Starliper 2006

To: Glenn Holcomb
U.S. Geological Survey
Office of Eastern Region Biology
11700 Leetown Road
Kearneysville, WV 25430

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Subject: Final Report for FY2004 Quick Response Project titled: Imperiled freshwater mussels: The need for research to prevent the introduction and transmission of pathogens and diseases. This project was supported by Region 5 for $32,400.

This research was conducted by:
Dr. Clifford Starliper, principal investigator, National Fish Health Research Laboratory
John Coll, US Fish and Wildlife Service Project Officer, Lamar Fish Health Unit, Northeast Fisheries Center
Dean Rhine and Patricia Morrison, US Fish and Wildlife Service, Ohio River Islands National Wildlife Refuge
Dr. Catherine Gatenby and Julie Devers, US Fish and Wildlife Service, White Sulphur Springs National Fish Hatchery

Final Report Prepared and Submitted by:
Dr. Clifford E. Starliper
Submitted on Review September 2006.
U.S. Geological Survey
Leetown Science Center
National Fish Health Research Laboratory
11649 Leetown Road
Kearneysville, WV 25430

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Products. At this time, products from this project are in various stages of preparation. The PI directed an undergraduate internship from a part of this project. He will present an oral presentation at the annual meeting of the West Virginia Academy of Science, April 2007. Two other meeting abstracts are presented on Pages 15-16; these currently are going through the LSC review processes. A draft review manuscript on the PI's mussel studies, including some of the information from this project has been written and is also in LSC review. Two additional manuscripts will be prepared in the near future that will

focus on objectives 1 and 2 in this project and will include previous related work by the PI.

Study Objectives and Results. Key findings relative to each of the three primary study objectives, as listed in the original study plan.

Objective #1. Develop methodology to examine and evaluate mussels for presence of pathogens without the need to sacrifice or harm them.

Results #1. In this report, from this point forward, we define our use of the term 'nonlethal' to also mean non-harmful or non-destructive, so the sampling process produces only minimal impact to the animal. In our initial studies here, we identified three 'candidate' nonlethal sites that will allow us to determine presence of a pathogen. Hemolymph, nonlethal fluid and mantle clip were selected because we thought each would have the least detrimental impact on the animals' subsequent health and survival. Furthermore, we selected these sites because we thought there would be a high probability that they will provide for isolation of pathogens and perhaps, comparable to that from lethal collections of fluids and tissues.

We did a preliminary study to evaluate the effects of the sample collection procedures alone. (Four) Groups of mussels, 10 *Fusconaia ebena* per group, were tested. We observed for any mortality after taking each of the three sites (fluid, hemolymph, mantle) individually and after taking all three samples from each mussel. After samples were taken, we placed the animals back into tanks fed with flow-through spring water (13°C) and observed them for three weeks. During this time, two mussels from the hemolymph bled groups died (2/20). We do not feel that this mortality was significant and was likely due to our taking too much hemolymph. In the two hemolymph groups, we bled up to 3 mL from a mussel, which far exceeds an amount necessary for bacterial culture or other assays. In our subsequent experiments for bacterial culture, we bled 0.4 mL per animal.

We used the pathogen transmission model developed by the PI (Starliper 2001) to introduce *Aeromonas salmonicida* to *Fusconaia ebena* and sampled by bacterial culture for the pathogen during depuration by the mussels. We compared our results for recovery of *A. salmonicida,* comparing nonlethal collected hemolymph, fluid, and mantle clip to lethal collection of fluid and total soft tissues. Our results were very encouraging for identification of a nonlethal site (see Tables 2 and 3). Nonlethal fluid was the best of the three nonlethal sites and was at least as good as the lethal collected sites. Equally as important, collection of nonlethal fluid involves no tissue invasive process and therefore, is innately the least stressful to mussels; mantle involves cutting the tissue and hemolymph involves a puncture with a needle. The PI is currently conducting further studies on these sites as part of another Region 5 Quick Response project.

To collect nonlethal fluid, we disinfected the outer shell surfaces with 100 ppm chlorine and gently pried open the valves, to about 5-10 mm apart at the anterior margin. We held

the animal over a sterile Petri plate with the open valves facing down and clean-caught the fluid that came out. With some mussels, we had to open and close the valves 2-5 times to 'milk' fluid from the animal. Even with relatively smaller animals, we had no problem in obtaining at least 0.5 mL or so, which is was adequate for our dilutions and plating. Our other two sites also require opening the valves. Hemolymph was collected from an adductor muscle, we used either side, which ever was the most easily accessible. We used 1 cc syringes and 22 G needles (1 – 1.25 inches long). About 0.4 mL of hemolymph was drawn. Several methods were attempted to remove the small piece of tissue, a scalpel, scissors and forceps were tried in various combinations. The easiest seemed to be to pinch a portion of the outer margin of the mantle using forceps with a twisting-pulling outward motion; this method also required the least opening of the valves. From Table 1, our mantle samples averaged less than a tenth of a gram. We did not disinfect the outer surface of the mantle tissue, as we did when we took all soft tissues in the lethal method. So, the isolation of *A. salmonicida* from mantle could have actually been influenced by mantle surface contamination with fluid. We chose not to disinfect because in a real situation we wouldn't because we would want the best chance to isolate a pathogen.

