Growth and survival of juvenile freshwater mussels in streams: Implications for understanding enigmatic mussel declines

Wendell R. Haag[1,6], **J. Jacob Culp**[2,7], **Monte A. McGregor**[3,8], **Robert Bringolf**[4,9], **and James A. Stoeckel**[5,10]

[1]US Forest Service, Southern Research Station, Center for Bottomland Hardwoods Research, 3761 Georgetown Road, Frankfort, Kentucky 40601 USA
[2]Kentucky Division of Water, 300 Sower Boulevard, Frankfort, Kentucky 40601 USA
[3]Kentucky Department of Fish and Wildlife Resources, Center for Mollusk Conservation, 3761 Georgetown Road, Frankfort, Kentucky 40601 USA
[4]Warnell School of Forestry and Natural Resources, University of Georgia, 180 E Green Street, Athens, Georgia 30602 USA
[5]School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, 203 Swingle Hall, Auburn, Alabama 36849 USA

Abstract: We used in situ exposures of juvenile mussels (96-d average, May–September) to investigate the causes of mussel declines. We measured survival and growth of mussels exposed to ambient conditions in 23 streams in Kentucky, USA. Our set of streams included both those that support diverse mussel assemblages (occupied streams) and those that have lost nearly their entire mussel fauna (defaunated streams). We used 2 types of enclosures in each stream: silos, which primarily expose mussels to water, and sediment cages, which provide greater exposure to sediments. We used both enclosure types because some contaminants are more prevalent either in water or sediments. We collected extensive water and sediment chemistry data (163 and 144 analytes, respectively) monthly at each stream and landscape data for each watershed. We found no evidence of acute toxicity. Survival averaged 90% in silos and was only 68% in cages. However, lower survival in cages appeared to be an artifact of the enclosure type (burial), and survival was unrelated to faunal health or water or sediment chemistry in either type of enclosure. In contrast, we found strong evidence that chronic stressors negatively affect growth in defaunated streams. Growth in both enclosure types was uniformly low in all defaunated streams (mean instantaneous growth, as mass = 0.005/d) compared with growth in occupied streams (0.026/d). Variation in water chemistry among streams was described by 2 principal components (PCs). PC2 described a gradient of increasing alkalinity, nutrients, total organic C, and temperature, which corresponded to the underlying geology and physiography in the study region. Growth was positively correlated with PC2, but defaunated streams were conspicuous outliers, suggesting that other factors further limited growth in these streams. PC1 described a gradient of higher row crop agriculture, pesticides, nitrate/nitrite, and karst influence. Mussel growth was negatively correlated with PC1, but agricultural contaminants were not present in all defaunated streams. Variation in sediment chemistry was described by only 1 PC that reflected underlying geology, similar to water chemistry PC2, and there were no consistent patterns of sediment contamination. Overall, lower total organic C and lower water temperature compared with the occupied streams were the main characteristics shared by the defaunated streams. This result suggests that mussel populations in relatively cool, unproductive streams are particularly vulnerable to chronic human impacts that negatively affect growth or other physiological processes.

Key words: biodiversity, conservation, water quality, sediment, contaminants, Unionoida

Freshwater mussel populations have declined precipitously across North America since the 1960s. These declines are enigmatic because they often occur in streams that lack obvious sources of impairment and continue to support relatively intact fish, snail, crayfish, and insect assemblages (Haag 2012). A common feature of these declines is a cessation of recruitment for most or all mussel species, which results in near-complete faunal loss when the remaining individuals die. However, causes of enigmatic mussel declines remain poorly understood.

E-mail addresses: [6]wendell.haag@usda.gov; [7]jacob.culp@ky.gov; [8]monte.mcgregor@ky.gov; [9]bringo@uga.edu; [10]jas0018@auburn.edu

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Laboratory studies have identified specific compounds to which mussels exhibit higher sensitivity than other organisms (e.g., ammonia, metals, chloride, some pesticides; Bringolf et al. 2007b, Wang et al. 2007, Gillis 2011). Other evidence suggests that larvae or juveniles are more sensitive to some contaminants than adults, which could explain the characteristic cessation of recruitment (Yeager et al. 1994, Augspurger et al. 2003, Strayer and Malcom 2012). Juveniles appear to feed mostly within the sediment, whereas adults tend to feed from the water column (Gatenby et al. 1996). Contaminant concentrations may differ between the sediment and overlying water, which could contribute to differential effects among life stages (Bartsch et al. 2003, Archambault et al. 2017). However, large-scale patterns of association between mussel responses in the wild and a large number of environmental factors remain uninvestigated.

We conducted a field study to examine potential causes of enigmatic mussel declines. We measured survival and growth of juvenile mussels during 96-d (average) exposures in 23 streams in Kentucky, USA. These streams included both those that have diverse and abundant mussel assemblages (hereafter occupied streams) and those that have lost their mussel fauna almost completely in recent decades (hereafter defaunated streams). We deployed mussels in 2 types of enclosures at each stream: silos, flow-through devices that expose mussels mainly to the water column, and sediment cages, which provide mussels more direct contact with sediments. We measured water and sediment chemistry in each stream during mussel exposures and quantified landscape variables in each watershed.

We asked the following questions: 1) does exposure of juvenile mussels to ambient conditions in streams have acute (survival) or chronic (growth) effects, and are these responses related to the general health of the mussel assemblage (defaunated or occupied); 2) are acute or chronic effects more severe in either the water or sediment exposures; and 3) are acute or chronic effects related to specific water chemistry, sediment chemistry, or landscape factors?

METHODS

Study streams

We chose 23 study streams based on 2 factors. First, we chose streams that historically supported diverse mussel assemblages but vary in current faunal health. Second, we focused on enigmatic declines, so we excluded streams where mussel declines appeared to result from obvious impacts such as impoundment, channelization, or severe water pollution (e.g., municipal or industrial discharge, extensive coal mining). We chose 1 site on each stream that coincided with long-term water quality monitoring sites established by the Kentucky Division of Water's (KDOW) Surface Water Monitoring Program (http://water.ky.gov/waterquality/Pages/WQMonitoring.aspx). The use of these sites allowed us to use water chemistry data generated by that program.

All of our study streams are affected by human activities and have lost some mussel species, but 7 have lost their mussel fauna almost completely in recent decades and are essentially defaunated (Table 1). Remnant mussel populations in defaunated streams are small and composed mostly of old individuals, suggesting that these populations are not viable. All other streams in our study support relatively healthy mussel populations with evidence of recent recruitment for most species, even though these streams have had some species loss. Seventeen of the 23 streams are considered impaired under Section 303(d) of the US Clean Water Act, but the varying sources of impairment do not indicate specific causes of mussel declines (Table 1). All but 2 of the streams (Green and Licking rivers) are unregulated. Study sites on the Green and Licking rivers are ~130 and 225 km downstream, respectively, from major dams (Green River Dam and Cave Run Dam). These dams influence flow but have little effect on temperature or water chemistry at the study sites because of the distance from the dams and the inputs of numerous tributaries.

Our study streams represented a wide range of habitat types (Table 1, Fig. 1). Streams ranged from small, 4th-order streams with watershed areas <160 km^2 to large streams draining >2000 km^2. The streams lie within 3 physiographic regions, each with different underlying geology and water chemistry (Fenneman 1938, Haag and Cicerello 2016). The Bluegrass section of the Interior Low Plateaus physiographic province lies on Ordovician limestone and has moderate to extensive karst. Bluegrass streams are well buffered and highly productive because of the high phosphate content and natural fertility of soils in much of the section. The Appalachian Plateaus physiographic province primarily lies on Pennsylvanian sandstone, although some streams have downcut into Mississipian limestones. Karst is present in the latter streams but absent elsewhere. Appalachian streams in Kentucky tend to be poorly to moderately buffered and the soils typically have low fertility. The Highland Rim section of the Interior Low Plateaus physiographic province is underlain mainly by Mississippian limestones and extensive karst occurs in much of this section. Highland Rim streams are moderately well to well buffered, and soil fertility is generally intermediate between the Bluegrass and Appalachian Plateaus. Land cover in the watersheds of our study streams ranges from heavily forested watersheds to primarily agricultural watersheds with pasture or row crops. Only 2 streams had >10% developed land in their watersheds (Floyds Fork = 21%, Elkhorn Creek = 20%). Coal mining occurs mainly in the Appalachian Plateaus, but oil and gas production occurs in all 3 regions.

Juvenile mussel rearing and field deployment

We reared juvenile *Lampsilis cardium* at the Center for Mollusk Conservation of the Kentucky Department of Fish and Wildlife Resources in Frankfort, Kentucky. *L. cardium*

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Table 1. Characteristics of study streams in Kentucky, USA. Defaunated streams are those that have lost nearly their entire mussel fauna since the 1960s; occupied streams continue to support diverse mussel faunas. Watershed area and landscape variables pertain to the entire watershed upstream of the study site. See Methods for details. Specific study site locations and other information is provided in Table S1.

