-wk-old mussels) and to determine whether species, age, or sediment type influences burrowing behavior.

METHODS

The six species evaluated included (1) Fatmucket *(Lampsilis siliquoidea*; size 0.4–5.0 mm [about 3, 7, 10, and 20 wk posttransformation]); (2) Notched Rainbow (*Villosa constricta;* size 6.0–8.0 mm [about 20 wk posttransformation]); (3) Washboard (*Megalonaias nervosa*; size 1.0–1.5 mm [about 6 wk posttransformation]); (4) Rainbow (*V. iris;* size 1.4–2.5 mm [about 7 wk posttransformation]); (5) Arkansas Fatmucket (*L. powellii*; size 0.9–1.3 mm [about 4 wk posttransformation]); and, (6) Oregon Floater (*Anodonta oregonensis*; size 1.5–2.3 mm [about 4 wk posttransformation]). Starting lengths of mussels were determined to the nearest 0.1 mm with a digitizing system using video micrometer software (Image Caliper, Resolution Technology, Dublin, OH, USA). Test organisms were obtained from Missouri State University cultures (Chris Barnhart, Springfield, MO, USA).

We conducted exposures using three sediments with different physical properties. Sediments evaluated included two commonly used control sediments: (1) West Bearskin Lake sediment, a sand/silt/clay mixture (49% sand) with a total organic carbon (TOC) content of about 3% obtained from northeastern Minnesota (Ingersoll et al. 1998), and (2) Spring River sediment, a predominantly fine sand (82% sand) with a TOC content of about 1% obtained from southwest Missouri (Besser et al. 2011) as well as (3) a coarse commercial sand with a diameter of $<$0.5 mm (Granusil, no. 4030) purchased from Menards (Eau Claire, WI, USA). Sediments were selected because they have been used successfully as control sediments in previous sediment toxicity exposures (Kemble et al. 2013; Ingersoll et al. 1998; Besser et al. 2011, 2015).

Experiments were conducted in clear 60-mL Monoject plastic syringes (Covidien, Mansfield MO, USA), which were modified by cutting the top off, leaving the top open to produce a 3.5-cm diameter opening (Fig. 1). Before the start of an exposure, test sediment was homogenized with a plastic spoon in a stainless-steel bowl. The syringe handle was pulled back to the 30-mL mark on the syringe, and 40 mL of one of three control materials was placed inside the syringe using a small scoop. About 10 mL of overlying water was gently poured over the sediment to maintain a flat sediment-water interface. The source of the overlying water was well water diluted with deionized water to a hardness of about 100 mg/L (as $CaCO_3$), an alkalinity of 85 mg/L (as $CaCO_3$), and a pH of about 8.2. A 24-h equilibration period was used to let sediments settle out of the water column before the introduction of mussels.

Up to 20 mussels were placed into each of the substrates (e.g., typically five mussels/replicate syringe with a total of four replicates/species/treatment) for either 4 or 24 h under static conditions. This stocking rate provided $\sim$306 mm^2 of

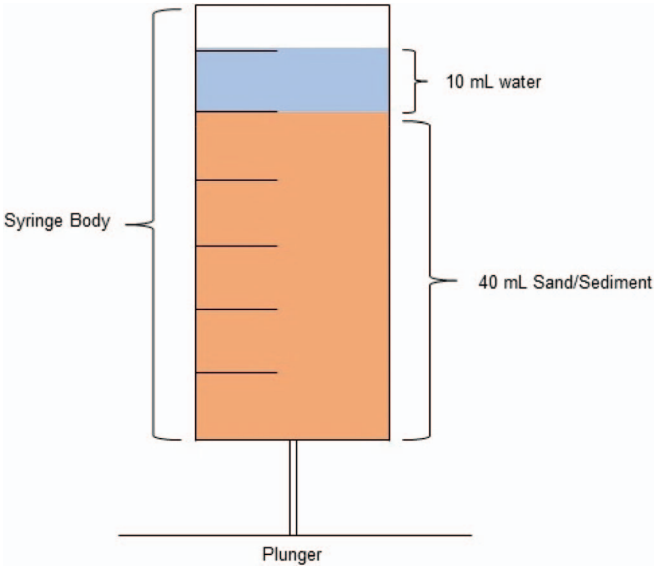

Figure 1. Design of the chambers (60-mL syringes) used to evaluate burrowing behavior of mussels in sediment.

surface area of sediment/mussel/chamber. For exposures using older Fatmucket Mussels and Notched Rainbow Mussels (5 mo old), we exposed one mussel/test chamber with additional replicate chambers/substrate tested (five replicates chamber/species). Using a pipette, mussels were stocked below the water surface of a syringe. We observed test chambers after stocking and recorded the time to complete burrowing. Chambers were then placed in a water bath at 23°C with a light intensity of about 500 lux (16L:8D photoperiod in the 24-h exposures). We did not feed mussels during the exposures. Average burrowing depth was the endpoint evaluated in exposures.

Water quality analysis was conducted on overlying water siphoned off at the end of the exposures. Given the small volume of overlying water, we were able to measure only dissolved oxygen, conductivity, pH, and total ammonia in most of the exposures. Ranges of the water quality parameters in the exposures were dissolved oxygen, 6.6 to 8.7 mg/L; conductivity, 238 to 935 µS/cm; pH, 7.86 to 8.50; total ammonia, 0.11 to 13.0 mg N/L; and unionized ammonia, 0.004 to 0.548 mg N/L (Appendix 1). The wide ranges of some water quality parameters are a result of using both artificial and natural sediments as a substrate.

At the end of the exposures, we recovered mussels from select sediment sections by siphoning off the overlying water with a pipette, then gently pressing the syringe plunger to the first 1-mL mark until a 1.7-mm section of sediment was exposed at the top of the syringe. The extruded sediment was then scraped off the top of the syringe using a stainless-steel spatula into a glass dish. Sediment in the glass dish was gently rinsed using a squirt bottle to break the small clump of sediment apart so the mussels could be counted in that section. We repeated this process using 1.7-mm sections of sediment until all the mussels had been recovered from the syringe. Average burrowing depth was calculated using the midpoint of a sampling section of sediment (i.e., 0.85 used for the 0–1.7 mm section) from a syringe. For the exposures with younger mussels (e.g., 3 wk old), sections of sediment were scraped into a 150-µm sieve and rinsed gently with test water. Material remaining on the sieve was rinsed into a small Petri dish. A microscope was used for counting mussels in the Petri dish from each 1.7-mm section.

Statistical analyses were performed using SAS statistical software (SAS/STAT version 9.2; SAS, Cary, NC, USA). Average burrowing depth of mussels was determined by (1) species, (2) mussel age, (3) sediment type, (4) study duration, or (5) a combination of these four. Differences in average burrowing depth of mussels were determined by analysis of variance (ANOVA). Burrowing depth data were transformed before ANOVA to improve normality as indicated by Shapiro–Wilk test (United States Environmental Protection Agency 2000; ASTM International 2019b). If transformations (square root or log) did not improve normality, data were rank transformed before analysis (Conover and Iman 1981).

RESULTS

Nearly all mussels burrowed into sediments after being stocked into the chamber, except one 20-wk-old Notched Rainbow collected on the surface of the West Bear Lake sediment after 24 h. Most mussels (90%) burrowed within 15 min of introduction to the test chambers. Shell size and age of mussels had no effect on the time it took for a mussel to completely burrow into a test sediment. Most mussels across species and age were recovered from the upper two layers of the three substrates (91% at sediment depths of 0.0 to 1.7 or 1.7 to 3.4 mm; Fig. 2, Appendix 2) with only 2% of mussels recovered at a depth >5.1 mm. No mussels were recovered from a sediment depth >6.8 mm. Mussels that were partially burrowed at the end of a bioassay were counted as being burrowed. Overall, there was a general trend for the older mussels to be recovered from a greater depth in sediment than younger mussels. However, this trend may be the result of fewer older mussels being tested and the fact that only one 20-wk-old mussel was tested per replicate. Similarly, we also observed a trend for mussels tested in the West Bear Lake treatment to be recovered at greater depths compared to the Spring River and Coarse Sand treatments (Fig. 3).

In our initial comparison of burrowing behavior with juvenile mussels we evaluated burrowing at two exposure times (4 and 24 h). However, our results showed there was no difference in average burrowing depth of mussels in any of the test sediments between the two study durations (Appendix 2). Therefore, all later exposures were conducted for 24 h only. Because there was no significant difference in mean mussel depth in the two study durations, we used replicate data from both the 4- and 24-h trials to determine mean burrowing depth where we had data for both exposure times.

Because the Fatmucket is a common test species it was the only species for which different age mussels were tested across

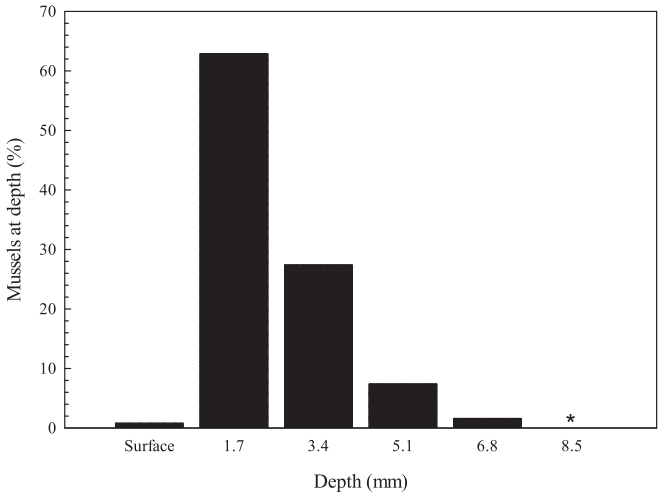

Figure 2. Percentage of all mussels recovered at a sampling depth. * = 0% recovered from a depth.

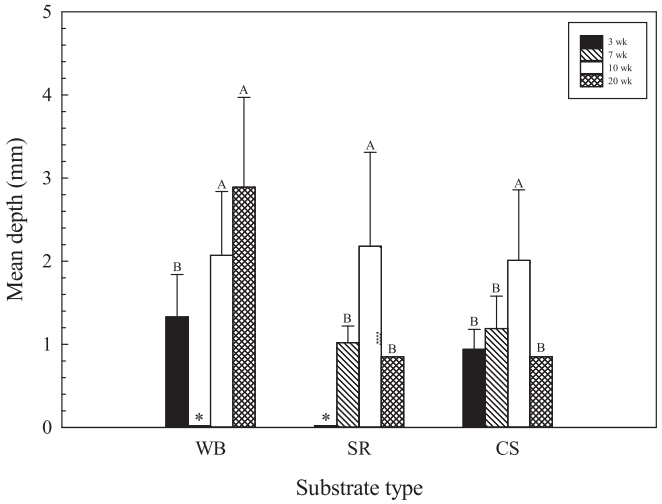

Figure 4. Percentage of Fatmucket mussels recovered at 4 or 24 h at sampling depths by sediment type. * = Age not tested. Different letters designate a significant difference in burrowing depth within a sediment type. WB = West Bear Lake sediment, SR = Spring River sediment, CS = Coarse Sand.

the three sediment types. The mean burrowing depth of 10- and 20-wk-old Fatmucket was significantly greater than the 3-wk-old Fatmucket in the West Bear Lake sediment. However, in the Coarse Sand and the Spring River sediment, only the 10-wk-old Fatmucket were recovered at a significantly greater depth compared to the other ages of Fatmucket tested (Appendix 3; Fig. 4).

Overall, 3- and 20-wk-old Fatmucket burrowed significantly deeper in the West Bear Lake sediment compared to the Spring River sediment and Coarse Sand. These age groups also burrowed more deeply than the 7- and 10-wk-old Fatmucket (Appendix 3). Mean burrowing depth of 7- and 10-wk-old Fatmucket was similar across all three sediment types (Appendix 3). We recovered 20-wk-old Fatmucket at a mean depth 3.3 times deeper in the West Bear Lake (2.89 mm) sediment than in either the Spring River sediment (0.85 mm) or Coarse Sand (0.85 mm). West Bear Lake sediment has more fines (higher silt and clay content) and is less dense than either the Spring River sediment or the Coarse Sand.

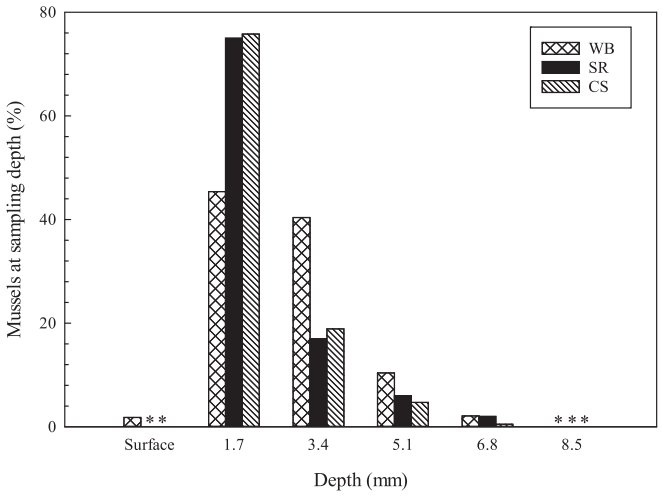

Figure 3. Percentage of mussels recovered 4 or 24 h at sampling depths by sediment type. * = 0% recovered from a sampling depth. WB = West Bear Lake sediment, SR = Spring River sediment, CS = Coarse Sand.

No significant difference in mean burrowing depth was detected between species (Arkansas Fatmucket, Rainbow, and Washboard) in multiple test sediments (Appendix 2). All 4-wk-old Arkansas Fatmucket were recovered at a depth of 3.4 mm or less in the three sediments. Individual Arkansas Fatmucket burrowed deeper in the Coarse Sand than in the two natural sediments, but there was no significant different in average burrowing depth of 4-wk-old Arkansas Fatmucket based on sediment type (Appendix 2). Similar to the Arkansas Fatmucket exposures, 6-wk-old Washboard were recovered at deeper depths in the Coarse Sand than in the West Bear Lake or Spring River sediments, but there was no significant difference in burrowing depth of Washboard in the three sediments (Appendix 2). Similarly, we observed no significant difference in mean burrowing depth of 7-wk-old Rainbow across the three sediments (Appendix 2).

There was no general pattern in mussel burrowing behavior when we compared different species of similar-age mussels across the three sediment types (Fig. 5). However, when we compared age groups (i.e., 3–4 wk, 6–7 wk, and 20 wk), we observed differences in burrowing depth between the ages tested. The 20-wk-old mussels burrowed significantly deeper in the West Bear Lake sediment compared to mussels 7 wk old or younger. In the Spring River sediment, 10-week-old mussels burrowed significantly deeper than the 4-, 6-, and 20-wk-old mussels. In the Coarse Sand treatment, mean burrowing depth of 6- and 10-wk-old mussels was significantly deeper than mean depths of the 3- and 20-wk-old mussels (Appendix 2).

Mussels of similar ages tended to burrow to similar

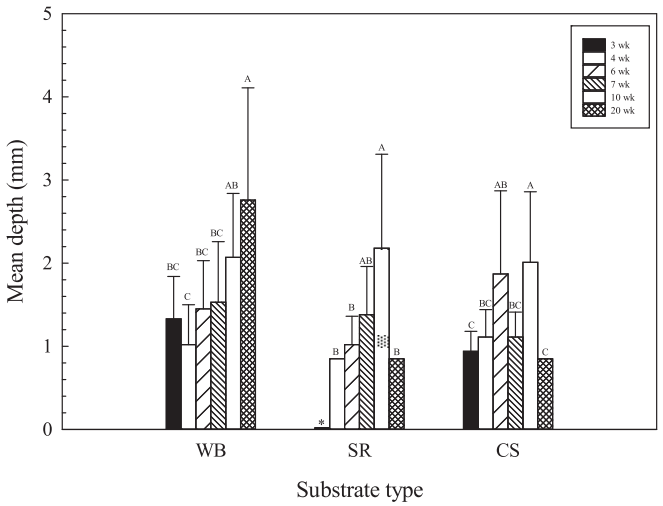

Figure 5. Mean mussel borrowing depth of all species tested at 4 and 24 h in three substrates by mussel age. Mean depths with different letters indicate a significant difference across age within a sediment type. * = Age not tested. WB = West Bear Lake sediment, SR = Spring River sediment. CS = Coarse Sand.

depths. Mean burrowing depth of the 3- to 4-wk-old mussels ranged from 0.85 to 1.33 mm in the West Bear Lake sediment and 0.94 to 1.11 mm in the Coarse Sand. A single 3-wk-old Fatmucket was recovered at a depth greater than 3.4 mm in the West Bear Lake sediment, but there was no difference in mean burrowing depth across the three species in the West Bear Lake treatment (Appendix 2). Similarly, we observed no significant difference in burrowing depth of the Arkansas Fatmucket and Fatmucket in the Coarse Sand. In exposures with 7-wk-old mussels, Rainbow were recovered at greater depths than the 7-wk-old Washboard in West Bear Lake and deeper than the Washboard and Fatmucket in Spring River. We recovered 18% of the Rainbow at a depth >3.4 mm in the West Bear Lake treatment, while 100% of the Washboard were recovered at <3.4 mm. However, there was no significant difference in mean burrowing depth of the two species (Appendix 2). Similarly, we recovered 10% of the 7-wk-old Rainbow at a depth of >3.4 mm in the Spring River sediment, while 100% of Fatmucket and Washboard were recovered at depths <3.4 mm. However, there was no significant difference in mean burrowing depth of the three species in the Spring River sediment (Appendix 2). In the Coarse Sand, 6-wk-old Washboard were recovered at a deeper average mean depth (1.85 mm) than either the 6-wk-old Fatmucket (1.19 mm) or 6-wk-old Rainbow (1.0 mm). Again, no significant difference in mean burrowing depth between the three species in the Coarse Sand was observed (Appendix 2). A similar pattern was observed with the older mussels. Notched Rainbow were recovered at a wider range of sediment depths compared to the 20-wk-old Fatmucket. However, there was no significant differences in mean burrowing depths of the two species in the West Bear Lake sediment (Appendix 2).

DISCUSSION

Based on the species and age of mussels tested in the current study, juvenile mussels up to an age of 20 wk readily burrow into sediment as was reported by Yeager et al. (1994). They reported 1- to 2-wk-old Rainbow burrowed within 20 min of being placed into the sediment with similar grain size characteristics of the natural sediments used in the current study. With the exception of one individual, mussels in the current study were completely burrowed within 15 min in all sediments and remained below the sediment surface for the duration of the study.

Burrowing depth varies based on the age of the mussel. The deepest we observed mussels in the syringes was 6.8 mm in the two natural sediments, similar to what Yeager et al. (1994) reported in feeding and burrowing studies in which juvenile Rainbow were recovered in the top 1 cm of sediment. Juvenile mussels have been recovered from much greater depths in field studies. Mussels 0–3 yr old were recovered from the top 8 cm of sediment (Neves and Widlak 1987) and Schwalb and Pusch (2007) recovered mussels up to a depth of 20 cm. However, juvenile mussels in these studies included older mussels, up to 3 yr in age. One potential limitation to burrowing depths observed in the current study was the size of the study chambers used (maximum depth, 36 mm). However, because the greatest observed burrowing depth was only 6.8 mm, we do not believe that space limitation in the syringe prevented the mussels from burrowing deeper. No mussels were recovered in the lowest sediment fraction of the syringe in any of our study Gough et al. (2013) and Archambault et al. (2014) also found mussels at shallow depths, indicating that space for vertical or horizontal movement was not a limitation. Gough et al. (2013) found that adult Pondhorn (*Uniomerus tetralasmus*), Giant Floater (*Pyganondon grandis*), and Southern Fatmucket (*Lampsilis straminea*) burrowed to shallow depths (a few centimeters) instead of moving to greater depths with reducing water levels. Archambault et al. (2014) found that under thermal stress, juvenile Pink Mucket (*Lampsilis abrupta*) and Eastern Lampmussel (*Lampsilis radiata*) did not burrow below the top stratum of sediment (2.5 cm).

In the current study, we exposed six different species of mussels and found no difference in burrowing depth or behavior between species of similar age. However, burrowing behavior of a mussel community depends on the diversity of the community (Allen and Vaughn 2009). When a mussel community was manipulated (i.e., density and diversity were manipulated by increasing the number of mussels or the number of species within a treatment), Allen and Vaugh (2009) observed significant differences in shell exposure (i.e., shell above sediment surface) and both vertical and horizontal movement between species. However, all the exposures conducted here were single-species exposures and differences in burrowing depth and behavior may have resulted had we tested with multiple species in a syringe.

Previous studies have shown that mussel density may affect vertical movement. Mussel density in the current study

was reduced from five mussels per chamber to one per chamber when we tested with larger mussels. In contrast to Allen and Vaughn (2009), it is unlikely, given the size of mussels tested, that burrowing depth with any of the six species tested was affected by density within a sediment.

Mussels in the current study showed no general pattern in burrowing behavior across the three sediment types (Fig. 5). If the physical characteristic of the sediment is important in determining the distribution, then relative ability of a mussel to burrow in different sediment types may be important in establishing and maintaining suitable habitats for survival, growth, and reproduction (Kat 1982). This is especially important for juvenile mussels in habitats prone to water-flow alterations, sedimentation, and erosion. While a clay substrate proved more difficult for the Eastern Elliptio and Giant Floater *(Anodonata grandis)* to right on than sand or gravel, both species and the Eastern Lampmussel (*Lampsilis radiata)* burrowed significantly deeper in clay in 30 min than the other two substrates (Lewis and Riebel 1984). Lewis and Riebel (1984). concluded that the substratum particle size had an influence on the ability and speed of righting and burrowing of unionid mussels. However, in the current study, only the 20-wk-old mussels were generally found at deeper depths in the West Bearskin Lake substrate. It is unclear if this result is due to the fact that West Bearskin Lake is a much finer substrate or if our sampling method artificially increased depth by pushing mussels deeper as we scraped a sediment section.

Similar to water temperature, photoperiod is thought to play a role on vertical migration of mussels. Vertical migration of adult Fatmucket was found to be more correlated with day length than with water temperature in both field populations and artificial streams (Perles et al. 2003). In the current study, the photoperiod was the standard 16:8 (light:dark) for all exposures (ASTM 2019a, 2019b). This photoperiod corresponds to a mid-April to mid-July time frame, when high densities of adult mussels have been reported at the sediment-water interface (Amyot and Downing 1991; Balfour and Smock 1995; Amyot and Downing 1997; Watters et al. 2001). Juvenile mussels are thought to stay in the substrate for the first couple years of life. Longer exposures in the syringes, along with alternating the photoperiod, could be conducted to determine whether day length or duration have an effect on burrowing behavior of young mussels (i.e., daily movement or depth used by young mussels).

Rapid burrowing by young mussels, as observed in this study, might provide protection from strong currents in a stream; remaining burrowed also might reduce the chance of being dislodged and relocated to less suitable habitat. Mussels 1 to 14 d old have been recovered at depths about two to three times deeper than in the current study (Yeager et al. 1994). One potential explanation is that the present study was conducted under static conditions while the other studies were done under flow-through conditions. Adult Freshwater Pearl Mussel (*Margaritifera margaritifera*) will burrow as deep as necessary to avoid being dislodged by the current (Thoms and Berg 1985). Schwalb and Pusch (2007) reported no significant difference in the distance moved among three species and found that the dynamics of surface densities of mussels could be explained by discharge, day length and water temperature, and those mussels may circumvent dislodgement in extreme flows by burrowing deeper into the sediment in riverine systems. These studies suggest that burrowing behavior is flexible in response to environmental conditions, so that may explain why we do not see a wide range of burrowing behavior in laboratory tests under controlled conditions.

Little research has been done evaluating juvenile mussel burrowing behavior. The current study examined burrowing behavior of several species of juvenile mussels under controlled conditions (i.e., testing with control sediments only, a set temperature). However, many of the factors that other investigators have found to affect older mussel burrowing behaviors could be evaluated with the methods and test apparatus used here. Study duration and species type did not affect burrowing depth of the mussel. However, additional studies with a longer duration may be needed to fully evaluate burrowing behavior over time. Also, due to the lack of mussel availability, we did not test all four ages with all the species. We also observed a general trend for older mussels to burrow to a greater depth than younger mussels. By burrowing to greater depths, older mussels may be exposed to different contaminants, or to lower levels of contaminants, than mussels remaining at or near the sediment-water interface, where contaminants tend to accumulate (Mulligan and Law 2013). Additional studies with older juveniles (>20 wk) in larger chambers also may help determine if and when juvenile mussels might inhabit the sediment-water interface and begin filtering overlying water. Additional studies could examine juvenile burrowing behavior in field-collected sediments evaluating mussel exposure of contaminants at the sediment/water interface to whole sediment and porewater throughout a sediment toxicity test. These additional studies would provide needed information about the benthic ecology of this imperiled group.

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Appendix 1. Overlying water quality data. NM: Not Measured, WB: West Bear Lake sediment, SR: Spring River sediment, CS: Coarse Sand.

Species	Age (wk)	Sediment	Exposure Time (h)	Temp (°C)	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	pH	Total Ammonia (mg/L)	Unionized Ammonia (mg/L)
Fatmucket	3	CS	4	23	8.6	342	NM	0.70	NM
Fatmucket	3	CS	24	23	8.5	238	NM	1.15	NM
Fatmucket	3	WB	4	23	8.1	486	NM	1.19	NM
Fatmucket	3	WB	24	23	7.7	383	NM	1.72	NM
Arkansas Fatmucket	4	WB	24	23	7.7	625	7.86	0.12	0.004
Arkansas Fatmucket	4	SR	24	23	7.1	774	7.98	0.45	0.020
Arkansas Fatmucket	4	CS	24	23	7.9	779	8.11	0.11	0.007
Oregon Floater	4	WB	24	23	7.8	593	8.07	0.38	0.021
Washboard	6	WB	24	23	8.2	583	NM	3.51	NM
Washboard	6	CS	24	23	8.6	725	NM	1.16	NM
Washboard	6	SR	24	23	8.1	533	NM	3.25	NM
Rainbow	7	CS	24	23	8.7	723	NM	0.15	0.000
Fatmucket	7	CS	24	23	8.3	709	NM	0.22	0.000
Fatmucket	7	SR	24	23	7.2	723	8.50	1.44	0.196
Rainbow	7	WB	24	23	7.4	581	NM	0.22	0.000
Rainbow	7	SR	24	23	7.2	747	NM	1.36	0.000
Fatmucket	10	WB	4	23	8.6	376	8.43	4.64	0.548
Fatmucket	10	CS	4	23	8.7	491	8.44	0.85	0.102
Fatmucket	10	SR	4	23	8.5	530	8.36	0.36	0.037
Fatmucket	10	WB	4	23	6.6	319	NM	5.37	NM
Fatmucket	10	WB	24	23	7.1	304	NM	5.93	NM
Fatmucket	10	CS	4	23	6.6	NM	NM	2.38	NM
Fatmucket	10	CS	24	23	7.2	735	NM	4.49	NM
Fatmucket	10	SR	4	23	6.7	401	NM	0.32	NM
Fatmucket	10	SR	24	23	7.1	445	NM	0.47	NM
Fatmucket	20	SR	4	23	NM	NM	NM	NM	NM
Fatmucket	20	CS	4	23	NM	NM	NM	NM	NM
Fatmucket	20	WB	4	23	8.0	334	NM	4.36	NM
Fatmucket	20	WB	24	23	7.3	347	NM	12.50	NM
Notched Rainbow	20	WB	4	23	8.0	338	NM	4.32	NM
Notched Rainbow	20	WB	24	23	7.3	339	NM	13.00	NM

Appendix 2. Raw mussel burrowing data. WB: West Bear Lake sediment, SR: Spring River sediment, CS: Coarse Sand.

Species	Age (wk)	Sediment	Exposure Time (h)	Rep	*N*	Percentage of Mussels at Burrowing Depths (mm)					
						Surface	0–1.7	1.7–3.4	3.4–5.1	5.1–6.8	6.8–8.5
Fatmucket	3	CS	4	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	4	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	4	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	3	CS	4	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	4	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	4	2	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	3	WB	4	3	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	3	WB	4	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	5	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	6	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	7	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	8	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	24	1	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	3	WB	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	24	3	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	3	WB	24	4	4	0.0	75.0	25.0	0.0	0.0	0.0
Fatmucket	7	CS	24	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	7	CS	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	7	CS	24	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	7	CS	24	4	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	7	SR	24	1	4	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	7	SR	24	2	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	7	SR	24	3	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	7	SR	24	4	4	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	10	WB	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	2	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	4	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	10	WB	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	2	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	10	WB	4	3	5	0.0	20.0	40.0	40.0	0.0	0.0
Fatmucket	10	WB	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	2	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	10	WB	4	3	5	0.0	20.0	40.0	40.0	0.0	0.0
Fatmucket	10	CS	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	CS	4	2	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	10	CS	4	3	5	0.0	40.0	40.0	0.0	20.0	0.0
Fatmucket	10	CS	4	4	5	0.0	0.0	20.0	80.0	0.0	0.0
Fatmucket	10	CS	4	1	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	CS	4	2	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	10	CS	4	3	5	0.0	20.0	80.0	0.0	0.0	0.0
Fatmucket	10	SR	4	1	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	SR	4	2	5	0.0	40.0	20.0	40.0	0.0	0.0
Fatmucket	10	SR	4	3	5	0.0	0.0	40.0	0.0	60.0	0.0
Fatmucket	10	SR	4	4	5	0.0	80.0	0.0	20.0	0.0	0.0
Fatmucket	10	SR	4	1	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	SR	4	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	10	SR	4	3	5	0.0	20.0	60.0	20.0	0.0	0.0
Fatmucket	10	WB	24	4	5	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	10	WB	24	5	5	0.0	0.0	60.0	20.0	20.0	0.0
Fatmucket	10	WB	24	6	2	0.0	0.0	100.0	0.0	0.0	0.0

Appendix 2, continued.

Species	Age (wk)	Sediment	Exposure Time (h)	Rep	*N*	Percentage of Mussels at Burrowing Depths (mm)					
						Surface	0–1.7	1.7–3.4	3.4–5.1	5.1–6.8	6.8–8.5
Fatmucket	10	CS	24	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	10	CS	24	5	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	CS	24	6	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	SR	24	4	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	10	SR	24	5	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	SR	24	6	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	20	SR	4	1	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	SR	4	2	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	SR	4	3	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	SR	4	4	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	CS	4	1	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	CS	4	2	1	0.0	100.0	0.0	20.0	0.0	0.0
Fatmucket	20	CS	4	3	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	CS	4	4	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	WB	4	1	1	0.0	0.0	0.0	100.0	0.0	0.0
Fatmucket	20	WB	4	2	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	4	3	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	4	4	1	0.0	0.0	0.0	100.0	0.0	0.0
Fatmucket	20	WB	4	5	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	24	1	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	24	2	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	WB	24	3	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	24	4	1	0.0	0.0	0.0	100.0	0.0	0.0
Fatmucket	20	WB	24	5	1	0.0	0.0	100.0	0.0	0.0	0.0
Rainbow	7	CS	24	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Rainbow	7	CS	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Rainbow	7	CS	24	3	5	0.0	80.0	20.0	0.0	0.0	0.0
Rainbow	7	CS	24	4	5	0.0	80.0	20.0	0.0	0.0	0.0
Rainbow	7	WB	24	1	5	0.0	80.0	0.0	20.0	0.0	0.0
Rainbow	7	WB	24	2	5	0.0	80.0	20.0		0.0	0.0
Rainbow	7	WB	24	3	4	0.0	50.0	0.0	50.0	0.0	0.0
Rainbow	7	WB	24	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Rainbow	7	SR	24	1	4	0.0	100.0	0.0	0.0	0.0	0.0
Rainbow	7	SR	24	2	4	0.0	50.0	50.0	0.0	0.0	0.0
Rainbow	7	SR	24	3	5	0.0	20.0	80.0	0.0	0.0	0.0
Rainbow	7	SR	24	4	5	0.0	60.0	0.0	40.0	0.0	0.0
Arkansas Fatmucket	4	CS	24	1	4	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	CS	24	2	5	0.0	80.0	20.0	0.0	0.0	0.0
Arkansas Fatmucket	4	CS	24	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Arkansas Fatmucket	4	CS	24	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	WB	24	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	WB	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	WB	24	3	5	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	WB	24	4	4	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	SR	24	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	SR	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	SR	24	3	4	0.0	100.0	0.0	0.0	0.0	0.0
Arkansas Fatmucket	4	SR	24	4	4	0.0	100.0	0.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	1	1	0.0	0.0	100.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	2	1	0.0	0.0	100.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	3	1	0.0	0.0	0.0	100.0	0.0	0.0

Appendix 2, continued.