Looking at the results in Tables 2 and 3, nonlethal fluid clearly shows the most promise as an alternative to lethal collections. Not only do we feel it is the least stressful for the animals to collect, our results on recovery of *A. salmonicida* demonstrate that this site is at least equal to lethal collection of fluid or the other tissue sites. Hemolymph was essentially ineffective. This was not too surprising because *A. salmonicida* is not a pathogen to *F. ebena* and it would not be expected to become systemic (i.e. bacterimia-like). With a systemic pathogen of mussels, hemolymph would certainly be worthwhile to visit in future studies. Our results with fluid, either lethal or nonlethal, corroborates previous work by the PI that 'fluid', which is inside the valves, but outside the soft tissues, is indicative of (mussel) internal microbial flora and is not simply a sample of water from their environment.

Additional points can be made from our observations. First, *A. salmonicida*, our bacterium of interest, was quickly depurated by the mussels. There was a two-log decrease in pathogen load from day 0, which contained up to 2.92×10^5 cfu/g, to day 5, which had the highest load in two nonlethal fluids at 2.07×10^3 cfu/mL. And the number of positives significantly decreased during this time. We had demonstrated in previous studies how quickly the bacterial flora of mussels changes, relative to environment (water) changes. And second, we demonstrated successful recovery of our target pathogen using a nonlethal procedure; we feel this is a milestone result.

Table 1. Physical measurements and data on *Fusconaia ebena* from a study comparing lethal vs. nonlethal sample collection for isolation of the fish pathogen *Aeromonas salmonicida*. Sampling on Days: 0, 5, and 9 relative to moving to clean tanks (initiation of depuration). Each value shown is the mean for six mussels. NL=nonlethal; g = grams; mm = millimeters; mL = milliliters; F,M,H = fluid, mantle, hemolymph (all three sites collected from each mussel); n/a = result doesn't apply.

Day-Site	total wt, g	Length, mm	Width, mm	Depth, mm	Tissues, g (%)[1]	Fluid, mL (%)[1]	Mantle wt, g
0-Lethal	95.7	60.5	54.0	35.5	9.83 (10.36)	10.67 (11.14)	n/a
0-NL fluid	155.3	73.5	64.3	44.8	n/a	4.23	n/a
0-NL mantle	127.6	69.0	59.3	41.5	n/a	n/a	0.037
0-NL hemolymph	133.3	70.3	61.2	41.0	n/a	n/a	n/a
0-NL F,M,H	160.9	75.5	65.7	43.8	n/a	3.38	0.047
5-Lethal	88.6	61.3	55.7	34.0	10.57 (12.02)	8.88 (10.12)	n/a
5-NL fluid	112.9	65.2	58.0	37.2	n/a	4.01	n/a
5-NL mantle	126.2	68.3	60.5	38.5	n/a	n/a	0.082
5-NL hemolymph	145.4	75.2	65.8	42.8	n/a	n/a	n/a
5-NL F,M,H	157.9	77.8	68.3	44.7	n/a	5.03	0.063
9-Lethal	75.6	57.8	52.7	32.7	8.19 (10.84)	7.35 (9.71)	n/a
9-NL fluid	78.3	56.3	51.7	32.5	n/a	1.98	n/a
9-NL mantle	83.7	60.2	55.0	33.0	n/a	n/a	0.062
9-NL hemolymph	88.7	60.5	54.3	35.7	n/a	n/a	n/a
9-NL F,M,H	107.9	65.2	58.7	41.2	n/a	3.52	0.077

[1]mean percent of total soft tissues weight (w/w) and collected fluid volume (v/w) comprising the total animal weight.

Table 2. Primary isolation of *Aeromonas salmonicida* from *Fusconaia ebena* relative to initiation of depuration[1]. Combine the data from this Table with the data from Table 1 for additional information. See footnote for an example[1].

Day 0 Site	No. *A. sal* positives of n = 6 *F. ebena*	Mean *A. sal* cfu/g or mL[2] for positives only	Mean cfu/g or mL[2] total bacteria for n = 6	Mean % *A. sal* cfu of total cfu (positives only)
L tissues	6	2.92×10^5	9.99×10^5	24.06
L fluid	6	6.34×10^4	1.91×10^5	15.90
NL fluid[3]	6	4.08×10^4	1.28×10^5	9.47
NL mantle[3]	5	1.64×10^3 (i.e. for n = 5)	3.92×10^5 (for n = 6)	3.85 (for n = 5)
NL hemolymph[3]	1	2.00×10^1	4.44×10^2	4.35
3 NL fluid[4]	6	6.86×10^3	3.79×10^4	9.42
3 NL mantle[4]	5	5.06×10^3	1.48×10^5	4.77
3 NL hemolymph[4]	0	0	1.43×10^3	0

Day 5 Site	No. *A. sal* positives of n = 6 *F. ebena*	Mean *A. sal* cfu/g or mL[2] for positives only	Mean cfu/g or mL[2] total bacteria for n = 6	Mean % *A. sal* cfu of total cfu (positives only)
L tissues	0	0	1.52×10^6	0
L fluid	2	6.66×10^2	1.52×10^5	1.81
NL fluid	1	4.00×10^3	3.18×10^5	13.65
NL mantle	0	0	1.14×10^4	0
NL hemolymph	0	0	6.87×10^2	0
3 NL fluid	2	2.07×10^3	7.94×10^4	9.43
3 NL mantle	2	2.64×10^1	2.01×10^4	0.14
3 NL hemolymph	0	0	6.79×10^3	0

Table 2. Continued.

