REPORT DRAFT ON PROJECT PROGRESS
March 29th, 2011
Covering June 2009 through June 2011

Combating Viral Hemorrhagic Septicemia and Improving Yellow Perch Aquaculture for the Great Lakes Region

CRIS Project Number: 3655-31000-020-00D
(Resources are split between ARS and SCA cooperators)

Research Management Unit: U.S. Dairy Forage and Aquaculture Research Unit/MWA/ARS/USDA

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I. Research Plan

The project goal is to investigate yellow perch (Perca flavescens) broodstock genetic lines for increased resistance to viral hemorrhagic septicemia virus (VHSv) through experimental challenges. The technologies and resources developed from this project will support innovation and improvement in perch aquaculture through the four following objectives: 1) further develop research tools to identify important biochemical pathways that underlie growth and susceptibility to the invasive fish pathogen viral hemorrhagic septicemia virus (VHSv), 2) undertake growth and disease genotype x environment studies to identify genotypes that show improved growth and VHSv resistance, 3) conduct physiological studies to characterize and identify genes affecting growth and immunity, and 4) develop a rapid, cost-effective, method for detecting VHSv and for characterizing the immune system in perch.
II. Team Personnel Roles and Activities

A. **Erin Crawford**, Technician (Dr. Willey) – Assisting in the development and optimization of primers and internal standards for fish housekeeping genes beta, actin, EF1-alpha, 18s RNA, RPL1, and target gene VHSv (F5R5)-all strains; PCR amplified RNA from target fish species and EPC cell lines for linearity comparisons between assays; Researching positive strand amplification for new replicating assay; Participated in USDA-APHIS phone conference call for StaRT-PCR development May 2010, and continuing collaborations.

B. **Lindsey Pierce**, Ph.D. candidate (Dr. Stepien) – Developed and optimized primers and internal standards for fish housekeeping genes beta-actin, EF1-alpha, 18s RNA, RPL1, and target gene VHSv (F5R5)-all strains; Extracted, reverse transcribed, and PCR amplified RNA from several fish species; Interfaced with muskellunge challenge program at Michigan State University and collected infected samples; Researched positive strand amplification for new replicating assay; Worked on detection and quantification of VHSv in juvenile muskellunge tissues from Michigan State University’s immersion challenge; Collaborated with Michigan DNR to sample and test gizzard shad for potential VHS outbreak (negative result); Presented VHS project results at AFS Sept. 2010, Lake Erie Fishery Genetics and Stock Structure Workshop for Managers Dec. 2010, Ohio State Annual Research Review Jan. 2011, 51st Annual Ohio Fish and Wildlife Management Conference Feb. 2011, 2nd Annual Midwest Graduate Symposium March 2011, Sigma Xi Oct. 2009, Midwest Fish and Wildlife Managers Association Feb. 2009, USDA Annual meeting in Washington D.C. with Dr. Stepien Dec. 2009; IAGLR May 2010; Made updates to VHS webpage; Twine Line interview for VHS information March 2010 with Dr. Stepien; Participated in USDA-APHIS phone conference call for StaRT-PCR development May 2010, and in ongoing collaborations.

C. **Vrushalee Palsule**, Technician (Dr. Stepien) – Hired Oct. 18, 2010 to accomplish VHS project laboratory objectives; Completed laboratory safety, biohazard disposal, and biosafety level 2 training; Extracted, reverse transcribed, and PCR amplified RNA from several fish species; Assisted in the development and optimization of primers and internal standards for fish housekeeping genes EF1-alpha, 18s RNA, RPL1, and target gene VHSv (F5R5)-all strains; Worked on detection and quantification of VHSv in juvenile muskellunge tissues from Michigan State University’s immersion challenge; Assisted Ph.D. student Pierce with gizzard shad sampling and testing from die-off at Mac-Ray Bay (negative result).

D. **Douglas Murphy**, Technician (Dr. Stepien) – Worked on DNA microsatellite discrimination among test strains of yellow perch to examine VHSv resistance; IACUC project paperwork; VHS lab work, supply ordering and primer design, VHSv fieldwork, lab work, and reporting assistance.

E. **Dr. Carol Stepien**- PI, Oversees all project activities; Organized and hosted the Lake Erie Fishery Genetics and Stock Structure Workshop for Managers that included VHS project work; Gave oral VHS presentation at the International Conference on Aquatic Invasive Species, Aug. 2010 in San Diego CA, presented results at annual American Society of Ichthyologists and Herpetologists annual conference July 2009 in Portland OR and at USDA CSREES/NIFA biannual aquaculture conference in
Washing D.C. Dec. 2009, and VHS patent presentation to University of Toledo board members Dec. 2009; Developed ongoing collaborations with the University of Wisconsin Milwaukee through visit and presentations Nov. 2009; Collaborating with Dr. Zhang of Ohio Department of Agriculture to test our VHS diagnostic; Collaborating with Dr. Mohamed Faisal of Michigan State University to conduct VHS challenge test experiments; Twine Line Sea Grant interview for VHS information and article publication March 2010; Participated in USDA-APHIS phone conference call for StaRT-PCR development and ongoing communications; Overseeing all grant writing and reports, plus all scientific papers and communications.