Species	Age (wk)	Sediment	Exposure Time (h)	Rep	*N*	Percentage of Mussels at Burrowing Depths (mm)					
						Surface	0–1.7	1.7–3.4	3.4–5.1	5.1–6.8	6.8–8.5
Notched Rainbow	20	WB	24	4	1	0.0	0.0	100.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	5	1	0.0	0.0	100.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	1	1	0.0	0.0	100.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	2	1	0.0	0.0	100.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	3	1	0.0	100.0	0.0	0.0	0.0	0.0
Notched Rainbow	20	WB	24	4	1	0.0	0.0	0.0	0.0	100.0	0.0
Notched Rainbow	20	WB	24	5	1	100.0	0.0	0.0	0.0	0.0	0.0
Oregon Floater	4	WB	24	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Oregon Floater	4	WB	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Oregon Floater	4	WB	24	3	5	0.0	100.0	0.0	0.0	0.0	0.0
Oregon Floater	4	WB	24	4	5	0.0	20.0	80.0	0.0	0.0	0.0
Washboard Mucket	6	WB	24	1	5	0.0	20.0	80.0	0.0	0.0	0.0
Washboard Mucket	6	WB	24	2	5	0.0	80.0	20.0	0.0	0.0	0.0
Washboard Mucket	6	WB	24	3	5	0.0	100.0	0.0	0.0	0.0	0.0
Washboard Mucket	6	WB	24	4	5	0.0	60.0	40.0	0.0	0.0	0.0
Washboard Mucket	6	CS	24	1	5	0.0	20.0	60.0	20.0	0.0	0.0
Washboard Mucket	6	CS	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Washboard Mucket	6	CS	24	3	5	0.0	20.0	40.0	40.0	0.0	0.0
Washboard Mucket	6	CS	24	4	5	0.0	80.0	20.0	0.0	0.0	0.0
Washboard Mucket	6	SR	24	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Washboard Mucket	6	SR	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Washboard Mucket	6	SR	24	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Washboard Mucket	6	SR	24	4	5	0.0	100.0	0.0	0.0	0.0	0.0

Appendix 3. Fatmucket (FM) mussel burrowing data. WB: West Bear Lake sediment, SR: Spring River sediment, CS: Coarse Sand.

Species	Age (wk)	Sediment	Exposure Time (h)	Rep	*N*	Percentage of Mussels at Burrowing Depths (mm)					
						Surface	0–1.7	1.7–3.4	3.4–5.1	5.1–6.8	6.8–8.5
Fatmucket	3	CS	4	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	4	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	4	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	3	CS	4	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	4	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	4	2	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	3	WB	4	3	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	3	WB	4	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	5	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	6	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	7	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	CS	24	8	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	24	1	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	3	WB	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	3	WB	24	3	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	3	WB	24	4	4	0.0	75.0	25.0	0.0	0.0	0.0
Fatmucket	7	CS	24	1	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	7	CS	24	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	7	CS	24	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	7	CS	24	4	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	7	SR	24	1	4	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	7	SR	24	2	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	7	SR	24	3	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	7	SR	24	4	4	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	10	WB	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	2	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	3	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	4	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	10	WB	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	2	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	10	WB	4	3	5	0.0	20.0	40.0	40.0	0.0	0.0
Fatmucket	10	WB	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	WB	4	2	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	10	WB	4	3	5	0.0	20.0	40.0	40.0	0.0	0.0
Fatmucket	10	CS	4	1	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	CS	4	2	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	10	CS	4	3	5	0.0	40.0	40.0	0.0	20.0	0.0
Fatmucket	10	CS	4	4	5	0.0	0.0	20.0	80.0	0.0	0.0
Fatmucket	10	CS	4	1	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	CS	4	2	5	0.0	40.0	60.0	0.0	0.0	0.0
Fatmucket	10	CS	4	3	5	0.0	20.0	80.0	0.0	0.0	0.0
Fatmucket	10	SR	4	1	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	SR	4	2	5	0.0	40.0	20.0	40.0	0.0	0.0
Fatmucket	10	SR	4	3	5	0.0	0.0	40.0	0.0	60.0	0.0
Fatmucket	10	SR	4	4	5	0.0	80.0	0.0	20.0	0.0	0.0
Fatmucket	10	SR	4	1	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	SR	4	2	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	10	SR	4	3	5	0.0	20.0	60.0	20.0	0.0	0.0
Fatmucket	10	WB	24	4	5	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	10	WB	24	5	5	0.0	0.0	60.0	20.0	20.0	0.0
Fatmucket	10	WB	24	6	2	0.0	0.0	100.0	0.0	0.0	0.0

Appendix 3, continued.

Species	Age (wk)	Sediment	Exposure Time (h)	Rep	*N*	Percentage of Mussels at Burrowing Depths (mm)					
						Surface	0–1.7	1.7–3.4	3.4–5.1	5.1–6.8	6.8–8.5
Fatmucket	10	CS	24	4	5	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	10	CS	24	5	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	10	CS	24	6	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	SR	24	4	5	0.0	80.0	20.0	0.0	0.0	0.0
Fatmucket	10	SR	24	5	5	0.0	60.0	40.0	0.0	0.0	0.0
Fatmucket	10	SR	24	6	5	0.0	40.0	40.0	20.0	0.0	0.0
Fatmucket	20	SR	4	1	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	SR	4	2	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	SR	4	3	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	SR	4	4	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	CS	4	1	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	CS	4	2	1	0.0	100.0	0.0	20.0	0.0	0.0
Fatmucket	20	CS	4	3	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	CS	4	4	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	WB	4	1	1	0.0	0.0	0.0	100.0	0.0	0.0
Fatmucket	20	WB	4	2	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	4	3	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	4	4	1	0.0	0.0	0.0	100.0	0.0	0.0
Fatmucket	20	WB	4	5	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	24	1	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	24	2	1	0.0	100.0	0.0	0.0	0.0	0.0
Fatmucket	20	WB	24	3	1	0.0	0.0	100.0	0.0	0.0	0.0
Fatmucket	20	WB	24	4	1	0.0	0.0	0.0	100.0	0.0	0.0
Fatmucket	20	WB	24	5	1	0.0	0.0	100.0	0.0	0.0	0.0

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REGULAR ARTICLE

COMPARISON OF SURFACE- AND PORE-WATER QUALITY BETWEEN TWO MARYLAND STREAMS WITH THE ENDANGERED DWARF WEDGEMUSSEL (*ALASMIDONTA HETERODON*)

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ABSTRACT

Degraded water quality, particularly elevated concentrations of ammonia, chloride, and toxic metals, can be harmful to freshwater mussels. We investigated whether the contraction in Dwarf Wedgemussel (*Alasmidonta heterodon*) distribution that occurred between 2002 and 2012 within Browns Branch (BB), a stream within a predominantly agricultural watershed on the Coastal Plain of Maryland, was associated with these and other water-quality factors. We measured surface- and pore-water concentrations of different forms of nitrogen, orthophosphate, anions, and dissolved metals at two sites in BB for 7 mo in 2014. The upstream site (BBUP) represented the lower extent of the current Dwarf Wedgemussel population, and the downstream site (BBDO) represented the lower extent of the species' distribution observed in a 2002 survey. As a comparison, we also sampled one site in Nanjemoy Creek (NANJ), a largely forested Coastal Plain watershed where Dwarf Wedgemussel distribution exhibited no change over the same 10-yr period. We tested the hypothesis that concentrations of potentially toxic analytes were significantly higher at BBDO than at BBUP and NANJ. Total ammonia nitrogen (TAN) was the only analyte consistent with this hypothesis in both surface and pore water. Concentrations of pore-water un-ionized ammonia (UIA-N) at BBDO were below the U.S. Environmental Protection Agency's Ambient Water Quality Criterion, but they frequently exceeded 0.2 µg/L, a concentration previously associated with a lack of mussel recruitment. We recommend conducting a new mussel survey of BB to assess current condition. If range contraction is still evident, more frequent and extended sampling should be performed, including capturing high-flow events to determine if pulses of ammonia and other pollutants occur.

KEY WORDS: *Alasmidonta heterodon*, water quality, ammonia, pore water

INTRODUCTION

Water quality degradation is commonly suspected of adversely affecting freshwater mussel populations (e.g., Brim Box and Mossa 1999; Strayer et al. 2004; Gascho Landis et al. 2012; Haag 2012; Gillis et al. 2017). Determining the precise physicochemical factors that negatively affect mussel populations is difficult because their complex life history makes them vulnerable to environmental stressors at multiple stages over long periods. Identifying stressors to mussels is a critical information need for effective conservation (Haag and Williams 2014).

Mussels are highly sensitive to ammonia; ions such as chloride, potassium, and sulfate; and metals such as copper, nickel, and zinc (Newton et al. 2003; Gillis 2011; Johnson et al. 2014; Wang et al. 2017). The un-ionized form of ammonia (UIA-N) is acutely toxic to early life stages of mussels (Newton et al. 2003; Wang et al. 2008), and elevated concentrations of UIA-N in pore water have been associated

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with mussel recruitment failure (Strayer and Malcom 2012). Consequently, the U.S. Environmental Protection Agency recently lowered its freshwater ambient water quality criteria for ammonia to be more protective of mussels (USEPA 2013). Nitrogenous pollution in streams primarily results from atmospheric deposition, point source effluent discharges, and agricultural practices that deliver pollution via surface-water runoff or groundwater infiltration (Boynton et al. 1995; Kemp et al. 2005).

The Dwarf Wedgemussel (*Alasmidonta heterodon*) once ranged from New Brunswick, Canada, to North Carolina, USA, but it now inhabits less than half of its formerly occupied streams, and most surviving populations are small (Strayer et al. 1996). In Maryland, it is found in a handful of Coastal Plain streams, including Nanjemoy Creek (NANJ) and Browns Branch (BB) (Bogan and Ashton 2016). Surveys in BB detected a contraction in the distribution of Dwarf Wedgemussel between 2002 and 2012 (Ashton et al. 2013). Dwarf Wedgemussel disappeared from the lower section of BB (hereafter BBDO) during this time, but its abundance nearly doubled in the upper section (hereafter BBUP). Mussel species richness and the distribution of most other species also declined in BBDO between 2002 and 2012. NANJ continues to support a population of Dwarf Wedgemussel, and no changes in its range or mussel species richness were observed in this stream over the last 25 years (J. M. McCann, Maryland Department of Natural Resources (MDNR), unpublished data).

We characterized surface-water and pore-water chemistry in BBUP, BBDO, and NANJ in 2014 to evaluate potential causes of the Dwarf Wedgemussel decline in BBDO. We hypothesized that concentrations of pollutants would be greater at BBDO, where Dwarf Wedgemussel abundance declined, than at BBUP and in NANJ, where Dwarf Wedgemussel has not declined. For later discussion, we abbreviate this hypothesis as BBDO $>$ BBUP $=$ NANJ.

METHODS

Study Area

BB and NANJ are on the Atlantic Coastal Plain in Maryland and flow into Chesapeake Bay (Fig. 1). The surface geology of the two watersheds is similar and relatively homogenous. Stream valleys are underlain by Tertiary sands, clays, and silts, and uplands are underlain by Quaternary sands, gravels, and clays (Cleaves et al. 1968). We extracted major land use categories within the upstream catchment of each study site from the 2001 and 2011 National Land Cover Dataset (Homer et al. 2007, 2015), following Ashton (2012). At all three sites, land use changed little between 2001 and 2011. In both time periods, the BBDO (2,409 ha) and BBUP (694 ha) catchments were primarily agricultural (about 70% of land cover, mainly in row crops, but also including pasture and poultry operations), with 19–26% forest and 1–8% urban. The Nanjemoy Creek catchment (4,106 ha) was predominantly forested (about 80%), with 7–14% agriculture and 2–6% urban.

In BB, we sampled water chemistry at one site in BBUP, at the lowermost extent of habitat occupied by the Dwarf Wedgemussel in 2012, and at one site in BBDO, 3.6 river km downstream, representing the downstream extent of habitat occupied by Dwarf Wedgemussel in 2002, prior to range contraction (Ashton et al. 2013; J. M. McCann, MDNR, unpublished data). At BBUP, the stream is approximately 2–5 m wide, and substrate consists of silt, fine sand, and fine gravel. At BBDO, the stream is 5–8 m wide, and substrate consists of sand, silt, and gravel. The study site in NANJ was located at the approximate center of the Dwarf Wedgemussel population in that stream, where the stream is 4–10 m wide and substrate is coarse sand and gravel.

Water Sampling and Analysis

We sampled each site about every 30 d from late April to early December 2014 (Table 1), to encompass critical periods of Dwarf Wedgemussel life history, including host-fish infection, juvenile metamorphosis and recruitment, and spawning (Michaelson and Neves 1995). We measured water temperature (°C), dissolved oxygen (DO, mg/L), pH, and conductivity (μSiemens/cm) with a YSI Model 55 multimeter (YSI Inc., Yellow Springs, OH, USA) at midchannel and middepth. We then collected two surface-water samples in trace-metal-clean-certified polyethylene 1-L bottles, one for anions and nutrients and the other for metals. Sample collection and handling procedures followed those of the Maryland Biological Stream Survey (MBSS 2007), such that neither filtration nor acidification was performed in the field. We placed samples on ice, maintained them in a refrigerator, and shipped them via express carrier in iced coolers to the University of Maryland Center for Environmental Science Appalachian Laboratory (UMCES, Frostburg, Maryland) for processing within 48 h of collection.

The sample for anions and nutrients was vacuum filtered using a 0.45-μm membrane filter and divided between two 125-ml polyethylene bottles. The anions sample was stored at 4°C, and the nutrients sample was stored at −20°C until analysis within recommended holding times. The anion sample was analyzed for chloride (Cl), nitrate-N (NO_3-N), and sulfate (SO_4) using ion chromatography (USEPA 1987) with a Dionex DX-120 instrument. The nutrient sample was analyzed for nitrite-N (NO_2-N), total ammonia-N (TAN), and orthophosphate (PO_4) using flow-injection colorimetry with a Lachat QuikChem 8000 (APHA 1998) (see Table 2 for detection limits). For dissolved-metals analysis, samples were withdrawn from the collection bottle in the lab using a sterile, 60-cc polyethylene syringe and filtered for dissolved metals with single-use 0.45-μm membrane syringe filters into trace-metal-clean polyethylene bottles. Filtered samples were acidified to a pH $<$ 2 with Optima-grade concentrated nitric acid. The following dissolved metals were measured by inductively coupled plasma mass spectrometry, using an

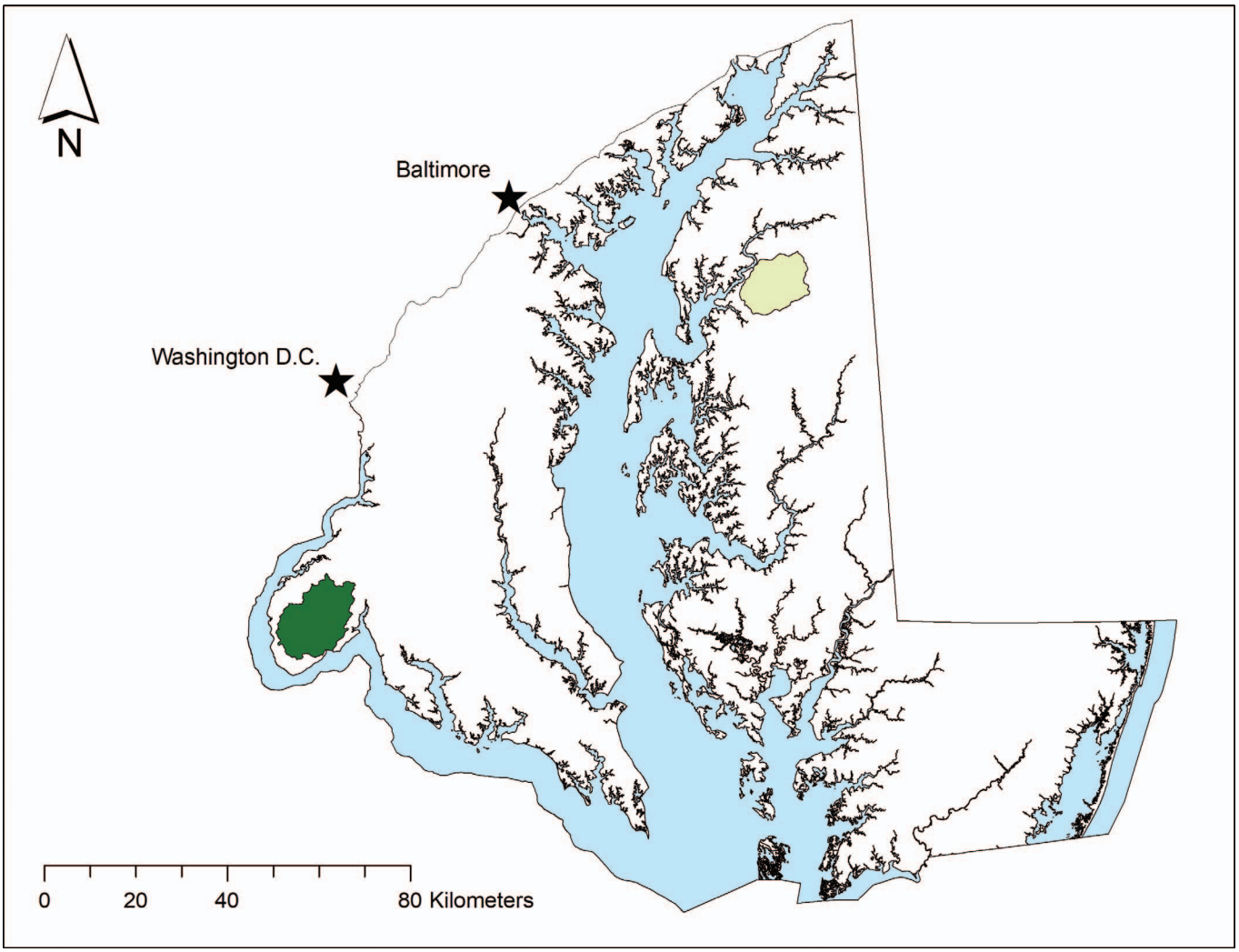

Figure 1. Atlantic Coastal Plain of Maryland, USA, showing Nanjemoy Creek (dark green) and Browns Branch (light green).

Agilent 7900 instrument equipped with an octopole reaction system to remove polyatomic interferences (USEPA 1998): aluminum (Al), antimony (Sb), arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), nickel (Ni), selenium (Se), silver (Ag), strontium (Sr), thallium (Tl), vanadium (V), and zinc (Zn) (see Table 3 for detection limits). UMCES employs a rigorous quality assurance/quality control program; results for a 10-sample proficiency test conducted during our project time period and analysis of an independent control sample for metals are presented in Appendix Tables A1 and A2.

We sampled pore water using sediment peepers (Teasdale et al. 1995; Strayer and Malcom 2012). The peepers (Fig. 2) were constructed from 225-ml polyethylene centrifuge tubes by drilling a 5-mm hole in the cap, beneath which we inserted a 1.2-μm polycarbonate filter. The filter was supported by vinyl mesh on the interior and protected from external damage by fiberglass mesh affixed to the cap with cyanoacrylate adhesive. Prior to deployment, the UMCES lab filled the peepers with deoxygenated, deionized water and shipped them to the U.S. Fish and Wildlife Service Chesapeake Bay Field Office laboratory (Annapolis, Maryland) in iced coolers. We maintained the peepers in a refrigerator for several days before taking them to the sites in iced coolers. Over the deployment period, pore water displaced the deoxygenated, deionized water by passive diffusion.

We deployed peepers by strapping three 225-ml peepers to a 100×10 cm high-density polyethylene slab with cable ties. We also strapped one 500-ml peeper to the slab so that we could insert the YSI probe into the peeper for water quality measurement. During our initial April sampling, we buried the slab about 10 cm deep in the substrate and anchored it in place with 1.2-cm-diameter steel rebar.

We retrieved peepers every 30 d, coincident with water sampling. On each sampling date, we exposed the slab and removed the peepers. We opened the 500-ml peeper and immediately measured pore-water temperature, pH, conductivity, and DO with the YSI probe. For the 225-ml peepers, we replaced the filter caps with solid caps, placed the peepers on

Table 1. Monthly surface- and pore-water temperature, dissolved oxygen, and ammonia data for Nanjemoy Creek (NANJ), lower Browns Branch (BBDO), and upper Browns Branch (BBUP). Abbreviations and units: Temp = water temperature (°C), DO = dissolved oxygen (mg/L), TAN = total ammonia-N (mg/L), UIA-N = un-ionized ammonia nitrogen (μg/L), AWQC = USEPA acute and chronic ambient water quality criteria for TAN (mg/L, USEPA 2013).

	Surface Water					Pore Water[a]				
Date	Temp	DO	TAN	UIA-N	AWQC	Temp	DO	TAN	UIA-N[b]	AWQC
NANJ										
24-Apr	12.8	10.50	0.013	0.007	41, 3.3	—	—	—	—	—
21-May	17.6	9.13	0.047	0.037	27, 2.4	17.8	6.91	0.056	0.045	27, 2.4
26-Jun	23.8	7.60	0.048	0.060	16, 1.6	23.4	0.83	0.039	0.048	18, 1.8
22-Jul	22.3	8.50	0.034	0.038	19, 1.9	22.2	0.43	0.267	**0.302**	19, 1.9
18-Sep	16.9	6.72	0.015	0.011	29, 2.6	17.0	2.43	0.225	0.172	29, 2.6
23-Oct	12.4	11.34	0.006	0.003	44, 3.6	12.6	0.93	0.047	0.026	41, 3.3
4-Dec	6.9	12.67	0.008	0.003	51, 4.9	6.9	—	0.007	0.002	51, 4.9
BBDO										
23-Apr	13.2	12.08	0.035	0.085	30, 3.0	—	—	—	—	—
20-May	16.2	10.38	0.124	0.379	23, 2.4	16.0	6.82	0.134	**0.403**	23, 2.4
25-Jun	20.3	9.52	0.092	0.381	17, 1.9	20.6	0.53	0.501	***2.12***	15, 1.8
22-Jul	22.2	9.45	0.072	0.341	14, 1.7	22.0	1.49	0.639	***2.99***	14, 1.7
28-Aug	20.0	7.86	0.068	0.275	17, 1.9	20.9	3.30	0.295	**1.27**	15, 1.8
1-Oct	16.8	10.40	0.094	0.301	21, 2.3	16.9	2.87	0.020	0.065	21, 2.3
5-Nov	10.8	10.52	0.089	0.181	35, 4.1	11.0	1.04	0.951	**1.96**	35, 4.1
BBUP										
23-Apr	12.3	12.65	0.007	0.033	24, 2.7	—	—	—	—	—
20-May	14.4	10.85	0.087	0.484	20, 2.4	14.4	8.10	0.106	**0.589**	20, 2.4
25-Jun	20.7	9.69	0.033	0.292	11, 1.5	21.0	6.40	0.003	0.027	11, 1.5
22-Jul	22.9	8.90	0.027	0.280	9.5, 1.3	21.7	1.12	0.091	**0.865**	10, 1.4
28-Aug	20.8	7.82	0.160	1.424	11, 1.5	—[c]	—	0.012	0.109	11, 1.5
1-Oct	17.2	10.33	0.014	0.096	16, 2.0	17.2	5.95	0.021	0.144	16, 2.0
5-Nov	11.0	10.24	0.022	0.094	26, 2.9	11.0	2.05	0.027	0.116	26, 2.9

[a]Pore-water samples were not collected in April and from NANJ in August.

[b]UIA-N pore-water concentrations exceeding the Strayer and Malcom (2012) threshold of 0.2 μg/L are in bold; those >2.0 are in bold italics.

[c]Pore-water temperature not measured; surface-water temperature used to estimate pore-water UIA-N.

Table 2. Mean (± 95% CI) ion and nutrient concentrations in seven, monthly surface-water samples from Nanjemoy Creek (NANJ), lower Browns Branch (BBDO), and upper Browns Branch (BBUP). Sites with different letters in parentheses have significantly different means (Tukey's HSD, $P < 0.05$). All units are mg/L except UIA-N (μg/L).

Analyte[a]	NANJ	BBDO	BBUP
Cl[b]	10.52 ± 2.89 (a)	20.16 ± 0.46 (b)	20.05 ± 3.43 (b)
TAN[c, d]	0.02 ± 0.02 (a)	0.08 ± 0.03 (b)	0.03 ± 0.02 (a)
UIA-N	0.02 ± 0.02 (a)	0.28 ± 0.10 (a)	0.39 ± 0.45 (a)
NO_3-N[d]	0.04 ± 0.03 (a)	5.55 ± 0.66 (b)	4.99 ± 0.57 (b)
NO_2-N[d]	0.003 ± 0.001 (a)	0.020 ± 0.010 (b)	0.020 ± 0.010 (b)
PO_4[d]	0.005 ± 0.002 (a)	0.010 ± 0.010 (a)	0.040 ± 0.010 (b)
SO_4[d]	2.54 ± 1.36 (a)	15.04 ± 1.41 (b)	30.02 ± 3.44 (c)

[a]Minimum detection limits (mg/L): Cl—0.020, TAN—0.002, NO_3-N—0.0019, NO_2-N—0.0019 mg/L, PO_4; 0.0011, SO_4—0.020.

[b]USEPA (2018) AWQC (mg/L): 860 (acute), 230 (chronic).

[c]See Table 1 for USEPA (2013) ammonia criteria.

[d]Southerland et al. (2005) categories (mg/L; L = low, M = Moderate, H = High) TAN: L < 0.03, M 0.03–0.07, H > 0.07; NO_3-N: L < 1.0, M 1.0–5.0, H > 5.0; NO_2-N: L < 0.0025, M 0.0025–0.01, H > 0.01; PO_4: L < 0.008, M 0.008–0.03; H > 0.03.

ice, and shipped them to UMCES. For the samples collected in May through July, UMCES analyzed anions in one peeper, nutrients in a second, and metals in a third. From August through the end of the study, UMCES followed the same procedure for anions and metals, but they analyzed nutrients from all three peepers (see Data Analysis). After retrieving the peepers, we replaced them with fresh ones and reburied the slab, attempting to minimize turbidity. We measured surface-water-quality parameters and collected surface-water samples before working with the peepers.

We monitored water temperature at each site throughout the study with Hobo Pro V2 data loggers (Onset Computer Corporation, Bourne, Massachusetts) programmed to record at 20-min intervals. We drove a piece of rebar into the substrate and affixed with cable ties one data logger about 5 cm above the substrate surface (for surface-water temperature) and one data logger buried about 5 cm below the substrate surface (for sediment temperature). We measured temperature because it influences the toxicity of chemical stressors, particularly ammonia (USEPA 2013), and because high temperatures can be lethal to mussels. We retrieved the loggers on the last day

Table 3. Mean (± 95% CI) dissolved metals concentrations (µg/L) in seven, monthly surface-water samples from Nanjemoy Creek (NANJ), lower Browns Branch (BBDO), and upper Browns Branch (BBUP). Sites with different letters in parentheses have significantly different means (Tukey's HSD, $P < 0.05$). Concentrations that exceed a USEPA AWQC[a] are in bold.

Analyte[b]	NANJ	BBDO	BBUP
Al	77.1 ± 44.4 (b)	23.1 ± 12.0 (a)	18.2 ± 9.7 (a)
Sb	0.02 ± 0.02 (a)	0.03 ± 0.03 (a)	0.02 ± 0.01 (a)
As	0.51 ± 0.16 (a)	0.36 ± 0.06 (a)	0.38 ± 0.07 (a)
Ba	31.8 ± 3.2 (a)	98.8 ± 9.7 (c)	79.1 ± 3.9 (b)
Be	0.06 ± 0.03 (b)	0.03 ± 0.01 (a)	0.04 ± 0.02 (a, b)
Cd	0.01 ± 0.01 (a)	0.04 ± 0.02 (a)	0.10 ± 0.05 (b)
Cr	0.34 ± 0.18 (a)	0.18 ± 0.08 (a)	0.21 ± 0.09 (a)
Co	0.63 ± 0.40 (a)	0.36 ± 0.21 (a)	0.38 ± 0.28 (a)
Cu	1.78 ± 0.87 (b)	0.79 ± 0.38 (a)	0.80 ± 0.42 (a)
Fe	**1,103 ± 398** (b)	201 ± 130 (a)	196 ± 131 (a)
Pb	0.35 ± 0.11 (b)	0.07 ± 0.04 (a)	0.05 ± 0.04 (a)
Mn	118.0 ± 65.0 (b)	54.0 ± 22.9 (a)	38.1 ± 21.1 (a)
Ni	1.29 ± 0.60 (a)	1.56 ± 0.55 (a)	2.77 ± 0.68 (b)
Se	0.10 ± 0.04 (a)	0.45 ± 0.05 (b)	0.49 ± 0.09 (b)
Ag	0.002 ± 0.00 (a)	0.002 ± 0.00 (a)	0.003 ± 0.00 (a)
Sr	30.0 ± 6.6 (a)	155.0 ± 7.0 (b)	251.0 ± 29.0 (c)
Tl	0.005 ± 0.00 (a)	0.02 ± 0.00 (b)	0.04 ± 0.01 (c)
V	0.59 ± 0.22 (a)	0.31 ± 0.06 (a)	0.42 ± 0.08 (a, b)
Zn	5.59 ± 2.48 (a)	3.20 ± 1.53 (a)	4.14 ± 1.83 (a)

[a]USEPA (2018) AWQC (µg/L) (acute, chronic): Al, Cu: could not be calculated due to lack of required water-quality parameters, As: 340, 150; Cr^{III}: 570,74; Cr^{VI}: 16,11; Fe: 1000 (chronic); Cd: 0.30, 0.16 (NANJ); 1.22, 0.48 (BBDO); 1.57, 0.59 (BBUP); Pb: 7.77, 0.30 (NANJ); 40.97, 1.60 (BBDO); 55.48, 2.16 (BBUP); Ni: 93.40, 10.41 (NANJ); 326.3, 36.31 (BBDO); 412.0, 45.84 (BBUP); Zn: 22.39, 22.97 (NANJ); 76.43, 79.19 (BBDO); 96.09, 99.74 (BBUP); Se: 3.1 (30-day); Ag: 3.2.

[b]Minimum detection limits (µg/L): Al, 0.200; Sb, 0.005; As, 0.006; Ba, 0.008; Be, 0.010; Cd, 0.003; Cr, 0.011; Co, 0.004; Cu, 0.281; Fe, 0.320; Pb, 0.019; Mn, 0.043; Ni, 0.011; Se, 0.018; Ag, 0.002; Sr, 0.028; Tl, 0.005; V, 0.051, Zn, 0.109.

of monitoring at each site and trimmed the data to June 1, 2014, through October 31, 2014, to include only the full months when all sites were monitored.

