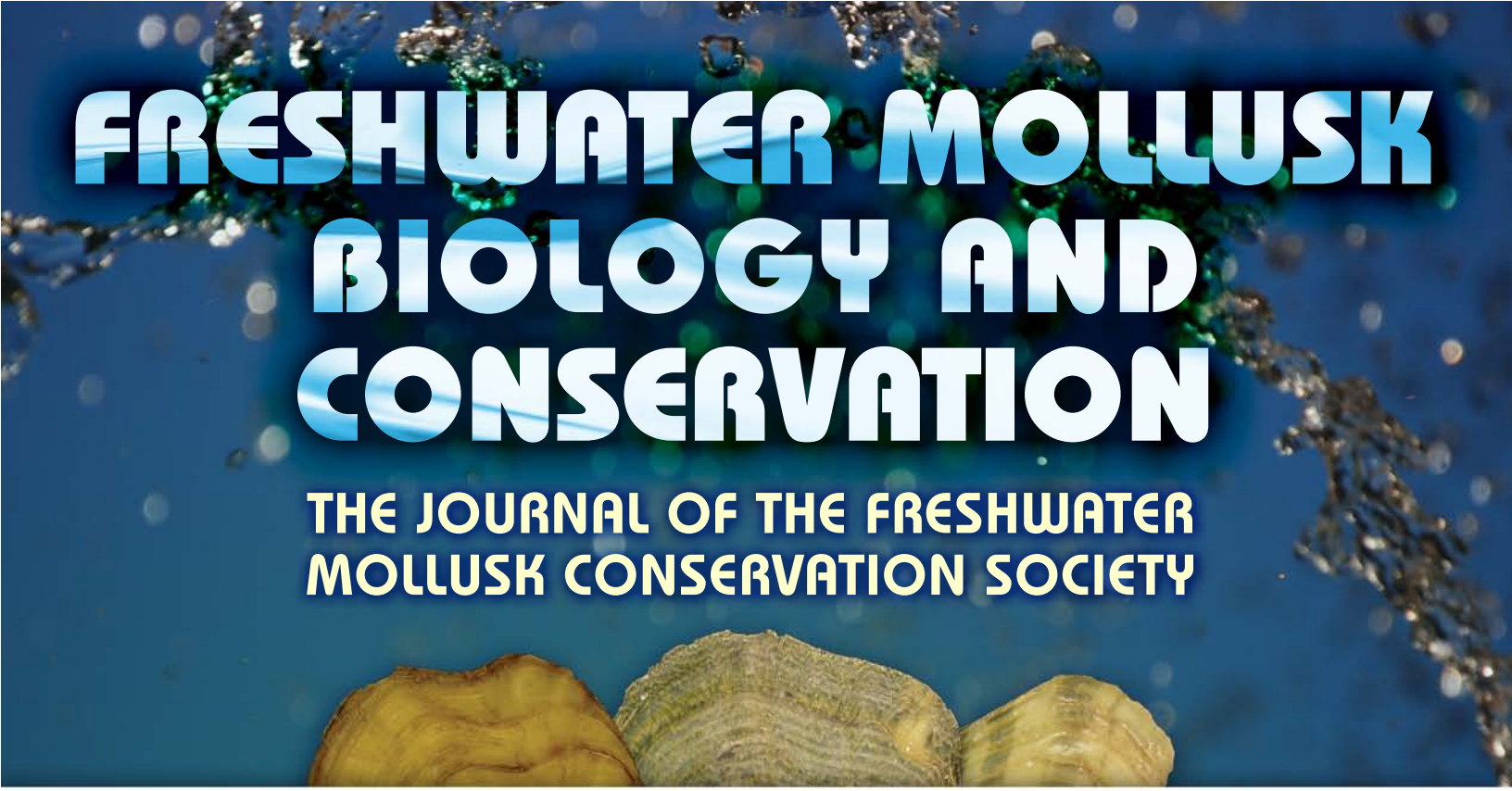

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REGULAR ARTICLE

STABLE ISOTOPE COMPARISON BETWEEN MANTLE AND FOOT TISSUES OF TWO FRESHWATER UNIONIDS: IMPLICATIONS FOR FOOD WEB STUDIES

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ABSTRACT

Unionid mussels are a key taxon for stable isotope studies of aquatic food webs, often serving as the primary integrator of the pelagic baseline. Past isotope studies with mussels have commonly used either foot tissue or mantle tissue, but no study has yet to quantify the relation of both carbon and nitrogen isotopes between these two tissue sources. This makes it difficult to justify cross-study comparisons when different tissue compartments and different species were used as the basis of food web models. Therefore, we collected foot and mantle tissues from two common mussel species, *Amblema plicata* and *Fusconaia flava*, from lotic and lentic sites in the Upper Mississippi and St. Croix rivers (Minnesota/Wisconsin). Paired tissue samples from each individual were analyzed for stable isotopes of nitrogen and carbon. There were strong relations between tissue types for both isotopes between species ($r^2 > 0.93$). Paired *t*-tests indicated that there were statistically significant differences between the tissue sources in some instances, but the difference (0.04–0.21‰) was less than the analytical precision of the mass spectrometer (circa 0.2–0.3‰). We conclude that the isotopic values from these two tissue sources are biologically comparable and recommend that researchers use the tissue source and extraction technique that minimizes stress to the mussels. We also tested for significant differences between species within a site for either isotope or tissue type and found no statistically significant difference between species with the exception of carbon in foot tissue at two sites. The highly correlated isotopic response supports the interchangeable use of both tissue compartments and both species. These findings support comparisons between studies whether the results were based on either of these tissues or the two species studied. Comparability will also simplify sampling designs, save time, and save money for processing samples without diminishing the usefulness of the data.

KEY WORDS: Mississippi River, St. Croix River, tissue comparison, freshwater mussel, threeridge, Wabash pigtoe

INTRODUCTION

Stable isotope analysis of food webs can be a powerful tool for understanding the effects of large-scale ecological changes, such as the introduction of invasive species and eutrophication (Thorp et al. 1998; Herwig et al. 2007; Delong 2010). Unionid bivalves are cornerstones of aquatic food web condition in stable isotope studies because they integrate the food web base over time, have slow turnover rates, and are not as sensitive to seasonal variability as other measurements of the pelagic food web base (Cabana and Rasmussen 1996; Post 2002). A measure of $\delta^{15}N$ from long-lived primary consumers, such as native mussels, allows calibration of site-specific background conditions that allow calculation of cross-site food chain length and other comparisons. Carbon isotope values from freshwater mussels can be used to identify sources of organic matter and to track the flow of carbon into primary consumers (Rounick et al. 1982; Finlay 2001; Brett et al. 2017), and nitrogen isotopes can be used as an indicator of nutrients entering the watershed (Lefebvre et al. 2009; Atkinson et al. 2014). Stable carbon and nitrogen isotopes can be used to

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calculate trophic niche space and to explore trophic relationships (Layman et al. 2007). In these and other ways, unionid mussels are a critical component for aquatic community stable isotope analysis to delineate food web dynamics. However, freshwater mussels as a group are sensitive to environmental degradation, with declining populations worldwide. Consequently, scientists increasingly employ nonlethal sampling methods for mussels, but this has resulted in the use of a number of different tissue-sampling protocols for isotope studies.

Stable isotope analysis requires sampling animal tissue or hemolymph and circa 1–2 mg (dry weight) of material is needed for mass spectrometer analysis. Given the conservation status of freshwater mussels, nonlethal and minimally invasive sampling methods for tissue collection are highly desirable. Three main tissue types are currently used for isotope studies of mussels: mantle tissue, foot tissue, and hemolymph from adductor muscles (McKinney et al. 1999; Gustafson et al. 2007; Weber et al. 2017). Mantle tissue biopsies have been shown to have no impact on long-term mussel survival (Berg et al. 1995). Foot biopsies and hemolymph extraction had no adverse effects on long-term survival of larger-bodied mussels (i.e., *Amblema plicata*, *Elliptio complanata*, *E. crassidens*; Naimo et al. 1998; Gustafson et al. 2005; Fritts et al. 2015), but the survival of a smaller-bodied species (*Villosa vibex*) was adversely affected by both methods (Fritts et al. 2015).

The widespread use of different tissues for isotopic studies of freshwater mussels has raised some concern that different tissues may produce different isotopic signatures and therefore limit the ability to compare isotope values across studies that have used different tissues (Weber et al. 2017). The published literature lacks a comprehensive comparison of variability between tissue sources for both carbon and nitrogen isotopes. Past studies that have conducted isotopic tissue comparisons between a combination of foot, mantle, and hemolymph have quantified the relation only among tissue sources for nitrogen isotopes (McKinney et al. 1999; Gustafson et al. 2007). However, carbon isotopes also provide valuable information about organic matter sources, including whether or not the sources are allochthonous or autochthonous, benthic or pelagic, and littoral or pelagic (Vander Zanden et al. 1999). Evaluating the relation of these isotopes among species can also provide insight into shared resource use, diet, and comparability of signatures among species at a site (Nichols and Garling 2000; Raikow and Hamilton 2001; Christian et al. 2004; Novais et al. 2016; Weber et al. 2017). Most food web models use both carbon and nitrogen isotopes in concert, which establishes the need for a more comprehensive assessment of tissue compartment comparisons for both isotopes.

The first objective of our study was to quantify the relation of carbon and nitrogen isotopes between foot and mantle tissue of two common freshwater mussel species (*Amblema plicata* and *Fusconaia flava*) in the Mississippi River Basin and to establish if these two tissue compartments produced comparable isotope results. The second objective of our study was to test for significant differences between these two species within a site for each isotope and tissue source. If the isotopic responses are statistically similar between the two species, this could allow for simplified sampling designs, particularly when a species is not able to be sampled consistently over the spatial gradient of interest.

METHODS

Study Location and Field Sampling

Freshwater mussels were collected from the Upper Mississippi and St. Croix rivers. Three main channel locations were sampled from Mississippi River Pool 2 (near St. Paul, MN), three sites from Lake St. Croix, three main channel sites between Lake St. Croix and the St. Croix Falls Dam, and one site above the St. Croix Falls Dam at Norway Point (Fig. 1). Each site was a stretch of relatively uniform habitat 1.5 km long. Scuba divers performed timed searches along each stretch in 2013 and 2014. *Amblema plicata* and *F. flava* were selectively sampled until a maximum of 25 individuals were collected for each species. We then selected five individuals per species per year from each site for tissue sampling and chose specimens as close as possible to the average size range of each species at each site. Sample sizes of five individuals have been reported to be sufficient and have low coefficients of variation for $\delta^{15}N$ (i.e., 5%; Gustafson et al. 2007). Given time constraints and field condition variability between years, some sites produced fewer than five individuals of a given species, and one site (i.e., M2MC2) contained specimens of only one of the target species. Two of the three sites within the Mississippi River had low mussel abundances, and therefore we chose to sample up to 10 individuals per species per year at the site with relatively high mussel abundances to increase our sample size in the Mississippi.

Tissue Sample Collection and Processing

Mantle samples were taken by gently prying open the mussel with a dull, flat-tipped sterile steel diving knife. Mantle tissue was held with a sterile duck billed forceps, and a 1 cm^2 section was snipped with sterile surgical scissors. Foot tissue was sampled in a similar way or with a biopsy needle (Bard Biopsy, Tempe, AZ). A subset of individuals had a duplicate sample collected from the same tissue source to evaluate variability within a tissue compartment. Tissue samples were immediately put on ice and transported to a –20°C freezer within 6 h. Frozen samples were transported to the Aquatic Resources Ecology Laboratory at Northland College (Ashland, Wisconsin) where they were dried at 60°C for 72 h and homogenized with mortar and pestle. Samples were then weighed, rolled into tin capsules, and shipped to Cornell University Stable Isotope Laboratory, Ithaca, New York. Stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) were determined with a Thermo Delta V isotope ratio mass spectrometer interfaced with a NC2500 elemental

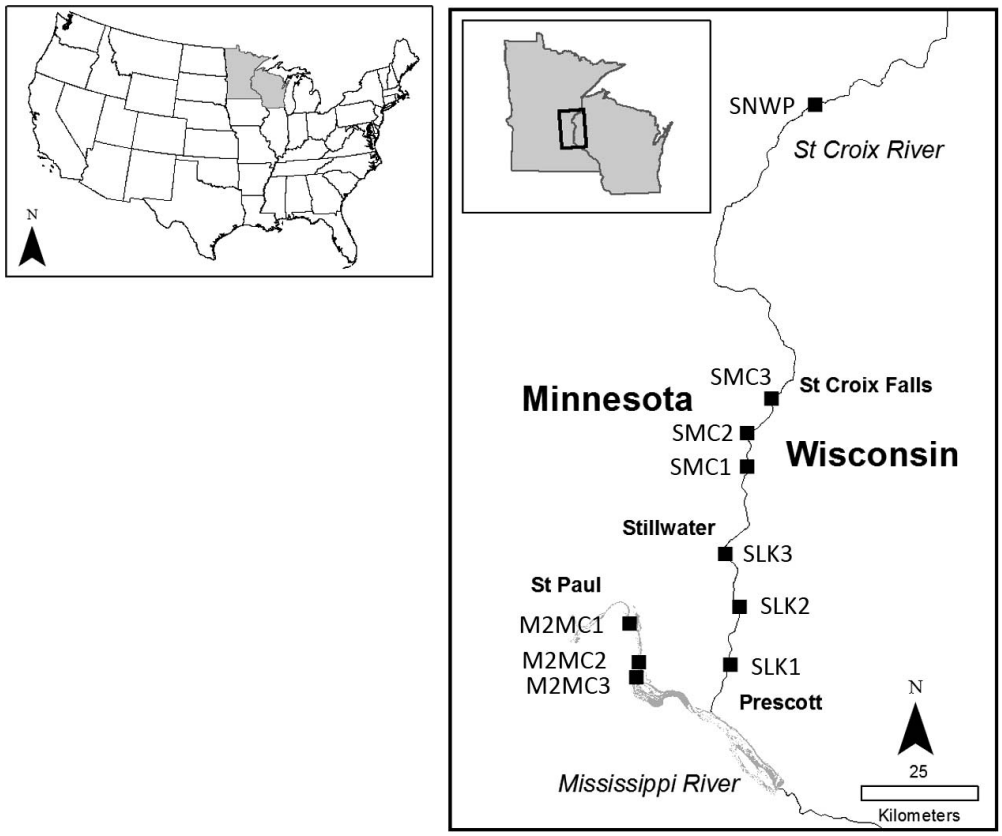

Figure 1. Sampling locations in the Upper Mississippi River basin, including seven sites in the St. Croix River and three sites in Pool 2 of the Mississippi River.

analyzer. In-house standards (mink animal material and methionine chemical standards) were run every 10 samples. In-house standards were routinely calibrated against international reference materials provided by the International Atomic Energy Association. Isotope data were expressed relative to Vienna PeeDee Belemnite for $\delta^{13}C$ and atmospheric air for $\delta^{15}N$. By convention, C and N isotope ratios are expressed as δ, the deviation from standards in parts per thousand (‰), according to the following equation:

$$\delta X(‰) = \left[(R_{\text{sample}}/R_{\text{standard}}) - 1\right] \times 1,000,$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Instrument precision for calibrating isotope ratios was 0.10–0.33‰ for $\delta^{13}C$ and 0.12–0.19‰ for $\delta^{15}N$.

Data Analysis

We used simple linear regression models to express relations between mantle and foot tissue results, in addition to performing paired *t*-tests (Infostat, Córdoba, Argentina). All data were assessed for normality. Residuals were normally distributed for both isotopes for the regression analysis, and the isotopic difference between tissues was normally distributed for both isotopes and for both species. We analyzed each species separately and combined them into a single dataset. We also tested the relation between duplicate tissue samples collected from the same tissue source within an individual. To evaluate the isotopic relationship between *A. plicata* and *F. flava*, we tested for differences between the species among locations for each isotope-tissue pairing using two-sample *t*-tests with Satterthwaite's approximation (Satterthwaite 1946; Oulhote et al. 2011). We also used a Bonferroni correction to account for multiple comparisons among locations (i.e., $\alpha = 0.05/9 = 0.0056$). Data from both years were combined for this analysis.

RESULTS

We sampled both foot and mantle tissue from 73 *A. plicata* and 87 *F. flava* (Table 1), with 56 replicates of the same tissue source within an individual to evaluate variability within a tissue compartment. All data are publicly available through ScienceBase (doi.org/10.5066/P9G92506). Among sampling locations, $\delta^{15}N$ values ranged from 5.76 to 12.63‰, and $\delta^{13}C$ ranged from –29.26 to –33.96‰. Mantle and foot tissue sources were positively correlated with regard to $\delta^{15}N$ and $\delta^{13}C$ for both species (Figs. 2, 3). Linear regression of $\delta^{15}N$ and $\delta^{13}C$ data resulted in respective r^2 values of 0.96 and 0.93 for *A. plicata* and 0.97 and 0.93 for *F. flava* (Table 2). Combining the data from both species resulted in r^2 values of 0.96 and 0.93 for $\delta^{15}N$ and $\delta^{13}C$ (Table 2).

Paired *t*-tests indicated that there was not a statistically

Table 1. *Amblema plicata* and *Fusconaia flava* sample size (*N*) and size parameters (mean ± SD) of specimens collected from the Mississippi and St. Croix rivers for this study.

Species	*N*	Length (mm)	Height (mm)	Width (mm)
A. plicata	73	84.9 ± 12.1	63.6 ± 5.3	43.9 ± 8.4
F. flava	87	60.1 ± 12.3	52.3 ± 7.2	38.2 ± 10.6

significant difference in $\delta^{13}C$ between tissues in *A. plicata* but $\delta^{15}N$ did differ, while both isotopes were significantly different in *F. flava* (Table 3). Foot tissue was slightly enriched over mantle tissue for both species for $\delta^{15}N$ (0.10–0.21‰). Mantle tissue was slightly enriched in $\delta^{13}C$ relative to foot tissue for *F. flava*, and there was no statistically significant difference between tissue sources for $\delta^{13}C$ in *A. plicata*. When analyzing both species combined, $\delta^{15}N$ was enriched in foot relative to mantle, and there was no difference between the tissue sources for $\delta^{13}C$. However, the overall difference between the tissues was only 0.04–0.21‰ for both isotopes, which is less than the analytical precision of the mass spectrometer (i.e., circa 0.2–0.3‰). There was no statistically significant difference between duplicate samples taken from the same tissue source (Table 4), and the regression relationships were very strong for both species, individually and combined ($r^2 = 0.92$–0.99; Table 5).

The comparison of isotopes between species across sites indicated that $\delta^{15}N$ was not significantly different between the species across all sites for both foot tissue and mantle tissue (Table 6, Fig. 4A, C). Carbon isotopes from foot tissue were significantly different between the species at two locations, one in the Mississippi River and one in Lake St. Croix, but not at any of the remaining locations, and $\delta^{13}C$ from mantle tissue was not significantly different between the species at any location (Table 6, Fig. 4B, D). Only one species (*F. flava*) was

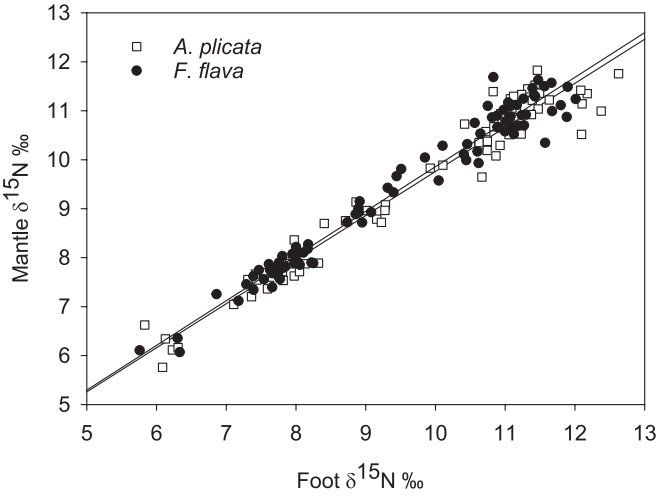

Figure 2. Simple linear regression of foot and mantle $\delta^{15}N$ per individual, with r^2 values of 0.955 for *Amblema plicata* and 0.967 for *Fusconaia flava*.

