Disease Prevention Research ~~Done in West Virginia~~ on Imperiled Freshwater Mussels (Unionoida) ~~Native to~~ North America.

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ABSTRACT

INTRODUCTION

Approximately 300 species of freshwater mussels are native to North America. The fauna is particularly rich in species diversity and in overall numbers of animals in the southeastern United States where water temperatures, food availability and diversity of the required fish hosts are prevalent. Mussels are recognized important components of ecosystems, as filter feeding animals they ~~filter~~ substantial amounts of water, but this can also leave them susceptible to adverse environmental impacts to the water. Within the past few decades, historical numbers have significantly declined, ~~and until the fauna is~~ now commonly recognized as one of the more imperiled in the United States. In fact, approximately 70 % of the native species are ~~officially~~ considered imperiled (Williams et al. 1993; i.e. listed as threatened, endangered, or special concern). Seventy-two species are currently listed by the U.S. Fish and Wildlife Service as threatened or endangered (http://ecos.fws.gov/tess_public/Speciesreport.do?groups-F&listingType=L). In West Virginia alone, currently there are 14 species of endangered mussels listed (http://ecos.fws.gov/tess_public/StateListing.do?status=listed&state=IN). A suite of causes, individually and in combination, have been identified as reasons for their decline, both directly to them and indirectly through impacts to their fish hosts. The life cycle of each species of mussel requires the use of a fish for transformation of embryonic glochidia into juveniles. Therefore, any adverse impact to the host fish affects the sustainability of mussel populations. Factors affecting the fish hosts include diseases, impoundments to prevent or limit their movements, and other environmental impacts. Mussels have often been referred to as important biological monitors for the overall health of aquatic ecosystems. This is in large part based on their relatively sedentary

existence and their filtering of water for respiration and food. However, this lends them susceptible to compromised environmental conditions such as those caused by pollutants or increased water temperatures and reduced dissolved oxygen concentrations often associated with decreased water flows. Disturbances to land adjacent to bodies of water that result in soil runoff and disturbances to river and lake bottoms that produce silt are obviously detrimental to mussels. The most serious threats to native mussel populations in recent years have resulted from the inadvertent introduction of the nonnative zebra mussel (*Dreissena polymorpha*) in the mid 1980's. Since, zebra mussels have become the most significant threat to native populations in rivers, lakes and streams. Zebra mussel colonizations have resulted in extirpation of native populations. In contrast with native species, zebra mussels do not utilize a fish host for reproduction. They also tolerate tremendously high numbers (i.e. high spatial density indices) within small areas. Reports of up to thousands of zebra mussels per square meter are not uncommon. They attach to solid structures or surfaces, including the shells of native animals and colonize to great numbers. These colonizations result in a poor prognosis for survival of native animals as they cannot compete with these high density colonies for oxygen and food, much less for reproduction. These combined threats to the future well-being of native mussels have precipitated an increased focus of conservation efforts by Federal and State agencies and private interests. These agencies are involved in a number of facets of mussel conservation including identifying ranges and population densities, identifying the fish host(s) for transformation, captive rearing and spawning and proper diet and feeding protocols. A significant part of these conservation efforts involves maintenance and propagation of threatened and endangered species at refuges free of the threats to their

survival. For most species, the ultimate goal for captive rearing is reintroductions into natural watersheds or augmentations of existing populations after peak numbers of zebra mussels subside.

CONSERVATION EFFORTS FOR NATIVE MUSSELS

Currently, a large number of the refuges for mussels are fish hatcheries that have been adapted to accommodate mussels. These hatcheries typically rear both a suite of fish species and a variety of mussel species. One of the larger facilities in the U.S. Fish and Wildlife Service for this is the White Sulphur Springs National Fish Hatchery (NFH) in White Sulphur Springs, West Virginia. For many years, this federal hatchery has served as one of the few national broodstock fish hatcheries for trout in the U.S. Fish and Wildlife Service's fish hatchery system. Millions of eyed eggs are produced and shipped to other facilities annually. This hatchery maintains an "A-1" classification within the Service's health inspection program, meaning the facility has a closed, specific pathogen-free water supply and fish have been determined to be pathogen and disease free for at least a number of years. Obviously, maintaining this classification is essential for the mission of this hatchery with respect to fish and the Federal fish hatchery system depends on this hatchery for productivity. Any compromise to the disease preventative strategy at White Sulphur Springs NFH is cause for great concern. So was the case early on with mussel relocation programs and specifically, when hatcheries were identified as refuges. This presented a particularly troublesome scenario to fishery managers and at White Sulphur Springs NFH, in particular. The primary reason for the concern was the fact that mussels collected for relocation to refuges were/are taken from open-water environment known to contain fish likely infected (and diseased) with any number of a variety of

pathogens. At that time, the important question was: what is the risk that mussels will act as vectors of pathogens to resident-hatchery fish populations? The National Fish Health Research Laboratory (NFHRL), in Leetown, WV became involved to address the serious concerns raised by fishery managers including: 1) can mussels harbor a fish pathogen? 2) can mussels act as fish pathogen vectors? 3) what is the risk that fish can become infected with a pathogen that would be introduced via mussels, and more important, what can be done to prevent pathogen vectoring?