Day 9 Site	No. *A. sal* positives of n = 6 *F. ebena*	Mean *A. sal* cfu/g or mL[2] for positives only	Mean cfu/g or mL[2] total bacteria for n = 6	Mean % *A. sal* cfu of total cfu (positives only)
L tissues	0	0	2.12×10^5	0
L fluid	0	0	2.60×10^4	0
NL fluid	1	1.32×10^3	7.65×10^4	0.47
NL mantle	0	0	6.42×10^4	0
NL hemolymph	0	0	2.16×10^3	0
3 NL fluid	0	0	9.85×10^3	0
3 NL mantle	0	0	4.35×10^2	0
3 NL hemolymph	0	0	5.40×10^3	0

[1]An example of combining the information in both tables would be to estimate the total number of viable *A. salmonicida* cells within one *A. plicata* by multiplying the mean cfu/g of L tissues by the mean total weight of tissues, then repeating the procedure for fluids and summing the two numbers.
[2]cfu/g applies to weighed samples: Lethal collected soft tissues (L tissues) and nonlethal collected mantle clips; cfu/mL applies to lethal and nonlethal fluid samples and hemolymph.
3collection of a single site from one animal and six animals per group for a total of 18 *F. ebena*
4collection of all three sites from one animal for a total of 6 *F. ebena*

Table 3. Overall summary of results. Comparison of lethal vs. nonlethal sampling for recovery of *Aeromonas salmonicida* from *Fusconaia ebena*. Number of *A. salmonicida*-positive mussels/the number of mussels assayed. This summarizes the results that went to form Table 2. Days are relative to moving the mussels to clean tanks to begin depuration; therefore, day 0 is the day they were moved. On that day 0, the mussels assayed by bacterial culture were removed from the pathogen-source tank and the remaining mussels were immediately moved to the clean tanks.

	A. salmonicida detected by lethal sampling. Soft tissues plus fluid	*A. salmonicida* detected by nonlethal sampling. One site per *F. ebena*	*A. salmonicida* detected by nonlethal sampling. Three sites from each *F. ebena*
Day 0	6/6 (six positive fluids)	12/18 (six nonlethal fluids; only one hemolymph positive	6/6 (six NL fluids; no hemolymphs positive)
Day 5	2/6 (two fluids)	1/18 (one NL fluid)	3/6 (two NL fluids)
Day 9	0/6	1/18 (one NL fluid)	0/6

Objective #2. Evaluate the effectiveness of the required 30-d minimum quarantine period (for zebra mussels) for allowing native mussels to depurate pathogens.

Results #2. Using the pathogen transmission model and *A. salmonicida*, we infected *F. ebena* and evaluated depuration over a 30-day period, which corresponds with a required 30-day quarantine of wild-caught animals for inspection of zebra mussels. Previously, similar studies were done by the PI using *Amblema plicata* at 13°C that were not fed and *F. ebena* at 20°C that were not fed. In this trial, we evaluated the effect of feeding on depuration by *F. ebena*, at 13°C; we intended to evaluate at 20°C, but our spring water heating system broke towards the end of our feeding acclimation period and because of high cost, could not be repaired. We had previously found very little difference in depuration at the two temperatures, except for slightly quicker infectivity and depuration at the higher temperature. One way of thinking is the lower temperature created a worst-case scenario, so we were comfortable with 13°C water temperature for this study. In this current study, mussels had not completely depurated *A. salmonicida* at 15 days (see Tables 5 and 6), but had cleared the pathogen by 30 days. Our overall result here, which was the fact that after a 30-day quarantine, mussels were no longer a source of the pathogen to fish corroborated results obtained from the two previous studies. Our results are encouraging in that they demonstrate mussels held in quarantine can conceivably rid of pathogens to a level to greatly reduce the risk of introducing pathogens to refuges or to wild populations during stocking. Furthermore, quarantining in pathogen-free water appears to greatly diminish introduction of pathogens to fish. It should be noted that *A. salmonicida* is not a pathogen to mussels; it was chosen for use in our model to study the dynamics of bacterial flora changes in mussels in response to depuration. It will be important to demonstrate similar responses to depuration using other pathogens, particularly pathogens to mussels. Our methodology provides a useful model for these future evaluations.

Prior to our establishment of the *A. salmonicida* epizootic in brook trout (*Salvelinus fontinalis*) which would serve as the source of the bacterium to *F. ebena,* we assayed fish and mussels by bacterial culture to ensure they were free of *A. salmonicida*. Total soft tissue(s) homogenates and fluids from ten mussels and mucus and kidneys from ten brook trout and twenty Atlantic salmon (*Salmo salar*) were examined. Colony forming units (cfu) were determined for total bacteria and suspect (blue) *A. salmonicida* colonies of mussel tissues and fluids. Suspect colonies were characterized further using standard biochemical tests. We determined that all of these mussels and both brook trout and Atlantic salmon were negative for *A. salmonicida*. Bacterial culturing was done as described in the study proposal (attached).

