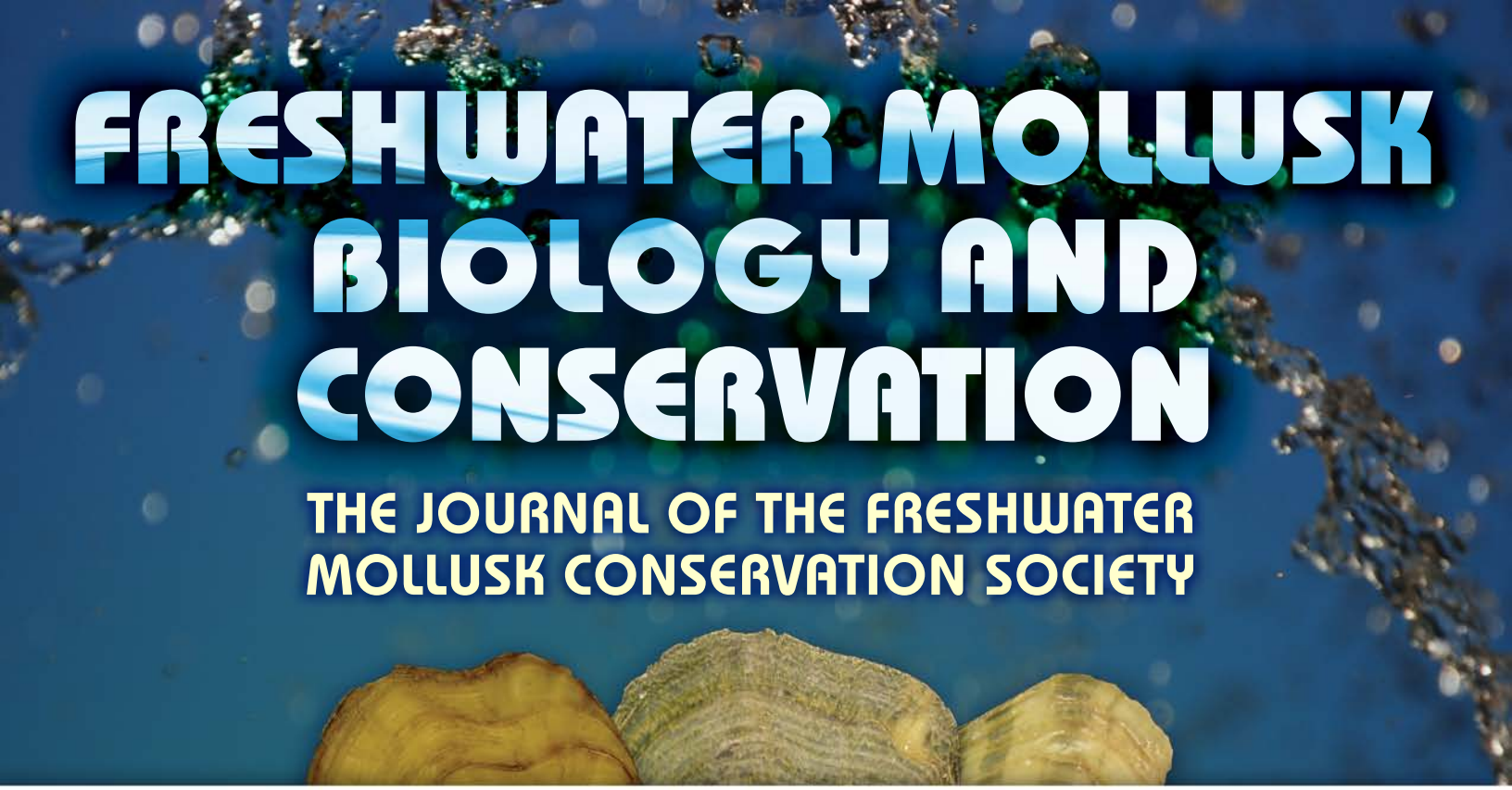

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A SPECIAL ISSUE OF FRESHWATER MOLLUSK BIOLOGY AND CONSERVATION
PROCEEDINGS OF THE 2018 FRESHWATER MOLLUSK HEALTH AND DISEASE WORKSHOP

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A SPECIAL ISSUE OF FRESHWATER MOLLUSK BIOLOGY AND CONSERVATION PROCEEDINGS OF THE 2018 FRESHWATER MOLLUSK HEALTH AND DISEASE WORKSHOP A FORWARD TO THE SPECIAL ISSUE

Megan E. Bradley[1] and Diane L. Waller[2]

[1] *U.S. Fish and Wildlife Service, Genoa National Fish Hatchery, S 5631 State Hwy 35 Genoa, WI 54632 USA*

[2] *U.S. Geological Survey, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, WI 54603 USA*

As the planet faces the loss of native freshwater mollusk diversity and declines of biomass, the conservation community's attention has shifted from the initial triage of preservation to a search for clearer mechanisms of these declines. In some water bodies, the loss of the mollusk fauna is linked to acute anthropogenic impacts (e.g., chemical spills), habitat destruction, and invasive species, but in many streams clear causation has remained elusive. Therefore, the topic for the 2018 Freshwater Mollusk Conservation Society Biennial Workshop, held in La Crosse, Wisconsin, from March 13 to 15, 2018, was freshwater mollusk health and disease.

Assessing the health of these organisms is difficult because the environment they inhabit is a challenging workplace for humans, there are few established benchmarks for physiological normalcy ("health") for the group, much less for individual species, and financial and personnel resources for research and monitoring are scarce. The goal of the workshop was to increase awareness of, and encourage expanded research on, freshwater mollusk health and the potential role of disease by (1) identifying knowledge gaps in assessing mollusk health, (2) providing information on health assessment and diagnostic tools for mollusks, (3) aligning sampling and relocation protocols with those for health and disease assessment, and (4) promoting interdisciplinary cooperation and communication to advance knowledge of freshwater mollusk health. Most presentations focused on bivalves, and coverage of freshwater gastropods was scant; the workshop program included only two presentations pertinent to gastropods, and, of the eight articles in this special issue, only one pertains to gastropod health or disease. Gastropod health and disease deserves increased attention.

The workshop represented a conversation among colleagues across organizations and continents, and this special issue features eight articles that encompass the topics discussed at the workshop. Waller and Cope provide an overview of the state of mussel health assessment and steps for advancing knowledge, which sets the stage for a review of enigmatic mussel declines and a new paradigm for investigation of their causes by Wendell Haag. Wengström et al. provide a perspective on die-offs of *Margaritifera* in Sweden. Andrew McElwain reviews the potential role of parasites and disease in mussel health, while Leis et al. and Goldberg et al. report survey results of the mussel microbiota and virome, respectively. Ciparis et al. evaluate condition indices for assessment of ion exposure, while Wolf et al. present the outcome of the disease risk assessment workshop session. Several presentations from the workshop are not represented by articles in this special issue, but we thank each presenter for their valuable contribution to the workshop and to the state of our knowledge on freshwater mollusk health. The workshop program is available at https://molluskconservation.org/EVENTS/2018Workshop/FMCS_2018%20program_finalREV.pdf.

Editor's Note: We thank Dr. Diane Waller for serving as Guest Editor for several of the articles in this special issue.

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REGULAR ARTICLE

THE STATUS OF MUSSEL HEALTH ASSESSMENT AND A PATH FORWARD

Diane L. Waller[1]* and W. Gregory Cope[2]

[1] *U.S. Geological Survey, Upper Midwest Environmental Sciences Center, La Crosse, WI 54603 USA*
[2] *Department of Applied Ecology, North Carolina State University, Raleigh NC 27695-7617 USA*

ABSTRACT

Declines of freshwater mussel (order Unionida) populations worldwide are attributed to habitat degradation, pollution, and invasive species, among other factors. However, these purported causes do not fully explain the enigmatic decline and large-scale die-offs of mussels that have occurred in apparently healthy streams across a wide geographic region. The roles of the microbiota and pathogens in mussel health have been understudied, and, as a result, few data exist to compare the microbiota of healthy mussels to that of stressed or dying mussels. Captive propagation and stocking programs have expanded across the globe without standard diagnostic protocols to assess health or potential diseases in hatchery-reared or wild stocks. Nonindigenous species, contaminants of emerging concern, and anthropogenic climate change could alter adversely the underlying processes that support mussel health, such as nutritional status and microbial composition, and these factors could increase the risk for outbreaks of opportunistic and emergent mussel disease. We propose a coordinated, collaborative, and multidisciplinary effort to advance methods for assessing freshwater mussel health. We identify research and resources needed to answer central questions surrounding mussel health, including identifying potential agents of disease, defining clinical signs of declining condition, refining stress-specific biomarkers for health assessment, and developing protocols specific for mussels.

KEY WORDS: unionid mussels, disease, biomarker, diagnostic, pathogen, mortality, decline

INTRODUCTION

The imperiled status of freshwater mussels (order Unionida) is well documented (Williams et al. 1993; Lydeard et al. 2004; Strayer et al. 2004; Régnier et al. 2009). The most commonly cited contributors to mussel declines are habitat destruction or alteration, pollution and poor water quality, impoundment, and invasive species (Strayer et al. 2004; Dudgeon et al. 2007; Downing et al. 2010; Haag and Williams 2014). However, these factors do not explain the declines and large-scale die-offs of mussels in otherwise healthy, unimpounded streams across a wide geographic region. The significant decline of mussels that occurred from the 1970s to 1990s has been described as "enigmatic" with characteristics suggesting a virulent and widespread factor specific to mussels (Haag and Williams 2014; Haag 2019).

One topic missing from most publications related to mussel conservation is organismal health and disease. The role of the microbiota and pathogens in mussel health has been understudied, and, as a result, their role in mussel declines is unknown. No clinical signs or biomarkers have been established to distinguish a healthy mussel from one that is of compromised health or dying. Although the suggestion that mussel mortality and declines could be pathogen related has not been widely considered among freshwater biologists, the effects of epizootics on other aquatic invertebrates are well documented. For example, fungal, bacterial, and viral diseases (Edgerton et al. 2002; Jiravanichpaisal et al. 2009; Longshaw 2011; Bower 2012) have adversely affected crayfish populations worldwide. Numerous diseases have significantly impacted marine bivalves, including ostreid herpesvirus and the protozoan disease bonamiasis in oysters (*Ostrea* spp.; Zanella et al. 2017). More recently, a Densovirus (Parvoviridae) has been associated with sea star wasting disease, the cause of extensive mortality among populations of 20 asteroid species in the Pacific Northwest (Hewson et al. 2014). In

*Corresponding Author: dwaller@usgs.gov

contrast, reports of pathogens in freshwater mussels are limited to those responsible for explosive epidemics in the Triangle-shell Pearl Mussel (*Hyriopsis cumingii,* family Unionidae) (see Zhong et al. 2016) in China. It seems unlikely that other freshwater mussel species are unaffected by comparable infectious agents.

We discuss the state of knowledge on freshwater mussel health assessment and disease and outline a strategy for advancing and expanding that knowledge. First, we provide an overview of research efforts on mussel health and disease in the past 30 years. We use "disease" throughout the article to refer to any impairment that interferes with or modifies normal function, including responses to environmental factors such as food availability, toxicants, and climate; infectious agents; inherent or congenital defects; or combinations of those factors (Wobeser 1981). Definitions of terms related to health assessment and disease used in the article are provided in Appendix A. Second, we discuss the growing need for a focused effort on health and disease research in mussels and describe the application and benefits of a holistic approach. We discuss existing approaches for monitoring health in other faunal groups and highlight their application to mussel conservation. Finally, we discuss research and resources needed to advance the state of knowledge of mussel health.

PERSPECTIVE ON FRESHWATER MUSSEL MORTALITY EVENTS

Questions about health and disease of mussels are not new. A 1986 workshop was prompted by a series of unexplained mussel die-offs that occurred between 1977 and 1986 in the Upper Mississippi (Blodgett and Sparks 1987; Thiel 1987), Tennessee (Ahlstedt and Jenkinson 1987; Jenkinson and Ahlstedt 1987), Powell, Neosho (Zale and Suttles 1987), Bourbeuse, and Meramec (Buchanan 1987) rivers (see also Neves 1987a). Some of these and other rivers in the eastern USA also were cited as areas of significant mortality in the 1940s and 1950s (e.g., Upper Mississippi River, Neosho River, Tennessee River), but details of these events were not provided (Latendresse 1987). The circumstances of each die-off varied, but there were five common threads: (1) other faunal groups were unaffected; (2) responses varied among mussel species; (3) mortality occurred in both adults and juveniles; (4) mortality often reoccurred several months or a year later, often in association with increased water temperature or gravidity; and (5) no contaminants, water quality issues, or parasites were associated with die-offs.

Several factors complicated efforts to identify causative agents of mortality. Mussel mortality often was reported incidental to other sampling events or by commercial harvesters, and considerable time elapsed between onset of the events and sample collection. Robust sample collection and analysis procedures were not delineated and followed, resulting in fragmented and opportunistic diagnostics. Diagnostics often were completed on dying mussels that were secondarily infected with opportunistic bacteria and fungi (Jenkinson and Ahlstedt 1987), or sampling was completed after mortality had subsided and no evidence of stress or infection was found in surviving mussels (Zale and Suttles 1987; Sparks et al. 1990). No pathology was found that was indicative of an infectious agent in dead or moribund specimens from six rivers with reported die-offs (Kern 1987). Findings were negative for a potential viral agent; however, the methods utilized fish cell lines to grow viruses (Thiel 1987). The most substantial result was that mussel health seemed to be correlated with total bacterial population, particularly the percentage of a yellow Gram-negative rod bacterium (Scholla et al. 1987); however, no further research was reported on this bacterium.

Other unexplained mussel declines and die-offs have occurred throughout North America and Europe and continue to the present day. Since the 1980s, mussel mortality has been reported from at least 18 sites in Oregon, Washington, California, and Idaho (E. Blevins et al., unpublished data). For example, the decline of the Western Pearlshell Mussel (*Margeritifera falcata*, family Unionidae) in Upper Bear Creek, Washington, was first observed in 2001, and continued mortality was documented in subsequent surveys (Brenner 2005; Thomas 2008). Tissue pathology and skewed sex ratios (4 males:1 female) were observed in mussels from the affected sites, but no specific cause of mortality was determined (Brenner 2005). In a follow-up study, *M. falcata* were relocated from an unaffected site to an affected site in the creek and monitored for the onset of mortality and associated changes in condition and tissue morphology (Thomas 2008). Relocated mussels died at the same time of the year as those in previous mortality events (i.e., fall), but early indicators of stress or the cause of mortality were not detected. Water samples collected upstream of the mortality site in late summer, preceding the onset of mortality, were toxic to fathead minnows in bioassay tests; however, no clear link was made between mussel mortality and water quality. More recent reports of mussel declines in the Pacific Northwest have come from the Crooked River, Oregon (2014 and 2018), and Chehalis River, Washington (2018) (E. Blevins et al., unpublished data), again, without explanation. In Europe, unexplained die-offs have been reported in populations of the Freshwater Pearly Mussel (*M. margaritifera*) in Sweden (Wengström et al. 2019).

Two recent mortality events in the USA are noteworthy because of their occurrence in high-value waters and the involvement of listed species. In late 2016, a die-off was observed in Ohio's Big Darby Creek, a State and National Scenic River, with mortality extending into spring 2017 (A. Sasson et al., unpublished data). All species of mussels were affected, including two federally endangered species (Clubshell, *Pleurobema clava*, and Northern Riffleshell, *Epioblasma torulosa*). In 2016, mass mortality of Pheasantshell (*Actinonaias pectorosa*) was reported from reaches of the Clinch River that historically have supported one of the most diverse mussel communities in the USA (Leis et al. 2018; J. Richard, unpublished data; C. Carey et al., unpublished data). Mortality

reoccurred in 2017, 2018, and 2019 and spread to additional species and sites.

The spatial and temporal extent of unexplained mussel declines and die-offs is not well documented, and a comprehensive review of the topic is beyond the scope of this paper. A review of enigmatic declines is provided by Haag (2019). Sufficient historic and current evidence of mussel declines exists to justify a greater focus on the topic of health and disease.

RESEARCH ON THE MICROBIOTA OF FRESHWATER MOLLUSKS

The 1986 mussel die-off workshop recommended increased research on mussel health (Neves 1987b), but those efforts have not been initiated. Published research on mussels in general has increased steadily over the past 30 years (Strayer et al. 2004; FMCS 2016), but research on the microbiota and infectious diseases remains scarce. Reports of freshwater mollusk infection and/or disease declined between 1970 and 2009, in contrast to significant increases in reports for other freshwater groups including amphibians, fishes, and crayfishes (Johnson and Paull 2011). Most mollusk reports (86%) dealt with snails infected by digenean trematodes. Since 1990, only about 95 disease-related articles have been published on freshwater bivalves (excluding Sphaeridae). Because of its economic value, *H. cumingii* was the subject of 16% of these articles, and *Dreissena* spp. (family Dreissenidae) were the focus of 35%. The remaining articles included one or more species of unionid mussels, excluding *H. cumingii*. Two reviews of freshwater mollusk disease highlighted the lack of information and the need for additional research on this topic (Grizzle and Brunner 2009; Carella et al. 2016).

The most important parasitic diseases of marine bivalves are nonciliate protozoans, including *Bonamia*, *Perkinsus*, *Haplosporidium*, and *Marteilia* (Zanella et al. 2017). The only reported protozoan infections of unionids are of Ciliophora (Grizzle and Brunner 2009; Carella et al. 2016), which are primarily ectoparasites of the gills and labial palps and have not been reported to cause serious pathology (McElwain 2019). Grizzle and Brunner (2009) suggested that pathogenic protozoans may be absent in freshwater mussels or have been overlooked. Detection of microparasites, such as haplosporidians, generally requires a histological exam accompanied by PCR or *in situ* hybridization (ISH) for verification (OIE 2016), and there are currently no reported assays specific to freshwater mussels. McElwain (2019) concluded that parasites have not been substantiated as a cause of mussel die-offs or declines. A review of endosymbionts (i.e., all organisms living in a host including parasites) of North American and European mussels found 239 studies over 168 years, but most mussel species (53%) have never been examined (Brian and Aldridge 2019). Only 48 of the 239 studies evaluated effects of endosymbionts on mussels; of those 48, none found a positive effect on the host and 72% found a negative effect. Nevertheless, Brian and Aldridge (2019) concluded that effects of endosymbionts on mussels are understudied and mostly unknown.

Early research related to bacteria and unionids focused on the risk of disease transfer between mussels and fish and the steps needed to reduce those risks. Initial methods were developed to assess microbial concentration in body fluids and whole-body tissues of mussels (Starliper et al. 1998; Starliper 2009) and to investigate transmission of bacteria between mussels and fish (Starliper 2001, 2005, 2008, 2009; Starliper and Morrison 2000). These studies demonstrated that wild mussels could harbor and potentially transmit fish pathogens (Starliper 2008; Starliper et al. 2008), and they established a quarantine period for mussels to depurate bacteria and reduce or eliminate the risk of pathogen transfer (Starliper 2001, 2005, 2009; Starliper and Morrison 2000). This research also provided baseline information on the microbiota of wild, apparently healthy mussels in the Clinch and Holston Rivers, which experienced previous die-off events (Starliper et al. 1998, 2008). An investigation of a recurrent mussel die-off in the Tennessee River, Alabama, was one of the first systematic efforts to examine the potential role of bacteria in mussel mortality (Starliper et al. 2011). The microbiota of moribund and apparently unaffected Ebonyshell (*Fusconaia ebena*) were sampled before, during, and after die-off events. Mean bacterial numbers were about 100 times greater in moribund mussels relative to unaffected mussels. The predominant bacteria found in both unaffected and moribund *F. ebena* were *Aeromonas* spp., but a link between disease and a bacterial agent was not established.

A limited number of studies have investigated the pathogenicity of specific bacterial species and strains to mussels. Ercan et al. (2013) challenged the Thick-shelled River Mussel (*Unio crassus*) with two fish pathogens, *Yersinia ruckeri* and *Lactococcus garvieae*, but they recovered no bacteria from hemolymph and reported no signs of pathology. *Aeromonas veronii* SJ-2 was isolated from moribund mussels associated with a large-scale mortality event of *H. cumingii* (Zhong et al. 2016). Mussels that were experimentally challenged with the same bacterium developed disease symptoms, demonstrating it as the causative agent. *Aeromonas* was one of the predominant bacterial groups recovered from mussels in several river systems associated with mortality events (Starliper et al., 2011; Leis et al. 2019), and this genus includes known pathogens of freshwater fish (Austin and Austin 2012), crayfish (Jiravanichpaisal et al. 2009), and marine bivalves (Zanella et al. 2017). Inoculation of *D. polymorpha* with four indigenous bacterial species, including three *Aeromonas* spp., at high concentrations and/or elevated water temperature caused mortality (Gu and Mitchell 2002). *Aeromonas* and other indigenous bacterial species deserve further consideration as potential mussel pathogens, particularly in association with environmental (e.g., elevated temperature, hypoxia) and endogenous stressors (larval brooding, spawning, high density).

Previous investigations of endogenous bacteria (e.g.,

Starliper et al. 1998; Chittick et al. 2001; Nichols et al. 2001; Starliper et al. 2008) focused on whole-body homogenates, the digestive gland, and surface structures, where bacterial species may be transient or similar to that of the environment. Antunes et al. (2010) compared culturable bacterial assemblages among fluid compartments (hemolymph, extrapallial fluid, and mucus) of wild Swan Mussels (*Anodonta cygnea*) with ambient water. Bacterial counts and the number of strains isolated were greatest from surface mucus samples and lowest in the hemolymph compartment, which is more isolated from the environment. Species found in ambient water (e.g., *Escherichia coli* and *Enterococcus* spp.) were not detected in mussel fluids, but entercocci were observed inside granulocytes, presumably taken up by phagocytosis. Leis et al. (2019) compared the endogenous microbiota among stable populations of mussels in the Upper Mississippi River basin and those experiencing reoccurring mortality in the Clinch River, Tennessee and Virginia. Surveys of culturable bacteria such as these allow for isolation of specific bacterial types/strains for further study of infectivity and pathogenicity or of potential benefits (host defense, probiotic production) to the host.

The bacterial species that are detected in culture-based surveys depends on the growth media and incubation conditions, as well as the tissue sampled. Thirteen different growth media were used to identify and isolate bacteria for mussel to fish contagion studies, but these methods targeted growth of fish pathogens (Starliper and Morrison 2000). Incubation of digestive gland samples from Eastern Elliptio (*Elliptio complanata*) at 20°C and 35°C revealed varying thermal preferences of bacteria species (Chittick et al. 2001), and identification of maximum bacterial diversity required incubation at both temperatures. Only limited comparisons can be made among surveys of mussel microbiota when sampling methods, media types, and incubation conditions differ.

Metagenomic analysis can characterize the microbiome of mussels without the limitations of culture conditions, but these analyses can be limited by the availability and accuracy of reference sequences available for identification. The microbiome of the digestive gland in the Alabama Rainbow (*Villosa nebulosa*) was characterized using 16s rRNA gene pyrosequencing (Aceves et al. 2018). The dominant operational taxonomic units were *Mycoplasm*-like but had $< 90\%$ similarity to available sequences for the genus *Mycoplasm*, and these bacteria may represent a new lineage. Studies such as this are important for growing the molecular database on the mussel microbiome; however, genomic identification of a bacterium may not provide information on its viability or virulence in mussels. Studies that combine culture-based and genomic methods may increase detection of bacterial species while enabling isolation, culture, and characterization of species of most interest.

Community characteristics of the microbiota (e.g., species richness, evenness) may be more indicative of mussel health than the presence or absence of specific bacterial species. Mussels collected during die-off events generally had lower bacterial species richness but higher loads of a few dominant species (i.e., lower evenness; Scholla et al. 1987; Starliper et al. 2011). Similarly, low evenness and low species richness of bacterial communities are associated with disease in oysters (Lokmer et al. 2016; Clerissi et al. 2018). The importance of a stable, diverse microbiota is well established for many organisms, including bivalves. Endogenous bacteria in hemolymph have shown antibiotic effects and enhance the immune response in marine bivalves (e.g., Defer et al. 2013; Desriac et al. 2014), and aquaculture facilities have increased the use of probiotics to reduce disease in marine bivalve cultures (see Prado et al. 2010). The importance of the gut microbiota in freshwater mussel health has received little attention. The microbiome of the digestive gland of wild *V. nebulosa* was altered after relocation to a hatchery environment (Aceves et al. 2018). *Villosa nebulosa* treated with antibiotics and subsequently challenged with a bacterial fish pathogen (*A. hydrophila*) showed no mortality, but bacterial species diversity was altered by antibiotic treatment (A. Aceves et al., unpublished data). These studies identified key bacterial phyla in *V. nebulosa*, but further investigation is needed to determine their role in the mussel host.

Bacteria may become opportunistic pathogens when environmental conditions (e.g., increased temperature, decreased flow regime) alter bacterial concentration or host defenses. Four indigenous bacterial species were pathogenic to *D. polymorpha* when mussels were inoculated with high concentrations or at elevated water temperature (Gu and Mitchell 2002). Unionid mussel die-off events are similarly associated with elevated water temperature and other stressors such as spawning, larval brooding, and decreased water flow (Neves 1987a; Starliper et al. 2011), conditions that may alter ambient microbial communities or the mussel immune system.

The least studied pathogens in freshwater mussels are viruses. The only virus known to cause disease in a freshwater mussel was detected in *H. cumingii* (see Zhang et al. 1986). This virus is relatively well studied because of its economic importance (Zhang et al. 2005; Ren et al. 2011, 2013, 2014; Zhong et al. 2011; Bai et al. 2016; Zhao et al. 2016). In contrast, a large number of viruses (including Herpesviridae, Iridoviridae, Picornaviridae, Papovaviridae, Birnaviridae, Retroviridae, and Reoviridae) are associated with diseases in marine bivalves (see Carella et al. 2016; Arzul et al. 2017; Zannella et al. 2017). Variants of oyster (*Crassostrea* spp.) herpesvirus (e.g., OsHV-1 and μvar) are associated with mass oyster mortalities on a global scale (Renault et al. 2014). Similar to bacterial disease outbreaks, high-density production and environmental factors (e.g., elevated temperature and salinity) are thought to influence the outbreak, and increase the spread, of viral diseases (Guo and Ford 2016; Zanella et al. 2017). Transfer of viral disease from shellfish to fish has also been demonstrated (Metcalf et al. 1979; Meyers 1984; Lees 2000).

Recently, a picorna-like virus was detected in an apparently healthy Wabash Pigtoe (*Fusconaia flava*) from the Upper Mississippi River (Goldberg et al. 2019). This virus

was not associated with pathogenicity, but the finding suggests that the scarcity of virus reports in mussels is due to a lack of investigative effort. Linking a virus with disease is problematic because of the lack of mussel cell lines to isolate and grow viruses. Primary cell cultures have been obtained from *D. polymorpha* (Quinn et al. 2009) and *Lamellidens marginalis* (Barik et al. 2004), but these cultures have not been sustained for more than several weeks. Recently, a hemocyte system was reported for assessing replication of the OsHV-1 virus in oysters (Morga et al. 2017). Detection of viruses in hemolymph or tissue samples by traditional assays, such as enzyme-linked immunosorbent assay (ELISA), is not possible because mussels do not produce antibodies in response to viral agglutinogens (Allam and Raftos 2015). However, molecular techniques can be used to determine tissue-specific location of viral particles, and these techniques can be augmented by histological examination.

RESEARCH ON HEALTH BIOMARKERS

Various tools and techniques exist to assess the relative health of native mussels. They often are referred to collectively as biomarkers; for our purposes, they are defined as a biological response at the molecular, cellular, biochemical, physiological, or behavioral level that can be related to exposure or susceptibility to, or effects of, some stressor. Newton and Cope (2006) reviewed biomarker research for mussels in the context of toxicology using a biomarker classification system developed for fish (Van der Oost et al. 2003). This classification groups biomarkers into 10 categories: biotransformation enzymes, oxidative stress, biotransformation products, amino acids and proteins, hematological, immunological, reproductive and endocrine, neuromuscular, genotoxic, and physiological and morphological. Many classes of biomarkers have been applied to mussels and have aided in the health assessment of these animals in both laboratory and ecosystem settings (Newton and Cope 2006), but additional studies are needed that focus on organismal health in the absence of a contaminant and characterize baseline conditions.

Since the review by Newton and Cope (2006), advances have been made in several key areas of biomarkers and health assessment tools, namely, those characterizing health status by analysis of hemolymph constituents such as enzyme and ion levels (Gustafson et al. 2005b; Burkhard et al. 2009; Archambault et al. 2013; Fritts et al. 2015a, b; Steinagel et al. 2018), behavioral endpoints such as mantle lure display and foot protrusion (Bringolf et al. 2010; Hazelton et al. 2013; Leonard et al. 2014; Hartmann et al. 2016), reproductive and endocrine endpoints (Morthorst et al. 2014; Leonard et al. 2017), and the use of -omics (e.g., metabolomics, proteomics, transcriptomics) techniques (Malecot et al. 2013; Leonard et al. 2014; Luo et al. 2014; Roznere et al. 2014; 2017; 2018; Bartsch et al. 2017). Recently, metabolomic studies of freshwater mussels have been used to identify shifts in key metabolites from stressors such as captivity and food limitation (Roznere et al. 2014), relocation (Bartsch et al. 2017; Roznere et al. 2017), and exposure to an estrogenic compound (Leonard et al. 2014). Other recent studies evaluated mussel gene responses to stress using transcriptomes (Wang et al. 2012; Cornman et al. 2014; Luo et al. 2014; Robertson et al. 2017; Jeffrey et al. 2018; Roznere et al. 2018; Waller et al. 2019).

Immunological measures are important indicators of health and disease status. Bivalves have wide-ranging cellular and humoral defense tools (Allam and Raftos 2015; Zanella et al. 2017) that can be general stress or pathogen-specific responses. Hemocyte count, phagocytic activity, natural killer-type activity, and lysozyme concentration were measured to assess immune responses of *E. complanata* (Gélinas et al. 2013) and *D. polymorpha* (Juhel et al. 2015) to cyanobacteria. Mahapatra et al. (2017) followed the hemocyte count, phagocytic activity, and nitric oxide generation of *L. marginalis* during starvation, and Steinagel et al. (2018) measured changes in hemocyte counts and morphology in response to translocation into captivity of Mapleleaf (*Quadrula quadrula*) and Threeridge (*Amblema plicata*).

Despite the advancements in tools to assess the relative condition of mussels, connections between biomarker responses and tangible outcomes for characterizing "good health" (i.e., normal or baseline status) are still needed.

THE NEED FOR AN INITIATIVE ON FRESHWATER MUSSEL HEALTH

The National Strategy for the Conservation of Native Freshwater Mollusks (herein the National Strategy), originally published in 1998 and updated in 2016, prioritized research and management needs for mollusk conservation (NNMCC 1998; FMCS 2016). "Disease" was mentioned only three times in the 1998 National Strategy and only twice in the 2016 National Strategy. "Health" is mentioned numerous times in both documents, but mostly in the context of population health (i.e., demographic attributes) or ecosystem or environmental health. Health of individual mussels is mentioned only twice in the 1998 National Strategy, with reference to producing healthy juveniles in captivity or avoiding disease in captive populations, and health of individuals is not mentioned in the 2016 National Strategy. Despite the scant mention of health and disease in both versions of the National Strategy, these topics are directly relevant to most of the 10 issues or problems identified by these documents.

Biomarker research in the last 20 years is a positive step, but no holistic framework presently exists for applying health assessment tools beyond a research setting. Biomarkers have not been used as routine tools for determining the condition of mussels in natural populations or in broodstock used for propagation. A holistic standardized approach to assessing mussel health is needed not only in response to mussel mortality events, but also for routine monitoring of mussel health in the wild, monitoring and evaluating propagation and restoration efforts, and determining the effects of environmental stressors in natural populations.

There is an immediate need to develop a suite of mussel-specific diagnostic tools for the investigation of mortality events. Past methods varied widely, relied primarily on those used for diagnosing fish disease, and emphasized bacterial culture. Standardized sample collection and diagnostic methods, including potentially more informative techniques such as genomics, are required for die-off events. Such methods would enable researchers to compare potential disease agents (e.g., virus, bacteria, parasites) and other characteristics among die-offs occurring at different locations.

Routine evaluation of biomarkers or other indicators of health in wild populations could provide early warning of population declines before they can be detected by traditional survey methods. Current population health assessments rely on measures of mortality, species richness, abundance, and sometimes demographic attributes (e.g., age structure and recruitment; FMCS 2016), but changes in these attributes may not be detectable until a decline is well underway. In contrast, metabolomics and transcriptomics can provide real-time data on a mussel's response to current conditions (Roznere et al. 2014; Fritts et al. 2015a; Jeffrey et al. 2018; Roznere et al. 2018; Waller et al. 2019). For example, expression of the chitin synthase gene was significantly reduced in mussels that were experimentally exposed to elevated carbon dioxide (Jeffrey et al. 2018; Waller et al. 2019). Down-regulation of the gene causes decreased shell growth, which was detectable in juveniles after only 28 days but was undetectable in slow-growing adults (Jeffrey et al. 2018). Changes in metabolites associated with energy use and production were detectable in *A. plicata* after only 1–2 weeks of food limitation (Roznere et al. 2014), but effects of food limitation on growth or survival may not be apparent for months or longer.

Mussel health monitoring combined with genomic analysis could identify individuals that are disease-resistant or resilient to environmental stressors, such as warming temperatures. Such an approach is becoming more common in shellfish and finfish aquaculture to reduce disease-related losses (see Houston 2017). This approach can help reduce disease potential in freshwater mussel propagation, and it has application for selection of stock for reintroduction, augmentation, and relocation.

A goal identified in the National Strategy is the use of propagation, augmentation, and relocation (PAR) without adversely affecting resident populations and their habitats (FMCS 2016). Propagation programs have expanded in the past 30 years; at least 19 facilities in the USA have produced $>$ 30 mussel species (Gum et al. 2011; Patterson et al. 2018), and 15 in Europe rear *M. margaritifera* (Gum et al. 2011). High-density, intensive culture in an artificial environment provides a likely scenario for epizootic outbreaks. Maintaining healthy individuals in culture facilities will depend on knowledge of potential pathogens, conditions that support immunocompetent animals, and the factors that favor optimal growth and health. Monitoring sublethal indicators, such as key metabolites (Roznere et al. 2014) and hemolymph parameters (Steinagel et al. 2018), could provide early warning of declining condition in captive mussels and help identify causes (e.g., nutritional deficiency, microbial imbalance, disease). Metagenomic analysis of gut microbiome and metabolomics could be used to compare responses of mussels to different diets and rearing conditions to optimize growth and production in propagation facilities (Roznere et al. 2014; Aceves et al. 2018).

Controlled PAR carries risks for both the resident and introduced mussels (Villella et al. 1998; Haag and Williams 2014; Wolf et al. 2019). Genetic impacts of stocking activities have been considered, but the risks of disease introduction are often overlooked (McMurray and Roe 2017). Many resource managers are diligent about preventing introduction of invasive mussels into native mussel habitat during relocation or stocking activities and follow a recommended quarantine period or disinfection procedure (Cope et al. 2003b). Health assessment and diagnostic tools are needed to determine the potential for transfer of infectious agents during field and hatchery operations and the need for quarantine procedures.

Early mussel relocation and restoration efforts had variable success (Cope and Waller 1995), owing in part to the lack of suitable criteria for site selection and follow-up monitoring, but subsequent research identified procedures and recommendations (Waller et al. 1993, 1995, 1999; Bartsch et al. 2000; Dunn et al. 2000; Cope et al. 2003a; Greseth et al. 2003) that vastly improved relocation success. Health assessment and diagnostic tools may further improve relocation success by providing assessments of the resident and relocated populations. For example, the survival, condition, and biochemical composition of resident and caged, translocated mussels were used to identify suitable source and destination streams for mussel relocation (Gray and Kreeger 2014). Survival was mostly indistinguishable among sites, but sublethal indicators (condition index) separated suitable from suboptimal sites. Furthermore, resident mussel condition was poor in one source stream, indicating that the presence of wild mussels did not necessarily indicate a suitable relocation site. Site selection for relocations and follow-up monitoring of mussel populations could benefit from more sensitive indicators of health to predict whether mussels are thriving, adapting, or stressed at a site well before gross responses (e.g., growth, reproduction, survival) are apparent (e.g., Roznere et al. 2018).

