Pathogens And Diseases Of Freshwater Mussels In The United States: Studies On Bacterial Transmission And Depuration

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Abstract

Unionid mussels are recognized as important contributors to healthy aquatic ecosystems, as well as bioindicators of environmental perturbations. Because they are sedentary, filter feeding animals and require hosts (i.e., fishes) to transform embryonic glochidia, mussels are susceptible to direct adverse environmental parameters, and indirect parameters that restrict the timely presence of the host(s). Their numbers have declined in recent decades to a point that this fauna is regarded as one of the most imperiled in North America. The most significant threat to populations of native unionids in recent years has been the introduction and spread of zebra mussels *Dreissena polymorpha*. Many federal and state agencies, and private interests are now engaged in mussel conservation efforts, including collecting selected imperiled species from impacted rivers and lakes and propagating them at refuges for future population augmentations. One essential consideration with mussel propagation and their intensive culture at refugia is the prevention of pathogen introductions and control of diseases. Currently, there are few reports of etiological agents causing diseases among freshwater mussels; however, because of increased observations of mussel die-offs in conjunction with transfers of live animals between natural waters and refugia, disease problems can be anticipated to emerge. This review summarizes research to develop bacterial isolation techniques, study pathogen transmission between fish and mussels, identify causes of seasonal mussel die-offs, and develop non-destructive methods for pathogen detection. These efforts were done to develop disease preventative techniques for use by resource managers to avoid potential large-scale disease problems in restoration and population augmentation efforts among imperiled populations.

Key words: Unionid, freshwater, mussel, disease, bivalve

Introduction

There are approximately 300 species of freshwater mussels native to North America with the southeastern United States being particularly noteworthy for species richness due to favorable water temperatures, food availability, and the presence of host fishes for transformation of glochidia. Mussels are important members of aquatic ecosystems. Mussel beds containing dense populations are capable of filtering substantial volumes of water, which promotes nutrient cycling and provides improved environments for both benthic plants and animals (Vaughn and Spooner 2006). Freshwater mussels are also recognized as sentinels for healthy aquatic ecosystems. Being sedentary animals that filter large volumes of water for respiration and food, this renders them very susceptible to potentially harmful environmental changes such as those from chemicals or pollutants or disturbances to land that results in soil runoff and bridge and dam construction that alters river and lake bottoms (Ellis 1936, Fuller 1974).

The numbers of native mussels have significantly declined in recent decades to a point where they are now one of the more imperiled faunas in the United States (Lydeard et al. 2004, Neves et al. 1997, Williams et al. 1993). Unionid mussels require a vertebrate host, primarily fishes, for transformation of embryonic glochidia into juveniles; therefore, impacts that affect the

presence of these host fishes also affects the sustainability of mussel populations. Factors that affect the fish hosts include diseases and impediments preventing or limiting their movements from being in proximity to female mussels when glochidia are released. The most serious cause of more recent native mussel population declines has been the introduction of the non-native zebra mussel (*Dreissena polymorpha*) during the mid-1980's and the subsequent prolific spread (Herbert et al. 1991, Nalepa 1994). Zebra mussels have since flourished in many lakes and rivers in North America, and have led to local extirpations of native populations (Nalepa et al. 1996, Ricciardi et al. 1998, Schloesser and Nalepa 1994). Zebra mussels tolerate extremely high spatial density indices, achieving for example, numbers in the hundreds of thousands per square meter (MacIsaac et al. 1991). Zebra mussels attach to solid surfaces including forming colonies on the shells of native mussels. Zebra mussels lead to poor survival of native animals by outcompeting for respiration and food, and for reproduction (Haag et al. 1993, Gillis and Mackie 1994). In contrast to the parasitic larval (glochidia) stage of native unionids, zebra mussel larvae are planktonic and do not involve vertebrate hosts for development. The combined threats to native mussels and poor prognosis for many native mussel populations in rivers impacted by zebra mussels, have catalyzed mussel conservation efforts. Recent mussel studies have focused on descriptions of the geographic ranges of species, abundance of current populations relative to historic records, and identification of the host fishes for transformation of mussel glochidia. Since collections of threatened and endangered mussels from natural environments for propagation at refuges continues to increase, improved husbandry and captive rearing techniques, particularly dietary requirements, are the current focus for much research.