Stream	Watershed area (ha)	Physiographic region	Species loss[1]	Forest (%)	Pasture/ hay (%)	Row-crop (%)	Intense karst (%)	Coal (tons/ha)	Stream impairment[2]
Defaunated streams									
Roundstone Creek	32,447	Appalachian Plateaus	78% (18)	64	27	<1	38	5.4	1, 2, 3, 6
Horse Lick Creek[3]	15,939	Appalachian Plateaus	87% (23)	82	12	0	28	12.9	1
Red River	141,300	Highland Rim	72% (32)	16	24	52	52	0	1, 3
Drakes Creek	124,383	Highland Rim	54% (35)	35	41	16	36	0	7
Nolin River[3]	96,161	Highland Rim	87% (31)	25	39	26	93	0	-
Little River	63,195	Highland Rim	82% (28)	30	22	37	85	0	2, 3, 4, 5, 7
Whippoorwill Creek	28,916	Highland Rim	85% (20)	15	24	54	75	0	1
Occupied streams									
South Fork Kentucky River	186,725	Appalachian Plateaus	11% (27)	82	11	<1	0	409.1	1
Rockcastle River	156,409	Appalachian Plateaus	25% (32)	64	27	<1	19	38.5	-
Tygarts Creek	71,224	Appalachian Plateaus	23% (31)	69	23	<1	25	6.6	2, 3, 7
Redbird River	49,841	Appalachian Plateaus	14% (22)	88	6	0	0	1093.9	1
Kinniconick Creek[4]	59,374	Appalachian Plateaus	9% (23)	84	12	0	3	0	-
Licking River	875,124	Bluegrass	21% (56)	49	42	2	18	69.7	1
South Fork Licking River	230,607	Bluegrass	26% (35)	20	68	4	53	0	-
Elkhorn Creek	127,034	Bluegrass	15% (20)	10	66	4	89	0	7
Eagle Creek	113,410	Bluegrass	35% (34)	53	40	<1	12	0	4
Beech Fork Salt River	112,887	Bluegrass	32% (44)	50	42	4	5	0	1
Floyds Fork Salt River	67,248	Bluegrass	26% (31)	42	31	5	15	0	1, 2, 3
Slate Creek	47,961	Bluegrass	26% (27)	41	49	2	4	0	1
Green River	447,086	Highland Rim	12% (67)	52	36	6	28	1.1	1, 7
Russell Creek	67,669	Highland Rim	42% (24)	41	45	6	30	0	-
Buck Creek	62,619	Highland Rim	32% (28)	41	51	2	31	<0.1	7
Little Barren River	59,225	Highland Rim	24% (21)	59	33	3	31	0	-

[1] Species loss pertains to the entire watershed for each stream and was calculated as (number of species recorded live or as recently dead shells from 1990–2015/total number of species recorded in all time periods) × 100; numbers in parentheses indicate total species richness (data from Haag and Cicerello 2016 except where otherwise noted).

[2] Kentucky Division of Water 303(d) impaired streams for 2014 (KDOW 2014), summarized pollutants and (sources): 1—*E. coli* or fecal coliform (agriculture, sewage, or unknown); 2—Nutrient/eutrophication (agriculture or sewage); 3—Sedimentation/siltation (agriculture or urban); 4—Nitrate/nitrite (agriculture or sewage); 5—Phosphorus (agriculture or sewage); 6—Dissolved oxygen (agriculture); 7—Fish consumption advisory (PCB or methylmercury). Impairment is given only for reaches including or in close proximity to our study site.

[3] Species loss for Horse Lick Creek is based on a 2017 survey (W. Haag and S. Price, unpublished data). Species loss for Nolin River is based on a 2016 survey (M. Compton, Kentucky State Nature Preserves Commission, personal communication).

[4] Kinniconick Creek technically is within the Bluegrass section of the Interior Low Plateaus physiographic province, but it lies within a mountainous area at the periphery of the section (Knobstone Escarpment) and stream habitats, land cover, and water chemistry closely resemble those of the Appalachian Plateaus to which the watershed is immediately adjacent.

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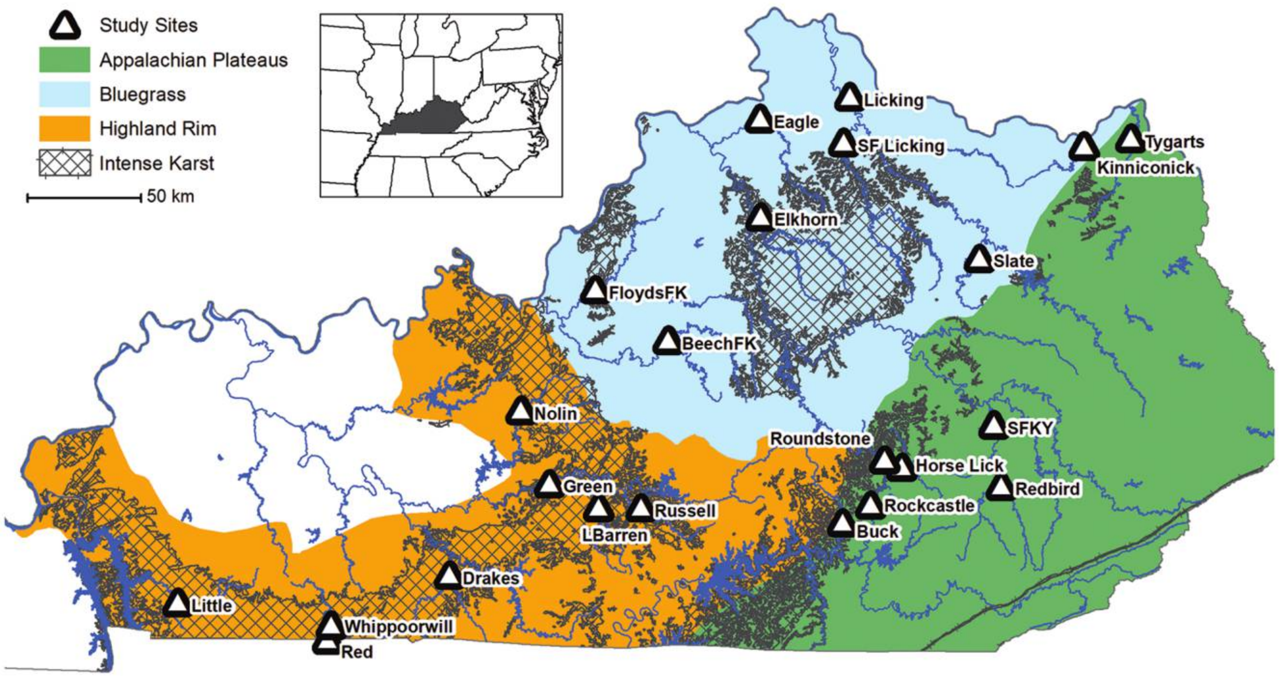

Figure 1. Map of Kentucky. The Appalachian Plateaus region is shaded green, the Bluegrass region is shaded blue, and the Highland Rim region is shaded orange. Triangles indicate the study site within each stream. Gray cross hatching indicates large, contiguous areas of intense karst, and smaller gray-shaded areas indicate localized areas of intense karst (e.g., headwaters of Tygarts Creek). Inset map shows location of Kentucky in the eastern USA.

is native to all of our study streams. The larvae (glochidia) of most freshwater mussels require a period as ectoparasites on fishes to complete metamorphosis from the larval to the juvenile stage. We harvested and combined the broods of 4 female *L. cardium* from the Licking River in Nicholas County, Kentucky, and infested Largemouth Bass (*Micropterus salmoides*) with these glochidia on 3 November 2014. We held the infested fishes in a recirculating aquarium system at 19 to 23°C, and juvenile mussels metamorphosed from 23 November to 4 December. We collected the juveniles and reared them from 24 to 26°C in 4-L trays within a recirculating aquaculture system that had biological and mechanical filtration. Juveniles were fed a mixture of commercial and cultured algae. By 15 May 2015, juvenile mussels averaged 6.4 mm in length (±0.3 standard deviation [SD], $n = 4016$) and 0.040 g (±0.006 SD, $n = 251$ batches of 16 individuals; mass estimates include the shell).

We deployed mussels in streams in 2 types of enclosures, silos and sediment cages (hereafter cages; Fig. 2). Silos consisted of concrete domes 26 cm in diameter and 14 cm high with a central chamber formed by a section of 4-inch (10.2-cm) SCH40 PVC pipe covered with a drain grate. Mussels were held in a smaller chamber made of 3-inch (7.6-cm) SCH20 PVC covered on both ends by 1-mm window screen. The smaller holding chamber fit into the central chamber of the silo and was secured with cable ties. Three pieces of bent 12.5-cm diameter steel rod were cast into the bottom of the silo to keep it ~3 cm off the stream bottom. Silos exploit the Bernoulli Principle by creating an upwelling current through the central chamber as stream water flows over the curved upper surface, which delivers food and oxygen to mussels in the chamber and carries away waste (C. Barnhart, personal communication). Silos expose mussels mainly to the water column but can accumulate variable amounts of fine sediment and sand.

We used polypropylene Whitlock-Vibert (W-V) boxes as cages in this experiment. These boxes are typically used to incubate salmonid eggs in streambeds (https://flyfishersinternational.org/Conservation/Projects-Programs/Whitlock-Vibert-Box). W-V boxes are 14 × 6.4 × 8.9 cm with 3.5 × 13-mm slots that have a large lower chamber and a smaller upper chamber. Mussels were too small to be contained in W-V boxes, so we enclosed mussels in 2-mm-mesh nylon bags within the small upper chamber of the boxes. Mussels in these cages were more directly and consistently exposed to sediments than were the mussels in the silos.