Nonindigenous species, such as *D. polymorpha* and *Corbicula* spp., may negatively impact mussels by altering nutrient flow and trophic pathways, the microbiota, and habitat availability and by attaching directly to native mussels (Strayer 1999; Ricciardi et al. 2002; Lohner et al. 2007; Higgins and Vander Zanden 2010). There are no reports of dreissenid mussels or *Corbicula* spp. transmitting disease to unionids, but research in this area is scarce. Secondary to serving as vectors of a pathogen, nonindigenous species may disrupt the established microbiome and immunocompetence in native mussel populations. Comparative studies among mussel communities with and without nonindigenous species, including measures of mussel health, could reveal previously undetected environmental alteration caused by the nonindig-

enous species and help evaluate their role in native mussel health and disease.

APPROACHES AND MODELS FOR MUSSEL HEALTH ASSESSMENT

Significant advancement in mussel health assessment will require a coordinated, collaborative, multidisciplinary effort to optimize resources and take advantage of expertise. In this section, we discuss three topics that may help facilitate this goal.

Adopting a Clinical Health Perspective

The application of basic clinical health assessment methods could provide a framework for mussel health assessment. For example, when people go to their physician for a check-up, certain parameters are routinely measured, including blood pressure, heart rate, blood chemistry, and urine chemistry, to evaluate their overall health status. The first step in developing clinical health assessment tools for mussels is identifying a suite of biomarkers or other measures that are likely to be most informative in a wide variety of contexts. The second step is determining what constitutes a "normal" or "healthy" mussel. This will require a dedicated research effort to characterize the baseline health attributes and reference range values for a number of mussel species and life stages across different geographic ranges. The third step is evaluating how health attributes change from baseline conditions in response to disease, exposure to contaminants, or other environmental stressors.

Adapting Existing Programs

Existing approaches and models of animal health assessment can be adapted or modified for mussels. For example, the U.S. Fish and Wildlife Service's (USFWS) system of eight National Fish Health Centers has well-established programs for monitoring both hatchery and wild fish. Although the focus of these programs is primarily on disease detection, the mission of the Centers includes monitoring physiological and nutritional status of organisms and environmental conditions as indicators of sublethal stress (https://www.fws.gov/wildfishsurvey/about/index.html, accessed September 26, 2019). Hatcheries undergo regular inspections to ensure that fish released into the wild or moved across state lines are disease-free. The National Wild Fish Health Survey component of the USFWS Fish Health Program samples for fish pathogens of concern at sites selected based on criteria such the presence of listed species, source of broodstock for hatchery propagation, and availability of other monitoring data (population parameters, contaminants, and environmental parameters) (https://www.fws.gov/wildfishsurvey/criteria.htm, accessed September 26, 2019). Similar selection criteria could be used to identify and prioritize sites to conduct annual mussel health monitoring. Most Centers have been underutilized for mussel health assessments, except in response to mass mortality events (e.g., Neves 1987b; Starliper 2011; Leis et al. 2018) or to certify mussels as free of fish pathogens. Incorporating mussels into existing programs at USFWS Fish Health Centers could occur with additional resources and modifications of sampling protocols.

The National Oceanic and Atmospheric Administration (NOAA) Mussel Watch Program uses bivalves, including dreissenids, to monitor contaminants and ecosystem health in coastal waters of the USA (https://inport.nmfs.noaa.gov/inport/item/39400, accessed September 26, 2019). The program has national oversight from NOAA but uses regional, state, and local groups to collect samples. In conjunction with established programs for water quality monitoring and fish and invertebrate surveys, an inland mussel watch program could be initiated using common, easily collected mussel species. Such an approach also might garner support for mussel health by highlighting their role as indicators of ecosystem health.

Existing mussel monitoring studies are an opportunity to simultaneously collect samples for health assessment. For example, a multi-agency Mussel Coordination Team uses a team of staff and volunteers to conduct annual surveys in the Upper Mississippi River basin at various reintroduction or augmentation sites for the federally endangered Higgins' Eye (*Lampsilis higginsii*) (https://www.mvp.usace.army.mil/Home/Projects/Article/571035/endangered-species-conservation-of-native-mussels/, accessed September 26, 2019). Events such as these are opportunities to conduct health monitoring at lower cost by using staff and resources already field deployed. Similar long-term monitoring programs on other river systems also could be adapted to include health monitoring (e.g., Jones et al. 2014; Ahlstedt et al. 2017).

Development and dissemination of standard protocols and diagnostic methods for mussels could use existing manuals as templates. The American Fisheries Society (AFS) Blue Book contains standard procedures for the detection, diagnosis, and inspection of pathogens of finfish and marine shellfish (AFS-FHS 2014). The Blue Book is a joint venture of the USFWS National Fish Health Centers and the AFS Fish Health Section and is based on published protocols and procedures from a variety of sources.

The "Histological Techniques for Marine Bivalve Mollusks and Crustaceans" is a comprehensive manual for examining marine shellfish and crustaceans that standardizes disease investigation (Howard et al. 2004). The manual includes guidance on each investigative step, beginning with specimen collection and shipping and extending to histological processing and staining techniques. Tissue-specific and pathogen-specific (e.g., *Cryptosporidum* and *Giardia* in shellfish) methods also are provided. The manual is a photomicrographic reference of normal histology and pathology and infectious agents. Histological references for mussels at this time are limited to McElwain and Bullard (2014), McElwain (2019), and Henley et al. (2019). Further efforts are needed to document pathology in freshwater mussels specific to disease, contaminants, and other environmental stressors and to compile these data into a comprehensive reference manual.

Adapting Existing Networks and Databases

The Freshwater Mollusk Conservation Society (FMCS) has members and committees in place to advance health assessment. The Guidelines and Techniques Committee could compile and review protocols related to mussel health assessment. For example, many state and federal agencies follow prescribed Hazard Analysis Critical Control Point plans to prevent the transfer of invasive species during field work. These plans could be compiled and modified by the committee to address protocols for reducing the risk of disease transfer during field work. FMCS was instrumental in updating the "Investigation and monetary values of fish and freshwater mollusk kills" handbook (Southwick and Loftus 2017). The existing guidelines, report forms, and notification network for reporting a kill could be supplemented with a framework for investigating specific causes of mortality, including sampling procedures and disposition of samples.

Communication and data sharing will be essential for coordinating health assessment and responding to mortality events. Existing communication networks and protocols can be modified for mussels. For example, Partners in Amphibian and Reptile Conservation organized a National Disease Task Force to facilitate communication, dissemination of outreach materials, reporting, and rapid response related to herpetofaunal disease (/http://parcplace.org/resources/parc-disease-task-team/, accessed September 26, 2019). The U.S. Geological Survey National Wildlife Health Center maintains a continuously updated online database for reporting ongoing and historical wildlife morbidity and mortality events (https://whispers.usgs.gov/home, accessed October 8, 2019). Similar rapid and wide communication about disease and other health issues is needed within the mussel community.

RESEARCH AND RESOURCES NEEDED

Progress in mussel health assessment will require more intentional, prioritized, and focused efforts to fill knowledge gaps and to implement procedures in management, propagation, and research programs. The central questions at this time are "How prevalent is disease in mussels and what are the causative agents?" and "What are the signs of declining health in a freshwater mussel?" In this section, we present four areas of research that are needed to advance an initiative on mussels and identify the resources that can support those efforts (Table 1).

Determine the Prevalence of Infectious Disease in Mussels, Identify Causative Agents, and Develop Diagnostic Tools for Their Detection

It is essential that we gain a better understanding of the occurrence and prevalence of mussel diseases in the wild and in captive facilities. The first step is to implement a coordinated effort to survey for potential pathogens from a wide variety of contexts using robust, informative methods. Additional research is needed to optimize and standardize tissue sampling and culture methods for detecting endogenous bacteria in mussels. Metagenomic analyses can detect a wider range of potential disease organisms without the limitations of culture methods. Detection and identification of potential pathogens by metagenomic techniques will require substantial funding. However, once the genome of a potential pathogen has been sequenced, primers or probes can be developed to detect the organism less expensively. Microbe-specific assays, such as quantitative polymerase chain reaction (qPCR) and ISH, have become routine for the detection of many fish and molluscan pathogens (AFS-FHS 2014; OIE 2016). Regardless of the method used, it is important to recognize that detection is not the equivalent of disease.

Initial assessments of pathogenicity can be conducted by comparing the mussel microbiota in different contexts to determine whether organisms are transient, endogenous, opportunists, or potential pathogens. Understanding the baseline prevalence of organisms in the microbiota is key to making these determinations. For example, increased prevalence of an organism above baseline conditions may indicate that an outbreak of a pathogen is occurring. Host species often differ in susceptibility to a pathogen; thus, baseline prevalence needs to be established for a wide array of mussel species.

Confirmation that a bacterium is the causative agent of a disease requires a modified version of Koch's postulates: isolation of the bacterium from the sick or affected mussel, growth in culture, and transmission to and disease production in a healthy host. Transmission studies conducted at the U.S. Geological Survey Leetown Science Center Fish Health Branch provided guidelines for evaluating infectivity of bacterial agents (Starliper and Morrison 2000; Starliper 2001, 2009). Concomitant to transmission studies, the relationship between bacterial concentration and pathology need to be examined.

Traditional diagnostic assays for virus pathology require cell lines to isolate and culture the virus; consequently, development of mussel cell lines is a high-priority need. In addition to viral screening, cell lines could be used to assess the effects of contaminants without sacrificing mussels and more quickly than whole-animal tests, enabling a more rapid response to an environmental event. Until mussel cell lines are developed, culture-independent molecular techniques can demonstrate a viral link to disease. Real time-polymerase chain reaction (RT-PCR) and ISH can detect viral genomic material and determine tissue-specific location of viral particles.

Although the literature suggests that parasites have seldom caused widespread or mass mussel mortality, information remains scarce on their occurrence and abundance under normal conditions. The effects of parasites on mussel health and the role of infection intensity and host condition have been studied for few parasites (Jokela et al. 1993; Taskinen and Saarinen 1999; Jokela et al. 2005; Saarinen and Taskinen 2005; Gangloff et al. 2008; Müller et al. 2015; McElwain et al. 2016; Pavluchenko and Yermoshyna 2017; Brian and Aldridge 2019; McElwain 2019). Surveys of mussel symbionts should broaden to include more mussel species and

Table 1. Research and resource needs for advancement of health assessment of freshwater mussels.

1. Determine the prevalence of infectious disease in mussels, identify causative agents, and develop diagnostic tools for their detection
• Characterize the exogenous and endogenous microbiota of freshwater mussels across spatial and temporal scales
• Hasten development of challenge models for microbes and mussels of interest; determine whether a bacterium or other pathogen is the causative agent of a disease
• Establish a continuous mussel cell line
• Build temporal-spatial data on the species-level taxonomic identities, intensity, prevalence, incidence, and pathology of mussel endosymbionts (including benign parasites, bacteria, and viruses, as well as obligate pathogens, exotic species, invasive species)
• Investigate the response of endosymbiont populations to changes in environmental variables (e.g., elevated water temperature, hypoxia, fluctuating flow regime)
• Determine how specific endogenous (reproductive status, nutritional status) and exogenous (contaminant exposure, elevated water temperature, hypoxia) factors affect the response of the host mussel to endosymbiont/parasitic infection
• Extend the search for microparasites of mussels using molecular, cellular, and histological detection tools
• Develop diagnostic tools (e.g., qPCR, in situ hybridization) to detect infectious agents
2. Establish standard measures of mussel health and specific indicators of disease and stress
• Standardize nonlethal sampling protocols for health monitoring and diagnostic assays
• Establish reference values/ranges for hemolymph chemistry and hemocyte numbers
• Establish reference values/ranges for physiological, cellular, and molecular biomarkers across species, sex, age, season, location
• Determine the sensitivity of biomarkers to varying levels of a stressor across species, sex, age, habitat, season
• Develop stressor-specific metabolomic profiles/fingerprints
• Correlate physiological, metabolic, and genomic responses to a specific stressor to understand mechanisms of disease
• Correlate laboratory and field studies of biomarker sensitivity
3. Understand the role of environmental variables in mussel health
• Investigate the response of a biomarker(s) to a stressor at varying environmental conditions (e.g., high temperature, hypoxia)
• Identify threshold levels or limits of key environmental factors (e.g., thermal limit) for development of a disease or stress response in mussels
• Investigate environmental factors that alter the microbiota and assess the effect on mussel health
• Determine the effect of nonindigenous species on native mussel health through alterations in microbiota, nutrient quantity and quality, introduction of pathogens, and habitat structure
4. Promote training and establish networks
• Incorporate topics on mussel health (e.g., nonlethal sampling, -omics, microbiology, risk assessment) into mussel conservation courses
• Incorporate freshwater mussels into courses on aquatic animal health
• Coordinate long-term monitoring programs to include water quality, hydrological, contaminant, population- and organismal-level data
• Encourage sharing of long-term monitoring data to identify population trends and correlation with environmental data
• Establish a central network for reporting mussel mortality and die-off events
• Establish response protocols for investigating mussel mortality events
• Develop a list of laboratories, including contact information and analytical capabilities, for submission and analysis of samples

quantification of occurrence, abundance, and effects on the host mussel. Understanding baseline parasite prevalence will be critical in determining the potential role of parasites in mussel mortality events. Molecular tools can be used to extend the search for previously undetected parasites and histological examination can determine pathogenicity.

Establish Standard Measures of Mussel Health and Diagnostic Indicators of Disease and Stress

An overarching goal of research toward health and diagnostic tool development is the need for nonlethal sampling methods and standardized protocols. Increased effort toward monitoring mussel health should not increase "take" of animals or induce mortality from handling. Mussel hemolymph, foot, and mantle samples can be collected without causing significant mortality (Naimo et al. 1998; Gustafson et al. 2005a, b; Fritts et al. 2015b, Bartsch et al. 2017). Refinement of these methods is needed to provide guidance on the sample type and volume or mass required for specific assays (e.g., Vodáková and Douda 2019) and the amount of tissue that can be sampled nonlethally based on individual body mass.

Currently, assessments of mussel health in situ are based on behaviors such as burrowing, siphoning, and response to handling or probing. These criteria are useful, but they are coarse and difficult to quantify, and they may be exhibited only after prolonged or acute stress. In toxicity tests and in situ exposures, growth or condition index are standard metrics for assessing health or fitness (e.g., Nobles and Zhang 2015; Waller et al. 2019; Ciparis et al. 2019). These measures require an extended period of exposure and may not provide information about specific mechanisms of impaired health.

Research on biomarkers in mussels has produced a suite of tools and endpoints that could serve as diagnostic tools to indicate specific stressors or disease in mussels. Van der Oost et al. (2003) proposed the following six criteria that must be satisfied in order for a biomarker to be useful: (1) the assay should be reliable, relatively inexpensive, and easy to use; (2) the response should be sensitive to the exposure in order to serve as an early warning parameter; (3) baseline data of the biomarker should be well defined in order to distinguish its response from natural variation; (4) the impacts of any confounding factors should be well established; (5) the underlying mechanism of the relationship between the response and exposure should be established; and (6) the relationship between the biomarker response and its long-term impact on the organism should be established. Biomarkers satisfying these criteria could provide specific, mechanistic assessments of mussel health. To that end, reference studies will be critical for determining variability of biomarker responses among tissue types, reproductive status, sex, and age within a species, in addition to interspecies and geographical variability (Ford and Paillard 2007; Hines et al. 2007; Viant 2007; González-Fernández et al. 2015; Hurley-Sanders et al. 2015). Developing stress-specific biomarkers will require a combination of controlled experimental studies (e.g., Luo et al. 2014; Nguyen et al. 2018) and field testing (e.g., Roznere et al. 2017; Grbin et al. 2019; Strubbia et al. 2019).

Understand the Role of Environmental Variables in Mussel Health

As in disease outbreaks in marine bivalves, environmental factors likely play a role in pathogen proliferation and mussel susceptibility to disease or other stressors. Likewise, the effects of many contaminants and toxins are dependent on environmental variables (e.g., water temperature, pH, dissolved oxygen) and the presence of other stressors (e.g., Wang et al. 2008; Wang et al. 2011; Beggel et al. 2017). Biomarker development and validation will require investigating the effect of environmental variables on biomarker responses. For example, biomarker responses in the Mediterranean Mussel (*Mytilus galloprovincialis*) varied according to geographic location and seasonal variability in environmental conditions, including pollution intensity (Grbin et al. 2019). Investigations such as this are needed for freshwater mussels to link environmental factors, stressors or disease, and biomarker responses. Changes in the mussel microbiome according to environmental conditions also may be important in evaluating mussel susceptibility to disease or stress.

Promote Training and Establish Networks

Training in health and disease is needed for mussel biologists. The National Strategy (FMCS 2016) recommends training and continuing education for mussel biologists, but health and disease topics are not specified. The USFWS National Conservation Training Center offers three courses on freshwater mollusks and one on fish health (NCTC 2018). Instruction on mussel health could be provided in a stand-alone course or incorporated as a module into existing courses, depending on the course objectives. Of equal importance is the need for staff at fish and wildlife health centers and veterinary colleges to gain knowledge and expertise on freshwater mussel biology and conservation.

A communication network is needed for reporting mussel mortality incidents and coordinating responses. Such a network could be hosted on the FMCS website and could provide at least two additional resources. The first is a list of laboratories and their analytical capabilities and sample submission procedures. The second is a clearinghouse of reference databases on mussel microbiota, metagenomics, parasites, biomarkers, and other topics that would enable a more robust investigation of mussel die-offs and declines.

CONCLUSIONS

The study of mussels has advanced substantially in many areas over the past several decades, but topics such as physiology, immunology, and basic biochemistry have received relatively little attention, largely due to limited financial resources and the lack of investigators conducting research in these areas. Improved tests, assays, and other diagnostic tools for assessing mussel health are needed to address disease, unexplained die-offs and declines, effects of contaminant exposures, changing climate, and many other issues relevant to mussel conservation. As with many other groups of organisms, it has been difficult to establish linkages between specific organismal responses and the effects on mussel populations or communities. Future mussel research could benefit from expanding the scope to all levels of biological organization (e.g., molecular to population or community) and learning from other more-established disciplines and frameworks like those from marine bivalves and fish health. Investment in propagation, surveys, recovery, and long-term monitoring should include resources for assessing the health and condition of the animals. A dedicated effort will be needed to advance the study of mussel health by developing a comprehensive, but realistic, plan for accomplishing these tasks.

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Table A1. Definitions of health-related terms.

Diagnosis	Determination of the nature of a disease (Stedman 2006).
Disease	Any impairment that interferes with or modifies the performance of normal function, including responses to environmental factors such as nutrition, toxicants, and climate; infectious agents; inherent or congenital defects; or combinations of these factors (Wobeser 1981).
Ectoparasite	A parasitic organism that lives on the surface of the host (Bush et al. 1997).
ELISA	Enzyme-linked immunosorbent assay is a test that detects and measures small molecules (e.g., antibodies, peptides, proteins) and infectious agents in fluids. The assay utilizes binding between a specific antigen and antibody for detection (Stedman 2006).
Emerging disease	One that has appeared in a population for the first time, that may have existed previously but that is rapidly increasing in incidence or geographic range, or that manifests itself in a new way (Okamura and Feist 2011).
Endogenous	Originating or produced from within the organism or one of its parts (Stedman 2006).
Endosymbiont	An organism that lives within another organism (Bush et al. 1997).
Epidemic (epizootic)	Significantly increased occurrence of a disease in an area or region (Bush et al. 1997).
Etiology	Study or theory of the factors that cause disease and the method of their introduction to the host; the causes or origin of a disease or disorder (Allen 2004).
Incidence	Rate at which a certain event occurs, e.g., the number of new cases of a specific disease occurring during a certain time period in a population at risk (Allen 2004).
Infection	Invasion and multiplication of parasitic organisms within the body (Stedman 2006); replication of organisms in host tissue, which may cause disease (Brachman 1996).
Infectious disease	Those that are caused by the entrance, growth, and multiplication of parasites or pathogens in the body and that may or may not be contagious (Okamura and Feist 2011).
Infectivity/ infectiousness	The characteristic of a disease agent that embodies capability of entering, surviving in, and multiplying in a susceptible host; the proportion of exposures in defined circumstances that result in infection (Stedman 2006).
In situ hybridization (ISH)	A technique that allows for precise localization of a specific segment of nucleic acid within a histologic section. The underlying basis of ISH is that nucleic acids, if preserved adequately within a histologic specimen, can be detected through the application of a complementary strand of nucleic acid to which a reporter molecule is attached (https://www.ncbi.nlm.nih.gov/probe/docs/techish/; accessed February 18, 2019).
Koch's Postulates	To establish the specificity of a pathogenic microorganism, it must be present in all cases of the disease, inoculations of its pure cultures must produce disease in animals, and from these it must again be obtained and propagated in pure culture (Stedman 2006).
Metabolome	Simultaneously quantifies multiple small molecule types, such as amino acids, fatty acids, carbohydrates, or other products of cellular metabolic functions. Metabolite levels and relative ratios reflect metabolic function, and out-of-normal-range perturbations are often indicative of disease (Hasin et al. 2017).
Microbiome	The genome of the microbiota of a given community (Hasin et al. 2017).
Microbiota	All of the microorganisms, including bacteria, viruses and fungi, in a community (Hasin et al. 2017).
Microparasite	A parasite that requires a microscope to be seen (e.g., viruses, bacteria, protozoans) (Bush et al. 1997).
qPCR	Quantitative polymerase chain reaction, also called real-time PCR (Kralik and Ricchi 2017).
Parasite	An organism that lives on or in another and gets its food from, or at the expense of, its host (Stedman 2006).
Pathogen	Any virus, microorganism, or other substance causing disease (Stedman 2006).
Pathogenicity	Ability of an agent to cause disease; pathogenicity is further characterized by describing the organism's virulence and invasiveness (Brachman 1996).
Pathology	The science and practice concerned with all aspects of disease, but with special reference to the essential nature, causes, and development of abnormal conditions, as well as the structural and functional changes that result from the disease processes (Stedman 2006).
Prevalence	The number of cases of a specific disease that are present in a given population at a specified time (Allen 2004).
Probiotic	Live microbial adjunct that has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response toward disease, or by improving the quality of its ambient environment (Verschuere et al. 2000).
Sensitivity	The proportion of individuals with a given disease or condition in which a test intended to identify that disease or condition yields a positive result (Stedman 2006).
Virome	Collection of nucleic acids, both RNA and DNA, that make up the viral community associated with a particular individual or ecosystem (McDaniel et al. 2008).
Virulence	Severity of infection, which can be expressed by describing the morbidity (incidence of disease) and mortality (death rate) of the infection (Brachman 1996).

APPENDIX REFERENCES

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REGULAR ARTICLE

REASSESSING ENIGMATIC MUSSEL DECLINES IN THE UNITED STATES

Wendell R. Haag[1]*

[1] *U.S. Forest Service, Southern Research Station, Center for Bottomland Hardwoods Research, 3761 Georgetown Road, Frankfort, KY 40601 USA*

ABSTRACT

Freshwater mussels have disappeared from many U.S. streams since the 1960s. These declines are enigmatic: there are no clear causes and other components of aquatic communities appear unaffected. I review the characteristics, spatial occurrence, timing, and potential causes of enigmatic mussel declines. They share some or all of the following characteristics: (1) fauna-wide collapse, affecting all species; (2) recruitment failure, leading to a senescent fauna; (3) no well-documented impact sufficient to affect all species rapidly; (4) specific to mussels; (5) recent occurrence, since the 1960s; (6) rapid action, often leading to faunal collapse within 10 yr; and (7) upstream progression in some cases. Enigmatic declines are largely restricted to upland regions south of maximum Pleistocene glaciation and north or west of the Gulf and Atlantic coastal plains, and they appear restricted to small- to medium-sized streams. In contrast, mussel declines with different characteristics are reported nationwide. Their consistent characteristics, restricted spatial occurrence, and similar timing suggest that enigmatic declines represent a distinct, diagnosable phenomenon. Many commonly invoked factors are not plausible explanations for enigmatic declines, and others are vague or poorly supported. Other factors are plausible in some cases (e.g., agricultural effects) but cannot explain declines across the affected area. I identified only two factors that could broadly explain enigmatic declines: disease and introduction of *Corbicula fluminea*, but these factors are poorly understood. The occurrence of enigmatic declines overlies the region with the highest mussel species richness on Earth, but I believe their severity and importance are underappreciated. Streams affected by enigmatic declines are vital research and management opportunities, deserving of increased attention; I propose ways that research can be focused to rigorously evaluate the specific mechanisms for these declines. Until we understand the causes of enigmatic declines, mussel conservation in affected areas is substantially hamstrung.

KEY WORDS: Unionida, conservation, extinction, disease, invasive species, sediment, fragmentation

INTRODUCTION

The dramatic and widespread decline of North American freshwater mussels is well recognized. Many mussel declines in the first half of the 20th century are clearly attributable to massive habitat destruction, mainly by dams. In contrast, more recent declines are enigmatic: there are no clear causes, and other components of the aquatic communities in these streams are relatively unaffected (Haag 2012). Despite more than three decades of research, we are still far from understanding the causes of such declines. Enigmatic declines are rarely viewed as distinct events; rather, they usually are considered part of a long, downward trend in mussel populations that began over 100 yr ago, and, as such, they are conflated with declines attributable to other, clearly supported causes. Explanations for enigmatic declines consist of a long list of potential threats or causal factors that has changed little over time. Hereafter, I refer to this body of explanations as "the conventional wisdom" (Table 1). Several factors in the conventional wisdom seem unrelated to enigmatic declines, the importance of many factors is untested, and the precise nature of other factors is unspecified. Nevertheless, much of the conventional wisdom has become accepted as proven fact.

Our understanding of enigmatic declines, and mussel declines in general, has been hampered by three related issues. First, a lack of clarity about the characteristics of enigmatic

*Corresponding Author: wendell.haag@usda.gov

Table 1. The conventional wisdom: factors invoked to explain mussel declines. Adapted from Bogan (1993), Strayer et al. (2004), and FMCS (2016).

Dams and Impoundment
Dredging and channelization
"Habitat degradation"
"Poor land use practices"
"Pollution," water quality degradation, contaminants
Sedimentation
Loss of riparian buffers
"Run-off," impervious surfaces
Eutrophication
Coal mining, oil and gas extraction
Exotic species
Hydrologic change
Overharvest
Lack of fish hosts; changes in fish assemblages
Climate change
Endocrine disrupters
Disease

declines makes it difficult to distinguish them from other types of declines and establish their spatial distribution and timing. Second, we are uncertain about whether enigmatic declines together represent a single, widespread phenomenon or a collection of largely unrelated events. Third, we have failed to critically evaluate the evidence for factors invoked to explain mussel declines and thus have tended to perpetuate poorly supported speculation about causes (see Downing et al. 2010). These issues have hampered the search for causes and may have encouraged management actions that have little chance of reversing declines.

I provide a critical analysis of enigmatic mussel declines and the factors invoked to explain them. First, I review the characteristics of enigmatic declines and assess their spatial occurrence and timing. Second, I evaluate how well the conventional wisdom explains these declines and discuss other potential explanations. Finally, I propose ways that mussel research and management can be focused to provide more specific information about the causes of enigmatic declines and more specific guidance for addressing them.

OVERVIEW OF ENIGMATIC MUSSEL DECLINES

In the first half of the 20th century, a frenzy of dam construction across the USA destroyed or radically altered thousands of kilometers of riverine habitat and profoundly affected aquatic communities. To date, most extinctions of North American mussel species are directly attributable to habitat destruction by dams (Haag 2012). Substantial mussel assemblages survived in some impounded streams, but they shifted to dominance of impoundment-tolerant species and now bear little resemblance to the pre-impoundment fauna (e.g., Garner and McGregor 2001). Fish assemblages and other aquatic organisms showed similar radical shifts after impoundment (Taylor et al. 2001). Throughout this period, mussels and other aquatic life also were nearly eliminated locally by severe water pollution or other specific, documented insults (Ortmann 1909; Forbes and Richardson 1913).

As late as the 1960s, many streams that escaped impoundment or other severe insults continued to support spectacular mussel faunas. We know about the condition of the fauna at that time in large part because of the efforts of two remarkable individuals, David H. Stansbery, of The Ohio State University, and Herbert D. Athearn, a private shell collector, both of whom collected mussels extensively across the eastern USA and whose large collections survive (Ohio State University Museum of Biological Diversity and North Carolina Museum of Natural Sciences, respectively). These and most other historical collections were not quantitative in any sense, and they have several potential sources of bias. First, sampling methods and effort are rarely recorded. Second, species that were common and widespread at the time (e.g., *Eurynia dilatata*) often appear to be underrepresented numerically in collections unlike rarer species for which most encountered individuals apparently were retained and catalogued (e.g., *Epioblasma* spp.). Third, many collections came mainly from muskrat middens, which may provide a biased depiction of the fauna that occurred at the site (Tyrrell and Hornbach 1998; Owen et al. 2011). Nevertheless, these collections clearly show that abundant, diverse, and largely intact mussel assemblages continued to exist across much of the USA (Table 2). Furthermore, these collections often contain a wide range of age classes, including juveniles.

Throughout this paper, I illustrate examples of enigmatic declines by comparing historical collections with contemporary survey data. Such comparisons must be made cautiously because of the unknown extent to which they are influenced by sampling artifacts at different times. To minimize this problem, the examples I provide consist of collections made at the same locations at different times, and I used only qualitative contemporary survey data. Contemporary qualitative survey methods are similar to methods used by Stansbery, Athearn, and others (Athearn 1969; J. Jenkinson, personal communication), and Stansbery trained or advised many contemporary mussel biologists. If anything, contemporary surveys probably are more exhaustive than historical surveys because today's agency-supported mussel programs provide resources that were largely absent in the past (Haag and Williams 2014).

Even considering potential sampling artifacts, collections from the 1960s contrast starkly with contemporary survey data. These comparisons show that the condition of the mussel fauna in many streams has deteriorated dramatically since the 1960s. In the Red River, Tennessee, species richness declined 44% between 1966 and 1990, the total number of individuals reported declined 90%, and a subsequent survey showed further deterioration (Table 2). Furthermore, the 1966 collection contains multiple age classes, but the 1990 survey reported that all live individuals were "very old," except for a

Table 2. Mussel assemblages in the Red River, Robertson County, Tennessee. Cell entries represent reported numbers of live individuals or recently dead shells. Sources: 1966, Ohio State University Museum of Biological Diversity, Division of Molluscs, Bivalve Collection Database (https://www.asc.ohio-state.edu/eeob/molluscs/terms_biv2.html, accessed February 14, 2019); 1990, Aquatic Resources Center (1993); 1998, Ray (1999).

	Year		
Species	1966	1990	1998
Amblema plicata	49	66	25
Cyclonaias tuberculata	12	22	7
Lampsilis cardium	5	3	5
Tritogonia verrucosa	2	3	5
Elliptio crassidens	5	14	3
Lampsilis fasciola	24	1	2
Eurynia dilatata	209	13	1
Potamilus alatus	6	1	1
Theliderma cylindrica	1	3	1
Alasmidonta marginata	11	0	0
Actinonaias pectorosa	11	6	0
Epioblasma triquetra	5	0	0
Epioblasma walkeri	376	0	0
Lasmigona costata	57	2	0
Leptodea fragilis	5	0	0
Medionidus conradicus	18	0	0
Obovaria subrotunda	420	1	0
Pleurobema oviforme	0	1	0
Pleurobema sintoxia	1	0	0
Pleuronaia dolabelloides	3	0	0
Ptychobranchus fasciolaris	22	1	0
Strophitus undulatus	15	0	0
Villosa iris	10	0	0
Villosa lienosa	11	0	0
Villosa taeniata	32	0	0
Villosa vanuxemensis	69	0	0
Total species	25	14	9
Total individuals	1379	137	50

single individual estimated at 8 yr old. In the Conasauga River, Georgia, species richness declined 72% between 1961 and 2005, and the total number of individuals declined 97% (Table 3). These are, at best, coarse estimates of declines in abundance, but they are similar to quantitative estimates from other streams. The Embarras River, Illinois, is one of the few streams for which pre-1980 quantitative data are available (as catch-per-unit-effort [CPUE]); overall mussel abundance in that stream declined 86% from 1956 to 1987 (Cummings et al. 1988). More recent quantitative data from other streams also show declines of similar magnitude (see subsequent). In the absence of quantitative data, mussel declines are usually reported simply as declines in species richness, but this metric alone does not fully illustrate their severity. Examining museum collections helps to better illustrate the catastrophic nature of these declines.

Table 3. Mussel assemblages in the Conasauga River at Lower Kings Bridge, Murray County, Georgia. Cell entries represent reported numbers of live individuals or recently dead shells. Sources: 1916, Florida Museum of Natural History Invertebrate Zoology Collection Database (http://specifyportal.flmnh.ufl.edu/iz/, accessed February 11, 2019); 1961, H. D. Athearn Museum of Fluviatile Mollusks collection catalog, Volume 3, North Carolina Museum of Natural Sciences mollusk collection; 2005, Johnson et al. (2005).

	Year		
Species	1916	1961	2005
Elliptio arca	12	46	0
Elliptio arctata	10	3	0
Epioblasma othcaloogensis	6	42	0
Epioblasma metastriata	11	1	0
Hamiota altilis	0	8	1
Lampsilis straminea	0	1	0
Lampsilis ornata	9	6	0
Leptodea fragilis	0	0	2
Medionidus parvulus	18	18	0
Pleurobema decisum	1	8	1
Pleurobema spp.[1]	225	26	1
Pyganodon grandis	2	0	0
Ptychobranchus foremanianus	17	7	0
Quadrula rumphiana	3	2	0
Strophitus connasaugensis	1	1	0
Toxolasma corvunculus	1	6	0
Tritogonia verrucosa	2	2	2
Villosa nebulosa	2	11	0
Villosa umbrans	3	13	0
Villosa vibex	3	8	0
Total species	18	18	5
Total individuals	316	215	7

[1]*Pleurobema* spp. includes *P. georgianum*, *P. hanleyanum*, and *P. stablile*.

CHARACTERISTICS OF ENIGMATIC MUSSEL DECLINES

Mussel declines or other changes in mussel assemblages can take many forms. I will begin this section by describing types of declines I do not consider "enigmatic declines." Obviously, the elimination of mussels and most aquatic life by well-documented, acute impacts such as a major chemical spill are not enigmatic (e.g., Schmerfeld 2006). Impoundment typically results in the loss of half or more of the original mussel fauna, but impoundment-tolerant species often increase in abundance, and other impoundment-tolerant species not present historically may colonize the stream (Garner and McGregor 2001). Loss of a fish host can eliminate a particular mussel species while leaving the remainder of the fauna relatively unaffected (Smith 1985; Fritts et al. 2012). Many unimpounded streams have lost a substantial portion of their historical mussel species richness but continue to support large populations of apparently adaptable species ("opportunistic species", Haag 2012; see "Fauna-Wide Collapse"). In one stream, overall mussel abundance declined slowly over 20 yr, but effects were disproportionate among species and recruitment continued (Hornbach et al. 2018). Some species have

Table 4. Characteristics of enigmatic mussel declines.

Fauna-wide collapse	Effects are not species-selective and result in loss of virtually the entire mussel assemblage.
Recruitment failure	Cessation of recruitment results in rapid loss of short-lived species followed by more gradual loss of long-lived species.
No smoking gun	Occurs in streams with no obvious, documented impacts even though a large number of factors may be invoked.
Specific to mussels	Other aquatic species, such as fishes, insects, snails and crayfishes, appear relatively unaffected.
Recent occurrence	Many began between the late 1960s and the 1990s, but some began more recently. However, there is little evidence of their occurrence prior to the 1960s.
Rapid action	Faunal collapse is evident within 10 yr.
Upstream progression	In some cases, faunal collapse proceeded upstream over 10–20 yr.

disappeared from nearly their entire historical range, even from streams that continue to support otherwise healthy mussel faunas (e.g., *Epioblasma rangiana*, *Pleurobema clava*, *P. rubrum*; Haag and Cicerello 2016; Stodola et al. 2017). These latter three types of declines are similar to enigmatic declines in that precise causes are unknown, but they differ in other ways, which I will describe subsequently. A final type of decline that I do not consider here is mussel die-offs. These remain truly enigmatic, and their relationship to enigmatic mussel declines—as I define them here—is unclear. However, die-offs often are relatively brief, transient events and may affect only certain species (Neves 1987; Jones and Neves 2007; J. Jones, personal communication).