Mussels were acclimated to a feeding protocol prior to the start of the experiment; the added food is in addition to the normal resident bacterial flora in the laboratory spring water supply and the detritus produced from feeding the brook trout and salmon. Algal cultures were prepared at the White Sulphur Springs National Fish Hatchery, (WV). Cultures were grown in a continuous culture (chemostat) system (Biofence™; Varicon

Aqua Solutions, U.K.) at inside ambient air temperature. The medium was an F/2 Guillard's formulation called Microalgae Grow Mass Packs™ (obtained through Aquatic Ecosystems, Apopka, Florida). Algal species fed were *Neochloris oleoabundans*, *Oocystis* spp., and *Bracteococcus grandis*. Depuration and cohabitation with bioindicator fish was done in 144-L flow through tanks. Our feeding protocol was as follows: a-the water supply valves to each tank were turned off; b- algal culture was poured into each tank and mixed; c-after 4 hr, the water supply valves were turned on. The calculated amount of culture added to each tank yielded 40,000 algal cells per mL of tank water. The 4 hr timeframe was generally selected (after discussions) as a compromise between enough time for *F. ebena* to feed while maintaining optimal DO for mussels and fish. Water flow to tanks was about 4-5 L per minute.

Isolation of *A. salmonicida* from tank water was accomplished by centrifuging 1 L of water (30 min @ 5,000 x g, 4°C), pouring off most of the supernatant and resuspending the pellet in the minimal amount of water left. Ten-fold dilutions of this were made and drop plated onto CBB plates. Colonies were converted into cfu/mL of tank water.

Results of the depuration experiment are summarized in Tables 5 and 6. The epizootic in fish to serve as the source of bacteria to mussels was established by IP injecting 12 brook trout (about 2 per pound) with *A. salmonicida* cells; six fish received 7.60×10^5 while the others received 7.60×10^4 cfu/fish. Within 4 days (13°C), 8 had died and about 50 clean (uninoculated) fish were added to the tank to become infected through cohabitation with the diseased fish. Ten days after the fish were IP injected, the first clean fish died and after *A. salmonicida* was confirmed, by culture, as the cause of death to this fish, 160 *F. ebena* were added to cohabit with diseased fish. Over the next three weeks, mortality to the fish continued and clean fish were periodically added to maintain a rate of mortality and maximum *A. salmonicida* shedding into the water column so the mussels could acquire a maximum number of cells via filtering. After the three weeks, 10 *F. ebena* were assayed for *A. salmonicida* infectivity and our desired 100 % prevalence level (10/10 *F. ebena* were *A. salmonicida* positive) was met. The remaining mussels were removed from the infection source tank and placed into five clean 144-L tanks (13°C; 30 mussels per tank) that were supplied with pathogen-free, flow through water, 4-5 L per minute. At this time, the depuration period commenced. After 1 day of depuration, 15 *F. ebena* were cultured (fluids and tissue homogenates) for *A. salmonicida*, 1 L of tank water was assayed and 50 bioindicator Atlantic salmon (about 10 cm long) were added to cohabit with the remaining (15) mussels. This process was repeated for the additional selected days through day 30. We considered complete *A. salmonicida* depuration by the mussels when three criteria were met: 1- *A. salmonicida* was not isolated from any of 15 *F. ebena* assayed; 2- *A. salmonicida* was not isolated from bioindicator Atlantic salmon; and 3- *A. salmonicida* was not isolated from tank water. Attempts at isolation of *A. salmonicida* from mussels and water are point-in-time evaluations, therefore, whether the pathogen is isolated or not depends solely on presence of the pathogen at that time and at a level above a minimum sensitivity of the bacterial culture assay. On the other hand, the bioindicator fish are in constant contact with the same water column as the mussels, so

there is a greater likelihood that the fish will become infected if *A. salmonicida* is being shed by the mussels.

Prevalence of *A. salmonicida* after 1 day of depuration remained at 100 % (Tables 5 and 6)), but the cfu/g of tissues and the cfu/mL of fluids decreased by more than 10-fold and 1,000-fold, respectively. Prevalence of *A. salmonicida* positive mussels remained the same at 5 days and again, cfu loads decreased more than 10-fold in tissues and fluids compared with those at 1 day. Beyond 5 days, we did not isolate *A. salmonicida* from the mussels or from the water. However, the bioindicator fish of day 10 and day 15 became infected with the pathogen and mortality commenced; *A. salmonicida* was biochemically confirmed as the cause of mortality to these fish.

At 30 days, depuration was complete. We did not isolate *A. salmonicida* from the mussels, the fish or the water. The end point for isolation of *A. salmonicida* in this study differed slightly with that found from a previous study where animals were not fed algae (Starliper 2001). In the previous study, the pathogen was not detected at 15 days or beyond. However, with both studies depuration had been completed within the 30 day quarantine and we consider this difference in endpoints to be insignificant as both studies yielded encouraging results, particularly when considering the high *A. salmonicida* cell loading density in mussels (about 2.24×10^7 cfu/g in tissues) at the start and at the 100 % prevalence level. This level of infectivity at the start can be considered a worst case scenario and we consider it highly unlikely for animals collected from open water sources, e.g. rivers and streams, to be infected with the pathogen at this high of a cell load.