Data Analysis

At the beginning of the study, we composited all peeper samples to ensure that we had adequate sample volume to complete all of the analyses. After the July sampling, we realized that we could complete the nutrient analyses with smaller sample volumes than expected, which allowed us to assess the variability between individual peepers. Thus, we began analyzing the three peepers for separate nutrient samples in August. We calculated the trimean (TM = 0.5 [Q2 + 0.5 (Q1 + Q3)] where Q = Quartile), instead of the arithmetic mean, to generate a more representative single estimate of pore-water nutrients for each sampling event.

We summarized monthly water-chemistry data as means with 95% confidence intervals (CI) in package *Rmisc* (Hope 2013) in R (R Core Team 2014). Distributions of 11 of the 75 analyte estimates (25 analytes at each site) for surface-water chemistry deviated from normality (Kolmogorov-Smirnov test, $D = 0.10–0.38$; $P = 0.003–0.99$). Distributions of 18 of the 75 analyte estimates for pore-water chemistry deviated from normality ($D = 0.11–0.49$, $P = 0.00005–0.99$). Most variables that exhibited non-normal distributions were dissolved metals with very low concentrations over a narrow range of values. However, more than half of the analytes had unequal variances among sites for both surface-water analytes (Levene's test, $F = 0.12–26.01$, $P = 0.000005–0.89$) and pore-water analytes ($F = 0.60–23.37$; $P = 0.0001–0.56$). Heterogeneous variances were observed for anions, nutrients, and metals.

Figure 2. Photograph of peeper assembly.

Since our objective was to determine if water-chemistry data fit a particular pattern and not if means were equal across all sites, we avoided the omnibus ANOVA and substituted multiple comparison tests to assess differences in chemical parameters between sites. We calculated Tukey HSD tests using the R package *multcomp* (Hothorn et al. 2008) from mean-square results of one-way ANOVAs with adjustment for heteroscedastic data (Long and Ervin 2000) using the R package *car* (Fox and Weisberg 2011). Due to the small experimental sample size and multiple statistical comparisons, we would expect to find significant differences in at least some chemical concentrations among sites that fit our hypothesized pattern of a stressor by chance under a null-hypothesis testing framework with a rigid alpha (e.g., $\alpha = 0.05$). Therefore, we placed similar weight on confidence intervals of mean concentrations in determining whether or not an analyte fits the pattern of BBDO > BBUP = NANJ. That is, results from HSD tests were not meant solely to firmly accept or reject, but instead to provide support for focusing on specific analytes to investigate in a more intensive study.

We compared mean analyte concentrations at each site with Ambient Water Quality Criteria (AWQC) (USEPA 2018). For hardness-dependent criteria (e.g., Ag, Cd, Cu, Ni, Pb, and Zn), we used hardness values measured previously by MBSS at our sites (NANJ = 16 mg/L as $CaCO_3$; BBDO = 66; BBUP = 87; https://dnr.maryland.gov/streams/Pages/dataRequest.aspx; accessed May 14, 2019). We compared TAN concentrations with AWQC for each sample event. We calculated these concentrations based on pH and temperature. We used temperature measurements obtained when our surface-water samples were collected. We did not use pH values collected simultaneously with surface-water samples because our values were not consistent with prior measurements at the sites by MBSS, which suggested probe malfunction. Instead, we calculated mean (± 95% CI) values from the antilog of pH measured previously by MBSS. The pH values we used for AWQC were 6.38 (± 0.22) for NANJ, 7.01 (± 0.11) for BBDO, and 7.33 (± 0.20) for BBUP.

We estimated the fraction of un-ionized ammonia (UIA-N µg/L) in surface and pore water following Thurston et al. (1979). In addition to AWQC, we compared pore-water UIA-N with the 0.2 µg/L UIA-N pore-water threshold for recruitment failure in *Elliptio complanata* proposed by Strayer and Malcom (2012). For nutrients, we compared surface-water concentrations with low, moderate, and high categories for Maryland streams (Southerland et al. 2005).

RESULTS

Temperature, Conductivity, and DO

Mean monthly (June through October) surface-water and sediment temperatures were nearly identical at BBDO (surface and sediment both = 18.9°C) and BBUP (surface = 18.9°C, sediment = 18.8°C). Mean monthly temperatures were about 1.0–1.5°C higher at NANJ (surface = 20.4°C; sediment = 20.2°C). The maximum surface-water temperatures observed at each stream were NANJ, 27.1°C; BBDO, 25.7°C; and BBUP, 24.6°C. Maximum sediment temperatures were NANJ, 25.3°C; BBDO, 24.8°C; and BBUP, 24.2°C.

Conductivity (µS/cm) in surface and pore water ranged from 39 to 149 at NANJ, 190 to 214 at BBDO, and 226 to 299 at BBUP. Surface-water DO exceeded Maryland's water quality criterion of 5 mg/L (MDE 2019) on all sample dates at all sites. In contrast, pore-water DO rarely exceeded this criterion, and all sites had values less than 3 mg/L (Table 1).

Ammonia

Surface-water and pore-water TAN concentrations did not exceed the AWQC at any site on any date (Table 1). The two highest-observed values were in pore water at BBDO (0.951 and 0.639 mg/L) and were 23% and 38% of the chronic AWQC, respectively. The maximum pore-water concentration at NANJ was 0.267 mg/L (14% of the chronic AWQC) and 0.106 mg/L (4% of the chronic AWQC) at BBUP. Compared with the Southerland et al. (2005) categories for Maryland MBSS surface-water data, TAN concentrations were low (<0.03 mg/L) and moderate (0.03 to 0.07 mg/L) at NANJ, moderate and high (>0.07 mg/L) at BBDO, and mostly low at BBUP.

Differences in mean TAN concentrations among sites for both surface water and pore water supported our hypothesis of BBDO > BBUP = NANJ (Tables 2 and 4). Differences in mean pore-water UIA-N concentrations among sites were consistent with our hypothesis, but differences in surface-water UIA-N were not. Pore-water UIA-N concentrations exceeded the 0.2 µg/L threshold of Strayer and Malcom (2012) in five of six measurements at BBDO, including two observations >2.0 µg/L (Table 1). Two of six measurements at BBUP (0.59 and 0.86 µg/L) and one of six at NANJ (0.30 µg/L) exceeded the threshold. In general, UIA-N concentrations were substantially lower in surface water than in pore water, except for two events at BBUP and one at BBDO.

Nitrate-N, Nitrite-N, and Phosphate

There are no AWQC for NO_3-N, NO_2-N, or PO_4. Differences in NO_3-N, NO_2-N, and PO_4 concentrations among sites did not support our hypothesis of BBDO > BBUP = NANJ. Mean surface-water NO_3-N and NO_2-N were both significantly higher at both BB sites compared with NANJ, and there were no significant differences between BBDO and BBUP (Table 2). For NO_3-N, both BB sites were categorized as high following Southerland et al. (2005). Mean pore-water NO_3-N at BBUP was significantly higher compared with results from BBDO and NANJ, which did not differ from each other (Table 4). Mean pore-water NO_2-N did not differ among sites. The mean surface-water PO_4 concentration at BBUP was significantly higher than the concentrations at NANJ and BBDO, which were similar to each other (Table 2). Pore-water PO_4 concentration was intermediate at BBDO and was not

Table 4. Mean (± 95% CI) ion and nutrient concentrations in six, monthly pore-water samples from Nanjemoy Creek (NANJ), lower Browns Branch (BBDO), and upper Browns Branch (BBUP). Sites with different letters in parentheses have significantly different means (Tukey's HSD, $P < 0.05$). All units are mg/L except UIA-N (μg/L). See Table 2 for detection limits.

Analyte	NANJ	BBDO	BBUP
Cl	5.89 ± 2.55 (a)	10.57 ± 4.71 (a)	18.56 ± 2.20 (b)
TAN	0.11 ± 0.12 (a)	0.42 ± 0.36 (b)	0.04 ± 0.05 (a)
UIA-N[a]	0.10 ± 0.12 (a)	**1.47 ± 1.16** (b)	**0.31 ± 0.35** (a)
NO_3-N	0.01 ± 0.01 (a)	0.82 ± 1.35 (a)	4.03 ± 1.28 (b)
NO_2-N	0.002 ± 0.001 (a)	0.06 ± 0.11 (a)	0.05 ± 0.05 (a)
PO_4	0.004 ± 0.002 (a)	0.03 ± 0.02 (a, b)	0.05 ± 0.02 (b)
SO_4	0.96 ± 1.11 (a)	5.15 ± 3.65 (a)	25.02 ± 4.18 (b)

[a]Mean ion and nutrient concentrations exceeding the Strayer and Malcom (2012) threshold of 0.2 μg/L are in bold.

significantly different from those concentrations at NANJ or BBUP (Table 4).

Chloride and Sulfate

Chloride concentrations did not exceed the acute or chronic AWQC in any samples. The maximum concentration was 26.2 mg/L in a surface-water sample from BBUP, about 10% of the chronic criterion of 260 mg/L (Table 2). Differences in mean Cl concentrations among sites did not support our hypothesis of BBDO > BBUP = NANJ. Surface-water mean concentrations were not significantly different between the two BB sites, but both were significantly higher (about double) than that at NANJ. Pore-water Cl mean concentrations were significantly higher at BBUP compared with those at BBDO and NANJ (Table 4).

There are no AWQC for SO_4. Differences in SO_4 concentrations among sites did not support our hypothesis of BBDO > BBUP = NANJ. In surface water, SO_4 differed significantly among all three sites with the highest value at BBUP and a much lower value at NANJ (Table 2). SO_4 varied similarly in pore water, but it was significantly higher at BBUP than at BBDO and NANJ, both of which had low values and did not differ from each other (Table 4).

Metals

Iron was the only metal detected that exceeded the AWQC. For surface water, the mean Fe concentration (1,103 μg/L) exceeded the chronic AWQC of 1,000 μg/L (there is no acute criterion) at NANJ, but values at BBDO and BBUP were much lower (Table 3). For pore water, the mean Fe concentration exceeded the chronic AWQC at NANJ and BBDO but not at BBUP (Table 5). Concentrations of no other metals closely approached AWQC. There were significant differences in mean concentrations of many metals among sites (Tables 3 and 5), but none supported our hypothesis of BBDO > BBUP = NANJ.

Table 5. Mean (± 95% CI) dissolved metals concentrations (μg/L) in six, monthly pore-water samples from Nanjemoy Creek (NANJ), lower Browns Branch (BBDO), and upper Browns Branch (BBUP). Sites with different letters in parentheses have significantly different means (Tukey's HSD, $P < 0.05$). See Table 3 for detection limits. Concentrations that exceed a USEPA AWQC (see Table 3) are in bold.

Analyte	NANJ	BBDO	BBUP
Al	79.6 ± 154.0 (a)	18.8 ± 30.4 (a)	23.8 ± 22.4 (a)
Sb	0.65 ± 0.70 (a)	0.18 ± 0.14 (a)	0.11 ± 0.11 (a)
As	2.24 ± 1.69 (a)	2.38 ± 2.46 (a)	0.80 ± 1.01 (a)
Ba	45.1 ± 21.3 (a)	61.5 ± 21.1 (a)	66.8 ± 8.1 (a)
Be	0.05 ± 0.05 (a)	0.01 ± 0.01 (a)	0.03 ± 0.01 (a)
Cd	0.01 ± 0.01 (a)	0.03 ± 0.04 (a, b)	0.06 ± 0.03 (b)
Cr	0.18 ± 0.23 (a)	0.11 ± 0.06 (a)	0.14 ± 0.07 (a)
Co	3.37 ± 3.06 (b)	1.89 ± 1.49 (a, b)	0.20 ± 0.17 (a)
Cu	0.56 ± 0.45 (a)	0.27 ± 0.06 (a)	0.59 ± 0.30 (a)
Fe	**1,220 ± 1,192** (a)	**1,753 ± 2,958 (a)**	79 ± 71 (a)
Pb	0.37 ± 0.50 (a)	0.10 ± 0.12 (a)	0.09 ± 0.07 (a)
Mn	1,249 ± 1,021 (b)	406 ± 375 (a, b)	16 ± 18 (a)
Ni	1.05 ± 0.80 (a, b)	0.96 ± 0.55 (a)	1.99 ± 0.59 (b)
Se	0.06 ± 0.06 (a)	0.10 ± 0.03 (a)	0.31 ± 0.11 (b)
Ag	0.003 ± 0.01 (a)	0.002 ± 0.01 (a)	0.006 ± 0.01 (a)
Sr	19 ± 8 (a)	69 ± 28 (b)	203 ± 27 (c)
Tl	0.005 ± 0.01 (a)	0.01 ± 0.01 (a)	0.04 ± 0.02 (b)
V	0.57 ± 0.53 (a)	0.40 ± 0.30 (a)	0.47 ± 0.23 (a)
Zn	20.1 ± 9.9 (a)	17.5 ± 9.6 (a)	15.4 ± 5.8 (a)

DISCUSSION

Nutrient concentrations in the agriculturally dominated landscape of eastern Maryland are among the highest in the nation (Denver et al. 2004). The region contains most of the state's extant Dwarf Wedgemussel populations, and most historical (but extirpated) populations in Maryland occurred in the region (Bogan and Ashton 2016). Concentrations of ammonia in sediment often increase in agriculturally dominated landscapes with high reactive nitrogen load (Strayer 2014), such as Browns Branch. We note, however, the similar percentages of agriculture in catchments of BBUP and BBDO in 2001 and 2011 and are thus unable to link land-use differences or changes with the observed range contraction.

Although TAN concentrations at BBDO did not exceed AWQC, conditions stressful to juvenile freshwater mussels may have occurred, based on pore-water UIA-N concentrations that exceeded the threshold of 0.2 μg/L (five of six measurements) proposed for *E. complanata* (Strayer and Malcom 2012). UIA-N concentrations also exceeded this threshold at BBUP and NANJ, but the frequency and magnitude of exceedance were much lower than at BBDO. Thus, elevated UIA-N can be considered a potential cause of Dwarf Wedgemussel range contraction in BB because it supports our hypothesis about the spatial distribution and concentrations of such a factor (BBDO > BBUP = NANJ). Furthermore, the concentrations reported in the peepers are long-term averages and may underestimate transient peak

concentrations to which juvenile mussels may be exposed (Strayer and Malcom 2012).

Mussels at BBDO may have been exposed to the combined stressors of high UIA-N and low DO in pore water. Low pore-water DO favors the long-term presence of ammonia because higher oxygen conditions are needed for oxidation of ammonia to nitrate (Kinsman-Costello et al. 2015). Low oxygen in sediments can result in death of juvenile mussels or increase their susceptibility to predators (Sparks and Strayer 1998). Although low DO was detected in pore water at all sites, TAN concentrations were much higher at BBDO. In general, the lower-DO pore water contained higher concentrations of ammonia than the higher-DO surface water. The highest UIA-N concentrations occurred in summer, but no sites experienced temperatures approaching the 29°C thermal limit proposed for Dwarf Wedgemussel (Campbell 2014).

Other factors not measured in this study could influence Dwarf Wedgemussel distribution and merit further examination. The only documented host fish of Dwarf Wedgemussel that co-occurs in Maryland is the Tessellated Darter (Ashton 2010). On average, their abundance was three times lower at BBDO compared with BBUP (M. J. Ashton, MDNR, unpublished data), which may limit Dwarf Wedgemussel reproduction and dispersal (McClain and Ross 2005). Additionally, many herbicides are commonly detected in agricultural streams of eastern Maryland (Denver et al. 2004). The effects of current-use pesticides on mussels are not as well studied as nutrients or metals (but see Bringolf et al. 2007). *Elliptio complanata* also has been found to integrate transgenic material via bacteria uptake near cornfields, which may weaken its immune system (Gagne et al. 2006; Douville et al. 2009). Whether this could occur in Dwarf Wedgemussels is unknown.

There are several sources of uncertainty in the study. First, the strength of our conclusions is limited because measurements were made only monthly within a single year. Additional sampling is necessary to further characterize inter- and intra-annual variation in pore-water and surface-water quality. Furthermore, the Strayer and Malcom (2012) threshold has not been replicated in other studies with *E. complanata* or examined for other species. Thus, Dwarf Wedgemussel may be more or less sensitive than *E. complanata* to pore-water UIA-N.

We recommend conducting a survey of Browns Branch to assess the current condition of the mussel fauna. If range contraction is still evident, more frequent and extended water-quality parameter and surface- and pore-water sampling should be performed, including capturing high-flow events to determine if pulses of ammonia and other pollutants occur. Sampling tributaries and areas of groundwater input also could identify sources of pollutants.

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Appendix

The University of Maryland Center for Environmental Science Appalachian Laboratory (UMCES, Frostburg, MD)

Table A1. Summary of UMCES results from 2014 proficiency test.

Analyte	Rating
Total ammonia nitrogen	Ideal
Nitrate-N	Ideal
Sulfate	Ideal
Chloride	Flagged low on 1 sample

employs a rigorous Quality Assurance/Quality Control (QA/QC) program, which includes ongoing monitoring and evaluation of precision and accuracy (analysis of duplicates, matrix spikes, method blanks, and independent control samples with acceptance criteria that must be met to accept analytical results), as well as participation in blind audits, proficiency tests (PT), and split-sample programs. Most of these practices are outlined in a report of the Chesapeake Bay Program Data Integrity Workgroup (EPA Chesapeake Bay Program 2017). Analysis of method blank, matrix spike, laboratory duplicate, and independent control sample results indicates acceptable laboratory performance. The results for some of the measured constituents from a 10-sample PT study conducted during the project time period (Table A1) and analysis of the independent control sample for metals (Table A2) help document the quality of UMCES' performance.

APPENDIX REFERENCE

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Table A2. Summary of UMCES results from the analysis of the independent control sample for metals. RSD: Relative standard deviation.

Analyte	Target (μg/L)	Mean	Count	Standard Deviation	Minimum	Maximum	% RSD
Be	10	10.4	17	0.34	9.9	10.9	3.27
Al	40	43.6	17	1.06	41.0	45.7	2.44
V	60	61.6	17	1.16	59.8	63.6	1.88
Cr	30	30.4	17	0.50	29.8	31.2	1.65
Mn	90	90.6	17	1.31	88.4	92.4	1.44
Fe	80	80.8	17	1.06	79.1	82.9	1.31
Co	60	61.9	17	0.98	60.1	63.1	1.59
Ni	80	82.6	17	0.56	81.7	83.6	0.68
Cu	40	41.8	17	0.37	41.1	42.3	0.88
Zn	50	51.3	17	0.73	50.2	52.6	1.41
As	20	20.7	17	0.35	20.3	21.4	1.68
Se	10	10.3	17	0.13	10.1	10.5	1.21
Sr	40	40.1	17	1.37	37.7	41.6	3.41
Ag	20	20.4	17	1.93	19.0	27.7	9.43
Cd	20	19.9	17	0.45	19.2	20.6	2.25
Sb	10	10.0	17	0.22	9.71	10.4	2.20
Ba	10	9.94	17	0.30	9.49	10.5	2.97
Tl	10	10.1	17	0.22	9.75	10.4	2.19
Pb	40	40.8	17	0.80	39.6	41.9	1.96

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REGULAR ARTICLE

PICKY PIGS PREFER PIGTOES: EVIDENCE FOR SPECIES-SELECTIVE FERAL PIG PREDATION ON FRESHWATER MUSSELS

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ABSTRACT

We observed evidence of predation on freshwater mussels during a field experiment. Mussels within the stream reach and experimental enclosures were dislodged from the sediment and shells were crushed whole, and the substrate and enclosures were extensively disturbed. Of the 12 mussel species detected in pre-experiment sampling, a Jacob's electivity index suggested that only two species (*Fusconaia cerina* and *Elliptio arca*) were positively selected for by the predator, with *F. cerina* being strongly preferred; other dominant species were avoided. We estimated that 1% of the mussel community and 6% of the *F. cerina* population was predated. We found that 70% of the experimental enclosures were disturbed, but those containing *F. cerina* were disturbed at a higher rate than other treatments. Water depth was a significant factor predicting disturbance of enclosures, and disturbance was not as severe for enclosures in deeper water. Based on characteristics of the event, we suggest that feral pigs (*Sus scrofa*) were responsible for the predation and disturbance. While only a small portion of the mussel community was predated, continued species and spatial selection could shift community structure and distribution. Feral pigs also may pose an indirect threat to mussel populations because substrate disturbance by rooting could decrease sediment stability.

KEY WORDS: feral pigs, freshwater mussels, selective predation, species selection, invasive species, community structure, predator–prey

INTRODUCTION

Selective predation plays a key role in structuring and regulating biological communities and processes (Schmitz et al. 2010). Predators exert top-down influence on multiple aspects of prey ecology, including behavior (Schmitz et al. 1997), metabolism and stoichiometry (Dalton and Flecker 2014), and life history (Reznick and Endler 1982). Predators may select based on prey size, morphology, nutritional value, defense mechanisms, or spatial distribution, resulting in varying magnitudes of predation pressure on different species or populations (Jokela and Mutikainen 1995; Watters 1995; Diggins and Stewart 2000). Over time, selective predation may result in shifts in community dynamics such as spatial distribution, species abundance, and diversity (Watters 1995; Tyrrell and Hornbach 1998; Diggins and Stewart 2000).

Freshwater mussels have many known predators including muskrats, otters, raccoons, turtles, catfish, and flatworms (Haag 2012). Lesser-known mussel predators are domesticated and feral pigs (*Sus scrofa*). Accounts of pig predation on mussels are scarce but have been reported for many years (Rafinesque 1820; Simpson 1899; Tudorancea 1972; Williams and Benson 2004). Feral pigs are an invasive species that have proliferated across the USA in the past few decades and now cause extensive economic and ecological damage (Mayer and Brisbin 2008; Ivey et al. 2019). They forage by rooting, which can result in severe disturbance in terrestrial and aquatic ecosystems (Kotanen 1995; Cushman et al. 2004; Barrios-Garcia and Ballari 2012). The expansion of feral pig populations in the USA poses an additional threat to the imperiled mussel fauna by direct predation and indirect effects of habitat disturbance.

There is little or no quantitative information about the magnitude of pig predation on mussels, species selectivity, or other features of this predator–prey relationship. We observed

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apparent pig predation on mussels during a field experiment in a lowland river in the southeastern USA. We used pre- and postpredation data on the mussel community present in the reach to evaluate species and water depth selectivity exhibited by the predator during foraging. We show evidence supporting pigs as the predator and discuss the consequences of pig predation for mussel conservation.

METHODS

Study Area

Our study was conducted on the Sipsey River, Alabama, a fifth-order alluvial river flowing mostly through the Eastern Gulf Coastal Plain physiographic province and draining into the Tombigbee River. The Sipsey River is unregulated, with extensive, forested floodplain wetlands, and it supports dense mussel aggregations (Haag and Warren 2010; Atkinson et al. 2019). Our observations were made within a 60-m reach of the river in Greene County, Alabama, that had been established previously for a field experiment (see Experimental Setup, below). The study reach consisted of a shallow run (maximum depth = 0.7 m) with gravel and sand substrate.

Experimental Setup

Our observations were made during an experiment designed to investigate the impact of mussel biodiversity on sediment processes, described briefly as follows (see Nickerson 2018 for additional details). The experimental setup consisted of 36 open-topped 0.25-m^2 enclosures designed to contain manipulated mussel assemblages. Enclosures consisted of a 25 × 25 × 15–cm frame of 5 × 5–cm lumber covered with steel mesh and buried so that the top edge was flush with the sediment surface. Enclosures were installed approximately every 4 m along eight cross-sectional transects spaced 6 m apart (Fig. 1). Enclosures were installed July 29, 2017. Prior to installation, each 0.25-m^2 area was excavated to a depth of 20 cm and sieved, and all naturally occurring mussels were identified and counted; 39 0.25-m^2 quadrats were excavated (representing 2.75% of total reach area), but ultimately, only 36 enclosures were installed. After installation, each enclosure was refilled with the sieved sediment and stocked with one of five experimental mussel assemblages: (1) only *Cyclonaias asperata*, (2) only *Fusconaia cerina*, (3) a 50/50 mixture of *C. asperata* and *F. cerina,* (4) sham mussel shells (empty valves glued together), and (5) a control with no mussels or shams. Mussel and sham assemblages were stocked at two densities, 24 and 48 individuals/m^2 (6 and 12 individuals/enclosure, respectively); this resulted in a total of nine treatments with four replicates each and a total of 216 stocked mussels. All experimental mussels and sham mussels were tagged with numbered fly-fishing line (Fig. 2A). Enclosures were stocked with mussels of similar size to standardize biomass within treatments.

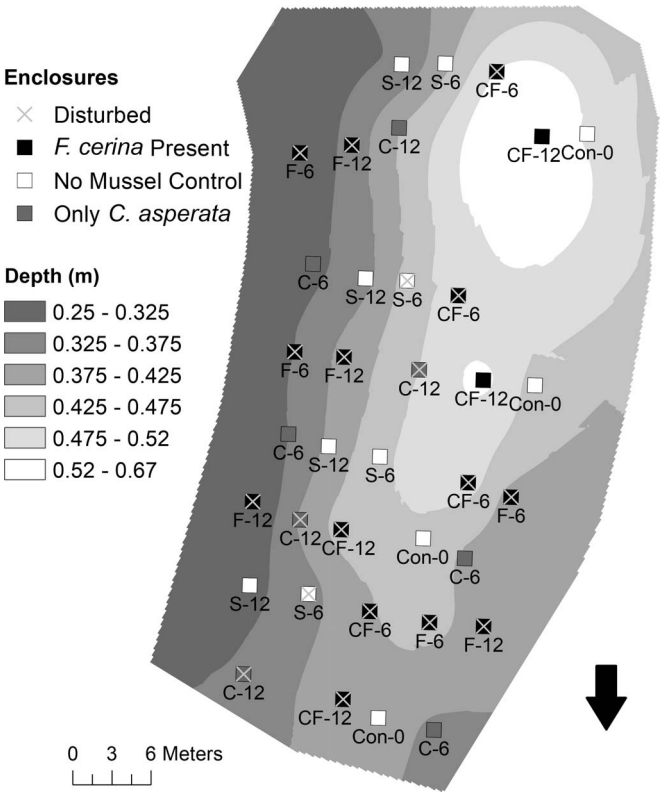

Figure 1. Map of the study reach in the Sipsey River, Alabama, showing placement of 0.25-m^2 experimental enclosures. Depth contours were interpolated from depth measurements at each enclosure. Alphanumeric codes indicates experimental treatment: F = *Fusconaia cerina* only, C = *Cyclonaias asperata* only, CF = 50/50 mixture of *F. cerina* and *C. asperata*, S = sham control, Con = no mussel control, 0 = no individuals, 6 = six individuals (24 individuals/m^2), and 12 = 12 individuals (48 individuals/m^2). Arrow indicates direction of stream flow.

Predation Event

We observed disturbance to a subset of enclosures and evidence of mussel predation during low-flow conditions on September 22, 2017, and again on September 26, 2017. Immediately upon discovering the disturbances, we identified which enclosures showed signs of disturbance, recorded which mussels were missing from enclosures, and returned individuals we found to their enclosures; many individuals could not be found and were recorded as missing. We collected all freshly dead shell material within and 4 m downstream of the study reach, as some fragments had drifted or were consumed outside of our established reach. Because many shells were crushed or disarticulated, we estimated the number of individuals predated based on the number of umbos recovered, with two umbos representing one individual. Fragments were confirmed as experimental individuals based on the presence of numbered ID tags, but the origin of all individuals (experimental or wild) could not be determined. We combined counts of disturbed enclosures and predated individuals from both events, and we did not consider differences in stocking densities among enclosures in our analyses.

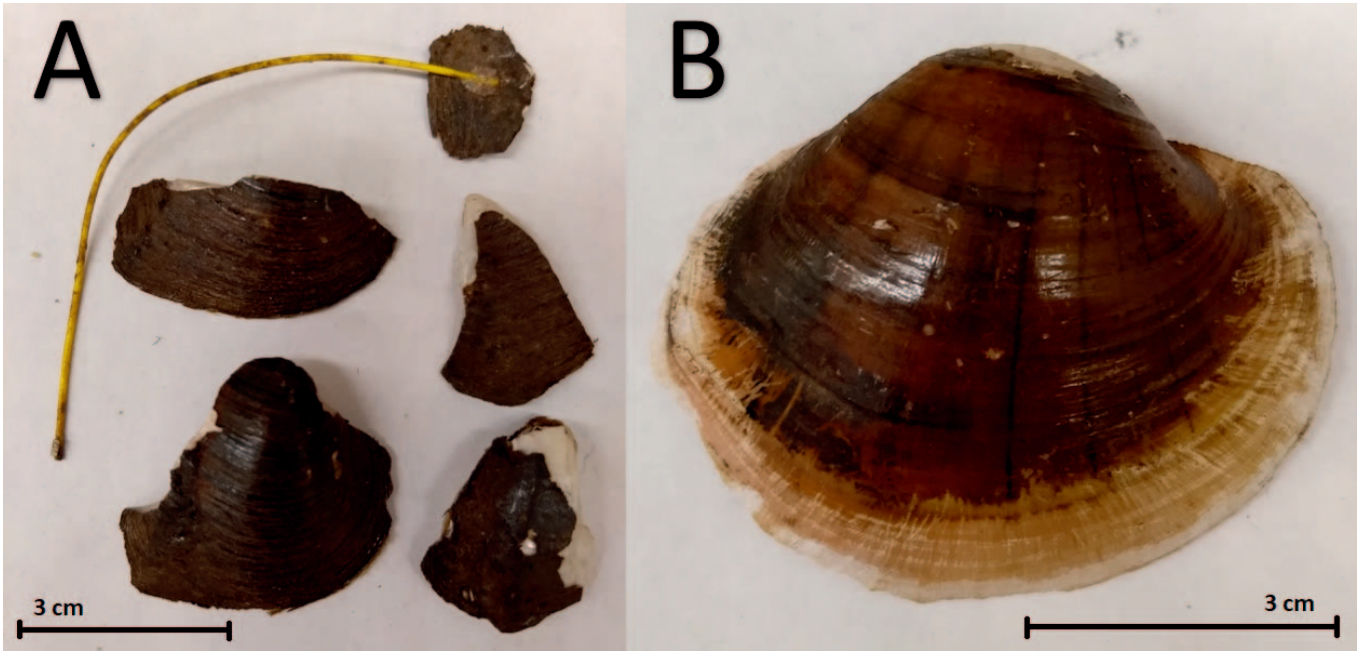

Figure 2. (A) Crushed shells of *Fusconaia cerina* recovered after apparent pig predation; top fragment shows attached fly-fishing–line tag. (B) Abrasion pattern on *Lampsilis ornata*, in which the shell margin was broken to access soft tissue.

Analysis of Selective Predation

We were unable to assess size selectivity in our analysis of selective predation due to standardization of individual size within enclosures. Rather, we focused on two other aspects of selective predation: species selectivity and water-depth limitation. We tested for evidence of species selection during the predation event by calculating Jacob's electivity index (Jacobs 1974) with species-abundance estimates from initial enclosure excavation representing available prey and counts of predated shell umbos representing consumed prey. Some of the mussels stocked into enclosures originated from outside the study reach; these individuals were included in estimates of available prey, but they constituted a small proportion of the mussel assemblage in the reach (Fig. 3). We used the "ivlev" function within the "selectapref" R package (Richardson 2017) to compensate for the difference in abundance between prey species, standardizing all scores between −1 and 1.