Each of these types of declines have characteristics that distinguish them from other, unrelated declines and that may inform our understanding of causal factors and mechanisms. Similarly, enigmatic declines appear to be a distinct type of decline that share some or all of a group of consistent characteristics (Table 4).

Fauna-Wide Collapse

One of the most consistent characteristics of enigmatic declines is that they affect most or all species in the mussel assemblage. This is a critical point. Mussel species often are viewed as "tolerant" or "sensitive" to various human impacts (e.g., Brim Box and Mossa 1999). Some mussel species adapt well to impoundment, while others do not (e.g., Garner and McGregor 2001; Haag 2012). Some species appear to tolerate other types of human degradation of streams, but the precise nature of degradation and mechanism for this tolerance are unknown. For example, about half of the 36 species reported historically from the Minnesota River, Minnesota, are now extirpated, but the river continues to support large populations of a few species (e.g., *Leptodea fragilis*, *Potamilus ohiensis*, *Truncilla truncata*, *Quadrula quadrula*, *Pyganodon grandis*; Sietman 2007).

Such differences in species' responses are not evident in enigmatic declines. In the Embarras River, abundance of virtually all species declined 66–100% (overall decline = 86%) between 1956 and 1987, with the single exception of *Leptodea fragilis*, which was relatively uncommon in both time periods (Fig. 1). Species often categorized as "tolerant" to human impacts declined dramatically (e.g., *Lampsilis siliquoidea*, 66%; *Pyganodon grandis*, 86%; *Quadrula quadrula*, 96%). The two most abundant species in 1987, *Lampsilis cardium* and *Cyclonaias pustulosa*, declined 71% and 83%, respectively. Without quantitative historical data for this river, those

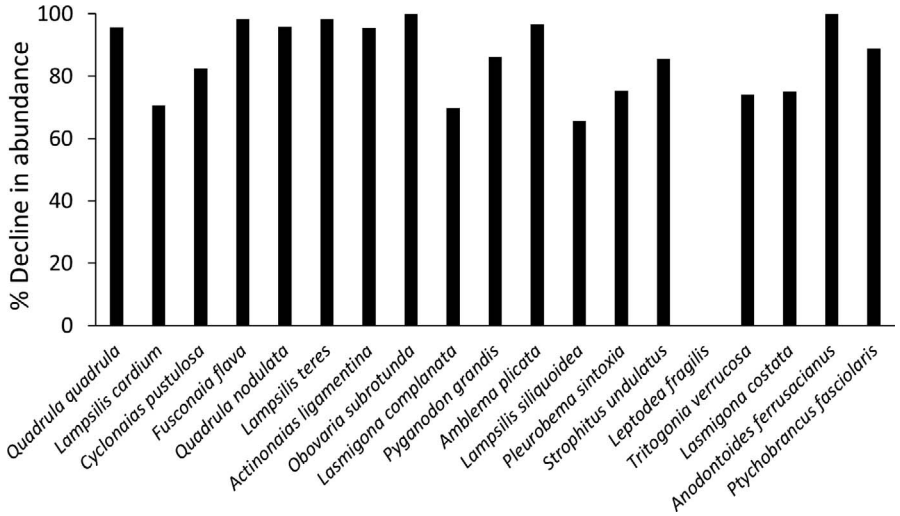

Figure 1. Percentage decline of mussel species in the Embarras River, Illinois, from 1956 to 1987. Thirty-nine species are reported from the river, but only the most abundant species are shown here. Data from Cummings et al. (1988).

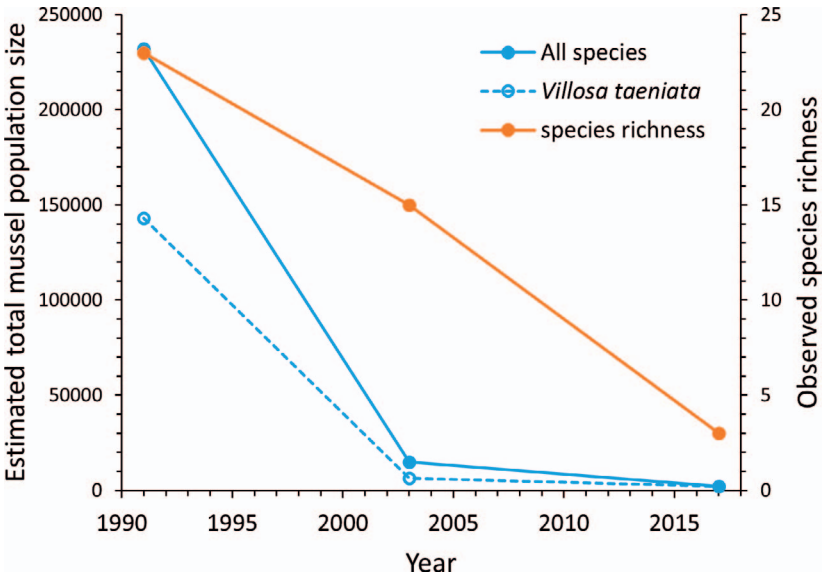

Figure 2. Declines in total mussel abundance; abundance of the dominant species, *Villosa taeniata*; and observed species richness in Horse Lick Creek, Kentucky, between 1991 and 2017. Data from Haag and Warren (2004) and W. Haag (unpublished data).

two species likely would have been viewed as "tolerant" based on their dominance in 1987, but this clearly was not the case. Similarly, *Villosa taeniata* was the most abundant species before and after an enigmatic decline in Horse Lick Creek, Kentucky, between 1991 and 2004, but its abundance declined 96%, similar to the overall mussel decline of 93% (Fig. 2). Because of the persistence of senescent adults (see subsequent), species richness typically declines more slowly than mussel abundance, initially masking the severity of the decline (Table 2 and Fig. 2). Despite the lack of quantitative historical baseline data in most streams, most well-documented examples of enigmatic declines show a near-complete collapse of the entire mussel fauna, ultimately resulting in a steep decline in species richness (e.g., Evans 2001; Warren and Haag 2005; Henley et al. 2013).

I found only two examples in which the mussel fauna survived a decline largely intact. Despite the 86% decline in mussel abundance in the Embarras River between 1956 and 1987, abundance appears to have stabilized subsequently, and species richness has changed little over time. CPUE and species richness were 47 individuals/h and 27, respectively, in 1956; 7/h and 25 in 1987; and 12/h and 26 in 2011 (Cummings et al. 1988; Shasteen et al. 2012b). Similarly, mussel abundance in the Sangamon River, Illinois, declined about 50% between 1956 and 1988, but abundance has stabilized and species richness has changed little (CPUE and richness, 1956: 22/h and 32; 1988: 9/h and 33; 2010: 13/h and 29; Schanzle and Cummings 1991; Price et al. 2012). I did not include the Sangamon River in my compilation of enigmatic declines (see "Spatial Occurrence of Enigmatic Mussel Declines") because of the less severe nature of that decline. In any case, these two examples contrast with the near-complete faunal loss seen in most streams.

Recruitment Failure

A mechanism of enigmatic declines appears to be a cessation of recruitment for all species. A preponderance of large individuals and a conspicuous absence of smaller size classes is reported consistently for enigmatic declines (e.g., Isom and Yokely 1968; Pinder and Ferraro 2012; Henley et al. 2013; Irwin and Alford 2018). Consequently, short-lived species often are the first to disappear, but long-lived species may persist for several decades (Henley et al. 2013; Table 2). Recruitment often is difficult to assess from survey data, but I have observed two clues in these streams that seem to be associated with recruitment failure. First, remaining individuals frequently are highly eroded, in contrast to the pristine condition of shells in healthy streams or historical collections. Second, muskrat middens are composed exclusively of *Corbicula fluminea*, presumably because remaining native mussels are scarce and large, exceeding the handling capability of muskrats (see Warren and Haag 2005).

Although some adults typically survive enigmatic declines, patterns of adult mortality are poorly known because the onset of these events is rarely witnessed. In some cases, relatively large numbers of aging individuals may persist in affected streams (e.g., Henley et al. 2013; personal observations), but baseline data on abundance are rarely available. Large numbers of recently dead adult mussels were reported during the onset of an enigmatic decline in the Little South Fork Cumberland River, Kentucky, in the early 1980s (Warren and Haag 2005), and a more recent enigmatic decline in the Little Tennessee River, North Carolina, was accompanied by massive adult mortality (Jarvis 2011). Contemporaneous observations such as these are scarce, but I provide additional discussion of this issue under "Timing of Enigmatic Declines." Regardless of their effects on adults, recruitment

failure in affected streams prevents recovery, ultimately leading to faunal collapse.

No Smoking Gun, Specific to Mussels

The most enigmatic characteristics of these declines are that they often occur in streams with no obvious impacts, and other aquatic species appear relatively unaffected. Aspects of the conventional wisdom typically are invoked to explain enigmatic declines, but conclusive evidence is rarely available. The decline in Horse Lick Creek was attributed to coal mining (Houslet and Layzer 1997; Haag and Warren 2004), but subsequent water and sediment sampling detected no evidence of coal mining effects (Haag et al. 2019). Furthermore, annual water quality sampling by the Kentucky Division of Water from 1998 to 2016 ranked the stream as "fully supporting aquatic life" (the highest possible ranking) in all years, and three assessments using the Kentucky Index of Biotic Integrity (IBI) during that period ranked the aquatic insect and fish assemblages as "good" or "excellent." Despite a near complete loss of the mussel fauna in the Buffalo River, Tennessee, the snail fauna remained intact, and an IBI ranked the fish fauna as "excellent" (Ahlstedt et al. 2017). Similarly, IBIs for aquatic insects and fishes in the Embarras River consistently rank the stream as "good–excellent," and it is widely used as a reference in bioassessments (Fausch et al. 1984).

Recent Occurrence, Rapid Action

I discuss aspects of the timing of enigmatic declines in more detail under "Timing of Enigmatic Declines." For now, it is sufficient to point out two characteristics about timing. First, enigmatic declines appear to have begun abruptly during, or shortly after, the 1960s, and there is little evidence of their occurrence prior to that time. Many enigmatic declines occurred between the late 1960s and the 1990s, a fact that is emphatically apparent upon examination of Stansbery's and Athearn's collections and other historical sources, but declines occurred later in some areas. Second, enigmatic declines appear to act rapidly, often leading to faunal collapse within 10 yr.

Upstream Progression

I am aware of two examples of upstream progression of enigmatic declines. Declines in the lower portion of Horse Lick Creek were documented about 1985, but the fauna in the middle and upper creek remained intact. The decline moved steadily upstream, and by 2003, it had moved 20 km into the headwaters at an average rate of 1.1 km/yr (Houslet and Layzer 1997; Haag and Warren 2004). Similarly, declines began about 1982 in the lower section of Little South Fork Cumberland River, but they moved steadily upstream about 50 km into the headwaters by 1997 at a rate of about 3.3 km/yr (Warren and Haag 2005). These streams have an unusually complete temporal and spatial sequence of survey data, which is available for few streams; consequently, it is unknown if upstream progression is a consistent characteristic of enigmatic declines.

SPATIAL OCCURRENCE OF ENIGMATIC MUSSEL DECLINES

I interviewed mussel biologists throughout the eastern USA and examined published literature and survey reports to compile a list of streams having the characteristics of enigmatic declines (Table 5). This list of streams is by no means comprehensive; rather, it is based on streams with which sources were familiar or for which published information was available. The confidence with which the severity, timing, and characteristics of declines in these streams can be assessed varies widely according to the nature of existing data. I omitted from this list streams where mussel declines are reasonably explained by a well-documented factor (e.g., major chemical spills, severe chronic pollution, direct impoundment-related effects), but undocumented insults of this nature may have occurred in some of the streams I do include. Despite these caveats, the occurrence of enigmatic declines showed a striking and surprising geographical pattern (Table 5 and Fig. 3).

Enigmatic mussel declines were largely restricted to uplands of the Interior Low Plateaus physiographic province, the Appalachian Highlands physiographic region south of the Ohio River (about 39° latitude), and the Ozark Plateaus and Ouachita physiographic provinces, mainly in northern and central Alabama, Arkansas, northern Georgia, Kentucky, Missouri, Tennessee, western Virginia, and West Virginia, with one example in southeastern Oklahoma. Enigmatic declines occurred throughout the Tennessee, Cumberland, Green, and Coosa river systems, other upland portions of the Mobile Basin (Black Warrior, Cahaba, and Tallapoosa river systems), portions of the Kanawha, Monongahela, and Kentucky river systems, and smaller tributaries of the Ohio River. West of the Mississippi River, enigmatic declines were reported in the White, Osage, Ouachita, Meramec, Red, and Arkansas river systems, and one smaller tributary of the Mississippi River (Salt River). Reports of enigmatic declines on the Atlantic Slope were limited mainly to streams in the Piedmont physiographic province in North Carolina, with two in the Potomac River system (Virginia and West Virginia). Outside of these areas, enigmatic declines were reported only in southern Illinois, northern Missouri, and eastern Iowa.

Enigmatic mussel declines were largely confined to areas south of the maximum extent of Pleistocene glaciation. With the exception of the Embarras, Salt, and Maquoketa rivers, enigmatic declines were not reported from the glaciated Central Lowlands physiographic province in Indiana, Illinois, Iowa, Ohio, Minnesota, or Wisconsin (B. Fisher, K. Cummings, J. Kurtz, B. Sietman, and T. Watters, personal communication). For example, mussel assemblages in the Little Wabash River, Illinois, remained relatively unchanged from 1956 to 2011. Mussel CPUE and species richness were

Table 5. Examples of potential enigmatic mussel declines in the eastern USA. Stream names are followed by the river system of which they are a part. Affiliations of individuals providing personal communications (pers. comm.) are provided in the Acknowledgments. Asterisks denote streams in which some recovery or stabilization has been documented.

Stream	Approximate Onset of Decline	Source
Alabama		
Terrapin Creek (Coosa)	1970–1990	Gangloff and Feminella 2007
Hatchet Creek (Coosa)	1970–1990	J. Moran, P. Johnson, pers. comm.
Shoal Creek (Coosa)	2000–2010	J. Moran, pers. comm.
Tallaseehatchee Creek (Coosa)	Before 2010	J. Moran, pers. comm.
Choctafaula Creek (Tallapoosa)	2000–2010	J. Moran, pers. comm.
Uphapee Creek (Tallapoosa)	2000–2010	J. Moran, pers. comm.
Little Cahaba River (Cahaba)	1970–1990	P. Johnson, pers. comm.
North River (Black Warrior)	1990–2000	O'Neil et al. 2011
Upper Black Warrior River tributaries	1990–2010	J. Moran, pers. comm.
Paint Rock River (Tennessee)	1970–1990*	P. Johnson, pers. comm.
Arkansas		
South Fork Ouachita River	1980–2000	J. Harris, pers. comm.
Upper Ouachita River	1990–2000	C. Davidson, pers. comm.
South Fork Saline River (Ouachita)	1990–2000	C. Davidson, pers. comm.
Middle Fork Saline River (Ouachita)	2000–2010	C. Davidson, pers. comm.
North Fork Saline River (Ouachita)	2000–2010	C. Davidson, pers. comm.
Alum Fork Saline River (Ouachita)	2000–2010	C. Davidson, pers. comm.
Caddo River (Ouachita)	1990–2000	C. Davidson, pers. comm.
Middle Fork Little Red River (White)	2000–2010	C. Davidson, pers. comm.
Illinois River (Arkansas)	2000–2010	C. Davidson, pers. comm.
Georgia		
Conasauga River (Coosa)	1970–1990	Evans 2001; Table 3
Etowah River tributaries (Coosa)	Before 1990	J. Wisniewski, pers. comm.
Coosawattee River tributaries (Coosa)	Before 1990	J. Wisniewski, pers. comm.
South Chickamauga Creek (Tennessee)	Before 1995*	P. Johnson, pers. comm.
Lookout Creek (Tennessee)	Before 1995*	J. Wisniewski, pers. comm.
Illinois		
Embarras River (Wabash)	1960–1985*	Cummings et al. 1988
Iowa		
Maquoketa River (Mississippi)	1980–1990	J. Kurth, pers. comm.
Kentucky		
Nolin River (Green)	1970–1990	Haag and Cicerello 2016
Drakes Creek (Green)	Before 1990	Haag and Cicerello 2016
Gasper River (Green)	Before 1990	Haag and Cicerello 2016
Little River (Cumberland)	Before 1980	Haag and Cicerello 2016
Rockcastle River (Cumberland)	1970–1980	Cicerello 1993; Table 6
Horse Lick Creek (Cumberland)	1985–2000	Haag and Warren 2004
Roundstone Creek (Cumberland)	1970–1990	Haag and Cicerello 2016
Buck Creek (Cumberland)	1980–2000	M. Compton, pers. comm.
Little South Fork Cumberland River	1980–2000	Warren and Haag (2005)
Cumberland River	1970–1990	Cicerello and Laudermilk 1997, 2001; Table 7
Red River (Kentucky)	1980–2000	M. McGregor, pers. comm.
Tygarts Creek (Ohio)	1990–2010	M. McGregor, pers. comm.
Little Sandy River (Ohio)	1990–2010	Haag and Cicerello 2016
Missouri		
Niangua River (Osage)	Before 2010	McMurray et al. 2018
Bourbeuse (Meramec)	1980–2000	Hinck et al. 2012
Meramec	1980–2000	Hinck et al. 2012
Little Black River (White)	1980–1998	Bruenderman et al. 2001
North Fork White River (White)	1985–2010	S. McMurray, pers comm.

Table 5, continued.

Stream	Approximate Onset of Decline	Source
Salt River system (Mississippi)	1985–2010	McMurray et al. 2017
Eleven Point River (White)	1985–2010	S. McMurray, pers comm.
Jacks Fork River (White)	1985–2010	S. McMurray, pers comm.
James River (White)	1985–2010	McMurray and Faiman 2018
North Carolina		
Little Tennessee River (Tennessee)	2003–2006	Jarvis 2011
Swift Creek (Tar)	1990–2000	S. McRae, pers. comm.
Tar River	1975–1990	S. McRae, pers. comm.
Swift Creek (Neuse)	1990–2000	S. McRae, pers. comm.
Little River (Neuse)	1990–2000	S. McRae, pers. comm.
Rocky River (Cape Fear)	1990–2000	S. McRae, pers. comm.
Waxhaw Creek (Catawba)	1980–2000	S. Fraley, pers. comm.
Oklahoma		
Blue River (Red)	1970–1990	Vaughn 1997
Tennessee		
Buffalo River (Tennessee)	Before 1965	Isom and Yokely 1968; Reed 2014
Duck River (Tennessee)	1970–1990*	Ahlstedt et al. 2017
Tellico River (Tennessee)	1980–2000	S. Fraley, pers. comm.
Harpeth River (Cumberland)	Before 1990	Irwin and Alford 2018
East Fork Stones River (Cumberland)	1970–1990	D. Hubbs, pers. comm.; Table 8
Red River (Cumberland)	1970–1990	Ray 1999; Table 2
Virginia		
Middle Fork Holston River (Tennessee)	1970–1990	Henley et al. 2013
North Fork Holston River (Tennessee)	2000–2010	J. Jones, pers. com
South Fork Holston River (Tennessee)	1980–2000	Pinder and Ferraro 2012
Copper Creek (Tennessee)	1980–2000*	Fraley and Alhstedt 2000
Big Moccasin Creek (Tennessee)	1980–2000	J. Jones, pers. com
New River (Kanawha)	1970–1990	J. Jones, pers. com
Aquia Creek (Potomac)	1990–2010	J. Jones, pers. com
West Virginia		
Upper Elk River (Kanawha)	1990–2010	J. Clayton, pers. comm.
Patterson Creek (Potomac)	1990–2010	J. Clayton, pers. comm.
South Fork Hughes River (Little Kanawha)	Before 2005	J. Clayton, pers. comm.
Kincheloe Creek (Monongahela)	1990–2010	J. Clayton, pers. comm.
Tygart River headwaters (Monongahela)	Before 1990	J. Clayton, pers. comm.

22 individuals/h and 29, respectively, in 1956, 20/h and 26 in 1988, and 18/h and 27 in 2011 (Cummings et al. 1989; Shasteen et al. 2012a). Similarly, enigmatic declines were not evident in the northeastern USA, including Pennsylvania, New York, and New England (R. Anderson and D. Strayer, personal communication; Strayer and Fetterman 1999; Raithel and Hartenstine 2006; Nedeau et al. 2000; Nedeau 2008).

Most surprisingly, enigmatic declines were not reported in most of the Gulf or Atlantic coastal plains, despite multiple reports of declines in adjacent upland regions. Many coastal plain streams in Alabama, Georgia, North Carolina, and South Carolina continue to support diverse and abundant mussel assemblages (J. Garner, J. Moran, T. Savidge, J. Wisniewski, personal communication). Streams in all of these areas have experienced changes in the mussel fauna or species losses due to various factors, known and unknown, but examples of unexplained, rapid, and complete faunal collapse are rare or nonexistent.

Assessing the occurrence of enigmatic declines is particularly difficult in Texas. Patterns of mussel declines in Texas are strikingly similar to those in the east: Coastal Plain streams continue to support diverse and abundant faunas, but many upland streams (e.g., those on the Edwards Plateau) now are essentially defaunated, despite having supported diverse faunas prior to the 1980s (Howells et al. 1997; C. Randklev, personal communication). However, these declines coincide with dramatic increases in water abstraction and aquifer depletion, leaving streams highly vulnerable to drought. Major hydrologic change is a plausible mechanism for mussel declines in Texas, but causal factors remain poorly understood.

Mussel declines are less well documented in the western USA, and this region has a limited mussel fauna. A recent

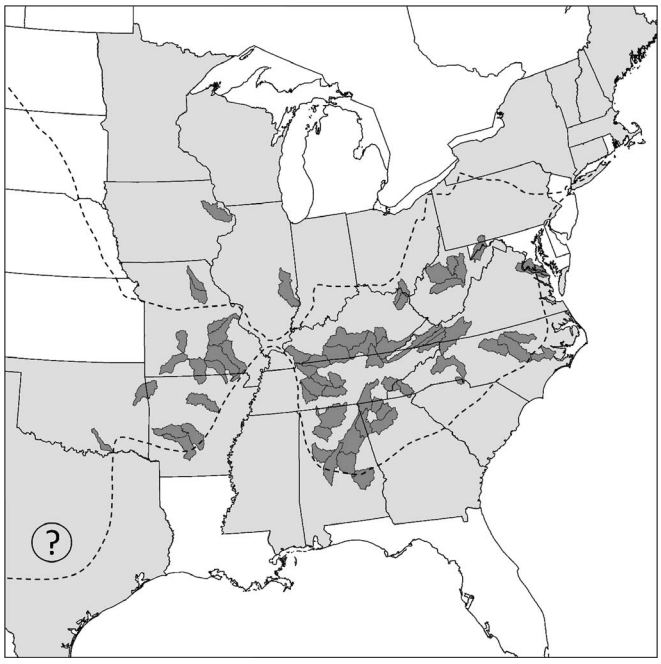

Figure 3. Map of the eastern United States showing the occurrence of enigmatic mussel declines. Shaded states are those for which the occurrence of enigmatic declines was assessed. Shaded polygons are eight-digit hydrologic units in which potential enigmatic declines are reported (see Table 5). The upper dashed line represents the maximum extent of Pleistocene glaciation; the lower dashed line represents the boundaries of the Gulf and Atlantic coastal plains. The question mark in Texas shows the approximate location of the Edwards Plateau and other upland regions that may have experienced enigmatic declines (see text).

assessment of Pacific coast river systems showed mussel declines in some areas (Blevins et al. 2017), but the characteristics of these declines, and the extent to which they are enigmatic or attributable to specific factors, remain unclear.

In addition to their restricted geographic scope, enigmatic declines are notable for their apparent occurrence only in small- to medium-sized streams. Few of the streams listed in Table 5 have watershed areas $>$2,000 km^2 (e.g., Conasauga, Embarras, Meramec, and Red [Tennessee] rivers), and some have watersheds $<$100 km^2 (e.g., Horse Lick Creek). Some that are depicted as separate events may reflect a single, larger phenomenon. For example, mussels have declined throughout the Rockcastle River system, including its tributaries Horse Lick and Roundstone creeks, and declines are evident throughout the upper Coosa and Ouachita river systems. Nevertheless, enigmatic declines are not reported from large rivers within the affected geographical area. Mussel species richness in the Ohio and Tennessee rivers is greatly reduced compared with historical richness, but these rivers continue to support large mussel populations (Payne and Miller 2000; Garner and McGregor 2001). The upper reaches of several watersheds have experienced widespread enigmatic declines, but their lower mainstem rivers continue to support extraordinary mussel assemblages, particularly beyond the point where those rivers flow off of uplands onto the Coastal Plain (e.g., Ouachita, Saline, and White rivers; Posey 1997; Davidson and Clem 2004).

Because of their severity, enigmatic declines in most affected streams are evident even from coarse, qualitative data. Declines in the Embarras and Sangamon rivers appear to be comparatively less severe (see "Fauna-Wide Collapse"). These declines are evident because of the unusual availability of historical abundance estimates, but they would not be detectable based on historical changes in species richness. It is possible that similar, less severe declines have occurred in other regions, but detecting them is difficult because of the lack of historical abundance estimates. Regardless, it seems clear that severe, enigmatic declines are restricted in distribution, but the reason for this is unknown.

TIMING OF ENIGMATIC MUSSEL DECLINES

Establishing the exact timing of enigmatic declines is usually impossible due to the nature of available data. Unusually complete collecting records from the Rockcastle and Cumberland rivers, Kentucky, and the East Fork Stones River, Tennessee, provide more precise assessments of the timing of these declines (Tables 6–8). Despite the qualitative nature of these data, they clearly show abrupt faunal collapse within 10 yr between the 1960s and the 1970s. In all three streams, the stark differences in the results of two surveys conducted only 10 yr apart likely cannot be explained solely by recruitment failure; rather, they suggest that high adult mortality also occurred during that period. These declines did not go unnoticed at the time. When I was a student of David Stansbery's in the 1980s, he once mused rhetorically, "Whatever happened to the East Fork Stones River?" Similarly, Herbert Athearn was aware of the decline in the Conasauga River. After a visit to the river in 1971, he recorded in his collection catalog, "This may be the last station I collect on this ailing stream" (H. D. Athearn Museum of Fluviatile Mollusks collection catalog, Volume 6, North Carolina Museum of Natural Sciences mollusk collection). This comment, made 10 yr after his 1961 collection (Table 3), also suggests the decline in the Conasauga River was rapid. Such precise estimates of timing are unavailable for most streams, but many experienced faunal collapse within a similar time period between the 1960s and 1990s (Table 5).

Other enigmatic declines appear to have begun substantially later, particularly in smaller streams. The decline in Horse Lick Creek, a tributary of the Rockcastle River, began in the 1980s, 10–15 yr after the decline began in the main stem (Tables 5 and 6). Mussel abundance (as CPUE) in the Little Black River, Missouri, declined about 80% between 1980 and 1998 (Bruenderman et al. 2001). Other recent declines are reported in Alabama, Arkansas, North Carolina, Missouri, and West Virginia. An unusually well-documented recent example is the Little Tennessee River, North Carolina, where the mussel fauna collapsed rapidly between 2003 and 2006 (Jarvis 2011). This example differs from others in Table 5 in that the decline appeared to have been most severe for *Alasmidonta*

Table 6. Mussel assemblages in the Rockcastle River at Livingston, Rockcastle County, Kentucky. A total of 29 species are reported from the site, but only the most abundant species are reported here. Cell entries represent reported numbers of live individuals or recently dead shells; dashes indicate that the species was not reported, but presence or absence is unclear. Sources: 1910, Wilson and Clark (1914); 1947, Neel and Allen (1964); 1963–1975, Ohio State University Museum of Biological Diversity, Division of Molluscs, Bivalve Collection Database (https://www.asc.ohio-state.edu/eeob/molluscs/terms_biv2.html, accessed February 14, 2019); 1982, Thompson (1985); 1990, Cicerello (1993).

	Year							
Species	1910[1]	1947	1963	1964	1967	1975	1982	1990
Eurynia dilatata	33	Common	209	139	35[2]	23	3	15
Villosa taeniata	6	Common	378	166	28	4	4	0
Medionidus conradicus	31[1]	Common	28	95	23	0	0	0
Venustaconcha troostensis	—	Common	44	24	7	2	0	0
Ligumia recta	1	Common	22	15	14	3	0	3
Lasmigona costata	8	Common	14	15	13	0	1	0
Ptychobranchus fasciolaris	1	Common	70	34	23	6	0	1
Actinonaias pectorosa	—	Common	15	14	8	1	0	0
Amblema plicata	1	Common	20	7	15	0	1	2
Lampsilis cardium	—	Common	48	13	1	0	2	2
Toxolasma lividus	—	—	22	6	1	0	0	0
Total species	18	18	24	20	17	10	10	9
Total individuals	458	—	1,056	573	184[2]	47	11	31

[1]Mussels overall described as "excessively abundant," and "in favored localities . . . *Medionidus conradicus* covered the entire bottom." Note that the sum of individuals reported for each species (including those not shown here) does not match the total individuals reported in this survey.

[2]Field notes report the species as "abundant" and most individuals were not retained; total individuals for this date does not include released *E. dilatata*.

spp., but these species dominated the fauna, and I include this example here despite the potential for selective effects.

Affected streams typically show little or no evidence of recovery. Two remarkable exceptions are the Duck River, Tennessee, and the Paint Rock River, Alabama, where mussel abundance has increased dramatically since a low point in the late 1970s and 1980s (Ahlstedt et al. 2017; P. Johnson, personal communication), and some recovery is evident in Copper Creek, Virginia (Hanlon et al. 2009). Apart from the Embarras River (see "Fauna-Wide Collapse"), I found no other documented examples of mussel recovery or stabilization after an enigmatic decline. The mussel faunas of Horse Lick Creek and the Cumberland, Red, Rockcastle, and East Fork Stones rivers continue to disappear (Fig. 2 and Tables 6–8), and other streams in Kentucky that experienced enigmatic declines in the 1970s or 1980s now are essentially defaunated (e.g., Little River, Nolin River; Haag and Cicerello 2016).

A critical question about enigmatic declines is whether they began abruptly after the 1960s, or if they are part of a longer, more gradual decline beginning in the early 1900s.

Table 7. Mussel assemblages in the Cumberland River below Cumberland Falls, McCreary County, Kentucky. A total of 22 species are reported from the site, but only the most abundant species are reported here. Cell entries represent reported numbers of live individuals or recently dead shells. Sources: 1910 and 1987, Cicerello and Laudermilk (1997); 1961 and 1972, Ohio State University Museum of Biological Diversity, Division of Molluscs, Bivalve Collection Database (https://www. asc. ohio-state.edu/eeob/molluscs/terms_biv2.html, accessed February 14, 2019).

	Year			
Species	1910	1961	1972	1987
Eurynia dilatata	122	113	2	7
Actinonaias pectorosa	73	161	1	~50
Lampsilis fasciola	16	20	0	0
Medionidus conradicus	Present	154	0	0
Ptychobranchus fasciolaris	81	35	1	5
Cyclonaias pustulosa	49	122	2	10
Tritogonia verrucosa	32	75	0	4
Villosa iris	0	27	0	0
Venustaconcha troostensis	5	7	1	0
Total species	20	16	5	10
Total individuals	810	810	7	88

Table 8. Mussel assemblages in the East Fork Stones River at Walterhill, Rutherford County, Tennessee. A total of 31 species are reported from the site, but only the most abundant species are reported here. Cell entries represent reported numbers of live individuals or recently dead shells; dashes indicate that the species was not reported, but presence or absence is unclear. Sources: 1911, Wilson and Clark (1914); 1965–1981, Ohio State University Museum of Biological Diversity, Division of Molluscs, Bivalve Collection Database (https://www.asc.ohio-state.edu/eeob/molluscs/terms_biv2.html, accessed February 14, 2019); 2002, D. Hubbs, personal communication.

	Year						
Species	1911[1]	1964	1965	1966	1976	1981	2002
Villosa taeniata	—	57	112	107	2	2	0
Lasmigona costata	8	27	93	5	25	34	0
Obovaria subrotunda	2	38	64	51	1	0	0
Epioblasma walkeri	70	18	39	84	0	0	0
Lampsilis fasciola	2	26	30	23	1	2	0
Amblema plicata	5	24	26	3	8	7	0
Pyganodon grandis	1	9	25	6	1	1	0
Fusconaia flava	5	15	22	4	2	0	0
Leptodea fragilis	—	5	12	0	0	1	0
Eurynia dilatata	—	7	10	0	6	1	0
Ptychobranchus fasciolaris	—	9	10	1	8	8	0
Lasmigona complanata	—	9	9	0	2	0	0
Total species	13	27	24	16	13	10	0
Total individuals	194	309	500	298	63	58	0

[1]Note that the sum of individuals reported for each species (including those not shown here) does not match the total individuals reported in this survey.

Collections from many streams in the 1960s and 1970s are remarkably similar in species composition to collections from the early 1900s (e.g., Hurd 1974; Jones and Neves 2007; Henley et al. 2013), but even crude historical estimates of abundance are scarce. The qualitative data in Tables 3 and 6–8 show considerable variation in mussel abundance among surveys attributable to river condition, collector efficiency, etc. For example, the higher abundance of *Pleurobema* in the Conasauga River in 1916 compared with 1961 is noteworthy. Overall, however, in all four examples, collections from the 1960s are remarkably similar to those from the early 1900s when compared with the major changes that occurred after the 1960s. Historical data such as these are available for few streams, but the spectacular museum collections from the 1960s strongly suggest that the mussel fauna in many places remained essentially intact until that time.

EXPLAINING ENIGMATIC MUSSEL DECLINES

An attempt to evaluate causes of enigmatic declines can benefit by placing these events in a broad context. I propose that enigmatic declines collectively represent a discrete, widespread phenomenon. This assertion is based on (1) the consistent characteristics shared by enigmatic declines, particularly the highly virulent, fauna-wide effects; (2) the restriction of these events to particular geographic regions and to small- to medium-sized streams, but their widespread occurrence within those regions; and (3) the rapid pace of declines and their sudden occurrence within a relatively narrow time frame since the 1960s. I further propose that enigmatic declines are largely unrelated to other factors that affect mussels, but they may occur in concert with those factors. I will elaborate on this assertion subsequently. This context is useful for evaluating the causes of enigmatic declines, but its validity is not necessarily a prerequisite for evaluating how well the conventional wisdom explains them.

Regardless of whether enigmatic declines are a distinct phenomenon, we can quickly eliminate several factors in the conventional wisdom (Table 1). Clearly, loss of fish hosts cannot explain enigmatic declines because the fish fauna in affected streams usually remains intact, as discussed previously. Even if changes in the fish fauna occur, these changes would have selective effects on particular species instead of fauna-wide effects including host generalists and specialists on many different fishes. Overharvest cannot explain enigmatic declines because few affected streams experienced commercial harvest of any kind, which was restricted mainly to large rivers, particularly since the 1960s (Haag 2012). Radical habitat alteration, such as channelization and dams, is eliminated by definition (no smoking gun), but indirect effects of dams are possible (see subsequent). The effects of climate change on mussels are poorly known, but those factors are expected to have selective effects depending on differences in thermal sensitivity among species (Galbraith et al. 2010).

Two major problems with the conventional wisdom for explaining any type of mussel decline is that many factors are vague, and the importance of some prominent factors is not well tested. Factors such as “habitat degradation,” “poor land use,” “pollution,” and “run-off” are cited repeatedly in studies of mussel declines (Strayer et al. 2004; Downing et al. 2010), but these terms provide neither a specific mechanism for those declines nor specific guidance for conservation. Sedimentation

is perhaps the most frequently cited explanation for mussel declines (e.g., Brim Box and Mossa 1999). Recent experimental or modeling studies support a role of elevated suspended sediment in mussel reproductive failure or population declines (Gascho Landis 2016; Hansen et al. 2016), but studies in the wild are conflicting. Increases in deposited fine sediment and substrate embeddedness are associated with recruitment failure of *Margaritifera margaritifera* in oligotrophic streams (Geist and Auerswald 2007; Denic and Geist 2015), but no such relationships have been found for unionids in eutrophic, warmwater streams (Strayer and Malcom 2012; Denic et al. 2014), which describes most or all streams in Table 5. At this time, the role of sedimentation in mussel declines remains poorly understood (reviewed by Haag 2012).