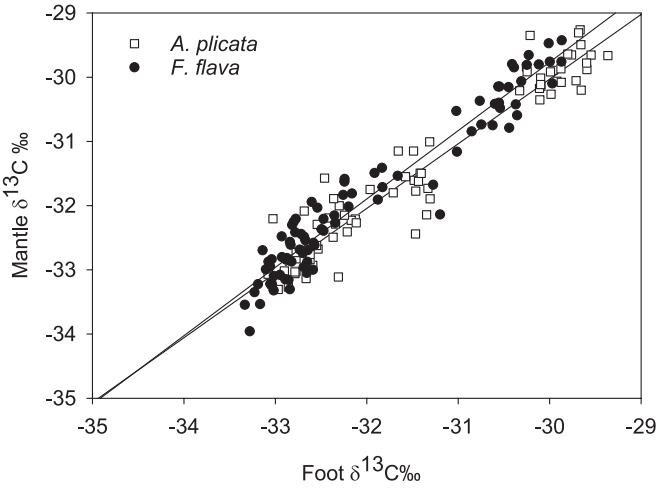

Figure 3. Simple linear regression of foot and mantle $\delta^{13}C$ per individual, with r^2 values of 0.925 for *Amblema plicata* and 0.934 for *Fusconaia flava*.

able to be collected at M2MC2, therefore species comparisons could not be conducted for this site.

DISCUSSION

This is the first study to compare both carbon and nitrogen isotopes between mantle and foot tissue compartments in freshwater mussels. For these two common and widely distributed species in the Upper Mississippi and St. Croix river systems, foot and mantle tissue sources were very similar with regard to $\delta^{15}N$ and $\delta^{13}C$. For the isotope/species combinations that had statistically significant per-mil differences in isotope composition between paired tissue compartments, the differences (i.e., 0.10–0.21‰) were within the range of instrumentation error (i.e., circa 0.2–0.3‰) and suggest that these differences would be unmeaningful in regard to food web analyses. We conclude that the tissue compartments are effectively interchangeable for carbon- and

Table 2. Simple linear regression results between the stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) of foot tissue versus mantle tissue for *Amblema plicata*, *Fusconaia flava*, and both species combined.

				Regression Coefficients	
Isotope	r^2	*F*	*P* Value	Intercept	Slope
A. plicata					
$\delta^{15}N$	0.955	1,490.34	<0.0001	0.757	0.900
$\delta^{13}C$	0.925	877.15	<0.0001	0.186	1.007
F. flava					
$\delta^{15}N$	0.967	2,472.81	<0.0001	0.733	0.913
$\delta^{13}C$	0.934	1,197.42	<0.0001	2.177	1.065
Combined					
$\delta^{15}N$	0.960	3,781.09	<0.0001	0.765	0.905
$\delta^{13}C$	0.927	2,007.00	<0.0001	0.642	1.019

Table 3. Stable isotope results of paired *t*-tests for foot versus mantle tissue for *Amblema plicata* ($N = 73$), *Fusconaia flava* ($N = 87$), and both species combined ($N = 160$). Difference (‰) = average difference between tissue types, and SD (dif) = standard deviation of the difference. Statistically significant differences are denoted in bold.

Isotope	Difference (‰)	SD (dif)	*t* Value	*P* Value
A. plicata				
$\delta^{15}N$	0.21	0.41	4.49	**<0.001**
$\delta^{13}C$	0.04	0.35	0.92	0.359
F. flava				
$\delta^{15}N$	0.10	0.32	2.83	**0.006**
$\delta^{13}C$	−0.11	0.32	−3.06	**0.003**
Combined				
$\delta^{15}N$	0.15	0.37	5.20	**<0.001**
$\delta^{13}C$	−0.04	0.34	−1.48	0.140

Table 4. Stable isotope results of paired *t*-tests for duplicate samples taken from the same tissue source from *Amblema plicata* ($N = 22$), *Fusconaia flava* ($N = 34$), and both species combined ($N = 56$). Difference (‰) = average difference between duplicate samples, and SD (dif) = standard deviation of the difference.

Isotope	Difference (‰)	SD (dif)	*t* Value	*P* Value
A. plicata				
$\delta^{15}N$	−0.01	0.36	−0.16	0.875
$\delta^{13}C$	−0.12	0.37	−1.51	0.147
F. flava				
$\delta^{15}N$	0.00	0.24	−0.06	0.950
$\delta^{13}C$	−0.01	0.12	−0.67	0.505
Combined				
$\delta^{15}N$	−0.01	0.29	−0.16	0.870
$\delta^{13}C$	−0.05	0.25	−1.63	0.108

nitrogen-stable isotopes for these two species within this study system.

While no studies have compared carbon isotopes between tissue sources, two studies have compared nitrogen isotopes among different tissues. *Elliptio* sp. foot tissue had slightly less spatial variability for nitrogen isotope signatures across small ponds as compared to mantle and adductor muscle tissue compartments (McKinney et al. 1999). Adductor muscle was enriched by 1.06‰ relative to mantle and foot tissues, and mantle tissue was enriched by 0.13‰ relative to foot tissue. Our findings for *A. plicata* and *F. flava* were the opposite, with foot tissue being more enriched in $\delta^{15}N$ relative to mantle tissue (i.e., 0.10–0.21‰). Like our study, the differences between foot and mantle tissues in the McKinney et al. (1999) study were less than the instrumental error and thus unmeaningful for the purposes of the food web analyses typically used.

Gustafson et al. (2007) found a strong positive relation (r^2 of 0.792) between nitrogen isotopes of foot tissue and hemolymph from *Elliptio complanata*. Foot tissue was generally enriched relative to hemolymph, but the actual difference in $\delta^{15}N$ between the tissues was not reported (Gustafson et al. 2007). We found even stronger correlations for $\delta^{15}N$ ($r^2 > 0.96$) and a difference of only 0.10–0.21‰ between mantle and foot tissue for *A. plicata* and *F. flava*. The tighter relation in our study compared to Gustafson et al. (2007) suggests that cross-study comparisons where foot or mantle tissues only were used might be more robust than comparisons that included hemolymph and other tissues.

The remarkably low variability in isotopic signatures among freshwater mussels within a location is one of the hallmarks that has made them an ideal taxon for isotopic baseline adjustment in food web studies (Cabana and Rasmussen 1996; Post 2002). Studying the isotopic relationship between/among species within a location can further enhance our understanding of the utility of freshwater mussels in isotope studies. A number of studies have indicated that $\delta^{15}N$ does not differ substantially among unionid species within a location (Nichols and Garling 2000; Raikow and Hamilton 2001; Christian et al. 2004; but see Weber et al. 2017), while the relationship of $\delta^{13}C$ between/among species has been more mixed (Nichols and Garling 2000; Christian et al. 2004; Weber et al. 2017). Unionid mussels have also been documented to differ in their carbon and nitrogen isotopic ratios relative to the invasive *Corbicula fluminea* (Atkinson et al. 2010).

Our data indicated that there was not a statistically significant difference for either isotope or tissue type between the species at nearly any site in this study. The equivalent isotopic signatures from these two unionid species by site justifies using one, rather than both, species for isotope studies in the Upper Mississippi and St. Croix rivers. These results also lay the foundation for using these two species interchangeably if one is not able to be sampled consistently over a spatial gradient in these Midwestern rivers. In our study system, this opens up more options for comparing food webs in the Mississippi and St. Croix Rivers over larger longitudinal (up and down the river and tributaries) and lateral (on-channel

Table 5. Simple linear regression results between the stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) of duplicate samples taken from the same tissue within an individual for *Amblema plicata*, *Fusconaia flava*, and both species combined.

				Regression Coefficients	
Isotope	r^2	*F*	*P* Value	Intercept	Slope
A. plicata					
$\delta^{15}N$	0.946	348.94	<0.0001	0.022	0.997
$\delta^{13}C$	0.924	243.96	<0.0001	2.741	1.092
F. flava					
$\delta^{15}N$	0.980	1,588.70	<0.0001	−0.297	1.031
$\delta^{13}C$	0.992	4,186.73	<0.0001	1.244	1.039
Combined					
$\delta^{15}N$	0.969	1,712.25	<0.0001	−0.174	1.017
$\delta^{13}C$	0.963	1,405.79	<0.0001	1.220	1.040

Table 6. Two sample *t*-test comparison of isotopes between species across sites. Statistically significant values after Bonferroni correction are denoted in bold. Sites that begin with M2 are from the Mississippi River Pool 2, and sites that begin with S are locations in the St. Croix River, listed from most downstream to most upstream. *N* = number of individuals per species per site, SD = standard deviation. Only one species was able to be collected at M2MC2, therefore species comparisons could not be conducted for this site.

	Amblema plicata			*Fusconaia flava*				
Site	*N*	Mean (‰)	SD (‰)	*N*	Mean (‰)	SD (‰)	*T*	*P* Value
δ^{15}N: Foot tissue								
M2MC1	19	11.15	0.18	19	11.13	0.31	0.27	0.7856
M2MC3	7	10.96	0.45	2	10.96	0.10	0.01	0.9937
SLK1	8	12.00	0.50	9	11.55	0.41	2.03	0.0602
SLK2	8	10.57	0.36	10	10.34	0.47	1.14	0.2707
SLK3	8	8.99	0.31	10	9.05	0.25	−0.43	0.6722
SMC1	5	7.87	0.22	10	7.88	0.31	−0.04	0.9725
SMC2	5	7.36	0.18	10	7.72	0.26	−2.77	0.0160
SMC3	8	7.86	0.27	10	7.67	0.41	1.12	0.2788
SNWP	5	6.11	0.18	3	6.13	0.32	−0.09	0.9335
δ^{15}N: Mantle tissue								
M2MC1	19	11.07	0.36	19	11.02	0.35	0.41	0.6856
M2MC3	7	10.79	0.39	2	10.74	0.10	0.19	0.8578
SLK1	8	11.10	0.42	9	11.00	0.44	0.48	0.6370
SLK2	8	10.10	0.30	10	10.18	0.43	−0.46	0.6543
SLK3	8	8.89	0.17	10	9.07	0.31	−1.51	0.1517
SMC1	5	7.90	0.28	10	7.87	0.29	0.20	0.8426
SMC2	5	7.34	0.22	10	7.73	0.21	−3.29	0.0059
SMC3	8	7.68	0.16	10	7.75	0.34	−0.53	0.6066
SNWP	5	6.20	0.32	3	6.18	0.15	0.12	0.9065
δ^{13}C: Foot tissue								
M2MC1	19	−29.88	0.27	19	−30.35	0.33	4.88	**<0.0001**
M2MC3	7	−29.93	0.22	2	−30.44	0.18	2.91	0.0227
SLK1	8	−32.67	0.32	9	−32.78	0.16	0.89	0.3858
SLK2	8	−32.16	0.23	10	−32.52	0.20	3.52	**0.0028**
SLK3	8	−31.61	0.36	10	−32.06	0.27	3.05	0.0077
SMC1	5	−32.71	0.19	10	−32.93	0.18	2.21	0.0458
SMC2	5	−32.86	0.08	10	−33.05	0.20	2.02	0.0640
SMC3	8	−32.34	0.44	10	−32.84	0.18	3.01	0.0147
SNWP	5	−31.39	0.07	3	−31.16	0.13	−3.28	0.0168
δ^{13}C: Mantle tissue								
M2MC1	19	−29.82	0.33	19	−30.13	0.45	2.49	0.0174
M2MC3	7	−30.03	0.14	2	−30.10	0.06	0.71	0.5029
SLK1	8	−32.50	0.36	9	−32.57	0.17	0.52	0.6138
SLK2	8	−32.03	0.20	10	−32.20	0.25	1.50	0.1521
SLK3	8	−31.41	0.27	10	−31.75	0.29	2.51	0.0232
SMC1	5	−32.97	0.18	10	−33.05	0.41	0.40	0.6922
SMC2	5	−33.14	0.14	10	−33.15	0.26	0.09	0.9317
SMC3	8	−32.61	0.34	10	−32.93	0.29	2.13	0.0488
SNWP	5	−31.84	0.37	3	−31.66	0.49	−0.59	0.5779

to off-channel) gradients, and we suspect this will also be useful for comparisons over time (Hornbach et al. 2018). The isotopic similarity of *A. plicata* and *F. flava* could simplify sampling designs and save time and money on sampling, all without diminishing the usefulness of the data.

This project advances the state of the science for isotopic studies in freshwater mussels by comparing two tissues for two isotopes in two species in two rivers over a large gradient of isotopic values. We argue that the similarity of the isotopic responses of foot and mantle tissue justifies retroactively comparing results between studies that have used these tissue types. However, we urge caution in overextending the implications of these findings outside of this geographic area, these species, or the range of isotopic values encountered in

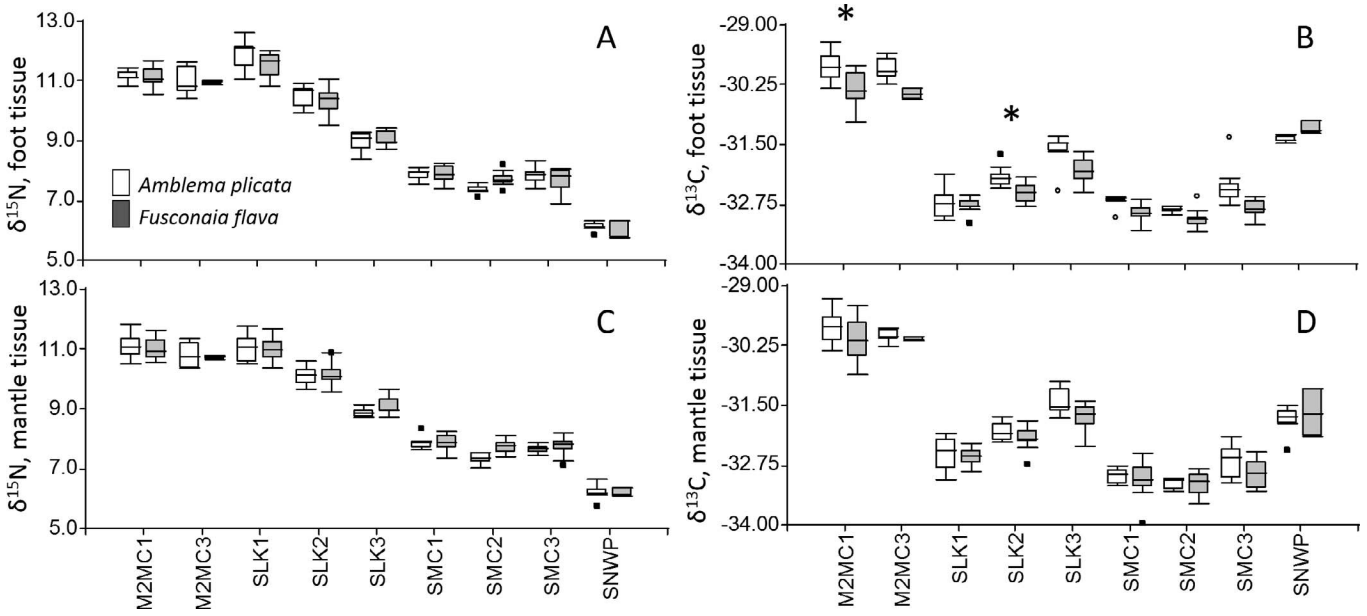

Figure 4. Box plots of a species comparison of isotopic values between *Amblema plicata* (white boxes) and *Fusconaia flava* (grey boxes) among sampling locations. Center horizontal lines are medians, upper and lower margins of the box are 25th and 75th percentiles, and the bars are the 5th and 95th percentile. Outliers are represented as individual points. A = foot tissue $\delta^{15}N$, B = foot tissue $\delta^{13}C$, C = mantle tissue $\delta^{15}N$, D = mantle tissue $\delta^{13}C$. Sites at which the species were significantly different are denoted with an asterisk above the paired boxes.

our study. Future research should evaluate the comparability of isotopic signatures between tissues of additional species from different geographic regions and habitats to evaluate the potential for species specific or location-based factors that may result in different patterns than what we observed in our study.

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REGULAR ARTICLE

COLLAPSE OF THE PENDLETON ISLAND MUSSEL FAUNA IN THE CLINCH RIVER, VIRGINIA: SETTING BASELINE CONDITIONS TO GUIDE RECOVERY AND RESTORATION

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ABSTRACT

In the 20th century, Pendleton Island (PI) in the Clinch River of southwestern Virginia was a singularly important location for conservation of freshwater mussels in North America, supporting at least 45 species. Comprising 55,500 m^2 of available habitat, PI is the largest contiguous patch of habitat for mussels in the unregulated reaches of the Clinch River in either Virginia or Tennessee. Mussel density at PI declined by 96% from its historical baseline of 25/m^2 in 1979 to ~1/m^2 in 2014, indicating a collapse of the fauna. We provide a quantitative description of the PI mussel assemblage collapse and establish baseline conditions for restoration scenarios. We examined long-term monitoring data collected at 15 sites in the Tennessee and Virginia sections of the river over a 35-yr period (1979–2014). While the mussel assemblage of PI has declined precipitously, density in the Tennessee section of the river has increased at an annual rate of 2.3% (1979–2004) and 1.3% (2004–14), stabilizing at a mean density of 29/m^2 over the last 10-yr period, a reasonable baseline density to gauge recovery and restoration at PI and at other disturbed sites in the river. Lost mussel abundance can and should be translated to ecosystem services loss at PI, representing more than 1.38 million mussels and tens of millions of lost mussel service years. When density of the PI mussel assemblage is projected forward 30 yr (2014–44), it returns to a baseline of 25/m^2 in 2036 only under a high-growth-rate scenario of 15% per yr. If realistic growth rate scenarios of 1% and 5% are used, density reaches 1.4/m^2 (~75,000 individuals) and 4.3/m^2 (~240,000 individuals), respectively, by 2044. These scenarios assume healthy nondegraded habitat conditions, which do not reflect current water and sediment quality at PI. Recovery of the assemblage to baseline densities will take decades and require active restoration of the fauna and habitat, including mussel translocations and stocking of hatchery-propagated juveniles.

KEY WORDS: Clinch River, Pendleton Island, freshwater mussels, recovery, restoration

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INTRODUCTION

Pendleton Island (PI) in the Clinch River of southwestern Virginia persisted as a globally significant habitat for freshwater mussels into the late 20th century, with 45 species recorded since 1979. One of the most-speciose habitats in the Clinch River, PI supports among the greatest species richness remaining in North America (Jones et al. 2014; Ahlstedt et al. 2016); only Speers Ferry has comparable documented richness (Ostby and Beaty 2017). Further, it may have supported among the greatest number of species documented in a single habitat in North America since the 1960s, a period following a boom in construction of large hydro-power and flood-control dams throughout the Tennessee and Cumberland river systems. Historically, these river systems supported globally significant endemism and species richness. In the late 20th century, PI was identified as a conservation priority because it supported numerous rare and endangered mussel species, including 24 species listed as federally endangered. Consequently, PI was a priority for state and federal government agencies and The Nature Conservancy, which purchased it in 1986 to help manage and protect the mussel fauna.