We deployed mussels in streams between 19 May and 18 June 2015. We placed 5 silos and 5 cages in each stream, and each device received 16 mussels. Prior to deployment we measured the length of each mussel, weighed each batch of 16 mussels (blotted wet mass), and haphazardly assigned 1 batch to each silo holding chamber or cage. We transported mussels to the field in aerated coolers filled with water and acclimated them to ambient stream temperatures

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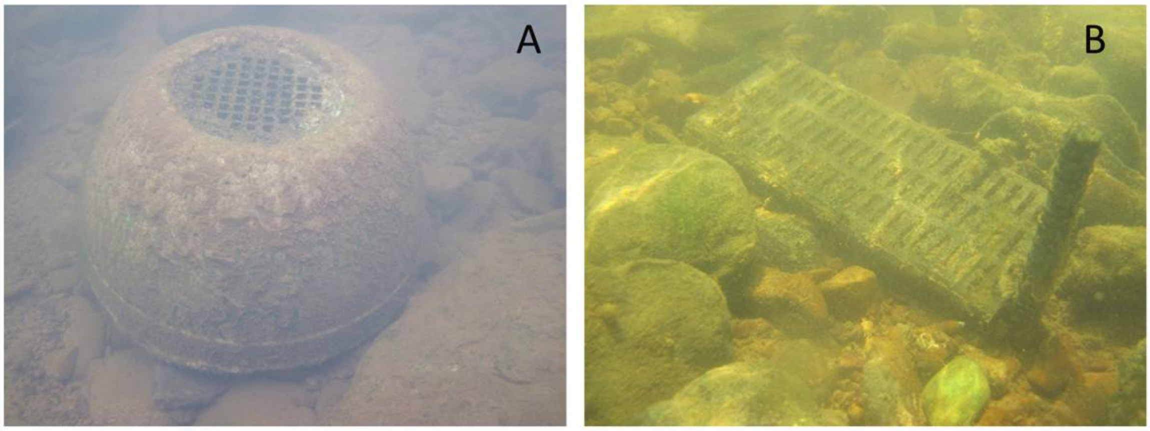

Figure 2. Silos (A) and sediment cages (B) used to hold mussels in streams. Photos show enclosures at the time of retrieval (W. R. Haag, photos).

by gradually replacing transport water with stream water. Temperature differences between the streams and the coolers were always <3°C and the acclimation process took ~45 min. We placed silos on stable gravel substrate in runs 0.5–1 m deep to ensure that silos had sufficient water flow and remained immersed as well as to reduce vandalism. We placed cages adjacent to silos, and all silos and cages were placed within a 5 to 10-m^2 area. We filled the lower chamber of cages with sediment from the site, placed mussels in the upper chamber, and buried cages so their tops were flush with the substrate surface. We then anchored the cages with rebar stakes and cable ties. We affixed an IBWetland temperature logger (Alpha-Mach Inc., Sainte-Julie, Quebec, Canada) to 1 silo and 1 cage at each stream. We programmed the loggers to record temperature every 75 min.

We retrieved silos and cages between 31 August and 9 September 2015. Exposure time averaged 96 d but ranged from 78 to 112 d among streams because high flows sometimes resulted in deployment delays. For the 19 streams unaffected by high flow, exposure time was between 90 and 106 d (Table S1). Upon retrieval of the enclosures we recorded the number of live mussels in each device, returned mussels to the laboratory on ice, and froze them at −18°C. Over the next 3 mo we thawed samples and measured individual length of each mussel and total blotted wet sample mass (including shell) of each batch of mussels.

Water and sediment chemistry

We collected water samples monthly, while mussels were deployed, from all streams in cooperation with KDOW staff. This resulted in 4 water samples for most streams, but high water prevented collection of some samples: we were only able to take 3 samples from 3 streams, and 2 samples from 2 streams (Table S1). We took water samples following the KDOW methodology and chain-of-custody documentation, which is based on USEPA standard methods (KDOW 2009). We collected 6 separate samples from each stream during each sampling event and cooled them to 4°C after collection. The samples included a 1-L sample for bulk variables (total suspended solids, Cl, SO_4, conductivity), a 500-ml sample for alkalinity/acidity, a 500-ml sample for nutrients fixed at pH < 2.0 with H_2SO_4, a 500-ml sample for metals fixed at pH < 2.0 with HNO_3, a 1-L sample for insecticides, and a 1-L sample for herbicides fixed at pH < 2.0 with HCl.

We also attempted to collect 2 sediment grab samples from each stream at roughly even intervals during the study (late June and August). However, we were unable to collect any samples from 1 stream (Nolin River), and we were able to collect only 1 sample from 13 streams (Table S1). We collected sediment samples following the National Water Quality Assessment program (Shelton and Capel 1994) and KDOW chain-of-custody documentation. Briefly, during each sampling event we used a scoop to collect 10 surficial grab samples from depositional areas, combined the samples, washed the composite sample in a 2-mm stainless steel sieve, and collected the material in a 500-ml wide-mouth bottle. After sampling a site, we washed all equipment with phosphate-free detergent and rinsed it with deionized water and ethanol.

Water and sediment samples were analyzed at the Kentucky Division of Environmental Program Support (KDEPS, Frankfort, Kentucky) following USEPA standard methods. The KDEPS analytical laboratory measured 163 analytes in water samples and 144 analytes in sediment samples (Table S2).

In addition to sediment grab samples, we used passive samplers (peepers) to measure the integrated concentrations of interstitial ammonia in the sediment over time (Strayer and Malcom 2012). Peepers consisted of 125-ml high-density polyethylene bottles with a dialysis membrane (6000–8000 daltons) placed over the bottle mouth and secured in place by the cap. The bottle cap had a large hole covered with window screen to expose but protect the

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membrane. We filled peepers with deoxygenated, deionized water and buried them in a horizontal orientation about 10 cm below the sediment surface. We deployed peepers in mid-August and retrieved them when we retrieved the enclosures. Longer deployments were not possible because the dialysis membranes degrade after ~2 w. We measured pH and temperature in each peeper immediately after retrieval, placed them on ice, and froze them upon returning to the laboratory. We shipped frozen peepers to the University of Georgia for measurement of dissolved aqueous ammonia by potentiometry with a calibrated high performance ammonia ion selective electrode and meter (Dual Star meter, Thermo Scientific Orion, Mansfield, Texas, USA; USEPA Method 1689).

Landscape data

We generated landscape and related data in ArcGIS version 10.2.2 with the Spatial Analyst extension (ESRI, Redlands, California, USA) and used MS Access version 14.0 (Microsoft, Redmond, Washington, USA) for data management. For each stream we established a polygon that encompassed the entire watershed upstream of the study site based on 8 and 10 digit hydrologic units (KDOW and USGS 2004a, 2004b). We obtained landscape data for each polygon from the 2011 National Land Cover Database (USGS 2014, Homer et al. 2015). We collapsed 11 landscape classes into the following 4 classes: developed (summed area of 4 classes), forest (3 classes), pasture/hay (3 classes), and row crop (1 class). We then calculated the proportional coverage of each class in each polygon and omitted classes that made up <1% of our watersheds (water, barren land, and wetlands). We obtained karst data from a 1:500,000-scale geologic map of Kentucky (KGS 1988) that classified karst into 3 categories: intense, prone, and none. We calculated the proportion of each polygon underlain by intense karst. We obtained coal mining data (total coal production in each watershed, all years: 1790–2014) from the Kentucky Mine Mapping Information System (KMMIS 2017). Finally, we obtained oil and gas production data (number of wells/ha) from the Kentucky Geological Survey (KGS 2017).

Data analysis

We calculated instantaneous growth (/d: ln[final mass in g/initial mass in g]/deployment period in d; Ricker 1975) in each enclosure based on mean individual mussel mass, estimated as total sample mass/number of live individuals in the retrieved enclosure. Our response variable for subsequent analyses was mean instantaneous growth (hereafter growth) across all replicates for each enclosure type (silo or cage) in each stream.

We reduced the number of water chemistry and landscape variables in the following ways. A total of 60 analytes were detected in water samples and 47 analytes were detected in sediment samples (Table S2). We omitted analytes detected at either ≤3 streams (e.g., many pesticides) or uniformly low levels near the detection limit (e.g., caffeine, several pesticides, and elements) from further analyses. Water hardness, calcium, conductivity, and alkalinity were highly correlated with each other ($r > 0.90$ for all comparisons) and provided a redundant depiction of a single facet of water chemistry; we retained only alkalinity. Reducing the dataset in this way left 30 water chemistry variables and 27 sediment chemistry variables, and we further reduced the water chemistry dataset for principal components analysis (PCA; see below). The proportion of developed land was low in all but 2 streams (see Study Streams), so we also omitted this variable from analysis. There was no correlation between mussel growth and watershed area ($r = 0.148$, $P = 0.610$, log watershed area), but sites were not chosen with regard to size and this variable was not included in our analyses.

We log-transformed all water and sediment chemistry variables except alkalinity and water temperature, which generally reduced skewness to <1 (median = 0.76), and we used the mean value for each analyte across all samples from a stream. Landscape variables based on proportions were logit- or arcsine-transformed, depending on the nature of the data.

We calculated Spearman correlation coefficients between growth and all retained water, sediment, and landscape variables (Table S3). We examined these correlations in the full dataset including all streams, the dataset excluding two defaunated streams (Horse Lick and Roundstone creeks), and the dataset excluding all seven defaunated streams. We did this because correlations between growth and several variables differed markedly among these subsets of streams (see Results). We do not base our major conclusions on correlations between growth and individual variables, but we use them to illustrate various patterns in the data.

We used PCA based on correlation matrices to examine patterns of water chemistry, sediment chemistry, and landscape variables. We examined water chemistry and sediment chemistry separately. Our initial analysis of water chemistry yielded PCs that were each weakly correlated with a large number of variables. This was because of slightly, but consistently, higher levels of metals in the Bluegrass as well as correlations between metals and other water chemistry factors in that region (see Results). Consequently, we omitted all metals and sulfate. The retained variables loaded on PCs in similar ways compared with the dataset including metals, but the reduced dataset provided a clearer picture of variation among streams. Our final dataset consisted of 21 variables that included 14 water chemistry variables, 6 landscape variables, and mean water temperature. Patterns of sediment chemistry were driven primarily by high metal concentrations in the Bluegrass (see Results) and were largely

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uninformative. For this reason, we did not analyze sediment chemistry with a dataset that included landscape variables and sediment temperature.