We tested for an effect of depth and species treatment on

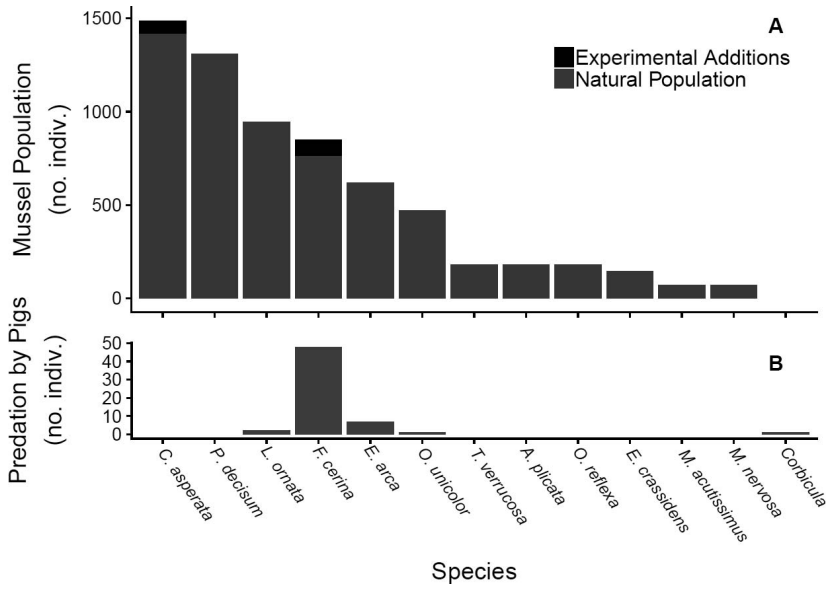

Figure 3. (A) Pre-predation mussel community structure in the experimental reach obtained from substrate excavation and including mussels stocked into experimental enclosures from outside the reach (black portion of histogram bars). (B) Apparent pig predation in the experimental reach.

enclosure disturbance, with disturbance quantified in three different ways: the proportions of dislodged, missing, or killed mussels in an enclosure. We tested for these effects using multiple linear regression with disturbance as the dependent variable and enclosure depth, species treatment, and the interaction term as the independent variables. We conducted separate multiple linear regressions for each measure of disturbance. We tested for differences among treatment combinations using Tukey post hoc tests. These analyses showed that species treatment was the only significant factor, and enclosures containing *F. cerina* were predated at a higher rate (see Results). Consequently, we explored potential depth selection further using linear and piecewise regressions including only those enclosures containing *F. cerina* (both *F. cerina*–only and mixed treatments) to eliminate noise that may have been introduced by the lower predation rate on other species treatments. Piecewise regression allows for the detection of a critical threshold or breakpoint, indicating that the relationship is not linear but changes abruptly at a threshold (Toms and Lesperance 2003). We compared linear and piecewise regressions to determine if a significant threshold depth existed. We used a Davies test to determine if breakpoints were significant and the relationship was better represented as multiple linear relationships (Muggeo 2016). We calculated linear regressions and conducted Tukey post hoc tests with the "aov," "lm," and "TukeyHSD" functions in base R; piecewise regressions and Davies test were calculated with the "segmented" and "davies.test" functions within the "segmented" R package (Muggeo 2008; R Core Team 2017).

RESULTS

Substrate within and outside of enclosures was heavily disturbed, indicating rooting, and mussels were dislodged from the enclosures or missing. Nineteen of 36 enclosures (52.8%) were disturbed, including 70.0% of the 24 enclosures containing mussels and 16.7% of the 12 enclosures containing sham mussels or no mussels.

Evidence of predation on mussels consisted of crushed shells and scratch marks on shells indicating severe abrasion (Fig. 2). In addition, many mussels were dislodged from enclosures but were not eaten. Crushed shells were found only within the stream channel and not on the shore. Initial excavation yielded 12 species and a total mussel population estimate of 6,516 individuals in the reach (Fig. 3A). We found a total of 59 predated individuals, including 48 *F. cerina*, seven *Elliptio arca*, two *Lampsilis ornata*, one *Obovaria unicolor*, and one *Corbicula fluminea* (Fig. 3B). These numbers correspond to 0.9% of all mussels predated, 5.6% of *F. cerina*, 1.1% of *E. arca*, and <0.4% of all other species. Of the 216 stocked mussels, 62.1% remained in the enclosures, 9.7% were dislodged from the sediment but not killed, and 28.2% were missing. Tags recovered from shell fragments confirmed that 27 of the missing *F. cerina* were predated, representing 44.3% of mussels missing from enclosures. All predated, tagged mussels were *F. cerina*, and these represented 25% of stocked individuals of that species. Only two sham mussels were confirmed predated, and both were *F. cerina* shells.

Enclosures were observed for 7 wk prior to the predation event. During that time, only five mussels became dislodged (mussels were replaced in the enclosures after dislodgement) and three mussels were lost. Three of the dislodged and two of the missing mussels were associated with a high-flow event at the beginning of the experiment that scoured a subset of enclosures.

Species Selection

General patterns of predation indicated strong selection for *F. cerina*. All eight enclosures containing only *F. cerina* were disturbed, and 75.0% of mixed-species enclosures were disturbed, but only 37.5% of the eight *C. asperata*–only enclosures were disturbed. Of the 108 stocked *F. cerina*, 40.8% remained in the enclosures, 11.1% were dislodged from the sediment, and 48.1% were predated or missing. Of the 108 stocked *C. asperata,* 83.4% remained in the enclosures, 8.3% were dislodged, and 8.3% were missing; none of the latter were confirmed predated.

Jacob's electivity index supported strong selection for *F. cerina*, which had the highest index score (0.70; Fig. 4). *Elliptio arca* was the only other species with a positive score (0.26), and all other species had negative scores, including species that dominated the wild community (e.g., *C. asperata*, *Pleurobema decisum*, *L. ornata*, and *O. unicolor*). *Corbicula fluminea* was not included in this analysis because it was not detected during the initial survey.

Depth Selection

Species treatment ($P = 0.001$) was the only significant variable for predicting dislodgement (depth, $P = 0.390$; depth $\times$ treatment, $P = 0.280$). A Tukey post hoc test showed that *F. cerina*–only ($P = 0.001$) and mixed ($P = 0.019$) treatments were both dislodged significantly more than *C. asperata*–only enclosures; *F. cerina*–only and mixed treatments ($P = 0.366$) were not significantly different from each other. Species treatment ($P < 0.001$) was the only significant variable for predicting the number of missing mussels (depth, $P = 0.283$; depth $\times$ treatment, $P = 0.265$). A Tukey post hoc test showed that *F. cerina*–only ($P < 0.001$) and mixed ($P = 0.008$) treatments had more missing mussels than *C. asperata*–only treatments, but they were not significantly ($P = 0.210$) different from each other. Species treatment ($P < 0.001$) was the only significant variable for predicting the number of killed mussels (depth, $P = 0.296$; depth $\times$ treatment, $P = 0.104$). A Tukey post hoc test showed that *F. cerina*–only ($P < 0.001$) and mixed ($P = 0.002$) treatments had more killed mussels than *C. asperata*–only treatments, but they were not significantly ($P = 0.136$) different from each other.

The proportion of individuals dislodged from *F. cerina*–containing enclosures was significantly and negatively corre-

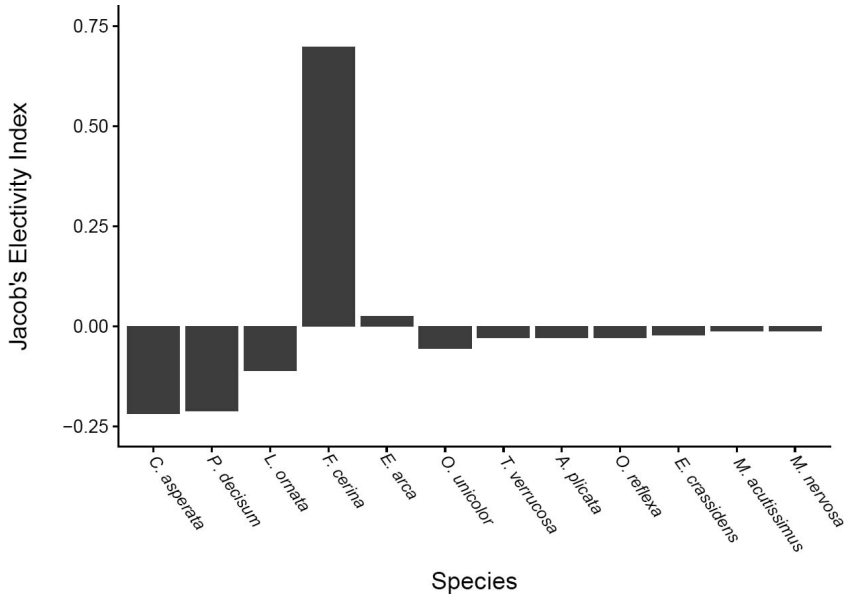

Figure 4. Jacob's electivity index scores for the 12 mussel species detected in the reach prior to predation.

lated with depth ($y = -2.03x + 1.73$, $P = 0.035$, $R^2 = 0.23$), as was the proportion of individuals killed ($y = -1.75x + 1.16$, $P = 0.007$, $R^2 = 0.37$). However, the proportion of individuals missing from *F. cerina*–containing enclosures was not significantly correlated with depth ($y = -1.46x + 1.38$, $P = 0.055$, $R^2 = 0.18$). Piecewise regression found breakpoints in depth in relationships for all three measures of disturbance, but the Davies test determined these breakpoints were not significant (dislodged, breakpoint in depth = 0.41 m, $P = 0.14$; missing, breakpoint = 0.49 m, $P = 0.13$; killed, breakpoint = 0.34 m, $P = 0.065$).

DISCUSSION

We did not directly observe the predation event, but several pieces of evidence support feral pigs as the culprit. First, we observed numerous pig tracks on the bank the day we discovered the event. We had monitored the experiment 3 d per week for the preceding 7 wk and did not observe pig tracks prior to the predation event. Second, the presence of crushed shells only within the stream channel is inconsistent with predation from smaller predators, such as muskrats. Muskrats, and other terrestrial predators, typically open the valves to consume the soft tissue, and deposit intact shells in middens on the shore (Tyrrell and Hornbach 1998; Diggins and Stewart 2000; Owen et al. 2011). Third, the pattern of disturbance and predation we observed indicates a large organism with a well-developed and strong crushing apparatus. Large catfish have bony crushing plates in their throat and are reported to crush heavy-shelled mussel species (Forbes 1888; Tiemann 2011). Apparent catfish predation was observed commonly in the Sipsey River prior to the proliferation of feral pigs in the watershed, and the appearance of these crushed shells is similar to those we observed (Haag 2012). However, crushed shells attributed to catfish predation occurred most frequently in deeper water under submerged logs or undercut banks, and their occurrence was not associated with notable substrate disturbance (Haag 2012; W. Haag, US Forest Service, personal communication). Our observations of crushed shells and substantial disturbance to the substrate is consistent with rooting and predation by feral pigs, and the lower rates of dislodgement and predation in deeper water supports a terrestrial predator. Our observations are similar to those of suspected pig predation during drought conditions, which presumably give pigs increased access to mussels (Williams and Benson 2004).

Predation during this event was highly selective. Of the 12 unionid species detected in the reach, feral pigs positively selected only *E. arca* and *F. cerina,* but *F. cerina* was highly favored and other dominant species in the reach appeared to be avoided (e.g., *C. asperata*, *P. decisum*, *L. ornata*). Furthermore, *C. asperata* and *F. cerina* were present in equal numbers in the mixed-species enclosures, yet *F. cerina* was selectively consumed in these enclosures even though both species were dislodged at similar rates. It is difficult to speculate why pigs so heavily favored *F. cerina* because its shell is similar in size, thickness, cubosity, and volume to *C. asperata* (see Owen et al. 2011).

Regardless of the basis for selectivity, pigs appeared to show a remarkable ability to detect the presence of *F. cerina*. Not only did pigs strongly favor this species, they disturbed enclosures containing *F. cerina* more frequently than other enclosure types. Visual clues are unlikely to be important because most mussels in the Sipsey River bury themselves in the substrate with only a small portion of the shell margin exposed (B. van Ee, personal observation). Pigs have well-developed olfactory and tactile capability in the snout, which helps them locate food in terrestrial environments (Allwin et

al. 2016). The ability of pigs to detect prey underwater is unknown, but other mammals have underwater olfactory capabilities (Catania 2006).

Effects of pig predation on mussel assemblages are largely unknown. Long-term selective predation can shift the composition and distribution of prey communities (Power 1984; Englund and Krupa 2000). Pigs consumed approximately 1% of the entire unionid community and approximately 6% of the *F. cerina* population in the experimental reach. Long-term selection for *F. cerina* could shift the community structure, and selective foraging in shallower areas could shift the community's spatial distribution. Indirect effects of pig predation also could influence mussel communities. Mussels dislodged from enclosures and scattered on the sediment surface could be vulnerable to other predators; they might be transported downstream by high flow; and they would be subject to increased stress and energy expenditures as a result of the need to rebury. Rooting by pigs also severely disturbs the streambed itself, which could decrease sediment stability and increase the erosion of previously stable substrate (Rafinesque 1820; Simpson 1899; Williams and Benson 2004; Butler 2006).

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LITERATURE CITED

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REGULAR ARTICLE

USE OF MORPHOMETRIC ANALYSES AND DNA BARCODING TO DISTINGUISH *TRUNCILLA DONACIFORMIS* AND *TRUNCILLA TRUNCATA* (BIVALVIA: UNIONIDAE)

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ABSTRACT

Closely related unionid species often overlap in shell shape and can be difficult to accurately identify in the field. Ambiguity in identification can have serious impacts on conservation efforts and population surveys of threatened and endangered species. *Truncilla donaciformis* and *Truncilla truncata* are sister species that overlap in their distributions and frequently co-occur in central North America. Because *T. donaciformis* is endangered in Canada and imperiled in some US jurisdictions, co-occurrence with the morphologically similar *T. truncata* means that misidentification could seriously impact status assessments and recovery efforts. The objectives of this study were to (1) establish species identifications of specimens using DNA barcoding (COI), (2) determine how well traditional morphometrics and geometric morphometrics accurately discriminate between the two species, and (3) determine the accuracy of field identifications relative to molecular and morphometric identifications. *Truncilla* specimens from four rivers in southern Ontario were photographed and visceral mass swabs were taken. Positive identifications of all specimens were obtained through DNA barcoding and comparison with sequences from GenBank. Traditional and geometric morphometric approaches were used to assign specimens to species. Assignments generated were compared to identifications based on mtDNA barcodes, with traditional and geometric morphometric analyses found to be 90% and 99% accurate in species identifications, respectively. This study confirmed the presence of *T. donaciformis* in Ontario's Thames River, and revealed that all *Truncilla* collected for this study from the other three rivers were *T. truncata*. This study reinforces the utility of combining geometric morphometric analyses and DNA barcoding for identifying problematic unionid specimens.

KEY WORDS: DNA barcoding, Laurentian Great Lakes drainage, morphometric analysis, shell shape, species identification

INTRODUCTION

Early delineations and descriptions of freshwater mussel species (order Unionida) were based on shell morphology (Watters et al. 2009), which can be expressed through coloration, shape, shell sculpture, or size. Some shell-shape characters have a clear genetic basis and are potentially adaptive (Inoue et al. 2013, 2014), but habitat and environment can also have major effects on shell morphology. Use of shell shapes led to overdescription of some species based on sometimes-subtle differences (Haag 2012). More recently, species descriptions have been based on internal soft-tissue anatomy and molecular tools, resulting in the synonymizing of many previously described species (Watters et al. 2009; Haag 2012). Sacrificing animals to examine soft tissues is often not

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an option when dealing with threatened and endangered species, so identifications of live animals are limited to external shell features and genetic methods (e.g., DNA barcoding; Hebert et al. 2003). Because failure to correctly identify species can have important implications for the conservation of unionid diversity, a major challenge in correctly identifying species is dealing with intraspecific variation in shell shape. As shell morphology remains the most common tool used in field survey identifications of freshwater mussels, this intraspecific variation can be problematic when attempting to differentiate among closely related species with similar shell morphologies.

Truncilla donaciformis (Lea, 1828; Fawnsfoot) and *Truncilla truncata* (Rafinesque, 1820; Deertoe) are sister species (Burlakova et al. 2019) with similar shell morphologies (Fig. 1). Both are described mostly using shell morphology that is variable and potentially nondiagnostic. Watters et al. (2009) qualitatively describes *T. donaciformis* as more elongate in shape, whereas *T. truncata* is more triangular and typically exhibits a prominent posterior ridge. Further confounding correct field identifications, both species exhibit some subtle sexual dimorphism, with females being more rounded along the ventral margin (Watters et al. 2009; Burlakova et al. 2019).

Both *T. donaciformis* and *T. truncata* are distributed throughout much of the Mississippi River and Ohio River drainages and parts of the Great Lakes drainage of North America (Watters et al. 2009). In Canada, *T. truncata* is relatively common in southwestern Ontario, while *T. donaciformis* is considered an endangered species (COSEWIC 2008). In the USA, *T. donaciformis* is considered imperiled in several U.S. states and may be declining across its range (NatureServe 2020). The distribution of *T. truncata* is similar to *T. donaciformis*, but it is more widely distributed throughout the USA and is considered less imperiled. As the distributions of *T. donaciformis* and *T. truncata* are mostly sympatric (Watters et al. 2009), and their morphological characters are known to overlap, differentiating between the species can be problematic. This potential ambiguity is even more concerning when one considers the disparity in conservation status of the two species. Misidentifications could lead to misspent resources and incorrect conservation and recovery strategies, resulting in a high cost in terms of both conservation capital and species outcomes (Shea et al. 2011).

A number of practices can be employed to improve the accuracy of identifications based on shell appearance. One such practice is morphometric analysis, which measures and quantifies shape. Traditional morphometrics (TM) in mollusks include the measurement and comparison of ratios between characters such as shell length, width, and height (e.g., Cyr et al. 2007). For some species, once a large sample size of confirmed specimens has been examined, TM can be reasonably accurate in differentiating species, but positive identification cannot be assured due to morphometric variation within populations (Cyr et al. 2007; Inoue et al. 2013, 2014). If the differences are great, traditional morphometrics are usually

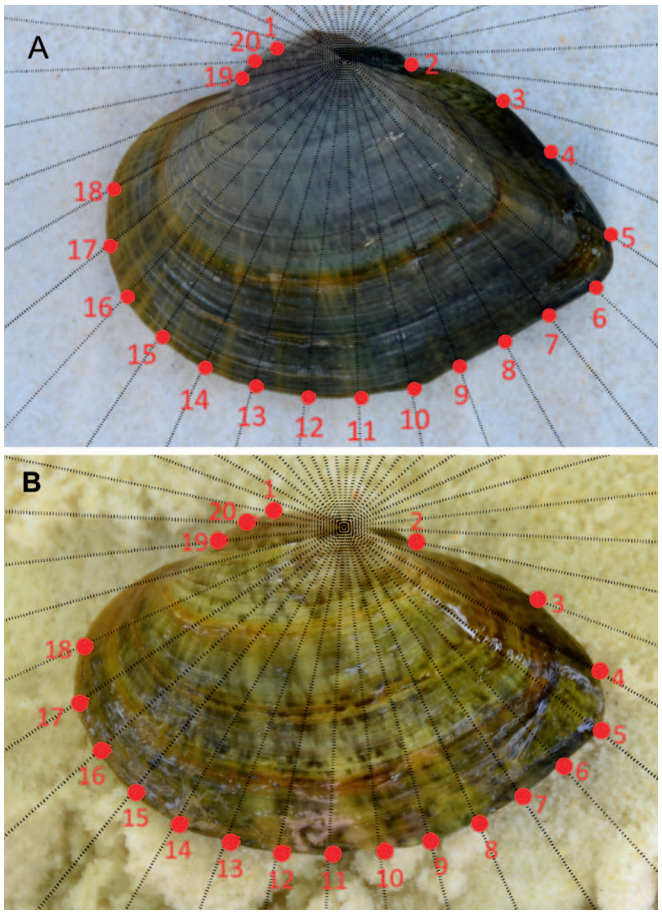

Figure 1. Positions of geometric morphometric type I and type II landmarks on the left valve of A) typical *Truncilla truncata* specimen and B) typical *T. donaciformis* specimen. Type I (anchor) landmarks: LM 1 were placed at the tip of the umbo, LM 2 at the posterior end of the hinge ligament. Type II landmarks: LM 3 to LM 18 were placed where a fan with 40 rays (anchored at midpoint between LM 1 and LM 2) crossed the shell margin; LM 19 and 20 were placed where rays from the fan cross the anterior side of the umbo.

sufficient for differentiating between groups, but if the differences are subtle, they can be confounded easily (Webster and Sheets 2010). Despite these drawbacks, taking shell measurements is quick, convenient, low-cost, and noninvasive, and these measurements are recorded in almost every field survey of unionid mussels. Geometric morphometrics (GM) involves the use of landmarks and pseudo-landmarks to assess the shape of an organism in two or three dimensions (Zelditch et al. 2012). Multivariate statistical analyses of the data are used to compare morphologies of the target specimens (Webster and Sheets 2010). Typically, these analyses are more robust and accurate than TM (Rohlf and Marcus 1993; Adams et al. 2004); their accuracy makes them an ideal method of differentiating between two occasionally ambiguous species. A reliable quantitative approach to species identification using a combination of morphometric analyses and DNA barcoding (see below) is preferable over "best guess" or "expert opinion."

In order to assess the utility and reliability of either

morphometric method, it is important to first independently confirm specimen identifications. In the last two decades, DNA barcoding has become an important tool for species identification (Hebert et al. 2003; Baird and Sweeney 2011). A (~650 bp) fragment of mitochondrial DNA encoding the cytochrome oxidase subunit 1 (COI) gene (Folmer et al. 1994) is often used as a reference sequence to which homologous sequences are compared, and barcoding with this sequence has been employed in the study of many animal groups including unionids (e.g., Inoue et al. 2014, 2018). Previously published *Truncilla* COI sequences (including those from Burlakova et al. 2019) on NCBI GenBank enable the positive identification of *T. donaciformis* and *T. truncata* specimens, which can help establish a group of specimens with confirmed identifications that can be used to discover morphological differences between the two species.

The objectives of this study were to (1) establish species identifications of specimens using DNA barcoding (COI), (2) determine how well TM and GM accurately discriminate between the two species, and (3) determine the accuracy of field identifications relative to molecular and morphometric identifications. We predict that GM will prove more accurate than TM in correctly differentiating between the species and that the combination of these techniques will elucidate areas of difference between the shell morphologies of *T. donaciformis* and *T. truncata*.

METHODS

We collected specimens of *Truncilla donaciformis* and *T. truncata* from the Ausable, Sydenham, Thames, and Welland rivers in Ontario, Canada, during targeted surveys completed in summer 2017 (Table 1 and Fig. 2). Swabs of the foot and mantle were taken from all live specimens and stored in lysis buffer (Sambrook et al. 1989). We photographed all field-collected specimens and returned them to the stream of origin after processing. Additional specimens were added to the morphometric dataset from the University of Michigan Museum of Zoology and the Ohio State University Division of Mollusks. The museum specimens used were lots from the Great Lakes drainage (Ontario, Michigan, and Ohio) and the Ohio River (the type locality for both species; Table 2 and Fig. 2). The left valve of each field- or museum-collected specimen was photographed in the same orientation for geometric morphometric analyses. For photographs, field-collected specimens (all live) were placed on a bed of fine sand, and a digital camera was mounted on a portable copy stand and placed at a 90° angle to the shell valve. Museum specimens (dead shell valves) were photographed using a digital camera mounted in a Stackshot apparatus with the valve placed on a piece of black fabric or modeling clay to ensure it was precisely at a 90° angle to the camera.

A 250 μL aliquot of the swab lysis buffer from each specimen was digested with 15 μL of proteinase K overnight at 56°C, followed by alcohol extraction and purification (Sambrook et al. 1989). Extracted DNA (stained with SYBR Green) was electrophoresed on a 1.5% agarose gel to confirm the success of the extraction and assess DNA quality. The female-lineage cytochrome c oxidase subunit I (COI) region of the mtDNA was amplified using the COI primers described in Campbell et al. (2005). Amplicon aliquots were stained with SYBR Green and electrophoresed in a 1.5% agarose gel to visualize fragment sizes and confirm successful amplifications. Reactions were purified using exonuclease I and shrimp alkaline phosphatase (EXOSAP). Polymerase-chain-reaction products mixed with EXOSAP were incubated at 37°C for 40 min, followed by 80°C for 20 min to denature any enzymes or remaining primers (as in Hewitt et al. 2019). Samples were Sanger sequenced by Eton Biosciences (etonbio.com) on an Applied Biosystems ABI 3730. The generated sequences were compared to those available on GenBank using BLAST (Altschul et al. 1990). The GenBank sequence with the highest percentage identity score resulting from the BLAST search was chosen as the most likely species and identified as such.

Table 1. Summary of field-collected and identified *Truncilla* specimens from rivers in Ontario, Canada.

Location	*T. truncata*	*T. donaciformis*	Uncertain *Truncilla*	River totals
Welland River	18	0	11	29
Ausable River	2	0	0	2
Thames River	33	28	10	71
Sydenham River	1	0	0	1
Totals	54	28	21	103

Traditional morphometric measurements for each specimen were length (maximum distance anterior to posterior) and height (maximum distance dorsal to ventral) measured to the nearest millimeter using Vernier calipers. For field-collected specimens, shell inflation (= width or maximum distance across left and right valves) was also measured. Length-to-height (L/H), length-to-width (L/W), and height-to-width (H/W) ratios were calculated for all field-collected specimens and then arcsine transformed to control for specimen size (as in Inoue et al. 2014).

For geometric morphometric analyses, an image of the left valve of each specimen was uploaded to the MakeFan program of the Integrated Morphometrics Package 8 (IMP8; Sheets 2014). Anchor (type I) landmarks were placed at the tip of the umbo and the point where the hinge ligament ends on the dorsal-posterior side of the valve (landmarks 1 and 2 on Fig. 1). Using MakeFan8, a 40-ray fan was placed at the midpoint between landmarks 1 and 2. Type II landmarks were placed at the point where each ray transected the edge of the shell, with landmark numbers starting at three and consecutively following the rays clockwise around the shell (Fig. 1). We chose to remove the point along the shell margin following landmark 18 because the curve at this point was highly variable among specimens and resulted in placement of the point at different parts of the shell (at the anterior curve of the shell margin, the anterior slope of the hinge, or the anterior of

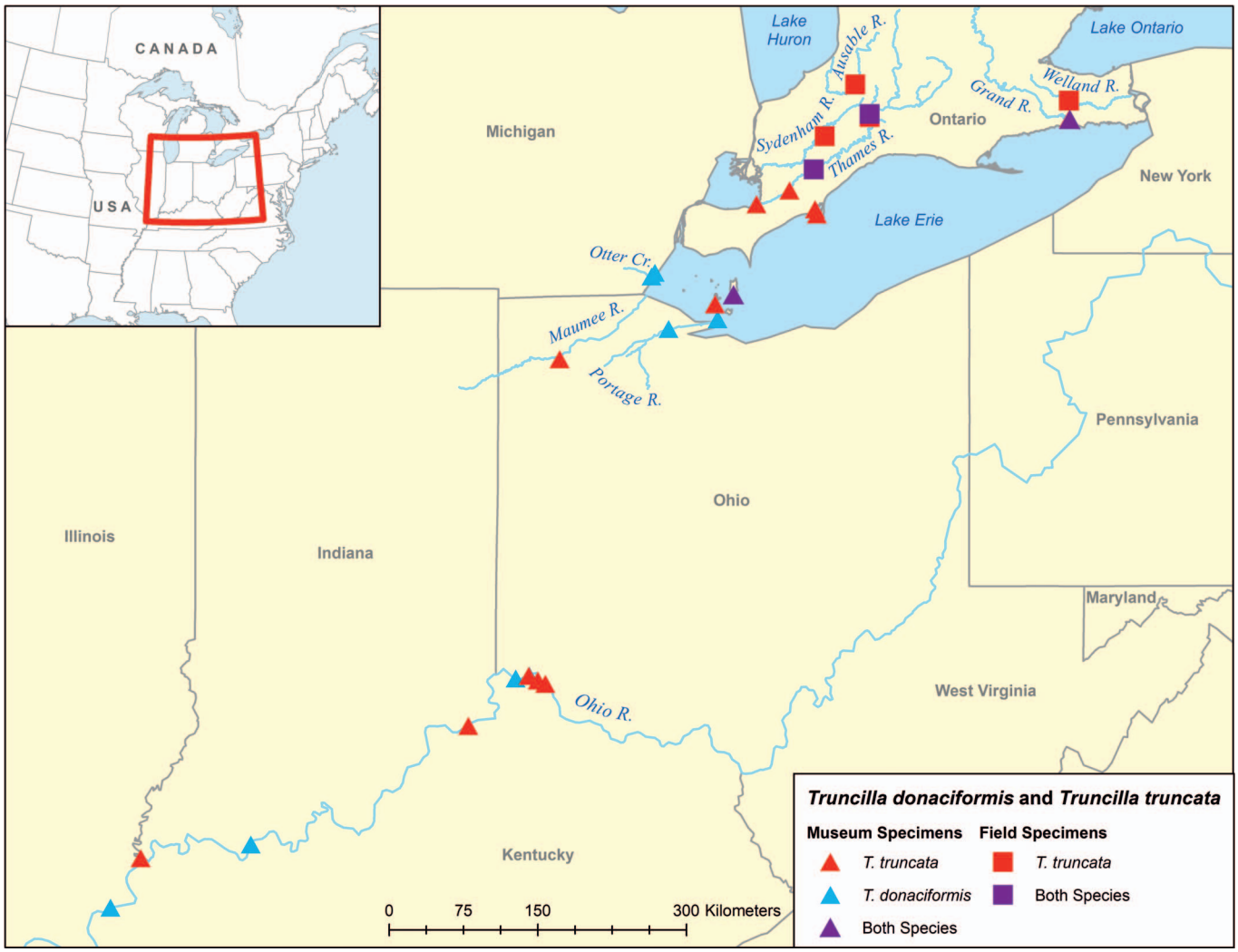

Figure 2. Map of *Truncilla donaciformis* and *T. truncata* field-collection site locations and museum specimen localities.

the raised umbo). Type II landmarks 19 and 20 were placed where the rays cross the anterior side of the umbo. The landmarks for each specimen were digitized using MakeFan8. CoordGen8 was used to implement a generalized Procrustes alignment of the digitized landmarks.

A principal component analysis (PCA) was applied to the transformed TM ratios (L/H, L/W, H/W) using XLSTAT v. 2018.6 (2018). Discriminant analysis (DA), implemented in XLSTAT using field-identified specimens, was employed to assess the utility of traditional morphometric measurements in species identification. The species memberships of unidentified specimens were predicted using the results of the DA and compared to the confirmed species identifications resulting from the NCBI BLAST search of the COI sequences. An alpha of 0.05 was used in all tests of significance.

CVAGen (in IMP8) was used to implement a canonical variates analysis (CVA, equivalent to the DA used with the TM dataset) to determine if and where significant differences in shell shape exist between the species and to visualize and quantify where the differences were greatest. Differences between shell shapes were visualized using a deformation grid and vectors on landmarks. Museum and field-collected specimens with confirmed identifications (using COI sequences) were used to create the CVA model. The unknown field-collected specimens were used to test the utility of the model using a jackknife assignment test based on the CVA results. We compared the predictions generated by the model to the confirmed species identifications resulting from the NCBI BLAST search of the COI sequences. The species memberships of the unidentified specimens were predicted using a jackknife assignment test based on the CVA results and compared to the confirmed species identifications resulting from the NCBI BLAST search of the COI sequences.