**Dr. James Willey**-coPI, Developed the actively replicating molecular biology test and supervised lab work; Holds the patents for the StaRT-PCR process being used; Presented VHS presentation to the University of Toledo board members for patent Dec. 2009; Participated in USDA-APHIS phone conference call for StaRT-PCR development May 2010 and ongoing communications; Supervises Erin Crawford and Ph.D. committee member for Lindsey Pierce.

**F. Dr. Douglas Leaman**-coPI, provided VHSv-IVb infected EPC and BF-2 cell pellets to the Willey lab for StaRT-PCR comparison; trained Ph.D. student Pierce in using real-time PCR; Ph.D. committee member for Lindsey Pierce; Assisted with protocol development for testing of StaRT-PCR and quantification of virus.

### III. Progress/Accomplishments to Date (in order of over-all project objectives):

**Objective 2:** Characterize critical pathways involved in growth and VHSv resistance in yellow perch through gene expression and physiological studies.

- **Sub-objective 2.A.** Develop transcriptomic sequences to support investigations on biochemical pathways involved in growth and VHSv susceptibility (Goetz, Shepherd and Stepien).

1. Primers have been designed for housekeeping genes beta-actin (F1R1) (F2R2) (F3R3), EF1-alpha (F1R1), 18s RNA (F1R1), RPL1 (F1R1) (F2R2), disease resistance/immune-responsive genes Vig 2 (F1R1) (F2R2) (F3R3), Complement component C3 (F1R1) (F2R2) (F3R3), leukotriene b4 (F1R1) (F2R2) (F3R3), interleukin 8 (F1R1) (F2R2), and target gene VHSv-all strains (F1R1) (F2R2) (F3R3) (F4R4) (F5R5) and VHSv-IVb specific strain (F1R1) (F2R2) (F3R3). Two or more sets of primers were designed to ensure informative results with the goal of choosing only one primer pair for each. To date, beta-actin (F1R1), EF1-alpha (F1R1), 18s RNA (F1R1), and VHSv-all strains (F5R5) were chosen for use in future experiments.

2. Primers to synthesize internal standards have been designed for housekeeping genes beta-actin (CT1), EF1-alpha (CT1) (CT2), 18s RNA (CT1), RPL1 (CT1) (CT2), and VHSv-all strains (CT4) (CT5). To date, beta-actin (CT1), EF1-alpha (CT2), 18s RNA (CT1), and VHSv-all strains (CT5) were chosen for use in future experiments.

3. New standardized mixture of internal standards (SMIS) was developed which includes beta-actin, EF1-alpha, 18s RNA, and VHSv5-all strains.
4. New SMIS is currently being validated via testing for true and relative accuracy, linearity, specificity, inhibition, and sensitivity.

- **Sub-objective 2.B.** Characterize the (adaptive and innate) immune response of perch exposed to the Midwest VHSV strain via disease challenge (Goetz, Leaman and New SY).
  1. We cloned three of the VHSV IVb proteins into eukaryotic expression vectors for expression in fish cells. Each is tagged with an appropriate epitope tag for expression analysis, and has been expressed in a variety of cell lines, including EPC fish cells and 293 human embryonic kidney cells.
  2. We acquired expression plasmids for fish IFN and fish MAVS to study the role of these pathways in cellular detection and resistance to VHSV. Both function fully in fish cells (EPC and BF2) and we have since demonstrated that both can confer upon fish cells absolute resistance to VHSV, thereby emphasizing the critical role of the IFN system in innate immunity to VHSV.
  3. We assessed the abilities of several VHSV-IVb proteins to perturb innate immune response pathways. We have demonstrated that IFN pretreatment of cells fully blocked VHSV replication. However, infection of cells followed by treatment with IFN 24 h later did not prevent VHSV replication or cytopathicity. This suggests that the virus can adversely impact cellular response to IFN to circumvent the antiviral mechanism and allow it to replicate even in immunocompetent animals.
  4. To assess the potential roles of VHSV M, P and NV proteins in this process, we performed a variety of cellular assays in which these viral proteins were expressed in conjunction with activators of the innate immune response (either MAVS or IFN), and downstream responses monitored by target promoter/luciferase constructs or endogenous gene expression. Results indicated that both M and P proteins inhibited cellular responses, while NV appeared to augment innate immune response.
  5. Results were extended to viral replication studies, which indicated that co-expression of M or P enhanced viral cytopathic effects and led to decreased IFN production by cells, consistent with our luciferase data. Our current results suggest that M protein is a general inhibitor of cellular transcription, while P may impact one or more specific step(s) in the response pathway. Studies aimed at identifying the sites of action of M (RNA transcription versus subcellular transport or stability of transcripts) are in progress, as is assessment of its putative pro-apoptotic function. P studies are a bit behind, but will focus on obvious sites of interaction, such as the innate immune pathways blocked by P proteins from other rhabdoviruses.
  6. Others have described NV protein as a “virulence factor” based on the observation that deleting it from the viral genome leads to impaired viral replication. However, when expressed in isolation, it appears to behave as a PAMP (pathogen associated molecular pattern) to stimulate the immune response. We are currently deciphering whether it is the protein or transcript that augments innate immune responses, by comparing the effects of coding versus non-coding versions of the mRNA in cells. Subsequent studies will be aimed at integrating these observations into NV’s potential role as a virulence factor (versus an indirect impact of its deletion) and whether this might be an important regulator of how strongly the host responds to VHSV rather than how effectively the virus replicates.
Objective 4: Develop and evaluate challenge assays, detection tools and vaccines for protecting yellow perch and other Great Lakes region species of fish from VHS.