We examined relationships between mussel growth and survival and water chemistry, sediment chemistry, temperature, and landscape factors with PC regression (Quinn and Keough 2002). We expected mussels in silos to be primarily influenced by water chemistry and mussels in cages to be primarily influenced by sediment chemistry. We therefore regressed growth and survival in silos on stream PC scores based on the dataset including water chemistry, water temperature, and landscape variables. We regressed growth and survival in cages on stream PC scores based on the sediment chemistry dataset without temperature and landscape factors (see previous). For both of these analyses, we conducted separate regressions for growth and survival.

PC2 for water chemistry depicted a gradient mainly reflecting underlying geology and related fundamental watershed characteristics, and there was a strong relationship between this component and growth (see Results). We evaluated this relationship further using Akaike's Information Criteria (AIC; Burnham and Anderson 2002) to evaluate a set of candidate models for predicting growth based on the 6 water chemistry variables that were most strongly correlated with PC2 (alkalinity, P, K, total Kjeldahl N [TKN], total organic C [TOC], and temperature). We conducted this analysis with and without the 7 defaunated streams because these streams were conspicuous outliers in the PC2-growth relationship. All analyses were conducted in SAS version 9.4 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Mussel survival and growth

We recovered 84% of silos (range among streams = 40–100%). Survival in silos averaged 90% across streams (Fig. 3). Silos with <50% survival were rare, and all streams had at least 1 silo with 100% survival. In general, silos within a stream had similar survival rates. Survival in silos did not differ between defaunated and occupied streams (mean survival, defaunated = 92.9%; occupied = 89.1%; $F_{1,\,21} = 0.27$, $p = 0.609$; arcsine survival).

We recovered 83% of cages (0–100%); no cages were recovered at Roundstone Creek. Survival was lower in cages than in silos and averaged 68% across streams (Fig. 3). Additionally, survival varied widely among cages within most streams. Approximately ½ of the streams had cages with both high and low survival (e.g., >80% and <30%), and all streams but 4 (Redbird and South Fork Kentucky rivers, Russell and Tygarts creeks) had at least 1 cage with >80% survival. Many cages were either buried under ≤2 cm of sediment or firmly impacted with fine sediment during the experiment, and these cages typically had high mortality. Survival in cages did not differ between defaunated and occupied streams (mean survival, defaunated = 88.4%; occupied = 59.8%; $F_{1,\,20} = 2.59$, $p = 0.123$; arcsine survival).

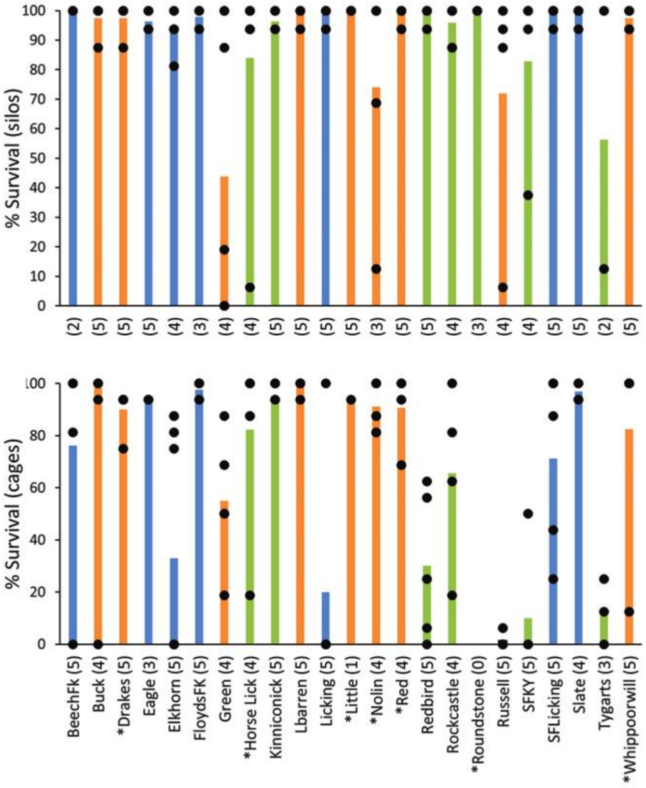

Figure 3. Mean juvenile mussel survival in silos and sediment cages after 96-d exposure (average) to ambient conditions in 23 Kentucky streams. Bars represent mean survival among enclosures at a stream and points represent survival in individual enclosures. No cages were recovered in Roundstone Creek. Bar color indicates physiographic regions: blue = Bluegrass, green = Appalachian Plateaus, and orange = Highland Rim. Numbers in parentheses on the x-axis are the number of enclosures recovered at each stream. Asterisks by stream names indicate defaunated streams.

Cage survival was not correlated with silo survival ($r = 0.22$, $p = 0.306$, $n = 23$; arcsine survival). Instances of low survival (<30%) in both silos and cages were observed only in the Green River and Horse Lick, Russell, and Tygarts creeks (Fig. 3).

Growth varied widely among streams (Fig. 4). Growth in both enclosure types was significantly lower in defaunated streams than in occupied streams (silos: mean growth, defaunated = 0.005/d, occupied = 0.028/d; $F_{1,\,21} = 63.85$, $p < 0.0001$; cages: mean growth, defaunated = 0.004/d; occupied = 0.023/d; $F_{1,\,20} = 20.87$, $p = 0.0002$). Growth and survival were not correlated in neither the silos ($r = 0.04$, $p = 0.855$, $n = 23$; arcsine survival) nor cages ($r = 0.08$, $p = 0.712$, $n = 22$).

The mean absolute increase in mass in silos varied among streams by 2 orders of magnitude, from 0.012 g

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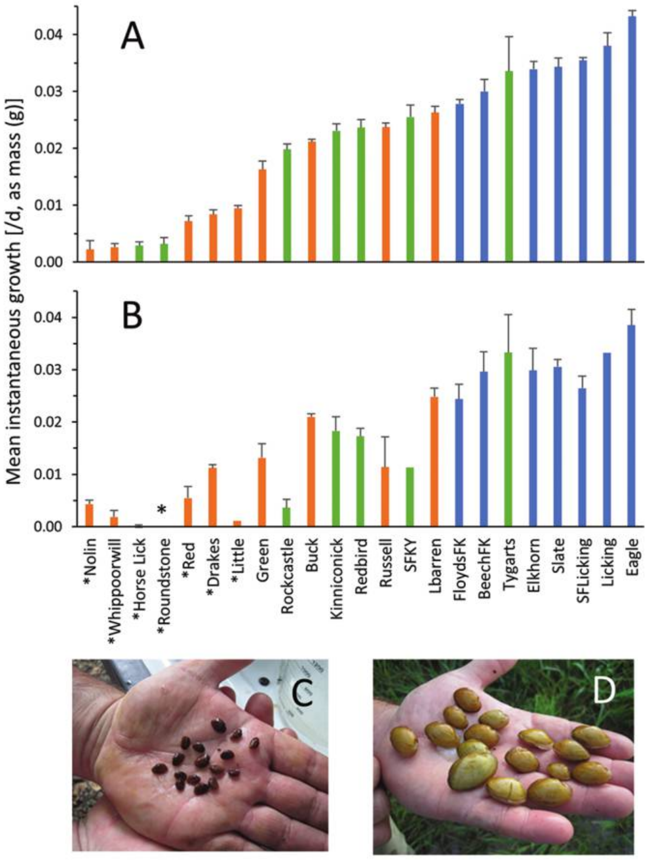

Figure 4. Mean (+SE) instantaneous growth of surviving juvenile mussels in silos (A) and sediment cages (B) after 96-d exposure (average) to ambient conditions in 23 Kentucky streams. No cages were recovered in Roundstone Creek (*). Symbol color indicates physiographic regions: blue = Bluegrass, green = Appalachian Plateaus, and orange = Highland Rim. Photos show mussels from a representative low-growth, defaunated stream (C, Drakes Creek), and a representative high-growth, occupied stream (D, Beech Fork) in silos at the end of the study. Asterisks by stream names indicate defaunated streams.

in Nolin River and Whippoorwill Creek to 2.109 g in Eagle Creek. The mean absolute increase in length also varied by 2 orders of magnitude, from 0.6 to 19.6 mm. Growth ranged from 0.0022/d in the Nolin River to 0.0432 in Eagle Creek. Growth was generally highest in the Bluegrass and lowest in the 7 defaunated streams (Fig. 4). Increase in length was consistent among individuals within a silo and the median coefficient of variation (CV) for length across all silos (n = 97) was 13% (range = 3–22%). We could not estimate within-silo variation in mass because we weighed all individuals in a silo together. Variability among silo replicates within a stream was also low (median CV, length = 17%, range = 3–143%; median CV, mass = 13%, range = 3–177%).

Growth in cages was broadly similar to growth in silos. Growth ranged from −0.0003/d in Horse Lick Creek to 0.0385/d in Eagle Creek, and growth was generally highest in the Bluegrass. All 7 defaunated streams had low growth (<0.012/d), but 3 streams that had high growth in silos had low growth in cages (Rockcastle and South Fork Kentucky rivers, Russell Creek). Growth in cages was significantly related to growth in silos ($F_{1,\,20} = 106.2$, $p < 0.001$, $R^2 = 0.84$), and the 95% confidence interval for the slope of the relationship included 1 (0.903 ± 0.183). However, silo growth was significantly higher than cage growth (paired t-test, $t = 4.22$, $p = 0.0004$), but this difference was small (mean difference, silos–cages = 0.005/d). Variation of growth within cages was low and similar to that in silos (median CV, length = 14%, range = 5–23%, n = 95), but variability among replicates within a stream was higher for cages than for silos (median CV, mass = 26%, range = 6–500%).