First discovered by Tennessee Valley Authority (TVA) biologists Steven Ahlstedt and Charlie Saylor during a 1978 reconnaissance survey, PI was immediately recognized for harboring a large, abundant, and diverse mussel assemblage. The following year, the site was quantitatively surveyed, and a mussel density of 25/m^2 was recorded (Ahlstedt et al. 2016). Unfortunately, the mussel assemblage has declined by 96% since then to its current density of $\sim$1/m^2 (Jones et al. 2014). The causes of the decline include degraded water and sediment quality related to coal-mining activities occurring in the watershed for more than 50 yr, with data, analyses, and discussion available in Krstolic et al. (2013), Johnson et al. (2014), Price et al. (2014), Zipper et al. (2014), and Cope and Jones (2016). These authors present the current state of scientific understanding that water and sediment quality in this reach of the river are adversely affected by increased levels of contaminants, including dissolved solids, trace metals, and polycyclic aromatic hydrocarbons, which in turn may limit mussel survival and reproduction. While the faunal collapse has been clearly documented (Jones et al. 2014; Ahlstedt et al. 2016), the magnitude of lost mussel abundance, species richness, and ecosystem services at PI has not been well quantified, and baseline conditions have not been set to gauge future recovery and restoration of this important assemblage.

The comprehensive survey of the unregulated Clinch River conducted by TVA from 1978 to 1983 demonstrated that mussel density and richness are highly heterogenous. Habitat patches such as PI are mussel hotspots in a river that features predominately exposed bedrock dotted with cobble/gravel bars of limited extent and variable long-term stability. Church (1997) hypothesized that heterogeneity was a product of underlying lithology, geologic structure, and channel form. He found that two types of reaches were more likely to support "high-quality" mussel assemblages. The first mostly occurred when the Clinch River flowed in the direction of geologic dip over limestone or dolomite geology; there, bedrock outcrops oriented perpendicular to flow trapped alluvium, creating long-term stable habitats capable of supporting mussel beds. The second—of which PI was a specific example—occurred where the river flowed over comparatively erosive shale formations. In these reaches, valley floors were wide and the river tended to braid. With 55,500 m^2 of riverbed available, PI is by far the largest contiguous habitat patch for mussels in the unregulated reaches of the Clinch River in either Virginia or Tennessee. In fact, it contains a similar amount of habitat as the three largest habitat patches in the Tennessee section of the river combined; Wallen Bend (WB), Kyles Ford, and Frost Ford (FF) contain a total of 58,765 m^2 of habitat (Jones et al. 2014). PI's extent, however, is deceptive, because it cannot be viewed in its entirety from the ground due to the size of the island, length of the channels, and the tree canopy, which obscures the view. The site is located in Scott County, Virginia, at river kilometer (RKM) 364.2 (RKM 0 is at the confluence with the Tennessee River), in a faunal transition zone between the headwaters and lower half of the river (Fig. 1). The site contains two main channels, 800–900 m long, defined by the main island, but it also contains smaller channels defined by smaller islands. As a result, PI contains a diversity of small-scale niche habitats, including shallow riffles and long runs with gravel-sand substrates, areas with boulders and slab rock, and stretches with ample large woody debris, which collectively provide ideal habitat conditions for high species richness of mussels and their host fishes (Fig. 2). Further, species richness may result from PI's location in a stream network connecting isolated mountain headwaters to the broad rivers of the Tennessee Valley and the lower Mississippi River.

Survey data exist to establish baselines for mussel density, abundance, and assemblage structure at PI. Long-term monitoring data collected from 1979–2014 at multiple sites in Tennessee and Virginia by Jones et al. (2014), Ahlstedt et al. (2016), and this study provide a context to establish quantitative baseline conditions and prevent what has been described as the "Shifting Baseline Syndrome," in which "each generation of fisheries scientists accepts as a baseline the stock size and species composition that occurred at the beginning of their careers, and uses this to evaluate changes. When the next generation starts its career, the stocks at that time serve as a new baseline. The result obviously is a gradual shift of the baseline" (Pauly 1995, 430). Historically, changes to the mussel assemblage at PI and other sites in the Clinch River have occurred and will continue to occur in the future, driven by myriad ecological and anthropogenic factors as well as by stochasticity. Therefore, it is critical that future changes to the fauna are measured against quantitative and parameterized baseline metrics so that the next generation of biologists can accurately assess population trends for respective species at PI and throughout the river.

The broad purpose of this study was to build on quantitative data collected from 1979 to 2004 (Ahlstedt et al. 2016) and 2004 to 2009 (Jones et al. 2014) by continuing long-term monitoring from 2010 to 2014 at key sites in the

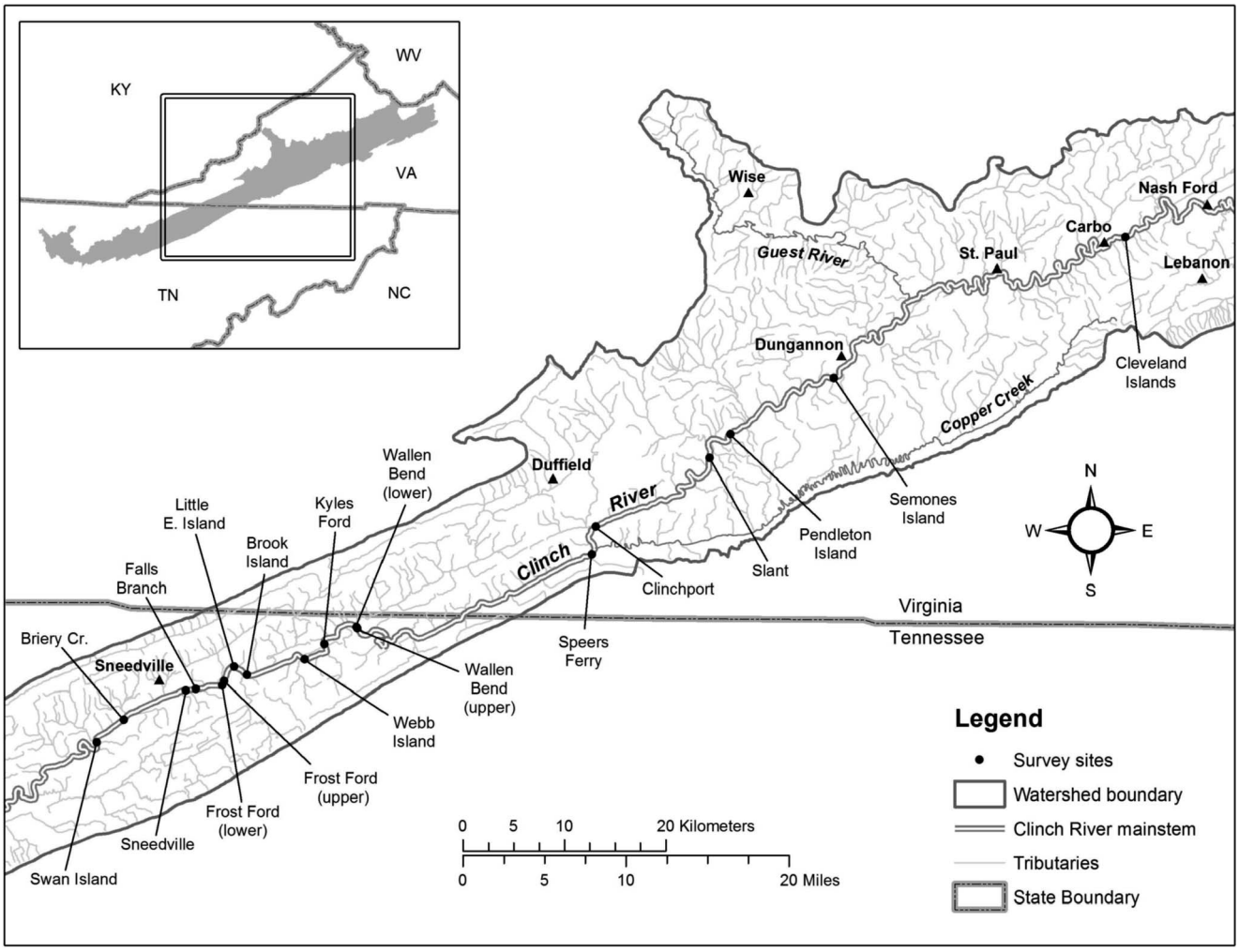

Figure 1. The 15 sites sampled during the study period from 1979 to 2014 in the Clinch River, Tennessee and Virginia, upstream of Norris Reservoir. Swan Island, upper Frost Ford, and upper Wallen Bend were sampled annually from 2004 to 2014, while remaining sites were sampled one to three times during the study period. Cleveland Islands, Slant, and Clinchport in Virginia were not sampled as part of the current study; data are available for these three sites in Jones et al. (2014), where the map was previously published.

Clinch River upstream of Norris Reservoir in Tennessee and Virginia, and to use the data to determine status and population trends of mussels throughout the river. More specifically, we used these data to set baseline conditions at PI for species composition and density so that recovery and restoration of the site's fauna can be accurately assessed in the future. Further, using four population growth rate scenarios to gauge recovery of the PI mussel assemblage in the future, we assessed lost mussel abundances, ecosystem services, and time to recovery over a 65-yr period (1979–2044).

METHODS

Establishing baseline conditions.—Two sections of the Clinch River made up the study area: (1) a 38.5-km section from Swan Island (RKM 271.1) upstream to Wallen Bend (RKM 309.6) in Hancock County, Tennessee, and (2) a 38.6-km section from Speers Ferry (RKM 339.7) upstream to Semones Island (RKM 378.3) in Scott County, Virginia, with a focus on the mussel fauna at PI (RKM 364.2) (Fig. 1). Since 1979, the mussel assemblage in the Tennessee section has been characterized by high abundance and assemblage stability, whereas the assemblage in the Virginia section has been characterized by low abundance and severe decline, including species extirpation (Jones et al. 2014). We analyzed mussel population densities at 12 sites in Tennessee and six sites in Virginia, which were initially surveyed from 2004 to 2009 (Jones et al. 2014), with a subset of those sites analyzed from 2010 to 2014 as part of the current study (Fig. 1 and Table 1). We did not sample three sites (Cleveland Islands, Slant, and Clinchport in Virginia) during the current study, but data and references are available for these sites in Jones et al. (2014). Hence, a total of 15 sites were sampled for the 2010–14 study period. Sites were sampled once or twice during the entire study period from 2004 to 2014, except for three

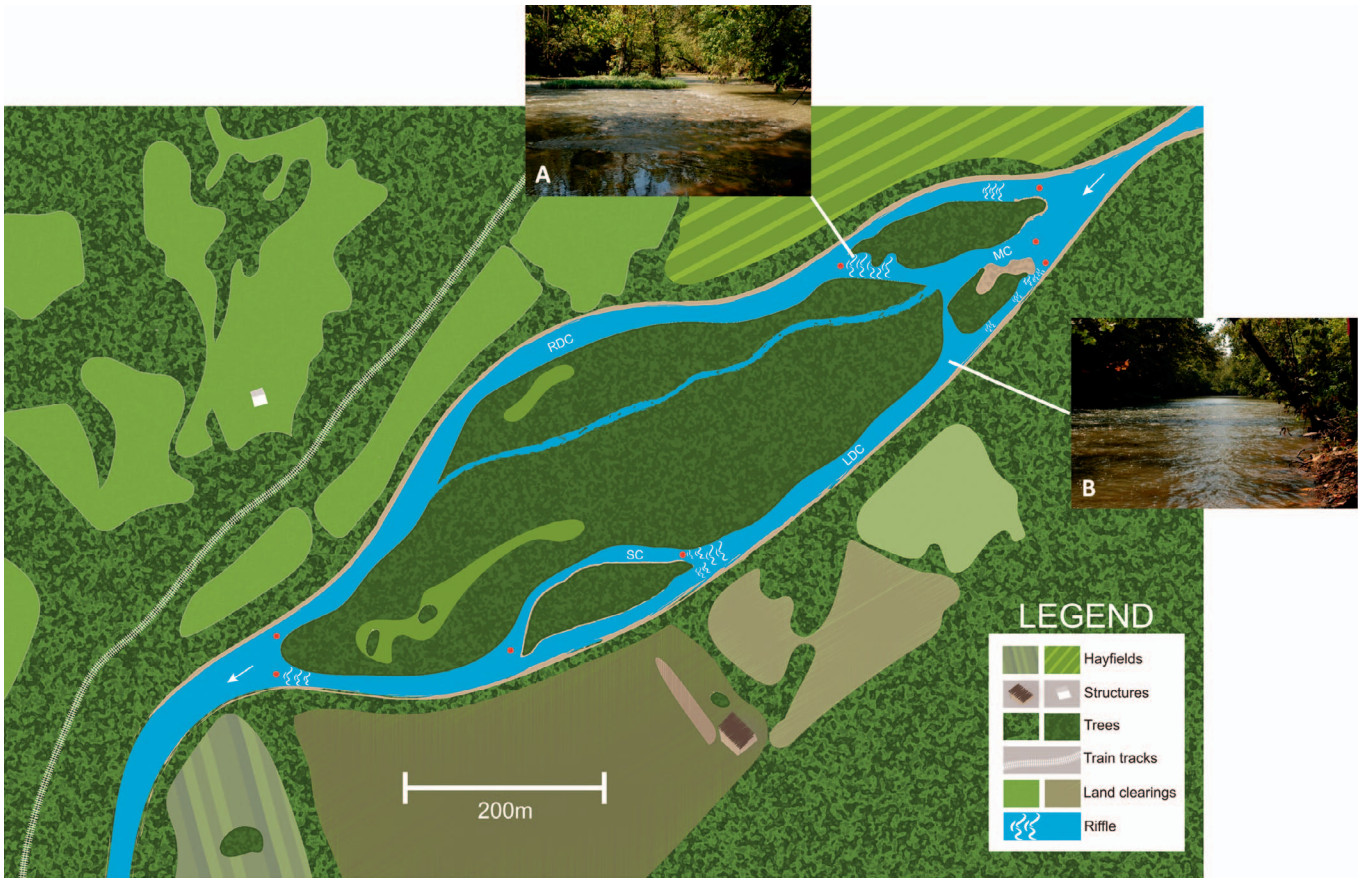

Figure 2. Graphic image of Pendleton Island, Clinch River, Scott County, Virginia showing right-descending channel (RDC), middle channel (MC), left-descending channel (LDC), side-channel (SC) of left-descending channel, and surrounding land features. RDC is 856 m long with mean width 25.7 m and LDC is 980 m long with mean width 24.9 m. Red dots indicate start and end points of the measured distance of each respective channel. Inset photograph A is a shallow riffle in RDC, and inset photograph B is a long run in LDC.

Tennessee sites, Wallen Bend (WB), Frost Ford (FF), and Swan Island (SI), which were sampled annually. These three sites were selected to establish and monitor baseline population trends in the Tennessee section of the river over the 10-yr period. To monitor population conditions in the Virginia section, Speers Ferry, Pendleton Island (PI), and Semones Island (SEI) were sampled in 2009 and then just PI and SEI in 2014. Most sites in Tennessee and Virginia also were sampled from 1979 to 2004 (Ahlstedt et al. 2016), and we combined those data with data from Jones et al. (2014) and the current study to analyze long-term population trends (1979–2014) in the Tennessee section of the river, and the long-term population trend at PI.

Because of the historical importance of the fauna at PI, we conducted additional analyses to establish and reconstruct baseline conditions for species density, abundance, and composition at the site using data from Jones et al. (2014), Ahlstedt et al. (2016), and the current study. In addition, we used data from several qualitative surveys conducted at PI to assess extant species richness at the site (Neves and Beaty 1996; Beaty and Neves 1997; Beaty and Neves 1998; Jones and Neves 1999; Ahlstedt et al. 2005). Historical mussel assemblage density for PI was set at 25/m^2, the maximum observed density at the site as measured in 1979 (Ahlstedt et al. 2016). Each species' frequency, percentage composition of the assemblage (proportion), density, and abundance at the site was reconstructed by averaging species composition data from 1979 at PI with composition data from all sites in Tennessee from 2004 to 2014. Using *Actinonaias ligamentina* as an example to calculate baseline for a species, we calculate area of PI (55,503 m^2) $\times$ baseline density (25/m^2) $\times$ mean proportion (0.10755) = 149,234 individuals at the site (Table 5). It was necessary to use recent composition data from the Tennessee section of the river because many of the species known from PI were uncommon to rare even in 1979. Further, percentage compositions of the common species already were skewed toward longer-lived and more tolerant species. Hence, an averaging approach combining data from sites and time periods was required to reconstruct the most probable proportion of each species at PI. Finally, decline and recovery of the mussel assemblage from an historical baseline of 25/m^2 was modeled first by linear regression of density data from 1979 to 2014 and then by projecting density over time from 2014 to 2044 using four growth rates—1%, 5%, 10%, and

Table 1. Site location, river kilometer, site area size, year sampled, sample size, and mussel density with 95% confidence intervals (CI) at sites sampled quantitatively with quadrats from 2004 to 2014 in the Clinch River, Tennessee and Virginia. The sample data are from [a]Ahlstedt et al. (2016), [b]Jones et al. (2014), and [c]Dennis (1989), and the current study includes data from 2010 to 2014. NA indicates that raw data were unavailable to calculate confidence intervals. *Quadrats were 0.5.

Site Location Name	River Kilometer (river mile)	Total Site Area (m^2)	Year(s) Sampled	No. (*N*) 0.25 m^2 Quadrats/yr	Mean Density/m^2	Lower 95% CI	Upper 95% CI
Swan Island (SI), Tennessee	277.1 (172.2)	5,760	1979[a]	40	7	NA	NA
			1988[a]	40	1.6	0.7	2.5
			1994[a]	40	10.6	NA	NA
			1999[a]	40	11.4	8.2	14.6
			2004[a]	40	29.4	22.2	36.6
			2004[b]	60	23.7	15.7	31.7
			2005[b]	60	15.1	8.8	21.4
			2006[b]	60	16.3	9.6	23
			2007[b]	60	22.5	16.4	28.6
			2008[b]	60	23.1	13.7	32.5
			2009[b]	72	28.2	20.6	35.8
			2010	80	27.5	20.5	36.9
			2011	80	26.7	19.9	35.9
			2012	80	34.2	25.4	46
			2013	80	17.7	12.6	25
			2014	80	15.7	11.5	21.4
Briery Creek, Tennessee	280.8 (174.5)	6,600	2006	40	12	8.9	15.1
Sneedville, Tennessee	287.6 (178.7)	2,016	2006	40	11.8	8.5	15
Falls Branch, Tennessee	288.7 (179.4)	5,334	2006	40	34.9	26.4	43.2
Frost Ford (FF), Tennessee	291.3 (181.0)	8,600	2007	72	9.2	7.2	11.2
Frost Ford (FF), Tennessee	291.8 (181.3)	15,050	2004[b]	60	31.4	27	35.8
			2005[b]	60	24.2	20.1	28.3
			2006[b]	60	27.1	22.6	31.6
			2007[b]	60	45.9	38.5	53.3
			2008[b]	60	68.4	58.8	78
			2009[b]	91	42.9	37	48.8
			2010	80	55.9	47.7	65.5
			2011	80	53.5	47.4	60.4
			2012	80	50	44.4	56.2
			2013	80	44.3	38.6	50.7
			2014	80	43.8	38.4	49.8
Little E. Island, Tennessee	293.7 (182.5)	11,200	2005	60	19.3	16.2	22.4
Brooks Island, Tennessee	295.3 (183.5)	~6,000	1979[a]	26	11.4	NA	NA
			1988[a]	26	9.7	6	13.4
			1994[a]	26	13.7	NA	NA
			1999[a]	26	40.8	32	49.6
			2004[a]	26	21.3	16.9	25.7
			2005[b]	60	21.5	13.6	29.2
Webb Island, Tennessee	301.7 (187.5)	4,576	2006	60	22.8	18.4	27.2
Kyles Ford, Tennessee	305.1 (189.6)	~15,000	1979[a]	41	31	NA	NA
			1988[a]	41	14.3	9.6	19
			1994[a]	41	37.6	NA	NA
			1999[a]	41	95.9	81.5	110.3
			2004[a]	41	74.3	60.3	88.3
			2004[b]	146	43.8	35.8	51.8

Table 1, continued.