The results from our first two objectives offer insight as for the future importance of quarantining and pathogen screening to prevent pathogen transmission from wild populations to resident, captive-reared imperiled mussels, and perhaps more important, control of pathogen transmission via stocking of captive-reared animals to augment wild populations. Presently, 15 federal and state mussel propagation facilities are rearing a suite of species for stream stocking (Jones et al. 2006). Captive propagation of mussels will certainly increase as more is learned about husbandry of these animals and as their fish hosts are identified. Although our results are considered somewhat preliminary, they set forth models that can be used to evaluate primary or opportunistic mussel pathogens if, or as, they are described.

Table 4. Physical data of experimental *Fusconaia ebena*, fed an algal culture diet and used to evaluate *Aeromonas salmonicida* depuration. Values are means for the number of animals examined.

	Number Examined	Length (mm)	Width (mm)	Depth (mm)	Total Wt. (g)	Volume Fluid (mL)	% fluid of total wt (v/w)	Wt Soft Tissues (g)	% tissues of total wt (w/w)	Valve Inner Area (cc)
Pre-Exam for *A. sal.* negative	10	71.80	61.80	40.40	143.74	12.45	8.65	9.45	6.61	25.30
A. sal. prevalence	10	68.40	58.90	39.70	129.33	6.59	4.95	8.60	6.64	23.80
After 1 day depuration	15	71.60	61.33	41.07	146.09	10.24	7.13	9.74	6.71	25.13
After 5 day depuration	15	71.40	61.60	40.27	141.07	12.13	8.62	9.61	6.77	27.80
After 10 day depuration	15	74.20	64.07	41.73	146.97	10.79	7.36	10.80	7.37	28.63
After 15 day depuration	15	69.00	60.60	40.47	125.95	10.20	8.14	8.78	7.00	29.27
After 30 day depuration	15	69.33	58.07	39.60	129.63	10.08	7.80	9.06	7.07	25.20

Table 5. Depuration of *Aeromonas salmonicida* by algal diet fed *Fusconaia ebena*: number of *A. salmonicida* positive *F. ebena* soft tissue homogenates and paired *F. ebena* fluids. Viable cell count data (cfu/g or cfu/mL) are the means for the number of animals assayed by bacterial culture onto CBB plate medium.

Purpose for Sampling:	No. *A.sal*-pos tissues / No. tested (%)	cfu/g *A.sal* in soft tissue homogenates	Total cfu/g in soft tissue homogenates	No. *A.sal*-pos fluids / No. tested (%)	cfu/g *A.sal* in fluids	Total cfu/g in fluids
Initial check: Ensure *A. sal.*-free	0/10 (0)	No cfu	1.21×10^6	0/10 (0)	No cfu	1.91×10^5
Prevalence check	10/10 (100)	2.24×10^6	3.63×10^6	10/10 (100)	3.40×10^7	3.41×10^7
After 1 day depuration	13/15 (86.7)	1.43×10^5	1.18×10^6	14/15 (93.3)	1.20×10^4	6.73×10^4
After 5 day depuration	12/15 (80)	1.39×10^4	1.47×10^5	12/15 (80)	1.00×10^3	1.92×10^4
After 10 day depuration	0/15 (100)	No cfu	3.33×10^4	0/15 (100)	No cfu	6.19×10^3
After 15 day depuration	0/15 (100)	No cfu	1.61×10^5	0/15 (100)	No cfu	7.53×10^4
After 30 day depuration	0/15 (100)	No cfu	5.37×10^5	0/15 (100)	No cfu	1.24×10^5

Table 6. Overall summary of algal fed *Fusconaia ebena* depuration of *Aeromonas salmonicida* through 30 days.

Bacterial Culture Sampling on CBB plate medium:	Was *A. salmonicida* isolated from *F. ebena* (tissues or fluids)? positives/assayed	Was *A. salmonicida* isolated from tank water?	Did bioindicator Atlantic salmon become infected with *A. salmonicida*?
After 1 day depuration	Yes, 15/15[1]	Yes, 1.25×10^2 cfu/mL tank water	Yes, with mortality and reisolation/confirmation of *A. salmonicida*
After 5 day depuration	Yes, 15/15[1]	Yes, 6.80×10^0 cfu/mL tank water	Yes, with mortality and reisolation/confirmation of *A. salmonicida*
After 10 day depuration	No	No	Yes, with mortality and reisolation/confirmation of *A. salmonicida*
After 15 day depuration	No	No	Yes, with mortality and reisolation/confirmation of *A. salmonicida*
After 30 day depuration	No	No	No

[1] In this Table, a mussel is positive if *A. salmonicida* was isolated from either the tissue homogenate or the fluid sample. This contrasts with the entries in Table 5.
For example, in Table 5 after 1 day there were 13/15 tissues and 14/15 fluids positive and in Table 6, we show 15/15 mussels positive. This means that for those two negative tissues, their paired fluids were positive.

Objective #3. Develop a laboratory challenge procedure to determine the effects of potential pathogens to mussels.

Results #3. We developed and tested waterborne (bath) challenge protocols for exposure of mussels to bacteria. We were unable to produce obvious stress or disease, or mortality with any of the challenges, even with up to 24 hr exposures with about 1×10^5 cfu/mL of tank water. This was the case with controls using uninoculated media, or from any of the various bacteria we exposed groups of mussels to. We evaluated isolates of environmental aquatic flora isolated from fish and two of the primary fish pathogens, *Aeromonas salmonicida* and *Renibacterium salmoninarum*. We believe our lack of success to produce mortality was due to not using a recognized bacterial pathogen of freshwater mussels. Until a pathogen to mussels is isolated, we do not believe that we will be capable to produce morbidity or mortality by artificial challenge procedures.