Mussel shells were bright yellow when they were placed in the streams. Mussels in streams with higher growth maintained that coloration, but shells in the 7 defaunated streams became darkly stained (Fig. 4). Shells in Kinniconick and Tygarts creeks had a 2-toned coloration pattern. Individuals in these streams were darkly colored in the center of their shells similar to slow-growing individuals, but had lighter coloration surrounding the dark central part of their shells similar to faster-growing individuals (Fig. 5).

Variation in water chemistry and landscape variables among streams

PCA of 21 water chemistry and landscape variables extracted 5 PCs with eigenvalues > 1. Only PC1 and PC2 were interpretable based on having eigenvalues that were

Figure 5. Juvenile mussels from Kinniconick Creek with 2-toned coloration such that the central portion of the shell is dark and the shell margin is yellow. Dark coloration generally coincided with slow growth for the mussels in our study, whereas light coloration coincided with high growth. Thus, 2-toned coloration may indicate growth inhibition early in the study followed by higher growth later in the study.

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greater than expected based on a broken-stick distribution (Jackson 1993). Together, PC1 and PC2 accounted for 60.5% of the variation in the dataset (Fig. 6; Table 2).

PC1 represented a gradient of streams with higher values for agricultural contaminants (atrazine, atrazine desethyl, metolachlor, dicamba, and nitrate/nitrite), karst, and row crop land cover contrasted with streams having low values for those attributes and higher forest cover. The 5 defaunated streams in the Highland Rim (Drakes and Whippoorwill creeks, Little, Nolin, and Red rivers) had the highest scores on PC1, whereas Appalachian streams had the lowest scores. Several other pesticides occurred at their highest concentrations in the Nolin River but were present at low concentrations or not detected in most other streams (2,4,5-T, 2,4-D, chloramben, clothianidin, dichloroprop, MCPA, MCPP, silvex). DDT or related compounds were detected in only 2 streams, where they were near the detection limit (Elkhorn Creek and Floyd's Fork, mean total DDT = 0.0125 and 0.0109 μg/L, respectively; detection limit = 0.0100).

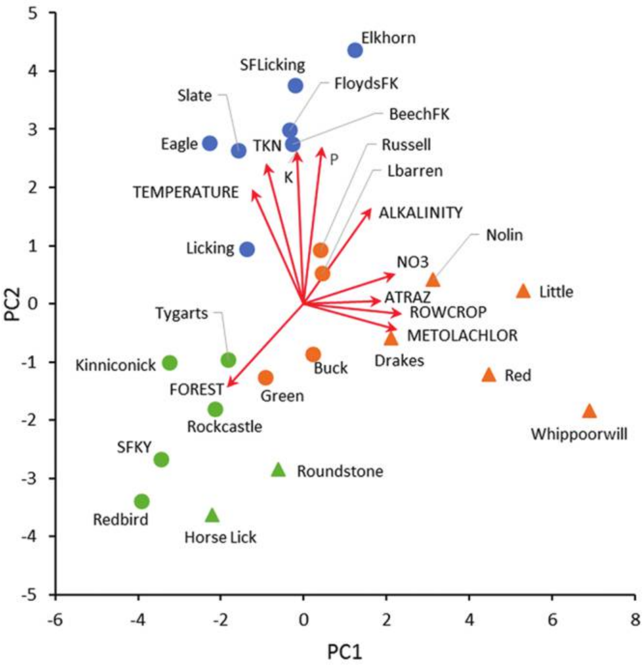

Figure 6. Principal components plot showing streams ordinated by water chemistry and land cover variables. Stream symbol color indicates physiographic regions: blue = Bluegrass, green = Appalachian Plateaus, and orange = Highland Rim. Triangles are defaunated streams, circles are occupied streams. Red arrows are vectors for variables that loaded strongly on principle component axes (see Table 2). Variable names are in capital letters (ATRAZ = atrazine; TKN = total Kjeldahl N). To increase figure clarity, we omitted the following variables, and include the pictured variable with the most similar value in parentheses: karst (NO_3); atrazine desethyl and dicamba (metolachlor); pasture (P); total organic C (TKN).

Table 2. Principal components loadings for water chemistry and landscape variables among 23 Kentucky streams. The percentage of variation explained by each principal component is given beside the component number. Correlations >0.60 are bolded.

Variable	PC1 36.8%	PC2 23.7%
Alkalinity (as $CaCO_3$, mg/L)	**0.72**	**0.63**
Atrazine (μg/L)	**0.76**	0.10
Atrazine desethyl (μg/L)	**0.78**	−0.06
Barium (μg/L)	0.39	−0.20
Chloramben (μg/L)	0.13	0.08
Chloride (mg/L)	0.57	0.37
Dicamba (μg/L)	**0.72**	−0.11
2,4-D (μg/L)	0.47	−0.06
Metolachlor (μg/L)	**0.89**	−0.04
Nitrate/nitrite (as N, mg/L)	**0.92**	0.28
Total organic carbon (mg/L)	−0.36	**0.78**
Phosphorus (mg/L)	0.29	**0.92**
Potassium (mg/L)	0.03	**0.87**
Total Kjeldahl N (mg/L)	−0.27	**0.77**
Mean water temperature (°C)	−0.43	**0.60**
% karst	**0.81**	0.21
% pasture	0.23	**0.79**
% row crop	**0.95**	0.05
%fForest	**−0.81**	−0.57
Oil and gas (no. wells/ha)	−0.11	−0.36
Coal production (tons/ha)	−0.56	−0.56

PC2 represented a gradient of streams having higher temperatures, higher values for variables related to nutrients or food availability (P, K, TKN, and TOC), and higher pasture/hay land cover contrasted with streams having lower values for those variables. This component separated streams based on physiography. All Bluegrass streams had the highest scores on this axis, Appalachian streams had the lowest scores, and Highland Rim streams had low–intermediate scores. Alkalinity loaded strongly on both PC1 and PC2, reflecting higher alkalinity in the Bluegrass and Highland Rim compared with the Appalachians.

Several metals or metalloids, especially Al, As, Cu, Mg, Ni, and Na, were slightly higher on average in the Bluegrass (Fig. S1). No streams with a history of coal mining had consistently elevated metals including 2 defaunated streams, Horse Lick and Roundstone creeks. Similarly, conductivity was not elevated in Horse Lick or Roundstone creeks (highest observed conductivity = 336 μS/cm in Roundstone Creek; mean conductivity, Horse Lick = 151 μS/cm, Roundstone = 253 μS/cm). Further, conductivity was not correlated with coal production ($r = –0.31$, $p = 0.108$). Instead, conductivity was essentially equivalent to alkalinity and hardness in our streams. Chloride was highest in the 2 streams with

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the highest urban land cover (Floyds Fork, Elkhorn Creek) and in the Nolin River (28–33 mg/L; 3–12 mg/L in all other steams).

Mean water temperature among streams varied from 20.1 to 25.0°C, but temporal patterns of temperature were similar in all streams (Fig. S2). All streams experienced a period of low water temperature from late June to mid-July that coincided with high rainfall and cool air temperatures during this period. Streams in which mussels showed apparent delayed onset of growth (2-toned shell coloration, Kinniconick and Tygarts creeks) had similar temperature patterns as streams that supported high mussel growth throughout the study.

Relationships of mussel survival and growth with water chemistry and landscape variables

Survival in silos was not related to water chemistry PC1 or PC2 (PC1: $R^2 = 0.02$, $p = 0.515$; PC2: $R^2 = 0.05$, $p = 0.351$, arcsine survival). However, PC1 and PC2 both explained a high percentage of the variation in growth among streams (Fig. 7). Growth was negatively related to PC1, indicating that mussels in streams with higher values of agricultural contaminants, more karst, and more row-crop land cover had lower growth ($R^2 = 0.29$, $p = 0.0075$). However, Horse Lick and Roundstone creeks, which had little or no agriculture and low PC1 scores, had low mussel growth and were strong outliers from this relationship (studentized residuals >2.0). Omitting these 2 streams from the regression yielded a stronger relationship that explained 58% of the variation in growth among remaining streams (Fig. 7A).

Growth was positively related to PC2, indicating that mussels in streams with higher nutrients, higher temperature, and higher pasture/hay land cover had higher mussel growth ($R^2 = 0.29$, $p = 0.0083$). However, this relationship provided a poor description of the data: variance of residuals decreased sharply with increasing predicted values of growth, there were several influential observations, and the 7 defaunated streams made up nearly all data points with negative residuals. Omitting the 7 defaunated streams yielded a relationship that explained a higher percentage of the variation in growth (Fig. 7B) with no influential observations and an even distribution of residuals. Growth in the 7 defaunated streams was 65 to 92% lower than predicted by this relationship, whereas growth in all other streams was within ±36% of predicted values (mean = 17%).

Bivariate relationships between growth and water chemistry and landscape factors reflected multivariate relationships (Table S3, Figs S1, S3). Anthropogenic factors that loaded strongly on PC1 (agricultural contaminants and row-crop land cover) were negatively correlated with mussel growth, and these correlations were stronger when Horse Lick and Roundstone creeks were omitted, suggesting that they were outliers from these relationships. Water chemistry factors that loaded strongly on PC2 (P, K, TKN, and TOC) were positively correlated with growth, and correlations for P and K were stronger when the 7 defaunated streams were omitted. Sulfate and several metals or metalloids (Al, As, Co, Cu, Fe, Na, and Ni) were positively correlated with growth. Coal production, oil and gas wells, and forest were not correlated with growth (Table S3, Fig. S3).