RESULTS

Field collections yielded photographs of 103 individuals: 54 identified as *T. truncata*, 28 as *T. donaciformis*, and 21 unidentified *Truncilla* from the Thames, Welland, Ausable, and Sydenham rivers in Ontario, Canada (Table 1). Museum

Table 2. Summary of *Truncilla donaciformis* ($n = 44$) and *T. truncata* ($n = 42$) specimens examined, photographed, and digitized at the Ohio State University Division of Mollusks and the University of Michigan Museum of Zoology. Museum collection lot numbers and number of specimens are listed for each species.

Ohio State University Division of Mollusks		University of Michigan Museum of Zoology	
T. donaciformis (museum lot–location–no. specimens)	*T. truncata* (museum lot–location–no. specimens)	*T. donaciformis* (museum lot–location–no. specimens)	*T. truncata* (museum lot–location–no. specimens)
8328–Grand R., Ontario–4	*550*–Lake Erie, Ohio–6	*63767*–Grand R., Ontario–1	*164427*–Ohio R., Ohio–3
15831–Lake Erie, Ohio–4	*64392*–Maumee R., Ohio–2	*198126*–Grand R., Ontario–3	*129769*–Ohio R., Illinois–3
66852–Portage R., Ohio–5	*85140*–Grand R., Ontario–2	*128791*–Lake Erie, Ontario–1	*31*–Ohio R.–3
58607–Ohio R., Ohio–4	*14937*–Lake St. Clair, Ontario–2	*70923*–Otter Cr., Michigan–5	*70941*–Ohio R.–1
	6577–Ohio R., Kentucky–5	*45014*–Lake Erie, Michigan–5	*185609*–Grand R., Ontario–4
	1661–Ohio R., Kentucky–5	*232560*–Ohio R., Ohio–1	*198129*–Thames R., Ontario–3
		62218–Ohio R., Kentucky–3	*70991*–Lake Erie, Ontario–1
		70863–Ohio R.–3	*227598*–Lake Erie, Ontario–1
		44852–Ohio R., Illinois–1	*71014*–Lake Erie, Ontario–1
		70915–Ohio R.–2	
		44824–Ohio R., Kentucky–1	
		59–Ohio R.–1	
17–*T. donaciformis*	22–*T. truncata*	27–*T. donaciformis*	20–*T. truncata*

specimens from the Ohio State University Division of Mollusks and University of Michigan Museum of Zoology were examined and photographed for a total of 44 *T. donaciformis* and 42 *T. truncata* specimens (Table 2). Photographs used for morphometric analyses have been submitted to MorphoBank (Project 3457, MorphoBank accession numbers M675689–M675794; http://morphobank.org/permalink/?P3457).

DNA Barcoding

Sequencing resulted in COI amplicons averaging 640 bp in length from all 103 individuals, with six unique haplotypes generated for *T. donaciformis* (GenBank accession nos. MT593033- MT593038) and four unique haplotypes generated for *T. truncata* (GenBank accession nos. MT594464–MT594467). A comparison of the COI sequences to those on GenBank using NCBI BLAST confirmed the identifications of 35 *T. donaciformis* and 68 *T. truncata* specimens. Based on the BLAST search results, one specimen from the Thames River (specimen no. 86; site TR-50) was *T. truncata* based on its COI sequence but was incorrectly identified in the field as *T. donaciformis*. Of the 21 unknown *Truncilla* specimens, 13 were identified as *T. truncata*, and eight as *T. donaciformis*.

Traditional Morphometrics

Principal components analysis of the traditional morphometric ratio data showed that the L/H ratio was the primary driver of differences between species shapes (Fig. 3). A *t*-test of the L/H ratios between species supported the hypothesis that the two groups' values were different ($t = -9.81$, $df = 100$, $P < 0.001$), but there was overlap between the species (Fig. 4). The H/W ratio explained less differentiation between the species, while the L/W ratio was not a diagnostic character between them.

The DA based on the traditional morphometric ratios revealed significant differences between *T. truncata* and *T. donaciformis* specimens confirmed with the COI sequence data. The DA correctly identified 93 of 103 specimens when compared to BLAST search results of the COI sequences. The misassigned specimens were three *T. truncata* and seven *T. donaciformis*. One of the specimens correctly predicted by the DA was the field-misidentified specimen from the Thames River (specimen no. 86). The DA also was used to assign the unknown *Truncilla* specimens to a species and was correct for 19 of 21 specimens when compared to BLAST search results of the COI sequences. The two misidentified specimens were a COI-identified *T. truncata* from the Welland River (site WLR05 specimen no. 10) and a COI-identified *T. donaciformis* from the Thames River (site TR-24 specimen no. 34). However, the latter specimen had only a 56.1% probability of assignment to the *T. truncata* group.

Geometric Morphometrics

The CVA revealed differences in shape along a single axis between the COI-confirmed species (Fig. 5). Differences in shell shape along CVA axis 1 were visualized using a deformation grid and landmark vectors (Fig. 6). The main differences were in length near the dorsal margin and in height along the ventral margin of the shell. The jackknife assignment test on the landmark-based CVA dataset resulted in 102 of the 103 field-collected specimens and all of the museum specimens being correctly assigned back to the COI-confirmed or expert-identified (for museum specimens) species. Of the

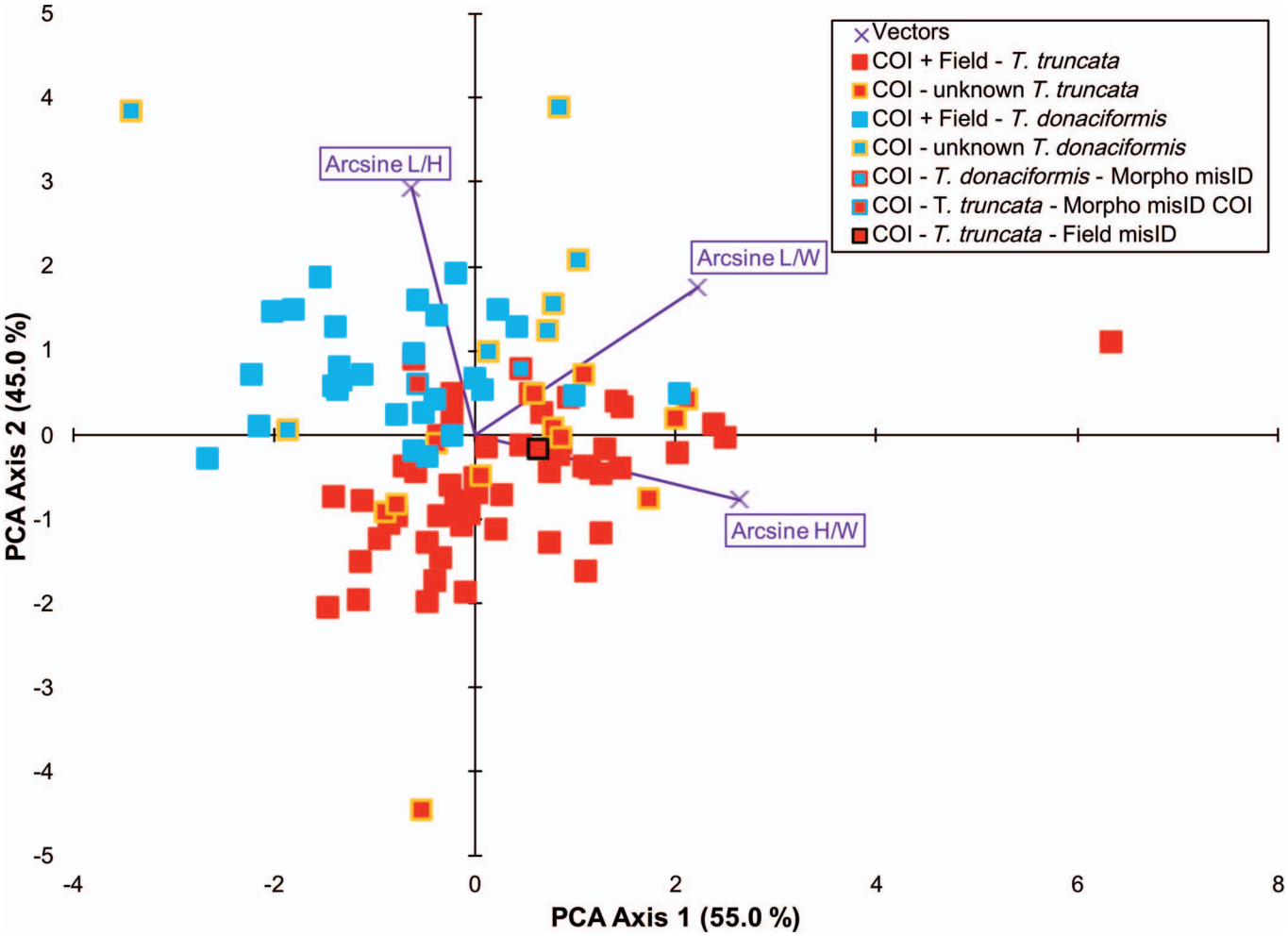

Figure 3. Principal components analysis (PCA) biplot for arcsine-transformed shell length-to-height (L/H), length-to-width (L/W), and height-to-width (H/W) ratios of field-collected *Truncilla* specimens. Species identifications were confirmed by comparing COI sequence data to sequences on GenBank.

specimens of uncertain species membership collected in the field, 20 of 21 were correctly identified based on their COI sequence. The misidentified specimen, from the Thames River (site TR-50 specimen no. 112), was a COI-identified *T. donaciformis* that was grouped with *T. truncata* using geometric morphometrics.

DISCUSSION

In identifying putative *Truncilla donaciformis* and *T. truncata* specimens, both geometric and traditional morphometrics were shown to be useful, providing a high degree of identification certainty when compared to definitive identifications based on DNA barcodes. Traditional morphometrics, paired with multivariate statistical analyses, were found to correctly identify 90% of the field-identified specimens and 90% of unknown specimens when compared to COI barcode identifications. Multivariate statistical analyses of landmark-based morphometric data provided greater identification accuracy with correct identifications of 99% of field- and museum-identified specimens and 95% of unknown specimens when compared to COI barcode identifications, although in both cases only a single specimen was incorrectly identified.

Shell morphology has long been used to differentiate and identify freshwater mussel species, with varying degrees of reliability (Watters et al. 2009). The utility and accuracy of shell morphology in that capacity has been assessed via mitochondrial DNA barcoding (Hebert et al. 2003; Baird and Sweeney 2011), and recent studies have upheld its merits in assigning individuals to species and groups (Inoue et al. 2013; Guarneri et al. 2014; Inoue et al. 2014; Barreto et al. 2016). However, because freshwater mussel morphology depends, at least partially, on environmental conditions and may vary considerably within species (Hornbach et al. 2010; Suzuki and Nagasawa 2013), care must be taken to systematically quantify the morphological characters being used to identify freshwater mussel specimens. In the sister species *T. donaciformis* and *T. truncata*, applying traditional and geometric morphometrics has provided quantifiable means of differentiating between the two. *Truncilla truncata* is described as being more triangular in shape, while *T. donaciformis* is more elongate following a continuum in the ratio of shell length to shell height (Watters

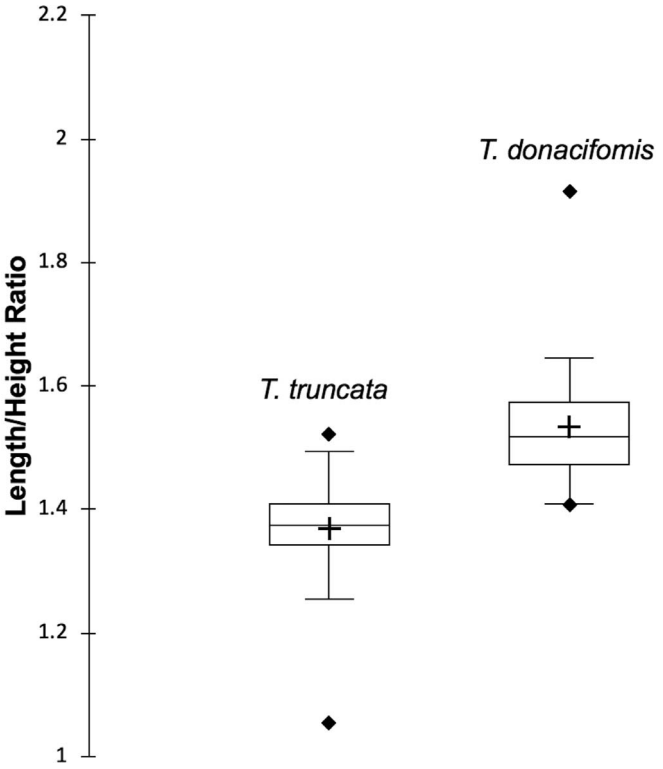

Figure 4. Box plot showing quartiles, means, and outliers of L/H ratios from field-collected *Truncilla* specimens.

et al. 2009). Length-to-height ratios >1.5 were 95% likely to be *T. donaciformis*, and ratios <1.4 were 95% likely to be *T. truncata*, but there was considerable overlap in the L/H ratio between *T. donaciformis* and *T. truncata* specimens. Additionally, the PCA identifies the shell H/W ratio as another important character in differentiating between the species, but this character is difficult to incorporate into field identifications.

Both morphometric techniques were able to differentiate between the two species with somewhat different reliability. Traditional morphometric data applied in a discriminant analysis was able to accurately differentiate between the two *Truncilla* species when compared to DNA barcodes, with an accuracy of 90%. Assignment tests of specimens using the landmark-based geometric morphometric dataset in the CVA provided improved accuracy over traditional morphometrics when compared to the DNA barcodes, with an accuracy of 99% among the specimens analyzed. The analysis of quantifiable morphological characters provided by these two morphometric techniques has the potential to provide researchers and mussel survey teams with greater confidence in assigning specimens to species compared to expert opinion based on qualitative assessment of characters. The general reliability of traditional morphometric techniques in differentiating between *T. truncata* and *T. donaciformis* may be sufficient for most specimens and studies, but we recommend using the more reliable geometric morphometric techniques whenever possible, especially for problematic specimens.

Traditional morphometrics using shell measurements, when applied appropriately, have the potential to provide researchers with a great deal more confidence in field identifications than qualitative assessments of shell characteristics (i.e., expert opinion). Conducted using measurements frequently taken in the field—length, width, and height—traditional morphometric data are relatively simple and cost-effective to analyze. The drawback to this ease is accuracy. Based on our findings, 10% of *Truncilla* specimens identified this way were inaccurately identified. This inaccuracy (i.e., false positives and false negatives) could have some consequences for species conservation efforts in terms of inaccurately estimating the distribution and status of the species, leading to misspent or unnecessary use of resources (Shea et al. 2011).

In contrast to traditional morphometric analyses, landmark-based geometric morphometrics provide a more robust dataset and yield greater accuracy in species identifications (Rohlf and Marcus 1993; Adams et al. 2004; Inoue et al. 2014). However, there is a cost for this accuracy; the methodology requires photos of each specimen, the digitization of landmarks onto those photos, the careful formatting of data, and the use of multivariate statistical analyses. These requirements add a great deal of time and, consequently, higher costs. However, those costs (in time and resources) might be negated by its increased reliability over traditional morphometric analyses; incorrect species identifications made by the latter could result in a waste of time and resources on populations that were erroneously assumed to exist. One relevant example of the implications of misidentification is in the Canadian status assessment of *T. donaciformis* (COSEWIC 2008), which cites historical records of *T. donaciformis* from Lyons Creek in the Welland River drainage, as well as a historical record from the Niagara River near the mouth of the Welland. Some of the specimens collected for the present study were taken from other locales in the Welland River and identified as possible *T. donaciformis*, but mtDNA barcoding and morphometric data both confirmed these as *T. truncata*. Without an examination of the shell specimens, our findings call into question whether *T. donaciformis* was ever really present in the Welland River drainage.

Perhaps the only way to identify a freshwater mussel specimen with near perfect certainty is with genetic techniques, but to do so for every specimen collected in a survey is costly and time-consuming, and it requires finding living animals. DNA barcoding techniques (Hebert et al. 2003) using COI and other mtDNA sequences have proven to be useful for identifying most unionoid species (Campbell et al. 2008; Boyer et al. 2011; Inoue et al. 2013, 2014; Keogh and Simons 2019), but there are some exceptions (i.e., within the genera *Elliptio* and *Pleurobema*; Inoue et al. 2018). Once a morphological database of specimens with confirmed identifications (i.e., using DNA barcodes) is established, geometric morphometric techniques have the potential to provide a relatively cost-effective and less technology-intensive alterna-

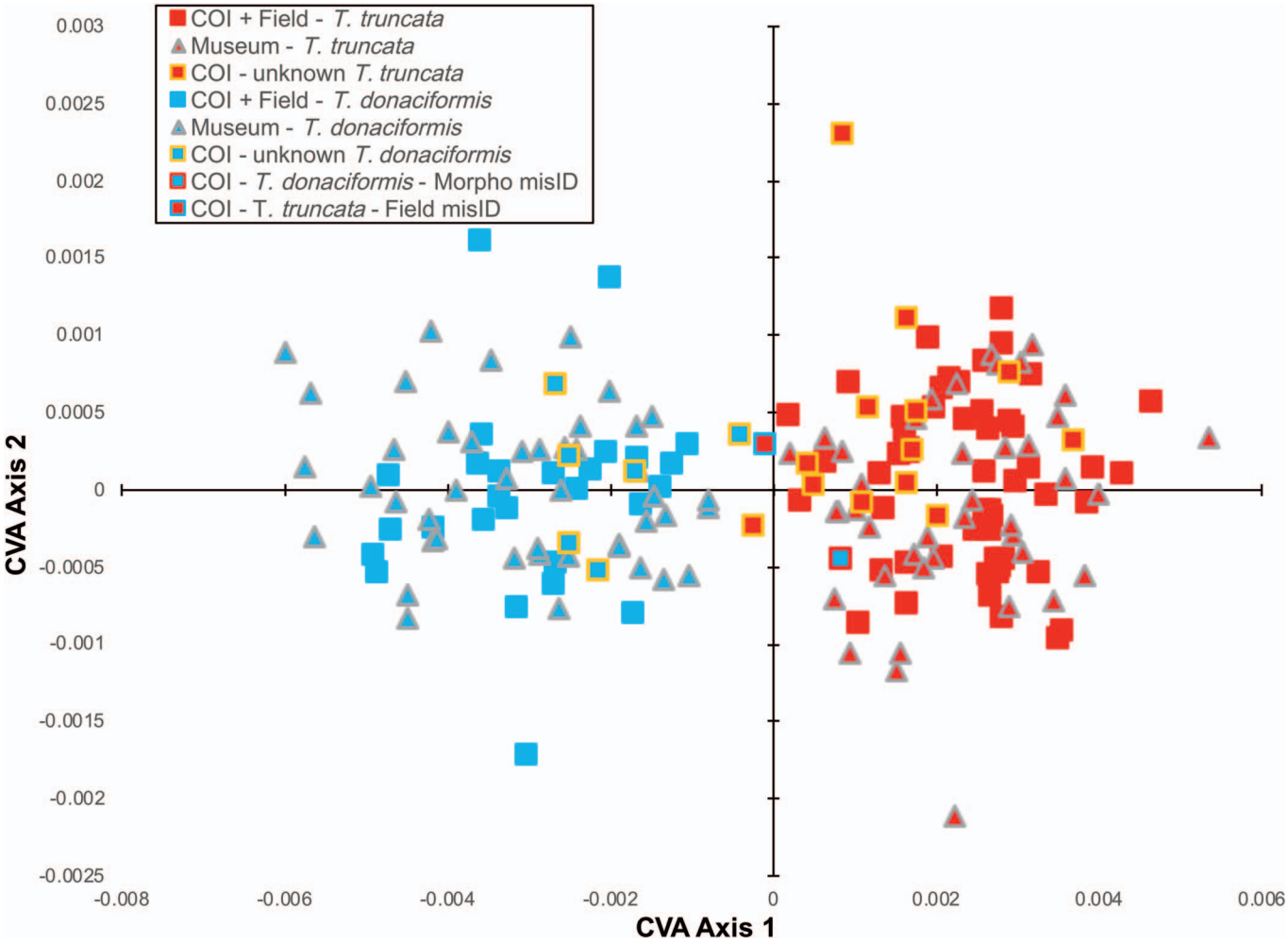

Figure 5. Canonical variates analysis biplot of *Truncilla* specimen shell shape using 20 landmarks. Species identifications were confirmed by comparing COI sequence data to sequences on GenBank or the identification of the museum specimens. The only significant differences in shape between the groups were found along CVA axis 1.

tive to DNA barcoding, with a high degree of accuracy that could be used with less reliance on taxonomic/genetic experts.

Misidentifying endangered *T. donaciformis* in Canada could have negative consequences for efforts directed at conservation of this endangered species, such as in the Welland River (COSEWIC 2008). We used traditional and geometric morphometric techniques, along with COI mtDNA sequencing, to confirm the presence of *T. donaciformis* in select Canadian watersheds presumed to be inhabited by the species. We can confirm definitively the presence of *T. donaciformis* in southwestern Ontario in the Thames River, but none of the specimens examined from the Ausable ($n = 3$), Sydenham ($n = 1$), or Welland rivers were found to be *T. donaciformis* using DNA barcoding or morphometric analyses. However, our sampling was by no means exhaustive, and *T. donaciformis* still may be present in these systems. No *Truncilla* specimens from the lower Grand River in Ontario, where *T. donaciformis* has recently been found alive (COSEWIC 2008), were collected in the field for this study, but Grand River shell specimens from museum collections (Table 2) did group with DNA barcode-confirmed specimens using landmark-based morphometric analyses, confirming that it was present historically. Museum specimens from Lake Erie grouped with the DNA barcode-confirmed specimens and verify the historical presence of *T. donaciformis* there.

In addition to definitively identifying unionids collected during field surveys in Ontario, our findings could prove useful for further investigations of *T. donaciformis* throughout its distribution. This study provides an accurate morphometric framework and dataset to be used in future surveys; our dataset is publicly available (DRYAD Link: https://doi.org/10.5061/dryad.rn8pk0p6m). Using a similar combination of morphometric and DNA barcoding techniques also could be useful for differentiating among other morphologically similar species. Examples include species in the notoriously difficult-to-identify genera *Pleurobema* and *Fusconaia* (Inoue et al. 2018), *Pyganodon* (Cyr et al. 2007), western North American *Anodonta* (Chong et al. 2008), and some members of the Lampsilini (Keogh and Simons 2019).

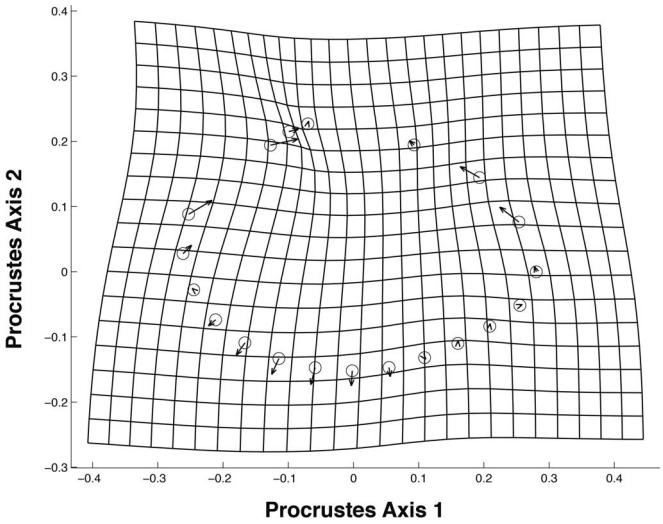

Figure 6. Deformation grid and vectors showing differences in Procrustes-superimposed shell shape along CVA axis 1 (Fig. 5). The differences in shape are denoted by the direction and relative magnitude of vectors and in the areas where the grid is most deformed from a square. The circles represent the mean shape of *T. donaciformis* specimens and the ends of the vectors represent the mean shape of *T. truncata* specimens.

ACKNOWLEDGMENTS

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REGULAR ARTICLE

MUSSEL COMMUNITY ASSESSMENT TOOL FOR THE UPPER MISSISSIPPI RIVER SYSTEM

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ABSTRACT

Upper Mississippi River (UMR) resource managers need a quantitative means of evaluating the health of mussel assemblages to measure effects of management and regulatory actions, assess restoration techniques, and inform regulatory tasks. Our objective was to create a mussel community assessment tool (MCAT), consisting of a suite of metrics and scoring criteria, to consistently compare the relative health of UMR mussel assemblages. We developed an initial MCAT using quantitative data from 25 sites and 10 metrics. Metrics fell in five broad groups: conservation status and environmental sensitivity, taxonomic composition, population processes, abundance, and diversity. Metric scoring categories were based on quartile analysis: 25% scoring as good, 50% scoring as fair, and 25% scoring as poor. Scores were meant to facilitate establishing management priorities and mitigation options for the conservation of mussels. Scoring categories assumed that a healthy mussel assemblage consists of species with a variety of reproductive and life-history strategies, a low percentage of tolerant species, and a high percentage of sensitive species; shows evidence of adequate recruitment, a variety of age classes, and low mortality; and has high abundance, species richness, and species and tribe evenness. Metrics were validated using a modified Delphi technique. MCAT metrics generally reflected the professional opinions of UMR resource managers and provided a consistent evaluation technique with uniform definitions that managers could use to evaluate mussel assemblages. Additional data sets scored a priori by UMR resource managers were used to further validate metrics, resulting in data from 33 sites spanning over 980 km of the UMR. Initial and revised MCAT scores were similar, indicating that data represent the range of mussel assemblages in the UMR. Mussel assemblages could be evaluated using individual metrics or a composite score to suit management purposes. With additional data, metrics could be calibrated on a local scale or applied to other river systems.

KEY WORDS: Mississippi River, mussel community health, Unionoida, freshwater mussels

INTRODUCTION

Native freshwater mussels (Order Unionida) are bioindicators of riverine ecosystem health because of their sensitivity to hydrophysical conditions, disturbance, and contamination, and their strong ecological ties to other components of aquatic communities and biotic and abiotic processes (Strayer et al. 2004, Vaughn 2010). Native freshwater mussels are ecologically significant because they transfer nutrients and energy from the water column to the sediments, stimulate production across trophic levels, stabilize substrates, provide habitat for other invertebrates and fish, and provide food for fish and mammals (Howard and Cuffey 2006, Vaughn 2017).

The Upper Mississippi River (UMR) historically harbored a diverse assemblage of native freshwater mussels (Van der Schalie and Van der Schalie 1950). Navigation pools (hereafter, pools) comprise the river reach between two adjacent dams, typically ranging from 20 to 40 km long and from 1 to 4 km wide. Freshwater mussel surveys in the UMR have documented 50 species; however, 10 of these species have been collected only as shell material in the last 40 yr, and 28 of the 40 extant species are federally listed or listed by bordering states as threatened or endangered (Dan Kelner, U.S. Army Corps of Engineers [USACE], 2020 oral

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communication). Mussel-assemblage composition in many areas of the UMR appears to have changed considerably from pre-European settlement times toward less-dense and less-species-rich assemblages dominated by contamination-tolerant habitat generalists (e.g., *Amblema plicata*, *Fusconaia flava*, Van der Schalie and Van der Schalie 1950, Theler 1987). These ongoing changes in abundance, species richness, and assemblage structure are driven by factors including human alteration of hydrology and hydrophysical habitat, contamination, exotic species, and past commercial harvest activities (Fuller 1980, Baker and Hornbach 2000). Perhaps most important, a series of 29 dams, constructed mostly in the 1930s for commercial navigation, dramatically altered habitat and hydrology.

Mussel conservation in the UMR is of great concern to the bordering states (Minnesota, Wisconsin, Illinois, Iowa, and Missouri) and federal agencies including the U.S. Fish and Wildlife Service (USFWS), the USACE, and the National Park Service (NPS). Natural resource managers in state and federal agencies expend considerable effort assessing the effects of management and regulatory actions (e.g., poolwide drawdowns, island construction) on mussels in the UMR system (defined as the UMR from Minneapolis, Minnesota to Cairo, Illinois; the Illinois Waterway from Chicago to Grafton, Illinois; and navigable tributaries). Natural resource managers in the UMR system need a quantitative means of evaluating the relative health of mussel assemblages to evaluate mussel resources, measure effects of management and regulatory actions, assess the efficacy of restoration techniques, and inform a variety of regulatory tasks.

Tools such as the Indices of Biotic Integrity exist for fish (e.g., Karr 1981) and macroinvertebrates (e.g., Blocksom and Johnson 2009), and they frequently are used to assess environmental conditions suitable for biota and to prioritize conservation actions. Metrics in fish and macroinvertebrate indices often include measures of sensitive and tolerant taxa, species richness and diversity, and taxonomic composition (Karr 1981, Lyons et al. 2001, Angradi et al. 2009, Blocksom and Johnson 2009). However, compared with freshwater mussels, most fish and invertebrates are short-lived and may respond more quickly to changes in environmental conditions, whereas mussels are likely to incorporate stressors over greater spatial and temporal extents (Newton et al. 2008). Moreover, assessment of mussel responses to stressors (e.g., degraded habitat, nutrient enrichment) is hindered because life-history traits and species-specific tolerances to contaminants and disturbances are largely unknown (Haag 2012, FMCS 2016). Our objective was to develop a mussel community assessment tool (MCAT) for natural resource managers to compare the relative health of mussel assemblages in the UMR. To meet this objective, we completed two phases: (1) creation of the MCAT through development of a suite of quantitative metrics and development of cut points using quartile analysis, and (2) validation of the MCAT through professional judgment and comparison with additional data from UMR resource managers. MCAT scores were developed to facilitate establishment of management priorities and mitigation options aligned to conservation goals.

METHODS

Phase 1

Criteria for data-set selection.—Data used to calculate metrics were from 25 sites within the UMR spanning 925 km from pools 2 to 26 (Figure 1, Table 1). Data sets came largely from Ecological Specialists, Inc. (a consulting firm specializing in freshwater mussel surveys) and from the USACE mussel database (USACE 2006). Data were collected either as part of long-term monitoring studies or for assessing potential effects of in-stream activity in support of permit applications under Section 404 of the Clean Water Act or Section 10 of the Rivers and Harbors Act. Most data were restricted to mussel beds within the UMR main stem, with few "nonbed" areas in the data sets. Thus, the inference from this study is largely limited to mussel beds. We used the mussel bed definition of Strayer et al. (2004): "aggregations of mussels where many or all of the species found co-occur at densities 10 to 100 times higher than those outside the bed."

To ensure consistency among data used to calculate metrics, we used only those data sets that had a sample size $\geq$20 0.25-m^2 quadrats, and only those samples with mussel-age data. All quantitative samples were collected by excavating the substrate within each 0.25-m^2 quadrat to a depth of $\leq$15 cm into either a 20-L bucket or bag with $\leq$6-mm mesh size. Each sample was rinsed through 6-mm and 12-mm mesh sieves, and live mussels and fresh-dead shells (shells with clean shiny nacre, Southwick and Loftus 2018) were separated from substrate and debris. We identified all live mussels and fresh-dead shells to species, and we measured most live mussels for length and age using external annuli counts. Although such counts may be less accurate than counts using internal annuli (Haag 2009), they can be done in the field, do not involve sacrificing animals, and are sufficiently accurate to identify younger ($\leq$5 yr old) and older ($\geq$15 yr old) mussels.

Data sets were within a spatial scale of $\geq$250 m^2, a spatial scale used for many management actions (e.g., island construction, dredging) and regulatory permit requests (e.g., Clean Water Act Section 404) in the UMR. Because *Dreissena polymorpha* has affected many UMR mussel beds, data sets were also all post-2000, after *D. polymorpha* became abundant in the UMR (circa 1995, Cope et al. 1997).