Our studies did provide us with information which can be employed in the future. To summarize, mussels are able to withstand fairly significant waterborne exposures. We believe waterborne challenge will be the accepted test exposure method. Injection challenges are injurious and invasive and are not as relevant as a method for exposure to mussels as with fish. With fish, injection can be viewed as similar to a natural injury such as rubbing against a rock or debris, which breaks the body surface barrier; this is not so with hard shell bivalves.

With another Quick Response project the PI is conducting, the PI recently isolated a bacterium that is a suspected pathogen to *F. ebena* from Pickwick Lake on the Tennessee River, in Alabama, Mississippi and Tennessee. We are currently characterizing isolates cultured from apparently diseased animals. With many, high cfu/g viable cell concentrations with this pathogen were isolated in pure culture. Once this bacterium is characterized, we will be using the protocols that we tested in this project for challenge studies with the other project.

Nondestructive Recovery Of *Aeromonas Salmonicida* From Challenge-Infected Ebonyshell Mussels (*Fusconaia Ebena*).

Clifford E. Starliper.

USGS National Fish Health Research Laboratory, 11649 Leetown Road, Kearneysville, WV 25430.

As captive-rearing of imperiled mussels expands, the detection and prevention of pathogen-induced diseases could become relevant. Determining the prevalence of pathogens is an initial step towards disease prevention. With imperiled fauna, the sampling procedures used must be nondestructive. In this study, the recovery of *Aeromonas salmonicida* from ebonyshell mussels (*Fusconaia ebena*) using nondestructive sampling (fluid, mantle clip, hemolymph) was compared to the recovery obtained using lethal methods (total fluid and soft tissues). *Aeromonas salmonicida* was introduced to the mussels using a pathogen transmission model. In an initial study, groups of *F. ebena* were observed for short-term (up to 3 wks) mortality resulting from the nondestructive sampling procedures. No mortality attributed to the procedures occurred. Other groups of *F. ebena* were infected with *A. salmonicida* (100% prevalence), placed in tanks for depuration and sampled over time to compare bacterium recovery obtained through the nondestructive vs. lethal sampling. Fluid and mantle sampling provided *A. salmonicida* recovery prevalences comparable to those obtained from lethal sampling. Hemolymph sampling was found to be ineffective. Fluid sampling offers the most promise. This method is the least invasive (stressful) to mussels and previous studies have shown the bacterial flora of fluid reflects the flora from within soft tissues.

EXAMINING MUSSELS FOR BACTERIAL PATHOGENS USING NONLETHAL METHODS, Clifford Starliper, USGS Leetown Science Center, Kearneysville, WV 25430.

Prevention of diseases caused by infectious agents is critical for captive propagation of aquatic animals. As captive-rearing of imperiled mussels expands, the detection and prevention of pathogen-induced diseases could become relevant. A preventative strategy will greatly reduce pathogen transmission between reared and wild populations. Determining the presence and prevalence of pathogens is an initial step towards prevention. With this imperiled fauna, any sampling must be nonharmful. In this study, recovery of the fish pathogenic bacterium *Aeromonas salmonicida* from ebonyshell mussels (*Fusconaia ebena*) using nondestructive sampling (fluid, mantle clip, hemolymph) was compared to the recovery obtained using lethal methods (total fluid and soft tissues). The three nondestructive sites were selected because their use was thought to have the least impact on mussels' subsequent health and survival, and because the prevalence of pathogen isolation was suspected to be comparable to that obtained from lethal sampling. *Aeromonas salmonicida* was introduced to the mussels using a pathogen transmission model. In an initial study, groups of *F. ebena* were observed for short-term (up to 3 wks) mortality due to the sampling procedures, themselves. No mortality attributed to the sampling procedures occurred. Other groups of *F. ebena* were infected (100% prevalence) with *A. salmonicida*, placed in tanks for depuration and sampled, over time, comparing the nonlethal to lethal sites for recovery of the bacterium. Fluid accounted for *A. salmonicida* recovery prevalence as good as either lethal site. Mantle clip was comparable to fluid; hemolymph was ineffective. Fluid sampling offers the most promise because it is the least invasive (stressful) to mussels and previous studies have shown the bacterial flora of fluid to correlate with the flora from within soft tissues.

PROJECT PLAN

LEETOWN SCIENCE CENTER
NATIONAL FISH HEALTH RESEARCH LAB

NUMBER: 01091 (amended October 22, 2003)

TITLE: Imperiled freshwater mussels: the need for research to prevent the introduction and transmission of pathogens and diseases.