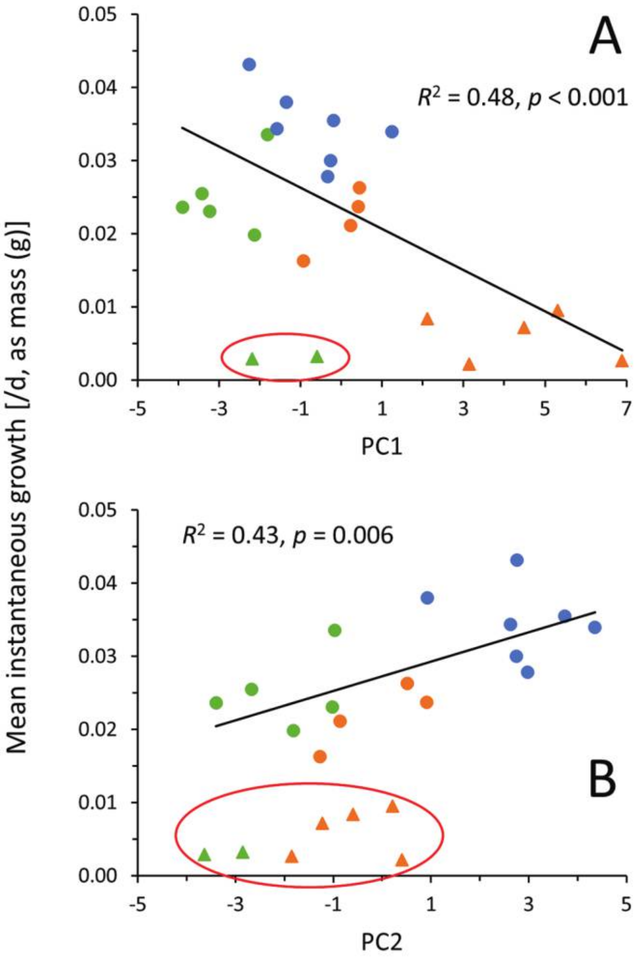

Figure 7. Principal components (PCs) regression of growth in silos on stream scores for water chemistry PC1 (A) and water chemistry PC2 (B) (see Fig. 6). Symbol color indicates physiographic regions: blue = Bluegrass, green = Appalachian Plateaus, and orange = Highland Rim. Triangles are defaunated streams, circles are occupied streams. The regression line for PC1 omits Horse Lick and Roundstone creeks (within ellipse). The regression line for PC2 omits all defaunated streams (within ellipse).

Defaunated streams had among the lowest TOC and water temperature, and all had a high percentage of karst in the watershed. TOC was 1.3 to 2.2 mg/L in defaunated streams compared with 1.7 to 5.4 in occupied streams (Fig. S1). The mean temperatures of 6 of the 7 defaunated streams were the coolest in our study (20.1–22.2°C). Only 1 defaunated stream, Drakes Creek, had a mean temperature (23.6°C) that fell within the range of occupied streams

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(22.4–25.0°C). Growth was strongly and positively related to water temperature (Fig. 8). A regression of growth on water temperature that included all streams had high explanatory power and predicted 0 growth at 20.2°C. A regression of growth on water temperature that excluded the 7 defaunated streams had less explanatory power and predicted 0 growth at ~17.8°C. This relationship was similar to relationships between growth, alkalinity, and nutrients, in that the 7 defaunated streams appear as outliers for which growth is substantially lower than expected at a particular temperature. However, we were unable to determine the most appropriate temperature model. The percentage of karst in the watersheds of defaunated streams was 27 to 93%; Elkhorn Creek and South Fork Licking River were the only occupied streams with >35% karst (Fig. S3).

Evaluation of 63 candidate models for predicting growth based on the variables most strongly correlated with PC2 (alkalinity, P, K, TKN, TOC, and temperature) showed that TOC and temperature were the most important predictors of mussel growth in silos (Table 3). When all streams were included, each of the 6 variables were included in at least 2 of the 10 best models, but TOC and temperature were included in all but 1 of the 10 best models. The model that did not include TOC had low support (temperature, alkalinity; $w_i = 0.017$). The model with the highest AIC weight included TOC, temperature, and alkalinity, but 2 other models had high support. All of the 10 best models explained a high percentage of variation in growth. Models that included only TOC or temperature had low support ($w_i < 0.016$).

TOC was of greater importance when defaunated streams were excluded, but these models explained less variation in growth than models that included all streams (Table 3). The model with the highest AIC weight included only TOC, and TOC appeared in all of the 10 best models. Water temperature appeared in only 4 of the 10 best models, and the model containing only temperature had weak support ($w_i = 0.0009$).

Sediment chemistry

PCA of 27 sediment variables extracted 4 components with eigenvalues > 1, and PC1 accounted for 65% of the variation in the data. PC2, PC3, and PC4 accounted for only 9.5, 6.5, and 4.4% of the variation in the data, respectively, and were uninterpretable because they had lower than expected eigenvalues based on a broken-stick distribution (Jackson 1993). PC1 separated streams based on higher levels of metals (especially Al, Ba, Be, Co, and Pb) in the Bluegrass vs lower levels of metals elsewhere. However, metals were only weakly correlated with PC1 (Table 4).

No pesticides were strongly correlated with PC1. Pesticides were more widely distributed in sediments than in water and were often present in streams with little agriculture in their watersheds or where pesticides were rare or absent in water samples (e.g., Horse Lick Creek, Rockcastle River, Roundstone Creek, Slate Creek). However, most pesticides were present at low concentrations near their detection limits, and higher concentrations were isolated occurrences limited to only 1 or 2 streams. Unlike in water samples, pesticides in sediments were not more prevalent

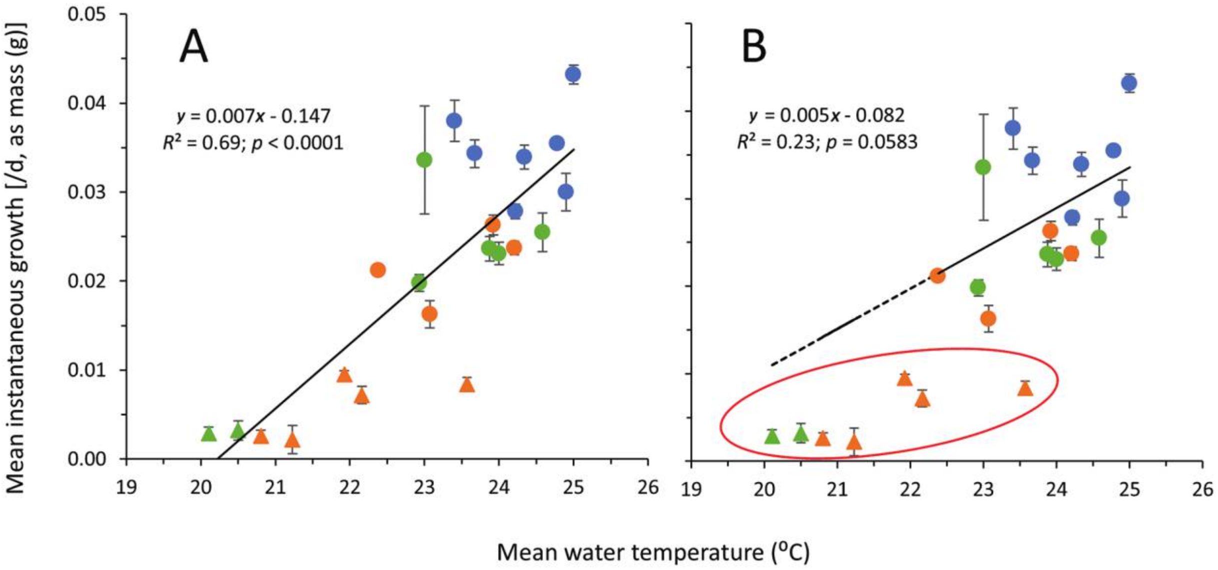

Figure 8. Mean (±1 SE) instantaneous growth in relation to water temperature. Symbol color indicates physiographic regions: blue = Bluegrass, green = Appalachian Plateaus, and orange = Highland Rim. Triangles are defaunated streams, circles are occupied streams. Relationship including all streams (A). Relationship excluding defaunated streams, which are located within the ellipse (B). The dotted portion of the regression line in (B) is extrapolated because it is beyond the range of values for the relationship.

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Table 3. Akaike information criteria (AIC$_c$) for the 10 best models for predicting mussel instantaneous growth based on alkalinity, P, K, total Kjeldahl N (TKN), total organic C (TOC), and mean stream temperature. ΔAIC$_c$ is the difference between the AIC$_c$ value of a given model and the model with the lowest AIC$_c$ value; w_i is the Akaike weight.