PATHOGEN AND DISEASE RESEARCH: ISOLATION OF BACTERIA

The risks for introduction of pathogens through movement of fish (or fish eggs) from natural waters to hatcheries or from one hatchery to another are well documented (Wedemeyer 2001). One of the first concerns of the NFHRL was to evaluate the potential regarding the risk for introduction of fish pathogens via relocation of mussels. Mussels that are collected from rivers for relocation to hatcheries (refuges) must first undergo a minimum 30-day quarantine to ensure they are free of zebra mussels (Chafee, 1997; Gatenby et al., 1998). Ideally, this quarantine perhaps offered an opportunity for mussels to depurate pathogens, and this would prevent vectoring. The NFHRL subsequently developed a model using the fish bacterial pathogen *Aeromonas salmonicida* to evaluate the effect of quarantining to eliminate that risk to vector. One of the first research projects, however, resulted in a reliable and reproducible protocol to isolate the bacteria present in mussels (Starliper et al., 1998). The procedure is done aseptically. The external surfaces of the valves (shells) are decontaminated, the valves are pried open just enough so the adductor muscles (on both sides of the animal) can be cut to permit the valves to be easily opened further. Fluid, which is (the term coined for) the liquid inside the valves,

but outside of the soft tissues, is clean-caught in a sterile Petri plate. We have shown that the fluids are influenced by, and representative of the bacterial flora within the animals' internal organs. Although the bacterial flora in mussels is dynamic in response to changes in their water-environment, the fluid is not simply a sample of that water. After all of the fluid is caught, the soft tissues are then excised for bacteriology. In our initial studies, we would excise the tissues and separate into two samples, one which consisted primarily of digestive tract and the second comprised all remaining tissues. It was relatively easy to separate tissues of larger animals, e.g. *A. plicata* weighing 150 grams or more; however, the task was more difficult with smaller animals. We have since determined the total colony forming units (i.e. viable bacterial cells; cfu) and bacterial flora profiles did not vary significantly between our two samples; perhaps, this was a function of our sampling procedure. Also, with the varying sizes of animals that we subsequently worked with, many of which were too small to easily separate, our standard procedure is to pool all soft tissues. The outer surfaces of the tissues are decontaminated by swirling in 200 mg/L sodium hypochlorite for about 30 seconds. This is done to eliminate, or greatly reduce the influence of bacteria that are likely to be on the surfaces, which intuitively would be the same as the fluid sample. The soft tissues are homogenized in an equal amount (weight/volume) of sterile 0.1 % peptone and 0.05 % yeast extract (pep-ye) for 1-2 minutes in a stomacher (e.g. Model 80, Seward Medical, London, UK). Fluids and tissue homogenates are diluted ten-fold through the 10^{-3} or 10^{-4} dilution in pep-ye and standard volumes of each dilution are used to drop inoculate bacteriological media. Media used for primary isolation varies with the specific need and can be basic general growth media or differential and selective media designed for specific pathogens. This protocol has since

been shown to be very effective for producing single, isolated bacterial colonies for enumeration and subsequent transfers, and it has been sensitive for primary isolation of specific pathogens in laboratory studies. Equally important, there have been no problems with fungal contamination on primary isolation plates, which is a very prevalent problem in field sampling that precludes evaluation of bacterial growth and isolation of representative colonial morphologies.

When the idea of introducing mussels to hatcheries for propagation was first devised, the assumption was that mussels would be fish pathogen vectors; however, to that point, a report of a fish pathogen being isolated from a mussel did not exist. Studies were specifically designed in an attempt to isolate a fish pathogen from mussels (Starliper et al., 1998; Starliper and Morrison, 2000). *Flavobacterium columnare*, the cause of columnaris disease to many cool and warmwater fishes, was isolated from *Amblema plicata* taken from the Ohio River, adjacent to Wood County, WV (Starliper et al., 1998). This was the first report of isolation of a fish pathogen from feral mussels and confirmed the suspicions of fishery managers that mussels collected from open water systems could indeed harbor fish pathogens.