Site Location Name	River Kilometer (river mile)	Total Site Area (m^2)	Year(s) Sampled	No. (*N*) 0.25 m^2 Quadrats/yr	Mean Density/m^2	Lower 95% CI	Upper 95% CI
Wallen Bend (WB), Tennessee	309.5 (192.3)	16,933	2007	120	22	18.7	25.4
Wallen Bend (WB), Tennessee	309.6 (192.4)	3,182	2004[b]	60	13.7	10.9	16.5
			2005[b]	60	12.9	10.2	15.6
			2006[b]	60	15.1	12.2	18
			2007[b]	60	21.3	18	24.6
			2008[b]	60	30.2	23.8	36.6
			2009[b]	60	28.5	23.2	33.8
			2010	80	27.3	23.2	32.2
			2011	80	17.2	13.9	21.3
			2012	80	18.8	14.5	24.3
			2013	80	17.2	13.9	21.2
			2014	80	18.6	14.6	23.8
Speers Ferry, Virginia	339.7 (211.1)	~4,000	1979[a]	40	3.7	NA	NA
			1988[a]	40	2.7	1.7	3.7
			1994[a]	40	2.9	NA	NA
			1999[a]	40	4.8	2.4	7.2
			2004[a]	40	4.7	2.6	6.8
			2009[b]	80	5	3.6	6.4
Pendleton Island (PI), Virginia	364.2 (226.3)	55,503	1979[a]	40	24.6	NA	NA
			1987[c]	*22	18.7	15	22.4
			1994[a]	40	11.2	NA	NA
			1999[a]	40	12.4	10	14.8
			2004[a]	40	4.6	2.9	6.4
			2009[b]	360	0.66	0.5	0.9
			2014	187	1.1	0.8	1.5
Semones Island (SEI), Virginia	378.3 (235.1)	~10,000	1983[a]	40	7.7	NA	NA
			1988[a]	40	4.6	NA	NA
			1994[a]	40	6.5	NA	NA
			1999[a]	40	4.2	2.7	5.7
			2004[a]	40	1.7	0.7	2.7
			2009[b]	124	0.61	0.3	0.9
			2014	133	0.69	0.6	0.8

15%—to determine when density would return to baseline. Additionally, we estimated mussel losses over time and into the future based on departures in density and abundance from baseline conditions, i.e., minus each year's density from 1979 to 2014 and from 2015 to 2044 under the four growth rate scenarios. Scientific and common names of mussels follow Williams et al. (2017).

Survey methods.—Sampling methodology followed that of Jones et al. (2014) and was conducted in late summer or early fall when water levels were low and young-of-the-year and older juvenile mussels had reached sizes adequate for detection (e.g., >10 mm). Most mussel species in the Clinch River prefer shallow water (<1 m) containing gravel shoals, which served as sample sites for our survey. This habitat is abundant in the river but interspersed with longer, slower-flowing deeper pools (>1 RKM) containing poorer quality mussel habitat. Typical lengths of gravel shoals were about 100–200 m but were occasionally longer. We determined the upstream and downstream limits of sampling sites, which are very discrete in the river, by visually inspecting substrate composition (e.g., noting an abrupt change from suitable gravel substrate to unsuitable bedrock or soft sediments), water depth, flow velocity, and general presence or absence of mussels. The same upstream and downstream boundaries were used at sites sampled annually. We measured, and subtracted from analyses, any small, exposed gravel bars and islands without mussels but within the immediate shoal area. Using a standard 100-m tape, we measured site dimensions (length and width) and then determined the total area (m^2) of the sample sites by multiplying mean river width, measured at 10–20 m intervals, by total reach length (Table 1). At PI in summer 2016, we measured stream width and length at 10-m intervals along the length of each channel using a rangefinder (Wildgame Innovations R400 HALO 6 × 24, Wildgame Innovations

Table 2. Abundance of live mussels of each species sampled during quantitative quadrat surveys conducted at Swan Island (SI), Frost Ford (FF), Wallen Bend (WB), and other sites (see Table 1) from 2004 to 2014 in the Clinch River, Hancock County, Tennessee. Data from 2004 to 2009 are from Jones et al. (2014). Sample sizes per year are available in Table 1.

	2004–2009				2010–2014			
Scientific Name	SI	FF	WB	Other Sites	SI	FF	WB	All Sites and Years
(1) *Actinonaias ligamentina*	401	173	148	318	184	184	120	1,528
(2) *Actinonaias pectorosa*	759	189	217	533	781	260	235	2,974
(3) *Alasmidonta marginata*	1	0	0	0	2	1	1	5
(4) *Amblema plicata*	1	0	1	0	0	0	0	2
(5) *Cyclonaias pustulosa*	3	0	0	4	7	5	0	19
(6) *Cyclonaias tuberculata*	11	35	18	24	11	33	9	141
(7) *Cyprogenia stegaria*	12	13	1	10	15	20	0	71
(8) *Dromus dromas*	84	62	2	26	104	67	0	345
(9) *Epioblasma brevidens*	40	51	30	59	53	65	20	318
(10) *Epioblasma capsaeformis*	80	1,616	363	464	198	1,763	314	4,798
(11) *Epioblasma triquetra*	15	4	2	8	17	12	1	59
(12) *Eurynia dilatata*	2	68	68	77	1	170	89	475
(13) *Fusconaia cor*	0	1	5	1	0	3	19	29
(14) *Fusconaia cuneolus*	0	7	13	4	1	10	17	52
(15) *Fusconaia subrotunda*	8	19	5	32	8	31	8	111
(16) *Hemistena lata*	9	28	0	21	12	29	5	104
(17) *Lampsilis fasciola*	18	28	34	52	28	56	40	256
(18) *Lampsilis ovata*	11	6	3	8	5	10	3	46
(19) *Lasmigona costata*	3	1	1	5	7	6	2	25
(20) *Lemiox rimosus*	1	15	8	17	0	28	1	70
(21) *Ligumia recta*	1	0	1	1	2	1	0	6
(22) *Margaritifera monodonta*	0	0	0	1	1	0	0	2
(23) *Medionidus conradicus*	167	1,105	574	973	289	1,305	648	5,061
(24) *Plethobasus cyphyus*	2	16	1	8	5	34	1	67
(25) *Pleurobema cordatum*	1	0	0	0	0	0	0	1
(26) *Pleurobema oviforme*	0	1	1	0	0	1	0	3
(27) *Pleurobema plenum*	0	14	0	7	3	27	0	51
(28) *Pleurobema rubrum*	1	0	0	0	1	0	0	2
(29) *Pleuronaia barnesiana*	1	1	0	1	1	2	0	6
(30) *Pleuronaia dolabelloides*	0	0	0	1	0	0	0	1
(31) *Potamilus alatus*	0	0	0	1	0	0	0	1
(32) *Ptychobranchus fasciolaris*	114	71	43	117	120	107	69	641
(33) *Ptychobranchus subtentus*	267	313	263	519	297	406	297	2,362
(34) *Strophitus undulatus*	0	1	0	3	1	5	0	10
(35) *Theliderma cylindrica*	1	5	1	6	3	9	0	25
(36) *Truncilla truncata*	0	0	0	1	0	0	0	1
(37) *Villosa iris*	1	20	21	29	12	44	22	149
(38) *Villosa vanuxemensis*	0	0	0	1	0	0	0	1
Total	2,015	3,863	1,824	3,332	2,169	4,694	1,921	19,818
Total species	28	27	25	33	29	30	21	38

Inc., Broussard, LA); we used the data to calculate the square area of suitable habitat.

We collected quantitative data by systematic 0.25 m^2 quadrat samples placed on transect lines positioned perpendicular along the width of the river. Both transects and quadrats were evenly spaced throughout the delineated shoal area. Quarter-meter quadrats were delineated using a 0.5 m × 0.5 m frame constructed of 12 mm diameter rebar welded at the corners. Using a mask and snorkel, surveyors visually searched for mussels while excavating substrate approximately 15–20 cm in depth within each quadrat. Live mussels were collected from quadrat excavations, placed in a mesh bag, and brought to the river bank for identification and measurement. We identified the mussels to species, and using digital calipers,

Table 3. Total count (from Table 2), proportion, density, and abundance category of live mussels for species sampled during quantitative quadrat surveys conducted at Swan Island, Frost Ford, Wallen Bend, and other sites (see Table 1) from 2004 to 2014 in the Clinch River, Hancock County, Tennessee. Categories include Abundant (>1/m^2), Common (<1–0.1/m^2), Uncommon (<0.1–0.01/m^2), and Rare (<0.01/m^2).

Species	Total Count	Proportion (%)	Density/m^2	Category
(1) *Medionidus conradicus*	5,061	25.54	7.47	Abundant
(2) *Epioblasma capsaeformis*	4,798	24.21	7.08	Abundant
(3) *Actinonaias pectorosa*	2,974	15.00	4.39	Abundant
(4) *Ptychobranchus subtentus*	2,362	11.92	3.49	Abundant
(5) *Actinonaias ligamentina*	1,528	7.71	2.26	Abundant
(6) *Ptychobranchus fasciolaris*	641	3.23	0.95	Common
(7) *Eurynia dilatata*	475	2.40	0.70	Common
(8) *Dromus dromas*	345	1.74	0.51	Common
(9) *Epioblasma brevidens*	318	1.60	0.47	Common
(10) *Lampsilis fasciola*	256	1.29	0.38	Common
(11) *Villosa iris*	149	0.75	0.22	Common
(12) *Cyclonaias tuberculata*	141	0.71	0.21	Common
(13) *Fusconaia subrotunda*	111	0.56	0.16	Common
(14) *Hemistena lata*	104	0.52	0.15	Common
(15) *Cyprogenia stegaria*	71	0.36	0.10	Common
(16) *Lemiox rimosus*	70	0.35	0.10	Common
(17) *Plethobasus cyphyus*	67	0.34	0.10	Common
(18) *Epioblasma triquetra*	59	0.30	0.09	Uncommon
(19) *Fusconaia cuneolus*	52	0.26	0.08	Uncommon
(20) *Pleurobema plenum*	51	0.26	0.07	Uncommon
(21) *Lampsilis ovata*	46	0.23	0.07	Uncommon
(22) *Fusconaia cor*	29	0.15	0.04	Uncommon
(23) *Lasmigona costata*	25	0.13	0.04	Uncommon
(24) *Theliderma cylindrica*	25	0.13	0.04	Uncommon
(25) *Cyclonaias pustulosa*	19	0.10	0.03	Uncommon
(26) *Strophitus undulatus*	10	0.05	0.01	Uncommon
(27) *Pleuronaia barnesiana*	6	0.03	0.009	Rare
(28) *Ligumia recta*	6	0.03	0.009	Rare
(29) *Alasmidonta marginata*	5	0.03	0.007	Rare
(30) *Pleurobema oviforme*	3	0.02	0.004	Rare
(31) *Margaritifera monodonta*	2	0.01	0.003	Rare
(32) *Amblema plicata*	2	0.01	0.003	Rare
(33) *Pleurobema rubrum*	2	0.01	0.003	Rare
(34) *Pleuronaia dolabelloides*	1	<0.01	0.001	Rare
(35) *Pleurobema cordatum*	1	<0.01	0.001	Rare
(36) *Potamilus alatus*	1	<0.01	0.001	Rare
(37) *Truncilla truncata*	1	<0.01	0.001	Rare
(38) *Villosa vanuxemensis*	1	<0.01	0.001	Rare
Totals	19,818	100	29.25	
Total ¼ m^2 quadrats excavated	2,721			

we measured for total shell length anterior to posterior (nearest 0.1 mm) before returning them to their approximate position of collection. Population densities (N/m^2) were calculated from the means of the quadrat samples at each site. Specifically, we multiplied mean density from 0.25 m^2 quadrat samples by four to derive an estimate of mean density per m^2 and then multiplied that figure by the total square area of each site to derive an estimate of mussel abundance. At PI, quadrat samples were collected in 2009 and 2014 from the entire left-descending channel, starting from the downstream end of the channel and ending at the upstream beginning of the channel (see Fig. 2 and red dots marked in left-descending channel). Ahlstedt et al. (2016) and Dennis (1989) collected quadrat samples from the upper third of the left-descending channel from 1979 to 2004.

Data analysis.—We used a generalized linear model (GLM) to test for significance of trends in the mussel assemblage time series data collected from 1979 to 2004 at

Table 4. Proportional percentage of each species in the Pendleton Island mussel assemblage in the Clinch River, Scott County, Virginia, from 1979 to 2014. Data are from quadrat sampling conducted by the following: [A]Ahlstedt et al. (2016), [B]Dennis (1989), [C]Jones et al. (2014), and [D]current study. Sample sizes per year are available in Table 1.

Scientific Name	1979[A]	1987[B]	1994[A]	1999[A]	2004[A]	2009[C]	2014[D]
(1) *Actinonaias ligamentina*	13.82	19.5	23.21	20.16	10.87	25.69	32.07
(2) *Actinonaias pectorosa*	14.63	21.9	30.36	34.68	39.13	17.08	13.20
(3) *Alasmidonta marginata*	0	0	0	0	0	0	0
(4) *Amblema plicata*	3.25	1.9	0.89	2.42	8.70	6.77	9.43
(5) *Cyclonaias pustulosa*	0	0	0	0	0	0	0
(6) *Cyclonaias tuberculata*	4.47	3.3	4.46	5.65	13.04	6.77	1.89
(7) *Cyprogenia stegaria*	0	0	0	0	0	0	0
(8) *Dromus dromas*	0	0	0	0	0	0	0
(9) *Elliptio crassidens*	0	0	0	0	0	0	0
(10) *Epioblasma brevidens*	0	0	0	0	0	1.69	1.89
(11) *Epioblasma capsaeformis*	3.25	0	0	0	0	0	0
(12) *Epioblasma gubernaculum*	0	0	0	0	0	0	0
(13) *Epioblasma triquetra*	0	0.5	0	0	0	0	0
(14) *Eurynia dilatata*	25.61	13.5	12.5	6.45	4.35	1.69	1.89
(15) *Fusconaia cor*	1.22	0	3.57	0	4.35	1.69	0
(16) *Fusconaia cuneolus*	4.47	11.6	2.68	5.65	0	0	0
(17) *Fusconaia subrotunda*	6.91	5.1	17.86	15.32	2.17	0	0
(18) *Hemistena lata*	0	0	0	0	0	0	0
(19) *Lampsilis abrupta*	0	0	0	0	0	0	0
(20) *Lampsilis fasciola*	0.81	1.4	0.89	2.42	0	1.69	3.77
(21) *Lampsilis ovata*	2.03	0.9	0	0	2.17	0	0
(22) *Lasmigona costata*	5.28	4.2	0.89	1.61	0	0	0
(23) *Lemiox rimosus*	0	0.5	0	0	0	1.69	0
(24) *Leptodea fragilis*	0.41	0	0	0	0	0	0
(25) *Ligumia recta*	0.41	0.5	0	0	0	0	0
(26) *Margaritifera monodonta*	0	0	0	0	0	0	0
(27) *Medionidus conradicus*	0.81	0	0	0.81	0	1.69	5.66
(28) *Plethobasus cyphyus*	0	0.5	0	0	0	0	0
(29) *Pleurobema cordatum*	0	0.5	0	0	0	0	0
(30) *Pleurobema oviforme*	0	0	0	0	0	0	0
(31) *Pleurobema rubrum*	0	0	0.89	0	0	0	0
(32) *Pleuronaia barnesiana*	0.41	6.5	0	0	0	0	0
(33) *Pleuronaia dolabelloides*	0	0	0	0	0	0	0
(34) *Potamilus alatus*	0.41	0	0	0	0	1.69	0
(35) *Ptychobranchus fasciolaris*	1.63	0.9	0.89	2.42	6.52	16.92	5.66
(36) *Ptychobranchus subtentus*	4.47	0.5	0.89	0.81	0	0	0
(37) *Strophitus undulatus*	0	0	0	0	0	0	0
(38) *Theliderma cylindrica*	5.28	0.9	0	0	0	1.69	0
(39) *Theliderma intermedia*	0	0	0	0	0	0	0
(40) *Theliderma sparsa*	0	0	0	0	0	0	0
(41) *Truncilla truncata*	0	0	0	0	0	0	0
(42) *Venustaconcha trabalis*	0.41	0.5	0	0	0	0	0
(43) *Villosa fabalis*	0	0	0	0	0	0	0
(44) *Villosa iris*	0	0.9	0	1.61	8.70	12.0	24.52
(45) *Villosa vanuxemensis*	0	0	0	0	0	1.69	0
Total individuals	246	206	112	124	46	59	53
Total species	21	21	13	13	10	15	10

Table 5. Relative proportion of each mussel species in the Clinch River at Pendleton Island (PI), Scott County, Virginia, and at sites in Hancock County, Tennessee, where A = data are from Table 3 and represent mean proportion of species at sites in Tennessee from 2004 to 2014; B = data are from Table 4 and represents proportion of each species in 1979 at PI; C = mean of data in columns A and B and represents proposed baseline proportion of each species for PI mussel assemblage; D = proposed baseline abundance of each species at PI based on a mussel assemblage density of 25/m^2.