Metrics.—For the MCAT, we considered a suite of 46 candidate metrics often used by UMR resource managers to evaluate mussel assemblages (Table 2). Candidate metrics fell into five broad groups of ecological attributes: conservation status and environmental sensitivity, taxonomic composition, population processes, abundance, and diversity (Table 2). Metrics were computed from 25 data sets collected within the main-stem UMR using SAS (v.9.2, SAS Institute, Inc., NC,

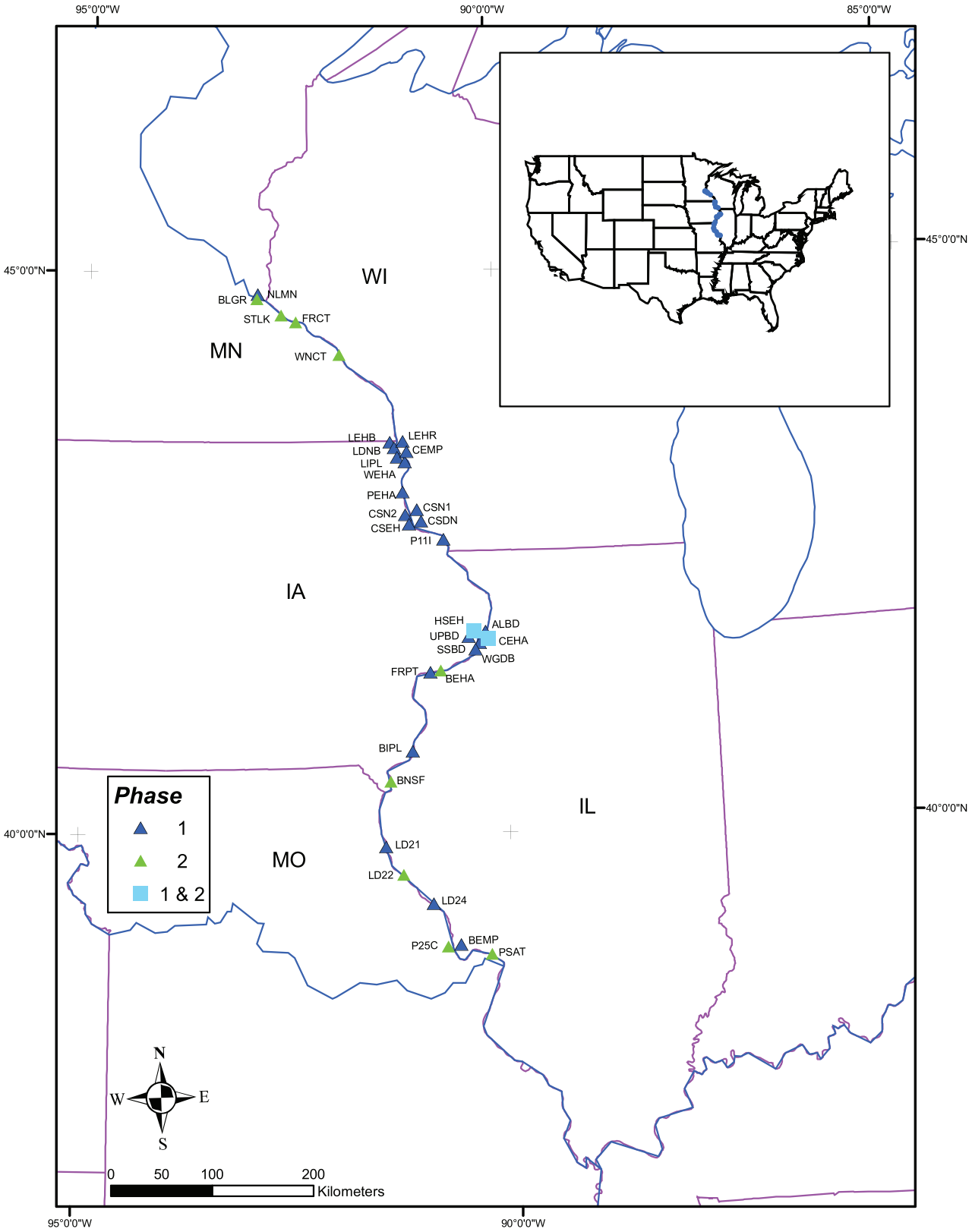

Figure 1. Sample locations of data sets used to develop (Phase 1) and validate (Phase 2) a mussel community assessment tool in the Upper Mississippi River (UMR). These sites spanned a range of 980 km, from navigation pools 2 to 26. Site names follow Table 1. The location of UMR sites within the USA is shown in blue in the inset.

Table 1. Site locations and description of data sets used in developing (Phase 1) and validating (Phase 2) the mussel community assessment tool in the Upper Mississippi River.

Site Name	Abbreviation	Phase	Navigation Pool	Year(s) Sampled	No. 0.25m^2 Quads	No. Live Mussels	Mean Density (no./m^2)	Data Source
Boulanger	BLGR	2	2	2011	118	71	2.4	USACE[a]
Nelson Mine	NLMN	1	2	2009	203	46	0.9	ESI
Sturgeon Lake	STLK	2	3	2014	90	474	21.1	MNDNR
4th Cut	FRCT	2	4	2014	80	236	11.8	MNDNR
West Newton Chute	WNCT	2	5	2012	196	372	7.6	MNDNR
Lansing downstream (bank)	LDNB	1	9	2005	33	9	1.1	ESI
Lansing EHA–bank	LEHB	1	9	2005	28	104	14.8	ESI
Lansing EHA–river	LEHR	1	9	2009	20	59	11.8	ESI
Lansing	LIPL	1	9	2005	65	71	1.1	ESI
Whiskey Rock EHA	WEHA	1	9	2009	123	167	5.6	USACE
Capoli Slough	CEMP	1	9	2009	188	132	3.0	USACE
Prairie du Chien EHA	PEHA	1	10	2007	351	418	4.8	USACE
Cassville downstream	CSDN	1	11	2002	357	1203	13.5	ESI
Cassville 1	CSN1	1	11	2006	74	193	10.4	ESI
Cassville 2	CSN2	1	11	2007	131	192	5.9	ESI
Cassville EHA	CSEH	1	11	2006	90	932	41.4	USACE
Pool 11 Islands	P11I	1	11	2005	179	458	10.2	USACE
Albany Bed	ALBD	1	14	2007–2008	180	252	5.6	ESI
Hanson's Slough EHA	HSEH	1	14	2007–2008	180	474	10.5	ESI
Hanson's Slough EHA	HSEH	2	14	2012	90	190	8.5	ESI
Up Bed	UPBD	1	14	2004–2008	488	1130	9.3	ESI
SS Bed	SSBD	1	14	2004–2008	487	535	4.4	ESI
Cordova EHA	CEHA	1	14	2004–2008	487	540	4.4	ESI
Cordova EHA	CEHA	2	14	2012	90	153	6.8	ESI
Woodwards Grove	WGBD	1	14	2007–2008	180	279	6.2	ESI
Buffalo EHA	BEHA	2	16	2014	150	218	5.8	USACE
Fairport	FRPT	1	16	2009	186	321	7.0	USACE
Burlington	BIPL	1	19	2008	131	115	3.5	ESI
BNSF	BNSF	2	19	2014	91	569	25.0	ESI
Lock and Dam 21	LD21	1	22	2009	40	48	4.8	ESI
Lock and Dam 22	LD22	1	22	2009	60	39	2.6	ESI
Lock and Dam 24	LD24	1	24	2006–2007	140	150	4.3	ESI
Pool 25 Chevrons	P25C	2	25	2012	100	43	1.7	USACE
Batchtown	BEMP	1	25	2003–2007	526	595	4.5	USACE
Piasa Toe	PSAT	2	26	2014	51	50	3.9	USACE

[a]USACE = U.S. Army Corps of Engineers; ESI = Ecological Specialists, Inc.; MNDNR = Minnesota Department of Natural Resources; EHA = essential habitat areas, defined as areas with density >10 unionids/m^2, *Lampsilis higginsii* constitutes at least 0.25% of the mussel assemblage, and the assemblage contains at least 15 other species with density >0.01/m^2 (USFWS 2004). All data are available from Heidi Dunn.

USA) and Primer-E (v.6, Plymouth Marine Laboratory, Plymouth, United Kingdom). Because data sets originally were collected for other purposes, some metrics could not be computed at all sites because of small sample size or questionable age data.

Our goal was to identify 10 metrics to serve as indices of the five broad groups, with one to three metrics in each group. First, we reduced the 46 metrics to 20 by prioritizing those that had sufficient distribution to discriminate among sites and that were less sensitive to sampling methods. We used Spearman correlation analyses to identify redundancy among metrics within broad groups. We sequentially discarded metrics having strong rank correlations ($P < 0.05$, $r > 0.6$) with other metrics in the same broad group. When selecting between candidates with strong correlations, we focused on those metrics that are least dependent on sample size or distribution.

Two metrics were selected within the broad group conservation status and environmental sensitivity. The percentage of species listed as threatened or endangered was selected as a measure of sensitive species. We calculated percent-listed species as the sum of individuals listed either

Table 2. List of candidate metrics explored for the mussel community assessment tool (MCAT) in the Upper Mississippi River (UMR). Broad MCAT metric groups are underlined. Metrics selected for use in the MCAT are bolded.

Conservation Status and Environmental Sensitivity
Percent species listed by federal or state agency[a]
Percent listed federally and bordering states[a]
Percent tolerant[b]
Taxonomic Composition
Percent *Lampsilis*
Percent *Quadrula*
Percent *Potamilus*
Percent *Amblema*
Percent *Truncilla*
Percent *Obliquaria*
Ratio *Amblema*:*Lampsilis*
Percent tribe Anodontini
Percent tribe Pleurobemini
Percent tribe Amblemini
Percent tribe Lampsilini
Percent tribe Quadrulini
Population Processes
Percent fresh-dead[c]
Percent mussels ≤5 yr
Percent mussels 6–10 yr
Percent mussels ≥10 years
Percent mussels ≥15 years
Percent mussels 11–20 yr
Percent mussels >20 yr
Mean age of mussels
Median age of mussels
Mean age of *Amblema* mussels
Median age of *Amblema* mussels
Mean age of *Lampsilis* mussels
Median age of *Lampsilis* mussels
Mean age of *Quadrula* mussels
Median age of *Quadrula* mussels
Abundance
Mean abundance
Variance of mean abundance
Standard deviation of mean abundance
Maximum total abundance
Median total abundance
Abundance at the 25th percentile
Abundance at the 75th percentile
Abundance at the 90th percentile
Diversity
Richness (number of species in the sample)
Pielou's evenness (J') at the species level[d]
Pielou's evenness (J') at the tribe level[d]
Shannon–Wiener diversity index (H')
Effective number of species (Hill's $N1$)
Expected number of species at a sample size of 39 mussels via rarefaction (ES_39)
Expected number of species at a sample size of 50 mussels via rarefaction (ES_50)
Expected number of species at a sample size of 100 mussels via rarefaction (ES_100)[e]
Expected number of species at a sample size of 200 mussels via rarefaction (ES_200)[e]

[a]Listed as a federal or state threatened or endangered species.

[b]Tolerant species in the UMR include *Amblema plicata* + *Quadrula quadrula* + *Obliquaria reflexa*.

[c]Percent fresh-dead = (no. of fresh-dead shells/[fresh-dead shells + live individuals]) × 100. Shells were considered fresh-dead if they had both valves attached, a flexible hinge line, and shiny nacre and if they were likely left by animals that died within the past few months.

[d]Standard-diversity indices were calculated using Primer E (v.6, Plymouth Marine Laboratory, United Kingdom).

[e]Rarefaction richness was calculated using EstimateS (v.9.1, Colwell 2013).

federally or by bordering states, divided by the total number of individuals, multiplied by 100. The percentage of tolerant species was selected as a measure of a disturbed assemblage. This metric was calculated as the sum of individuals of *A. plicata*, *Quadrula quadrula*, and *Obliquaria reflexa* (abundant species in UMR mussel beds), divided by the total number of individuals, multiplied by 100.

One metric was selected to represent taxonomic composition. The percent tribe Lampsilini measured the dominance or lack of dominance by one tribe. This was calculated as the number of individuals in the tribe Lampsilini, divided by the total number of individuals, multiplied by 100.

Three metrics were selected to represent population processes. The percentage of fresh-dead mussels was used as an index of recent mortality and was selected as a measure of recent stress on a mussel assemblage. We calculated percent fresh-dead mussels as the number of fresh-dead shells, divided by the number of fresh-dead and live individuals, multiplied by 100. The percentage of ≤5-yr-old mussels represents recruitment into an assemblage over the last 5 yr and was calculated as the number of individuals ≤5 yr old, divided by the total number of individuals, multiplied by 100. The percentage of ≥15-yr-old mussels is a measure of older individuals in the assemblage and was calculated as the number of individuals ≥15 yr old, divided by the total number of individuals, multiplied by 100.

The metric selected for abundance was abundance at the 75th percentile (Q75, 3rd quartile). Quartiles provide more information on the spread of data than simply the mean or median. This metric represents abundance in the densest part of a sample area and was calculated by ranking abundance from all samples and selecting the value that was exceeded in 25% of the samples.

Three metrics were selected to measure diversity: Pielou's evenness (J') at the species level, evenness at the tribe level, and rarefaction richness at 100 individuals (ES_100).

Evenness measures the distribution of species or tribes within an assemblage and was calculated as $J' = H'/H'_{max}$, where H' is Shannon diversity index and H'_{max} is the maximum possible H' (every species/tribe is equally represented):

$$H' = -\sum_{i=1}^{R} p_i \ln p_i,$$

where p_i is the proportion of individuals of the i^{th} species (Ludwig and Reynolds 1988).

Rarefaction richness at 100 individuals is the expected number of species with a sample size of 100 individuals estimated by rarefaction (Colwell et al. 2012). Because the number of species is highly related to the number of individuals collected, rarefaction richness allows richness to be compared on the basis of an equal number of individuals (Colwell et al. 2012). Rarefaction richness was calculated using EstimateS (v.9.1, Colwell 2013).

Frequency histograms of individual metric values were plotted, and a quartile analysis was used to determine critical values (hereafter referred to as cut points) for dividing data sets into scoring categories, with ~25% of sites scored in the poor category, 50% in the fair category, and 25% in the good category for each individual metric. Typically, the 2nd and 3rd quartiles were combined for the fair scoring category.

Phase 2

Metrics were validated in three ways. First, we compared (via agreement or proximate agreement) MCAT-derived scoring categories derived from a subset of the initial data sets with the professional judgment of UMR natural resource managers. Second, we compared cut points derived from Phase 1 with cut points derived from Phases 1 and 2 data sets combined. Third, we compared multivariate patterns among sites using principal components analysis (PCA) with the professional judgment of UMR natural-resource managers.

We used a modified Delphi technique (i.e., on the basis of expert opinion, Zuboy 1981, Mukherjee et al. 2015) to compare the MCAT metrics with the resource managers' professional judgment. Independent scores from UMR natural-resource managers were compared with scores derived from MCAT metrics for a subset of Phase 1 sites and for newly identified data sets. We organized a workshop in La Crosse, Wisconsin during February 2015 that was attended by 10 UMR natural-resource managers from state and federal agencies (Minnesota Department of Natural Resources, Wisconsin Department of Natural Resources, Iowa Department of Natural Resources, Missouri Department of Conservation, NPS, USACE, and USFWS). Before the workshop, we provided each participant with three high-scoring and three low-scoring data sets randomly selected from the Phase 1 data but not the Phase 1 metrics or scoring categories. The six data sets were Lansing essential habitat area (EHA)-river, Capoli Slough, Prairie du Chien EHA, Cassville downstream, Burlington, and Batchtown (Table 1).

For each data set, participants were provided raw data (i.e., species, length, and age in each sample), summarized data for the site (i.e., number and relative abundance of each species), and general site information (i.e., UMR pool number, sample size, coordinates, area sampled). We asked workshop participants to use their professional judgment and the method typically used by their agency to score each site as poor, fair, or good for the site overall (overall composite score), and for each broad metric group (i.e., conservation status and environmental sensitivity, taxonomic composition, population processes, abundance, and diversity; broad metric group composite score). At the workshop, we assembled scores from participants and discussed processes used to score test data sets, as well as strengths and weaknesses of each metric and potential alternative metrics.

To match the level of scoring done by workshop participants for each site, we computed Phase 1 broad metric group composite scores and an overall site composite score. Poor, fair, and good category scores for individual metrics were converted to a numerical score of 0–6 (poor = 0; fair = 3; good = 6) on the basis of Phase 1 cut points (Table 3). Broad metric group composite scores were computed as the mean of the component metrics, and the overall site composite score was computed as the mean of broad metric group composite scores. Composite scores 0–2.0 were considered poor, 2.1–4.0 were considered fair, and 4.1–6.0 were considered good.

We estimated the percent agreement between workshop-participant scores (i.e., professional judgment) and Phase 1 scores (number of participant scores agreeing with MCAT, divided by number of participants, multiplied by 100). We also estimated the proximate agreement between participant scores and the Phase 1 scores to evaluate differences across a broader continuum. For proximate agreement, the Phase 1 broad group composite scores and overall site composite scores were judged similar to a workshop-participant categorical score (good, fair, poor) if they fell within the trisected numerical scoring range for each scoring category expanded by 1.0 point (i.e., 0–3.0 = poor or nearly poor, 1.6–4.5 = fair or nearly fair, 3.1–6.0 = good or nearly good).

Workshop participants were asked to provide a list of additional data sets (Phase 2 data sets) that might be used in the Phase 2 validation effort. From these candidate data sets, we randomly selected four data sets from the upper pools (pools 1–8), three from the middle pools (pools 9–17), and three from the lower pools (pools 18–26) that met the criteria developed in Phase 1. Each contributor of a data set was asked to a priori score the overall mussel assemblage as poor, fair, or good. Individual MCAT metrics for these new sites were calculated as in Phase 1. However, four sites had an insufficient number of individuals to compute ES_100 by simple rarefaction; in these cases, we applied a sample-based Bernoulli product model (Colwell et al. 2012) to extrapolate species-richness curves. Workshop participant scores (i.e., a

Table 3. Final metrics and individual metric cut points within scoring categories (poor, fair, good) for Phase 1, Phase 2, and combined data of the mussel community assessment tool in the Upper Mississippi River.

	Poor			Fair			Good		
Broad Metric Group	Phase 1	Phase 2	Combined	Phase 1	Phase 2	Combined	Phase 1	Phase 2	Combined
Conservation status and environmental sensitivity									
% Listed species	<1.0	<0.4	<0.6	1.0–3.6	0.4–5.6	0.6–3.6	>3.6	>5.6	>3.6
% tolerant	>61.5	>59.6	>62.7	40.4–61.5	38.0–59.6	38.3–62.7	<40.4	<38.0	<38.3
Taxonomic composition									
% Tribe Lampsilini	<26.2 or >56.3	<13.1 or >59.2	<17.2 or >56.4	26.2–37.2 or >47.5–56.3	13.1–27.9 or >35.6–59.2	17.2–34.7 or >39.5–56.4	>37.2–47.5	>27.9–35.6	>34.7–39.5
Population processes									
% Fresh-dead	>7.8	>4.1	>6.7	3.6–7.8	2.0–4.1	2.6–6.7	<3.6	<2.0	<2.6
% ≤5 yr old	<22.9	<13.1	<19.8	22.9–48.8	13.1–49.9	19.8–49.3	>48.8	>49.9	>49.3
% ≥15 yr old	<0.6 or >8.7	<0.9 or >52.0	<0.8 or >16.0	0.6–1.9 or >5.3–8.7	0.9–3.8 or >5.9–52.0	0.8–2.4 or >5.6–16.0	>1.9–5.3	>3.8–5.9	>2.4–5.6
Abundance									
Abundance at 75th quartile (no./m^2)	<8	<8	<8	8–12	8–16	8–13	>12	>16	>13
Diversity									
Species evenness	<0.700	<0.651	<0.665	0.700–0.780	0.651–0.722	0.665–0.780	>0.780	>0.722	>0.780
Tribe evenness	<0.731	<0.719	<0.719	0.731–0.801	0.719–0.810	0.719–0.823	>0.801	>0.810	>0.823
ES_100	<13.4	<10.2	<11.5	13.4–15.8	10.2–12.9	11.5–15.7	>15.8	>12.9	>15.7

priori ranking of Phase 2 data sets) were compared with an overall composite score based on the MCAT.

Data from Phases 1 and 2 were combined (combined data sets) and used to generate combined-frequency histograms for each individual metric, and quartile cut points for scoring categories were updated. Combined-data cut points were compared with Phase 1 cut points to assess their validity. The percent change in cut points was calculated by dividing the difference between Phase 1 and combined-data cut-point values by the overall range of values for that metric.

We used PCA of the MCAT metric values in the combined data sets to explore multivariate patterns among sites. Only sites with a full suite of metrics were analyzed, and data from sites sampled over multiple years were averaged before analysis. Percentage data were arc-sine transformed, and all data were normalized to account for differences in measurement scales before correlation-based PCA ordination. Only PCA axes with eigenvalues >1 were interpreted.

RESULTS

We used 35 data sets from 33 sites meeting a priori criteria in the MCAT, 25 data sets from Phase 1 plus 10 data sets in Phase 2 (Table 1). Two of the Phase 2 data sets (Hanson's Slough EHA and Cordova EHA) were from sites also included in Phase 1, and data from both phases were combined, resulting in a combined 33 data sets. Data sets were collected between 2002 and 2014 in 14 pools spanning a range of ~ 980 km from Pool 2 just south of Minneapolis–St. Paul, Minnesota to Pool 26 just north of St. Louis, Missouri (Figure 1, Table 1). Average abundance across these data sets ranged from <1 to about 41 mussels/m^2. Phase 1 data sets included a concentration of sites in pools 9–14, lack of poor-quality sites, and lack of sites with a high percentage of ≤5-yr-old individuals. Phase 2 data sets included sites in the upper, middle, and lower pools, two poor-quality sites, and two sites with >75% individuals ≤5 yr old. Phase 2 data values also were well distributed among the Phase 1 values for all metrics, and the similarity in distribution of values and cut points with the additional data sets added credibility to the metric cut points developed in Phase 1 (Figures 2–5; Table 3).

Phase 1

Ten metrics deemed useful for assessing the relative health of mussel assemblages were identified: percent listed species, percent tolerant species, percent Lampsilini, percent fresh-dead, percent ≤5 yr old, percent ≥15 yr old, Q75 abundance, species evenness, tribe evenness, and ES_100 (Table 3). The percent-listed-species metric ranged from 0 to 12% (Figure 2A). The upper quartile of sites (good scoring category) had >3.6% listed species, and the lower quartile (poor scoring category) had <1.0% listed species. The percent-tolerant-species metric ranged from 11 to 83% (Figure 2B), with the good category having <40% tolerant species and the poor category having >62% tolerant. The percent-tribe-Lampsilini metric ranged from 11 to 78% (Figure 3A). The mid-quartile (>37 to 48%) was scored in the good category. The low (<26%) and high (>56%) extremes were scored in the poor category. The percent-fresh-dead metric ranged from 0 to 39%

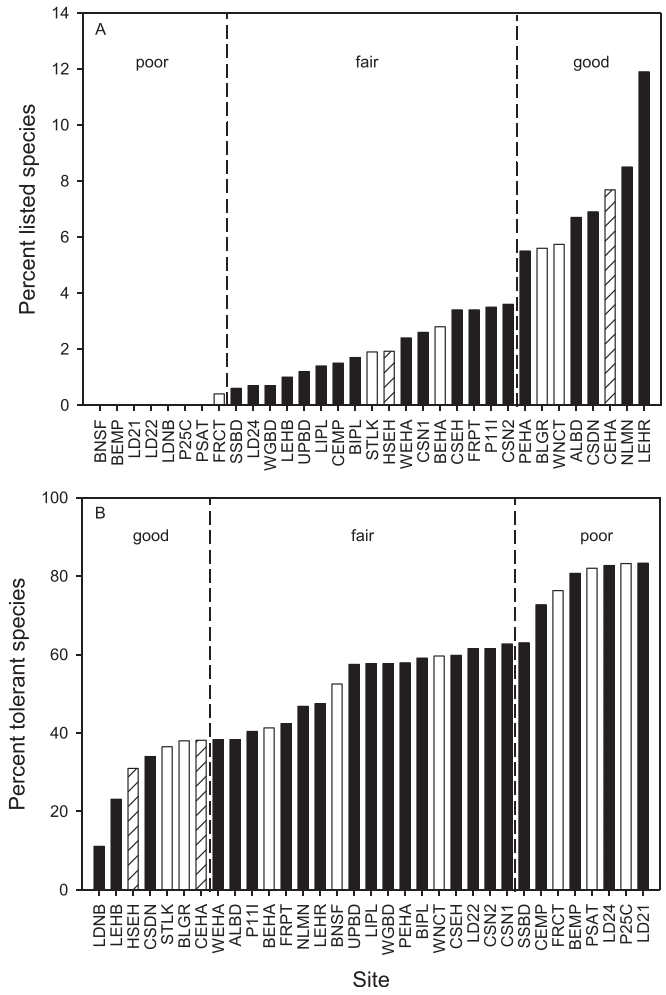

Figure 2. Frequency curves for the percent listed species (A) and the percent tolerant species (B) for the mussel community assessment tool in the Upper Mississippi River for the combined (Phase 1 and Phase 2) data sets. Site names follow Table 1. Dashed lines delineate good, fair, and poor scoring categories using combined data cut points. Data sets from Phase 1 are solid bars, Phase 2 open bars, both Phases hatched bars

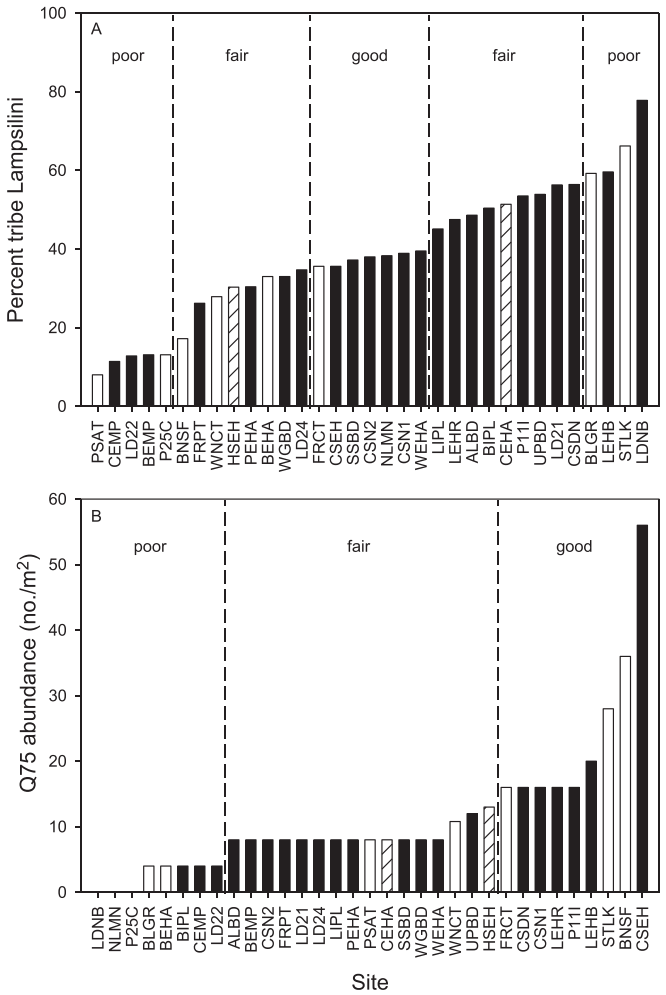

Figure 3. Frequency curves for the percent tribe Lampsilini (A) and the abundance at the 75th percentile (Q75 abundance; B) for the mussel community assessment tool in the Upper Mississippi River for the combined (Phase 1 and Phase 2) data sets. Site names follow Table 1. Dashed lines delineate good, fair, and poor scoring categories using combined data cut points. Data sets from Phase 1 are solid bars, Phase 2 open bars, both Phases hatched bars

(Figure 4A). The lower quartile of sites (good) had <4% freshly dead mussels and the upper quartile (poor) had >8%. The percent-≤5-yr-old metric ranged from 5 to 55% (Figure 4B). The upper (good) and lower quartile (poor) of sites had >49% and <23%, respectively. The percent-≥15-yr-old metric ranged from 0 to 19% (Figure 4C). The mid-quartile (>2 to 5%) was scored as good, and the extremes (<0.6 or >9%) were scored as poor. The Q75-abundance metric ranged from 0 to 56 mussels/m^2 (Figure 3B). The upper (good) and lower quartile (poor) of sites had >12/m^2 and <8/m^2, respectively. The species-evenness metric ranged from 0.5 to 0.9 (Figure 5A). The upper quartile (good) was >0.8 and the lower (poor) quartile was <0.7. The tribe-evenness metric ranged from 0.6 to 0.9 (Figure 5B). The upper (good) and lower (poor) quartiles were <0.8 and >0.7, respectively. The rarefaction-richness (ES_100) metric ranged from 8 to 18 (Figure 5C); the upper (good) and lower (poor) quartiles had >16 and <13 species, respectively.

Phase 2

In assessing data sets, workshop participants generally agreed with the MCAT (Table 4). Any disagreement stemmed from variable interpretations of the broad metric groups, agency priorities, and expectations of scoring categories based on personal experience with specific river reaches rather than evaluation of the data set. Phase 2 data sets also were scored similarly between workshop participants and the MCAT. Metric values for Phase 2 data sets were generally in the same range as the Phase 1 data sets (Figures 2–5). Scoring cut points based on combined data were similar to cut points based on Phase 1 (Table 3).

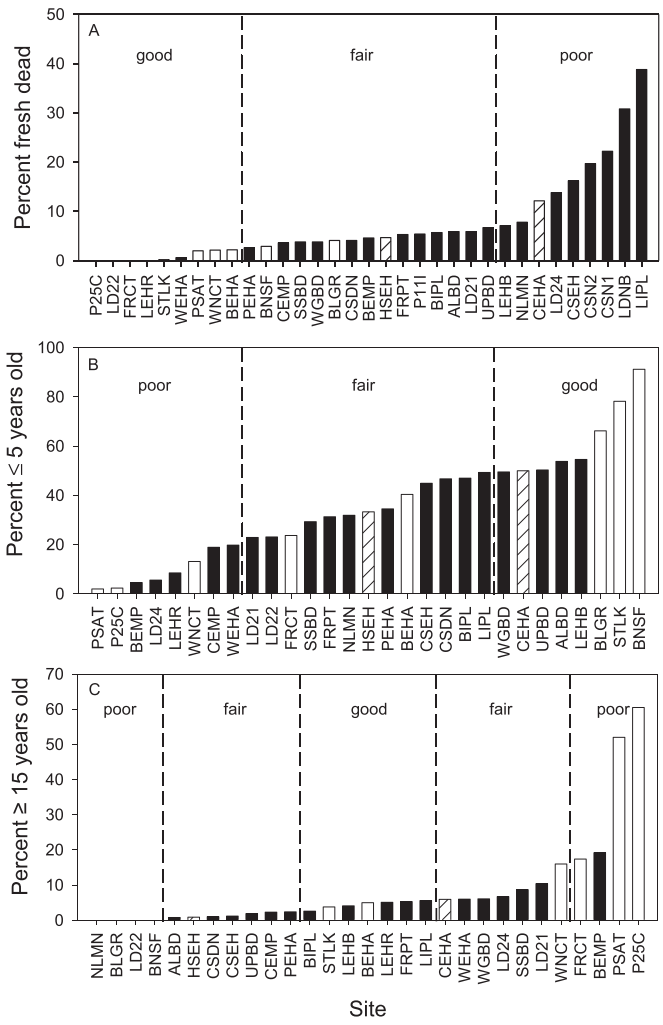

Figure 4. Frequency curves for the percent fresh-dead mussels (A), the percent ≤5 yr old (B), and the percent ≥15 yr old (C) for the mussel community assessment tool in the Upper Mississippi River for the combined (Phase 1 and Phase 2) data sets. Site names follow Table 1. Dashed lines delineate good, fair, and poor scoring categories using combined data cut points. Data sets from Phase 1 are solid bars, Phase 2 open bars, both Phases hatched bars

Conservation status and environmental sensitivity.—Workshop participants used variable criteria to evaluate this broad metric group, but their scores generally agreed with MCAT scores. Most participant scores and Phase 1 MCAT scores agreed across all sites (Table 4). The percentage of participants scoring the sites nearly the same as the Phase 1 MCAT was the highest for any broad metric group (80 to 90%). Some participants primarily evaluated this metric group on the basis of the presence or absence of threatened and endangered species, whereas others also considered abundance or age composition of listed species. Participants varied in focal species evaluated at sites, ranging from a focus on only federally listed species, to consideration of both state and federally listed species, to consideration of listed species as well as other species perceived to be rare in a given reach.