Refuges for rearing mussels include fish hatcheries that have been modified to accommodate both the mussels and their fish hosts for transformation. Typically, these hatcheries rear a suite of sport and restoration fish species. Since mussels are collected from open-water sources and are relocated to the hatcheries, there is ongoing concern that the mussels could vector fish pathogens to the resident hatchery fish. There is also the concern for pathogen vectoring to resident populations of hatchery-reared mussels and ultimately, to wild populations of both the fish and mussels through stocking practices. Host predisposing factors promoted by intensive culture may increase the consequences of pathogen introductions by heightening the hosts' susceptibility. Although there are currently no recognized diseases of cultured freshwater mussels, because they are now routinely moved from aquatic environments to hatcheries, it is reasonable to anticipate that some of the same pathogen transmission and disease issues encountered in fish culture will be-come increasingly relevant to mussel propagation. Much is known of the diseases that occur in marine bivalves driven primarily by their commercial importance in aquaculture. It is prudent now to adapt some of the same disease prevention practices done in fish and marine bivalve culture to freshwater mussel husbandry. The goal for captive propagation of native freshwater mussels is to introduce these animals into natural watersheds and augment existing populations, thus it is imperative to maintain disease-free status. This review summarizes mussel research done at the National Fish Health Research Laboratory to address the potential for pathogen vectoring to hatchery-resident fish during relocation. Also, determining whether such mussels actually har-

bored fish pathogens that could be transmitted to fish was of specific interest. Additional studies were done to delineate how readily mussels acquire and depurate a fish pathogen from the water column and whether a pathogen could in fact be transmitted between mussels and fish. As mussel propagation efforts continue to increase, research needs specific to mussels, including nondestructive sampling and description of their etiological agents, have become more relevant.

A reliable procedure was developed to improve the recovery of bacteria from mussels (Starliper et al. 1998). To perform this procedure, after recording the morphometric data, the external surfaces of the valves (shells) were brushed clean and disinfected with 200 mg/L sodium hypochlorite. The valves were pried apart and the adductor muscles were cut to allow full opening. The fluid inside the valves, but outside of the soft tissues, was collected in a sterile Petri dish (e.g., 15 × 100 mm). The soft tissues were excised and swirled in 200 mg/L sodium hypochlorite for 30 seconds to ensure that the resulting bacteria isolated are from within the tissues and not from an external, environmental source. The tissues were rinsed for 15-sec in sterile pep-ye broth that contains 0.1% peptone (Becton, Dickinson and Company, Sparks, MD) and 0.05% yeast extract (Becton, Dickinson and Company, Sparks, MD). The soft tissues were weighed, diluted 1:2 (weight:volume) in pep-ye broth and homogenized for 1 – 2 min in a Model 80 Stomacher (Seward Medical, London, United Kingdom). $Log_{(10)}$ dilutions were prepared from both the fluids and the soft tissue homogenates in pep-ye broth and volumes (e.g., 0.025 mL) of each dilution are drop-inoculated onto the surface of bacteriological media. Selection of the bacteriological media used can be tailored to recover the total bacteria or specific bacteria of interest (Starliper and Morrison 2000, Starliper et al. 1998, 2008). Individual bacterial colonies that result may be enumerated (which are reported as cfu/mL of fluid or cfu/g of soft tissues), selected, and identified using standard microbiological characterization methods (Griffin 1992, Holt et al. 1994, Janda and Abbott 1998, Koneman et al. 1992, MacFaddin 2000, Murray et al. 1999). This procedure was employed in an effort to determine if feral mussels could harbor common fish bacterial pathogens (Starliper and Morrison 2000). A battery of general, specific and differential bacteriological media were employed to recover *Aeromonas salmonicida*, *Renibacterium salmoninarum*, *Edwardsiella* spp., *Yersinia ruckeri*, and Flavobacteria. Six collections, representing six species of mussels were made throughout one sampling season, which is typically June through October or November from the Ohio River, adjacent to Wood, Co., West Virginia. Fluids and soft tissue homogenates were collected and processed as previously described. Although none of the targeted fish pathogens were recovered during this study, *Flavobacterium columnare*, cause of columnaris disease in many cool and warmwater fishes, was previously recovered from *Amblema plicata* from the Ohio River, West Virginia (Starliper et al. 1998) and more recently from *Villosa iris* from the Clinch River, Virginia (Starliper et al. 2008), which clearly demonstrated that mussels could harbor a viable fish bacterial pathogen. As would be anticipated, a variety of bacteria commonly encountered in freshwater aquatic environments can be recovered from apparently healthy mussels (Table 1, Starliper et al. 2008). The total bacterial load recovered from mussel soft tissues typically ranges between 1.77×10^5 and 3.55×10^6 cfu/g, whereas the bacterial totals from fluids are approximately ten-fold less (Starliper et al. 2008). These numbers of total

bacteria appear to remain relatively consistent from different mussel species and watersheds (Starliper and Morrison 2000, Starliper et al. 1998); however, the flora in mussels is dynamic in response to environmental changes in water (Starliper et al. 1998).

Table 1. List of bacteria that are commonly recovered from unionid mussels within freshwater environments. Data are from *Actinonaias pector-osa*, *Lexingtonia dolabelloides*, and *Villosa iris* in the Holston and Clinch Rivers, Virginia (Starliper et. al. 2008).