THE BACTERIAL FLORA OF MUSSELS AND DEPURATION

The dynamic nature of the bacterial flora was studied (Starliper et al., 1998; Starliper and Morrison, 2000). The results of these studies offered our first insight as to whether the 30-day quarantine for zebra mussels could also serve to allow mussels the opportunity to depurate fish pathogens, thus preventing them to act as vectors. The bacterial flora of mussels was examined before and after their exposure to a new and different water supply. It was shown that the bacterial loads or the total number of colony

forming units (cfu) per mL of fluid or per gram of soft tissues was stable; however, the profiles of the bacterial species isolated from animals were very responsive to the water change. Large-scale changes to the bacterial profiles occurred within 24 hours, which were encouraging data that hinted at their potential to depurate pathogens. In fact, when the previously mentioned isolation of *F. columnare* was made from an *A. plicata* (Starliper et al., 1998), this animal had been taken directly from the Ohio River and assayed. Cohorts were collected at the same time and location and were placed in NFHRL flow-through, specific pathogen free spring water. In a study to evaluate the effect of the water change on the bacterial flora, we examined our first group of animals after 24 hr. Sampling of other groups continued on various days through day 30. No other isolations of *F. columnare* were made beyond that initial isolation. However, our isolation of *F. columnare* did demonstrate that mussels could harbor fish pathogens. More important, our failure to isolate the pathogen after the water change demonstrated that mussels could depurate fish pathogens, and after only 24 hrs in the water at NFHRL.

Throughout the 1990's, the number of rivers affected by zebra mussels was increasing and within affected rivers there was an imminent and advancing wave of zebra mussel infestations. The prognosis for survival of the native animals ahead of this threat was poor. For many of those populations that contained few numbers and perhaps had already experienced mortality due to other causes, there was the likelihood for extirpation and this scenario was being repeated in other rivers. Often, the only option to save local populations was to relocate them to refugia (e.g. White Sulphur Springs NFH) with the goal of future restoration or population augmentation. At that time, no data existed on bacterial pathogens specific to mussels and on potential pathogens that mussels might be

exposed to when placed at the hatchery. A study was done to determine if any of the bacteria resident in water and on fish at White Sulphur Springs NFH might be pathogenic to mussels. The predominant flora was isolated from the mucus of 100 rainbow trout (*Oncorhynchus mykiss*); 15 bacterial isolates were grown in pure cultures and used in waterborne challenges to groups of mussels. Additional challenges were also done using two recognized bacterial pathogens of fish, *Aeromonas salmonicida* and *Renibacterium salmoninarum*, a Gram-negative and a Gram-positive, respectively. Challenges with up to 1×10^6 cfu/mL and for up to 24 hours exposure were done and no mortality or obvious adverse effects, i.e. weak and slow valve closures in response to stimuli, were noted in the observation periods that followed the challenges. However, we were successful to reisolate *A. salmonicida* from that group of mussels in the observation period and shortly after challenge; this finding proved to be a pivotal result as it led to the eventual development of a model to study pathogen contagion/transmission between fish and mussels. The model also provided an excellent tool to study pathogen depuration dynamics in mussels. Through our development of the model, we found that *A. salmonicida* is readily transmissible between fish and mussels through cohabitation in the same tanks and via the water column. Thus far, we have only used *A. salmonicida* with the model because some salmonid species are extremely susceptible to this pathogen (e.g. calculated LD_{50} values of less than one cell per fish) and they would provide a very sensitive bioindicator.

The model requires the establishment of an (artificial) acute epizootic of furunculosis. Either brook trout (*Salvelinus fontinalis*) or Atlantic salmon (*Salmo salar*) are injected intraperitoneally with viable *A. salmonicida* cells (in a range of 10 and 1,000