Aquatic habitats and ecosystems doubtless are degraded by sediment and other results of human land use (e.g., Waters 1995), but there are two logical flaws in using them to explain enigmatic mussel declines. First, these factors are expected to have long-term, cumulative, and broad-based effects on aquatic ecosystems corresponding to well over a century of intensive human alteration of the landscape. For example, over 75% of conversion of forest lands to other uses occurred prior to 1900, and many watersheds in areas affected by enigmatic declines were clear cut prior to 1920 but are now reforested (Clark 1984; USDA Forest Service 2001). Long-term, cumulative, and broad-based effects are not concordant with the abrupt, rapid decline of mussel populations seen since the 1960s and the lack of similarly rapid effects on other components of those ecosystems. Second, long-term degradation of stream habitats should have predictably selective effects on aquatic species, resulting in a homogenization of those faunas (McKinney and Lockwood 1999). Such homogenization is seen in impounded streams, where mussel faunas are dominated by a highly predictable group of impoundment-tolerant species with similar life history traits (Haag 2012). Similarly, mussel species losses in Midwestern rivers are attributed to long-term increases in sediment loads, but those rivers continue to support large populations of a characteristic group of species that apparently can thrive under such conditions (Sietman 2007). Outcomes of enigmatic declines also are highly predictable, but only in the characteristic decline or loss of the entire fauna, including species that typically tolerate habitat degradation.

Other, more specific factors in the conventional wisdom remain highly plausible explanations for enigmatic declines. Inputs of agricultural contaminants such as pesticides and nitrogenous fertilizers increased exponentially since the 1960s, coincident with the advent of enigmatic declines (Vitousek et al. 1997; Nowell et al. 1999). Research suggests an especially important role of unionized ammonia, which is acutely toxic to mussels but less toxic to other aquatic organisms, potentially explaining the mussel-specific effects of enigmatic declines (Augspurger et al. 2003; Wang et al. 2007; see also Strayer and Malcom 2012). This is a compelling mechanism in some cases. For example, the Red River, Tennessee, the Little and Nolin rivers in Kentucky, and the Conasauga River in Georgia are in intensely agricultural regions and show elevated nitrogen loading, which creates conditions favorable for ammonia formation; pesticide contamination is also prevalent in these streams (Sharpe and Nichols 2007; Haag et al. 2019).

Despite the compelling case for a role of agricultural contaminants, intensive agriculture is of limited occurrence in many affected streams, particularly in the Appalachian, Ozark, and Ouachita highlands. Horse Lick Creek and the Little South Fork have little row-crop agriculture in their largely forested watersheds, and agricultural contaminants are absent or present at low concentrations (Haag et al. 2019). Initially, declines in these two streams were attributed to coal mining, which was plausible because of the advent of mining activity in the lower portions of both watersheds in the late 1970s and 1980s (Houslet and Layzer 1997; Warren and Haag 2005). However, mining became a more tenuous explanation in these streams as the declines moved upstream beyond mined areas (see "Upstream Progression"). Coal mining is a likely cause of mussel declines in some areas, particularly those affected by severe pollution such as acid-mine drainage (e.g., Clayton et al. 2015), but I did not consider declines in streams with documented coal mine pollution as enigmatic (e.g., Powell River, Virginia; Zipper et al. 2016). The lack of satisfactory explanations for enigmatic declines in many streams such as Horse Lick Creek and the Little South Fork calls into question the veracity of factors used to explain similar declines at similar times in other streams.

An important implication of viewing enigmatic declines as a distinct phenomenon is that it compels us to search for factors common to all affected streams. Even though enigmatic declines appear restricted to specific regions, the affected areas encompass a wide diversity of landscapes and land uses. Consequently, enigmatic declines typically are explained by invoking whichever factors from the conventional wisdom appear plausible in a particular stream, whether or not supporting information is available. Enigmatic declines in agricultural regions typically are attributed to agricultural contaminants, sediment, and related effects, while declines in urbanizing watersheds are explained by issues such as proliferation of impervious surfaces. Enigmatic declines without obvious or satisfactory explanations often are attributed to multiple, often vague factors. Haag and Warren (2004) explained the mussel decline in Horse Lick Creek as "likely a result of ongoing contamination from reclaimed and abandoned coal mines, as well as possible contamination from other, unidentified sources." Out of 45 peer-reviewed papers dealing with mussel declines, more than half invoked multiple factors, and up to eight factors were invoked in a single paper (Strayer et al. 2004).

We cannot rule out the possibility that enigmatic declines are caused by multiple, varying factors in different streams. However, the multiple-factor explanation seems unlikely for two reasons. First, most or all of the factors in the conventional wisdom are present throughout much of the USA, and it is difficult to imagine how they could be so harmful in the

affected region but not in others. Second, the probability of all of these factors coming into play to produce such disastrous effects suddenly and virtually simultaneously across a large, heterogeneous area seems very low.

If we assume that enigmatic declines are caused by a single factor, this leads us to consider if any factors in the conventional wisdom or elsewhere can reasonably explain enigmatic declines in all affected streams. Such a factor needs to satisfy two requirements: (1) it is present in all affected streams and (2) it was absent prior to the 1960s.

Stream fragmentation and associated effects of isolation and small population size are not usually considered in the conventional wisdom. However, nearly all streams affected by enigmatic declines are isolated to some extent by impoundments or other stretches of highly modified stream habitats, and fragmentation generally occurred prior to the 1960s, thus potentially satisfying both requirements. Fragmentation is a likely mechanism for the selective disappearance of large river species in the lower reaches of smaller streams because these populations were probably sustained by immigration from mainstem rivers, which are now impounded (Haag 2009). In contrast, for several reasons, fragmentation is an unlikely explanation for the rapid, fauna-wide collapse characteristic of enigmatic declines. First, mussel assemblages eliminated from mainstem rivers by impoundment differed substantially from assemblages in unimpounded tributaries. Assemblages in Cumberland River tributaries such as the Rockcastle and Stones rivers were dominated by or included *Villosa taeniata*, *Lampilis fasciola*, *Medionidus conradicus*, and other species that were rare or absent in the mainstem (Wilson and Clark 1914; Neel and Allen 1964), making it unlikely that they were sustained by mainstem populations. Second, a biogeographic analysis of the Cumberland River system based on regional species-area relationships showed that tributaries should have been large enough to support nearly their entire historical mussel assemblage even after loss of mainstem populations (Haag 2012); these streams have largely maintained their fish and snail faunas after isolation. Third, like long-term habitat degradation, effects of fragmentation should be gradual and selective. Initially abundant species, particularly those not sustained by mainstem populations, should decline more slowly (or not at all) than species initially present as small populations; such patterns are not seen in enigmatic declines. Finally, nearly all streams in the United States are fragmented and isolated to some extent (Benke 1990), begging the question: why are enigmatic declines restricted to certain regions?

I am aware of only two factors that fit the requirements stated above. The first is disease. Disease is rarely considered as a factor in mussel declines, except for its potential role in mussel die-offs (Neves 1987). At this time, few potential pathogens of freshwater mussels have been identified in North America, and none have been linked conclusively to mussel declines or die-offs (reviewed in Grizzle and Brunner 2009 and Haag 2012). Disease could explain the rapid pace of enigmatic declines, but several important issues about this explanation need to be examined. First, most identified bivalve pathogens are highly species-specific (e.g., Allam et al. 2006). To explain enigmatic declines, a pathogen would need to be both highly virulent to all unionid species and nonvirulent to nonnative *Corbicula fluminea*, which persists in affected streams. Second, the persistence of aging, adult mussels suggests that a pathogen would need to be particularly virulent to younger life stages. Third, and importantly, there would need to be a mechanism that restricts the effects or occurrence of a pathogen to the affected geographic regions. Even within those regions, some streams continue to support apparently healthy mussel populations, including large, impounded streams that receive flow from affected streams (e.g., Garner and McGregor 2001). Nevertheless, disease is an understudied factor that deserves more attention. Raising these issues here is not meant to discount disease as a potential factor; rather, these issues should be viewed as the basis for testable hypotheses about their mode of action.

The other factor that could explain enigmatic declines is the invasive Asian Clam, *Corbicula fluminea*. Several mechanisms by which *Corbicula* could negatively affect native mussels have been proposed, including food competition; ingestion of mussel sperm, glochidia, and juveniles; habitat disturbance by burrowing; and water quality degradation associated with periodic, mass *Corbicula* die-offs (reviewed by Strayer 1999). Even if *Corbicula* does not directly affect native mussels, it could be a vector for disease. Compared with another invasive bivalve, *Dreissena polymorpha*, *Corbicula* has received little attention as a possible explanation for mussel declines, and some authors have effectively dismissed this possibility (e.g., Vaughn and Spooner 2006; Haag 2012). *Dreissena* does not occur in most streams affected by enigmatic declines, but *Corbicula* occurs throughout the affected region (Foster et al. 2019).

The arrival of *Corbicula* coincides remarkably closely with the advent of enigmatic declines. *Corbicula* first appeared in Stansbery's collections from the Rockcastle, Cumberland, and East Fork Stones rivers in 1967, 1972 and 1970, respectively, almost precisely at the time that native mussel populations crashed in those streams (Tables 6–8). *Corbicula* was first reported in the Conasauga River in 1970, one year before Athearn described the stream as "ailing" (Foster et al. 2019). In the Little South Fork Cumberland River, *Corbicula* moved upstream about 20 km between 1981 and 1987, which closely followed the upstream progression of the native mussel decline (data from Starnes and Bogan 1982; Anderson et al. 1991). Arrival of *Corbicula* in the Little Tennessee River, between 2002 and 2004, was followed immediately by an abrupt decline in mussel abundance, including an 80% decline in *Alasmidonta raveneliana* by 2006 (Jarvis 2011; S. Fraley personal communication). Most studies of *Corbicula*–native mussel interactions are dated and anecdotal (see Strayer 1999), but a growing body of experimental evidence shows a strong potential for food competition with native mussels (Hakenkamp and Palmer 1999; Yeager et al. 2000; Ferreira-Rodríguez and Pardo 2017; Ferreira-Rodríguez et al. 2018).

Finally, *Corbicula* is mostly absent in the northern USA, which could explain the absence of enigmatic declines in that region (but see subsequent).

There are at least two issues related to invoking *Corbicula* as a mechanism for enigmatic declines. Competition with *Corbicula* should be stronger for juvenile mussels than adults to explain adult persistence in affected streams; such selective effects are plausible if juveniles have higher energetic requirements than adults. As with disease, the most important issue is that *Corbicula* occurs throughout the Coastal Plain, where enigmatic declines are not documented, and in streams in affected areas that continue to support mussel populations (e.g., Miller et al. 1986; Garner and McGregor 2001). To my knowledge, a mechanism by which native mussels could co-occur with *Corbicula* in some areas but not in others has not been proposed. One possibility is that smaller or less productive upland streams may have lower food resources, and mussels in these streams may be more vulnerable to food competition with *Corbicula*. Again, as with disease, these issues can form the basis of testable hypotheses. In my opinion, *Corbicula* is the most compelling single explanation for enigmatic mussel declines, and this potential factor deserves increased attention.

MOVING FORWARD: FOCUSING RESEARCH AND MANAGEMENT EFFORTS

Below I provide my perspective on how research and management can be focused to better understand and address mussel declines. My suggestions pertain most specifically to enigmatic declines, but they are relevant to any poorly understood decline or change in mussel assemblages.

Deemphasize the Conventional Wisdom

The most important initial step toward better understanding mussel declines is to acknowledge explicitly that we do not understand the causes of those declines in many cases. Mussel biologists should refrain from speculating about the causes of declines when no specific mechanisms are proposed and little or no supporting evidence is available. The conventional wisdom can provide a basis for testable hypotheses, but I believe that habitual recitation of vague or untested factors has hampered mussel conservation for two reasons. First, apart from propagation, most mussel conservation actions involve addressing "poor land use," sedimentation, or related factors. These actions are likely to benefit streams broadly, but the precise role of these factors in mussel declines is poorly known, and they are unlikely causes of enigmatic declines. Second, habitual recitation of the conventional wisdom either has convinced policy-makers (and even many mussel biologists) that causes of mussel declines are understood, or it has confused them due to the myriad factors that are often invoked. A frank acknowledgment that causes remain largely unknown is more likely to encourage funding and creative research, ultimately leading to more effective and targeted conservation strategies.

Revisit Previously Ignored or Poorly Understood Factors

An important need is for more research on potentially widespread, but largely ignored factors such as disease and *Corbicula*. It is also important that other poorly studied factors receive more critical evaluation. I have argued that sedimentation is an unlikely factor in enigmatic declines, but this assertion needs evaluation, and sedimentation may be important in other contexts. The prominence of sedimentation in the conventional wisdom may have discouraged additional research because investigators have the impression that its effects on mussels are well understood. Given the widespread increases in sediment in streams, this factor sorely deserves a fresh look.

Develop Better Assessment Approaches

Most existing information about potential causes of mussel declines comes from either (1) correlative or observational field studies or (2) laboratory toxicological studies. Field studies nearly always focus on correlations or qualitative associations of assemblage- or population-level responses with various factors. For example, a study may correlate land use at a specific time with species richness in a watershed. Such approaches are informative, but they rarely provide concrete information about specific factors or mechanisms, and they are not repeatable or replicable. They also are limited by the potentially long response time of mussel assemblages and populations to various factors; current assemblage condition may be a function of past events, and recovery may be a slow process. Another weakness of these approaches is that they do not allow us to assess present conditions in streams that have lost their mussel fauna and whether the causal factor for the decline is still in effect. Results of toxicological studies can be readily applied to the field by assessing exceedance of a contaminant above a critical level, but contaminant effects in the wild may be influenced by many environmental factors.

These approaches represent two opposite ends of the research spectrum, and both are essential, but the link between these approaches is underrepresented in our knowledge base. The link is measuring specific responses (e.g., survival, growth, physiological condition) of individual mussels to ambient conditions in the wild (e.g., Bartsch et al. 2003; Gagné et al. 2004; Nobles and Zhang 2015; Haag et al. 2019). This approach also may be correlative, but it provides a real-time assessment of mussel responses to current conditions (whether or not wild mussel populations exist), it is repeatable and replicable to a much greater extent than assemblage- or population-focused approaches, and it allows evaluation of toxicological results in a natural context. The availability of large numbers of propagated juvenile mussels makes this approach feasible with a minimal impact on wild populations. Studies of this nature typically are conducted by housing

mussels in enclosures. However, detailed monitoring of wild or reintroduced individuals and their responses to ambient conditions also provides opportunities to evaluate specific hypotheses about causes of mussel declines (e.g., Jones et al. 2012; Clayton et al. 2015; Stodola et al. 2017). For example, assessing changes in individual mussel performance over time in response to management actions meant to reduce sediment could provide valuable information about the effectiveness of such actions.

Don't Abandon Degraded Streams

The focus of much mussel research and management is on remaining high-quality mussel assemblages. It is essential to protect these assemblages, but degraded streams, particularly those with no clear source of impairment, are vital opportunities for research and conservation. For research, these streams are opportunities to identify and study factors that have severe, negative effects on mussel assemblages. For conservation, these streams represent hundreds of kilometers of potentially recoverable habitat. Recovery plans for nearly all threatened and endangered species stipulate creation of additional populations, and indeed, this is the only way to significantly reduce extinction risk. For many species, suitable locations for establishing additional populations do not exist unless streams affected by enigmatic declines can be rehabilitated. Mussel biologists (myself included) have tended to walk away from streams after loss of the mussel fauna. Horse Lick Creek and the Little South Fork have received little attention since the early 2000s, in contrast to the intense activity that occurred in those streams when they supported important mussel faunas. The elimination of the mussel fauna from a stream for unknown or poorly understood reasons should spur intensified research and management activity, not abandonment.

SUMMARY

Although mussel declines in general are well recognized, the severity and importance of enigmatic declines are underappreciated by the conservation community. One reason for this may be their restriction to specific regions, which are unfamiliar to many biologists. However, their occurrence closely overlies the region with the most species-rich mussel fauna on Earth. Enigmatic declines throughout that region have profoundly deepened the mussel conservation crisis in a few decades. For example, nearly all unimpounded tributaries of the Cumberland River have experienced enigmatic declines, placing that system's unique species and assemblages in imminent danger of extinction. Another reason for the underappreciation of enigmatic declines may be our failure to recognize them as a distinct, diagnosable phenomenon. I make the case that characteristics of enigmatic declines support such a view, but this assertion needs further evaluation. Regardless, it is essential that we discover the causes of these declines, including the reasons for their puzzling restriction to smaller streams and specific geographic areas. Until enigmatic declines are better understood, mussel conservation in affected areas is substantially hamstrung, and conservation in other areas faces the possibility that the scope of enigmatic declines will expand.

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REGULAR ARTICLE

MASS MORTALITY EVENTS IN FRESHWATER PEARL MUSSEL (*MARGARITIFERA MARGARITIFERA*) POPULATIONS IN SWEDEN: AN OVERVIEW AND INDICATION OF POSSIBLE CAUSES

Niklas Wengström[1,2,6]*, Håkan Söderberg[3], Johan Höjesjö[1], and Anders Alfjorden[4,5,6]

[1] *University of Gothenburg, Department of Biological and Environmental Sciences, Box 463, SE-405 30 Gothenburg, Sweden*
[2] *Swedish Anglers Association, Sjölyckan 6, 416 55 Gothenburg, Sweden*
[3] *The County Administrative Board of Västernorrland, Pumpbacksgatan 19, 871 86 Härnösand, Sweden*
[4] *National Veterinary Institute (SVA), Department of Animal Health and Antimicrobial Strategies, 75189 Uppsala, Sweden*
[5] *Uppsala University, Department of Organismal Biology, Norbyvägen 18D, 752 36 Uppsala, Sweden*

ABSTRACT

The freshwater pearl mussel *Margaritifera margaritifera* is an endangered species in Sweden with more than 600 known populations distributed in 16 out of 21 counties. Only approximately one-third of these populations are considered viable and healthy with signs of recent juvenile recruitment. From 2011 to 2017, research documented an increased mortality in this species, of up to 100% in some populations, but no etiological cause of these mortalities has been identified. With this paper, we provide current knowledge of locations where mass mortality of freshwater pearl mussel has been found in Sweden and discuss possible causes. Postmortem sampling and histopathological findings from two counties in 2016–17 detected lesions in digestive glands indicating a reduced capacity for nutrient uptake. Results from these macroscopic and microscopic investigations also indicate a reduction in, or a lack of, reproductive output compared with reference populations.

KEY WORDS: *Margaritifera margaritifera*, die-offs, mortality, pathology, emaciated

INTRODUCTION

The Swedish fauna of freshwater mussels consists of seven native species (*Margaritifera margaritifera, Unio crassus, Unio pictorum, Unio tumidus, Anodonta anatina, Anodonta cygnea*, and *Pseudanodonta complanata)* and four nonnative species (*Dreissena polymorpha*, *Mytilopsis leucophaeatea, Rangia cuneata*, and *Sinanodonta woodiana;* von Proschwitz et al. 2017). All native species can be found in streams and lakes except for *M. margaritifera* and *U. crassus*, which only occur in streams. According to the Swedish Red List of Threatened Species, the conservation status of *A. anatina, A. cygnea*, and *U. tumidus* is listed as least concerned, *U. pictorum* and *P. complanata* as near threatened, and *U. crassus* and *M. margaritifera* as endangered (EN; Artdatabanken 2018a).

Margaritifera margaritifera has a Holarctic distribution and can be found in North America (common name: eastern pearlshell) and Europe (common name: freshwater pearl mussel [FPM]; Graf and Cummings 2007). Sweden has more than 600 populations of *M. margaritifera* distributed across the country in 16 of 21 counties (Söderberg et al. 2008; Fig. 1). The species is listed as EN because of limited juvenile (individuals <50 mm in length) recruitment and habitat loss (Artdatabanken 2018a). Juvenile recruitment occurs in 50% of

*Corresponding Author: niklas.wengstrom@sportfiskarna.se
[6]These authors contributed equally to this work.

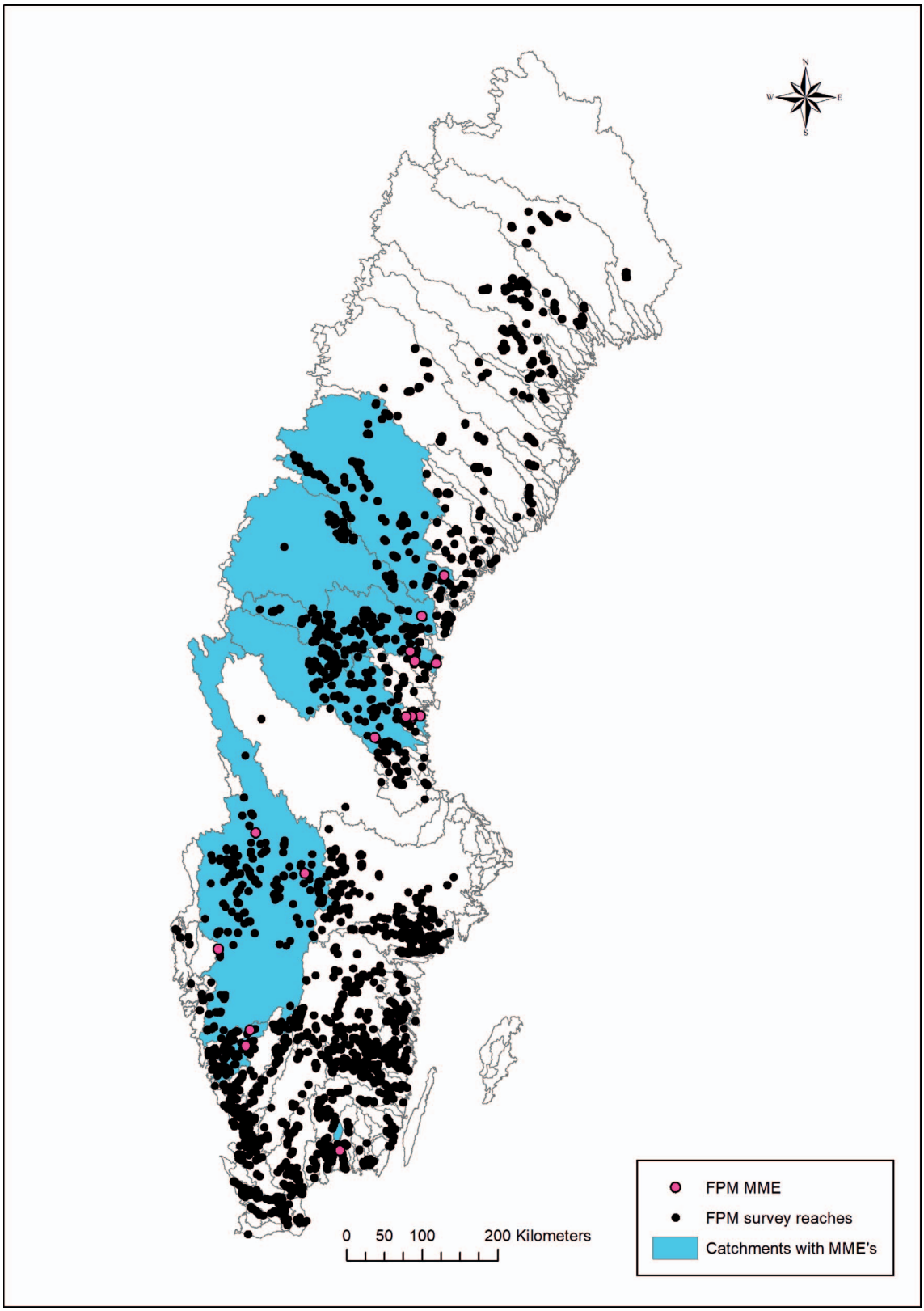

Figure 1. Locations of all freshwater pearl mussel (FPM) survey reaches in Sweden (black dots). Catchments with mass mortality events (MMEs) are indicated in blue and reaches with MMEs are indicated with red dots.

all populations, but only one-third of these are considered viable in terms of recent recruitment and proportion of juveniles (>20% of the population <50 mm in length; Söderberg et al. 2008). Recruitment failure is associated with turbidity and sedimentation of fine substrates and habitat alteration caused by historical use of streams for logging, mills, and hydropower plants (Österling et al. 2010; Degerman et al. 2013). Acidification of streams and lakes became one of Sweden's biggest environmental problems in the late 1960s, exacerbating habitat loss as it not only impacted FPM recruitment but also negatively affected brown trout (*Salmo trutta*), the host fish of FPM (Hesthagen et al. 1999; Taskinen et al. 2011). In recent years, the discovery of mass mortality events (MMEs) in *M. margaritifera* populations due to unknown causes has become a new threat (Fig. 1). Our definition of an MME is a >20% decrease in the number of individuals in a reach (minimum length of 3 m) between two or more surveys. Such mass mortality events in FPM populations have been reported from different countries and often the causes of these die-offs are unclear (Blodgett and Sparks 1987; Strayer et al. 2004; Downing et al. 2010; Southwick and Loftus 2017; Sousa et al. 2018). An MME affects all life stages in a population and can remove a substantial part of a population in a short time (Fey et al. 2015).

In this paper we report the occurrence of MMEs in Swedish populations of *Margaritifera margaritifera* and address possible causes by comparing histological examinations of mussels from four streams with die-offs to mussels from streams without MMEs.

METHODS

Since the 1980s, *Margaritifera margaritifera* populations have been sampled with both quantitative and qualitative methods in regional monitoring programs performed by county administrative boards (CABs). In preliminary surveys, every 20 to 50 m of a stream are searched with an aqua scope for 5 to 10 min. These preliminary surveys are used to determine the presence of mussel beds (densities >1 mussel/m^2). For the quantitative method, 15–18 randomly picked reaches (≤20 m in length) are searched within the known distribution of a population within a stream, and all mussels seen on the bottom are counted. In order to assess the demographic distribution of the population, the lengths of 15 mussels from each reach are measured to the nearest millimeter. For the qualitative surveys, the reach in a stream with the highest density (>1 mussel/m^2) and highest numbers of juveniles is sampled. In streams with low densities and no juvenile recruitment, the reach with the highest number of adults is sampled. A maximum of 100 individuals are collected and measured for length. All collected data are reported to the national database for freshwater mussels (Artdatabanken 2018b). Results of these surveys are compared with data from earlier surveys from the same streams and reaches using data from the national database. In total, 17 streams are included in this study (Table 1).

During recent field surveys (2016–17), *M. margaritifera* ($n = 21$) from four streams were transported alive to the National Veterinary Institute (NVI; Uppsala, Sweden) in boxes with water from the sampling sites; they were kept cold using cooling blocks. We collected mussels from two healthy reference populations (Älgån, Ålakarsbäcken) where no signs of MMEs have been reported and from two populations (Stampebäcken, Lillån) where MMEs have been reported. At the NVI, each animal was measured for length (mm) and wet weight (g) including shell (after emptying the body cavity of stored water). Each mussel was opened by cutting the anterior and posterior adductor muscles with a scalpel and letting out all water left from the mantle cavity. After removing the animal from the shell (by detaching the adductor muscles and mantle) we measured its soft body weight (g). We photographed each mussel using a mobile phone camera (Microsoft Lumia 640 LTE, file type: JPG-format; Redmond, WA USA) and inspected the mantle, gills, foot, and adductor muscles. Ocular characteristics, such as coloration and transparency of mantle, body thickness, condition of foot, coloration of digestive gland, and filling of gonads, were noted.

We examined cross sections of the bodies of these animals for signs of pathological alterations of the gonads or digestive glands. Two cross sections of each animal, including gonad, stomach, digestive gland, mantle, and gill, were collected and fixed in Davidson's freshwater fixative. Sampled organs were processed according to the standard routines for histological sectioning of samples embedded in paraffin blocks (Howard and Smith 1983). Sections of 5 μm were cut and stained with H&E. Stained sections of each sampled specimen were inspected by microscopy using both low power (Olympus SZ binocular stereo zoom microscope; Tokyo, Japan) and high-power magnification (Nikon labophot; Tokyo, Japan). Photos were taken with a Canon EOS 500D attached to a microscope by a lens-adaptor (Martin Microscope Company MM-SLR adaptor S/N:0468; Easley, SC USA).

RESULTS

Between 2006 and 2018, MMEs occurred in 17 streams belonging to nine different catchments from southern and central Sweden (Fig. 1). In five catchments, more than one stream had an MME and in catchment 46/47, all known populations suffered MMEs (Table 1). The mortality between two surveys ranged from 22% to 100%, and in 10 of the streams, the mortality was more than 90% (Table 1). In streams with more than one surveyed reach, MMEs were detected at all reaches in all cases except Örasjöbäcken, where an MME was detected at only one of 21 reaches (Tables 1 and 2). Length measurements indicate that 47% of the streams had recent juvenile recruitment (Table 2).

Table 3 lists ocular characteristics, such as coloration and transparency of mantle, body thickness, activity of foot, coloration of digestive gland, and filling of gonads. We found

Table 1. Streams with mass mortality events (MMEs) in Sweden and their association with catchments, counties, and streams. Mortality is based on the difference in numbers of live Freshwater Pearl Mussel (FPM) individuals between the survey years. The number of reaches per stream is associated with the different monitoring methods used in Sweden.

Catchment ID	No. of FPM Populations in the Catchment	County Code	Stream Name	Group	Year of MME Detection	Survey Years	% Mortality	No. of Reaches
38	43	Y	Tannån	MME	2016	2006, 2016	95	1
40	6	Y	Örasjöbäcken	MME	2013	2015–17	70	1
40	6	Y	Örasjöbäcken	Reference	—	2007, 2015	3	21
42	46	Y	Rännöån	MME	2004	2006, 2016	100	1
42	46	Y	Tälgslättån	MME	2017	2005, 2017	96	1
42/43	2	Y	Galtströmmen	MME	2017	2005, 2017	—*	1
46/47	3	X	Enångersån	MME	2011	2005, 2017	99	15
46/47	3	X	Grottsjöbäcken	MME	2012	2012–17	95	1
46/47	3	X	Grängsjöbäcken/ Tolockbäcken	MME	2012	2012–17	97	1
46/47	3	X	Lövåsbäcken	MME	2012	2012–17	100	1
46/47	3	X	Mjusbäcken	MME	2012	2012–17	100	1
48	57	X	Lillån	MME	2017	2011, 2016	69	15
84	2	K	Husörenbäcken	MME	2017	2005, 2017	97	5
105	15	O	Kovraån/Lillån	MME	2016	2005, 2017	84	1
105	15	O	Viskan	MME	2016	2015–17	93	1
108	52	O	Teåkersälven	MME	2017	2016, 2017	54	18
108	52	S	Stampbäcken	MME	2015	2009, 2015	81	15
108	52	S	Värån	MME	2013	2014, 2017	22	19

FPM = freshwater pearl mussel; MME = mass mortality event; County Codes: K = Blekinge, O = Västra Götaland, S = Värmland, X = Gävelborg, Y = Västernorrland; * = data deficiency.

anatomical differences between mussels from sites with MMEs (Stampebäcken, Lillån) and those without (Älgån, Ålakarsbäcken). The mussels from healthy populations ($n = 6$) typically had firm, thick bodies, compact feet, and well-developed gonads filled with protruding gametocytes (Fig. 2a, b, e, f, j). In contrast, mussels from populations with MMEs ($n = 15$) were thinner, with more relaxed bodies, and with the foot halfway or fully released. Further, several individuals showed low or no presence of gonads (Fig. 2c, d, h, i, j). In many cases, the digestive glands in healthy mussels were filled with dark green, protruding food (Fig. 2b, g) and the inside of the shells were smooth and iridescent. In comparison, mussels from sites experiencing MMEs had digestive glands that were nearly empty and so they appeared pale (light green to pale brown) and more transparent (Fig. 2d, j). Individuals from one stream with MMEs (Stampbäcken) exhibited multiple small cavities in the mother of pearl layer on the inside of the shell, and one specimen also had small nodules on the otherwise smooth inside shell surface.

The microscopic investigation supported the observed external differences. We found different lesions in digestive glands and other organs in mussels from MMEs. When gonads were sectioned in specimens from a population with MMEs, the gametocytes/oocytes were often absent or low in numbers (Fig. 3g, j). These findings clearly differ when compared with a reference population (Fig. 3a, b, d), where one can clearly see follicles filled with oocytes. Declining populations in areas with MMEs generally showed empty follicles without gametocytes or follicles filled with other cell types. In some cases, hypertrophy, hyperplasia, or enlarged vacuolated epithelial cells could be detected in digestive glands in individuals from the MME population (Fig. 3g, h, k, l). Many digestive gland cells showed no presence of granular or vesicular content in the tubules. In some specimens, cellular infiltration was seen in the connective tissue areas surrounding the tubular digestive glands, expanding the distance between each tubular gland (Fig. 3k, l). In comparison with a normal tubular gland from the reference population (Fig. 3e), a fine band of connective cells separated the tubular glands and there was no cellular infiltration. In the external parts of the foot and body, where connective and muscle fibers dominate, we observed signs of hypertrophied muscular fibers or cellular swelling, as well as increased cellular infiltration, in some of the mussels from MMEs (Fig. 3i) compared with reference mussels (Fig. 3c, f).

DISCUSSION

Mass mortality events of freshwater mussel populations in Sweden have been reported only in *M. margaritifera* populations, and these have occurred only in southern and

Table 2. Streams with mass mortality events and measurements of percentage of juveniles; average, minimum, and maximum lengths; and number of dead and live mussels for each survey year.

Stream Name	Survey Year	% Juveniles	Average Length (mm)	Minimum Length (mm)	Maximum Length (mm)	No. of Dead Mussels	No. of Live Mussels
Tannån	2006	0	97	74	114	4	42
Tannån	2016	0	106	98	114	31	2
Örasjöbäcken*	2015	0	99	71	123	93	253
Örasjöbäcken*	2016	0	100	68	118	111	142
Örasjöbäcken*	2017	0	100	16	114	35	107
Örasjöbäcken*	2018	0	—†	—	—	60	58
Örasjöbäcken – reference	2007	4	81	30	111	11	377
Örasjöbäcken – reference	2015	34	67	18	110	16	417
Rännöån	2006	1	99	49	120	6	129
Rännöån	2016	0	—	—	—	6	0
Tälgslättån	2005	0	104	69	142	1	89
Tälgslättån	2017	0	96	92	112	29	4
Galtströmmen	2005	12	91	15	154	0	177
Galtströmmen	2017	3	96	33	144	10	431
Enångersån	2005	8	87	23	126	—	628
Enångersån	2017	—	—	—	—	—	3
Grottsjöbäcken	2012	20	—	34	—	69	20
Grottsjöbäcken	2017	0	—	—	—	0	2
Grängsjöbäcken	2012	—	—	—	—	26	33
Grängsjöbäcken	2013	—	—	—	—	34	3
Grängsjöbäcken	2014	—	—	—	—	18	2
Grängsjöbäcken	2015	—	—	—	—	10	0
Grängsjöbäcken	2016	—	—	—	—	0	0
Grängsjöbäcken	2017	—	—	—	—	0	1
Lövåsbäcken	2012	—	—	—	—	169	54
Lövåsbäcken	2014	—	—	—	—	36	0
Lövåsbäcken	2017	—	—	—	—	0	0
Mjusbäcken	2012	—	—	—	—	2	217
Mjusbäcken	2013	—	—	—	—	91	100
Mjusbäcken	2014	—	—	—	—	186	6
Mjusbäcken	2015	—	—	—	—	150	0
Mjusbäcken	2017	—	—	—	—	0	0
Lillån	2011	2	89	30	120	93	5,987
Lillån	2016	—	—	—	—	0	1,856
Husörenbäcken	2005	87	45	38	51	21	20
Husörenbäcken	2017	—	—	—	—	0	1
Kovraån/Lillån	2005	—	—	—	—	17	365
Kovraån/Lillån	2016	—	—	—	—	106	59
Viskan	2015	—	—	—	—	70	350
Viskan	2016	—	—	—	—	300	29
Viskan	2017	—	—	—	—	300	5
Teåkersälven	2016	0	—	—	—	—	553
Teåkersälven	2017	0	—	—	—	—	407
Stampbäcken	2009	0	68	54	80	—	5,314
Stampbäcken	2015	3	63	42	70	—	1,012
Värån	2014	0	94	56	117	27	1,642
Värån	2017	0	96	70	120	—	1,279

*Measurements taken from dead mussels.

†Dash indicates data deficiency.

Table 3. Biological parameters and macroscopic findings from examination of freshwater pearl mussels (*Margaritifera margaritifera*) from two unaffected streams (ref.) and two streams with mass mortality events.