Taxonomic composition.—Workshop-participant scores agreed or nearly agreed with the Phase 1 MCAT scores at only two sites, and agreement with the MCAT was the lowest for any broad metric group (Table 4). Measures used by workshop participants to evaluate taxonomic composition ranged widely and included combinations of evenness, richness, presence or number of rare species, number of sensitive species, number of individuals in each tribe, presence of each tribe, richness in each tribe, and a balance between Amblemini and Lampsilini. This variability in interpretation likely contributed to the disagreement in scores.

Population processes.—Most workshop participants' scores and Phase 1 MCAT scores strongly agreed (≥50%) for four of the six sites (Table 4). Proximate agreement was >50% of participant scores for all sites. Criteria used by workshop participants to evaluate population processes generally focused on the age structure of the assemblage. Participants often used measures of recent recruitment, such as the total number of species represented by mussels ≤5 yr old and the percentage of the overall assemblage composed of mussels ≤5 yr old. Presence of older individuals also was considered. Despite the variability in defining population processes, workshop participant and MCAT scores were similar.

Abundance.—Most workshop participant scores agreed with the Phase 1 scores for five of the six sites (Table 4). Workshop participants generally scored abundance by considering the mussel density in samples with some spatial considerations. Some considered the overall density of the site compared with other sites within a given river reach, but others evaluated sites on the basis of whether samples indicated the presence of dense patches of mussels. However, these comparisons were generally qualitative (i.e., they did not compute any specific percentile of the distribution).

Diversity.—Workshop participant scores strongly agreed with each other for three of the six sites, but most disagreed with the Phase 1 MCAT scores for four of five sites (ES_100 could not be computed for Lansing EHA) (Table 4). Proximate agreement between participants and Phase 1 MCAT scores was ≥50% for three of the five sites. Participants used widely differing criteria when evaluating sites for diversity, including the percentage of the assemblage comprised of *A. plicata*, qualitative assessment of evenness, representation of all tribes, frequency of each species within samples, and degree of patchiness within a site. However, workshop-participant scores for diversity closely matched the individual-metric ES_100 scores (four of five sites), suggesting that participants may have relied on species richness rather than evenness measures when scoring sites.

Metric values and cut points.—Workshop participants agreed that Phase 1 cut points were within the range of their professional judgment. Most Phase 2 metric values fell within the range of Phase 1 metric values (Figures 2–5). Phase 2 data sets expanded the range of values slightly for four of the 10 metrics: percent Lampsilini, percent ≤5 yr old, percent ≥15 yr old, and ES_100. For most metrics, the scoring category cut points changed <10% between Phase 1 and the combined data

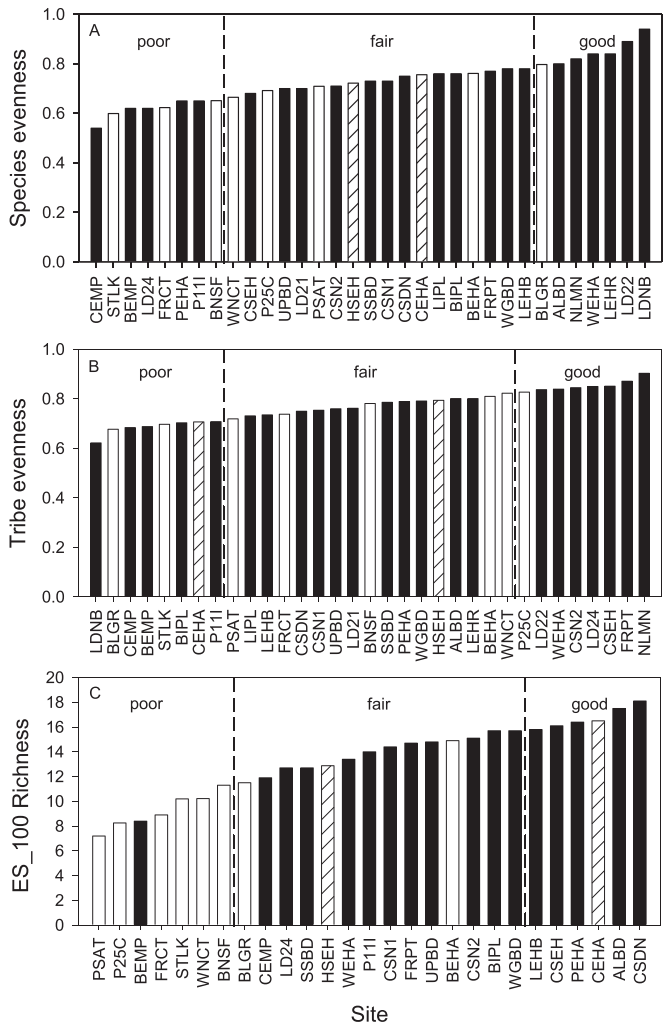

Figure 5. Frequency curves for species evenness (A), tribe evenness (B), and the expected species richness at a sample size of 100 mussels estimated by rarefaction (ES_100, C) for the mussel community assessment tool in the Upper Mississippi River for the combined (Phase 1 and Phase 2) data sets. Site names follow Table 1. Dashed lines delineate good, fair, and poor scoring categories using combined data cut points. Data sets from Phase 1 are solid bars, Phase 2 open bars, both Phases hatched bars

set (Table 3). The change was slightly greater (10 to 20%) for percent Lampsilini, percent $\geq$15 yr old, and ES_100.

Principal components analysis.—Generally, patterns resulting from the PCA reflected site scores by workshop participants (Figure 6). Sites ranked poor by participants plotted to the left, fair sites plotted in the middle, and good sites plotted to the right on the PCA axis 1. The first three principal components were interpreted (eigenvalue $>$1) and accounted for 45, 15, and 14% of the variation in the data, respectively. Metrics with high loadings in the first principal component were percent listed species, percent tolerant species, percent Lampsilini, percent $\geq$15 yr old, and ES_100. Metrics with high loadings in the second principal component were percent $\leq$5 yr old, Q75 abundance, species evenness, and tribe evenness. Metrics with high loadings in the third principal component were percent fresh-dead mussels, Q75 abundance, and tribe evenness.

DISCUSSION

Indices of biological integrity are typically motivated by a desire to improve understanding of the ecological condition of sites or systems, and to assess the degree of environmental impairment (Karr 1981, Lyons et al. 2001, Angradi et al. 2009, Blocksom and Johnson 2009). Biological integrity refers to a site or water body's ability to support and maintain a balanced, integrated, adaptive community of organisms having a species composition, diversity, and functional organization comparable with natural habitats (Karr and Dudley 1981). The MCAT is uniquely focused on evaluating the conservation value of native freshwater mussel assemblages, rather than extrapolating scores to overall site or system ecological health.

Few other assessment tools have been developed for mussel assemblages (but see Szafoni 2002). Illinois' Freshwater Mussel Classification Index contains four metrics (species richness, presence of intolerant species, total abundance, and percent live species with individuals $\leq$30 mm or $\leq$3 yr old) that are summed to one index value that is used to identify priority areas for mussel conservation (Szafoni 2002). The strength of the MCAT lies in (1) using quantitative data to derive robust cut points that can change as information accumulates, (2) providing resource managers with 10 well-defined metrics across five assemblage characteristics that can be used individually or aggregated to one overall index value, depending on conservation objectives, and (3) providing resource managers with a consistent, quantitative means of evaluating mussel assemblages to aid decision making.

Our analysis indicates that the most robust mussel assemblages in the examined data sets have the following characteristics: $>$4% listed species, $<$38% tolerant species, 35 to 40% Lampsilini, $\leq$3% fresh-dead mussels, $>$49% mussels $\leq$5 yr old, 2 to 6% mussels $\geq$15 yr old, $>$13 mussels/m^2 in the 75th quartile, a species evenness $>$0.8, a tribe evenness $>$0.8, and $>$16 species in a sample of 100 individuals (ES_100). These characteristics are similar to those reported by Haag and Warren (2010) in their assessment of the traits of self-sustaining mussel assemblages in southern streams. They characterized self-sustaining mussel assemblages as having high retention of historical species richness, a gradual increase in species richness from upstream to downstream, widespread occurrence of most species, low dominance and high evenness, high abundance of many species, and frequent recruitment for all species.

The 10 selected MCAT metrics appeared to adequately reflect how UMR resource managers evaluate mussel assemblages. Overall summary scores were similar between the MCAT and UMR resource managers participating in the workshop. Principal components analysis of sites based on the MCAT metrics also ranked sites similarly to workshop participants. Additionally, *Lampsilis higginsii* EHAs, which are sites that were selected by the *L. higginsii* recovery team as

Table 4. Mussel community assessment tool (MCAT) score and percentage of workshop participants independently scoring six sites as good, fair, or poor for broad MCAT metric groups. Individual metrics were converted to numerical values (poor = 0; fair = 3; good = 6). Broad metric group scores were computed as the mean numerical score of the individual metric scores. Proximate agreement is the percentage of participant scores similar to the MCAT score on a 0–6 numerical scale. Workshop participant and MCAT broad metric group scores were considered similar (proximate) if they fell within the trisected numerical scoring range expanded by 1.0 point (i.e., 0–3.0 = poor or nearly poor; 1.6–4.5 = fair or nearly fair; 3.1–6.0 = good or nearly good). Bolded participant ratings indicate the percent agreement with the Phase 1 MCAT score. Site-name descriptions as in Table 1.

		Participant Rating (%)			
Site Name	Phase 1 Score	Good	Fair	Poor	Proximate Agreement (%)
Conservation status and environmental sensitivity					
Lansing EHA[a]	Good	**60.0**	30.0	10.0	90.0
Capoli Slough	Poor	20.0	40.0	**40.0**	80.0
Prairie du Chien EHA[a]	Good	**70.0**	20.0	10.0	90.0
Cassville downstream	Good	**90.0**	10.0	0.0	90.0
Burlington	Fair	10.0	**60.0**	30.0	90.0
Batchtown	Poor	10.0	10.0	**80.0**	80.0
Taxonomic composition					
Lansing EHA[a]	Good	**40.0**	60.0	0.0	40.0
Capoli Slough	Poor	0.0	50.0	**50.0**	50.0
Prairie du Chien EHA[a]	Fair	70.0	**20.0**	10.0	30.0
Cassville downstream	Fair	80.0	**10.0**	10.0	20.0
Burlington	Fair	30.0	**70.0**	0.0	70.0
Batchtown	Poor	10.0	50.0	**40.0**	40.0
Population processes					
Lansing EHA[a]	Fair	22.2	**33.3**	44.4	55.6
Capoli Slough	Fair	0.0	**66.7**	33.3	66.7
Prairie du Chien EHA[a]	Good	**55.6**	22.2	22.2	77.8
Cassville downstream	Fair	77.8	**11.1**	11.1	88.9
Burlington	Good	**77.8**	22.2	0.0	100.0
Batchtown	Poor	0.0	33.3	**66.7**	100.0
Abundance					
Lansing EHA[a]	Good	**80.0**	20.0	0.0	80.0
Capoli Slough	Poor	0.0	50.0	**50.0**	50.0
Prairie du Chien EHA[a]	Fair	30.0	**60.0**	10.0	70.0
Cassville downstream	Good	**80.0**	20.0	0.0	80.0
Burlington	Poor	0.0	60.0	**40.0**	40.0
Batchtown	Fair	0.0	**70.0**	30.0	100.0
Diversity					
Lansing EHA[a]	.	20.0	70.0	10.0	.
Capoli Slough	Poor	10.0	50.0	**40.0**	40.0
Prairie du Chien EHA[a]	Fair	90.0	**10.0**	0.0	10.0
Cassville downstream	Fair	90.0	**10.0**	0.0	100.0
Burlington	Poor	30.0	50.0	**20.0**	70.0
Batchtown	Poor	10.0	40.0	**50.0**	50.0
Overall composite					
Lansing EHA[a]	.	55.6	44.4	0.0	.
Capoli Slough	Poor	0.0	66.7	**33.3**	100.0
Prairie du Chien EHA[a]	Fair	77.8	**22.2**	0.0	100.0
Cassville downstream	Good	**100.0**	0.0	0.0	100.0
Burlington	Fair	11.1	**88.9**	0.0	88.9
Batchtown	Poor	11.1	22.2	**66.7**	66.7

[a]Essential habitat areas (EHAs) are defined as areas with density >10 unionids/m^2, *Lampsilis higginsii* constitutes at least 0.25% of the mussel assemblage, and the assemblage contains at least 15 other species with density >0.01/m^2 (USFWS 2004).

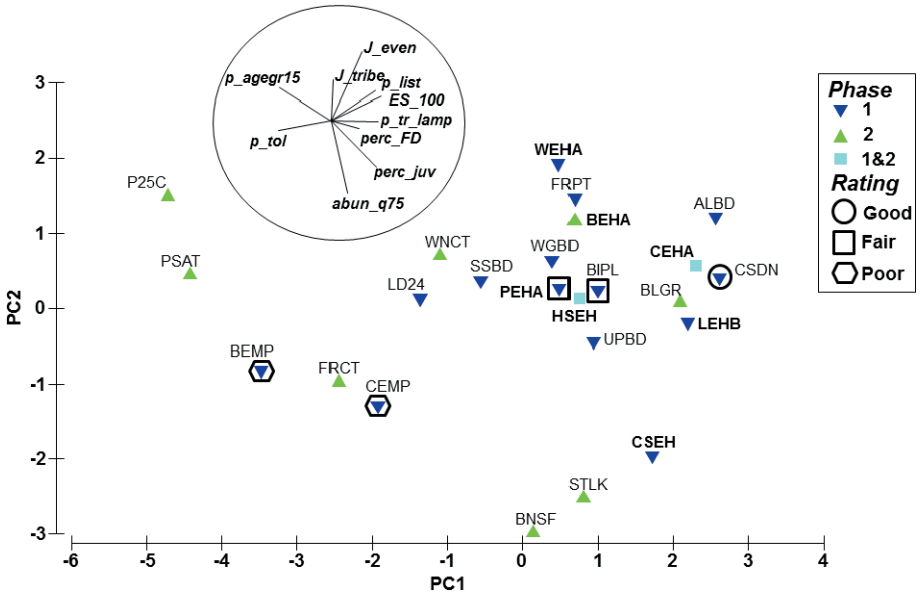

Figure 6. Principal components analysis (PCA) ordination of the mussel community assessment tool metric data for Phase 1 and Phase 2 sites. Only the first two axes (PC1 and PC2) are shown, which account for 60% of the total variance. Hollow symbols represent the predominant rating of test sites by workshop participants. Site names follow Table 1. Essential habitat areas (EHA) are bolded. EHAs are considered high-quality mussel assemblages, defined as areas with density >10 unionids/m^2, *Lampsilis higginsii* constituting at least 0.25% of the mussel assemblage, and the assemblage containing at least 15 other species with density >0.01/m^2 (USFWS 2004). The circular inset depicts the vector loadings for individual metrics: p_pool_list = percent listed species, p_pol_tol = percent tolerant, p_tr_lamp = percent tribe Lampsilini, perc_FD = percent fresh-dead, perc_juv = percent ≤5 yr old, p_agegr15 = percent ≥15 yr old, abun_q75 = abundance at the 75th percentile, J′_even = species evenness, J′ tribe = tribe evenness, and ES_100 = expected species richness at a sample size of 100 mussels estimated by rarefaction.

high-quality mussel assemblages (USFWS 2004), all plotted on the positive side of axis 1 in the PCA. Variation in scores by workshop participants stemmed largely from inconsistent group and individual metric definitions rather than from disagreements in cut points for scoring sites. Collectively, these findings indicate that the MCAT reflects the professional judgment of resource managers with respect to mussel assemblages in the UMR.

Metrics

Metrics of species sensitivity and environmental tolerance are often included in biotic indices (Karr 1981, Lyons et al. 2001, Angradi et al. 2009, Blocksom and Johnson 2009). Percent listed species was used as a surrogate for sensitive species, similar to the Illinois index described above. Although there has been considerable progress in evaluating the sensitivity of mussels to environmental contaminants, toxicity data are available for only a fraction of species (Cope et al. 2008, FMCS 2016). Listed species are those that state or federal regulatory agencies have determined are imperiled because of sensitivity to environmental conditions (e.g., physical disturbance, poor water quality) or because they are at the edge of their natural range (IL DNR 2020, MN DNR 2020). Higher percentages of these species in an assemblage indicate more pristine environmental conditions, likely reflecting a higher-quality assemblage.

Biological communities frequently show skewed species-abundance distributions, with a few numerically dominant species and many rare species (Kunte 2008). Species that dominate under degraded conditions are often the most tolerant (Karr 1981). Three species dominated mussel assemblages across the 980-km study reach: *A. plicata*, *O. reflexa*, and *Q. quadrula*. Dominance by a few species often indicates human effects or other stressors (Haag and Warren 2010). Stressors may affect many species simultaneously, causing decreases in rare species and subsequent increases in common species (Haag 2012). Although little information is available on tolerance of mussel species to impaired conditions, tolerant species are often more abundant in areas with silt accumulation, low velocity, and high temperature (Miller and Payne 1998, Spooner and Vaughn 2009, Bartsch et al. 2010).

A healthy assemblage should contain diverse behavioral and life-history traits, which often align with mussel tribes (Haag 2012). The percent-tribe-Lampsilini metric was selected to represent taxonomic composition for the MCAT. Twenty-one of the 50 species known from the UMR are in tribe Lampsilini (Graf and Cummings 2007), and 20 of the 21 Lampsilini are opportunistic or periodic species (Haag 2012). Opportunistic traits, such as rapid growth, early maturity, short life span, and high reproductive output enable a species to colonize a site rapidly and to persist in unpredictable environmental settings (Winemiller 2005, Haag 2012). For example, Randklev et al. (2019) found that opportunistic species, such as *Lampsilis* sp., were proportionally more

abundant in reaches where the adverse effects of dams were prominent. Thus, assemblages dominated by Lampsilini may indicate less-stable habitat.

Self-sustaining mussel assemblages should contain multiple size and age classes and have a recruitment rate that meets or exceeds the mortality rate (Haag and Warren 2010). The metrics percent fresh-dead, percent ≤5 yr old, and percent ≥15 yr old were selected for the MCAT as indices of population processes. The percent fresh-dead mussels in an assemblage can be used as a measure of recent mortality; in our analysis, <3% fresh-dead shells typically were observed in higher-quality mussel assemblages. For most mussel species, once maturity is reached, the mortality rate is low (Haag 2012). Mean estimated annual mortality of the three most common species in a reach of the UMR was 11% in *A. plicata*, 19% in *O. reflexa*, and 18% in *Cyclonaias pustulosa* (Newton et al. 2011). A high percentage of fresh-dead shells may indicate relatively recent mortality from a chronic or acute water-quality event, substrate deposition or scouring, high level of *D. polymorpha* infestation, disease, or other factors (Southwick and Loftus 2018).

Areas that contain both young and old mussels are likely to be areas of persistent mussel assemblages (Ries et al. 2016). The percent-≤5-yr-old metric represents recruitment into an assemblage over the last 5 yr and has been used commonly to describe recent recruitment in the UMR (e.g., Newton et al. 2011, Ries et al. 2019). Age at maturity varies from 0 to 11 yr old among species, but most species mature at ≤6-yr old and many mature between 2 and 4 yr old (Haag 2012). In our analysis, higher-quality mussel assemblages contained ∼50% ≤5-yr-olds. Similarly, the percentage of the population consisting of juveniles ≤5 yr old ranged from 40 to 62% across three reaches of the UMR (Newton et al. 2011).

Longevity of mussels also varies considerably among species, but generally ranges from 15 to 40 yr (Haag 2012). Low recruitment, coupled with a high percentage of older individuals, may indicate a nonreproducing assemblage due to conditions that are no longer suitable for recruitment (Haag 2012, Ries et al. 2016). Areas with many juveniles and few older individuals may indicate newly forming areas with suitable habitat (areas where juveniles are deposited by fish or by local hydraulic conditions) or ephemeral habitats (areas that may be destroyed by the next flood or drought, Ries et al. 2016). Recent observations in the UMR indicate that assemblages with >75% juveniles may represent a transient or new assemblage (H. Dunn, personal observation). Variation in life-history strategies are important to consider when interpreting age metrics.

Often, areas with locally high abundance are considered to be of higher quality relative to areas with low abundance (Szafoni 2002, USFWS 2004). The results of our workshop showed that most resource managers rely on mean abundance if quantitative data are available. However, mean abundance is sensitive to nonnormal distributions (e.g., skewness, outliers) and strongly affected by sampling design that may or may not account for spatial patterns of mussels or include various proportions of bed and nonbed areas. Thus, abundance at the 75th percentile may better reflect densities in the core of a mussel bed and should allow data sets containing at least part of a good mussel area to score higher. Given that mussels are distributed patchily across several scales (Ries et al. 2016, 2019), this metric should allow patches of high abundance to score higher.

Biological diversity is composed of two components: species richness and species evenness (Bock et al. 2007). The latter is an estimate of the dominance of an assemblage by a few species (Ludwig and Reynolds 1988). Several studies indicate that evenness is a useful metric in mussel-assemblage analyses (Haag and Warren 2010, Zigler et al. 2012, Hornbach et al. 2017). For example, Haag and Warren (2010) reported evenness values ranging from 0.82 to 0.88 across six high-quality mussel assemblages in the Sipsey River, Alabama. These values are similar to those reported in the MCAT (∼0.80) across high-quality sites. Thus, high evenness values are often a characteristic of robust mussel assemblages. Because the number of species and the number of individuals are highly correlated, observed richness is often a downward-biased estimate of true richness (Colwell et al. 2012). Rarefaction curves estimate the number of species that one would expect to find, on average, after x individuals are sampled (Gotelli and Colwell 2001). ES_100 accounts for the effect of sample size better than using raw species richness. This advantage is especially important when evaluating data from multiple sources, obtained for different purposes, and with differing sampling designs—as was done in the MCAT. Rarefaction curves are becoming more frequent in studies of mussel assemblages (e.g., Daniel and Brown 2013, Miller et al. 2017).

MCAT Application

A strength of the MCAT is in providing a series of consistent and quantitative metrics for managers to use when evaluating mussel assemblages. We view the MCAT as an important step toward developing a suite of useful metrics to assess the relative health of mussel assemblages in the UMR and elsewhere. However, the distribution of metrics and the decision points for scoring metrics need to be interpreted carefully because of limitations in the data. We attempted to apply reasonable decision points, but a sample size of 33 data sets is relatively small. Although we applied criteria to reduce sampling variability among sites and attempted to select metrics that were relatively insensitive to sampling design, concerns about sampling design cannot be dismissed.

We also recognize that our data represent a single snapshot of each site. Because some mussel species are long-lived, population and assemblage responses to environmental stressors might have substantial lag times that may complicate interpretation of metrics and their application in management decisions. Metrics can be improved adaptively by re-evaluating decision points or adding or replacing metrics as new data become available. For example, inclusion of metrics

describing functional guilds, such as thermal and reproductive guilds, may add considerable value to the MCAT once more species are categorized (Barnhart et al. 2008, Gates et al. 2015). Application of a standardized design for sampling mussels (see Newton et al. 2011) may improve the development of MCAT metrics. Last, metrics derived for the UMR may apply to other systems with modification and calibration. For example, a tolerant-species metric could consider those species having increased abundance over time or that overwhelmingly dominate mussel assemblages in a given river.

The creation of multiple metrics will provide more information to resource managers than a single composite score. For example, sites with high diversity but low density might have a high conservation priority in reaches depauperate in species. Conversely, sites with high density but low diversity might merit conservation importance if management goals prioritize specific ecosystem functions, such as water filtration. Preserving mussel assemblages with differing attributes may enhance the ecological integrity of rivers. Individual metrics may help managers identify potential problems. For example, although an assemblage may score "good" on most metrics, a "poor" recruitment score may be an early warning sign of a declining assemblage.

Any ecological model constructed for conservation purposes, such as the MCAT, can provide a common framework for assessing mussel assemblages and subsequent conservation decisions. More important, such frameworks can facilitate discussion of management decisions, especially when biologists or agencies disagree. Discussing the strengths and weaknesses of natural resource decisions using formalized models is often more beneficial than an ad-hoc approach and can lead to adaptive improvements to both the model and resultant decisions (Starfield et al. 1994).

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REGULAR ARTICLE

POPULATION GENETICS OF A COMMON FRESHWATER MUSSEL, *AMBLEMA PLICATA*, IN A SOUTHERN U.S. RIVER

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ABSTRACT

Myriad anthropogenic factors have led to substantial declines in North America's freshwater mussel populations over the last century. A greater understanding of mussel dispersal abilities, genetic structure, and effective population sizes is imperative to improve conservation strategies. This study used microsatellites to investigate genetic structure among mussel beds and estimate effective population sizes of a common North American mussel species, *Amblema plicata*, in the Little River, Oklahoma. We used five microsatellite loci to genotype 270 individuals from nine mussel beds distributed throughout the river and one of its tributaries, the Glover River. Our results indicate that subpopulations of *A. plicata* in the Little River are genetically similar. Upstream subpopulations had less genetic diversity than sites located downstream of the confluence of the Glover and Little rivers. Downstream subpopulations were primarily assigned to the same genetic group as upstream subpopulations, but they were admixed with a second genetic group. Low flows during droughts likely influenced the observed genetic structuring in *A. plicata* populations in the Little River. Additionally, downstream subpopulations may be admixed with a genetically distinct population of *A. plicata*, which may account for the increased genetic diversity. Estimates of effective population sizes (N_e) of large mussel beds were low compared to the total abundance (N) of *A. plicata*. While our data have limitations, they provide important information on the spatial scale at which conservation plans should focus and the population sizes that should be sustained through relocation and restocking programs.

KEY WORDS: Unionid mussels, genetic structure, effective population size, dams, fragmentation, dispersal

INTRODUCTION

Freshwater mussels (Bivalvia: Unionoida, hereafter "mussels") are a highly diverse and imperiled group of animals. With approximately 300 species, North America has the highest diversity of mussels. Roughly 70% of endemic species are considered threatened, vulnerable, endangered, or extinct (Lydeard et al. 2004), and even common species are decreasing in abundance (Anthony and Downing 2001; Vaughn et al. 2015). Declines in mussel populations can be attributed to a variety of factors, such as habitat destruction and fragmentation, introduction of invasive species, pollution, and commercial exploitation of shells for the pearl and pearl button industries (Lydeard et al. 2004; Strayer et al. 2004). Conserving the remaining mussel fauna is a priority, but without understanding the basic population biology of mussels, developing successful conservation plans may be impossible. One emerging conservation tool is the propagation and restocking of mussels (FMCS 2016). To use this tool successfully, we need to understand the spatial scale of genetic structuring and effective population sizes of mussel beds.

Mussels often occur in dense aggregations or beds that are separated by stretches of river with no or very few mussels (Strayer et al. 2004). Mussel larvae (glochidia) are obligate ectoparasites on fish, but adults are sedentary (Barnhart et al. 2008). Thus, gene flow among and within beds must occur either via movement of glochidia attached to host fish, or through the downstream drift of sperm, juvenile mussels, or glochidia before they are attached to fish (Schwalb et al. 2011; Ferguson et al. 2013; Irmscher and Vaughn 2015, 2018).

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Mussel beds that are connected through gene flow can be considered one large metapopulation with individual beds serving as subpopulations (Vaughn 2012). Thus, the overall genetic structure in mussels should be the sum of connectivity among subpopulations due to gene flow and the isolation of subpopulations due to dispersal barriers such as impoundments or stretches of unsuitable habitat (Galbraith et al. 2015).

Genetic structure can indicate the spatial scale at which gene flow is occurring. Habitat disturbances and fragmentation can influence the genetic structure of mussel beds by blocking gene flow among beds (Watters 1996; Strayer et al. 2004; Newton et al. 2008; Schwalb et al. 2011; Galbraith et al. 2015). Multiple studies have evaluated genetic structure in unionid mussels (Berg et al. 1998; Kelly and Rhymer 2005; Elderkin et al. 2006; Berg et al. 2007; Zanatta and Wilson 2011; Galbraith et al. 2015; Jones et al. 2015; Inoue and Berg 2017; Hoffman et al. 2018), but few studies have estimated effective populations sizes of mussel beds and compared these estimates to total bed populations (Inoue et al. 2015).

Our goal was to better understand connectivity between mussel beds and the effective population sizes of these beds. We used microsatellites to evaluate population genetic structure and effective population size in a common, widespread mussel species, the threeridge mussel (*Amblema plicata*), in a medium-sized south-central U.S. river known for its diverse and relatively healthy mussel and fish populations (Vaughn and Taylor 1999; Vaughn 2003; Matthews et al. 2005). The Little River is fragmented by both large and low-head dams (Vaughn and Taylor 1999; Allen et al. 2013), which might restrict gene flow and result in distinct genetic clusters of individuals upstream and downstream of these dams. Our objectives were to assess the population genetic structure of *A. plicata* in the Little River and estimate the effective population size of each sampled mussel bed.

METHODS

Study Area, Species, and Sampling

Amblema plicata is a common, wide-ranging mussel species found throughout central and eastern North America, and it is one of the most abundant species in the Little River (Vaughn and Taylor 1999). *Amblema plicata* have been estimated, based on growth rate, to reach sexual maturity at six years of age (Haag 2012). Male *A. plicata*, like all unionids, broadcast their sperm into the water column to fertilize females downstream (Haag 2012). After fertilization and larval development, female *A. plicata* release larval threads, which are mucus threads with attached glochidia, into the water column to infect host fish (Haag 2012). *Amblema plicata* is a host generalist but typically uses fish in the sunfish (Centrarchidae) and perch (Percidae) families (Freshwater Mussel Host Database 2017).

During the summers of 2015 and 2016, we collected *A. plicata* tissue samples from eight mussel beds in the Little River and one mussel bed in the Glover River, a tributary of the Little River, in southeast Oklahoma (Fig. 1). Three high-gradient sites were above the confluence of the Little and Glover rivers, while six low-gradient sites on the Little River were below the confluence of the rivers. The Little River is influenced by two large impoundments and two small low-head dams. Its main stem is impounded by Pine Creek Dam (constructed in 1969), while the Mountain Fork River, a major tributary of the Little River, is impounded by Broken Bow Dam (constructed in 1968), a hypolimnetic release dam (Vaughn and Taylor 1999; Matthews et al. 2005). Cold water from Broken Bow Dam has eliminated most mussels in the lower Mountain Fork River and the lower Little River below the confluence of these two rivers (Vaughn and Taylor 1999). The Glover River is unimpounded and enters the Little River approximately 30 km downstream of Pine Creek Dam. The two low-head dams are located on the main stem of the Little River, one between the outflow of Pine Creek Reservoir and the confluence with the Glover River, and the other between the Glover and Mountain Fork rivers' confluences with the Little River (Fig. 1).

