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REGULAR ARTICLE

THE STATUS OF MUSSEL HEALTH ASSESSMENT AND A PATH FORWARD

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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

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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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