Stream Name	Survey Year	Date	ID	Length (cm)	Weight (g) Whole Specmen	Weight (g) Soft Body	Gonads	Foot	Digestive Gland	Shell Inside
County of Värmland										
Älgån (ref.)	2017	November 1	5436	10.2	60.3	17.6	Large, protruding	Compact, withdrawn	Dark green, protruding content	Smooth, iridescent
Ålgån (ref.)	2017	November 1	5437	9.2	50.5	14.0	Large, protruding	Compact, withdrawn	Dark green, protruding content	Smooth, iridescent
Ålgån (ref.)	2017	November 1	5438	8.3	33.7	11.6	Large, protruding	Compact, withdrawn	Dark green, protruding content	Smooth, iridescent
Ålgån (ref.)	2017	November 1	5439	8.4	34.4	10.6	Large, protruding	Compact, withdrawn	Dark green, protruding content	Smooth, iridescent
Stampe-bäcken	2017	November 1	5440	6.3	12.6	3.4	Not detected	Relaxed,* fully released	Pale brown, no content	Small cavities, iridescent
Stampe-bäcken	2017	November 1	5441	7.0	24.1	4.5	Not detected	Compact, halfway released	Pale green, semitransparent, no content	Small cavities, iridescent
Stampe-bäcken	2017	November 1	5442	7.2	26.6	7.8	Small, semi-protruding	Compact, halfway released	Dark green, protruding content	Smooth, iridescent
Stampe-bäcken	2017	November 1	5443	6.9	20.4	5.3	Large, protruding	Compact, halfway released	Medium green, no content	Rough with nodules and small cavities
Stampe-bäcken	2017	November 1	5444	7.0	23.5	7.3	Large, protruding	Compact, withdrawn	Dark green, protruding content	Smooth, iridescent
Stampe-bäcken	2017	November 1	5445	7.1	26.5	6.4	Not detected	Relaxed, fully released	Pale green, no content	Small no. of cavities, iridescent
Stampe-bäcken	2017	November 1	5446	7.2	24.6	6.2	Not detected	Relaxed, fully released	Pale green, no content	Small no. of cavities, iridescent
County of Gävleborg										
Alakars-bäcken (ref.)	2016	June 15	1826	—†	132.4	45.2	Large, protruding	Compact, withdrawn	Dark green, protruding content	Smooth, iridescent
Alakars-bäcken (ref.)	2016	June 15	1827	—	153.3	41.7	Large, protruding	Compact, withdrawn	Dark green, protruding content	Smooth, iridescent
Lillån	2016	June 15	1818	11.1	109.1	30.7	Not detected	Relaxed, fully released	Pale brown, no content	Smooth, iridescent
Lillån	2016	June 15	1819	8.6	40.6	9.7	Not detected	Relaxed, fully released	Pale green, no content	Smooth, iridescent
Lillån	2016	June 15	1820	10.0	74.9	20.2	Not detected	Relaxed, fully released	Pale green, no content	Smooth, iridescent
Lillån	2016	June 15	1821	10.5	70.8	22.2	Not detected	Relaxed, fully released	Pale brown, no content	Smooth, iridescent
Lillån	2016	June 15	1822	10.8	81.7	23.7	Not detected	Relaxed, fully released	Pale brown, no content	Smooth, iridescent
Lillån	2016	June 15	1823	9.4	66.1	15.8	Not detected	Relaxed, fully released	Pale green, no content	Smooth, iridescent
Lillån	2016	June 15	1824	8.8	47.2	13.5	Not detected	Relaxed, fully released	Pale green, protruding content	Smooth, iridescent
Lillån	2016	June 15	1825	11.1	132.4	45.2	Not detected	Relaxed, fully released	Pale brown, no content	Smooth, iridescent

*This specimen died shortly before examination.

†Dash indicates data deficiency.

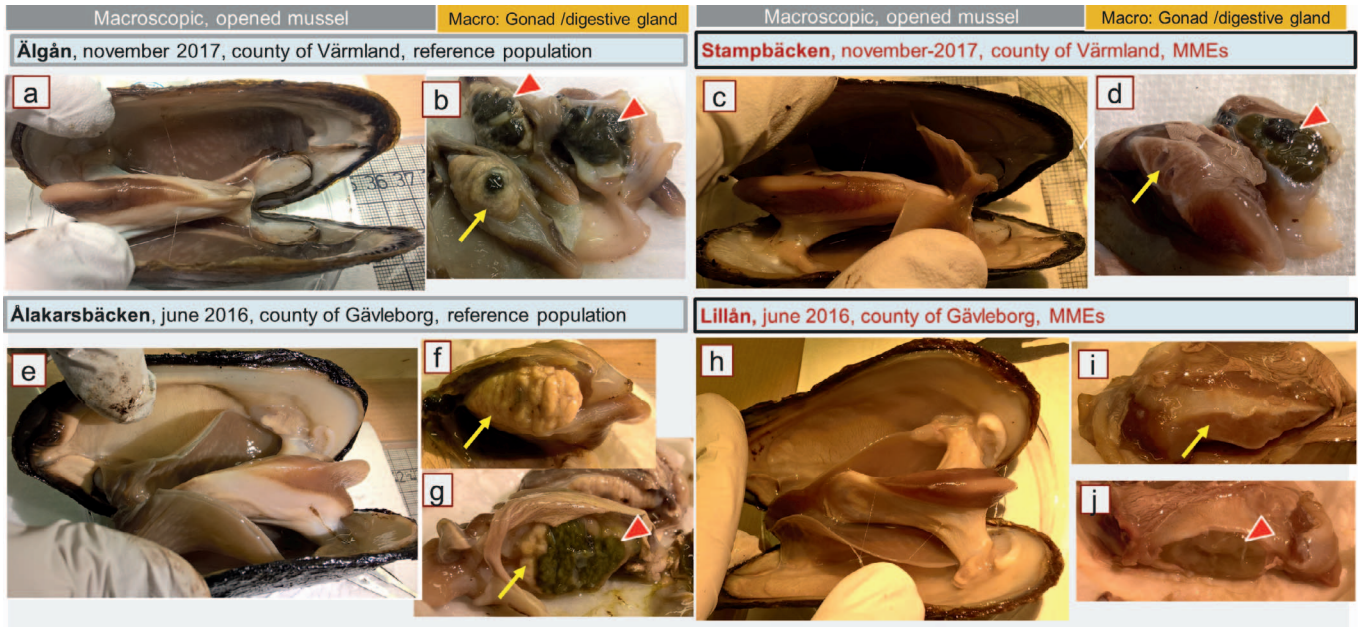

Figure 2. Macroscopic observation of *Margaritifera margaritifera* from reference populations (Ålgån, Ålakarsbäcken; left) and those from populations experiencing mass mortality events (Stampebäcken, Lillån; right). Differences between gonads are highlighted with yellow arrows, while differences between digestive glands are highlighted with red arrow heads.

central Sweden. As in the USA, the causes of MMEs in Sweden are uncertain (Downing et al. 2010). The geographic pattern in Sweden is unclear and warrants further investigation. Mass mortality events have not been detected in Västerbotten and Norrbotten, the two most northern counties in Sweden, but it is possible they have been missed, as reaches are investigated only every 6 yr. That was the case with Örasjöbäcken, where investigators found an isolated MME in 2013 while walking between two monitoring reaches. Between 2007 and 2015, the number of live animals increased by 10% at the 21 survey reaches in Örasjöbäcken and the proportion of juveniles increased from 4% to 34% (Table 2). In the isolated reach, the mortality was 70% between 2013 and 2017. This isolated MME needs further investigation.

In this study, we found some novel results regarding differences in histopathological structure in mussels from sites with and without MMEs. Individuals from MME sites seemed to be emaciated and may have lost the capacity to use and digest energy resources. This may be explained by alteration or damage to the epithelial cells of the digestive glands. The observed lesions in digestive glands and other organs are likely to adversely impact digestion and assimilation of food, thus indirectly affecting reproduction by decreasing energy resources and storage of ions and metabolites necessary for production of offspring. We also observed the absence, or decreased volumes, of gonads, findings that indicate a negative impact on the reproductive capacity of these mussels. The causes of our findings are still unresolved. Infections transmitted from vectors or conspecifics have been documented to affect reproductive capacity in other mussel species via direct or indirect castration (Rice et al. 2006; Lafferty and Kuris 2009), destruction of the gonads by intracellular infections of oocytes (Ngo et al. 2003), or targeting of epithelial cells that are important for food absorption and uptake of nutrients (Villalba et al. 1993). In the latter study, *Marteilia refringens* infections reported in the Mediterranean mussel (*Mytilus galloprovincialis*) were significantly linked to gonad development. Adipogranular (ADG) cells, which store energy and promote the initiation of gonad development, were investigated in mussels from farming areas. A negative correlation was found between the degree of parasitic infection and the abundance of ADG cells in the mantles of these mussels. The conclusion was that *Marteilia refringens* infection was clearly associated with the inhibition of ADG cells and also the development of the gonads in both males and females (Villalba et al. 1993). Whether or not this could account for the observed pattern in our study is unclear as no obvious infectious diseases or parasites were detected.

The qualitative survey method examines only one reach in a stream so it is impossible to state whether the whole stream is affected or just a part of the stream. In some of the streams, it is also difficult to say when the start of the MME occurred because of the 6-yr monitoring interval and the fact that monitoring started in only the mid-1990s. In all streams with more than one surveyed reach, all reaches were affected except for Örasjöbäcken. This finding suggests that MMEs tend to occur throughout a given stream rather than being limited to single reaches. Thus, causes are likely to be characteristic of a stream. The five most common causes of MMEs identified in

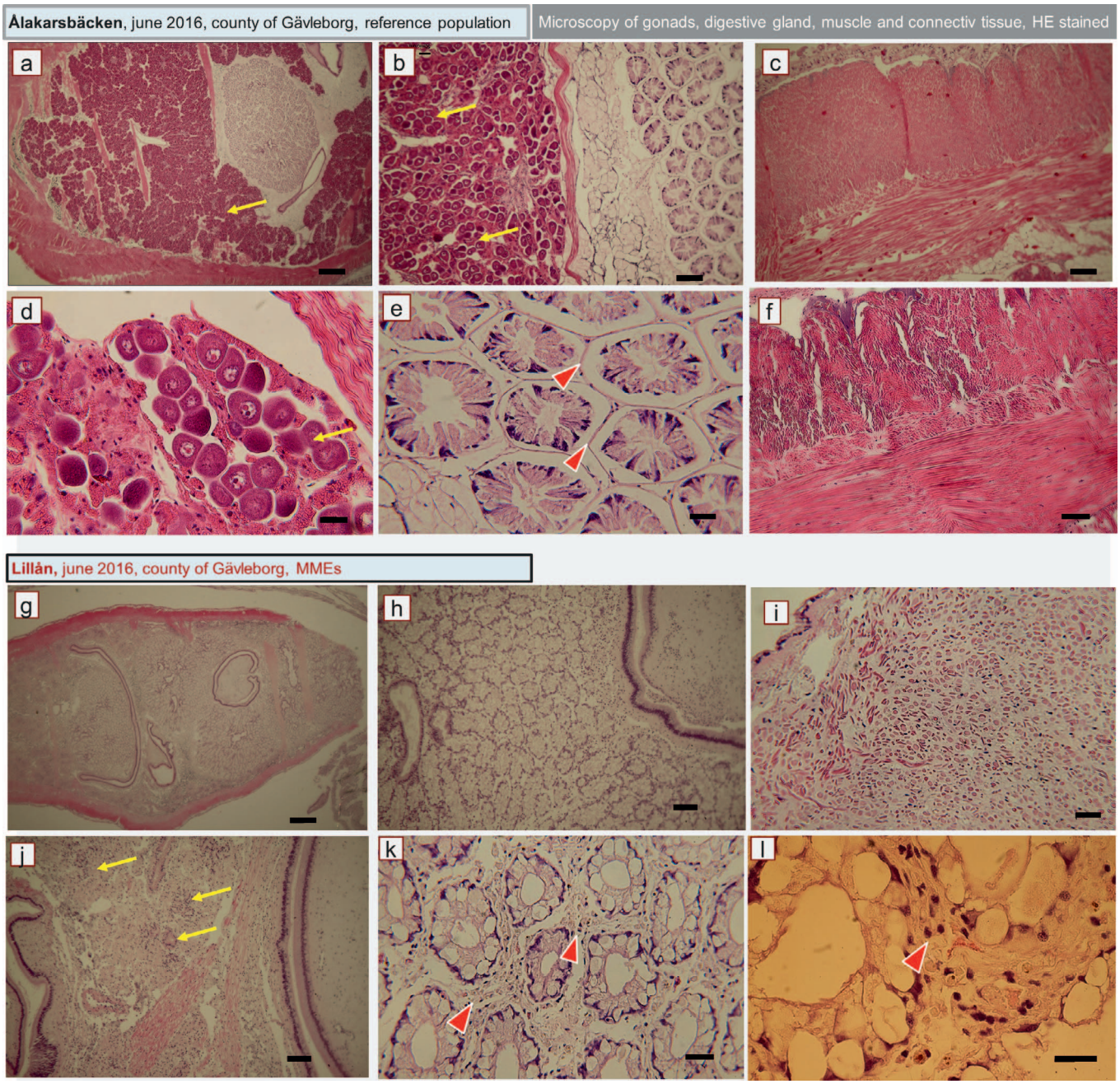

Figure 3. Histopathology of fixed cross sections of *Margaritifera margaritifera*. Differences in morphology and the presence of lesions were detected when comparing reference individuals (a–f) with those collected from sites experiencing mass mortality events (MMEs; g–l). Tissue sections were stained with H&E. Scale bars are as follows: a,g = 1,000 µm, b,c,h,i,j = 100 µm, d,e,f,k = 40 µµm, l = 20 µm. Cellular infiltration between the digestive glands is highlighted by red arrowheads (k,l). This infiltration is absent in specimens from the reference population (e). Normal gonads are highlighted with yellow arrows (a,b,d), but were not detected in many mussels from sites experiencing MMEs (j). The external layer of the foot had thick bundles of muscle fibers that were tightly packed in the reference mussels (c,f) but hypertrophied in many of the MME individuals (i).

the USA are pollution, habitat destruction, hydrologic change, presences of dams, and lack of host fishes (Downing et al. 2010). Populations dominated by single, old cohorts of mussels might suffer MMEs if these cohorts die of natural causes. Old and senescing FPM populations are common in Sweden. However, our length data suggest that multiple cohorts are affected, and the histological analyses indicate other reasons besides age-related death. At this point, we are unable to determine what factors account for MMEs in Swedish streams.

During the last decade, mortality of up to 100% in Swedish populations has been reported but the possible causes of MMEs in *M. margaritifera* populations are still unresolved. However, we detected lesions in digestive glands indicating a

reduced capacity for uptake of nutrients, and both macroscopic and microscopic investigations indicated reduced reproductive capacity compared with reference populations. Clearly, more investigations are needed to determine the causes of declines in Sweden's mussel populations.

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REGULAR ARTICLE

A COMPARISON OF BACTERIA CULTURED FROM UNIONID MUSSEL HEMOLYMPH BETWEEN STABLE POPULATIONS IN THE UPPER MISSISSIPPI RIVER BASIN AND POPULATIONS AFFECTED BY A MORTALITY EVENT IN THE CLINCH RIVER

Eric Leis[1]*, Sara Erickson[1], Diane Waller[2], Jordan Richard[3], and Tony Goldberg[4]

[1] *La Crosse Fish Health Center, Midwest Fisheries Center, U.S. Fish and Wildlife Service, 555 Lester Ave, Onalaska, WI 54650 USA*

[2] *Upper Midwest Environmental Sciences Center, U.S. Geological Survey, 2630 Fanta Reed Rd, La Crosse, WI 54603 USA*

[3] *Southwestern Virginia Field Office, U.S Fish and Wildlife Service, Southwestern Virginia Field Office, 330 Cummings Street, Abingdon, VA 24210 USA*

[4] *University of Wisconsin–Madison, Department of Pathobiological Sciences and Global Health Institute, 1656 Linden Drive, Madison, WI 53706 USA*

ABSTRACT

The diagnosis of bacterial disease in freshwater unionid mussels has been hindered by a lack of baseline information regarding the microbial communities associated with these animals. In this study, we cultured and identified bacteria from the hemolymph of stable mussel populations from Wisconsin portions of the upper Mississippi River basin and compared the results to those from mussel populations experiencing a mortality event in the Clinch River in Virginia and Tennessee. Several bacterial genera were consistently identified across mussel species and locations, appearing to be part of the natural bacterial flora. One noteworthy bacterial species identified from the Clinch River was *Yokenella regensburgei*, which occurred in relatively high prevalence during the mortality event but was absent from samples acquired afterward. Its role in the mortality event, if any, is unknown but deserves further investigation. We suggest that future studies of freshwater mussel health incorporate hemolymph as a sample type due to its relative separation from the aquatic environment, its role in the circulatory system, and the fact that it can be collected nonlethally.

KEY WORDS: freshwater mussel, Unionidae, microflora, bacteriology, hemolymph, disease

INTRODUCTION

Freshwater mussels are exposed to the microorganisms that they filter and accumulate from the aquatic environment. Bacteria are a food source, but also can be found in body tissues outside of the gut, including the hemolymph, in apparently healthy animals (Starliper et al. 1998, 2008; Antunes et al. 2010). In general, the characteristic bacterial flora of freshwater mussels is largely unknown, despite the emergence of microbiome research examining correlations between bacterial and archaeal communities and health and resilience across a variety of animal species (e.g., gut biota of humans and fish, livestock, etc.; see Ingerslev et al. 2014; Ghanbari et al. 2015; Reese et al. 2018; Trinh et al. 2018). In mussels, bacterial diversity in fluids and tissues has been associated with healthy, responsive animals (Starliper et al. 2008), whereas high bacterial loads have been associated with sick or moribund animals (see Sparks et al. 1990, Starliper et al. 2011).

*Corresponding Author: eric_leis@fws.gov

Historically, mass mortality events of freshwater mussels have occurred that suggest infectious causes (Neves 1987), but in most cases, the causative agent has not been identified (see review in Grizzle and Brunner 2009). While samples may be collected and analyzed from such events, the lack of data regarding the normal microbiota of healthy mussel populations has made it difficult to identify potential pathogens as well as other potential commensal or mutualistic relationships (Starliper et al. 1998; Starliper 2008). The reports by Starliper, focused on populations from the southeastern USA (Starliper et al. 1998; Starliper and Morrison 2000; Starliper 2001, 2005, 2008, 2011; Starliper et al. 2008, 2011), constitute much of the knowledge base regarding the bacterial communities associated with freshwater mussels. To develop much-needed specific diagnostic assays, we must better understand mussel-microbe interactions and identify pathogens, tasks feasible only with bacteriology data from diverse unionid species across broader geographic regions.

Standardized diagnostic methods for freshwater fish typically utilize the kidney for the collection of bacteriological samples (USFWS and AFS–FHS 2012) due to its function as a filtration organ. However, similar methods are lacking for freshwater mussels, primarily because of limited attention to the diseases of these taxa (Grizzle and Brunner 2009). Typically, previous bacteriology studies of unionids have utilized lethal sampling to collect fluids and mixed tissue or whole body homogenate samples (Starliper et al. 1998, 2008, 2011; Starliper and Morrison 2000; Starliper 2001, 2005), while others compared the microbiota between specific tissues (Sparks et al. 1990; Chittick et al. 2001; Nichols et al. 2001; Antunes et al. 2010). Although bacteria were cultured from most tissue types, interpretations are confounded by lack of organ specificity (mixed tissue or whole body homogenates) as well as the risk to sample integrity due to the closeness of the internal organs to the aquatic environment and the disinfection procedures used to reduce contamination. Moreover, whole body and soft tissue samples generally require sacrifice of the mussel, which should be avoided, especially for imperiled fauna.

A sample type that has received less attention in the assessment of freshwater mussel health is hemolymph (Sparks et al. 1990; Starliper 2008; Antunes et al. 2010). This fluid, which plays an important role in immunity as well as many other critical functions, makes up approximately 50% of the weight of mussel tissue (Thorp and Covich 2010). The interaction of hemolymph with the organs and tissues of the mussel, its relative compartmentalization from the aquatic environment, and the accessibility through the adductor sinus for nonlethal sampling provide many advantages (Gustafson et al. 2005; Burkhard et al. 2009). Furthermore, this sample may be particularly useful in examining potential septicemia (Sparks et al. 1990).

In this study, we cultured and identified bacteria from the hemolymph of unionid mussels from apparently stable populations in the Wisconsin portion of the Upper Mississippi River (UMR) basin as well as from samples obtained from a mussel mortality event in the Clinch River in Tennessee and Virginia. Our primary objective was to determine the community composition of the culturable bacteria present within these populations and the prevalence of specific taxa.

METHODS

We collected a variety of freshwater mussel species on June 16, 2017, August 23, 2017, August 29, 2017, October 6, 2017, and October 26, 2017, from the Wisconsin stretches of the La Crosse River (43°54′52.51″N, 91°4′34.93″W), Chippewa River (44°45′38.80″N, 91°40′44.80″W), Lake Onalaska (a backwater lake of Pool 7 of the UMR; 43°53′45.85″N, 91°16′10.41″W), Black River (43°52′18.65″N, 91°14′42.39″W), and Goose Island (Pool 8 of the UMR; 43°44′46.20″N, 91°13′34.17″W), respectively (Fig. 1). We obtained samples from Pheasantshell (*Actinonaias pectorosa*) mussels from the Tennessee reaches (Wallen Bend, 36°35′2.65″N, 83°0′49.96″W; Kyle's Ford, 36°33′57.05″N, 83°2′29.57″W) of the Clinch River on November 2, 2017, during an active mortality event (Richard 2018) and postmortality event on February 1, 2018, again from Kyle's Ford as well as Sycamore Island (Virginia, 36°37′16.36″N, 82°49′6.20″W) (Fig. 1). Mussels were hand-collected and held in source water until a sufficient sample size (∼20–30) was acquired for each site. During the postmortality sampling event, we processed approximately half of the Pheasantshells in the field and, due to inclement weather, transported the others in source water to the laboratory, where they were held (up to 72 h) before sampling. Following collection, we used either a reverse pliers or a child's nasal speculum and stopper to open the shell slightly. The anterior adductor mussel was cleaned using an individually wrapped sterile rayon swab soaked in 70% isopropyl alcohol. We withdrew approximately 100 μL of hemolymph from the anterior adductor muscle using a 1 mL insulin needle and syringe. The hemolymph sample was then sinuously streaked onto a tryptic soy agar (TSA) plate using a sterile inoculation loop. TSA plates were incubated for 1 wk in a 21°C incubator. Bacterial colonies with unique macroscopic morphologies were sampled from each plate using a sterile bacteriology loop and placed in sterile 2.0 mL microconical screw-cap collection tubes. Following the manufacturer's instructions, we then extracted DNA using 100 μL of PrepMan™ Ultra Sample Preparation Reagent (ThermoFisher Scientific). Polymerase chain reaction (PCR) primers targeting the 16S rRNA gene (Table 1) were used to amplify and sequence this gene from each isolate. The master mix consisted of 46 μL Platinum PCR Supermix as well as 100 pmol of each selected forward and reverse primer (Table 1). Two μL of extracted DNA was added to the master mix for each reaction. PCR products were exo-SAP purified, and Sanger sequencing was performed by the Whitney Genetics Laboratory (U.S. Fish and Wildlife Service; Onalaska, WI). We edited sequences using Geneious (version 11.1.5) and conducted BLASTn queries using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Accession numbers reported in the Supplemental Data represent the top listed, named species that shared the most similarity to our

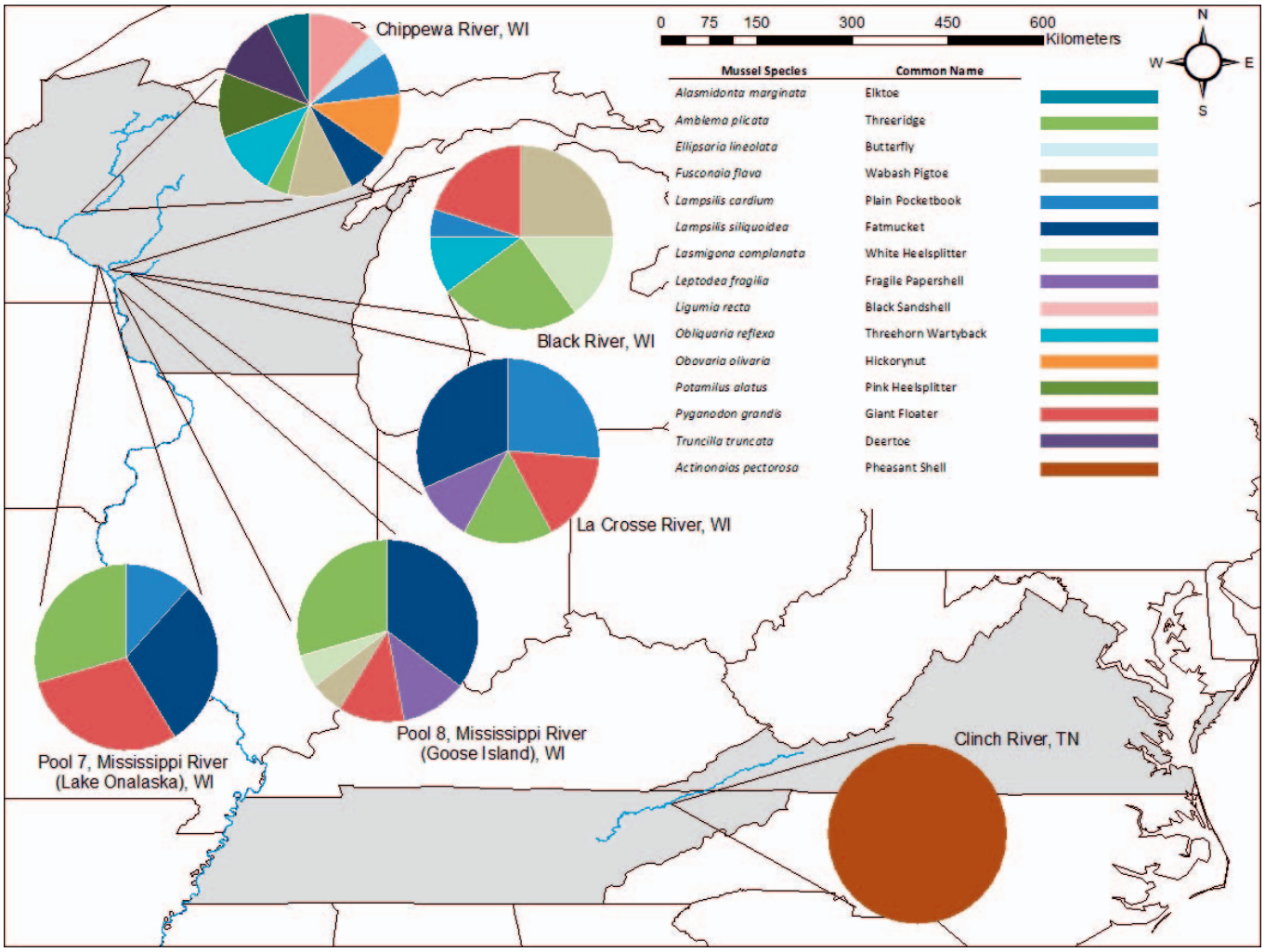

Figure 1. Relative proportion of mussel species sampled at each location.

query. Ambiguities are also reported (i.e., multiple species, or in a few cases genera, that shared the same degree of similarity).

RESULTS

We obtained unionid mussels ($n = 99$) representing 14 species from five sites in the Upper Mississippi River Basin (Fig. 1). We cultured bacteria representing 47 genera (Table 2) from the hemolymph of 73 mussels (74%), identifying 190 colonies through molecular methods. Two colonies were not identified. The most prevalent bacterial genera from the UMR overall were *Bacillus* spp. (19%) and *Aeromonas* spp. (21%). Most genera had a prevalence <10%, and approximately half were single-incidence isolates. Mussels sampled from the UMR basin were healthy in appearance with the lone exception being one gaping Plain Pocketbook (*Lampsilis cardium*) from the La Crosse River; we identified only *Pseudomonas* spp. from this animal. We detected bacteria displaying high levels of similarity to two fish pathogens, *Yersinia ruckeri* and *Aeromonas salmonicida*. We identified *Y. ruckeri* from one Black Sandshell (*Ligumia recta*) and one Three Horn Wartyback (*Obliquaria reflexa*) from the Chippewa River (Supplemental Data). We identified *A. salmonicida* from one Plain Pocketbook, one Wabash Pigtoe (*Fusconaia flava*), two Deertoe (*Truncilla truncata*), one Fat Mucket (*Lampsilis siliquoidea*), and one Hickorynut (*Obovaria olivaria*) in the Chippewa River; one Giant Floater and one Plain Pocketbook from the Black River; two Fat Muckets and one Wabash Pigtoe from the Goose Island

Table 1. Primers used in PCR amplification and sequencing of 16S rRNA genes of bacterial isolates cultured from unionid mussels.

Primer	Sequence (5′–3′)	Reference
8F	AGAGTTTGATCCTGGCTCAG	Turner et al. 1999
27F	AGAGTTTGATCMTGGCTCAG	Lane 1991
518F	TACCAGGGTATCTAATCC	Faisal et al. 2017
800R	CCAGCAGCCGCGGTAATACG	Faisal et al. 2017
1160F	AATCATCACGGCCCTTACGC	Faisal et al. 2017
1387R	GGGCGGWGTGTACAAGGC	Marchesi et al. 1998
1492R (I)	GGTTACCTTGTTACGACTT	Turner et al. 1999
1541R	AAGGAGGTGATCCAGCCGCA	Loffler et al. 2000

Table 2. Bacteria cultured and identified from hemolymph collected from mussels in the upper Mississippi River basin.

Location	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
La Crosse River	**Fatmucket**	6	*Agrococcus*	17
	Lampsilis siliquoidea		*Bacillus*	33
			Erwinia	17
			Exiguobacterium	33
			Kocuria	17
			Microbacterium	17
			Arthrobacter	17
	Fragile Papershell	2	*Bacillus*	50
	Leptodea fragilis		*Pseudomonas*	50
	Giant Floater	3	*Bacillus*	67
	Pyganodon grandis		*Chryseobacterium*	50
			Aeromonas	50
			Pseudomonas	50
	Plain Pocketbook	5	*Acinetobacter*	20
	Lampsilis cardium		*Brevundimonas*	20
			Chryseomicrobium	20
			Comamonas	20
			Exiguobacterium	80
			Microbacterium	20
			Pseudarthrobacter	20
	Threeridge	3	*Bacillus*	67
	Amblema plicata		*Brevundimonas*	33
			Erwinia	33
			Exiguobacterium	33
			Stenotrophomonas	33
			Luteimonas	33
Chippewa River	**Black Sandshell**	3	*Aeromonas*	33
	Ligumia recta		*Deefgea*	33
			Yersinia	33
	Deertoe	3	*Aeromonas*	67
	Truncilla truncata		*Bacillus*	33
			Brevundimonas	33
			Chromobacterium	33
			Enterobacteriaceae (*Serratia, Yersinia, Rahnella*)	33
			Microbacterium	33
			Pseudomonas	67
			Sporosarcina	33
	Elktoe	2	*Aeromonas*	50
	Alasmidonta marginata		*Chromobacterium*	50
			Leuconostoc	50
			Pantoea	50
			Pseudoxanthomonas	50
			Stenotrophomonas	100
	Fatmucket	2	*Acidovorax*	50
	Lampsilis siliquoidea		*Aeromonas*	50
			Chromobacterium	50
	Hickorynut	3	*Aeromonas*	33
	Obovaria olivaria		*Bacillus*	33
	Pink Heelsplitter	3	*Agrobacterium* (*Rhizobium*)	33

Table 2, continued.

Location	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
	Potamilus alatus		*Bacillus*	33
			Moraxella	33
			Pseudomonas	33
	Plain Pocketbook	2	*Aeromonas*	67
	Lampsilis cardium		*Agrobacterium* (*Rhizobium*)	33
			Bacillus	33
			Chromobacterium	67
			Bacillales (*Viridibacillus, Bacillus, Lysinibacillus, Kurthia, Paenibacillus*)	33
	Threehorn Wartyback	3	*Acinetobacter*	33
	Obliquaria reflexa		*Aeromonas*	100
			Brevundimonas	100
			Lysinibacillus	33
			Vogesella	33
			Yersinia	33
	Threeridge	1	*Sphingomonas*	100
	Amblema plicata			
	Wabash Pigtoe	3	*Aeromonas*	33
	Fusconaia flava			
Lake Onalaska	**Fatmucket**	5	*Aeromonas*	20
	Lampsilis siliquoidea		*Thermomonas*	20
	Giant Floater	5	*Cellulomonas*	20
	Pyganodon grandis		*Microbacterium*	20
	Plain Pocketbook	2	*Cellulomonas*	50
	Lampsilis cardium		*Cellulosimicrobium*	50
			Microbacterium	50
			Pseudomonas	50
	Threeridge	5	Alpha proteobacterium	20
	Amblema plicata		*Bacillus*	20
			Bosea	20
			Curtobacterium	20
			Fictibacillus	20
			Flavobacterium	20
			Pseudomonas	20
			Pseudoxanthomonas	20
			Sphingopyxis	20
Black River	**Giant Floater**	4	*Aeromonas*	25
	Pyganodon grandis		*Brevundimonas*	25
			Enterobacteriacea (*Erwinia, Pantoea*)	25
			Staphylococcus	25
	Plain Pocketbook	1	*Aeromonas*	100
	Lampsilis cardium		*Stenotrophomonas*	100
	Three Ridge	5	*Bacillus*	20
	Amblema plicata		*Pseudomonas*	40
			Staphylococcus	20
			Stenotrophomonas	20
	Threehorn Wartyback	2	*Deefgea*	50
	Obliquaria reflexa		*Staphylococcus*	50
	Wabash Pigtoe	5	*Acidovorax*	20

Table 2, continued.

Location	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
	Fusconaia flava		*Bacillus*	20
			Flectobacillus	20
			Morganella	20
			Rhodococcus	20
			Serratia	20
			Staphylococcus	20
	White Heelsplitter	3	*Acidovorax*	33
	Lasmigona complanata		*Aeromonas*	66
Mississippi River	**Fatmucket**	6	*Acidovorax*	17
	Lampsilis siliquoidea		*Acinetobacter*	33
			Aeromonas	50
			Arthrobacter	17
			Bacillus	50
			Chryseobacterium	17
			Microbacterium	33
			Pseudomonas	17
			Shewanella	17
			Staphylococcus	17
	Fragile Papershell	2	*Chitinibacter*	50
	Leptodea fragilis			
	Giant Floater	2	*Bacillus*	50
	Pyganodon grandis		*Bosea*	50
			Chryseobacterium	50
			Rheinheimera	50
	Threeridge	5	*Stenotrophomonas*	50
	Amblema plicata		*Brevundimonas*	20
			Microbacterium	20
			Stenotrophomonas	60
			Variovorax	20
	White Heelsplitter	1	*Acidovorax*	100
	Lasmigona complanata		*Bacillus*	100
			Flavobacterium	100
			Staphylococcus	100
			Stenotrophomonas	100
	Wabash Pigtoe	1	*Pseudarthrobacter*	100
	Fusconaia flava		*Aeromonas*	100

backwater of Pool 8 in the UMR; one Giant Floater from the La Crosse River; and three Pheasantshells from the Clinch River (Supplemental Data). Note that some ambiguity (see Supplemental Data) was observed in the identifications of *A. salmonicida*, likely due to the diversity of genetically similar taxa within Aeromonad species.

During an active mortality event, we sampled 19 Pheasantshells from the Clinch River in Tennessee and cultured bacteria from 89% of the hemolymph samples. Again, *Bacillus* (16%), *Aeromonas* (42%), and *Pseudomonas* (21%) were among the most prevalent genera (Table 3). We also identified *Yokenella regensburgei* from 42% of the Pheasantshells; this bacterium was not observed in samples obtained from the UMR.

In the postmortality sampling event of 14 Pheasantshells, we cultured bacteria from 100% of the samples with the most prevalent isolates identified as *Bacillus* spp. (53%) and *Pseudomonas* spp. (53%) (Table 3). It was noteworthy that *Y. regensburgei* was not identified from this later sampling event.

DISCUSSION

Hemolymph from 74% of the mussels from the UMR, 89% of Pheasantshells sampled during the mortality event, and 100% of Pheasantshells sampled after the mortality event yielded at least one bacterial colony. In both geographic areas, *Bacillus*, *Pseudomonas*, and *Aeromonas* were among the most

Table 3. Bacteria cultured and identified from hemolymph collected from mussels in the Clinch River.