cells per fish). Once these injected fish begin to die (typically within a week, even using a low dosage of cfu), non-injected (clean) fish are added to the tank. The clean fish will become infected naturally via horizontal transmission within 2 weeks. Once the clean fish begin to die and *A. salmonicida* is confirmed as the cause of death, mussels are added to the tank to cohabit with the fish. Mussels uptake *A. salmonicida* cells shed by the fish during the epizootic. During this time of cohabitation, clean fish are periodically added to maintain peak fish mortality in the tank and therefore, maximum shedding of cells made available to the mussels. Within 2-4 weeks, bacterial culture for *A. salmonicida* from the mussels will reveal a 100 % prevalence of infection. Mussels were then moved out of the cohabitation (epizootic) tank and placed in tanks supplied with pathogen free, flow through water; this initiates depuration. These 'pathogen-primed' mussels allowed us to study pathogen transmission and vectoring dynamics. In our studies thus far, we demonstrated that depuration over a time relative to the quarantine for zebra mussels (30 days) was sufficient for the mussels to rid of *A. salmonicida*. We have shown that *Amblema plicata* (and another species; data not shown) successfully rid of the pathogen within the time for quarantine, and at two different water temperatures, 13°C and 20°C. We considered depuration to be complete when *A. salmonicida* was not isolated from the mussels or their tank water, and bioindicator fish remained clean. It is important to note that this model resulted in a maximum pathogen uptake by mussels from 'naturally' diseased fish and this could be considered a worst case scenario. Although mussels collected from rivers can be infected with a (fish) pathogen, they would not be exposed to the high challenge dose of pathogenic cells as produced from our challenge model. Therefore, it is possible that the duration of the quarantine would be even more effective

for depuration, possibly within less time given the much lesser number of cells at the start. This is encouraging for fishery and mussel resource managers. These studies do, however, bear repeating with actual mussel pathogens, should they be identified, to determine if transmission between mussels can be prevented. Examples where this data would be important include a study of pathogen transmission of mussels from rivers to captive reared populations at refugia, and vice-versa.

Other studies on mussels at the NFHRL have been done or have been initiated to evaluate disease risks to mussels and to develop disease preventative measures for mussels held in captivity and to non-captive populations. Our initial studies focused more on the risks of pathogen vectoring to fish. But, as mussel conservation activities continue to increase, particularly relocation, propagation, and stocking of hatchery-reared animals, the need for studies that focus on development of mussel disease preventative methods becomes more apparent. Although there are practically no reports of infectious agent diseases of mussels and pathogen transmission among wild and captive animals, the possibility that these issues could become important is also apparent. Much has been written on these same pathogen and disease issues on fishes, for example on pathogenicity and transmission, on geographic ranges, host susceptibilities, etc. Furthermore, much has been learned about disease preventative measures in fishes. Because fish and mussels share an aquatic medium environment, a reasonable assumption can be made that some of these same disease issues might become more relevant to mussel resource managers in the future. Current projects at the NFHRL address two principal research needs. One is to identify causes of mussel dieoffs that occur in some rivers and the other is to develop nonlethal sampling techniques. Within the past several

years there has been a substantial increase in mussel population surveys in terms of numbers of surveys being conducted and the number of persons conducting those surveys. Natural mussel dieoffs in certain rivers have been observed and in large part, the causes for these dieoffs have not been identified. Some of the data collected during dieoffs have been previously associated with dieoffs in fish epizootics. For example, mussel dieoffs at certain geographic locales occur seasonally, which is usually related to a change in water temperature; varying degrees of host specificity have been noted and in at least one instance, mortality has been primarily to gravid females of one species, presumably explained by spawning stressors.

The NFHRL's primary strategy to investigate the role of infectious agents in mussel dieoffs is to conduct periodic sampling throughout a mussel collection season and determine the bacterial flora of nondiseased animals in that affected part of the river. This will provide a picture of the expected, normal bacterial flora which will allow us to recognize suspected bacterial pathogens present in moribund animals during a dieoff. In addition, at the same time as a dieoff, healthy cohorts from an adjacent regions of the river not experiencing mortality will be evaluated. In the diseased mussels, we will be using bacterial culture and based on experiences with dieoffs in fish, we will be looking for pure cultures of the same bacterium from most of the mussels examined. This data will be combined with observations of the previously mentioned parameters, i.e. seasonality, host specificity, etc. The importance of this work is once a pathogen of mussels is known, then periodic health inspections can be conducted, similar to what is done by the U.S. Fish and Wildlife Service for hatchery reared fish. Determining the presence of a pathogen and control-prevention strategies can be implemented. This will

greatly reduce the risk of introductions of pathogens to refuges, and back to native populations stocked as a part of restoration or population augmentation activities.

The current procedure for primary isolation of bacteria from mussels calls for sacrificing the animals. Clearly, methods that require sacrificing threatened and endangered species is unacceptable. This method is only good for those animals occurring abundantly and these are presently not recognized candidates for conservation activities. A study was recently begun to assess selected sample sites collected from mussels through nonlethal and nondestructive methods for pathogen screening. Studies are being done to ensure that the procedures, themselves, to collect the samples do not cause obvious detrimental affects to mussels. Related to this, we are employing the pathogen exposure method and *A. salmonicida,* as previously described, to compare the prevalence of isolation of this pathogen by bacterial culture of nonlethal sites to those of standard lethally collected sites.

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