Species	A	B	C	D
(1) *Actinonaias ligamentina*	7.71	13.82	10.755	149,234
(2) *Actinonaias pectorosa*	15.0	14.63	14.815	205,569
(3) *Alasmidonta marginata*	0.03	0	0.015	208
(4) *Amblema plicata*	0.01	3.25	1.63	22,617
(5) *Cyclonaias pustulosa*	0.01	0	0.005	69
(6) *Cyclonaias tuberculata*	0.71	4.47	2.58	35,799
(7) *Cyprogenia stegaria*	0.36	0	0.18	2,498
(8) *Dromus dromas*	1.74	0	0.87	12,072
(9) *Elliptio crassidens*	0	0	0	0
(10) *Epioblasma brevidens*	1.6	0	0.8	11,101
(11) *Epioblasma capsaeformis*	24.21	3.25	13.73	190,514
(12) *Epioblasma gubernaculum*	0	0	0	0
(13) *Epioblasma triquetra*	0.3	0	0.15	2,081
(14) *Eurynia dilatata*	2.4	25.61	14.005	194,330
(15) *Fusconaia cor*	0.15	1.22	0.685	9,505
(16) *Fusconaia cuneolus*	0.26	4.47	2.365	32,816
(17) *Fusconaia subrotunda*	0.56	6.91	3.725	51,687
(18) *Hemistena lata*	0.52	0	0.26	3,608
(19) *Lampsilis abrupta*	0	0	0	0
(20) *Lampsilis fasciola*	1.29	0.81	1.05	14,570
(21) *Lampsilis ovata*	0.23	2.03	1.13	15,680
(22) *Lasmigona costata*	0.13	5.28	2.705	37,534
(23) *Lemiox rimosus*	0.35	0	0.175	2,428
(24) *Leptodea fragilis*	0	0.41	0.205	2,845
(25) *Ligumia recta*	0.03	0.41	0.22	3,053
(26) *Margaritifera monodonta*	0.01	0	0.005	69
(27) *Medionidus conradicus*	25.54	0.81	13.165	182,674
(28) *Plethobasus cyphyus*	0.34	0	0.17	2,359
(29) *Pleurobema cordatum*	0.01	0	0.005	69
(30) *Pleurobema oviforme*	0.02	0	0.01	139
(31) *Pleurobema rubrum*	0.01	0	0.005	69
(32) *Fusconaia barnesiana*	0.03	0.41	0.22	3,053
(33) *Pleuronaia dolabelloides*	0.01	0	0.005	69
(34) *Potamilus alatus*	0.01	0.41	0.21	2,914
(35) *Ptychobranchus fasciolaris*	3.23	1.63	2.43	33,718
(36) *Ptychobranchus subtentus*	11.92	4.47	8.185	113,573
(37) *Strophitus undulatus*	0.5	0	0.25	3,469
(38) *Theliderma cylindrica*	0.13	5.28	2.705	37,534
(39) *Theliderma intermedia*	0	0	0	0
(40) *Theliderma sparsa*	0	0	0	0
(41) *Truncilla truncata*	0.01	0	0.005	69
(42) *Venustaconcha trabalis*	0	0.41	0.205	2,844
(43) *Villosa fabalis*	0	0	0	0
(44) *Villosa iris*	0.75	0	0.365	5,065
(45) *Villosa vanuxemensis*	0.01	0	0.005	69
Total				1,387,574

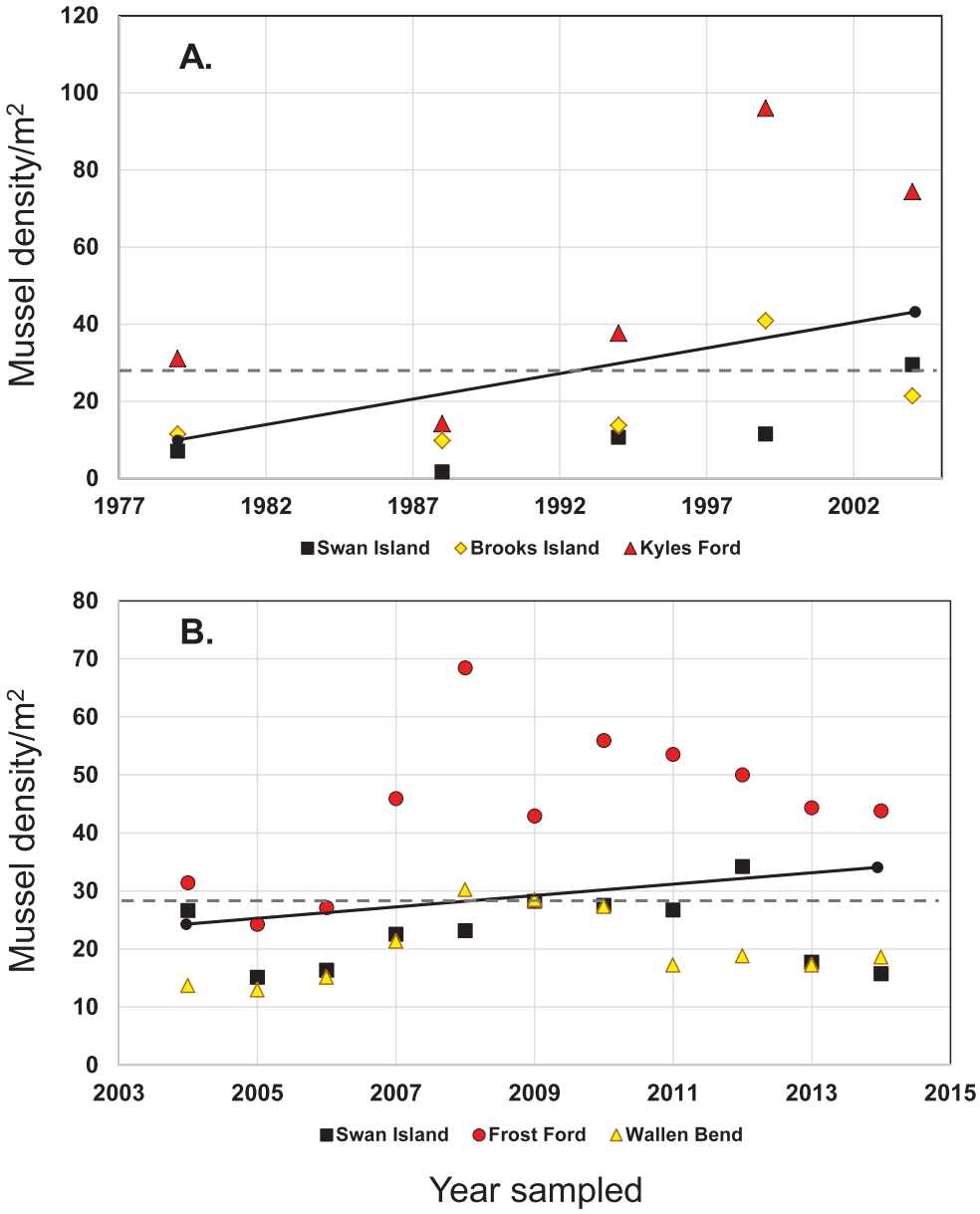

Figure 3. Panel A: Time series of mean mussel density from 1979 to 2004 at three sites, Swan Island, Brooks Island, and Kyles Ford, in the Clinch River, Hancock County, Tennessee; data are from Ahlstedt et al. (2016). Solid line is linear regression [y = 1.3982x + (–2,758.9174)] of increasing density over all sites ($P < 0.001$) and years, and broken horizontal line is mean (27.32/m^2) over all sites and years. Panel B: Time series of mean mussel density sample annually from 2004 to 2014 at three sites, Swan Island, Frost Ford, and Wallen Bend, in the Clinch River, Hancock County, Tennessee; data are from Jones et al. (2014) and from the current study. Solid line is linear regression [y = 0.8861x + (–1,751.0382)] of increasing density over all sites ($P = 0.003$) and years, and broken horizontal line is mean (29.1/m^2) over all sites and years. Density data from all sites are available in Table 1.

SI, Brooks Island, and Kyles Ford and from 2004 to 2014 at SI, FF, and WB, and from 1979 to 2014 at PI by utilizing data from Ahlstedt et al. (2016), Jones et al. (2014), and the current study. A GLM also was used to test for significance of trends in density for six mussel species at SI, FF, and WB from 2004 to 2014. We tested data for normality using the Shapiro-Wilk test; data were not normally distributed and modeled in the GLM using a Poisson distribution and the log-link function. We used simple linear regression to produce trend lines for assemblage density data. To test for differences in mussel assemblage density at PI between the 2009 and 2014 sample years, we used a two-sample *t*-test assuming unequal variances. All statistical tests were implemented using the program R (R Core Development Team 2006).

RESULTS

Baseline conditions, Clinch River, Tennessee.—Mussel density significantly increased ($P < 0.001$) at monitoring sites

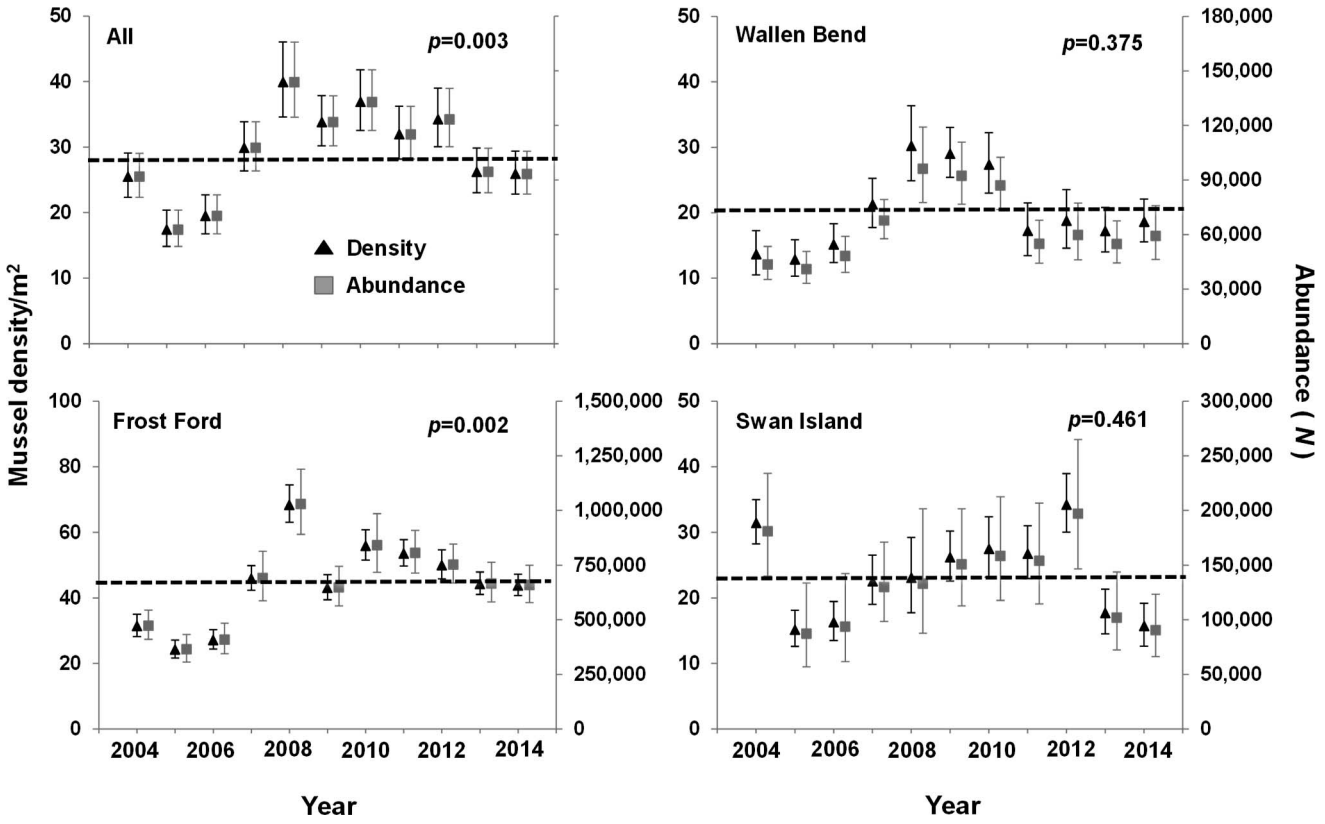

Figure 4. Time series of mean mussel density from 2004 to 2014 at Swan Island, Frost Ford, and Wallen Bend in the Clinch River, Hancock County, Tennessee. Error bars represent 95% confidence intervals, where nonoverlapping intervals among sample years indicate significant ($P < 0.05$) differences. Broken line is the mean of all years. Data from 2004 to 2009 are from Jones et al. (2014).

in the Tennessee section of the Clinch River over a 25-yr period from a mean of 16.5/m^2 in 1979 to a mean of 41.7/m^2 in 2004; mean density at sampled sites over this period was 27.1/m^2 (Table 1 and Fig. 3). Using the regression equation in Figure 3 [y = 1.3982x + (–2,758.9174)], predicted density in 1979 was 8.1/m^2, and in 2004 it was 43.1/m^2. The corresponding mussel assemblage growth rate over this period was 81%, an increase of 2.26% per year. Site density was lowest at SI in 1988 at 1.6/m^2 and highest at Kyles Ford in 1999 at 95.9/m^2 (Table 1). Of the three sites (SI, FF, WB) sampled annually from 2004 to 2014, mussel density significantly increased ($P = 0.003$) at monitoring sites over this 10-yr period in the Tennessee section of the Clinch River from a mean of 23.9/m^2 in 2004 to a mean of 26.0/m^2 in 2014; mean density at sampled sites over this period was 29.1/m^2 (Table 1 and Fig. 3). Using the regression equation in Figure 3 [y = 0.8861x + (–1,751.0382)], predicted density in 2004 was 24.7/m^2, and in 2014 it was 33.6/m^2. The corresponding mussel assemblage growth rate over this period was 27%, an increase of 1.3% per year. Mean density and abundance were highest at FF at 44.3/m^2 and 666,715 mussels, where density increased significantly ($P = 0.002$) over the study period, followed by SI at 22.8/m^2 and 131,328 mussels and WB at 20.1/m^2 and 63,958 mussels, where density remained stable and did not significantly increase or decrease (Fig. 4). Densities and abundances fluctuated at these three sites, reaching their highest levels at FF and WB in 2008 and SI in 2012 and their lowest levels at all three sites in 2005. By 2014, mussel density at all three sites had returned to at or below each site's mean density.

Including samples from 2004 to 2009 (Jones et al. 2014), a total of 38 mussel species were collected live in quadrat samples at the 12 sites in the Tennessee section of the Clinch River from 2004 to 2014 (Table 2). All species sampled over this 10-yr period also were sampled from 2004 to 2009; therefore, no additional species were sampled from 2010 to 2014, with the following six species not collected during this latter period: *Amblema plicata*, *Pleurobema cordatum*, *Pleuronaia dolabelloides*, *Potamilus alatus*, *Truncilla truncata*, and *Villosa vanuxemensis*. We sampled a total of 30 species at SI, with 28 species sampled from 2004 to 2009 and 29 species sampled from 2010 to 2014. A total of 30 species were sampled at FF, with 27 species sampled from 2004 to 2009 and 29 species sampled from 2010 to 2014. A total of 28 species were sampled at WB, with 25 species sampled from 2004 to 2009, and 21 species sampled from 2010 to 2014. We sampled a total of 33 species at other sites from 2004 to 2009, with the aforementioned six species sampled only at these sites and not at SI, FF, and WB.

Five lampsiline species were abundant, each typically

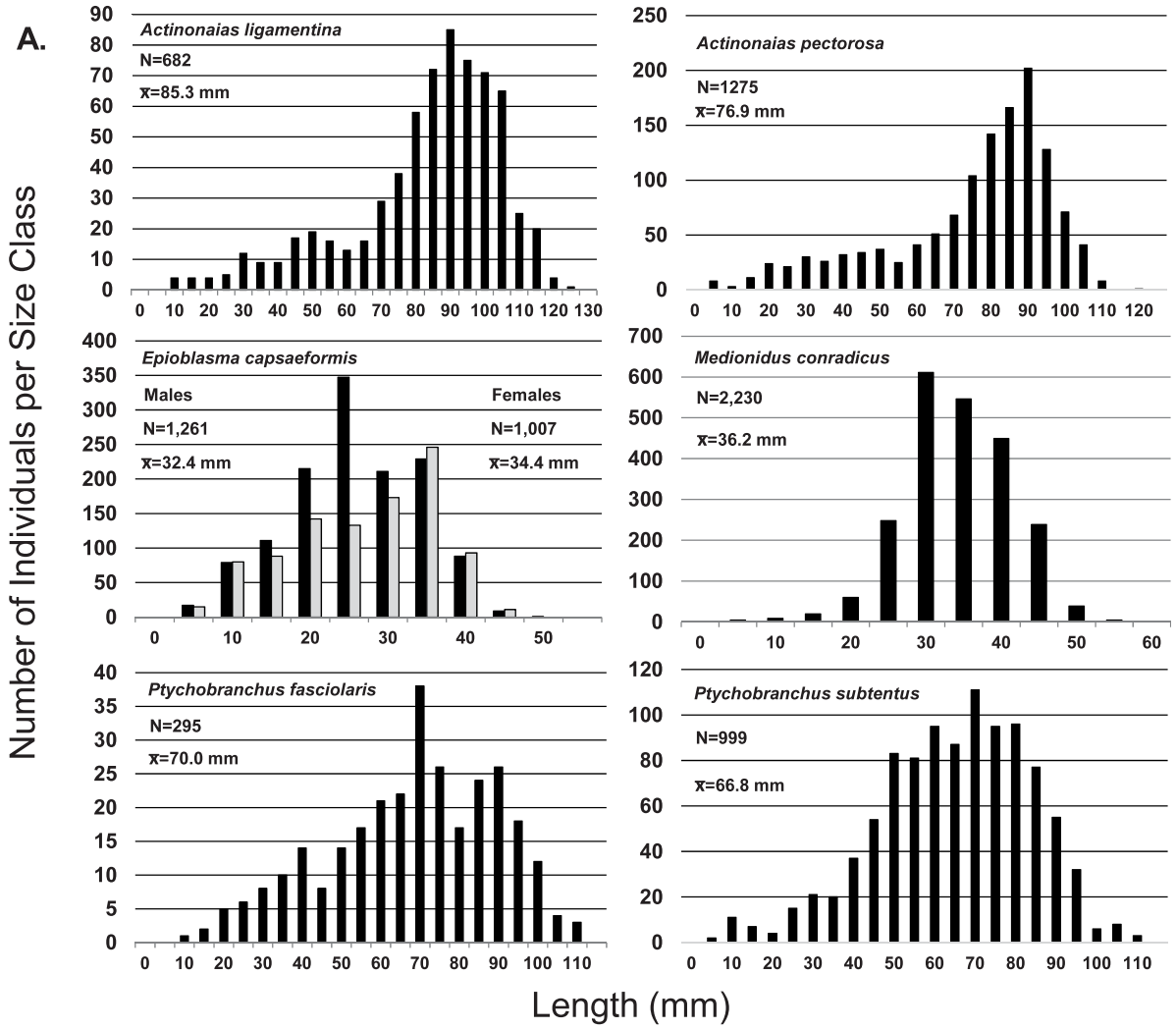

Figure 5. Panel A: Length frequency histograms of the six most abundant species sampled from 2010 to 2014 in the Clinch River, Hancock County, Tennessee. Panel B: Length frequency histograms of the six most abundant species sampled in 2009 and 2014 at Pendleton Island in the Clinch River, Scott County, Virginia.

occurring at a density >1/m^2 and together making up >80% of the assemblage: *Medionidus conradicus* (25.5%), *Epioblasma capsaeformis* (24.2%), *Actinonaias pectorosa* (15.0%), *Ptychobranchus subtentus* (11.9%), and *A. ligamentina* (7.7%). In addition, *Ptychobranchus fasciolaris* (3.2%) was common (Table 3). These six species' size-class frequency distributions indicate they have been recruiting annually from 2004 to 2014, with smaller, younger mussels well represented in samples (Fig. 5). Individuals of the relatively short-lived *M. conradicus* and *E. capsaeformis* <20–30 mm were typically ~3–4 yr old (Scott 1994; Jones and Neves 2011), while individuals <50–70 mm of the latter four longer-lived species were typically ~4–5 yr old (Scott 1994; Henley et al. 2001). Densities of these six species varied among sites and years at the three sites sampled annually (Fig. 6). Density of *A. ligamentina* at SI ranged from a low of 2.6/m^2 in 2014 to high of 7.1/m^2 in 2004; at FF, it ranged from 1.4/m^2 in 2005 to 2.5/m^2 in 2010; at WB, it ranged from 0.9/m^2 in 2012 to 2.3/m^2 in 2006. Density of *A. pectorosa* at SI ranged from a low of 5.1/m^2 in 2006 to a high of 11.5/m^2 in 2012; at FF, it ranged from 1.2/m^2 in 2005 to 3.0/m^2 in 2014; at WB, it ranged from 1.0/m^2 in 2004 to 2.9/m^2 in both 2007 and 2009. In contrast, density of *E. capsaeformis* fluctuated greatly at all three sites. At SI, its initial density was 0.7/m^2 in 2004; it reached a low of 0.1/m^2 in 2006, increased to 3.5/m^2 in 2012, and then decreased to 1.3/m^2 by 2014. Similarly, at FF, the initial density of *E. capsaeformis* was 7.5/m^2 in 2004; it reached a low of 5.4/m^2 in 2005, increased to 40.0/m^2 in 2008, and then decreased to 13.9/m^2 by 2014. The same pattern prevailed at WB, where initial density was 1.9/m^2 in 2004; it decreased to 1.3/m^2 in 2006, increased to 8.9/m^2 in 2008, and then decreased to 3.0/m^2 by 2014. The density of *M. conradicus* also fluctuated greatly at each site. At SI, its initial density was 1.9/m^2 in 2004; it reached a low of 0.5/m^2 in 2005, increased to 4.6/m^2 in 2012, then decreased to 1.6/m^2 by 2014. At FF, its density reached a low of 9.3/m^2 in 2006 and a high of 16.6/m^2 in 2010, and at WB, it reached a low of 3.8/m^2 in 2004 and a high of 11.5/m^2 in 2010. By comparison,