Date	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
November 2017	**Pheasantshell** *Actinonaias pectorosa*	19	*Aeromonas*	42
			Micrococcaceae (*Arthrobacter*/*Pseudarthrobacter*)	5
			Bacillus	16
			Enterobacteriaceae (*Yokenella*/*Klebsiella*)	5
			Flavobacterium	5
			Lysinibacillus	5
			Massilia	5
			Moraxella	5
			Pseudomonas	21
			Streptococcus	11
			Yokenella	42
February 2018	**Pheasantshell** *Actinonaias pectorosa*	14	*Arthrobacter*	7
			Bacillus	53
			Cellulomonas	7
			Exiguobacterium	7
			Klebsiella	7
			Kocuria	7
			Massilia	7
			Microbacterium	13
			Paeniglutamicibacter	7
			Planococcus	7
			Pseudomonas	53
			Sanguibacter	7
			Sphingomonas	7
			Streptomyces	7

prevalent bacterial genera identified from mussel hemolymph. Many of the species identified from the UMR and Clinch Rivers also had been reported previously from unionid mussels in the Mississippi, Illinois, Clinch, and Tennessee rivers (Sparks et al. 1990; Starliper et al. 2008, 2011).

Aeromonas spp., a group known for varying levels of pathogenicity (Sreedharan et al. 2011), were identified with the highest prevalence (42%) during the peak of the mortality event on the Clinch River. This genus was not identified during the postmortality sampling and was reported from only 21% of the mussels sampled from apparently healthy populations in the UMR. In previous studies, *Aeromonas* spp. have been among the most prevalent bacteria cultured from both healthy and diseased mussels (Sparks et al. 1990; Atunes et al. 2010; Starliper et al. 2011). We suggest that future work investigate associations between *Aeromonas* spp. and unionid health and disease, especially studies examining bacterial growth and mussel immune function under stressful conditions.

Yersinia ruckeri and *A. salmonicida* are important fish pathogens with regulatory implications. Fish infected with these bacteria are not only at risk for disease, but they may not be approved for stocking, thereby putting them at risk for depopulation. Since captive mussel propagation efforts typically occur in fish hatcheries, we suggest testing mussels for these pathogens before incorporating them into hatchery operations. Additionally, strong consideration should be given to depurating the mussels, in isolation, before introducing them to hatchery facilities, because this has been shown to effectively eliminate *A. salmonicida* (Starliper 2005).

The diversity of the microbial community in the hemolymph may be an important indicator of population health. Measures of bacterial diversity reportedly decrease in stressed Zebra mussels (*Dreissena polymorpha)* (Gu and Mitchell 2002) and diseased Pacific oysters (*Crassostrea gigas)* (Lokmer et al. 2016). In fact, the diversity of microbial communities in the hemolymph of Pacific oyster was a predictor of response to environmental stress (Lokmer et al. 2016). Our results provide baseline data on microbial diversity

in native mussel populations in the UMR for comparison, especially if future stressful events occur.

An important next step is to compare microbial community composition in nonnative bivalves that co-occur with native mussels, such as dreissenids and *Corbicula*. Other studies have investigated bacterial communities associated with Zebra mussels, but none, to our knowledge, have concurrently examined native mussels (e.g., Frischer et al. 2000; Gu and Mitchell 2002; Winters et al. 2010, 2011). Zebra mussels have caused significant shifts in bacterial community structure (Frischer et al. 2000; Lohner et al. 2007), which could have consequences for the stability of native mussel microbiota. Similarly, *Corbicula* are efficient filter feeders that reduce bacterial abundance in streambeds (Hakenkamp et al. 2001) and could also potentially alter the microbial community composition.

Bacteria have been routinely isolated from the hemolymph of aquatic invertebrates in varying stages of health (see the table in Zhang et al. 2018). It is therefore plausible that mussels are under constant invasion from bacteria in the aquatic environment. However, the consistent presence of taxonomically related bacteria across mussel species and geographic locations suggests a characteristic unionid hemolymph microbiome. Members of the genus *Bacillus* have several characteristics that appear probiotic in nature. For example, several *Bacillus* spp. (including some sharing high levels of similarity to species identified in this study; see Supplemental Data) convert urea into calcium carbonate (Wei et al. 2015; Anbu et al. 2016), a major component of the freshwater mussel shell. Furthermore, members of this group also are known for their antimicrobial properties (Yilmaz et al. 2006). The well-studied *Bacillus subtilis* is a calcium carbonate producer that has been used as a probiotic in chicken feed to thicken eggshells and inhibit pathogens (Fathi et al. 2018; Hosseindoust et al. 2018). This species also has been shown to produce fructooligosaccharides (Silva et al. 2016), which reportedly increase calcium absorption in mammals (Morohashi et al. 1998). *Bacillus subtilis* also has been recommended as a probiotic in shrimp culture due to its inhibition of *Vibrio*, a common shellfish pathogen (Vaseeharan and Ramasamy 2003). Similarly, other genera were identified that have species and/or strains with similar potential probiotic characteristics: *Exiguobacterium* (production of the shell component chondroitin, Bhotmange and Singhal 2015), *Brevundimonas* (calcium carbonate production, Wei et al. 2015), *Chromobacterium* (violacein production, Durán and Menck 2001), *Sporosarcina* (calcium carbonate production, Wei et al. 2015; Kim et al. 2016), *Pseudomonas* (calcium carbonate production, Li et al. 2015), *Stenotrophomonas* (production of osmoprotective and antifungal properties, Wolf et al. 2002), *Lysinibacillus* (calcium carbonate production, Lee et al. 2017; chondroitin production, Bhotmange and Singhal 2015; antimicrobial properties, Ahmad et al. 2014), *Acinetobacter* (calcium carbonate production, Zamarreno et al. 2009), and *Microbacterium* (calcium carbonate production, Xu et al. 2017).

Many of the bacteria isolated from unionid mussels were similar genetically to genera with species and/or strains targeted for bioremediation efforts (see Supplemental Data). There are many examples describing the use of environmental bacteria, including some genetically similar to the hemolymph isolates, with the potential for environmental detoxification (Schippers et al. 2005; Hegazi et al. 2007; Genovese et al. 2008; Seeger et al. 2010; Chatterjee et al. 2011; Irawati et al. 2012; Wanjohi et al. 2015; Huët and Puchooa 2017; Poornima and Velan 2018). Historical issues with contamination have been documented in both the Mississippi (Schramm 2004) and Clinch river (Price et al. 2014) systems. Although water quality in the UMR has improved significantly since the 1970s (Schramm 2004), the presence of these bacterial species in freshwater mussels in the UMR may be a response to persistent pollutants, especially in the sediments where mussels reside. The microbiome of an animal plays a critical role in chemical detoxification within the host (see the review in Adamovsky et al. 2018), and we do not know the extent to which the bacteria residing within the mussels may be providing this service. Future research examining whether the species and strains of bacteria associated with freshwater mussel hemolymph are indeed active in the detoxification of aquatic pollutants will be critical in examining this aspect of symbiosis as well as to assess whether mussel microbiomes may be an indicator of environmental pollutants.

In the Clinch River, *Y. regensburgei* was identified from 42% of the Pheasantshells sampled during an active mortality event but, interestingly, was not detected from the same population just a few months later. Isolates from the *Yokenella* genus have been shown to degrade hydrocarbons from soils contaminated with oily sludge (Bhattacharya et al. 2003); its presence could indicate elevated levels of contaminating hydrocarbons during the period of peak mortality, levels that may have subsided thereafter. Interestingly, *Y. regensburgei* also was identified during the peak of a mortality event involving Ebonyshell (*Fusconaia ebena*) from the Tennessee River, Alabama (Starliper et al. 2011). In human medicine, *Y. regensburgei* is considered an opportunistic pathogen (Lo et al. 2011; Jain et al. 2013); it also has been identified from a variety of environmental samples including well water and the gastrointestinal tracts of insects (Kosako et al. 1984). Such observations warrant further investigations of the relationship of *Y. regensburgei* with freshwater mussels, perhaps including *in vivo* exposures of mussels to hydrocarbons and experimental assessment of the mitigating effects (if any) of *Y. regensburgei* on toxicosis.

The occurrence of a bacterial species in both apparently healthy and sick mussels does not necessarily indicate either a commensal or pathogenic relationship. Changes in the environment, condition of the host, and balance of the microbial community can facilitate pathogenesis. Additionally, the virulence of a bacterial species can vary significantly

among strains (see, e.g., Olivier 1990). Indigenous bacteria isolated from Zebra mussel whole body homogenates were pathogenic when administered in high doses and under elevated water temperatures (Gu and Mitchell 2002). Studies of the pathogenicity of suspect bacteria under different conditions are needed to elucidate the mechanisms and conditions that encourage bacterial pathogenesis in freshwater mussels.

In our study, the TSA media and culture conditions undoubtedly limited the diversity of bacterial species that were identified. Incubation temperature, time, and media are all important factors to consider when attempting to recover specific bacteria of interest (Starliper and Morrison 2000) or to maximize growth of greater microbial diversity. For example, incubation of digestive gland samples from *Elliptio complanata* at both 20°C and 35°C yielded a greater number and type of isolates than at a single temperature (Chittick et al. 2001). Additional research is needed to determine optimal media and culture conditions for growth of bacteria from freshwater mussels. Furthermore, research using metagenomic analysis will help identify unculturable species as well as examine functional profiles of all hemolymph bacteria, especially in regard to pathways pertaining to calcium carbonate production and pollutant detoxification.

CONCLUSIONS

Our study established reference data on the diversity of culturable bacteria from the hemolymph of unionid mussels across multiple species and geographic regions in the USA. Hemolymph proved highly suitable for assessing the microbiota of freshwater mussels by nonlethal methods. Isolates genetically similar to two potential fish pathogens, *A. salmonicida* and *Y. ruckeri*, were detected in mussels from two sites in the upper Mississippi River basin. *Yokenella regensburgei* was identified from Pheasantshell mussels during a mortality event, and further work is necessary to determine the importance of this bacterium.

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NOTE

A NOVEL PICORNA-LIKE VIRUS IN A WABASH PIGTOE (*FUSCONAIA FLAVA*) FROM THE UPPER MISSISSIPPI RIVER, USA

Tony L. Goldberg[1]*, Christopher D. Dunn[1], Eric Leis[2], and Diane L. Waller[3]

[1] *Department of Pathobiological Sciences, University of Wisconsin-Madison, 1656 Linden Drive, Madison, WI 53706 USA*

[2] *U.S. Fish and Wildlife Service, La Crosse Fish Health Center-Midwest Fisheries Center, 555 Lester Avenue, Onalaska, WI 54650 USA*

[3] *U.S. Geological Survey, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, WI 54603 USA*

ABSTRACT

Unionid mussels are threatened by multiple environmental stressors and have experienced mass mortality events over the last several decades, but the role of infectious disease in unionid health and population declines remains poorly understood. Although several microbial agents have been found in unionids, to date only one virus has been documented—Lea plague virus (*Arenaviridae*) in propagated Triangle Shell mussels (*Hyriopsis cumingii*) in China. We used next-generation DNA sequencing to screen hemolymph of seven individuals of five unionid species from the Upper Mississippi River basin, USA for viruses. We identified the complete polyprotein gene of a novel picornalike virus in one individual of the Wabash Pigtoe (*Fusconaia flava*). The virus is a member of the Nora virus clade of picornalike viruses and is most closely related to viruses from arthropods in China. We did not detect viruses in another Wabash Pigtoe or in animals of the other four species. It is premature to make inferences about the role of this virus in the health of Wabash Pigtoes or other unionid species or the origin or transmission of this virus. Nevertheless, to our knowledge, our results represent the first report of a virus in wild North American unionids. Technologies based on next-generation DNA sequencing should prove useful for identifying new viruses and investigating their role in unionid health and disease.

KEY WORDS: Unionidae, *Fusconaia flava*, Wabash Pigtoe, Mississippi River, virus, next-generation DNA sequencing

*Corresponding Author: tony.goldberg@wisc.edu

INTRODUCTION

Freshwater mussels (order Unionida) face mounting threats from habitat loss and alteration, invasive species, poor water quality and pollutants, hydrologic changes, and other stressors (Strayer et al. 2004; Dudgeon et al. 2006; Downing et al. 2010; Haag and Williams 2014). Unexplained mortality events have been documented since at least the 1970s, but their causes remain poorly understood (Haag and Williams 2014). Unionids are susceptible to a variety of metazoan, protozoan, fungal, and viral infections (Carella et al. 2016), which may contribute to mussel mortality as primary or secondary factors. Recently, we described a coordinated effort to investigate potential pathogens associated with unionid mass mortality events (Leis et al. 2018).

Viruses are likely culprits in mass die-offs of wildlife species, accounting for a higher percentage of disease-associated events across all animal taxa than other classes of pathogens (Fey et al. 2015). Viruses are also more likely to emerge (appear in new places, new hosts, and new clinical contexts) than other classes of pathogens because of their error-prone replication and ensuing ability to mutate, evolve, and "jump" to new species (Woolhouse et al. 2005). Viruses are major causes of mortality in marine bivalves (Zannella et al. 2017). To our knowledge, the only virus described from unionids to date is Lea plague virus, an arenavirus (family *Arenaviridae*) responsible for mass mortality of Triangle Shell mussels (*Hyriopsis cumingii* Lea) in southern China (Carella et al. 2016); these mussels are cultivated at high density for freshwater pearl production. We surveyed five unionid species from the Upper Mississippi River basin, USA to investigate whether viruses may be present in North American unionids.

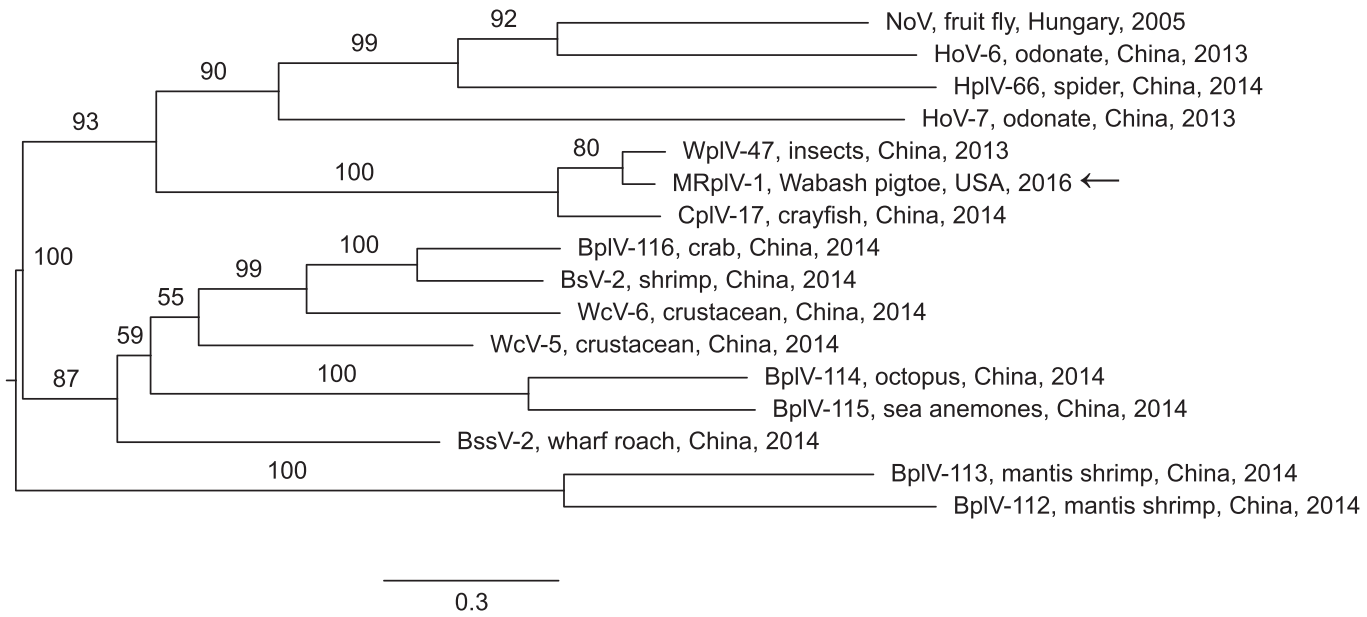

Figure 1. Phylogenetic tree of picornalike viruses. The major glycoprotein nucleic acid sequences of each virus were aligned using the codon-based Prank algorithm (Loytynoja 2014) implemented in the program TranslatorX (Abascal et al. 2010), with the Gblocks algorithm (Castresana 2000) applied to remove poorly aligned regions. The maximum-likelihood method implemented in the computer program PhyML (Guindon et al. 2010) was then applied to the resulting 1,332-position nucleic acid alignment, with the model of molecular evolution estimated from the data. Taxon names indicate abbreviated virus names (see below), host, country, and year of collection. The novel picornalike virus from the Wabash Pigtoe is indicated with an arrow. Numbers beside branches show statistical confidence of clades based on 1,000 bootstrap replicates of the data. Scale bar indicates nucleotide substitutions per site. Taxon abbreviations and GenBank accession numbers: NoV: Nora virus (NC_007919); HoV-6: Hubei odonate virus 6 (NC_033071); HplV-66: Hubei picornalike virus 66 (NC_033133); HoV-7: Hubei odonate virus 7 (NC_033232); WplV-47: Wenzhou picornalike virus 47 (NC_033150); MRplV-1: Mississippi River picornalike virus 1 (MK301250); CplV-17: Changjiang picornalike virus 17 (KX884555); BplV-116: Beihai picornalike virus 116 (NC_032635); BsV-2: Beihai shrimp virus 2 (NC_032594); WcV-6: Wenling crustacean virus 6 (NC_032810); WcV-5: Wenling crustacean virus 5 (NC_032839); BplV-114: Beihai picornalike virus 114 (NC_032633); BplV-115: Beihai picornalike virus 115 (NC_032618); BssV-2: Beihai sea slater virus 2 (NC_032622); BplV-113: Beihai picornalike virus 113 (NC_032559); BplV-112: Beihai picornalike virus 112 (NC_032571).

METHODS

We sampled a total of seven individuals: one Threeridge (*Amblema plicata*) and two Wabash Pigtoes (*Fusconaia flava*), collected from the Mississippi River north of Brownsville, Minnesota (43° 43.137′ N, 91° 15.373′ W) on September 16, 2016, and one Threeridge, one Giant Floater (*Pyganodon grandis*), one Plain Pocketbook (*Lampsilis cardium*), and one Fatmucket (*Lampsilis siliquoidea*), collected from the La-Crosse River below Neshonoc Dam in Wisconsin (43° 54.874′ N, 91° 4.586′ W) on September 30, 2016. We opened the mussels slightly with reverse pliers and collected a single, approximately 1-mL hemolymph sample from each animal using a needle and syringe inserted into the anterior adductor muscle sinus, which is a nonlethal sampling method (Gustafson et al. 2005). We then transferred the hemolymph to a microcentrifuge tube, placed it on ice during transportation, and stored it at –80°C until the samples were processed for molecular analysis. This sampling was part of a pilot monitoring effort to characterize microbes in the hemolymph of mussels across the Upper Mississippi River basin.

To identify viruses in hemolymph, we used a virus discovery method based on next-generation DNA sequencing (NGS). NGS methods are "agnostic"—they can detect not only known viruses but also unknown viruses that are genomically similar to known viruses, without prior knowledge of which viruses may be present (Munang'andu et al. 2017). These methods have revolutionized the study of invertebrate viruses, revealing their extraordinary diversity and deep evolutionary history (Shi et al. 2016; Wolf et al. 2018).

We used published methods optimized for detecting viruses of all genomic compositions in fluids and tissues, including those of aquatic organisms (Sibley et al. 2016; Toohey-Kurth et al. 2017). Briefly, we extracted total nucleic acids from 200 µL of hemolymph using the QIAamp MinElute virus spin kit (Qiagen Inc., Valencia, CA, USA) and converted RNA to double-stranded complementary DNA (dscDNA) using the Superscript dscDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) with random hexamer priming. We then prepared dscDNA for paired-end NGS on an Illumina MiSeq instrument (MiSeq Reagent Kit v3, 2x150 cycle, Illumina, San Diego, CA, USA) using the Nextera XT DNA sample prep kit (Illumina). NGS reads were quality trimmed and analyzed for similarity to viruses in the GenBank database as described by Sibley et al. (2016) and Toohey-Kurth et al. (2017).

RESULTS

We obtained a total of 31,907,949 sequence reads (average 4,558,278 reads per individual mussel) with an average length of 109 base pairs after quality trimming. We did not detect any viruses in the animals from the La Crosse River or in the Threeridge and one Wabash Pigtoe from the Mississippi River. Sequences from the other Wabash Pigtoe mapped to a picornalike virus with approximately 12-fold coverage, yielding a complete open reading frame of 6,990 nucleotides encoding a putative viral polyprotein gene of 2,329 amino acids (GenBank accession number MK301250). The virus is a member of the Nora virus-related clade of picornalike viruses, named for the Nora virus of *Drosophila* fruit flies (Habayeb et al. 2006), which have genomes of approximately 10,000 bases of single-stranded, positive-sense RNA and infect a diverse array of aquatic, marine, and terrestrial invertebrates (Shi et al. 2016). The virus is most closely related to the Wenzhou picornalike virus 47 strain WHCCII11151 (GenBank accession number NC_033150) found in unspecified insects in China in 2013 (Shi et al. 2016). It is more distantly related to the Changjiang picornalike virus 17 strain CJLX30705 (GenBank accession number KX884555) found in unspecified crayfish in China in 2014 (Shi et al. 2016) (Fig. 1).

DISCUSSION

The presence of a virus in a North American unionid is not surprising, given the ubiquity of invertebrate viruses worldwide (Shi et al. 2016; Munang'andu et al. 2017; Wolf et al. 2018). At present, no inferences should be made about the role, if any, of this virus in the health of Wabash Pigtoes or any other species it may infect. The phylogenetic similarity of the Mississippi River picornalike virus 1 to arthropod viruses from China is interesting as evidence of the global distribution of the Nora virus clade of picornalike viruses, but because current data on these viruses are geographically biased, inferences about transmission or geographic spread also are premature. However, our detection of this virus in the hemolymph of only one mussel of seven indicates that such viruses are not present in all animals, even of the same species at the same place and time. We have not previously detected a virus similar to the Mississippi River picornalike virus 1 in any other sample sequenced in our laboratory despite analyzing hundreds of samples from diverse sources, supporting the conclusion that our results do not represent contamination.

Our results suggest that NGS-based methods will be useful for identifying viruses in unionids and for investigating the role, if any, of viruses in mortality events. We are currently applying such methods to investigate unionid mass mortality events in the Clinch River, Tennessee (Leis et al. 2018). Applying these methods to carefully selected groups of mussels of different health and disease states across different geographic regions should provide useful information for understanding how viruses may contribute to unionid declines in general.

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NOTE

ARE PARASITES AND DISEASES CONTRIBUTING TO THE DECLINE OF FRESHWATER MUSSELS (BIVALVIA, UNIONIDA)?

Andrew McElwain

Department of Biological Sciences, College of Liberal Arts and Sciences, State University of New York at Oswego, 392 Shineman Center, Oswego, NY 13126 USA, andrew.mcelwain@oswego.edu

ABSTRACT

Freshwater mussels (Mollusca: Bivalvia: Unionida) consist of 843 species in six families, but many are imperiled. Significant causes of mussel declines include contaminants and loss of substrate. Potentially, etiological agents are also contributing factors, but parasites and pathogens of freshwater mussels are understudied relative to those affecting marine bivalves. Published accounts of viral pathogens have been reported exclusively from *Hyriopsis cumingii* (Unionidae) in China. There are limited records of possible bacterial and fungal pathogens from unionids in the USA and Finland. Parasitic and commensal organisms generally include ciliates (Ciliophora), trematodes (Platyhelminthes: Aspidogastrea and Digenea), roundworms (Nematoda), moss animals (Ectoprocta, Entoprocta), oligochaetes and leeches (Annelida: Sedentaria: Clitellata), mites (Arthropoda: Acari), copepods (Arthropoda: Copepoda), insects (Arthropoda: Insecta), and fish eggs (Chordata: Actinopterygii). Parasites injure the host through attachment or feeding or when they invade host tissue to complete their life cycles (e.g., digeneans). Commensals are small organisms living in or on mussels that may use the mantle cavity or shell as a refuge or substrate, and commensals also may feed on particulates that have been gathered by their molluscan host. Typically, however, the relationship between the two parties is subject to speculation (e.g., leeches). We are in the midst of a biodiversity crisis, and this minireview highlights the relationships among these organisms and the need to understand the health of wild and captive mussels.

KEY WORDS: freshwater mussels, Unionida, parasites, diseases, gross pathology, histopathology

Freshwater mussels are a globally distributed group of about 843 species in six families (Graf and Cummings 2007; Williams et al. 2017). Approximately 29 species have gone extinct in the USA as a result of human activities and many other mussel species have declining populations (Haag 2012). These declines are thought to result primarily from human activities that fall into one of four general categories. The first includes activities (such as dam construction) that change the physical habitat of rivers and lakes. The second includes activities that contaminate the benthos with chemical and physical waste from industrial and municipal sources (e.g., Hornbach 2001; Grabarkiewicz and Davis 2008). The third category is the extensive harvesting of mussels for the button and pearl industries, which has contributed to declines in some species, especially in the USA (Haag 2012). The fourth category is the introduction of nonnative aquatic molluscs, such as the zebra mussel (*Dreissena polymorpha*) or Asian clam (*Corbicula fluminea*), which compete with native mussels for food and available substrate or which foul waters, harming indigenous species (Cummings and Graf 2010). Additionally, nonnative molluscs potentially can introduce nonnative etiological agents that might negatively affect native molluscs (Prenter et al. 2004). Although we lack data on the presence of nonnative etiological agents in freshwater mussels, *Perkinsus marinus* and *Haplosporidium nelsoni* are good examples of introduced pathogens that have affected significantly the USA marine shellfish industry (Burreson and Ford 2004; Villalba et al. 2004). Because of declining populations and mass mortality events known as "die-offs" and "mussel kills," the health of wild and hatchery-reared mussels is a growing concern (Neves 1987; Fleming et al. 1995; Lydeard et al. 2004).

In an effort to shed light on the possibility of etiological agents as causative factors of mussel declines, Grizzle and Brunner (2009) reviewed the literature regarding parasites and infectious diseases reported from freshwater bivalves. Most of the cited literature are observations of single-celled eukaryotic organisms and metazoans that may engage in either a commensal or parasitic relationship with unionids or margaritiferids in North America and Europe. The other four families in Unionida are underrepresented in the parasite and disease

literature. Additionally, there appears to be almost no peer-reviewed literature on viral, bacterial, or fungal infections in freshwater mussels. A few exceptions include reports of RNA and DNA viruses infecting the digestive system of *Hyriopsis cumingii* in China (Grizzle and Brunner 2009; Lei et al. 2011). In 1931 mussel propagation personnel reported "adult mussels became sterile through bacterial attacks on larval mussels" (Pritchard 2001). Intracellular microorganisms have been observed in histological sections of the digestive gland of *Elliptio complanata* in the USA, but it was unclear if they were prokaryotic or eukaryotic (see Fig. 5 in Chittick et al. 2001). Fungal hyphae were observed in the marsupia of *Unio pictorum*, *U. tumidus* (Pekkarinen 1993a), and *Pseudoanodonta complanata* in Finland (Pekkarinen 1993b). These latter three studies or observations appear to have been overlooked by Grizzle and Brunner (2009). It is possible that some parasite or disease records may be missed because they appear in literature in which characterizing parasites or diseases was not the primary objective, such as reports on surveys that detail population or community structure. Pekkarinen (1993a) reported fungi and ciliates in the marsupium associated with degenerating glochidia, but it was unclear if the fungi were pathogenic or saprophytic. Overall, parasitic or commensal organisms have been reported primarily in wild mussels; there is little information about disease problems that may occur in a hatchery.

Ciliates (Ciliophora), trematodes (Platyhelminthes: Aspidogastrea, and Digenea), moss animals (Ectoprocta, Entoprocta), roundworms (Nematoda), oligochaetes and leeches (Annelida: Sedentaria: Clitellata), mites (Arthropoda: Acari), copepods (Arthropoda: Crustacea: Copepoda), insects (Arthropoda: Insecta), and fish eggs (Chordata: Actinopterygii) are associated with the soft tissues or shells of freshwater mussels (Grizzle and Brunner 2009; Wisniewski et al. 2013). Aspidogastreans, digeneans, nematodes, mites, insect larvae, and fish eggs either infect mussels or have been reported as injurious agents. Interestingly, the eggs of both mites (*Najadicola ingens*) and fishes (*Rhodeus sericeus*) sometimes may obstruct the water tubes of a marsupium and prevent or hinder the development of glochidia (Stadnichenko and Stadnichenko 1980; McElwain et al. 2016 and references therein). Ciliates, oligochaetes, leeches, insect larvae, and copepods have been found in the mantle cavity of mussels, but the relationship between these organisms and their hosts is poorly understood. Perhaps their presence was not associated with tissue damage or perhaps the authors did not provide many supporting details concerning injuries. For example, the larvae of several midge species (Chironomidae) have been found in the mantle cavity of unionids (Roback et al. 1979). Some species, such as *Baeoctenus bicolor*, appear to injure gill and mantle tissue, whereas others, such as *Orthocladius dorenus*, do not (Gordon et al. 1978; Roback et al. 1979). Other noteworthy examples include the observations of Antipa and Small (1971). Transmission electron microscopy revealed the remnants of unionid gill cells in the food vacuoles of *Conchopthirius curtu*, but there was no evidence of tissue damage associated with attached ciliates. Curiously, Coker et al. (1921) reported *Chaetogaster limnaei* feeding on mussel parasites but provided no other supporting details. Overall, few studies have used light or electron microscopy to document the pathological changes to tissues associated with pathogens, parasites, or commensals.

Since the publication of the work of Grizzle and Brunner in 2009, a few noteworthy studies have been published regarding parasites in freshwater mussels. Levine et al. (2009) reported *Gomphus militarus* (Arthropoda, Insecta, Odonata) as potentially feeding on the gills of *Popenaias popeii* (Unionidae), a critically endangered species restricted to two populations in the Rio Grande basin (Carman 2007). Some mussels were missing the entire outer gills or all four gills. It is unclear how often odonates occur in the mantle cavity of mussels, as there appears to be no other literature on this topic (Grizzle and Brunner 2009). Lopes et al. (2011) found third-stage larvae of *Hysterothylacium* sp. (Nematoda, Anisakidae) in the pericardial cavity of *Diplodon suavidicus* (Hyriidae) in Brazil and presented photographs of nematodes coiled in the pericardial cavity. In the early 20th century, *Ascaris* sp. or *Ascaris*-like worms were reported in the digestive tract of unspecified unionids in the USA, but there were no accompanying species descriptions, no information about pathology, and no indication that any specimens were deposited in a museum (Clark and Wilson 1912; Wilson and Clark 1912; Coker et al. 1921). McElwain et al. (2016) described histopathological changes associated with the eggs and larvae of *Unionicola* sp. from *Strophitus connasaugaensis* and provided a literature review regarding pathologies associated with *Unionicola* spp. in unionids. Similarly, Abdel-Gaber et al. (2018) described injuries to the tissues of *Coelatura aegyptiaca* (Unionidae), *Mutela rostrata*, and *Chambardia rubens* (Mutelidae) associated with eggs and larvae of *Unionicola tetrafurcatus*. Müller et al. (2015) described histopathological changes to the gonad and hepatopancreas associated with *Rhipidocotyle campanula* and *Phyllodistomum* sp. Few studies have demonstrated tissue damage associated with digeneans in unionids.

Parasite-induced pearl formation, shell deformities, and neoplasms received little or no treatment by Grizzle and Brunner (2009). Mussels may form pearls in response to digeneans (Clark and Wilson 1912; Wilson and Clark 1912; Gentner and Hopkins 1966; Hopkins 1934), mites (Dallas 1858; Baker 1928; Edwards and Vidrine 2013), and midge larvae (Forsyth and McCallum 1978; Pekkarinen 1993a), and pearls may occur in various soft tissues, especially the mantle. Interestingly, some small organisms can become embedded in the nacre or may otherwise cause an increased localized deposition of nacre, and such protuberances are referred to as blister pearls (Jameson 1902). Shell deformities of freshwater mussels include protuberances, infoldings, and misshapen shells. Some anomalies are thought to be the result of an injury to the mantle that disrupts the normal process of shell formation, such as a small animal traveling between the shell and mantle. Some deformities may be the result of damage to the shell that is later repaired (Beedham 1971; Forsyth and

McCallum 1978; Roper and Hickey 1994; Parmalee and Bogan 1998; Strayer 2008). There are also reports of shell erosion as a result of friction or a low pH (Kat 1982; Roper and Hickey 1994; Parmalee and Bogan 1998; Nedeau 2008; Haag 2012). However, some shell deformities are more difficult to explain (Pekkarinen 1993a; Strayer 2008). Pekkarinen (1993a) reported a pustular disease affecting the posterior portion of the periostracum and nacre of *Anodonta anatina*, *Unio pictorum*, and *U. tumidus* in the Vantaa River, Finland. The author speculated that some of the pustules may have formed in response to chironomid larvae, but it is unclear how these invaders might cause protuberances of the periostracum. Pustules commonly occurred among *A. anatina* and were occasionally observed among *U. pictorum* and *U. tumidus*. Strayer (2008) reported a widespread shell deformity affecting *E. complanata*, *Alasmidonta undulata*, *Pyganodon cataracta*, *Lasmigona costata*, and *L. compressa* in streams in New York's Hudson River valley and Southern Tier regions. Affected mussels displayed a truncated posterior shell margin (the exposed portion of a mussel shell when the animal is normally buried), but the causative agent/mechanism behind this aberration remains indeterminate. Possible agents/mechanisms include: (1) the exposed portion of the shell was worn down, (2) the shell formation process was corrupted by a chemical contaminant or a pathogen that damaged the mantle, or (3) the mussels were irritated by a chemical contaminant that caused the mantle to periodically retract. Strayer (2008) estimated the prevalence of the deformity to be $>10\%$ at some sites. Several authors have reported tumors arising from tissues in the mantle cavity, mostly among *Anodonta* spp. Williams (1890) reported an adenomyoma from the mantle of *A. cygnea*. Collinge (1891) reported a tumor arising from the mantle–gill junction in *A. cygnea*. The tumor seemed to impair nacrezation since the affected animal lacked nacre in the posterior portion of the shell. Butros (1948) reported a connective tissue tumor from the labial palp of *A. imblicata*. Pauley (1967a, 1967b) observed adenomas from the foot of *A. californiensis*. Pekkarinen (1993b) described hyperplastic lesions that grossly resembled tumors in the marsupial gill. Overall, the literature indicates that neoplasms may occur in $<1\%$ of mussels in a given population.

Some metazoans may damage somatic tissues or more directly impair fecundity by infecting the gonad or by obstructing the marsupial water tubes, but these appear to be isolated or rare events (Pauley and Becker 1968; Gordon et al. 1978; Huehner and Etges 1981; Grizzle and Brunner 2009; Levine et al. 2009; Müller et al. 2015; McElwain et al. 2016). Parasites typically exhibit an aggregated distribution among hosts; most hosts are infected with a small number of parasites, whereas only a small number of hosts in a given population are colonized by large numbers of parasites (Poulin 2011). Therefore, it seems unlikely that metazoan parasites would be responsible for widespread declines. Furthermore, the literature does not provide a clear indication as to the cause of die-offs and or mussel kills. It seems more likely that a microbial pathogen, rather than a metazoan parasite, would be a causative agent of, or a contributing factor to, a mussel kill or die-off, but there is little evidence of this in the published literature aside from viral diseases affecting *H. cumingii* in China (Grizzle and Brunner 2009). Furthermore, our understanding of mussel health is limited because the primary literature contains few documented examples of microscopy used to characterize the gross and histopathological changes to tissues associated with parasites, commensals, or diseases.