BACKGROUND AND JUSTIFICATION:

About 68-69 % of the (297) mussel species native to North America are considered imperiled (Williams et al. 1993). The U.S. Fish and Wildlife Service (USFWS) is now significantly involved in a number of freshwater mussel conservation projects, including propagation of threatened and endangered species for reintroduction and stock enhancement of dwindling populations, and species preservation. The Service's work on conservation of mussels is national in scope and is particularly relevant to those USFWS Regions with the greatest diversity in mussel populations and species richness, Regions 3, 4, and 5. There are a number of causes for the dwindling native mussel populations including waterway related construction, loss of riparian boundaries and the invasion of the zebra mussel *Dreissena polymorpha* (Ellis, 1936; Fuller, 1974; Gillis and Mackie, 1994; Hebert et al., 1991; Kat, 1982; Keller and Zam, 1990; Nalepa, 1994; Ricciardi et al., 1996; Schloesser, et al. 1996). Because these mussels originate from open waters (e.g. Ohio, Clinch, Holston Rivers) there is great risk for introduction of pathogens and diseases to the imperiled mussels, which may already be maintained at a refuge/facility. Furthermore, there is likely an even greater risk to vector/introduce fish pathogens to resident hatchery fish. As well, we do not understand the disease risks that hatchery propagated mussels pose to wild mussels when released into streams as part of the restoration efforts. There is a critical need for research to address issues related to pathogens, diseases, and the nature of vectoring from mussels to fish, and mussels to mussels. This information is pivotal to prudent health and disease management plans that must be developed for each fish hatchery considering propagation of mussels. Disease and mortality prevention is critical for the successful conservation of imperiled mussel species at hatcheries and in the wild. Most importantly, objectives reached through this project include development of methodologies and technology that will be transferred to USFWS fish health centers for implementation, thereby protecting fish and mussels propagated within the FWS Fish Hatchery System.

INFORMATION NEEDS TO BE ADDRESSED:

Imperiled mussels are being propagated at a number of USFWS and State refugia and this activity is likely to increase significantly. Imperiled species includes those listed within the Endangered Species Act. This proposal and forthcoming results will be applicable to

all mussel species, including wild populations. This proposal describes research that is proactive and preventative in nature; in other words, as we embark on species restoration of mussels, we want to get a head start on many of the anticipated disease problems by understanding how we can prevent them. As the USFWS develops and expands its mussel propagation programs, Fish Health Centers need the appropriate technology development to adequately address current and future needs.

OBJECTIVES/HYPOTHESES:

The proposal objectives listed here are in succession to studies done by the PI to date on the major issue of conservation of native freshwater mussels. These objectives reflect current USFWS research needs of resource managers.

1) Develop methodology to examine and evaluate (listed and non-listed) mussels for presence of pathogens without the need to sacrifice or harm them.

2) Evaluate the effectiveness of the required 30-d minimum quarantine period (for zebra mussels) for allowing native mussels to depurate pathogens

3) Develop a laboratory challenge procedure to determine the effects of potential pathogens to mussels.

PROCEDURES:

Methods, techniques and Experimental design.

Native mussels for this project will be collected by diving and/or brailing. They will be quarantined for a minimum of 30 d at the quarantine facility at the Ohio River Islands National Wildlife Refuge, and then transported to the NFHRL. A specific algae diet for mussels will be produced at White Sulphur Springs; this hatchery is already culturing algae to feed mussels being propagated there. The NFHRL is designed specifically for research of infectious and pathogenic diseases of aquatic species e.g. fishes and mussels. The lab has complete wet and dry lab facilities. Water is spring sourced; flow through with a constant temperature of 12-13°C. All effluent water is decontaminated. Previous publications by the PI describe the methodology for isolation of bacteria from mussels and a model to infect mussels with *A. salmonicida* so pathogen transmission can be studied (Starliper et al. 1998; Starliper and Morrison 2000; Starliper 2001). These publications will serve as the basis to meet the objectives in this proposal. Mussels will be fed and maintained in optimal conditions for all studies. For objective #1, mussels will be exposed to *A. salmonicida* until a 100% positive prevalence, and then transferred to tanks supplied with specific pathogen-free water and allowed to begin depuration. Mussels will be examined for re-isolation of the pathogen at various times during depuration. Tissues will be collected by non-lethal and compared to the "traditional" method of sacrificing the animals. The effect upon the animals of the non-lethal

collection process, itself, will be evaluated. For objective #2, mussels will be exposed to the pathogen to 100% positive prevalence and relocated to tanks supplied with pathogen-free water. At predetermined times; mussels will be assayed for presence of *A. salmonicida* by bacterial culture and cohabitation with fish susceptible to the bacterium to serve as a bioindicator. Depuration will be considered successful when *A. salmonicida* is not reisolated from mussels, water or the sentinel fish. For objective #3, the PI has isolated two bacterial species suspected to have been involved with mussel epizootics in the Holston River. However, no current methodology exists for in-vivo disease reproduction, a requisite to confirm a true pathogen. Methodology will be developed to expose healthy mussels and reproduce clinical signs or mortality.