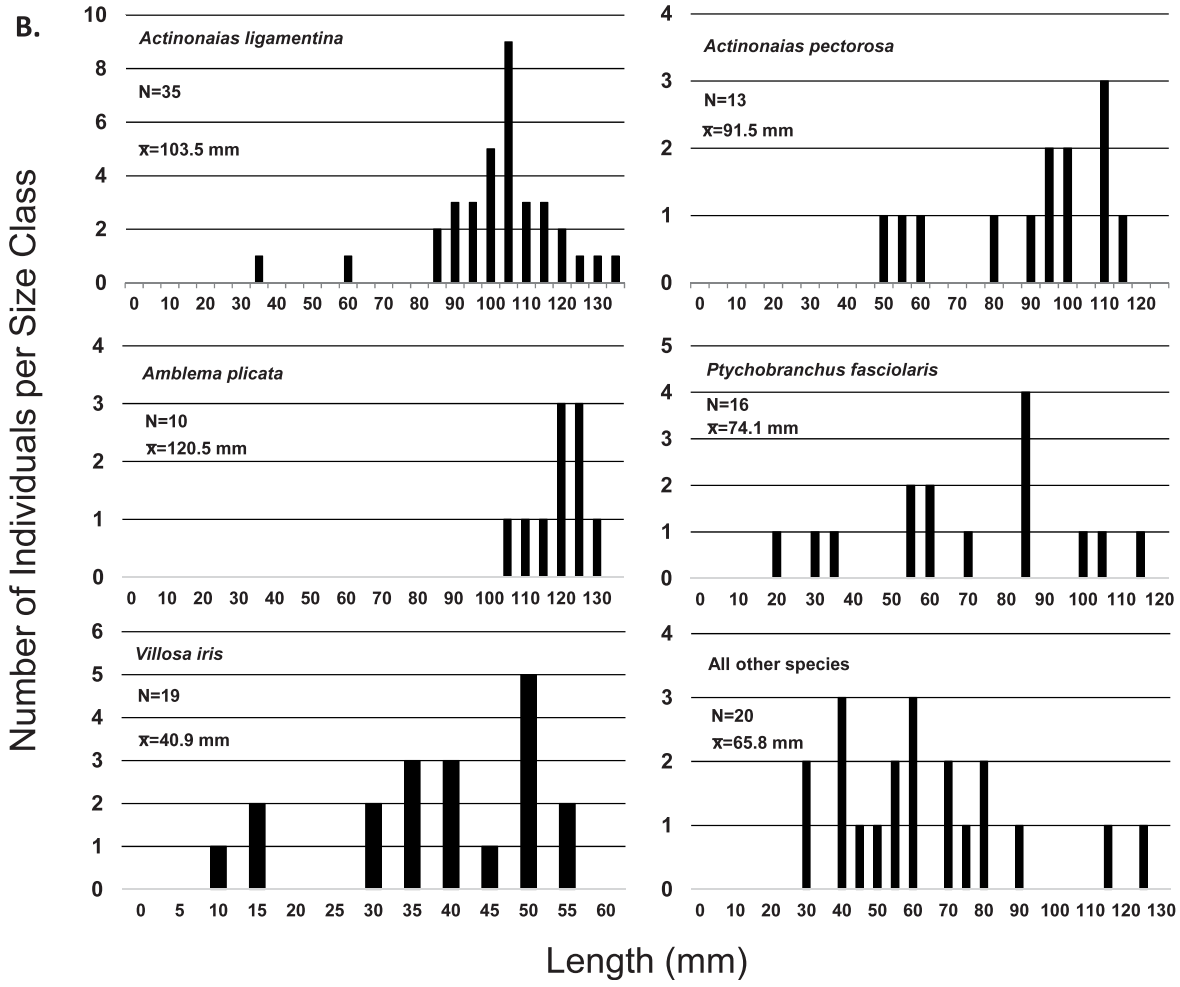

Figure 5, continued.

densities of *P. fasciolaris* were lower and more similar among sites and years, ranging at SI from 0.9/m^2 in 2005 to 1.7/m^2 in 2006, at FF from 0.4/m^2 in 2004 to 1.7/m^2 in 2013, and at WB from 0.3/m^2 in 2005 to 0.9/m^2 in 2012. Densities of *P. subtentus* also were similar among sites and years, ranging at SI from 1.6/m^2 in 2006 to 5.6/m^2 in 2004, at FF from 2.4/m^2 in 2007 to 4.9/m^2 in 2010, and at WB from 1.4/m^2 in 2005 to 4.7/m^2 in 2010. Despite the annual fluctuations at these three sites, densities of all six species remained stable and did not statistically increase or decrease from 2004 to 2014 (Fig. 6). All other species were common, uncommon, or rare in Tennessee, typically occurring at densities $<$1/m^2 per site, and collectively making up $<$10% of total abundance (Table 3). Specifically, 21 species were uncommon or rare, occurring at densities $<$0.10/m^2 and represented more than half of assemblage richness.

Baseline conditions, Pendleton Island, Virginia.—Of the 45 mussel species known from the Clinch River at Pendleton Island (PI), 29 have been collected live in quadrat samples from 1979 to 2014 (Table 4). The remaining 16 species, with the exception of *Villosa fabalis* (shell only), have been collected live from 1979 to 2014 but not in quadrat samples. Many of these species (e.g., *Epioblasma torulosa gubernaculum*, *Lampsilis abrupta*, *Quadrula intermedia*, and *Quadrula sparsa*) were represented by the collection of a single live individual in 1982 and 1983 during numerous visits to the site to assess species richness (R. J. Neves, USGS retired, personal communication). However, at least 29 species have been collected live or as fresh-dead shells during qualitative surveys since 1994 and thus are likely still extant at the site (see the Appendix). The greatest number of species sampled quantitatively was 21, which occurred in 1979 and 1987 when quadrat sample sizes were lower (40 and 22, respectively). In contrast, during the most recent sampling efforts (conducted in 2009 and 2014), a total of 15 and 10 species were observed, respectively, when quadrat sample sizes were much higher (360 and 187, respectively). Five species made up most of the relative abundance at PI in 2009 and 2014: *A. ligamentina* (2009 = 25.7%, 2014 = 32.1%), *Villosa iris* (2009 = 12.0%, 2014 = 24.5%), *A. pectorosa* (2009 = 17.1%, 2014 = 13.2%), *P. fasciolaris* (2009 = 16.9%, 2014 = 5.7%), and *A. plicata* (2009 = 6.8%, 2014 = 9.4%) (Table 4). Based on observed

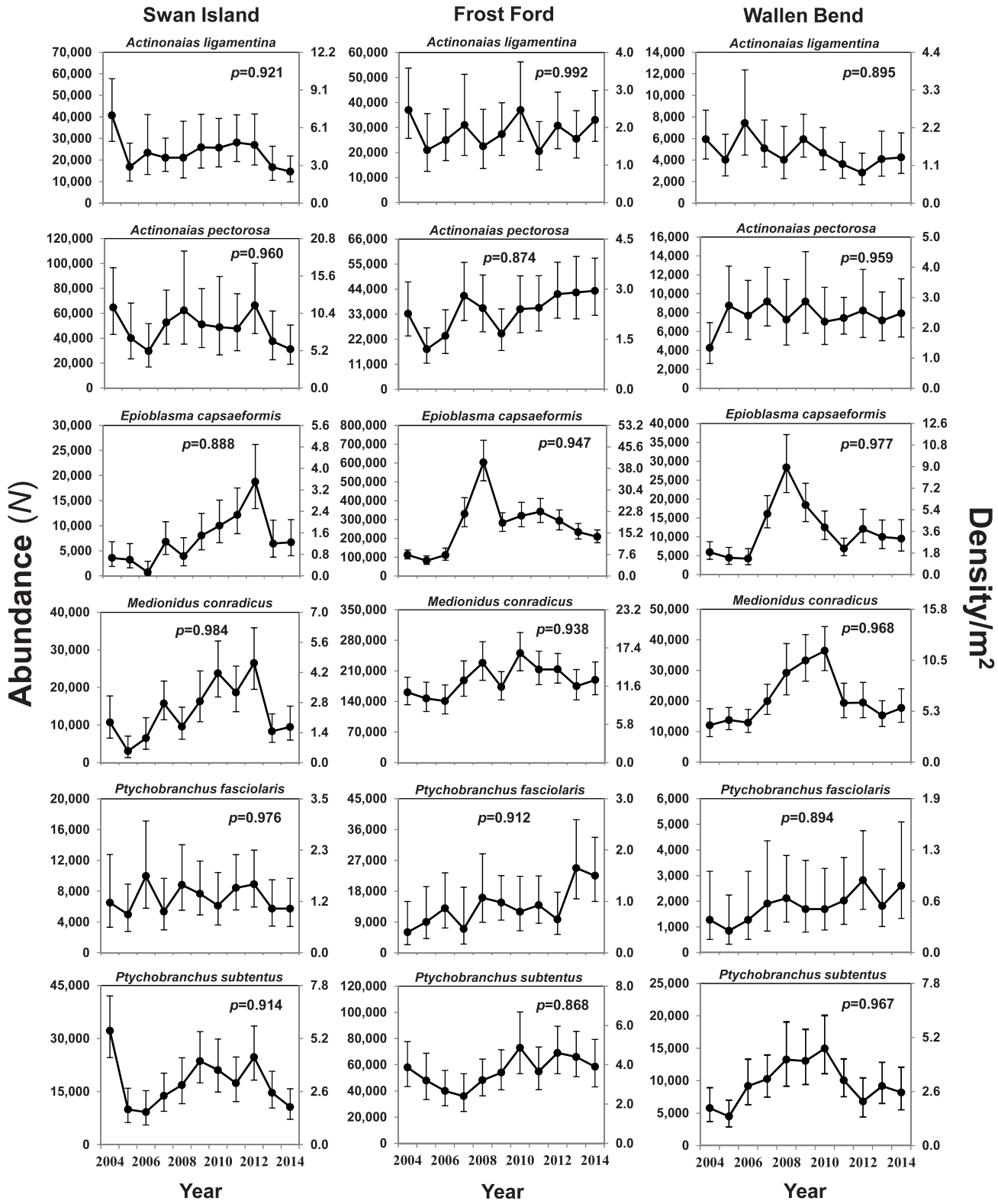

Figure 6. Time series of mean mussel density of the six most abundant species sampled annually from 2004 to 2014 at Swan Island, Frost Ford, and Wallen Bend in the Clinch River, Hancock County, Tennessee. None of the time series were significant over the study period. However, error bars represent 95% confidence intervals, where nonoverlapping intervals among sites or sample years indicate significant ($P < 0.05$) differences. Data from 2004 to 2008 for *Epioblasma capsaeformis* are from Jones and Neves (2011), and data from 2004 to 2009 for the other five species are from Jones et al. (2014).

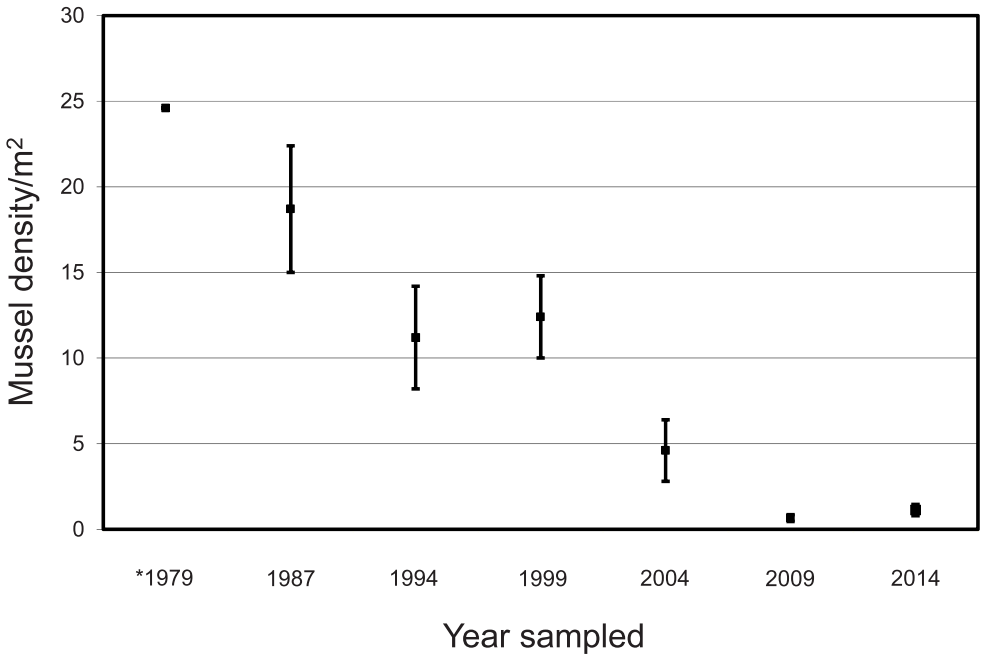

Figure 7. Time series of mean mussel density from 1979 to 2014 at Pendleton Island, Clinch River, Virginia, showing a severe and statistically significant decline in density ($P < 0.001$); data from 1979 to 2004 were collected by Ahlstedt et al. (2016) using a random survey design. Error bars represent 95% confidence intervals, where nonoverlapping intervals among sites or sample years indicate significant ($P < 0.05$) differences. Error bars are present for 2009 and 2014. *Raw data from 1979 were unavailable, but SE and 95% confidence intervals were likely similar to other sample years at the site with similar mean densities, e.g., the sample taken in 1987.

size-class frequency distributions and the presence of individuals <30–50 mm long, four of these species show evidence of recent recruitment (Fig. 5B). No small individuals of *A. plicata* were observed in either sampling year, but small and young individuals (<5 yr) of other species (e.g., *Lampsilis fasciola* and *M. conradicus*) were observed.

Our data, combined with that of Jones et al. (2014) and Ahlstedt et al. (2016) (see Fig. 7), demonstrate the following pattern in density and abundance of the mussel assemblage at PI: (1) in the late 1970s, density was ~25/m^2 with an abundance of nearly 1.4 million individuals, (2) from 1979 to 2009, density and abundance significantly declined ($P < 0.001$) to 0.7/m^2 and an abundance of ~37,000 individuals, and (3) from 2009 to 2014, density remained low but increased to 1.1/m^2 with an abundance of 50,000–60,000 individuals, significantly higher ($P = 0.005$) than the 2009 sample (Table 1 and Fig. 8). Mussel density upstream at Semones Island was ~8/m^2 in the early 1980s and then declined to 0.6/m^2 in 2009 (Jones et al. 2014) and 0.7/m^2 in 2014 (Table 1).

Projecting density and abundance forward 30 yr (2014–44), the mussel assemblage of PI returns to a baseline of 25/m^2 only if we assume a high growth rate of 15% per yr. If we assume a growth rate of 10% per year, density reaches 17.4/m^2 and an abundance of ~968,000 individuals in 2044. Under lower and arguably more realistic growth rate scenarios of 1% and 5%, density reaches just 1.4/m^2 (~75,000 individuals) and 4.3/m^2 (~240,000 individuals), respectively, by 2044.

Finally, we based mussel losses over time on departures in density and abundance from the baseline of 25/m^2 and an abundance of 1,387,575 mussels (Fig. 8). Losses can be viewed as follows: (1) the absolute loss (difference) in density or abundance from baseline in a specific year, (2) the total cumulative loss in density or abundance over a specified time period, (3) loss of ecosystem services provided by mussels in a specific year, and (4) the cumulative loss of services provided by mussels over a specified period. In 2014, for example, the absolute loss in mussel density was 24/m^2 with a loss in abundance of 1,332,072 mussels. In 2014, the ecosystem services provided by an equivalent of 1,332,072 mussels were lost; however, from 1979 to 2014, the cumulative ecosystem services provided by an equivalent of 27,514,980 mussels were lost. When these losses are projected forward in time, cumulatively, they can be staggering. For example, under the 1% growth rate scenario, from 1979 to 2044, the ecosystem services provided by an equivalent of 67.2 million mussel years would be lost (Fig. 8, panel B).

DISCUSSION

Patterns of Mussel Abundance and Species Richness in Tennessee

As demonstrated by Jones et al. (2014), Ahlstedt et al. (2016), and again in our study, it is evident that two broad and ongoing patterns of mussel abundance occur in the Clinch River, one of relative stability and high abundance in

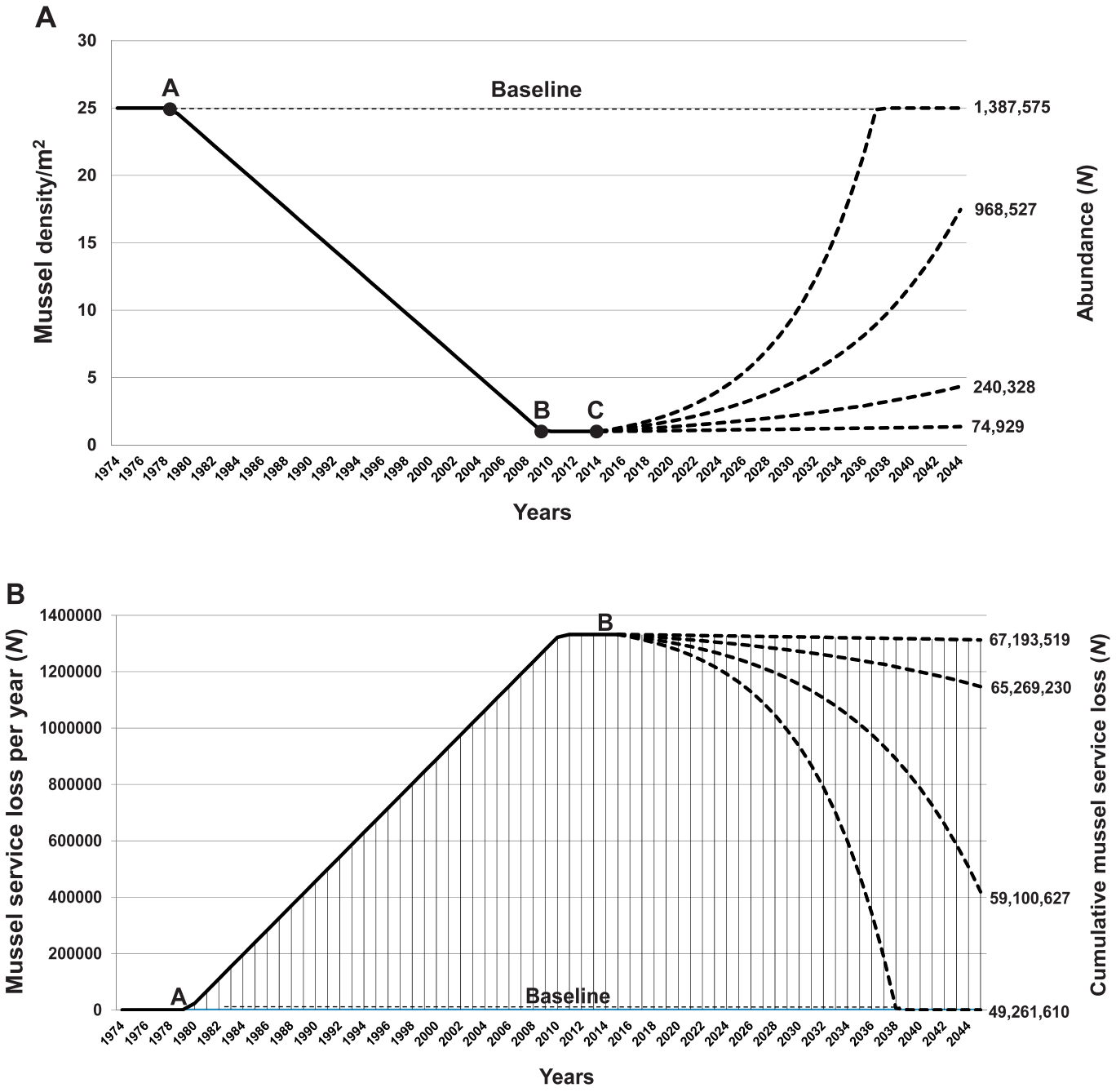

Figure 8. Panel A: Historical baseline mussel density of 25/m^2 at Pendleton Island, Scott County, Virginia, is represented by horizontal broken line beginning in 1979 and projected into the future until 2044. Point A represents the measured density of 25/m^2 sampled by in 1979 by Ahlstedt et al. (2016), and Point B represents the measured density of ~1/m^2 sampled in 2009 by Jones et al. (2014). Declining solid straight line between points A and B is a linear regression (y = 1,568 − 0.7797*X) of the mean mussel density values from 1979 to 2009 shown in Figure 7. The horizontal straight line between points B and C represents a mean mussel density of ~1/m^2 from 2009 to 2014. The broken curved lines represent the mussel assemblage increasing over time from 2014 until 2044 at a rate of 1%, 5%, 10%, and 15%, respectively. Panel B: Inverse mussel density curves of the mussel assemblage at Pendleton Island are shown to illustrate per year and cumulative mussel service losses from 1979 to 2044, using the above growth rates, where point A is 1979 and point B is 2014.