To unravel the potential causes of mussel kills or die-offs, I recommend that gross anatomical and histological characteristics of normal and infected or diseased mussels be compared and photographed during health assessments. To this end, investigators should consult Löw et al. (2016) for a detailed description of the periostracum and nacre of a normal shell and the gross external and internal anatomy of healthy soft tissues. Gross pathology studies visually documented the following: insects (Beedham 1971; Forsyth and McCallum 1978; Levine et al. 2009), mites (Humes and Jamnback 1950; McElwain et al. 2016), tumors (Butros 1948; Pauley 1967b), and die-offs (Pauley 1968; Neves 1987). Images of aberrant shells have been published in Beecher (1883), Baker (1901), Williams (1969), Forsyth and McCallum (1978), Kat (1982), Pekkarinen (1993a), Roper and Hickey (1994), Parmalee and Bogan (1998), Nedeau (2008), Strayer (2008), Haag (2012), and Edwards and Vidrine (2013). Regarding histology, McElwain and Bullard (2014) is a comparative and comprehensive histological atlas for Unionidae. Correspondingly, several studies have included images of histopathological changes to tissues associated with pathogens, parasites, commensals, tumors, and die-offs. These are as follows: viruses (Zhiguo et al. 1986; Jianzhong et al. 1995; Lei et al. 2011), intracellular microorganisms (Chittick et al. 2001), aspidogasters (Pauley and Becker 1968; Bakker and Davids 1973; Fredericksen 1972; Huehner and Etges 1981; Huehner et al. 1989; Rosen et al. 2016), digeneans (Kniskern 1952; Chittick et al. 2001; Müller et al. 2015), insects (Beedham 1971), mites (Mitchell 1955; Baker 1976; McElwain et al. 2016; Abdel-Gaber 2018), fish eggs (Stadnichenko and Stadnichenko 1980), tumors (Butros 1948; Pauley 1967a; Pauley 1967b; Pekkarinen 1993b), and die-offs (Pauley 1968).

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REGULAR ARTICLE

AQUATIC DISEASE RISK ANALYSIS: APPLICATIONS FOR THE CONSERVATION AND MANAGEMENT OF FRESHWATER MOLLUSKS

Tiffany M. Wolf[1]*, Philip Miller[2], Alex Primus[1], and Dominic A. Travis[1]

[1]*Veterinary Population Medicine, University of Minnesota, St. Paul, MN 55108 USA*
[2]*Conservation Planning Specialist Group, International Union for the Conservation of Nature, Apple Valley, MN 55124 USA*

ABSTRACT

Wildlife disease concerns are global and broad in scope and involve a wide diversity of expertise from multiple disciplines. In the realm of freshwater mollusk conservation, there is a paucity of information on pathogens of freshwater mollusks or pathogens of other species they might harbor. Consequently, it is a daunting task to manage and mitigate disease in freshwater mollusks. Disease risk analysis (DRA) is a structured, evidence-based process that aids decision making in the face of uncertainty by characterizing the potential impact of infectious and noninfectious diseases on ecosystems, wildlife, domestic animals, and people. In March 2018, as part of the 11th biennial meeting of the Freshwater Mollusk Conservation Society, a team from the University of Minnesota College of Veterinary Medicine's Risk Analysis Unit and the Conservation Planning Specialist Group (CPSG, previously the Conservation Breeding Specialist Group: http://www.cpsg.org) of the International Union of the Conservation of Nature Species Survival Commission (IUCN SSC) offered a mini-workshop series; its aim was to help conservationists, animal resource managers, and industry professionals integrate science and policy to frame, characterize, and manage health risks using international standards in DRA. Participants worked through the initial stages of DRA to examine the risks of disease introduction into aquatic systems as a result of freshwater mollusk translocation. They formulated and prioritized problems in the larger effort to (1) train the community in DRA, (2) leverage funding for further work, and (3) begin communicating with policy makers in this area. Here we report the results of this working group activity and demonstrate the utility of the DRA process in addressing concerns, real and perceived, regarding the risk of diseases associated with freshwater mollusk conservation activities.

KEY WORDS: aquatic, disease, freshwater mollusks, freshwater mussels, hazard analysis, reintroduction, risk assessment, translocation, restoration

INTRODUCTION

"Risk" is the potential of losing something of value, weighed against the potential to gain something of value (Von Neumann 1947). In the health sciences, it is defined as the probability of an adverse event occurring in a defined population over a specified time interval. At its most basic level, "risk" can be represented through the following basic equation:

$$\text{Risk} = \underline{\text{Likelihood}}\ (\text{of an outcome}) \times \underline{\text{Consequence}}\ (\text{should it occur}).$$

Risk can be characterized or measured in different ways: qualitatively (e.g., characterized as "high," "medium," or "low"), semiquantitatively (e.g., rated on a scale of 1–5), or quantitatively (assigned a probability factor or percentage). When conducting a formal "risk analysis," the outcome should be reported transparently, providing information regarding the level of uncertainty (how sure or unsure one

*Corresponding author: wolfx305@umn.edu

is) surrounding the estimate, as well as full disclosure of the assumptions and data sources used during the process.

Disease risk analysis (DRA), particularly in the context of endangered species management, is an inherently complex process, characterized by incomplete information about the system of interest, diverse (and often competing) objectives held by different stakeholders engaged in or influenced by management activities, and insufficient mechanisms for proper collaboration and communication between the scientific community and the public (Westley and Miller 2003). Recognizing this complexity is a crucial early step in effectively applying DRA tools and processes to wildlife conservation planning. Further, applying an analysis methodology that is thorough, evidence-based, inclusive, and transparent will add significant rigor and value to the process of species conservation planning. This improved process forms at least part of the scientific foundation for generating effective disease management policy to enhance long-term species viability.

Ideally, DRA methods are implemented within a framework of structured decision making to inform species conservation planning (Gregory et al. 2012). Structured decision making (SDM) provides an organized approach to identifying multiple objectives around a given problem of interest, rigorously evaluating consequences of alternative management options, assessing trade-offs among the various alternatives, and communicating decision rationale in a clear and transparent manner. We recognize the value of incorporating SDM elements within a formal DRA process to guide freshwater mollusk conservation management, and we recommend thoughtful consideration and application of appropriate SDM tools as an extension of the DRA elements discussed in this paper.

Risk Analysis: Conducting the Process

An in-depth explanation of comparative risk analysis and assessment is beyond the scope of this discussion; a review of the references and links in Box 1 will provide the user with ample background in terms of the language and methods employed. For this report, we utilize the format supported by World Organisation for Animal Health and International Union of the Conservation of Nature (OIE and IUCN) presented in Jakob-Hoff et al. (2013) (Fig. 1):

(1) Problem formulation
(2) Hazard identification
(3) Risk assessment
(4) Risk management
(5) Implementation and review.

However, the basic process of risk analysis can be divided into conceptually similar steps, regardless of the standard used.

The first phase, *problem formulation*, consists of generally outlining the question, issue, or policies being considered, as well as a stakeholder analysis and communication plan. This includes outlining the general pathways and disease categories of concern. The goal of the *hazard identification* process (second phase), is to establish *specifically* which hazards (diseases) are of priority concern and the particular pathways by which they may be introduced. The result of phases 1–2 is the development of a more specific question (not unlike a scientific hypothesis) that can be modeled moving forward. This subsequent analytical model is built and tested during *risk assessment* (phase three) and results in an estimation of the probability or likelihood that each important hazard (i.e., disease) is introduced into the system as well as the associated implications (consequences). The goal of *risk management* (phase four) is to outline and test scenarios that reduce both the likelihood and implications of the risks defined during the assessment. The final phase of the process, *implementation and review*, involves the development of a clear action plan outlining a process and timeline for the evaluation and review of the established risk management plan. The involvement of all potentially affected parties in the overall stakeholder engagement process (e.g., problem formulation, pathway and hazard prioritization, data collection and evaluation, result discussion and dissemination, management option evaluation, etc.) is the goal of *risk communication*. This is an important, but often overlooked, aspect of the *risk analysis* continuum and should take place throughout the entire process.

Figure 1. Phases of the disease risk analysis process as outlined by Jakob-Hoff et al. (2013).

Freshwater Mollusk Conservation and DRA

While most wildlife conservation efforts have focused on charismatic vertebrates such as mammals, birds, reptiles, and amphibians, relatively little attention has been paid to invertebrates, which represent more than 97% of extant animal

Box 1. Development of the Standards for Disease Risk Analysis in Wildlife

Several standards and processes exist today for assessing risk that may be applied to environmental, domestic animal, or free-ranging wildlife settings. The US Environmental Protection Service (EPA) has guidelines available on its website for both human health and ecological risk assessments (https://www.epa.gov/risk, accessed October 25, 2018). Of relevance, the EPA definition of ecological risk assessment is "the process for evaluating how likely it is that the environment may be impacted as a result of exposure to one or more environmental stressors such as chemicals, land change, disease, invasive species and climate change" (https://www.epa.gov/risk/ecological-risk-assessment, accessed October 25, 2018). Recognizing invasive species as one of the largest threats posed by formal and informal trade, as well as anthropogenic forces such as land use decisions and climate change, Invasive Species Specialist Group, part of the International Union for Conservation of Nature's Species Survival Commission (IUCN SSC), created a dedicated web page highlighting a myriad of risk assessment resources useful in this area (http://www.issg.org/risk_assessment_resources.htm, accessed October 25, 2018).

In the 1990s, the World Organisation for Animal Health (OIE) implemented a standard methodology to be applied globally when assessing infectious disease risks of animals, including those in the aquatic environment (World Organisation for Animal Health [OIE] 2018). Additionally, since 1992, IUCN's Conservation Planning Specialist Group (CPSG, formerly CBSG) has been facilitating collaboration between experts in zoo and wildlife veterinary medicine, disease ecology, and population management to develop a set of methods and tools for realistic and rigorous analysis of disease risks in wildlife and at the wildlife–domestic animal–human interface. In 2010, recognizing that the range of concerns in relation to wildlife disease had broadened well beyond those associated with animal movements, the OIE and IUCN co-sponsored the publication of the "Manual of Procedures for Wildlife Disease Risk Analysis" (Jakob-Hoff et al. 2013) and its companion, the IUCN "Guidelines for Wildlife Disease Risk Analysis" (World Organisation for Animal Health [OIE] and International Union for Conservation of Nature 2014). The intent of these publications is to support the implementation of risk assessment and management processes and tools when making decisions regarding biodiversity conservation and wildlife health at the interface of domestic animal and public health with respect to infectious diseases. In an attempt to support interdisciplinary collaboration, inform decision making, align language, and limit confusion, the IUCN adopted the terminology and framework of the OIE in regard to wildlife risk analysis. Recognizing the broad array of methods and tools available, the format supported by OIE and IUCN presented in Jakob-Hoff et al. (2013), which included input from experts encompassing all of the above, is utilized in this report.

species on the planet, play crucial roles in ecosystems and environmental stability, and seem to be just as vulnerable to external stressors (Brusca and Brusca 1990; Ponder 1999). Nonmarine mollusks (i.e., mollusks that live in terrestrial and/or freshwater environments) seem to be particularly vulnerable to population declines and extinction (Lydeard et al. 2004; Strayer and Dudgeon 2010; Lopes-Lima et al. 2017). Of the 750 recorded extinctions of animal species since the year 1500 AD, approximately 40% of these were mollusks, and the majority of these were nonmarine mollusks (www.iucnredlist.org, accessed October 24, 2018). The susceptibility of freshwater mollusks to population decline is most apparent in North America (mussels of the order Unionida, Superfamily Unionoidea), where more than 70% of the native species are considered endangered, threatened, or of special concern and 37 species are presumed or possibly extinct (Williams et al. 1993; Master et al. 2000; Lydeard et al. 2004). The reasons for these declines include environmental degradation, pollution, water-flow regulation and water extraction, fisheries overexploitation, and nonnative species introductions (Strayer and Dudgeon 2010; Lopes-Lima et al. 2017).

Conservation efforts for aquatic macroinvertebrates such as unionid mussels often start with restoring habitat and imposing harvest restrictions. In many cases, however, such changes by themselves do not restore previous species composition and population densities (Palmer et al. 2010; Jourdan et al. 2018). Supplemental strategies, such as captive propagation, augmentation, and reintroduction—practices often used in vertebrate conservation projects, in which individuals are moved to sites within current or historical distribution of the species—increasingly are being used for aquatic invertebrate groups, including unionid mussels. The differences between such approaches, as well as the considerations that should be addressed when contemplating them, have been discussed elsewhere (McMurray and Roe 2017). Given the limited data currently available on the success of unionid augmentations and reintroductions, it is difficult to evaluate the utility of these practices in the conservation of the group (Lopes-Lima et al. 2017; Jourdan et al. 2018). Although a brief search did not reveal any reports of disease resulting from unionid reintroductions or augmentations, this is a recognized risk associated with wildlife translocations, including those of mollusks (Cunningham 1996; Hoftyzer et al. 2008; Jones and Creeper 2019). If unionid translocations are going to continue to be used as a conservation tool for this group, a more thorough and prescribed analysis of the risks involved with the practice

seems appropriate. Such risk assessments may not only help quantify the risks involved, but they also could help identify strategies that might reduce those risks.

In this spirit, we conducted a pilot DRA stakeholder engagement workshop under the auspices of the 11th biennial Workshop of the Freshwater Mollusk Conservation Society. The workshop consisted of participants in the overall three-day conference on Freshwater Mollusk Health and Disease (Supplement 1), convened and supported by the United States Fish and Wildlife Service's (USFWS) Genoa National Fish Hatchery (Mussel Propagation Team), USFWS Midwest Fishery Resource Office and Fish Health Center, and the United States Geological Survey USGS Upper Midwest Environmental Sciences Center and designed and delivered via a collaboration of CPSG and the University of Minnesota College of Veterinary Medicine Disease Risk Analysis Unit. The audience was split into three training sessions, which included an introduction to and participation in the process. The following is a summary of these training exercises.

METHODS

The DRA training workshop was held in association with the 11th biennial Workshop of the Freshwater Mollusk Conservation Society, in La Crosse, Wisconsin, on March 13–15, 2018. Its goals were to help attendees (1) understand DRA terminology, standards, and methods, (2) introduce and practice key elements of the DRA process, and (3) understand how risk analysis can facilitate science-based management and policy. All attendees participated in one of three iterations of the DRA training workshop, each of which lasted three hours and consisted of an in-depth introduction into the DRA process, a case study that enabled participants to actively engage in the process, and a final discussion on approaches and tools available for risk assessment, implementation, and monitoring. For the case studies, participants were divided into small working groups. Here we summarize output from the case studies, which included three primary activities from the first two phases of the DRA, problem formulation and hazard identification.

Problem Formulation

Groups were asked to consider the question: What is the risk of the spread of infectious disease with mollusk translocation? We use the term translocation as "the intentional movement and release of a living organism where the primary objective is a conservation benefit," as published by IUCN in "Guidelines for Reintroductions and Other Conservation Translocations" (IUCN/SSC 2013). Groups were given approximately 10 minutes to determine if this was the "right" question to consider or if there was a more important or pressing issue on which to focus. We then instructed the groups to refine the question, giving specificity to their species or populations of concern or to the geographic location in which they were working. They also were challenged to further define the goals and scope of their DRA and to consider a more specific pathway and question if they saw the need to dig deeper (realizing that sometimes an organized discussion leads to a decision to do nothing at present).

Hazard Identification and Prioritization

We gave working groups approximately 30 minutes to list all possible hazards (diseases) of concern associated with the problem of interest and identify criteria (e.g., mortality, morbidity, transmissibility) with which to rank those hazards for further analysis. As groups considered the hazards, they had an opportunity to further refine the problem on which they were focusing, including establishing any assumptions or limitations under which they were working and the acceptable level of risk. The latter was defined as the level at which stakeholders would require management action options, given the basic premise that "zero risk" does not occur or occurs only rarely. Zero potential morbidity or likelihood of disease transmission is often not realistic.

In developing criteria for ranking hazards, groups were asked to consider the likelihood of the risk as well as the magnitude or consequences of the hazard. In other words, they were asked to consider the potential for movement of a pathogen or disease along with movement of freshwater mollusks, as well as the recognized impact of the disease on their population(s) of interest. The goal of this stage of the activity was to help groups work through and communicate clearly their criteria for prioritizing hazards for further analysis in the DRA, criteria that could be communicated easily to stakeholders and partners.

System Mapping and Identifying Critical Control Points

In the final stage of the DRA activity, we allotted 40 minutes for working groups to create a conceptual diagram of the system or pathway for freshwater mollusk translocation. They mapped the specific stages of the translocation process, from collection of mollusks at a source site to release of mollusks into a destination site. Along the pathway, groups were asked to consider what activities would increase or decrease the hazard risk and where strategies for mitigation could be implemented to reduce risk. The latter were defined as critical control points, in which specific procedures could be used to reduce the hazard to the predetermined acceptable level of risk. Specific outputs of this activity included a system map that identified critical control points (e.g., Fig. 2) and a description of the specific procedures considered for risk reduction at each point along the pathway.

The duration of the workshop did not allow time for groups to work through additional stages of the DRA (e.g., risk assessment, development of a risk management plan, or design for implementation and monitoring), although an overview on approaches to these steps was discussed. To conclude the

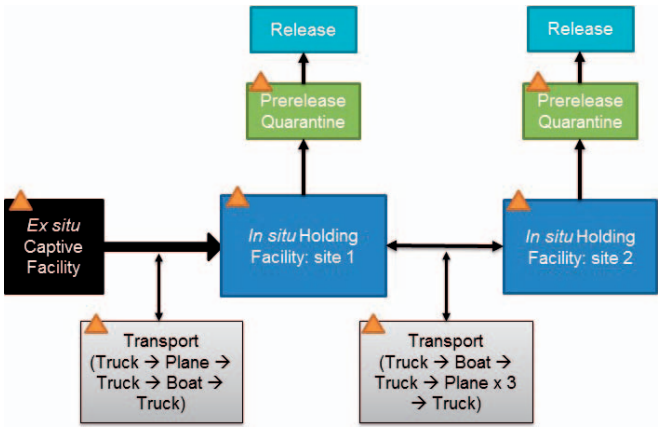

Figure 2. A generalized concept map illustrating the pathway from the captive propagation of a wildlife species to its release into the wild. This map shows how individual animals move from an ex situ captive propagation facility (black) to two separate in situ holding facilities (blue) prior to release. Transport between facilities is detailed (gray), as is an additional stage for quarantining (green) individual animals immediately prior to release (aqua). Critical control points (orange triangles) have been identified as locations along the pathway where disease risk may change and strategies for mitigation can be implemented.

DRA activity, each group reported on their problem formulation, hazard identification and prioritization, and system mapping, which are summarized below.

RESULTS

A total of 14 groups, composed of 5–10 participants each, worked through the initial DRA stages: (1) problem formulation, (2) hazard identification and prioritization, and (3) mapping the system with identification of critical control points for risk mitigation. Over the three separate iterations of the DRA workshop, approximately 110 conference attendees participated, representing national/federal ($n = 36$), state ($n = 20$), county ($n = 3$), or tribal ($n = 1$) governmental, nongovernmental ($n = 3$), university ($n = 26$), for-profit ($n = 15$), and museum and zoo ($n = 3$) organizations.

Problem Formulation

The first activity asked the working groups to refine the question, characterizing the population of interest, assumptions, scope of analysis, and acceptable level of risk. All groups felt the proposed question was important, and many maintained the question as written, while other groups refined it by incorporating greater specificity. Generally, the specificity introduced was a particular species on which the group was focused as the population at risk (e.g., *Quadrula fragosa*, *Epioblasma obliquata*) or the locations associated with mollusk translocation. Generally, particular pathogens were not identified in the question, although one group focused specifically on the risk of introducing viral hemorrhagic septicemia (VHS), a reportable viral pathogen of fish species, to the geographic region into which the mollusks were being moved (Kim and Faisal 2011). Almost all groups identified the augmented community of mollusks as the population at risk, and many included an ex situ captive propagation population as well, particularly if that population contained individuals originating from different locations. Two groups also considered native fish communities or fish species as other populations at risk. Such was the case for the group analyzing the risk of VHS introduction with mollusk translocation and for a second group that considered channel catfish an important host species for glochidial development within the region where mollusks were being introduced. Yet another group also considered humans, in addition to mollusks and fish, as a potential population at risk for disease exposure.

Assumptions outlined by the various groups demonstrated common themes. Most groups noted that source populations had some level of disease and that, subsequently, there is a risk that individual mollusks harboring viable pathogens could be unwittingly collected and translocated. One group refined this assumption further; they noted that a pathogen could remain viable through the translocation pathway such that transmission to the captive or augmented population, assumed to be free of disease currently, could occur, resulting in an observable impact of disease on the captive or augmented populations. Where a specific pathogen or pathogens were identified as the focus of the DRA, the group made a clear assumption that these were the most important pathogens to consider for analysis. Groups that considered the incorporation of quarantine, or the existence of a holding facility, as a means of disease detection and mitigation assumed that activities conducted within quarantine or holding (e.g., disease screening and surveillance) would be effective in detecting pathogens of interest, allowing for actions that could reduce disease introduction. One group that considered more than infectious pathogens as disease risks explicitly stated their assumption that copper (a hazard of concern) would be detectable in the sediment at the release site and that a population of host fish for the introduced mussels also would be present at the release site. The group that focused on VHS assumed that the pathogen was present at the source site and absent at the release site, based on the reported geographic distribution of the disease in native fish. Almost all groups noted that a major limitation to the DRA was the general lack of information on the spectrum of pathogens that mollusks might harbor, which challenged the depth at which groups could take the DRA.

Only three groups reported the intended scope of the DRA. One group discussed the scope of the problem, where unknown die-off events of host fish were occurring in the propagation facility. They assumed that mussels collected as broodstock were harboring fish pathogens. Consequently, they also were considering any shared, potentially contaminated equipment and fish or water released from the facility as a component of the DRA. A second group considered the DRA a cost-benefit analysis, intending to determine if successful translocation and reestablishment of a thriving mollusk

population was worth the risk of introducing disease, based on the recognized consequence to the existing aquatic populations within the destination location. The third group identified the scope of their DRA based on the taxonomic groups of interest and locations of the translocation activities.

Often, the acceptable level of risk and the perceived risk of introducing disease with mollusk translocation were reported as the same thing and considered to be high. For example, even in these early stages of the DRA process, which required groups to reflect on the process of translocation, most recognized a general lack of existing standards for biosecurity in preventing disease transmission in the community. This deficiency seemed to be partly due to a large amount of uncertainty surrounding important aquatic or mussel pathogens, the role of mollusks in their transmission, and the role of disease in the decline of aquatic, and more specifically mollusk, populations. Thus, the general consensus among groups was that, despite these unknowns, a higher level of risk would be tolerated where survival and reestablishment of mollusk populations is highly dependent on translocation activities.

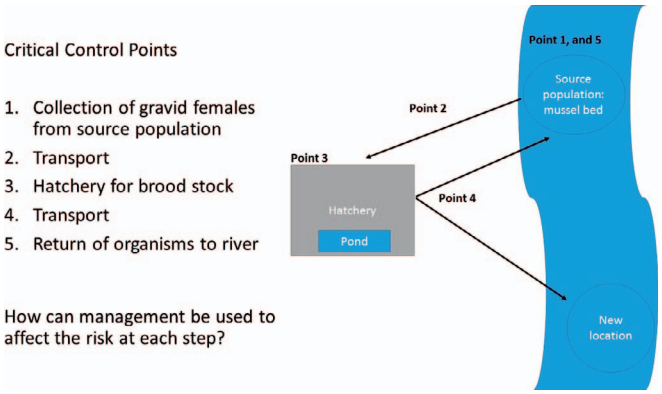

Figure 3. Representative conceptual map of the steps involved in the translocation and augmentation of freshwater mollusk populations. The diagram is one of several systems maps produced by the working groups during the DRA workshop and represents the general steps of the translocation process that were considered by most groups. Each critical control point identified by the working group is a place along the process where disease risk may increase or decrease as a result of activities at that location along the pathway.

Hazard Identification and Prioritization

Working groups identified a number of infectious and noninfectious hazards to consider with freshwater mollusk translocation. Infectious hazards, the primary focus of most groups, often were classified at a broad level—viruses, parasites, bacteria, and fungi, for example—although in a few cases, specific pathogens or pathogen groups were the focus (e.g., *Tetrahymena glochidiophila*, digenean trematodes, ciliated protozoans). Several groups also identified as hazards fish or other aquatic pathogens—in particular, VHS and *Aeromonas salmonicida*— that mollusks might harbor. Noninfectious hazards included the unintended transport of native and nonnative, nontarget species; copper contamination; genetically linked diseases; and/or habitat differences that might influence health and survival.

Criteria for prioritizing hazards are generally based on (1) the likelihood of risk, which in the case of an infectious hazard would be the likelihood that infectious mollusks were translocated, thus potentially introducing pathogens to a new site or augmented site, and (2) the consequence or magnitude of pathogen introduction. Commonly listed criteria for prioritization included hazard presence in source and augmented populations, presence of susceptible species in captive facilities or at release sites, capacity to detect and/or control hazards along the pathway, the transmission route, virulence, and infectious and latent periods of pathogens, the interaction of translocation activities and infection outcomes (e.g., stress on susceptibility or infectiousness), and potential for pathogen dispersal in contaminated water (from source or captive facilities). As groups weighed the consequence of disease introduction, they considered primarily pathogen characteristics such as species specificity and morbidity and mortality impacts on mollusk and other aquatic species populations in both captive facilities and in situ populations. Similar considerations were given to the risk of translocating nontarget native and nonnative species, particularly the presence of species in either source or destination sites and the ability to detect and mitigate nontarget species transfers within the translocation pathway.

System Mapping and Identifying Critical Control Points

The translocation pathway mapped by most groups included collection of freshwater mollusks from a source population, transport to one or more holding or propagation facilities, followed by transport to either an augmented population or a new location (e.g., Fig. 3). In most cases, the destination location was different from the source location, although one group that considered a hatchery facility where aquatic species or mollusks from other locations were maintained as a potential source for disease introduction augmented the population from which the captive broodstock originated. Generally, locations for disease to originate along the pathway were considered to be the collection site and rearing facility, particularly in cases where the water source for the rearing facility differs from that of the collection site.

Most groups identified holding or propagation facilities as an optimal place to integrate quarantine for disease screening and/or mitigation (although these facilities were not necessarily functioning in this capacity). Some groups maintained that quarantine with disease screening should occur when new mollusks enter the facility, to reduce the risk of disease entering the captive facility, and again before transporting mollusks to their destination location, to reduce risk to the augmented population. Another option for disease screening is

prior to collection, in which a sample of the population might be screened for the hazards of concern. Groups also identified quarantine of host fish used in rearing facilities as an important measure for reducing the risk of fish pathogens entering the propagation facility and impacting the broodstock or being transported with mollusks to new locations upon release. Good biosecurity, which includes the decontamination of water leaving the captive facility, was identified as an important factor in reducing disease risk within these sites.

Groups also identified transportation events as critical control points, where additional measures can be implemented to mitigate disease: equipment cleaning and disinfection, water changes and decontamination, examination and/or testing of mollusks for evidence of disease, minimization of transport stress through water quality and temperature regulation, and removal of nontarget species. These measures were deemed particularly important for disease mitigation in cases where a captive facility, which might provide a quarantine mechanism, is not part of the translocation pathway. Groups also recommended that, where seasonal pathogens are of concern, transport activities might be limited to times of the year when pathogen risk is lowest. Groups agreed that the key to identifying changes in disease risks and responding to those changes with measures that mitigate risks and protect all aquatic populations is to carefully monitor source, captive, and augmented/reintroduced populations for disease.

DISCUSSION

The March 2018 DRA workshop, although limited in time and scope, successfully introduced mollusk conservation biologists and population managers to an internationally accepted, structured approach to considering disease risks in conservation planning. While weighing risks of adverse outcomes in population management was not a novel concept, considering the risk of disease in this context generally was. Overall, feedback on the workshop was positive, and many participants thought that a structured approach to problem formulation and hazard ranking was useful. This process highlighted a number of commonalities:

- There are differing approaches to outlining very similar problems, depending upon the "lens" of the person addressing the problem. A multidisciplinary, workshop approach to problem definition can resolve differences in language and methodology to create a collaborative problem definition and/or picture that can be used by the community to move forward.
- As is common when addressing almost all problems associated with wildlife diseases, the paucity of data available on mollusks, or on their role as disease vectors, limits the amount of "data-driven" decisions that can be made, increasing reliance upon qualitative data and expert opinion. However, despite the large amount of uncertainty in regard to the unknowns, most participants recognized the need to consider disease and its impacts in association with conservation activities.
- The systems mapping revealed multiple conceptually similar opportunities for disease mitigation (e.g., quarantine of new arrivals or prerelease, biosecurity of facility and equipment, decontamination of discharged water, etc.) that can be implemented even without a priori knowledge of a specific pathogen of concern.

At the very least, this process is a useful tool for addressing an identified problem with an eye toward developing evidence-based, scientifically rigorous solutions. A representative group of experts in this community now possess first-pass answers to the following:

- Are there cases or scenarios where the risk of infectious disease is perceived to be important enough to be assessed formally?
  - If so, what are those scenarios?
- Who are the stakeholders and does the stakeholder community collectively want to engage in this process?
- What are the potential pathways and consequences of concern?
- What are the criteria for prioritizing pathways and potential pathogens?
- Moving forward, is there a list of priority problems for this community to address using the process outlined above?

Most importantly, the question initially posed to all participants, "What is the risk of the spread of infectious disease with mollusk translocation?," was considered important for the mollusk conservation community to continue to discuss. We encourage the community to continue to identify and define specific situations where science can contribute to examining and mitigating disease risks for wildlife management.

ACKNOWLEDGMENTS

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REGULAR ARTICLE

EXPOSURE TO ELEVATED CONCENTRATIONS OF MAJOR IONS DECREASES CONDITION INDEX OF FRESHWATER MUSSELS: COMPARISON OF METRICS

Serena Ciparis[1,2]*, Garrett Rhyne[2], and Ty Stephenson[3]

[1] *U.S. Fish and Wildlife Service, Virginia Field Office, Gloucester, VA 23061 USA*
[2] *Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, VA 24061 USA*
[3] *Blacksburg High School, Blacksburg, VA 24061 USA*

ABSTRACT

Energy storage is critical for gametogenesis and successful spawning in bivalve mollusks. However, it often is overlooked as an endpoint in toxicological studies of freshwater mussels. Energy storage can be assessed through direct measurement of energy substrates or the use of the condition index (CI) as an indicator of overall nutritional status. Our study focused on the CI of adult *Lampsilis fasciola* exposed to treatment conditions designed to mimic the Powell River (Virginia, USA), which historically supported an exceptionally diverse freshwater mussel community. Coal mining operations have impacted the upper Powell River, and low-flow specific conductance frequently exceeds 900 µS/cm. We used four treatments in a full-factorial design to evaluate mussel responses to diluted pond water (control), simulated Powell River water, control sediment, and Powell River sediment. We measured glycogen content of mantle tissue and CI and compared several CI metrics. Exposure to simulated Powell River water caused a significant decrease in several CI metrics compared to control water. There was no effect of sediment type, nor was there any effect of sex; both males and females lost body mass in simulated Powell River water. However, males had significantly lower glycogen content of mantle tissue, indicating females likely were using other sources of energy to compensate for salinity stress. Comparison of CI metrics demonstrated that dissection was necessary to discern the effect of major ions on energy storage and that the use of tissue weight (g)/shell cavity capacity (g) had lower variability than tissue weight (g)/shell cavity volume (mL). The observed decrease in CI of adult *L. fasciola* after exposure to elevated concentrations of major ions has implications for maintaining mussel populations in the Powell River and in other rivers with rapidly increasing salinity.

KEY WORDS: conductivity, mining, bivalve, Unionidae, condition, glycogen

INTRODUCTION

Central Appalachian streams and rivers that drain watersheds with intensive coal mining have undergone dramatic changes in water and sediment quality. Frequently, they have elevated concentrations of major ions (SO_4^{2-}, HCO_3^-, Mg^{2+}, Ca^{2+}, Na^+, Cl^-, and K^+) and trace elements, as well as substrate alterations, such as increased proportions of fine sediment and sand (Pond et al. 2008; Bernhardt et al. 2012; Griffith et al. 2012). The upper Powell River watershed (Virginia, USA) has experienced extensive disturbance from coal mining. Surface mining has occurred in more than one-third of the watershed (Zipper et al. 2016), and deep mines are present under almost all of the surface-mined areas (DMME 2015). Since the 1960s, a steady increase in concentrations of major ions, measured as total dissolved solids (TDSs), has occurred in the Powell River (Zipper et al. 2016). As of the mid-2000s, measured specific conductance in the upper Powell River frequently exceeds 900 µS/cm (VDEQ 2015). This value exceeds derived extirpation concentrations (95th centile) for the majority of mayfly genera in central Appalachian streams, and it is well above the proposed 300 µS/cm benchmark for protection of aquatic life in this region (USEPA 2011). Sediment quality in the Powell River also has been affected by

*Corresponding Author: serena_ciparis@fws.gov

coal mining. The presence of excess fine sediment (measured as embeddedness) likely impairs benthic aquatic life in the upper Powell River (MapTech 2011). In addition, the sediment contains elevated concentrations of nickel and naphthalene (MapTech 2011), contaminants often associated with coal particles (Stout and Emsbo-Mattingly 2008; Van Aken et al. 2015), which are visible in the sediment and which make up 1–5% of it by weight (Wolcott 1990).

The Powell River is part of the Upper Tennessee River watershed, an area of exceptionally high freshwater mussel diversity. Long-term monitoring of the river has documented declining mussel species richness and densities, with the decline greatest in its upstream reaches (Wolcott and Neves 1994; Johnson et al. 2012; Ahlstedt et al. 2016). There is a spatial association between areas with the highest in-stream TDS concentrations and the greatest declines in freshwater mussel assemblages (Zipper et al. 2016). However, simulated Powell River water (944 mg/L TDS, 1190 μS/cm) did not cause a significant decrease in survival or growth of juvenile freshwater mussels compared to diluted pond water (Ciparis et al. 2015). These results suggest either that major ions are not the primary cause of the observed mussel declines in this river or that they cause physiological changes in mussels that were not captured by using only survival and growth in juvenile mussels as toxicological endpoints.

Energy storage is directly linked to gametogenesis in bivalves (e.g., Bayne et al. 1982; Fearman et al. 2009). Thus, maintaining populations depends on the balance between energy availability and the energetic cost of metabolism in individuals. When the difference between availability and cost is positive, excess energy can be used for growth, storage, and, ultimately, reproduction, but when the difference is negative, energy reserves are depleted (Bayne and Newell 1983; Widdows and Johnson 1988; Van Haren and Kooijman 1993). Changes in environmental salinity can affect energy storage in bivalves. Depletion of stored energy has been documented in marine bivalves exposed to low-salinity water (Kumar et al. 2015; Bertrand et al. 2017) and in the freshwater clam *Corbicula fluminea* exposed to high-salinity water (Bertrand et al. 2017). However, to our knowledge, changes in energy storage in freshwater mussels as a result of exposure to elevated salinity, particularly the mixture of major ions found in mining-impacted streams, have not been evaluated.

Evaluating energy storage in bivalves as a response to seasonal or environmental changes requires distinguishing between energy reserves in soft tissues and structural biomass (Van Haren and Kooijman 1993). Energy storage in soft tissues can be measured directly, as glycogen, lipid, or protein content (e.g., Bayne 1982; Kumar et al. 2015; Bertrand et al. 2017). Energy storage also can be measured indirectly using the entire animal. Energy reserves or "fatness" of oysters was first defined as a condition index (CI) in the early 1900s, and it was measured as the proportion of internal shell volume occupied by soft body tissue (Crosby and Gale 1990). Crosby and Gale (1990) reviewed the methodology for calculating CI in bivalves and found a lack of uniformity in applied measurements and formulas, preventing comparisons across studies. After testing three commonly used formulas, they recommended a standardized method, calculated as CI = dry soft tissue weight (g) × 1000/internal shell cavity capacity (g). However, review of recent literature for freshwater bivalves still shows wide variation in the methodology used to calculate CI, with soft tissue weight (wet or dry) divided by either shell length (Blaise et al. 2017), shell length ^3 (Spooner and Vaughn 2009), shell weight (Payton et al. 2016; Bertucci et al. 2017; Zhao et al. 2017), shell cavity volume (Nobles and Zhang 2015; Otter et al. 2015), total dry weight (Ganser et al. 2015), or total wet weight (Michel et al. 2013), with or without the use of scaling factors (×10, ×100, etc.).