Variables in model	Adjusted R^2	AIC$_c$	ΔAIC$_c$	w_i	Evidence ratio
All streams					
TOC, temperature, alkalinity	0.84	−78.289	0.000	0.244	-
TOC, temperature, P	0.84	−77.639	0.649	0.177	1.38
TOC, temperature, alkalinity, P	0.84	−76.221	2.068	0.087	2.81
TOC, temperature, alkalinity, TKN	0.84	−75.696	2.593	0.067	3.66
TOC, temperature, alkalinity, K	0.83	−75.415	2.874	0.058	4.21
TOC, temperature, K, TKN	0.83	−74.987	3.302	0.047	5.21
TOC, temperature	0.81	−74.952	3.337	0.046	5.30
TOC, temperature, K, P	0.83	−74.743	3.546	0.042	5.89
TOC, temperature, K	0.82	−74.732	3.556	0.041	5.92
Temperature, alkalinity	0.79	−72.920	5.369	0.017	14.65
Defaunated streams excluded					
TOC	0.56	−80.386	0.000	0.157	-
TOC, temperature	0.57	−79.287	1.099	0.090	1.73
TOC, TKN	0.56	−78.962	1.424	0.077	2.04
TOC, P	0.55	−78.779	1.607	0.070	2.23
TOC, alkalinity	0.54	−78.294	2.092	0.055	2.85
TOC, K	0.53	−78.166	2.220	0.052	3.03
TOC, alkalinity, P	0.56	−77.568	2.818	0.038	4.09
TOC, temperature, alkalinity	0.55	−77.193	3.193	0.032	4.94
TOC, temperature, TKN	0.54	−77.133	3.253	0.031	5.09
TOC, temperature, P	0.54	−76.879	3.507	0.027	5.78

in areas with high row crop land cover, and no streams had consistently high sediment pesticide levels. Legacy pesticides and associated metabolites such as DDT, aldrin, and dieldrin were not detected in sediments in any stream, with the exception of endrin aldehyde, which was detected in only 1 sample (Kinniconick Creek, 3.38 μg/kg).

We found elevated interstitial ammonia in only 2 peepers, 1 each from 2 streams (Little River = 0.28 μg/L; Tygarts Creek = 0.38 μg/L). Ammonia concentration was <0.10 μg/L in all other peepers.

Sediment temperature was nearly identical to water temperature. Mean temperature was not significantly different between cages and silos (paired t-test, $t = 0.59$, $p = 0.562$; mean difference, silos–cages = 0.04°C), and a linear regression of cage temperature on silo temperature yielded essentially a 1:1 relationship (slope = 1.009 ± 0.102; intercept = −0.239 ± 2.376). Growth in cages was positively related to sediment temperature when all streams were included, but temperature explained less of the variation in growth in cages ($R^2 = 0.49$, $p = 0.0003$) than in silos (see Fig. 8). These relationships predicted 0 growth at similar temperatures (20.3°C for cages and 20.2°C for silos). Growth in cages was not related to sediment temperature when defaunated streams were excluded ($R^2 = 0.08$, $p = 0.279$).

Survival in cages was not related to sediment chemistry PC1 ($R^2 = 0.05$, p = 0.337, arcsine survival). Growth in cages was significantly related to stream scores for sediment PC1 ($R^2 = 0.48$, $p = 0.0006$), but omitting defaunated streams only modestly improved explanatory power ($R^2 = 0.51$). Most metals and metalloids were positively correlated with mussel growth, and it made little difference whether or not defaunated streams were excluded (Table S3).

DISCUSSION

Acute vs chronic effects

The high mussel survival in silos in all streams indicates a lack of acute water-borne toxicity during our study. Survival in cages similarly provides little evidence of acute sediment toxicity. Survival in cages was lower than in silos, but cage survival was not related to the health of the mussel fauna: we observed uniformly high survival in several defaunated streams (Little, Nolin, and Red rivers; Drakes Creek) and low survival in some occupied streams (Beech Fork, Licking, Redbird, and South Fork Kentucky rivers). Moreover, survival was not related to water or sediment chemistry for silos or cages. The lack of evidence for acute toxicity is surprising and informative because the expectation of acute

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Table 4. Principal components loadings for sediment chemistry variables among 23 Kentucky streams. The percentage of variation explained by the principal component is given below the component number. All units are based on sediment dry mass.

Variable	PC1 65%
4-Nitrophenol (μg/kg)	0.07
Aluminum (mg/kg)	−0.23
Antimony (mg/kg)	−0.20
Barium (mg/kg)	−0.23
Beryllium (mg/kg)	−0.23
Cadmium (mg/kg)	−0.13
Calcium (mg/kg)	−0.19
Chromium (mg/kg)	−0.17
Cobalt (mg/kg)	−0.23
Copper (mg/kg)	−0.21
Dicamba (μg/kg)	−0.03
Iron (mg/kg)	−0.22
Lead (mg/kg)	−0.24
Magnesium (mg/kg)	−0.21
Manganese (mg/kg)	−0.22
Mercury (mg/kg)	−0.18
Molybdenum (mg/kg)	−0.17
Nickel (mg/kg)	−0.22
Picloram (μg/kg)	−0.07
Potassium (mg/kg)	−0.21
Selenium (mg/kg)	−0.22
Silver (mg/kg)	−0.13
Sodium (mg/kg)	−0.21
Solids, %	0.18
Thallium (mg/kg)	−0.15
Vanadium (mg/kg)	−0.20
Zinc (mg/kg)	−0.22

effects of contaminants has motivated many previous efforts to identify causes of mussel declines (reviewed by Keller et al. 1997).

In contrast to survival, mussel growth was strikingly concordant with stream faunal health. The 7 defaunated streams had uniformly low growth in both the silos and cages, which suggests that growth reduction associated with chronic exposure to contaminants or other factors is a mechanism for mussel declines. Reduced juvenile growth and fitness is consistent with the lack of recruitment often associated with enigmatic mussel declines. We have no measurements of body condition or other indicators of fitness from our animals, and we do not know how low growth influences long-term survival. However, in mussel hatcheries, low growth rates in the range we observed in defaunated streams is abnormal and usually results in mortality within a few months (M. McGregor, unpublished data, C. Barnhart, personal communication).

Water vs sediment exposures

The general similarity of growth in silos and cages indicates that mussel response to ambient conditions is not specific to water or sediment exposures. Differences in survival and growth between silos and cages probably represent artifacts based on enclosure type rather than responses to stream conditions. Silos experienced low survival only infrequently, probably because of localized flow conditions that resulted in anoxia within silo chambers. Cages appeared more vulnerable to these artifacts based on their lower and more variable survival at most sites. Cages tended to become buried, which could create anoxia and prevent confined mussels from remaining in their normal position near the water-sediment interface. Slightly lower growth in cages also may be related to generally poorer conditions in cages, but overall differences in growth between silos and cages were small.

Sediment chemistry explained little of the variation in mussel growth. The relationship between growth and PC1 for the sediment variables is probably an artifact of the slightly higher levels of metals in productive Bluegrass streams. Sediment toxicity is invoked as a cause of mussel declines in some areas because contaminant concentrations can be higher in sediments where juvenile mussels feed (e.g., Cope et al. 2008, Besser et al. 2015, Archambault et al. 2017). The lack of association between sediment chemistry and mussel survival or growth in our study was surprising, and, given the prevalence of row-crop agriculture in some watersheds, we were surprised by the rarity of water-insoluble pesticides that can adsorb to sediment particles.

Water chemistry factors related to growth

The positive relationship between mussel growth and PC2 appears to reflect expected responses to higher water temperature, alkalinity, and nutrients or food availability (P, K, TOC, TKN). Our study streams are nutrient enriched to varying degrees by human activities. However, differences in nutrients, temperature, and alkalinity also reflect fundamental characteristics of the streams based on their physiography and underlying geology. For example, soils in the Bluegrass are fertile and well-buffered because of the phosphate-rich limestone bedrock, in contrast with the poorer, sandstone-derived soils of the Appalachians. These fundamental differences among physiographic regions have a large effect on biological communities in Kentucky streams (Pond et al. 2003). Lower growth is expected in all defaunated streams because of their cooler temperatures and lower productivity. However, growth in defaunated streams is well below that predicted by the growth-PC2 relationship, suggesting that other factors can override these otherwise strong, fundamental determinants of growth.

The important conservation question is: what factors are responsible for the apparently abnormally low growth in

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defaunated streams? PC1 reflects contaminants associated with intensive row-crop production (pesticides and nitrates/nitrites). The negative relationship between growth and PC1 is compelling because intensive agriculture is often implicated in mussel declines (e.g., Poole and Downing 2004, Sharpe and Nichols 2007). We cannot determine whether these contaminants are causal agents of low mussel growth or associated with unmeasured growth-reducing factors. Pesticides have variable effects on mussels, and their chronic effects on mussel growth and survival are poorly understood (e.g., Keller et al. 1997, Bringolf et al. 2007a, b).

Of the contaminants we measured, nitrate/nitrite loaded most strongly onto PC1, and mussel growth was uniformly low in streams with nitrate/nitrite concentrations >2 mg/L. Acute sensitivity of juvenile mussels to nitrate is low, and the median lethal nitrate concentrations for North American *L. siliquoidea* and *Megalonaias nervosa* and European *Anodonta anatina* and *Unio crassus* in the laboratory were 2 orders of magnitude higher than maximum concentrations in our study (Douda 2010, Soucek and Dickinson 2012). However, field studies found reproducing populations of several European species only in streams with NO_3 concentrations <2 mg/L (Köhler 2006, Zettler and Jueg 2007, Douda 2010; but see Denic et al. 2014), which is remarkably similar to our results.

NO_3 could be more detrimental to mussels in the field than in the laboratory because high NO_3 may represent conditions favorable for ammonia formation, particularly in sediments (Strayer 2014). Unionized ammonia (NH_3) is acutely toxic to juvenile mussels, which are more sensitive to NH_3 than most other aquatic organisms that have been tested (Augspurger et al. 2003, Mummert et al. 2003, Wang et al. 2007). NH_3 also is associated with chronic effects on mussels, such as decreased growth of *L. cardium* in the laboratory (Newton and Bartsch 2007) and lack of recruitment in wild populations of *Elliptio complanata* (Strayer and Malcom 2012). We detected elevated interstitial NH_3 in only 2 samples, but the short deployment period of our peepers (2 wk) means that we could have missed spikes of NH_3 occurring at other times during the study.