Tennessee, and one of severe decline and low abundance at PI and other sites in a 68-km section of the river from St. Paul, Virginia, downstream to approximately Clinchport, Virginia (RKM 411.5 to 343.3). In Tennessee, mussel abundance has steadily increased since the late 1970s, and beginning around 2004, it stabilized at the current density of nearly 30/m^2. In this section of river, mussel abundance varies among sites, years, and species, but we consider these fluctuations largely natural, driven by environmental factors such as stream discharge and fluctuations in host fish abundance, and

generally not by anthropogenic stressors (Jones and Neves 2011).

Of the 38 mussel species sampled quantitatively in the Tennessee section of the river, six species made up the majority of mussel abundance from 2004 to 2014, and they exhibited marked differences in their population dynamics over time. For example, populations of *E. capsaeformis* and *M. conradicus* have been more variable, characterized by obvious periods of increase and decrease, and have driven much of the variability in assemblage density over time. The increase in abundance of *E. capsaeformis* was due to high recruitment of juveniles in 2008 and 2009, likely in response to favorable survival and growing conditions for both the species and its host fishes during the low stream discharges that occurred in the summers of 2007 and 2008 (Jones and Neves 2011). In contrast, populations of *A. ligamentina*, *A. pectorosa*, *P. fasciolaris*, and *P. subtentus* also made up a large percentage of the assemblage density in Tennessee, but their numbers were less variable; their populations were best characterized as stable over the study period. For all six species, recruitment occurred regularly, with juvenile mussels a dominant and easily measurable part of their population age class structure.

The remaining 32 species were much less abundant, and their population trends were difficult to discern because sampling variability masked population changes. Even under seemingly excellent water quality and habitat conditions in the Tennessee section of the river, these species occurred at densities $<1/m^2$. Thirteen of these species are listed as endangered; the Clinch River populations represent some of the best or only remaining populations of each respective species rangewide, and they are critical to the species' long-term survival. However, many of these species are locally uncommon or rare and have much larger populations in other sections of the river upstream in Virginia, in tributaries to the river, and in streams outside of the Clinch River drainage. For many of these species, the presence of a few small individuals in quadrat samples provides evidence of recruitment; hence, we expect them to persist as relatively small populations over the coming decades.

Setting Baseline Conditions at Pendleton Island, Virginia

The mussel assemblage at PI now occurs at a density far below its historically documented baseline of nearly $25/m^2$. Based on our interpretation of existing data, we believe that assemblage density prior to 1979 was likely higher. PI is the largest site in the river upstream of Norris Reservoir and historically contained the highest species richness. Thus, prior to habitat degradation, and assuming normal habitat conditions such as the current conditions in Tennessee, these two factors—large habitat size and historical species richness—suggest that PI is capable of supporting a mussel assemblage at even higher densities. Sites in Tennessee certainly have supported much higher mussel densities, for example, $95.9/m^2$ at Kyles Ford in 1999 and $68.4/m^2$ at FF in 2008 (Table 1). It is possible that the assemblage at PI was in decline prior to 1979. Shorter-lived species such as *E. capsaeformis* and *Leptodea fragilis* were rare then, and by 1983, the assemblage comprised mostly larger and older mussels, suggesting that recruitment of juveniles was failing (R. J. Neves, U.S. Geological Survey, retired, personal communication). Thus, a density of $25/m^2$ should be considered the minimum baseline for the mussel assemblage at PI.

Based on quadrat sampling from 1979 to 2014, species composition at PI has shifted in two ways. First, fewer species are present in the samples. In 2009 and 2014, species once common at the site were absent from sampling, as were many of the rare and endangered species that were regularly detected there. Notable examples include *Fusconaia cuneolus*, *Fusconaia subrotunda*, *Lasmigona costata*, and *P. subtentus*, all of which were common in the late 1970s and early 1980s. Other species, such as *Ligumia recta*, *Plethobasus cyphyus*, *Pleurobema rubrum*, and *Venustaconcha trabalis*, which were always rare at the site, are now extremely rare or are extirpated. Many of the extremely rare species that were never detected in quadrat samples but were detected by qualitative sampling techniques used at the site over the years, are likely extirpated, including *Hemistena lata*, *L. abrupta*, *T. truncata*, *Theliderma intermedia*, and *Theliderma sparsa*, none of which have been seen live in decades. Second, the mussel assemblage today is dominated by species that are either long-lived and/or tolerant to disturbances in the Upper Tennessee River system, namely, *A. ligamentina*, *A. pectorosa*, *A. plicata*, *L. fasciola*, *P. fasciolaris*, and *V. iris*. Proportional species compositions and abundances for PI, shown in Table 5, can be used as targets to evaluate recovery and restoration of the site in the future.

Recovery and Valuation of Mussels at Pendleton Island, Virginia

Assuming environmental conditions improve at PI, it will take decades for the mussel fauna to naturally recover. Because of the extirpation or extinction of some species at the site and throughout the river, and assuming populations comprising the assemblage grow at a low annual rate (1–2%), recovery to baseline density and species richness most likely is not possible over the next 30 yr without human intervention. The relatively intact mussel assemblage in the Tennessee section of the river, for example, grew at only ~2% per year from 1979 to 2014 (Fig. 3). It seems prudent and justified to assume low but arguably realistic population growth rates into the future at PI. Clearly, the magnitude of lost mussel resources and services at PI is extremely large, and our analysis does not include assessment of other sites (e.g., Semones Island) in the ~68-km affected reach, which would easily double estimated losses. Both valuation and a better understanding of the ecological consequences of these lost mussel resources and services are required so that natural resource managers can better understand the cost, recovery, and restoration needs of the fauna.

As a natural resource, mussels can be valued in a variety of ways, including for the market price of their shells, for the broad range of ecosystem services they provide (including biofiltration, bioturbation of sediments, nutrient cycling and storage, habitat/habitat modification, environmental monitoring, food for other species, food for humans, products from shells such as jewelry, cultural value, existence value; see Vaughn 2017 for review) and for the cost to replace them if they are destroyed by a chemical spill or other anthropogenic disturbance (Southwick and Loftus 2017). Due to the high number of endangered species that occur in the Clinch River, and because the waterway is a designated mussel sanctuary in Tennessee, it is currently illegal to harvest mussels from the river in either Tennessee or Virginia. Therefore, no legal commercial market exists to put a dollar value on the mussel resource at PI. Moreover, while ecosystem services provided by mussels are likely substantial—increased productivity of benthic communities, sequestration of excess nutrients and contaminants, and food resources provided to both commercially and recreationally valuable fishes and other animals—the dollar values per type and unit of service are not available yet for mussels. For example, ecosystem services provided by healthy oyster reefs, excluding oyster harvesting, have been valued at $5,500 to $99,000 per hectare per year (Grabowski et al. 2012), yet corresponding estimates of the dollar value per hectare per year of a healthy mussel bed have not been established. Although we are unable to place a dollar value on the ecosystem services that could be provided by restored mussel assemblages, we can make attempts to estimate their replacement costs, for example, the cost to produce them at a hatchery to a size suitable for stocking (typically 20–30 mm and 1–3 yr old). Currently, prices range between $25.97 to $129.30 per mussel, depending on the ease or difficulty in producing individuals of a species (Southwick and Loftus 2017). Thus, just the replacement costs to return mussel populations to baseline levels at PI—not considering the value of lost ecosystem services—would easily be in the tens of millions of dollars.

SUMMARY AND CONCLUSIONS

Collection of long-term quantitative density data over the last 35 yr (1979–2014) has been critical to determining assemblage-level and population-level Clinch River mussel density trends in Tennessee and Virginia. It is now clear that the mussel assemblage in Tennessee has increased in density over this period and has stabilized at a mean density of $\sim$29/m^2. The long-term monitoring data collected in Tennessee can serve as a baseline to judge species recovery and restoration efforts in upstream reaches of the Clinch River in Virginia and in other streams throughout the Tennessee River drainage. The mussel fauna at PI has declined by 96% from its historically documented baseline density of 25/m^2 in 1979 to its current density of $\sim$1/m^2. It is possible that the significant increase in density at PI we observed from 2009 to 2014 represents natural recovery at the site. However, future sampling will be needed to confirm if this increase in density is a true upward trend. The lost mussel abundance and ecosystem services at the site represent more than a million mussels and tens of millions of lost mussel service years. Recovery of the assemblage to baseline or higher densities likely will take decades and will require active restoration of the fauna and their habitat. With exception of the presumed extinct *Epioblasma gubernaculum*, species extirpated from PI could be restored to the site using population sources downstream in the Tennessee section of the river and from other rivers. However, if water and sediment quality remain poor and do not improve over time, natural recovery and active restoration of the mussel assemblage at PI and nearby sites may not be possible for decades and could represent a permanent loss of ecological integrity and ecosystem services in the Clinch River.

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Appendix

Table A1. Counts of live mussels per species collected in the Clinch River at Pendleton Island, Scott County, Virginia, during qualitative surveys conducted from 1994 to 2016. Sample data are from [1]The Nature Conservancy unpublished data of survey of shell middens at Pendleton Island in 1994, [2]Neves and Beaty (1996), [3]Beaty and Neves (1997), [4]Beaty and Neves (1998), [5]Jones and Neves (1999), [6]Ahlstedt et al. (2005), [7]Jones and Beaty unpublished data (2014), and [8]Jones and Beaty unpublished data (2016).

Species	1994[1]	1996[2]	1997[3]	1998[4]	1999[5]	2004[7]	2014[7]	2016[8]
(1) *Actinonaias ligamentina*	51	51	81	77	95	138	107	214
(2) *Actinonaias pectorosa*	14	59	19	33	56	126	28	131
(3) *Alasmidonta marginata*	0	0	0	0	0	0	0	0
(4) *Amblema plicata*	21	140	78	35	68	88	21	66
(5) *Cyclonaias pustulosa*	0	0	0	0	0	0	0	0
(6) *Cyclonaias tuberculata*	3	10	16	9	12	37	18	36
(7) *Cyprogenia stegaria*	0	0	0	0	0	0	0	0
(8) *Dromus dromas*	0	0	0	0	0	0	0	0
(9) *Elliptio crassidens*	0	0	0	0	0	0	0	0
(10) *Eurynaia dilatata*	14	1	0	2	0	0	7	31
(11) *Epioblasma brevidens*	0	0	0	0	0	0	0	2
(12) *Epioblasma capsaeformis*	0	0	0	0	0	0	0	0
(13) *Epioblasma gubernaculum*	0	0	0	0	0	0	0	0
(14) *Epioblasma triquetra*	0	0	0	0	0	0	0	1
(15) *Fusconaia cor*	6	2	3	3	0	6	0	1
(16) *Fusconaia cuneolus*	17	8	2	2	4	3	1	2
(17) *Fusconaia subrotunda*	6	25	17	56	44	10	3	7
(18) *Hemistena lata*	0	0	0	0	0	0	0	0
(19) *Lampsilis abrupta*	0	0	0	0	0	0	0	0
(20) *Lampsilis fasciola*	7	0	0	1	0	14	4	4
(21) *Lampsilis ovata*	2	0	11	2	0	1	1	0
(22) *Lasmigona costata*	2	0	6	3	6	0	1	2
(23) *Lemiox rimosus*	0	0	0	0	0	3	0	0
(24) *Leptodea fragilis*	0	0	0	0	0	0	0	0
(25) *Ligumia recta*	2	0	2	0	1	0	0	1
(26) *Margaritifera monodonta*	0	0	0	0	1	0	0	0
(27) *Medionidus conradicus*	1	0	0	0	0	0	0	2
(28) *Plethobasus cyphyus*	1	2	0	1	0	3	1	0
(29) *Pleurobema cordatum*	0	0	0	0	0	0	0	0
(30) *Pleurobema oviforme*	5	1	6	0	1	0	1	0
(31) *Pleurobema rubrum*	1	0	0	0	0	0	0	0
(32) *Pleuronaia barnesiana*	0	3	0	2	0	0	0	0
(33) *Pleuronaia dolabelloides*	2	0	0	8	6	0	0	0
(34) *Potamilus alatus*	3	23	18	9	0	0	0	0
(35) *Ptychobranchus fasciolaris*	2	8	5	8	5	16	15	66
(36) *Ptychobranchus subtentus*	0	0	5	3	0	6	0	5
(37) *Strophitus undulatus*	0	0	0	0	0	0	0	0
(38) *Theliderma cylindrica*	5	0	1	0	3	1	0	1
(39) *Theliderma intermedia*	0	0	0	0	0	0	0	0
(40) *Theliderma sparsa*	0	0	0	0	0	0	0	0
(41) *Truncilla truncata*	0	0	0	0	0	0	0	0
(42) *Venustaconcha trabalis*	0	0	1	0	0	0	0	0
(43) *Villosa fabalis*	0	0	0	0	0	0	0	0
(44) *Villosa iris*	10	0	0	0	0	2	7	17
(45) *Villosa vanuxemensis*	4	0	1	0	0	1	1	1
Totals	179	333	272	254	302	455	216	590
Number of species (29)	22	13	17	17	13	18	15	19

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REGULAR ARTICLE

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THE *AMBLEMA PLICATA* TRANSCRIPTOME AS A RESOURCE TO ASSESS ENVIRONMENTAL IMPACTS ON FRESHWATER MUSSELS

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ABSTRACT

High-throughput sequencing technologies, such as RNA sequencing (RNA-Seq), have greatly enhanced our ability to sequence and characterize the transcriptome of nonmodel organisms. The ability to study expression of thousands of genes in highly threatened yet understudied organisms holds great potential for advancing the field of conservation biology. Despite rapid gains in our analytical abilities and understanding of the physiological underpinnings of the organism, genomic resources remain limited for nonmodel organisms such as freshwater mussels, one of the most imperiled groups of animals worldwide. Here we provide the first characterization of the transcriptome of the North American freshwater mussel *Amblema plicata* (threeridge) using an RNA-Seq approach. Gill tissue samples were collected from mussels in the Muskingum River in Washington County, Ohio, USA. RNA was extracted and sequenced on the Illumina HiSeq 2500 sequencer with output as 100-base-pair paired-end reads. De novo assembly of sequenced reads was performed using Trinity. Assembled transcripts were used as BLASTx queries against the National Center for Biotechnology nonredundant database, and functional annotation using gene ontology (GO) terms was performed using Blast2GO. Transcriptome assembly produced 264,027 transcripts. Of these transcripts, 54,331 (20.58%) received BLAST hits and 22,223 were annotated with GO terms. We provide examples of identified candidate genes that may be useful for studying physiological responses of freshwater mussels to various environmental stressors, such as temperature, hypoxia, and pollutants. The *A. plicata* transcriptome improves the genomic resources available for freshwater mussels, and may aid in the development of molecular tools, with the ultimate goal of increasing our understanding of freshwater mussel physiology and improving conservation techniques.

KEY WORDS: animal stress, conservation, gene expression, transcriptomics, unionid bivalves

INTRODUCTION

The advent of high-throughput, next-generation sequencing technologies has decreased the cost and time involved in genomic and transcriptomic data acquisition and has greatly facilitated genetic studies in nonmodel organisms (Ekblom and Galindo 2011). RNA sequencing (RNA-Seq) enables researchers to identify a species' transcriptome (the expressed portion of the genome) and characterize changes in that transcriptome through development or in response to various environmental conditions (Wang et al. 2009). Although the genome of most nonmodel organisms has not been fully sequenced and annotated, advances in methodologies, such as de novo assembly of RNA-Seq data, allow us to characterize the transcriptome of understudied species of interest through comparison with better-studied model species to infer possible gene function. Several studies have now reported comparative transcriptomic characterizations of previously understudied taxa (e.g., Riesgo et al. 2012; Francis et al. 2013), including the copepod *Tigriopus californicus* (Schoville et al. 2012), green spotted puffer fish (*Tetraodon nigroviridis*; Pinto et al. 2010), and Pacific white shrimp (*Litopenaeus vannamei*; Zeng et al. 2013). Our ability to sequence, functionally annotate, and

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study the expression of thousands of genes in almost any organism holds great potential for advancing the field of conservation biology (Allendorf et al. 2010; Garner et al. 2016; Corlett 2017). For example, transcriptomics can be used to identify markers for pathogen resistance (Harper et al. 2016) or to select source populations for reintroduction by predicting differences in stress responses to environmental change (He et al. 2016). Unfortunately, freshwater mussels (Bivalvia: Unionidae), one of the most endangered faunal groups worldwide, have few available transcriptomic resources (Wang et al. 2012; Bai et al. 2013; Cornman et al. 2014; Luo et al. 2014; Patnaik et al. 2016).

Freshwater mussels are relatively sessile filter feeders that rely almost solely on physiological adaptations to mitigate environmental stressors. The center of freshwater mussel biodiversity is found in North America, but more than half of native species are considered threatened, endangered, or extinct and their numbers are decreasing rapidly (Lydeard et al. 2004; Strayer et al. 2004; Haag and Williams 2014). These animals continuously and simultaneously face persistent and widespread anthropogenic forces, including exposure to toxic contaminants, excessive nutrient inputs, sediment loading from agricultural activities, competition from zebra mussels and other invasive species, hydrologic regime alterations caused by impoundments, and global climate change (Richter et al. 1997; Watters 2000; Strayer et al. 2004). Captive propagation, reintroduction, and ecosystem restoration are a few of the many conservation efforts used in the attempt to conserve freshwater mussels (Strayer and Dudgeon 2010; Haag and Williams 2014). However, there is a pressing need to develop and implement additional health assessment and monitoring methods. Studying gene expression may prove an effective strategy for understanding how these animals respond to a multitude of environmental stressors and conservation efforts (such as animal translocation) at the biomolecular level.

We have sequenced and characterized the first transcriptome of the North American freshwater mussel *Amblema plicata* (threeridge) using gill tissue from individuals collected in the wild, and we provide it as a publicly available resource. *Amblema plicata* is a species with stable populations but it is also congeneric with *Amblema neislerii* (fat threeridge), which is federally endangered in the USA and listed as critically endangered by the International Union for Conservation of Nature (Bogan 1996). Widespread in the Mississippi and Laurentian drainages, as well as parts of the Mobile River, *A. plicata* has one of the largest distributions of any unionid (Williams et al. 1993). In contrast, *A. neislerii* is endemic to the Apalachicola system, a much smaller range, and has suffered from impoundments and water drawdown (Box and Williams 2000; USFWS 2003). The transcriptome provided here can be used to improve conservation of the internationally monitored *A. neislerii*, as well as other closely related and threatened freshwater mussels in the family Unionidae. In addition to our transcriptomic characterization, we discuss genes that are likely to be of interest to investigators studying the physiological responses of freshwater mussels to various environmental stressors, such as temperature, hypoxia, and pollutants. The *A. plicata* transcriptome expands the genetic tool kit available for monitoring and managing freshwater mussels, one of the most endangered, yet understudied, groups of animals.