The primary objective of our study was to evaluate the effect of exposure to elevated concentrations of major ions found in the Powell River on energy storage, assessed as CI and glycogen content, in adult *Lampsilis fasciola*, a native freshwater mussel. A secondary objective was to assess potential effects of coal-contaminated sediment from the Powell River on metrics of energy storage in *L. fasciola*. Our final objective was to evaluate several methods of measuring CI for differences in sensitivity to treatment effects and precision. We included measurements on live mussels in this comparison for potential application to imperiled species of freshwater mussels, for which nondestructive measurement techniques are preferred.

METHODS

We obtained *Lampsilis fasciola,* approximately 2 yr old, from the Aquatic Wildlife Conservation Center (AWCC; Virginia Department of Game and Inland Fisheries, Marion, Virginia, USA) in July 2013, which produced them from one gravid female collected from the Clinch River, Virginia, USA. Hatchery-reared host fish (*Micropterus salmoides*) were infested in May 2011, and excysted juvenile mussels were collected in late May through June 2011. Juveniles were held in downwelling bucket systems followed by outside troughs. The South Fork Holston River (Marion, Virginia, USA) was the water source for both systems. Mussels were transferred to the Freshwater Mollusk Conservation Center (FMCC; Virginia Tech, Blacksburg, Virginia) in coolers with aerated river water. Mussels were held at the FMCC in flow-through systems using water and sediment from an on-site pond until October 2016; they were approximately 5 yr old at the time of this study (mean length = 40.68 ± 2.37 mm, mean weight = 11.59 ± 1.48 g). They were sexually mature (>3 yr old; Zale and Neves 1982) but smaller than wild 5-yr-old *L. fasciola* collected from the Clinch River (mean length ∼ 50 mm; Jones and Neves 2011).

We designed a full-factorial study to evaluate the effects of elevated concentrations of major ions and coal-contaminated sediment on freshwater mussels. The four treatments included (1) control water and control sediment (CWCS), (2) control water and Powell River sediment (CWPS), (3) simulated Powell River water and control sediment (PWCS), and (4) simulated Powell River water and Powell River sediment (PWPS).

Treatment Preparation

Pond water from the FMCC pond was filtered through a 5-μm polypropylene microfiber filter (Vortex Filter, Filter Specialists, Inc., Michigan City, IN, USA). A 50:50 mixture of filtered pond water:deionized water was used as the control water and as a base water to prepare the simulated Powell River water. A previous study demonstrated excellent mussel survival in the 50:50 mixture, compared to poor survival in 100% pond water when used in closed exposure systems (Ciparis et al. 2015). The target TDS concentration for simulated Powell River water was 950 mg/L, similar to the target concentration for simulated Powell River water (942 mg/L) derived in Ciparis et al. (2015). This represents recent TDS concentrations measured during low-flow conditions at a Virginia Department of Environmental Quality (VDEQ) long-term monitoring station on the Powell River, at Big Stone Gap, Virginia (6BPOW179.20; for location information see Fig. 1 in Ciparis et al. 2015). Nominal ion concentrations (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}, and HCO_3^-) for the Powell water treatments (Table 1) were based on recipes developed by Ciparis et al. (2015), adjusted for the diluted base water used in the current study. Control and simulated Powell River water were prepared weekly. We prepared treatments from base waters using certified American Chemical Society (ACS) reagent-grade salts. Potassium chloride (KCl), potassium bicarbonate ($KHCO_3$), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), calcium carbonate ($CaCO_3$), magnesium sulfate heptahydrate ($MgSO_4*7H_20$), calcium chloride dihydrate ($CaCl_2*2H_2O$), and sodium sulfate (Na_2SO_4) were purchased from Fisher Chemical (Fair Lawn, NJ, USA). We purchased calcium sulfate ($CaSO_4$) from Sigma-Aldrich (St. Louis, MO, USA). For simulated Powell River water, salts were mixed into 17 L of base water in 18-L buckets and held in a water bath (see below) for 24 h prior to water exchanges. Control water also was held in 18-L buckets in a water bath for 24 h.

Two types of sediment were used in the exposure. We obtained control sediment from the FMCC mussel culture system used for older juvenile mussels (grow-out phase); we collected Powell River sediment from the Powell River at Big Stone Gap, Virginia, USA (36.8635 N, −82.7855 W) using a stainless-steel shovel and plastic bucket. Sediment from this area of the Powell River has visible coal particles and previously documented elevated concentrations of nickel (mean = 26 mg/kg, max = 49 mg/kg, $n = 9$) and naphthalene (1.2 mg/kg, $n = 1$) (MapTech 2011). Control sediment and Powell River sediment were held for two weeks at 4°C to minimize activity of indigenous animals and microbes. After the holding period, we poured the Powell River sediment into a long, clear plastic bin and used forceps to remove large debris and *Corbicula fluminea* shells. We mixed the sediment thoroughly with a stainless-steel spoon and distributed it into 16 4-L glass jars, at a target volume of 750 mL, equivalent to a height of 5 cm (Hazelton et al. 2014). Control sediment also was distributed into 16 4-L jars at the same target volume. We placed water from the FMCC pond in each jar (approximately 3 L) and held the jars in the exposure system (see below) for 1 wk with constant aeration. Ammonia concentrations were measured on day −1 and determined to be negligible. The pond water was exchanged for treatment water in each jar on day 0.

Prior to distribution in the jars, six subsamples were collected from each sediment type for estimation of organic content based on loss-on-ignition (500°C for 12 h) from dried (60°C for 3 days) samples. Organic content was 2.9 ± 0.3% in Powell River sediment and 1.3 ± 0.1% in control sediment. To document presence of coal particles in the sediment, we determined a crude estimate of contribution to sediment dry weight. Coal particles were visually identified in the inorganic fraction of the Powell River sediment subsamples (postignition), separated using forceps, and weighed; we then determined the proportion of total sediment dry weight as coal. This method estimated 1.5 ± 0.6% coal in the sediment (dry wt. basis), which is similar to previously documented amounts (Wolcott 1990).

Mussel Exposure System

Experimental units consisted of 4-L glass jars with 3 L of water and 750 mL of sediment. Each jar had an airline affixed with a glass pipet to maintain oxygen near saturation. We placed each jar in an 18-L bucket containing approximately 3 L of water; we placed four buckets into each of four 757-L containers filled with water to serve as a temperature control bath (water bath). Temperature (target = 22°C) was maintained in the water baths using aquarium heaters. Each water bath contained one replicate (jar) of each treatment randomly arranged ($n = 4$ for all treatments). We used a blocked design to account for any temperature differences between water baths.

On day 0 (October 24, 2016), we randomly selected a total of 32 mussels from the original cohort. At the time of selection, each mussel had a unique Hallprint shellfish tag (Hallprint Inc., Hindmarsh Valley, South Australia) affixed to the shell. Shell shape was used for initial determination of the sex of each mussel. Females were gravid at the time of the study, and sex was confirmed using the presence (F) or absence (M) of glochidia in marsupial gills observed after gently opening the shell. We randomly assigned two mussels, one male and one female, to each jar. Prior to placement in the jars, each mussel was weighed to the nearest 0.0001 g using a digital scale and measured to the nearest 0.1 mm using dial calipers; we measured the volume of the mussel to the nearest 1 mL using pond water displacement in a graduated cylinder.

During the exposure, mussels were fed daily by adding 0.9 mL of a 1:1 algal cell ratio from two premixed commercial micro-algae diets (Nanno 3600 and Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) to each jar at a concentration of 20,000 cells/mL. This feeding regime was derived from a base mixture previously used for juvenile mussels (Carey et al. 2013; Ciparis et al. 2015), adjusted to a feeding rate

Table 1. Ions as nominal concentrations in control water (CW) and simulated Powell River water (PW) and mean measured concentrations ($n = 6$ weekly measurements, standard deviation in parentheses) in four treatments, consisting of either CW or PW and control sediment (CS) or Powell River sediment (PS). Total dissolved solids (TDS) is the sum of all ion concentrations.

	CW	CWCS	CWPS	PW	PWCS	PWPS
	Nominal	Mean	Mean	Nominal	Mean	Mean
Ca^{2+}	15.6	22.1 (0.60)	23.4 (0.92)	86.0	30.2 (6.6)	34.5 (7.2)
K^{+}	1.15	1.63 (0.09)	1.79 (0.12)	6.00	7.28 (0.85)	7.36 (0.70)
Mg^{+}	15.7	16.2 (0.99)	16.0 (1.1)	49.0	53.0 (5.2)	52.3 (4.7)
Na^{+}	2.70	5.88 (0.83)	6.06 (0.55)	114	179 (12)	179 (12)
SO_4^{2-}	7.75	18.1 (2.2)	18.6 (1.8)	452	472 (32)	477 (30)
HCO_3^{-}	110	114 (5.6)	133 (5.6)	229	163 (6.0)	179 (15)
TDS[a]	**158**	**183**	**204**	**955**	**924**	**948**

[a]Includes the nominal concentration of Cl^{-} of 5.1 for all CW treatments and 19.1 for all PW treatments; Cl^{-} could not be measured due to a faulty probe.

(~10,000 cells/mL/mussel) used for similarly sized *L. fasciola* (Hazelton et al. 2014). For each jar, a 100% water exchange occurred weekly. Water was gently siphoned in and out of each jar in order to minimize disturbance of the sediment. We measured temperature (°C), specific conductance (μS/cm), dissolved oxygen (% saturation), and pH just prior to, and 24 h after, water exchanges using a YSI 556 Multi-Probe Sensor (YSI Inc., Yellow Springs, OH, USA). We sampled concentrations of NH_3-N, alkalinity, and elements (Na, K, Mg, Ca, and S) just prior to weekly water exchanges. A sample (25 mL) from each jar was filtered (0.45-μm pore size) and aliquots were combined into a pooled sample for each treatment. Ammonia (NH_3-N) was measured weekly using a HACH DR/2400 meter (Hach, Inc., Loveland, CO, USA) following the manufacturer's methods. Total alkalinity (mg/L $CaCO_3$) was measured weekly using a standard titration method and was converted to HCO_3^{-} using the equation mg/L HCO_3^{-} = mg/L $CaCO_3 \times 1.22$. Element concentrations were measured weekly. The Virginia Tech Soil Testing Laboratory measured elements in solution using Inductively Coupled Plasma Atomic Emission Spectrometry (Spectro ARCOS ICP, Spectro Analytical Instrumentation, Kleve, Germany) following standard methods (USEPA Method 200.7 [USEPA 1994] and APHA Method 3120 [APHA 2012]) and standard operating and quality assurance/quality control (QA/QC) as detailed in Ciparis et al. (2015). Sulfate concentration was calculated from measured total S; all S was assumed to be present as SO_4^{2-}.

On day 40 (December 3, 2016), mussels were removed from each treatment; they were weighed and measured, and their volume recorded, as described above. All mussels were dissected. Wet tissue weight was measured to the nearest 0.0001 g using a digital scale. The viscera and a section of mantle tissue (target 0.25 g, mean 0.28 g ± 0.08 g SD) were removed from each mussel, weighed, placed individually in 1.5-mL microcentrifuge tubes, and immediately frozen. The remaining tissue was reweighed, and the tissues and mussel shells were dried at 60°C. We measured and recorded dry tissue weight and shell weight. The proportion of water in the tissue and total wet weight were used to determine the total dry weight, prior to removal of digestive gland and mantle tissue. Finally, we determined the cavity volume by filling one of the valves of each mussel full of water, measuring the amount, and doubling it. We maintained consistent meniscus shape and height. We did not use the method of water displacement to determine shell cavity volume (Crosby and Gale 1990) due to the low density of the shells, which prevented accurate measurement of displacement.

Glycogen Determination

Mantle tissue was homogenized in 300 μL sodium citrate buffer (0.1 M, pH 5), placed in a boiling water bath at 100 SC for 5 min, and centrifuged at 10,000 g for 5 min. We added duplicate 100 μL aliquots of the supernatant to a microplate and 5 μL of 1% amyloglucosidase to one replicate to hydrolyze glycogen to glucose (Carr and Neff 1984). The plate was incubated at 25°C for 12 h. We quantified glucose in treated and untreated supernatants using a glucose oxidase assay (Sigma Glucose [G-O] Assay Kit, Sigma-Aldrich). Glucose concentration was determined spectrophotometrically at 540 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and normalized to a glucose standard curve. Glycogen content was determined as the amount of glucose produced by treatment with amyloglucosidase. Glycogen content in each sample was normalized to wet weight of extracted mantle tissue. Analysis of a glycogen standard (*Mytilus edulis* [blue mussel] tissue; Sigma-Aldrich) demonstrated a mean recovery of 91% (±3% relative standard deviation [RSD]).

Data Analysis

We calculated several metrics of mussel body condition. On live mussels, we calculated two metrics on day 0 (initial) and day 40 (end): (1) Fulton's K = (mussel W/L^3) × 10, where W = weight of the entire mussel in g, L = length in cm, and 10 is a scaling factor (Heinke 1908; Nash et al. 2006) and (2) MW:MV = mussel W/mussel V, where W = weight of the entire mussel in g, and mussel V = volume in mL of water

displaced by the live mussel. On dissected mussels, we calculated two CI metrics using both wet and dry tissue weights: (1) TW:SV = tissue W/shell V, where tissue W = weight of tissue in g and shell V = shell cavity volume in mL, measured as the amount of water held by one valve of the shell × 2 and (2) TW:SC = tissue W/shell cavity capacity, where tissue W is tissue weight in g and shell cavity C is the weight of the shell cavity in g, determined by subtracting the weight of the dry shell from the weight of the whole mussel. A final metric, described as a body component index (Crosby and Gale 1990), was calculated as TW_{dry}:SW, where SW is the weight of the dry shell.

We conducted statistical tests using SAS software (SAS 9.4, SAS Institute, Inc., Cary, NC, USA) with a significance level of $\alpha = 0.05$. We compared each metric of mussel condition and glycogen content of mantle tissue between treatments using a mixed model with a normal distribution (Proc GLIMMIX). Similarly, mussel lengths (initial and end) and growth (as change in shell length during the exposure) were compared between treatments. All measurements were normally distributed (Shapiro-Wilk W test, $P > 0.05$), with the exception of glycogen content of mantle tissue, which was $\log_{10}$ transformed prior to analysis in order to fit the normal distribution. The initial model contained three predictors—water type (CW or PW), sediment type (CS or PS), and sex—as well as all possible interactions (water×sediment, water-sex, sediment×sex, and water×sediment×sex). Water bath (block) was included as a random variable, and jar was the subject of measurement. Final models contained only significant predictors. We evaluated any significant interactions as pairwise comparisons of all relevant treatment combinations, using a Tukey post-hoc test with P values adjusted for multiple comparisons. We determined the relative standard deviation (RSD) as RSD = (standard deviation/mean) × 100 for each metric of condition calculated for dissected mussels, because one objective of the study was to evaluate methods of determining mussel condition.

RESULTS

Total dissolved solids concentrations, as the sum of measured ions, were similar to nominal concentrations (Table 1). Simulated Powell River water TDS concentrations were within 3% of nominal concentrations. Control water TDS concentrations exceeded nominal concentrations by 16–28%, due to lack of rainfall influencing pond water composition at the time of the study. Differences in concentrations of individual ions in the simulated Powell River water compared to nominal concentrations were due to either elevated concentrations in the base water (e.g., SO_4^{2-}), incomplete solubility of Ca-containing salts, or the accidental replacement of $NaHCO_3$ (recipe) with Na_2CO_3 (used). Despite these minor differences between nominal and measured ion concentrations, a five-fold difference in both TDS concentrations and specific conductance between control water (~250 µS/cm) and simulated Powell River water (~1,250 µS/cm) was achieved

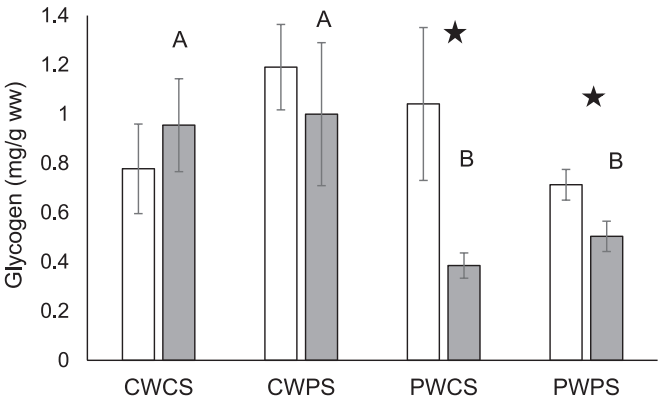

Figure 1. Mean glycogen content in mantle tissue of female (open) and male (gray) mussels in each treatment ($n = 4$) at the end of the exposure (day 40). Error bars are standard errors of the mean. Each treatment consisted of one water type and one sediment type, where CW = control water, PW = simulated Powell River water, CS = control sediment, and PS = Powell River sediment. Letters indicate a statistically significant difference between water type for males only ($P = 0.020$) and a ★ indicates a statistically significant difference between males and females in the PW treatments ($P = 0.038$).

(Tables 1 and 2). There was little variation in measured ion concentrations and specific conductance throughout the study (Tables 1 and 2).

Water quality measurements were consistent over time and were within acceptable ranges for toxicity tests with freshwater mussels. Dissolved oxygen was maintained at >95% saturation throughout the study. Mean measured temperatures were similar between treatments; all were within 0.1°C of the 22°C target temperature (Table 2). Measured pH was stable within each treatment over the course of the study (Table 2). Ammonia-N concentrations during the 1-wk acclimation period for sediment within the jars were low, ranging from 0.01 to 0.09 mg/L on day −1. During the exposure, NH_3-N was below detection in all treatments from day 7 to day 40 (Table 2).

Survival in all treatments was 100% for the entire study. Mussel length (initial and end) and growth, as change in shell length over the course of the study, were similar between treatments, with no relationship to water type, sediment type, or significant interaction. Mean mussel growth was ≤ 0.2 mm in all treatments; negligible growth was expected given the ages of the mussels and the relatively short duration of the study.

For live mussels, initial weight:length ($K_{initial}$) and weight:volume ($MW:MV_{initial}$) metrics were similar between treatments, with no relationship to water type, sediment type, or significant interaction (Table 3). There was a significant effect of sex on $K_{initial}$ (GLIMMIX, $P < 0.0001$), with no significant interactions. Females had a higher $K_{initial}$ (1.86 ± 0.05 SE) compared to males (1.58 ± 0.01 SE). This effect was maintained at the end of the exposure; K_{end} was significantly higher (GLIMMIX, $P = 0.0006$) in females (1.81 ± 0.04 SE) compared to males (1.61 ± 0.03 SE), with no significant interactions between sex and other variables. There was no

Table 2. Mean concentrations of water quality parameters, with standard deviation in parentheses, for each treatment ($n = 4$ replicates) measured just prior to each water change. Treatments: control water + control sediment (CWCS), control water + Powell sediment (CWPS), simulated Powell water + control sediment (PWCS), and simulated Powell water + Powell sediment (PWPS).

Parameter	CWCS	CWPS	PWCS	PWPS
Temperature (°C)	22.1 (0.79)	22.0 (0.79)	22.1 (0.78)	22.0 (0.82)
Specific conductivity (μS/cm)	250 (7.6)	256 (10)	1,263 (50)	1,274 (42)
pH	7.01 (0.14)	7.00 (0.12)	7.15 (0.14)	7.16 (0.12)
NH_3-N (mg/L; days 7–40)	<0.01	<0.01	<0.01	<0.01

effect of sex (or interactions) on either $MW:MV_{initial}$ or $MW:MV_{end}$. At the end of the exposure, there was no effect of water type, sediment type, or an interaction on either K_{end} or $MW:MV_{end}$ (Table 3).

For dissected mussels, there was a statistically significant effect of water type on all metrics of body condition, regardless of whether they were determined using wet or dry tissue weights (Table 3). Mussels exposed to simulated Powell River water had significantly lower TW:SV and TW:SC compared to mussels exposed to control water (GLIMMIX, $P < 0.030$), with no effect of sediment type or significant interaction (Table 3). There was no effect of sex nor significant interactions with sex. The body component index, TW_{dry}:SW, was also significantly lower for mussels exposed to simulated Powell River water compared to control water (GLIMMIX, $P = 0.007$; Table 3), with no effect of sediment type, sex, or significant interactions.

We assessed measurement variability of the two CI metrics recommended by Crosby and Gale (1990). Within each treatment, relative standard deviation was lower for TW:SC compared to TW:SV when metrics were calculated using either wet or dry tissue (Table 4).

For glycogen content of mantle tissue, there was a significant effect of water type (GLIMMIX, $P = 0.014$) with no effect of sediment type or significant interaction. There was also a significant effect of sex on glycogen content in mantle tissue (GLIMMIX, $P = 0.036$) and an interaction between water type and sex that was not statistically significant at $\alpha = 0.05$ (GLIMMIX, $P = 0.066$) but warranted further exploration (Fig. 1). Pairwise comparisons revealed that males had significantly lower mantle glycogen content compared to females in simulated Powell River water (adjusted $P = 0.038$) and that males exposed to Powell River water had significantly lower mantle glycogen content than males exposed to control water (adjusted $P = 0.020$) (Fig. 1). Females and males had similar glycogen content in control water (adjusted $P = 0.99$), and females exposed to simulated Powell River water had similar glycogen content to females exposed to control water (adjusted $P = 0.94$) (Fig. 1).

DISCUSSION

Compared to control water, exposure to elevated major ion concentrations in the simulated Powell River caused a decrease in the proportion of the body cavity occupied by tissue (CI) and the body-component index for both male and

Table 3. Mean (standard error) of metrics of mussel condition measured in each treatment ($n = 4$) and overall means of treatments within each water type. Each treatment consisted of one water type and one sediment type, where CW = control water, PW = simulated Powell River water, CS = control sediment, and PS = Powell River sediment. Asterisks indicate metrics were significantly lower in PW compared to CW treatments; there were no significant effects of sediment type or interactions between water and sediment type for any metric. For metrics, K = (weight [g]/length [cm]^3) × 10, MW = weight of live mussel (g), MV = volume of live mussel (mL), TW = tissue weight (g), SV = shell cavity volume capacity (mL), SC = shell cavity capacity, determined as MW minus shell weight, and SW = shell weight. Subscripts indicate whether the measurement was performed on day 0 (initial) or day 40 (end) for live mussels, and whether TW was for wet or dry tissue of dissected mussels. The horizontal line separates measurements on live and dissected mussels.

	Treatment				Water Type	
	CWCS	CWPS	PWCS	PWPS	CW	PW
$K_{initial}$	1.69 (0.12)	1.72 (0.06)	1.81 (0.12)	1.66 (0.11)	1.70 (0.06)	1.74 (0.08)
K_{end}	1.67 (0.09)	1.72 (0.07)	1.76 (0.10)	1.69 (0.09)	1.70 (0.15)	1.72 (0.18)
$MW:MV_{initial}$	1.47 (0.06)	1.34 (0.05)	1.35 (0.07)	1.36 (0.09)	1.40 (0.04)	1.35 (0.05)
$MW:MV_{end}$	1.50 (0.09)	1.48 (0.07)	1.45 (0.09)	1.39 (0.03)	1.49 (0.06)	1.42 (0.04)
$TW:SV_{wet}$	0.41 (0.03)	0.45 (0.03)	0.39 (0.03)	0.37 (0.02)	0.43 (0.02)	0.38 (0.02)*
$TW:SC_{wet}$	0.34 (0.02)	0.37 (0.02)	0.32 (0.02)	0.32 (0.01)	0.35 (0.01)	0.32 (0.01)**
$TW:SV_{dry}$	0.044 (0.004)	0.052 (0.006)	0.037 (0.004)	0.039 (0.004)	0.048 (0.004)	0.038 (0.003)**
$TW:SC_{dry}$	0.036 (0.002)	0.042 (0.004)	0.030 (0.003)	0.033 (0.002)	0.039 (0.003)	0.031 (0.002)**
$TW:SW_{dry}$	0.045 (0.005)	0.052 (0.008)	0.035 (0.005)	0.038 (0.003)	0.048 (0.004)	0.037 (0.003)**

*$P = 0.030$.

**$P \leq 0.0086$.

Table 4. Relative standard deviation for each metric calculated for dissected mussels in each treatment ($n = 4$). Each treatment consisted of one water type and one sediment type, where CW = control water, PW = simulated Powell River water, CS = control sediment, and PS = Powell River sediment. For metrics, TW = tissue weight (g), SV = shell cavity volume capacity (mL), and SC = shell cavity capacity, determined as MW (weight of live mussel in grams) minus shell weight. Subscripts indicate whether TW was for wet or dry tissue of dissected mussels.

	CWCS	CWPS	PWCS	PWPS
$TW:SV_{wet}$	13.9	11.7	16.1	10.8
$TW:SC_{wet}$	8.95	8.82	12.7	7.44
$TW:SV_{dry}$	17.2	22.1	22.3	19.7
$TW:SC_{dry}$	11.4	19.1	20.0	14.7

female *L. fasciola*. Differences between water types were comparable for indices calculated using wet and dry tissue weights, indicating that tissue mass was lower in mussels exposed to simulated Powell River water. These results demonstrate that the mixture of major ions present in the Powell River likely results in a reduction in total energy storage in adult freshwater mussels, producing a measureable reduction in CI. There was no effect of Powell River sediment on CI of *L. fasciola* compared to control sediment. We did not measure contaminant concentrations in the sediment used in the exposure, but previous sampling of sediment in the upper Powell River in the vicinity of the collection site showed elevated concentrations of naphthalene and nickel (MapTech 2011), and the estimated coal content of the sediment used in the study (1.5%) was similar to previous studies of the Powell River (Wolcott 1990). Exposure to coal fines in sand caused apparent energetic stress in female *Villosa iris*, measured as significantly higher proportion of resorbing oocytes compared to controls (Henley et al. 2015). However, unlike coal particles in the Powell River sediment, the coal fines evaluated in Henley et al. (2015) were not weathered and were suspended in the water column, which may have increased bioavailability of coal-associated contaminants. Results of our study suggest that the bioavailability of coal-associated contaminants in Powell River sediment may be limited, but the study design does not allow their exclusion as a potential stressor to freshwater mussels inhabiting the river. Instead, these results clearly demonstrate that the elevated concentrations of major ions in the simulated Powell River water reduces the condition index of adult freshwater mussels, a measurable adverse physiological effect.

When compared to mussels in control water, all mussels exposed to simulated Powell River water had lower tissue mass, but only males had lower glycogen content in mantle tissue. This indicates that in order to compensate for increased salinity stress, males were using energy stored as glycogen whereas females likely were using energy stored in another form. At the start of this study (October), female mussels had obviously inflated marsupial gills containing glochidia, which is consistent with the classification of *L. fasciola* as bradytictic and indicates spawning and fertilization occurred previously. In bivalves, oocyte resorption occurs after spawning as a normal part of the gametogenic cycle (Kennedy and Battle 1964; Dorange and Le Pennec 1989; Henley et al. 2015). Cyclic resorption of atretic oocytes provides an efficient mechanism of nutrient recycling, particularly for lipids and proteins (Pipe 1987). Untimely resorption of developing oocytes also has been observed in marine and freshwater bivalves exposed to contaminants, and it is likely related to an energetic deficit (Bayne et al. 1981; Henley et al. 2015). In our study, females exposed to simulated Powell River water potentially were using energy stored in resorbing oocytes to compensate for increased stress, which explains a reduction in CI similar to males but not a concurrent loss of glycogen reserves. This finding is consistent with observations of energy use in marine mussels; when subjected to starvation during the period of gametogenesis and spawning (winter), mussels used stored protein, followed by lipid and carbohydrate, and when subjected to starvation during gametogenic quiescence (summer), mussels used only stored carbohydrate (Bayne and Newell 1983). Limited study of gametogenesis in *L. fasciola* from one tributary in the Upper Tennessee River watershed found the presence of early-stage glochidia in gill marsupia in early September, suggesting spawning in late August (Zale and Neves 1982), which is consistent with observations of gravid females during this study. Residual gametes were present several months after spawning, and active gametogenesis occurred throughout the year (Zale and Neves 1982). Thus, *L. fasciola* exposed to simulated Powell River water potentially could have resorbed either atretic oocytes or developing oocytes to compensate for increased energetic demands. However, definitively determining the use of this pathway would require histological evaluation or direct measurement of energy substrates in the viscera, which were beyond the scope of this study.

The mechanism for increased energy use by adult *L. fasciola* when exposed to elevated concentrations of major ions in simulated Powell River water remains unclear. One possibility is that *L. fasciola* exposed to simulated Powell River water closed their valves to avoid exposure, inducing anaerobic catabolism of energy reserves. Shell closure is a common avoidance response in freshwater mussels exposed to high concentrations of toxicants (Cope et al. 2008). The salinity in the current study was <1 ppt, and *L. fasciola* remained buried in all treatments for the duration of the exposure. Limited observations indicated that the mussels were also actively siphoning in all treatments. Blakeslee et al. (2013) found that the freshwater mussel *Elliptio complanata* could acclimate to 1 ppt (psu) salinity within 7 days, maintaining oxygen consumption rates similar to controls, with no shell closure observed up to 4 ppt salinity. Although mussel behavior was not specifically documented in the current study, shell closure and resulting anaerobic catabolism do not appear to be the primary mechanism for a decrease in metrics related to energy storage in *L. fasciola* exposed to simulated Powell River water.

Increased energy expenditure for osmoregulation is another potential mechanism for the observed decrease in metrics related to energy storage in *L. fasciola* exposed to the elevated concentrations of major ions in simulated Powell River water. Freshwater bivalves are generally osmoconformers when exposed to water with moderately elevated salinity (Dietz et al. 2000; Ruiz and Souza 2008; Griffith 2017). As extracellular osmotic pressure increases, bivalves increase intracellular concentrations of inorganic and organic (amino acids) osmolytes to maintain cell volume (Jordan and Deaton 1999; Ruiz and Souza 2008). The intracellular amino acids are generated from both increased synthesis and protein catabolism, and the increased activity of the associated transaminases and proteolytic enzymes likely has a high energetic cost (Bishop et al. 1994). In addition to regulating cell volume, there is likely an energetic cost for maintaining individual ions at concentrations necessary to avoid ionoregulatory imbalance. Maintaining intracellular concentrations of Na^+, K^+, Ca^{2+}, and H^+ depends at least in part on the activity of energy-dependent ATPases and is also affected by cotransport of other ions, including HCO_3^-, Cl^-, and Mg^{2+} (Byrne and Dietz 2006; Griffith 2017). Thus, the ion concentrations in simulated Powell River water may have increased the energetic cost of osmoregulation for *L. fasciola* by necessitating increased intracellular concentrations of amino acids and increased transport of individual ions to maintain ionic homeostasis.

Sulfate concentrations were particularly high in the simulated Powell River water, reflecting conditions in the river and in other mining-impacted rivers in central Appalachia (e.g., Pond et al. 2008). The exact mechanism of SO_4^{2-} uptake and transport in freshwater invertebrates is unclear (Griffith 2017). When exposed to elevated SO_4^{2-} concentrations, mayflies take up SO_4^{2-} rapidly (Scheibener et al. 2017); freshwater mussels take up SO_4^{2-} more slowly, but the concentration in the hemolymph eventually becomes isoionic with the exposure water (Dietz et al. 2000). Mayflies exposed to elevated concentrations of SO_4^{2-} had reduced time to emergence, attributed to the energetic cost of active SO_4^{2-} excretion (Buchwalter et al. 2018). There also may be an energetic cost of SO_4^{2-} excretion in freshwater mussels, as both freshwater mussels and mayflies appear to transport SO_4^{2-} using anion exchange (Dietz et al. 2000; Buchwalter et al. 2018). In rainbow trout, the SO_4^{2-}/anion exchanger (SLC26A1) in the renal proximal tubule is colocalized with both Na^+,K^+-ATPase and vacuolar-type H^+-ATPase (Katoh et al. 2006), providing a potential mechanism for increasing energy expenditure with increasing SO_4^{2-} excretion. In our study, the apparent energetic stress associated with osmoregulation observed for mussels exposed to the simulated Powell River water, coupled with the elevated concentration of SO_4^{2-} in the exposure water, suggests that the mechanism of SO_4^{2-} transport in freshwater mussels warrants further study, because it potentially could be an energetically demanding process.

The elevated concentrations of major ions in simulated Powell River water caused a significant reduction in metrics related to energy storage in adult *L. fasciola*, in contrast to results of a previous study in our laboratory, which showed no significant effect of this water on growth of juvenile *Villosa iris* (Ciparis et al. 2015). Food availability may affect the response of freshwater invertebrates to the energetic demands of salinity stress; individuals with optimal nutrition may be less sensitive compared to individuals with suboptimal nutrition (Buchwalter et al. 2018). The ratio of food availability to energy demands may have been greater for juvenile *V. iris,* because the feeding rate used in Ciparis et al. (2015) was optimized to promote mussel growth. In contrast, the feeding rate for adult *L. fasciola* was based on limited published information of feeding regimes for similarly sized individuals (e.g., Hazelton et al. 2014). The feeding rate was sufficient for maintenance, as indicated by 100% survival of *L. fasciola* in all treatments, but the food availability may not have been sufficient to meet additional energetic demands from exposure to the simulated Powell River water. Differences in responses to the elevated concentrations of major ions between the two studies also could be related to life stage; the juvenile mussels (age 3–5 mo) were undergoing rapid shell growth, which requires sequestering of ions, predominantly Ca^{2+} and HCO_3^- with smaller amounts of Na^+, K^+, Mg^{2+}, Cl^-, and SO_4^{2-} (Marin et al. 2012). Sequestration could reduce extracellular concentrations of these ions and thus the energetic demands of osmoregulation upon exposure to water with elevated ion concentrations. Finally, CI and other measures of energy storage in mussel tissues may be more sensitive than measurements involving only the mussel shell (e.g., shell growth).

In general, toxicity tests with juvenile freshwater mussels are considered protective of adult mussels for acute effects (Cope et al. 2008). However, toxicological studies focusing only on survival and growth of juveniles fail to capture potential effects of nonlethal concentrations on the reproductive potential of freshwater mussels. There is a direct relationship between energy storage and reproductive potential, because bivalves use stored glycogen during gametogenesis (Bayne et al. 1982; Pipe 1985), and females under energetic stress will actively resorb oocytes (Bayne and Newell 1983; Henley et al. 2015). The finding of significantly lower CI, a metric of energy storage, in *L. fasciola* exposed to simulated Powell River water has direct implications for sustainability of mussel populations in the river. In the upper Powell River, a decline in mussel recruitment has been observed since 1980 (Wolcott 1990; Johnson et al. 2012), and reproductive failure of adults is one potential contributing factor. Thus, energy storage is an ecologically relevant endpoint for freshwater mussels, which are long-lived and generally reproduce annually.

Evaluation of metrics related to energy storage in freshwater mussels showed differences in sensitivity. Metrics calculated on live mussels were not significantly different between treatments, in contrast to measurements on dissected mussels. This is likely due to shell weight dominating total body weight measurements, overshadowing the relatively small changes in tissue weight between treatments. In addition,

the significant difference in Fulton's K between males and females highlights a potential pitfall of the use of weight:-length ratios in sexually dimorphic mussel species, particularly if sex is not included as a covariate. Given the imperiled status of many species, measurements on live freshwater mussels often are preferred, but our results suggest that CI metrics on live mussels may not accurately assess the impacts of environmental stressors on energy storage. On dissected mussels, results were similar for metrics using wet and dry tissue weights, indicating both are suitable for measurement of condition index. Generally, dry tissue weight is preferred because it removes water in the tissue as a source of variability (Crosby and Gale 1990), but if further analysis of the tissue precludes drying, use of wet tissue weight appears to be an acceptable method for assessing CI. Both TW:SV and TW:SC had similar differences between treatments, but variability in TW:SC was lower, which supports the findings of Crosby and Gale (1990). Although shell cavity capacity (SC) is technically the weight of tissue and water held by the shell cavity, it provides a close approximation of the shell cavity volume due to water comprising the majority of the weight of a live mussel and water's specific gravity. Shell cavity capacity can be measured more precisely than shell cavity volume (SV), as demonstrated by this study and by Crosby and Gale (1990). Therefore, for future studies of energy storage in freshwater mussels, we recommend the use of TW_{dry}:SC. A scaling factor of 1000 was recommended by Crosby and Gale (1990), but this was developed for oysters, which generally have heavier shells compared to freshwater mussels. A scaling factor of 100 appears more appropriate for freshwater mussels, to bring the calculated CI close to 1 (Nash et al. 2006). As we have demonstrated, the body component index (TW_{dry}:SW) may show similar responses as CI to environmental stressors, but Crosby and Gale (1990) caution against its use for assessment of temporal changes in nutritive status, particularly for bivalves with active or variable shell growth.

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