Acinetobacter junii/johnsonii
Acinetobacter lwoffii
Acinetobacter radioresistens
Aeromonas hydrophila
Aeromonas caviae
Brevundimonas vesicularis
Chryseobacterium indologenes
Chryseomonas luteola
Citrobacter koseri
Comamonas testosterone
Enterobacter intermedius
Flavobacterium columnare
Corynebacterium-like
Hafnia alvei
Klebsiella spp.
Moraxella spp.
Ochrobactrum sp.
Pantoea spp.
Pasturella spp.
Plesiomonas shigelloides
Proteus vulgaris
Providencia rettgeri
Pseudomonas fluorescens
Ralstonia pickettii
Serratia fonticola
Serratia liquefaciens
Serratia marcescens
Shewanella putrefaciens
Sphingobacterium multivorum
Sphingomonas paucimobilis
Stenotrophomonas maltophilia

A model system was developed to study bacterial pathogen transmission between fishes and mussels (Starliper 2001, 2005). The fish used in these studies were brook trout (*Salvelinus fontinalis*) or Arctic char (*S. alpinus*). The mussels were threeridge (*A. plicata*) or ebonyshells (*Fusconaia ebena*). Disease caused by *A. salmonicida* was established in experimental groups of fish by intraperitoneal injection of the bacterium. Once the disease was established and the fish began to die, non-injected fish were placed in the tank to become horizontally infected. The injected fish typically succumb prior to any deaths occurring among the fish that become infected through horizontal exposure to the pathogen. When mortality commenced in the horizontally-exposed fish, mussels were placed in the tank to cohabit with the fish and acquire *A. salmonicida* by siphoning the water column. Within 2-3 weeks after they were introduced to the tanks, a 100% incidence of *A. salmonicida* was readily shown by bacterial culture in samplings of about 10 mussels. At this time, the remaining *A. salmonicida*-positive mussels were moved to different (158-L) tanks to initiate *A. salmonicida* depuration for a defined 30-day period. During the 30 days, groups of mussels were sampled for *A. salmonicida* and clean sentinel brook trout or Arctic char were introduced to cohabit with the mussels. Pathogen depuration was considered to be complete when *A. salmonicida* was not isolated from the mussels or cohabitation-tank water effluent (cultured by 1-L centrifugation and plating) and the indicator fish remained free of infection. The 30-d depuration period was selected because native mussels are quarantined for at least this long to ensure that they are free of zebra mussels before they are placed at refugia (Chafee 1997, Gatenby et al. 1998). After only 1 d of depuration, the prevalence of *A. salmonicida* in mussels decreased from 100% to $\leq$ 30%, and depuration of *A. salmonicida* was completed in *A. plicata* and *F. ebena* within 15 d of the 30 d observation periods (Starliper 2001, 2005).

It has been shown that the bacterial flora within mussels changes rapidly (Nichols et al. 2001, Starliper et al. 1998). Nichols et al. (2001) showed that endosymbiotic microbes (i.e., bacteria) were not present in mussels and Starliper et al. (1998) demonstrated that the bacterial flora in mussels changed significantly within 24-hr of a change in water supply. The finding that the bacterial flora rapidly responds to changing water supplies could be used favorably as a means to minimize the risk for introductions of pathogens. Since mussels collected from rivers for refugia are quarantined to guard against the spread of zebra mussels (Chaffee 1997, Gatenby et al. 1998), it is thought that if this were done in specific pathogen-free water, the quarantine might offer the opportunity for the mussels to depurate both fish and mussel pathogens. Laboratory studies have demonstrated how readily *A. salmonicida* was transmitted between fish and mussels through simple cohabitation experiments (Starliper and Morrison 2000, Starliper 2001, 2005), indicating possible pathogen vectoring by wild-caught mussels to hatchery fish. However, the results that showed the pathogen was effectively depurated within 30-d were encouraging. Furthermore, the fact that *F. columnare* was not recovered from cohort *A. plicata* after only 1 d of depuration also showed how readily the fish pathogen was eliminated apparently by siphoning pathogen-free water (Starliper et al. 1998).