Agricultural contaminants could also indirectly affect mussels by altering food resources necessary for growth (see Graymore et al. 2001). Human enrichment of streams is assumed to increase mussel growth because enrichment increases food availability (Strayer 2008, Fritts et al. 2017). However, the converse idea that human impacts on streams could have eliminated essential food items has received little attention.

Despite the compelling association of low growth and row-crop agriculture, low growth was not associated with agricultural effects in two other defaunated streams. The Horse Lick and Roundstone creek watersheds have little agriculture of any kind and agricultural contaminants were rarely detected, yet these streams had low growth and were conspicuous outliers from the growth-PC1 relationship. Mussel declines in these streams and elsewhere in the Appalachian Plateaus have been attributed to coal mining (Harker et al. 1980, Layzer and Anderson 1992, Warren and Haag 2005). Both the Horse Lick and Roundstone watersheds have a history of coal mining, but other streams with a higher intensity of mining supported higher mussel growth (Redbird and South Fork Kentucky rivers), and we found no correlation between growth and intensity of coal mining. Characteristic effects of coal mining on streams such as elevated major ions, metals, and conductivity are associated with mussel declines (Price et al. 2014, Gangloff et al. 2015, Zipper et al. 2016). We observed none of these effects in Horse Lick or Roundstone creeks; instead, they were distinguished primarily by cooler temperatures, lower nutrients, and high forest cover similar to other Appalachian streams that supported higher mussel growth (Rockcastle, Redbird, and South Fork Kentucky rivers; Tygarts Creek). Our sampling may have missed spikes of conductivity, but elevated conductivity and major ions are routinely observed in coal-influenced streams (Gangloff et al. 2015), and continuous monitoring in Horse Lick Creek in 2016 revealed no periods of elevated conductivity (M. Croasdaile, personal communication). Our study excluded streams with obvious and severe coal mining impacts (e.g., acid-mine drainage), which probably negatively affect mussels and other aquatic organisms. However, we found no evidence of coal mining effects in any stream, and our data do not support the previous supposition of coal mining as a cause of mussel declines in Horse Lick and Roundstone creeks.

Other factors previously implicated in mussel declines were also unrelated to mussel growth in our study. High salinity wastewater from oil and gas extraction is implicated in mussel declines (Warren and Haag 2005, Patnode et al. 2015), but we found no associations between growth and oil and gas extraction or related water quality factors (e.g., chloride, barium; see Kondash et al. 2014). Total suspended solids (TSS) concentrations higher than those found in our study predicted mussel extirpation in a simulation model (Hansen et al. 2016), but we found no correlation between TSS and growth. Elevated metals are associated with mussel declines in several areas (Keller et al. 1997, Johnson et al. 2014). We found positive correlations between growth and several metals, although this is probably an artifact of higher growth in the productive Bluegrass streams, and the metal concentrations in these streams were lower than those considered harmful to mussels (e.g., Wang et al. 2007; Table S3).

All 7 defaunated streams shared the following characteristics: low TOC, low water temperature, and a high percentage of karst. These factors were largely confounded in our study probably because groundwater inputs from karst are cool and typically low in TOC. We do not know if karst plays a direct role in growth reduction or if this relationship

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is spurious. Karst could allow contaminants to enter groundwater quickly via sinkholes and other conduits and arrive at streams before sunlight and soil microorganisms degrade them. However, no such contaminants were identified in all defaunated streams.

Mussel growth is strongly related to temperature (Hanlon 2000, Carey et al. 2013). The strong relationship between temperature and growth in our study could indicate that defaunated streams are too cold to support mussel growth, but this explanation seems unlikely for several reasons. First, these streams once supported diverse and abundant faunas, and it is unlikely that stream temperatures have decreased in the last few decades. Second, even in our coolest stream, Horse Lick Creek, reduced mussel growth was observed during the onset of mussel declines in the lower portion of the creek (including our study site) in the 1980s and 1990s. However, normal growth continued in the upper portion of the stream, which was unaffected by the decline at that time (Houslet and Layzer 1997). Third, onset of growth in *L. fasciola* occurs at ~15°C (Hanlon 2000), and the predicted onset of growth from our data set excluding defaunated streams (17.8°C) is closer to this value than that predicted by our full dataset (20.2°C). Moreover, growth at our defaunated streams was substantially lower than predicted by a temperature-growth relationship for *L. fasciola* (Carey et al. 2013; Figure S4). Thus, mussel growth in defaunated streams appears to be lower than expected based on temperature alone.

Higher TOC could broadly represent greater food resources for mussel growth. Lower food availability and temperature may predispose mussels to chronic effects of contaminants or other stressors, but we did not identify any stressors common to all defaunated streams. Our defaunated streams are in no sense coldwater, oligotrophic streams. Instead, they probably are on the lower end of temperature and productivity gradients seen in warmwater streams in the eastern US. Conditions associated with low growth differed subtly from those in streams that supported higher growth (e.g., temperature differences of 1–2°C and small differences in TOC). This raises the possibility that associations between temperature, productivity, and mussel declines have been overlooked in other areas.

Conclusions

Our results show that growth is a sensitive endpoint for assessing mussel responses to stream conditions. Mussel growth at a site is an integrated response to the full range of physical and biotic variables that are influenced by processes occurring throughout the watershed upstream of the site. The 2-toned shell coloration pattern we found in shells from Kinniconick and Tygarts creeks suggests that conditions that support growth can change abruptly, but we were unable to determine the nature of these changes. Further, it is unclear why darkly stained shells were associated with low growth.

The association of growth reduction with agricultural contaminants is compelling and suggests a potential mechanism and causal factor for mussel declines in some areas. However, we did not identify any contaminants that can explain reduced mussel growth and mussel declines in all our defaunated streams. Other, unidentified factors may be responsible for mussel declines in the non-agricultural Horse Lick and Roundstone creeks, but the previous supposition of coal mining effects is not supported by our data. Alternatively, it is possible that the association between agricultural contaminants and growth is spurious, and a single, pervasive—but as yet, unidentified—factor affects all of our defaunated streams, as well as other streams that have experienced mussel declines. If so, future studies should pay particular attention to investigating factors that may have inordinate effects in cooler, less productive streams.

Our results should be viewed with several caveats. First, our study used juvenile mussels reared under hatchery conditions with glochidia from a single source population ~150 km upstream of our study site in the Licking River. Genetic preadaptation to specific stream conditions can influence juvenile mussel performance in stream exposure experiments (Denic et al. 2015). Consequently, high growth in the Licking River and elsewhere in that basin (South Fork Licking River and Slate Creek) could be a result of adaptations of our juveniles to the conditions in that basin. However, we saw similarly high growth in other river basins (Salt and Kentucky) and another physiographic region (Tygarts Creek). All experimental animals have an evolutionary history, but we used hatchery-reared animals to minimize the bias that can result from the environmental histories of wild-collected individuals. A 2nd caveat is that we used 1 mussel species, and additional studies are needed to evaluate the generality of our results across taxa. Finally, 2015 was an unusually wet summer in our study area, and future studies are needed to evaluate the generality of our results in other years and stream flow conditions.

In situ exposures of juvenile or adult mussels have been used for at least 20 y to investigate the effects of various environmental factors on mussel survival or fitness (e.g., Warren et al. 1995, Bartsch et al. 2003, Gagné et al. 2004, Nobles and Zhang 2015, Bartsch et al. 2017), but this technique is underused given its broad applications. The technique is especially valuable for measuring sublethal or chronic effects, such as those reflected by growth, because it is difficult to reproduce the full range of natural conditions that could influence mussel physiology and performance. In our study, treatment artifacts associated with silos were minimal, and silos provided informative depictions of growth based on stream conditions. Mussels in sediment cages have more direct contact with the sediments in which they normally occur, but cages are more prone to treatment artifacts that

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do not necessarily reflect stream conditions. These treatment artifacts could have been more prevalent than usual in our study because of high flows and high sediment transport during 2015. Juvenile mussels are ideal for in situ exposures because they may be more sensitive than adults to some contaminants, and their higher growth rate makes it easier to detect differences in growth. Recent advances in mussel culturing make available large numbers of juveniles of similar size, age, and origin, which allows fully replicated studies with minimal impacts on wild populations.

When growth is used as an experimental endpoint, it is important to account for natural determinants of growth, particularly when comparing among streams that differ in physiography or other fundamental factors (see Denic et al. 2015). In situ exposure of hatchery-reared mussels in silos, cages, or other devices carries a risk of introducing those animals and other associated organisms into study streams. It is important to use appropriate biosecurity to lessen the risk of introducing disease and to avoid using species or genotypes that are not native to the study area (Jones et al. 2006, Patterson et al. 2018). Mussels are often described as canaries in the coal mine because of their apparent high sensitivity to environmental degradation. The use of in situ mussel exposures offers the ability to quantify these responses in a natural context, which will provide a better understanding of the effects of stream impairment on ecosystem integrity.

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Author contributions: WRH conceived the study design with input from all authors, led the field work, and analyzed the data. JJC assisted with study site selection and field work and coordinated water and sediment chemistry analysis. MAM coordinated production of juvenile mussels and construction of silos. RB coordinated ammonia analysis in peepers. JAS provided logistical support and input on all aspects of the study. WRH wrote the first draft of the manuscript with input from all authors; WRH and JAS revised the manuscript.

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