METHODS

Sample Collection

We collected three adult *A. plicata* from the Muskingum River in Washington County, Ohio, USA below Devola Lock and Dam #2 (39.468703 N, 81.489303 W) on August 7, 2015. Upstream of this location is mostly valley with limited agriculture in the floodplain and a few small towns. The river is impounded by a series of low-head dams and associated locks. This species was chosen because it is common, not listed by state or federal agencies, and found in a wide variety of habitats. We gently pried open their shells with reverse pliers and collected 11–21 mg of gill tissue from each individual. Each tissue sample was placed in a 2-mL RNase-free cryotube, snap frozen in liquid N_2, and stored at –80°C.

RNA Extraction and Sequencing

Tissue samples were mechanically disrupted and homogenized using a Mini-BeadBeater-8 (BioSpec Products Inc., Bartlesville, OK, USA). Using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), RNA was extracted and its concentration and integrity were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) at The Ohio State University Comprehensive Cancer Center (Columbus, Ohio, USA). All samples had an RNA integrity number value $>$8.9. RNA-Seq library preparation and sequencing were performed by the Molecular and Cellular Imaging Center at the Ohio Agricultural Research and Development Center (Wooster, Ohio, USA). RNA-Seq libraries were prepared using the Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina, Inc., San Diego, CA, USA). Libraries were sequenced on the Illumina HiSeq 2500 Sequencer with output as 100-base-pair (bp) paired-end reads.

Transcriptome Assembly and Annotation

Quality of sequencing data was assessed with FastQC (version 0.11.5; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic (version 0.36; Bolger et al. 2014) was used to scan raw reads with a sliding window of four bases and trim read ends when the average Phred quality score dropped below 15, which corresponds to a probability of an incorrect nucleotide call that is equal to 10^{-15}. For downstream analyses, we used only those reads with a minimum of 70 bp remaining after quality trimming. De novo assembly of trimmed reads was performed with Trinity (version 2.3.2; Grabherr et al. 2011) using default parameters.

Table 1. Summary statistics for sequencing and transcriptome assembly.

Statistic	Value
Raw reads produced by Illumina sequencing	69,737,622
Estimate of reads used in final assembly	96.65%
Total assembled transcripts	264,027
Total assembled bases	190,222,260
Mean transcript length	720 bp
Median transcript length	376 bp
N50	1,255 bp
Minimum transcript length	200 bp
Maximum transcript length	16,161 bp
GC content	35.75%

N50 = 50% of transcripts are equal to or larger than this value; bp = base pairs; GC, guanine–cytosine.

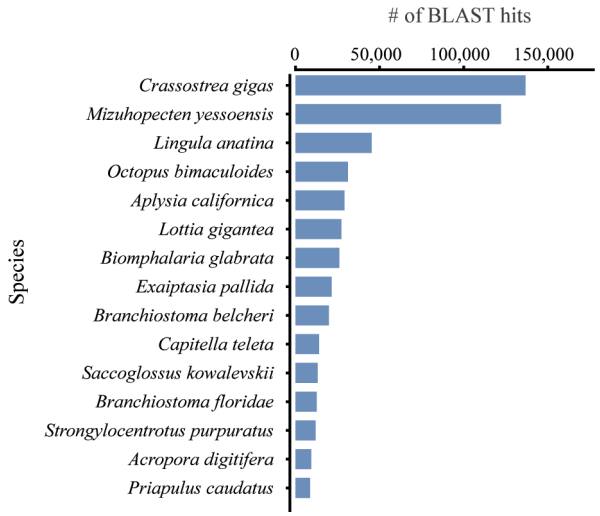

Figure 1. Distribution of BLAST hits for the top 15 species.

To assess the quality of the transcriptome assembly, we estimated the percentage of raw reads represented in the Trinity assembly by mapping with Bowtie 2 (version 2.1.0; Langmead and Salzberg 2012) using default parameters; we assessed assembly completeness according to conserved metazoan ortholog content using benchmarking universal single-copy orthologs (BUSCO; version 2.0; Simão et al. 2015).

Transcripts assembled by Trinity were used as BLASTx queries against the National Center for Biotechnology Information nonredundant database (downloaded July 13, 2017) with a word size of six (the number of nucleotides used by the algorithm to detect regions of similarity between sequences), an expect value (E-value) cutoff of 1E-5 (the number of matches expected to occur by chance alone), and a hit threshold number of 20 (maximum number of matches). Functional annotation of transcripts using gene ontology (GO) terms and InterProScan was performed with Blast2GO (version 4.1.9; Conesa et al. 2005; Götz et al. 2008) using default parameters.

RESULTS

Illumina sequencing produced 69,737,622 raw reads. After trimming, high-quality reads were assembled into 264,027 transcripts with a mean length of 720 bp, N50 of 1,255 bp (50% of transcripts are equal to or larger than this value), and guanine–cytosine content of 35.75% (Table 1). Bowtie2 calculated a 96.65% read alignment to the transcriptome assembly. BUSCO analysis indicated that the assembly produced 811 (82.9%) complete, 116 (11.9%) fragmented, and 51 (5.2%) missing BUSCOs. The Illumina sequence data were archived in GenBank under accession number SRP133691. Both the raw data and transcriptome assemblies can be found under BioProject PRJNA436349.

Of the 264,027 transcripts assembled by Trinity, 54,331 (20.58%) received BLAST hits, and 22,223 of these were annotated with GO terms. The taxonomic distribution of annotation data revealed that five of the top six species were also mollusks (Fig. 1). These species included the Pacific oyster (*Crassostrea gigas*), Japanese scallop (*Mizuhopecten yessoensis*), California two-spot octopus (*Octopus bimaculoides*), California sea slug (*Aplysia californica*), and owl limpet (*Lottia gigantea*). Transcripts were assigned by Blast2GO to one or more of the GO domains: "biological process" (14,869), "molecular function" (17,096), and "cellular component" (13,370). More than half of the transcripts within biological process were assigned to the second-level categories "cellular process," "metabolic process," and "biological regulation" (Fig. 2). The most common categories within molecular function were "binding" and "catalytic activity" (Fig. 2), and those in cellular component included "cell," "cell part," "membrane," and "organelle" (Fig. 2).

We identified numerous genes in the transcriptome that may be useful in gene expression-based studies of freshwater mussels' responses to myriad environmental stressors and discuss examples in the next section (Table 2). Although the genes discussed are likely to be useful in studies of freshwater mussels, they are provided only as examples. Researchers interested in selecting genes for further study are advised to consult the Supplementary Data for an exhaustive list of transcriptome annotations. CLICK HERE.

DISCUSSION

Linking Environmental Stressors and Gene Expression

We provide a publicly available transcriptome resource for the freshwater mussel *A. plicata* and give examples of identified genes whose expression can be studied in response to environmental stressors. Such stressors damage nucleic acids, proteins, carbohydrates, and lipids, as well as the larger cellular structures consisting of these macromolecules, resulting in adverse health effects (Kültz 2005). In response,

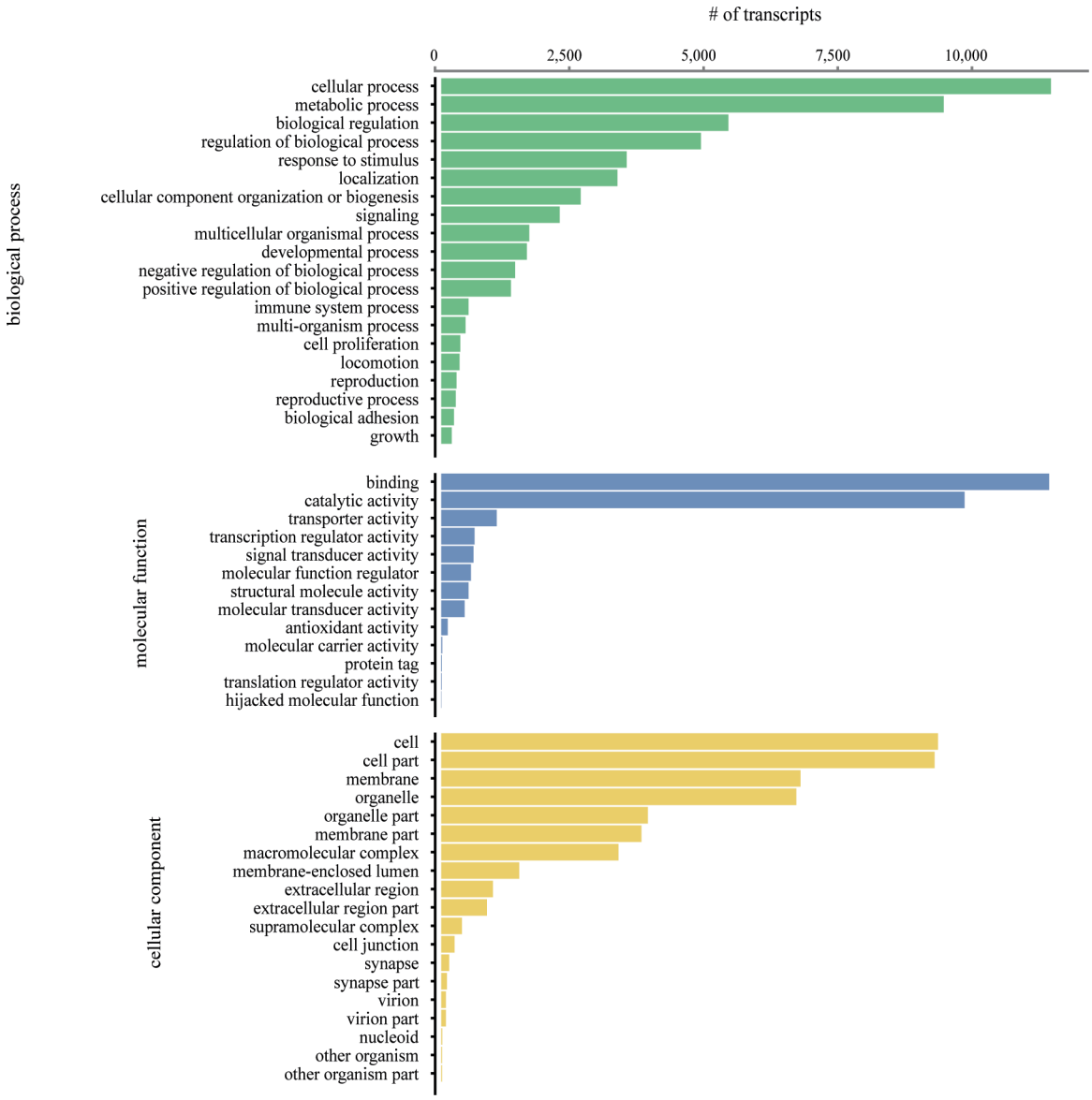

Figure 2. Number of transcripts assigned to gene ontology terms from biological process, molecular function, and cellular component domains.

organisms have evolved various cellular stress response pathways to combat and mitigate damage and restore homeostasis. These pathways are initiated when a signal activates a specific transcription factor that relocates to the nucleus and upregulates expression of target genes (Simmons et al. 2009). The roles of these target genes vary, ranging from neutralizing reactive oxygen species to assisting in the refolding of denatured proteins, but all have cytoprotective functions. In the following sections, we discuss responses to temperature stress, hypoxia, and pollutants, three common stressors to freshwater mussels. We specifically discuss genes that have been confidently assembled and identified in the *A. plicata* transcriptome and that could be used for differential expression studies. These and the many other sequenced genes accessible in the Supplementary Data are needed to create custom oligonucleotide primers that can be used to conduct targeted gene expression studies using quantitative PCR techniques, or to design DNA probes for microarrays to simultaneously assay the expressive state of multiple genes. For further information about the design of qPCR and microarray techniques for conservation applications, see Gibson (2002) and Tymchuk et al. (2010). Studying changes in gene expression can increase our knowledge of how an organism reacts to a certain stressor and its level of sensitivity, which can then be used to improve management and conservation efforts.

Temperature.—Temperature stress is a universal threat among metazoans and is one of the most important factors influencing the behavior and physiology of ectotherms (Angilletta et al. 2002). Bivalves are especially vulnerable to extreme temperature changes since they are sessile organisms and have limited ability to seek out different microhabitats.

Table 2. Examples of protein homology identified in the transcriptome of *Amblema plicata* that may be useful in studies of responses to environmental stressors. Descriptions of function are adapted from UniProt. The reported E-value is the lowest value for transcripts matching that homologue. See Supplementary Data for transcripts and associated annotations.

Putative Homologue (Protein)	Function	E-Value
Heat shock protein 90	A molecular chaperone.	0E0
Heat shock 70 kDa protein 12A	A molecular chaperone.	0E0
Heat shock 70 kDa protein 12B	A molecular chaperone.	0E0
Heat shock factor	A transcription factor that activates expression of heat shock proteins.	6.05E-114
Hypoxia-inducible factor 1 alpha	A transcription factor that activates expression of genes in response to hypoxia.	0E0
Catalase	An enzyme that protects cells from hydrogen peroxide by converting it to oxygen and water.	0E0
Glutathione *S*-transferase	An enzyme that conjugates glutathione to various substrates for detoxification.	7.9E-149
Superoxide dismutase	An enzyme that protects cells from superoxide radicals by converting them to oxygen and hydrogen peroxide.	9.36E-126
Glyceraldehyde 3-phosphate dehydrogenase	An enzyme that participates in the glycolysis pathway.	9.72E-152
Triosephosphate isomerase	An enzyme that participates in the glycolysis pathway.	4.96E-104
Metallothionein	A protein that binds heavy metals.	1.73E-36

Heat shock proteins (HSPs) are molecular chaperones that were first discovered to be induced in *Drosophila melanogaster* in response to heat stress (Ritossa 1962; Tissières et al. 1974). Further studies confirmed the role of HSPs in thermotolerance in other organisms (Snutch et al. 1988; Airaksinen et al. 1998; Clark et al. 2008; Waagner et al. 2010). For example, the marine mussel *Mytilus californianus* has higher levels of HSP70 when found in warmer areas compared with those individuals living in cooler areas (Helmuth and Hofmann 2001). HSPs and other molecular chaperones are essential for survival at elevated temperatures since they ensure the correct folding of newly synthesized proteins and assist in refolding or degrading misfolded proteins accumulated during stress (Feder and Hofmann 1999; Kregel 2002). Other genes whose expression has been studied in response to heat stress include heat shock factor (e.g., in zebrafish; Råbergh et al. 2000) and those involved in combating reactive oxygen species, such as Cu/Zn–superoxide dismutase (Cu/Zn-SOD) (e.g., in the bumblebee *Bombus ignitus*; Choi et al. 2006) and catalase (e.g., in the snakehead *Channa punctata*; Kaur et al. 2005). All of these genes have been identified in the *A. plicata* transcriptome and can be incorporated into gene expression-based studies of freshwater mussel responses to temperature stress. Anthropogenic disturbances such as dam construction, clearing of riparian vegetation, irrigation, channelization, and industrial activities can influence lake and stream temperatures (Poole and Berman 2001; Hester and Doyle 2011). Furthermore, freshwater mussels will become increasingly threatened as climate change continues (Strayer and Dudgeon 2010). The incorporation of gene expression-based studies will provide insight into organismal response to temporary and chronic exposure to temperature stress and, consequently, guide management decisions such as species translocation and habitat restoration.

Hypoxia.—Hypoxia, a decreased level of oxygen availability, is a common stressor of aerobic animals that rely on oxygen for energy production and metabolic function (Giaccia et al. 2004). Freshwater mussels may experience oxygen depletion during certain management practices, such as translocation between habitats (Waller et al. 1995); during eutrophication, when excessive amounts of nutrients cause oxygen depletion (Mallin et al. 2006); or during aerial exposure, as a result of drought-induced decline in water levels (Golladay et al. 2004). We identified several genes in the *A. plicata* transcriptome that can be used to study freshwater mussel responses to hypoxic conditions. Hypoxia-inducible factor (HIF), which consists of the hypoxia-inducible α subunit and the constitutively expressed β subunit, acts as the master regulator of oxygen homeostasis, and the expression of this transcription factor during hypoxic conditions results in upregulation of target genes (Semenza 2002; Greijer et al. 2005). Hypoxia has been shown to induce expression of HIF-1α in the Pacific oyster *Crassostrea gigas* (Kawabe and Yokoyama 2012) and the blue mussel *Mytilus galloprovincialis* (Giannetto et al. 2015). Because an important aspect of the hypoxia response is regulation of glycolysis, the expression of genes coding for such proteins as glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase have also been found to increase in response to low oxygen (Fields et al. 2014). Climate change has increased the frequency and severity of droughts in some regions, such as the southeastern USA (Mazdiyasni and AghaKouchak 2015), a hot spot for freshwater mussel diversity (Haag 2012) and home to the federally endangered *A. neislerii*. Already, water drawdown for upstream municipalities has resulted in the stranding of this listed species in some areas (unpublished work). Drought conditions have caused dramatic declines in mussel abundance (Haag and

Warren 2008) and shifts in species composition (Galbraith et al. 2010). Gene expression-based studies can increase our understanding of how freshwater mussel physiological responses vary in response to droughts of varying intensity and duration, in combination with heat stress, and in different habitats.

Pollutants.—Water-quality degradation is among the most important causes of freshwater mussel declines (Strayer et al. 2004). Pollutants may include nutrients from agricultural runoff, pathogens, organic compounds such as sewage and pesticides, and inorganic compounds such as heavy metals (Schwarzenbach et al. 2010). Metallothioneins have received interest in toxicology since they bind heavy metals and may protect the organism against metal toxicity (Amiard et al. 2006). Metal exposure has been shown to increase metallothionein concentrations in numerous invertebrates, including annelids (e.g., a freshwater oligochaete; Deeds and Klerks 1999), mollusks (e.g., the freshwater mussel *Pyganodon grandis*; Giquère et al. 2003), and crustaceans (e.g., a copepod; Barka et al. 2001). Glutathione *S*-transferase is also important in detoxification processes and was found to be significantly higher in blue mussels (*Mytilus edulis*) living in polluted waters next to a thermoelectric power plant (Manduzio et al. 2004). The upregulation of many proteins can be induced by more than one specific stressor. For example, HSPs, which play a protective role during heat stress (as discussed above), are activated in response to other stressors as well, including heavy metals and pesticides (Lee et al. 2006), and they are often used as indicators of stress levels in toxicological studies (Gupta et al. 2010). Similarly, gene expression levels of Cu/Zn-SOD increased in the bumblebee *B. ignitus* in response to low and high temperatures and bacterial infection (Choi et al. 2006). Bacterial infection also altered gene expression levels of Cu/Zn-SOD in the scallop *Chlamys farreri* (Ni et al. 2007). Because freshwater mussels are filter feeders, they are constantly exposed to a wide range of pollutants. The long-lived, sessile nature of freshwater mussels also makes them useful indicators of water quality (Naimo 1995). With the growing interest in effects of contaminant mixtures and contaminants of emerging concern (de Solla et al. 2016; Montes-Grajales et al. 2017), gene expression-based studies can increase our understanding of the mode of action of various chemical and biological pollutants and their interactions.

CONCLUSION

We have provided a publicly available transcriptome of *A. plicata* and discussed how this resource can be used for gene expression-based studies in response to common stressors such as temperature, hypoxia, and pollutants. Because transcriptome profiling is relatively expensive, this transcriptome provides researchers a resource from which to select candidate genes for designing microarrays or conducting real-time quantitative PCR to conduct in situ or lab-based conservation work. Such targeted sequencing will allow relatively inexpensive studies of gene expression among multiple individuals and a wide variety of environmental conditions in both natural and experimental settings. Furthermore, the corresponding increase in genomic information for closely related species will enable a more in-depth functional characterization of the genes in the *A. plicata* transcriptome that currently lack annotations. Transcriptomes of nonmodel organisms also can be used for many other applications, such as detection of alternative splicing, development of molecular markers (e.g., single-nucleotide polymorphisms), gene discovery, and identification of conservation units, making them effective tools in evolutionary and population genetics analyses (Ekblom and Galindo 2011).

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