Nondestructive bioassays have been used in freshwater mussel studies. Berg et al. (1995) developed a mantle biopsy technique with *Quadrula quadrula* and *Actinonaias ligamentina*. Henley et al. (2006) used a swab-stroke procedure to obtain DNA from *Q. pustulosa* and Gustafson et al. (2005) used a syringe and needle to obtain hemolymph from the adductor muscle. None of these procedures resulted in significant mortality in observation periods that followed sample collections; therefore, a study was done to assess three non-lethal sample sites for recovery of *A. salmonicida* (Starliper 2008). Groups of *A. plicata* and *F. ebena* were infected with *A. salmonicida* to a 100 % incidence by cohabitation with infected fish and sampled during a 30-d depuration period. As depuration proceeded, the numbers of *A. salmonicida* were declining in the remaining groups of mussels, which provided a more robust comparison between non-lethal and lethal samples to detect the bacterium. Three non-lethal sample sites were evaluated: 1-hemolymph, collected by gently opening the valves and obtaining 0.2 – 0.5 mL from the adductor muscle using a syringe and needle (i.e., 22 G); 2- inner fluid, collected using the clean-catch procedure previously described; and 3, a biopsy of approximately 3 × 5 mm of mantle tissue. Lethal fluids and soft tissue homogenates were collected, and dilutions were made of all samples in pep-ye broth and used to inoculate bacteriological media as previously described. There was no mortality among the mussels during the six-week observation period after the nondestructive samples were taken. The nondestructive procedures were shown to be comparable to methods that require sacrificing the animals for recovery of *A. salmonicida* (Starliper 2008). These results indicate the potential value in nondestructive sample collections for assessment of pathogens in mussel populations. Although none of the three procedures caused mortality, the fluid collection is considered the least traumatic to mussels because it is a noninvasive procedure. Hemolymph and mantle clips are tissue invasive procedures and could create a port of entry for infectious agents.

Increasingly, mussel die-offs with apparent natural causes are being observed in the United States (Neves 1987, Starliper et al.

2008, 2009). These die-offs are characterized by large numbers of dead and moribund specimens that were limited to relatively localized geographic areas. Because the die-offs were often noted incidentally, accurate mortality data have been difficult to collect and the number of populations affected is not known. With those that have been observed, the mortality, which occurs over a period of days to 2-3 weeks, differs from acute death rates typically seen in events with non-infectious causes, such as critically low dissolved oxygen or toxic substance exposure, in which acute mortality would be within a shorter period. Confounding attempts to identify causes, in open waters the evidence is quickly diluted and carried away with the flowing water. Anecdotal observations from these die-offs indicate possible roles of etiological agents. Die-offs occur seasonally and in conjunction with increased water temperatures, and when many mussel species are spawning and releasing glochidia. Mussel host-disease specificity has been noted. Mussel die-offs in the Middle Fork Holston and Clinch Rivers, Virginia have involved primarily gravid female slabside pearlymussel (*Lexingtonia dolabelloides*), a candidate species for federal protection, and to lesser extent the pheasantshell (*Actinonaias pectorosa*), rainbow mussel (*Villosa iris*), and the endangered shiny pigtoe (*Fusconaia cor*, Starliper et al. 2008). Ebonyshells (*F. ebena*), specifically post-spawned females, are the primary affected host in Pickwick Lake, Tennessee River, Alabama (Starliper et al. 2009), where significant die-offs were noted in 2002 and have since occurred biennially. Characteristics of moribund animals from all die-offs were similar; the most obvious sign being delayed and weakened hinge/valve closing in response to external stimuli. In attempts to isolate a pathogen(s) as the cause of the die-offs, bacterial isolations from healthy cohorts served as controls. From Pickwick Lake, the bacteria from healthy *F. ebena* were typical of that encountered from other mussels and drainages (Table 1). The total bacterial counts from fluids and soft tissues were in the range of about 1×10^5 to 1×10^6 cfu/mL or cfu/g, respectively. In contrast, the total bacteria recovered from diseased *F. ebena* were 100- to 1,000-fold greater in numbers, and primary cultures were pure or nearly pure with the same bacterium from all specimens from that year. However, the predominant bacteria from 2006 and 2008 were not consistent. The predominant bacterial species from 2006 mussels was *Aeromonas sobria*, whereas the primary bacterium from 2008 was *Yokenella regensburgei* (Starliper et al. 2009). Both of these bacteria may be recovered from aquatic environments and healthy mussels. It was thought that the diseased mussels were compromised by a combination of stressors and their bacterial profiles indicated possible opportunistic pathogen involvement or secondary pathogens to another etiology, which at the current time remains undescribed.

Maintaining good quality health of captive mussels is essential for a successful propagation program. Healthy mussels will reflect the historic genetic integrity of wild populations, maintain high condition factors (Paterson et al. 1997, 1999) and remain disease-free (Villella et al. 1998, Jones et al. 2006). Optimal mussel condition factor likely plays an important role in resistance to diseases. Also, scheduled pathogen and disease examinations, or inspections, could become a widespread and useful tool to reduce the risk of pathogen introductions and the spread of diseases.

Conclusions

Freshwater mussel conservation efforts have increased dramatically in recent years. Dis-

eases to mussels are currently not recognized as widespread in feral populations and are not associated with the precipitous decline of the fauna. Study results indicate that pathogens and diseases have the potential to impact conservation of this imperiled fauna. It is important to implement disease preventative strategies as part of mussel conservation efforts, much of which can be adapted from fisheries management techniques, particularly for mussel refugia that also maintain fish.

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