Tissue samples from 30 individual *A. plicata* were collected from each site for a total of 270 samples. Tissue samples were collected from the first 30 individuals found within quadrats at large sites; if fewer than 30 individuals were collected within quadrats, then the remaining individuals were located through semiquantitative timed searches. At small mussel beds, tissue samples were collected randomly from 30 *A. plicata* individuals that were located during hour-long semiquantitative time searches. Five of the nine sites (sites: 1, 5, 6, 7, and 8) were large mussel beds (>50 m long). These sites were quantitatively sampled with quadrats (Vaughn et al. 1997). Twenty 0.25 m^2 quadrats were placed randomly along transects throughout the mussel bed and excavated to a depth of 15 cm. The density (mussels/m^2) of *A. plicata* was calculated using the quadrat data, and the total abundance of *A. plicata* in each large mussel bed was estimated by multiplying the density by the area of each bed. Semiquantitative timed searches (Vaughn et al. 1997) were conducted for an hour at four small mussel beds (<50 m long; sites: 2, 3, 4, and 9). Mussels were located tactilely or visually while snorkeling or scuba diving over the mussel bed. We collected approximately 20 mg of mantle tissue from each mussel and stored it in 95% ethanol. We also measured the shell length of every mussel sampled.

DNA Extraction and Genotyping

DNA was extracted using the methods of the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany). We successfully amplified nine microsatellite loci using primers developed for *Amblema neislerii*: Anec101, Anec114, Anec122, Anec126, Aned103, Aned104, Aned108, Aned126, and Aned140 (Díaz-Ferguson et al. 2011) and a variety of different polymerase-chain-reaction (PCR) conditions described in Table A1. Additionally, we tested two other loci that did not successfully amplify with PCR (Anec103 and

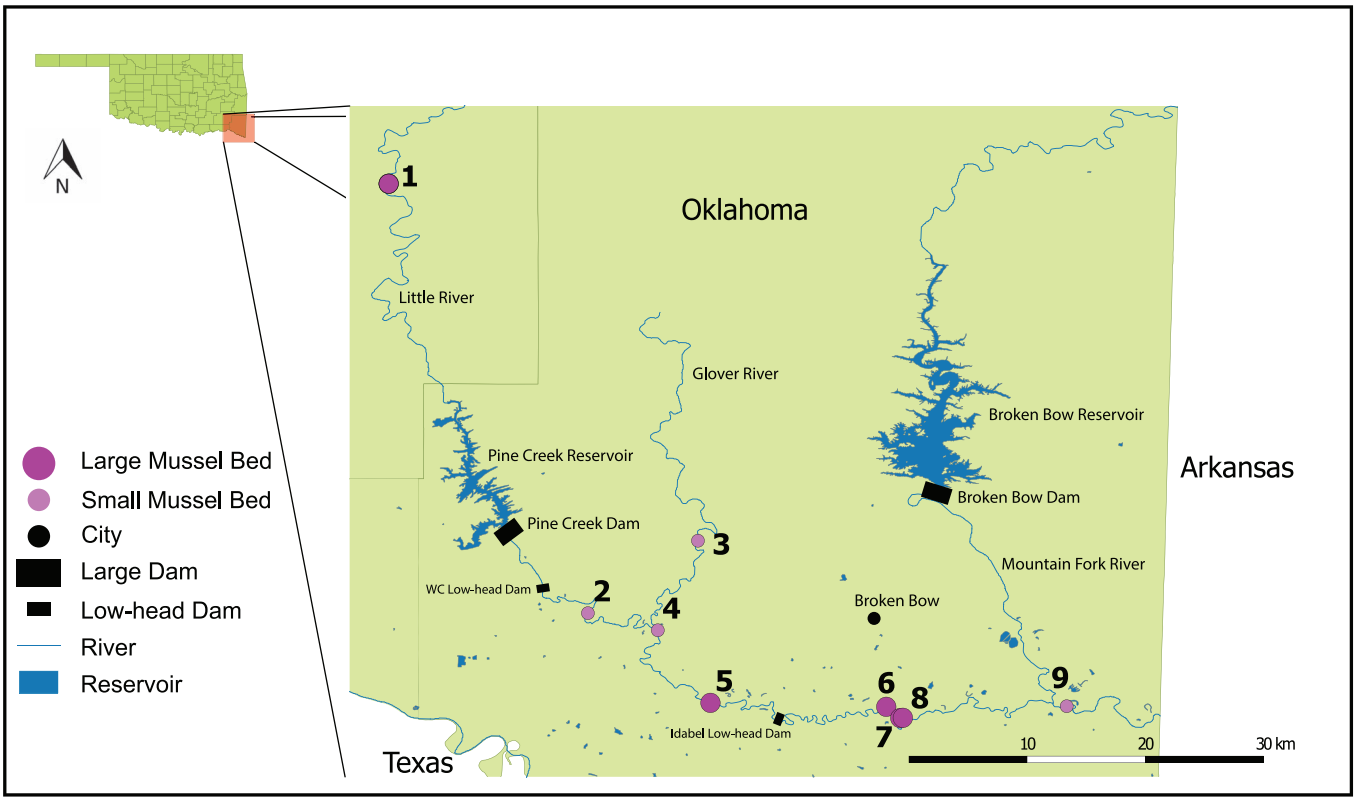

Figure 1. Sampling sites in the Little River drainage, Oklahoma, USA.

Aned132). We used the ILS600 red size standard (Promega, Madison, Wisconsin, USA) and analyzed the PCR products on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, California, USA). Alleles were binned and scored in GeneMapper V3.7 (Applied Biosystems).

Data Analysis

We used GenAlEx 6.502 to calculate expected (H_e) and observed (H_o) heterozygosities and number of alleles per locus and site (genetic diversity) and to check for deviations of genotype frequencies from Hardy-Weinberg expectation (HWE; Peakall and Smouse 2006, 2012). We checked for linkage disequilibrium within and among mussel beds with GENEPOP V4.6 (Raymond and Rousset 1995; Rousset 2008). We estimated null-allele frequencies with MICRO-CHECKER (van Oosterhout et al. 2004). Subpopulation pairwise F_{ST} was calculated with GENEPOP V4.6 (Raymond and Rousset 1995; Rousset 2008). We ran exact G-tests to check for significant allelic differentiation and genotypic differentiation (F_{ST} values) in GENEPOP V4.6. Due to the genetic similarity and geographic proximity (Table 3) of sites 6, 7, and 8, we combined them into a single subpopulation with 90 individuals before we tested for isolation-by-distance (IBD) and evaluated genetic structure. We ran paired Mantel tests with 9,999 permutations in GenAlEx 6.502 (Peakall and Smouse 2006, 2012) comparing pairwise F_{ST} and river distance between sites (measured with the path function in Google Earth Pro) to analyze IBD across all sites. Pairwise geographic distances from the combined subpopulation of sites 6–8 were taken from the midpoint of the distance between sites 6 and 8.

We used STRUCTURE (Version 2.3.4; Pritchard et al. 2000), which uses a Bayesian clustering method to assign individuals to populations and infer genetic structure, to evaluate population genetic structure. Across all runs, we assumed independent allele frequencies and allowed for individuals to be admixed among subpopulations. We used the sampling location of each individual as prior information to assist clustering (LOCPRIOR model). Each run had an initial burn-in period of 50,000 and was followed by an additional 100,000 Markov Chain Monte Carlo (MCMC) replicates. We ran 10 iterations for each value of K (genetic clusters). Values of K ranged from 1 to 7 and were based on the number of subpopulations. STRUCTURE HARVESTER (V0.6.94; Earl and vonHoldt 2012) was used to determine the number of genetic clusters (K) that best fit the data. The value of K that corresponds to the greatest $P(X|K)$ value was identified as the number of genetic clusters in the study area, which according to Evanno et al. (2005) is a good predictor of the real number of genetic clusters. We used the FullSearch algorithm in CLUMPP (Version 1.1.2; Jakobsson and Rosenberg 2007) to find the optimal alignment of 10 replicate cluster analyses from STRUCTURE with $K = 2$, and we used DISTRUCT (Version 1.1; Rosenberg 2004) to graphically

Table 1. Genetic diversity metrics per locus and site for *A. plicata* in the Little River. Figure 1 shows the locations of each site. n = number of individuals genotyped per locus. H_o = observed heterozygosity. H_e = expected heterozygosity. Bold font indicates departures from Hardy-Weinberg expectation. Values in the rightmost column are for means across all nine sites.

		Site									
Locus	Metric	1	2	3	4	5	6	7	8	9	All
Anec101	n	30	30	30	30	30	30	30	30	30	270
	No. of alleles	15	6	11	7	8	16	13	10	11	27
	H_o	**0.80**	**0.33**	**0.50**	**0.20**	**0.40**	**0.53**	**0.43**	**0.33**	**0.37**	0.43
	H_e	0.85	0.70	0.80	0.77	0.78	0.88	0.85	0.79	0.81	0.80
Anec114	n	30	30	30	30	30	30	30	30	30	270
	No. of alleles	6	9	10	13	12	11	12	9	11	16
	H_o	0.80	0.90	0.87	0.93	0.87	0.90	0.77	0.90	0.83	0.86
	H_e	0.78	0.84	0.82	0.87	0.89	0.85	0.88	0.85	0.86	0.85
Anec122	n	30	30	30	30	30	30	30	30	30	270
	No. of alleles	2	2	2	2	2	3	3	2	3	4
	H_o	0.50	0.27	0.10	0.47	0.23	**0.30**	0.30	0.30	0.27	0.30
	H_e	0.47	0.28	0.10	0.44	0.34	0.35	0.26	0.30	0.24	0.31
Anec126	n	30	30	30	30	30	30	30	29	30	269
	No. of alleles	12	20	20	20	24	19	24	19	22	31
	H_o	0.90	1.00	**0.83**	0.90	0.87	**0.80**	**0.90**	0.83	0.97	0.89
	H_e	0.38	0.92	0.91	0.93	0.94	0.93	0.94	0.93	0.95	0.92
Aned103	n	28	30	30	30	30	29	28	26	27	258
	No. of alleles	4	7	7	8	7	7	6	5	5	12
	H_o	**0.21**	0.30	**0.37**	**0.37**	**0.10**	**0.28**	**0.32**	**0.19**	**0.22**	0.26
	H_e	0.6	0.55	0.68	0.7	0.76	0.73	0.65	0.73	0.59	0.66
Aned104	n	30	30	30	30	30	30	30	30	30	270
	No. of alleles	8	12	8	12	14	13	15	12	11	19
	H_o	**0.57**	**0.33**	**0.37**	**0.43**	**0.57**	**0.50**	**0.37**	**0.53**	**0.27**	0.44
	H_e	0.78	0.87	0.82	0.87	0.89	0.90	0.92	0.83	0.88	0.86
Aned108	n	30	30	30	30	30	30	30	30	30	270
	No. of alleles	13	16	18	15	20	20	16	26	25	29
	H_o	**0.57**	**0.57**	**0.63**	**0.63**	0.67	**0.60**	**0.47**	0.80	0.80	0.64
	H_e	0.80	0.89	0.89	0.86	0.90	0.94	0.90	0.94	0.94	0.90
Aned126	n	30	30	30	30	30	30	30	30	30	270
	No. of alleles	8	11	6	11	11	13	14	14	13	18
	H_o	0.43	0.70	0.23	0.53	0.63	**0.67**	**0.70**	**0.63**	0.77	0.59
	H_e	0.38	0.60	0.22	0.59	0.60	0.68	0.68	0.68	0.75	0.57
Aned140	n	30	30	30	30	30	30	30	30	30	270
	No. of alleles	7	9	7	10	11	14	12	10	13	16
	H_o	0.87	**0.70**	0.63	**0.60**	0.83	0.90	**0.70**	0.80	0.73	0.75
	H_e	0.71	0.76	0.73	0.82	0.83	0.87	0.84	0.85	0.80	0.80
Mean	n	29.78	30	30	30	30	29.89	29.78	29.44	29.67	269
	No. of alleles	8.33	10.22	9.89	10.89	12.11	12.89	12.78	11.89	12.67	19.11
	H_o	0.63	0.57	0.50	0.56	0.57	0.61	0.55	0.59	0.58	0.57
	H_e	0.70	0.71	0.66	0.76	0.77	0.79	0.77	0.77	0.76	0.74

represent the individual assignment scores of all 270 individuals across the seven subpopulations. Upstream beds (sites 1–3) and downstream beds (4–9) were grouped together to form two populations and checked for evidence of recent population declines using a Wilcoxon test in BOTTLENECK (Version 1.2.02; Cornuet and Luikart 1996) under the infinite-allele and two-phase models with 1,000 iterations.

We used NeEstimator (Version 2.01; Waples and Do 2008; Do et al. 2014) to calculate the effective population size (N_e) of each mussel bed using the linkage disequilibrium (LD) method with a critical value of 0.05. We calculated the proportion of reproductively active *A. plicata* individuals (N_e/N) in each large mussel bed by dividing the effective population size by the total abundance.

Table 2. Null allele frequencies per locus across the nine sites. Negative null allele frequencies indicate a heterozygote excess at a given locus and site. Bold font indicates the presence of null alleles at a given locus and site due to a significant excess of homozygotes ($P < 0.05$), which is calculated using Fisher's combined probability test.

	Site								
Locus	1	2	3	4	5	6	7	8	9
Anec101	0.03	**0.26**	**0.19**	**0.35**	**0.24**	**0.19**	**0.25**	**0.27**	**0.27**
Anec114	−0.01	−0.04	−0.03	−0.04	0.02	−0.03	0.06	−0.04	0.02
Anec122	−0.03	0.02	−0.05	−0.03	0.13	0.06	−0.16	0.00	−0.14
Anec126	−0.01	−0.05	0.04	0.02	0.04	**0.07**	0.02	**0.06**	−0.01
Aned103	**0.29**	**0.20**	**0.22**	**0.23**	**0.41**	**0.29**	**0.23**	**0.34**	**0.28**
Aned104	**0.14**	**0.30**	**0.28**	**0.25**	**0.18**	**0.22**	**0.30**	**0.18**	**0.34**
Aned108	**0.14**	**0.18**	**0.14**	**0.13**	**0.13**	**0.18**	**0.24**	**0.08**	**0.08**
Aned126	−0.22	−0.14	−0.12	0.07	−0.03	0.00	−0.01	0.04	−0.03
Aned140	−0.12	0.04	0.06	**0.14**	0.00	−0.03	**0.08**	0.02	0.03

RESULTS

We genotyped 30 *A. plicata* individuals from each bed for a total of 270 individuals. The number of alleles ranged from four to 31 across loci and beds (Table 1). Genetic diversity was higher across the four downstream subpopulations (mean number of alleles per locus [±SE] = 18.33 ± 2.78) than at the three upstream subpopulations (14.11 ± 2.53). Mean observed heterozygosities ranged from 0.50 to 0.63 and mean expected heterozygosities ranged from 0.66 to 0.79 among sites (Table 1). Because deviations from HWE due to heterozygote deficiencies occurred at six or more sites for four loci (Anec101, Aned103, Aned104, and Aned108), and these loci also had null alleles at high frequencies (Table 2), they were not included in subsequent analyses. The remaining five loci deviated from HWE at three or fewer sites with null alleles present at low frequencies at two or fewer sites. We found no evidence of large allele dropout or scoring errors due to stuttering. There was no evidence of linkage disequilibrium between loci across all subpopulations. Linkage disequilibrium between two or fewer loci was detected within six sites. Microsatellite genotypes of all 270 individuals can be obtained by contacting the authors.

Pairwise F_{ST} values ranged from –0.0035 to 0.0596, with significant ($F_{ST} \neq 0$, df = 10, $P < 0.05$) allelic and genotypic differentiation at 13 of the 21 subpopulation pairs, while pairwise geographic distances between sites ranged from 12.40 km to 155.80 km (Table 3). The paired Mantel test did not find a significant relationship between genetic (F_{ST}) and geographic (river km) distance within the Little River ($R = 0.51$, $P = 0.09$), suggesting a lack of isolation-by-distance. Although there was significant ($F_{ST} \neq 0$) genotypic differentiation between upstream (1–3) and downstream (4–7) subpopulations ($F_{ST} = 0.0102$, df = 10, $P < 0.001$), analysis of population genetic structure revealed a single genetic cluster ($K = 1$) among the seven *A. plicata* subpopulations. Downstream subpopulations exhibited almost no genetic structure among sites, and they are genetically similar to upstream subpopulations (Fig. 2). Individual-based population assignment scores indicated that downstream subpopulations had a higher degree of admixture between two genetic groups (blue and orange); however, both the upstream and downstream subpopulations were overwhelmingly assigned to the same genetic group (blue). There was no evidence of recent population bottlenecks in upstream and downstream populations.

Large mussel beds had *A. plicata* densities ranging from 3.5 to 9.4 individuals/m^2 and estimated total abundance ranging from 1,572 to 61,776. Small beds had catch per unit effort ranging from 34 to 82 individuals/hr (Table 4). The effective population sizes of the five large beds ranged from 81.4 (95% CI: 28.7–Infinite) at site 7 to Infinite at sites 5 and 6; and the effective population sizes of small beds ranged from

Table 3. Pairwise geographic distances in river kilometers and F_{ST} values above and below the diagonal, respectively. Bold font indicates significant genetic differentiation between subpopulations ($F_{ST} \neq 0$, df = 10, $P < 0.05$).

Subpopulation	1	2	3	4	5	6–8	9
1		74.56	102.66	86.96	101.96	133.01	155.80
2	**0.0332**		28.10	12.40	27.40	58.45	81.23
3	**0.0596**	**0.0351**		15.94	30.94	61.99	84.77
4	**0.0162**	0.0066	**0.0462**		15.00	46.05	68.83
5	**0.0239**	0.0040	**0.0291**	−0.0016		31.05	53.83
6–8	**0.0324**	0.0041	**0.0318**	**0.0061**	−0.0035		22.79
9	**0.0478**	0.0112	**0.0377**	**0.0114**	−0.0028	0.0006	

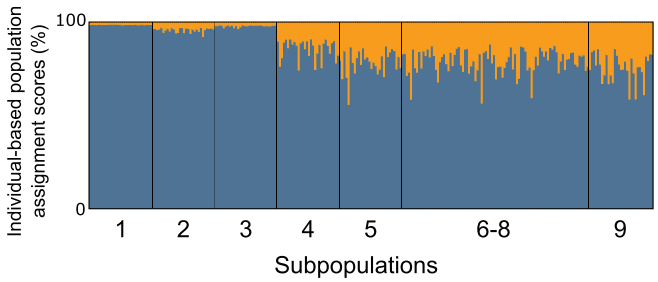

Figure 2. Bayesian clustering analysis of *A. plicata* genetic structure among seven subpopulations in the Little River, with $K = 2$.

100.8 (95% CI: 21.9–Infinite) to Infinite (Table 4). The mean proportion of individuals breeding (N_e/N) among three large mussel beds was 0.071, but values were highly variable.

DISCUSSION

Our results indicate that upstream and downstream subpopulations of *A. plicata* in the Little River are genetically similar. The three subpopulations upstream of the confluence of the Glover and Little rivers were overwhelmingly assigned to one genetic group, while the four downstream subpopulations were admixed between two genetic groups with 70–80% of each individual-based population assignment score being assigned to the same genetic group as upstream subpopulations. While most studies have found little or no within-river genetic structuring of mussel populations where stream flows are consistent and unfragmented (Szumowski et al. 2012; Ferguson et al. 2013; Galbraith et al. 2015; Jones et al. 2015; Inoue and Berg 2017), our study and one other have found genetic structuring at microsatellite loci among mussel populations within a stream. Inoue et al. (2015) found genetic differences in upstream and downstream populations of *Popenaias popeii* in the Black River of New Mexico. Although we found no evidence of a recent population bottleneck at the upstream sites, the low mean number of alleles across loci at upstream sites suggests that these sites have lower genetic diversity than downstream sites. Two possible mechanisms underlying these differences in genetic diversity are (1) restricted gene flow between upstream and downstream subpopulations during periods of drought and (2) loss of rare alleles by genetic drift associated with decreases in upstream population sizes during droughts. Droughts are common and cyclical in the south-central USA and have been shown to lead to decreases in mussel population sizes in rivers in this region (Galbraith et al. 2010; Atkinson et al. 2014; Vaughn et al. 2015). Upstream reaches of the Little River are smaller and higher gradient than downstream reaches, and during droughts riffle areas can become dry or very shallow (Vaughn 2003; Matthews et al. 2005). Gene flow among mussel beds requires sufficient flow for the movement of fish hosts, juveniles, sperm, and/or unattached glochidia. Irmscher and Vaughn (2015) found that the movement of fish hosts in the Little River was restricted during droughts. Thus, low-water conditions during droughts may restrict gene flow between upstream and downstream populations or decrease upstream population sizes and exacerbate genetic drift. We did not observe genetic structuring among sites in the lower river (below the confluence with the Glover River), and this is likely because there is sufficient gene flow among these sites. Downstream sites were more genetically diverse than upstream sites, which may be due to admixture from another genetically distinct population of *A. plicata* from further downstream.

Although the Little River is fragmented by both small low-head dams and large dams, as well as the reservoirs formed by them, we did not see evidence of interrupted gene flow, but this could be due to the long generation times of mussels. Pine Creek Dam (constructed in 1969) impounds the river itself and thus impedes host-fish dispersal between beds upstream and downstream of the dam. Broken Bow Dam (constructed in 1968) is a hypolimnetic release dam on a major tributary of the Little River, the Mountain Fork River. Cold water constantly flowing into the Little River via the Mountain Fork has caused significant declines in mussel abundance downstream from the release (Vaughn and Taylor 1999), along with preventing host-fish dispersal. Finally, small low-head dams on the Little River

Table 4. Demographic metrics for *A. plicata* at each site. Area, density, total number of *A. plicata* individuals, and proportion of individuals breeding were estimated only for large beds where quantitative sampling using quadrats was completed. Small beds are indicated with an asterisk by the site number; relative abundance (as CPUE = catch per unit effort) was measured for these. N = total number of individuals. N_e = effective population size. N_e/N = proportion of individuals breeding. Negative N_e values can be explained by sampling error and interpreted as an infinite N_e (Do et al. 2014).

Site	Area (m^2)	Density (mussels/m^2)/CPUE (mussels/hr)	N	N_e (95% CI)	N_e/N
1	449	3.5	1,572	327.9 (22.0–Infinite)	0.209
2*	—	75	—	119.6 (28.4–Infinite)	—
3*	—	41	—	100.8 (21.9–Infinite)	—
4*	—	82	—	−610.8 (49.7–Infinite)	—
5	2,598	3.6	9,353	−223.5 (67.7–Infinite)	—
6	4,949	4.0	19,796	−219.3 (79.9–Infinite)	—
7	4,949	9.4	46,060	81.4 (28.7–Infinite)	0.002
8	7,020	8.8	61,776	168.7 (34.9–Infinite)	0.003
9*	—	34	—	157.2 (38.3–Infinite)	—

main stem also may restrict fish movement. However, we did not find distinct genetic clusters upstream and downstream of any of the dams; rather, we found that sites upstream and downstream of Pine Creek Dam (sites 1–3) assigned to the same genetic group. These populations are now isolated, but we likely did not see the genetic signal yet because the time of isolation is relatively short given the long generation times of mussels. Many mussel species, including *A. plicata*, are long-lived organisms with long generation times (Haag and Rypel 2010). In a study of growth and longevity of mussels in southeast Oklahoma using dendrochronological techniques, maximum ages of adult *A. plicata* from three rivers ranged from 53 to 79 years old (Sansom et al. 2016). Thus, there have likely not been enough generations for differentiation to occur upstream and downstream of large dams through the loss of alleles due to genetic drift. Low-head dams may not completely block gene flow because the Little River experiences frequent high flows (Matthews et al. 2005) and fish hosts may be able to freely migrate over them during floods. Other studies also have failed to show the isolating effects of dams on genetic structure in mussels, again, likely a consequence of the long generation times of mussels (Kelly and Rhymer 2005; Szumowksi et al. 2012).

Few studies have compared the effective population sizes of mussel beds to the estimated total population size (N_e/N). Mean estimates of N_e/N ranged from 0.10 to 0.11 from 192 published estimates across 102 species (Frankham 1995). We found that estimates of N_e for *A. plicata* were small relative to N estimated by quantitatively sampling mussels. Proportions of breeding mussels in the three large beds where N was estimated and the estimated N_e was not infinite, were highly variable, ranging from 0.002 to 0.209 (mean $N_e/N = 0.071$). Other broadcast-spawning species have widely variable N_e/N ratios. A freshwater mussel species (*Popenaias popeii*) endemic to the Rio Grande drainage in the United States and Mexico had an N_e/N ratio of 0.12 in the Black River in New Mexico (Inoue et al. 2015). The estimated N_e/N ratio for Pacific oysters (*Crassostrea gigas*) was less than 10^{-6} (Hedgecock et al. 1992). Another broadcast-spawning species, sea bass (*Atractoscion nobilis*), had N_e/N ratios ranging from 0.27 to 0.40 (Bartley et al. 1992). Our results are corroborated by other studies of mussels that have found relatively low values of N_e. For *Lampsilis cariosa* from three river drainages in Maine, N_e ranged from 150 to 1,850 individuals across nine sites (Kelly and Rhymer 2005), while *Lampsilis cardium* exhibited N_e from 1.5 to 205.8 individuals across eight sites in Ohio (Ferguson et al. 2013). The effective population size of *Quadrula fragosa* in the St. Croix River was estimated to be roughly 150 individuals (Roe 2010). Estimates of effective population sizes are more informative when compared to total population sizes. Restocking programs should be designed to ensure that N is sufficiently large to lead to values of N_e that are high enough to offset the effects of genetic drift on target mussel populations.

This study contributes to our understanding of the population genetics of a common mussel species, but there are limitations to the results. While only nine of 11 loci were successfully amplified with PCR, an additional four loci consistently deviated from HWE due to heterozygote deficiencies, likely due to high frequencies of null alleles. Additional loci would provide more resolution when evaluating genetic structure and estimating effective population sizes. Furthermore, to date, all microsatellite studies of *A. plicata* genetic structure have used primers developed for *A. neislerii* (Díaz-Ferguson et al. 2011). Primers developed specifically for *A. plicata* may amplify more successfully.

This study provides important information on the genetic structure and effective population size of a common mussel species, which can be used to help manage and conserve not only common species but rare ones as well. Galbraith et al. (2015) found that mussel genetic structure did not vary as a function of rarity. Because sampling for common species is less time-intensive and thus less expensive than sampling for rare ones, common species could be used as surrogates for rare species when attempting to understand the population genetic structure of mussels.

We found that *A. plicata* subpopulations within a large extent (156 km) of the Little River were genetically similar, but genetic structuring was present within this reach and is likely influenced by flow conditions and possibly admixture of downstream subpopulations with a genetically distinct subpopulation. Although our data have limitations, our results provide useful information on the spatial scale at which conservation plans should focus and the population sizes that should be sustained through relocation and restocking programs. In stretches of river with genetically similar beds, individuals could be translocated from healthy beds to beds that are declining (Galbraith et al. 2015). Additionally, managers could use individuals from stable beds to propagate mussels for stocking into beds that are suffering from declines. Restocking programs should be designed to ensure that total population sizes are high enough to lead to effective population sizes high enough to offset the effects of genetic drift. For any mussel conservation program to be successful, there must be high-quality, unfragmented habitat into which to translocate or restock mussels. An understanding of the mussel population genetics of a region is important before implementing conservation strategies, including propagation and relocation.

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Appendix A: PCR Reaction Mixes and Conditions

Table A1. PCR reaction mixes and conditions for all nine loci. [a]James Cureton, University of Oklahoma, personal communication; [b]Galbraith et al. (2015).

Locus	PCR Reaction Mix Per Sample	PCR Conditions
Anec101[a]	6.25 μl GoTaq® Green Master Mix (Promega)	[a]30 s at 94°C
	1.50 μl labeled primer (10 μM, forward)	4 cycles of [10 s at 94°C, 20 s at 58°C, 90 s at 72°C]
	1.50 μl unlabeled primer (10 μM, reverse)	4 cycles of [10 s at 94°C, 20 s at 56°C, 90 s at 72°C]
	1.75 μl ddH_2O	4 cycles of [10 s at 94°C, 20 s at 54°C, 90 s at 72°C]
	1.50 μl DNA	15 cycles of [10 s at 94°C, 20 s at 52°C, 90 s at 72°C]
Anec122[a]	6.25 μl GoTaq® Green Master Mix (Promega)	15 cycles of [10 s at 94°C, 20 s at 50°C, 90 s at 72°C]
	1.00 μl labeled primer (10 μM, forward)	10 min at 72°C
	1.00 μl unlabeled primer (10 μM, reverse)	
	1.75 μl ddH_2O	
	1.50 μl DNA	
Aned104[a]	6.25 μl GoTaq® Green Master Mix (Promega)	
	0.75 μl labeled primer (10 μM, forward)	
	0.75 μl unlabeled primer (10 μM, reverse)	
	1.75 μl ddH_2O	
	1.50 μl DNA	
Aned126[a]	6.25 μl GoTaq® Green Master Mix (Promega)	
	0.40 μl labeled primer (10 μM, forward)	
	0.40 μl unlabeled primer (10 μM, reverse)	
	1.75 μl ddH_2O	
	1.00 μl DNA	
Aned140[a]	6.25 μl GoTaq® Green Master Mix (Promega)	
	1.00 μl labeled primer (10 μM, forward)	
	1.00 μl unlabeled primer (10 μM, reverse)	
	1.75 μl ddH_2O	
	1.50 μl DNA	
Aned103[b]	6.25 μl GoTaq® Green Master Mix (Promega)	[b]10 min at 94°C
	0.75 μl labeled primer (10 μM, forward)	35 cycles of [45 s at 94°C, 60 s at 60°C, 60 s at 72°C]
	0.75 μl unlabeled primer (10 μM, reverse)	7 min at 72°C
	1.75 μl ddH_2O	
	1.50 μl DNA	
Aned108[b]	6.25 μl GoTaq® Green Master Mix (Promega)	[b]10 min at 94°C
	1.50 μl labeled primer (10 μM, forward)	35 cycles of [45 s at 94°C, 60 s at 54°C, 60 s at 72°C]
	1.50 μl unlabeled primer (10 μM, reverse)	7 min at 72°C
	1.75 μl ddH_2O	
	1.50 μl DNA	
Anec114[b]	0.25 μl TopTaq™ DNA Polymerase (Qiagen)	[b]10 min at 94°C
	1.00 μl 10× buffer	35 cycles of [45 s at 94°C, 60 s at 51°C, 60 s at 72°C]
	0.80 μl 800 μM dNTPs	7 min at 72°C
	0.33 μl labeled primer (1 μM, forward)	
	0.33 μl unlabeled primer (1 μM, reverse)	
	5.29 μl ddH_2O	
	2.00 μl DNA	
Anec126[b]	0.25 μl TopTaq™ DNA Polymerase (Qiagen)	[b]10 min at 94°C
	1.00 μl 10× buffer	35 cycles of [45 s at 94°C, 60 s at 48°C, 60 s at 72°C]
	0.80 μl 800 μM dNTPs	7 min at 72°C
	0.33 μl labeled primer (1 μM, forward)	
	0.33 μl unlabeled primer (1 μM, reverse)	
	5.29 μl ddH_2O	
	2.00 μl DNA	

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