Isolation of Bacteria

Primary bacterial culture of *A. salmonicida* from fish and mussel tissues will be done following Starliper et al. (1998) and Starliper (2001). Mucus and kidney tissues from each fish will be homogenized and serially ten-fold diluted in 0.1% peptone-0.05% yeast extract (pep-ye) and 0.025 mL drops from each dilution will be placed on the surface of tryptic soy agar supplemented with 0.01% coomassie brilliant blue (CBB). Presumptive *A. salmonicida* colonies will be enumerated and confirmed according to standard biochemical identification techniques (MacFaddin 1980; Koneman et al. 1992). Depending on the size of the mussels, two or three samples will be collected from each mussel for isolation of the pathogen, pallial fluid, gut and ot; for mussels that are too small, soft tissues will be combined. Physical data will be collected from each, and the outer shell surfaces will be gently scrubbed using 200-ppm sodium hypochlorite. The valves will be opened and the pallial fluid will be collected. All soft tissues will be excised and portioned into gut, which will be comprised primarily of digestive tract and ot, which will contain the balance of the soft tissues. Each of the gut and ot tissue samples will be surface disinfected by a 30 sec dip in 200-ppm sodium hypochlorite followed by rinsing in pep-ye. Then, each will be homogenized and diluted in pep-ye; quantities of each dilution will be used to inoculate CBB plates. Presumptive, blue *A. salmonicida* colonies will be enumerated and confirmed as previous. Resulting bacteria will be quantified by enumeration and reported as colony forming units (cfu) per gram of tissue or mL of fluid.

DATA:

The PI will be responsible for collection and maintenance of data. Records, file folders, laboratory notebooks, computer disks and other pertinent forms of data will be identified by title or project plan number. Deviations from the approved project plan will be brought to the attention and agreed upon by the investigator and laboratory director; work will proceed with approval. If warranted, a written record of the change will be made using the LSC project plan amendment form.

Critical data and Analysis

Origin and physical data of collected mussels, total bacterial counts, fish and mussel mortality, viable cell enumeration from bacterial analyses and bacteriological characterizations will comprise the primary data. Raw bacterial counts will be converted to cfu/g or cfu/mL. Basic statistical treatment e.g. means, minimum, maximum, etc. will summarize data. If the PI deems necessary, significant differences will be determined with various tests including t-tests (ANOVA) and Tukey's method for mean differences.

Acceptance/Rejection Criteria

Non-lethal tissue collection that results in mortality of mussels will constitute unacceptable methodology.

Location of Data

Data will be kept in the office and laboratory of the principal investigator.

LOCATION:

Wet and dry laboratory work will be done at the NFHRL. Data analyses and preparation of reports, manuscripts, and presentations will be done at the NFHRL. Field mussel collections and algae culture will be done by the cooperators/partners (refer to the cooperators/partners section).

SCHEDULE:

October 2003 - September 2005: Collect and analyze data. Preparation of reports, manuscripts and presentations for scientific (i.e. mussel and fisheries) meetings will be ongoing.

September 2005 – September 2006: Complete data analyses and preparation of reports and manuscripts.

DURATION OF STUDY:

Begin: November 1, 2003.

End: September 31, 2006.

SAFETY:

The principal investigator and biological laboratory technician are aware of the safety room, the laboratory safety manual and have completed the required training. No electro

shocking, isotopes or hazardous chemicals will be used in this study. Generally, bacteria encountered are not human pathogens, but it is standard practice in the investigator's laboratory to handle all bacteria as if they are. This cautious approach also leads to quality control of the bacterial studies by maintaining purity and avoiding contamination and thus, maximizing productivity. Spent bacteriological media and cultures will be destroyed by steam sterilization. The PI has read and understands material safety data sheets for reagents and chemicals to be used in the study. Lab coats and gloves will be worn as necessary.

ANIMAL WELFARE:

Spent fish and mussel carcasses will be destroyed via incineration. Clean-stock and experimental fish and mussels will be cared for and maintained as per laboratory guidelines and under the purview of the laboratory's animal welfare officer.

COOPERATORS/PARTNERS:

The USFWS Service Project Officer is John Coll, Director, Fish Health Unit, USFWS Region 5, Northeast Fisheries Center. PO Box 155, Fish Hatchery Road, Lamar, Pennsylvania 16848.

Support to receive money for this project came from three FWS Regions, including Ecological Services, Fisheries and Refuges. The partners for this research project are Dr. Catherine M. Gatenby, Project Leader, USFWS White Sulphur Springs National Fish Hatchery, Dean Rhine, Manager, USFWS Ohio River Islands National Wildlife Refuge and Shane Hanlon, USFWS Southwest Virginia Field Office. The specific roles of the partners will be the collection, quarantine, and providing of native freshwater mussels and an algal diet to maintain optimal health of the animals. The FWS partners' expertise in the location of mussel beds in the Ohio River and proper husbandry of the captive animals is essential to this project. There are three distinct objectives listed for this project and because each involves the use of a primary fish pathogen, *Aeromonas salmonicida*, and model (described by the PI), the experiments must be completed at the NFHRL, a facility designed to accommodate research involving viable pathogens. The USFWS partners will provide the aforementioned efforts as both in-kind services and using supplies and expendables, etc. necessary bought using funding for this project.

KEY STAFF AND BUDGET:

Dr. Clifford E. Starliper

2004 Region 5, USFWS FY04 SSP. Funded at $32,400.

EXPECTED PRODUCTS:

The PI maintains informal contact (i.e. phone calls and email) with many of the Federal and State persons and agencies that work in this arena. So progress and results will be widely known, as the PI's previous mussel-pathogen work has. The PI has a record of disseminating results and technical assistance.

Depending on the outcome of this research, at least one manuscript will be prepared for submission to a peer-reviewed journal. The results will be presented at professional meetings such as those of the Freshwater Mussel Conservation Society and Eastern Fish Disease Workshop. Reports will be submitted as required.

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