• Sub-objective 4.A. Continue to develop a standard VHS disease challenge model to characterize the disease state (mortality, viral titer and histopathology) in yellow perch following VHSv challenge (Goetz, Stepien, Willey and New SY).

We are testing yellow perch (*Perca flavescens*) spleen and kidney tissues using our newly developed StaRT-PCR assay to detect presence/absence and quantify viral molecule number of VHSv in experimentally infected fish.

1. Primers were designed and optimized by us for housekeeping genes beta-actin (F1R1) (F2R2) (F3R3), EF1-alpha (F1R1), 18s RNA (F1R1), and target gene VHSv-IVb (F1R1) (F2R2) (F3R3) and VHSv-all strain (F1R1) (F2R2) (F3R3) (F4R4) (F5R5). Three housekeeping genes were incorporated for greater accuracy when quantifying VHSv. To date, beta-actin (F1R1), EF1-alpha (F1R1), 18s RNA (F1R1), and VHSv-all strains (F5R5) were chosen for use in future experiments.

2. Primers to synthesize internal standards were designed for housekeeping genes beta-actin (CT1), EF1-alpha (CT1) (CT2), 18s RNA (CT1), RPL1 (CT1) (CT2), and VHSv-all strains (CT4) (CT5). To date, beta-actin (CT1), EF1-alpha (CT2), 18s RNA (CT1), and VHSv-all strains (CT5) were chosen for use in future experiments.

3. Internal standards for housekeeping genes and VHS gene N (target gene) were developed and optimized. Primers and internal standards were designed to measure all strains of VHS.

4. Primer set was tested with human viruses vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV), fish rhabdovirus infectious hematopoietic necrosis virus (IHNV), and various VHSv isolates Ia: F-1, 07-71, KRRV, IVa: Makah, Orcas, Bogachiel, Cod 91, Elliott Bay, Obama, and IVb: MI03 for specificity (i.e., will only detect VHSv). Results indicate VHSv-all strains primer is specific for VHSv, showing amplification of all VHS strains.

5. We successfully extracted, reverse transcribed, and PCR amplified RNA from several fish species: white perch (*Morone americana*), smallmouth bass (*Micropterus dolomieui*), round goby (*Neogobius melanostomus*), muskellunge (*Esox masquinongy*) and yellow perch (*Perca flavescens*). We additionally extracted, reverse transcribed gene-specific reverse transcription, and PCR amplified RNA from muskellunge (*Esox masquinongy*) and cell culture pellets from epithelioma papulosum cyprini (EPC) infected cell lines.

6. Sensitivity testing of the primers is currently underway. Thus far, we have measured down to 10 molecules of internal standard in a PCR reaction over 11 orders of magnitude. We are continuing to conduct experiments to test analytical characteristics of StaRT-PCR.

• Sub-objective 4.B. Develop and validate a standardized reverse transcriptase polymerase chain reaction (StaRT-PCR) test to quantify live, replicating VHS virus that is transmissible among infected fish (Stepien and Willey).

We are developing key molecular genetic tools with the quality control necessary to detect the virus not only rapidly but also reliably and accurately. Additionally, this test will have the ability
to distinguish between actively replicating virus (i.e., is transmissible). This objective by our team is headed by co-PI Dr. James Willey, with technician Erin Crawford and Ph.D. candidate Lindsey Pierce (the latter is from Dr. Stepien’s laboratory).

1. Research has been conducted to determine which strand of each of our primer sets (forward or reverse) amplifies the positive VHSv strand. Positive strand primers were selected for gene-specific reverse transcription to detect active virus. To date, beta-actin (F1R1), and VHSv-IVb (F2R2) were chosen for use in future experiments, with the forward primer amplifying the positive strand in each primer set. An alien tag sequence was added to the VHSv-IVb R2 primer to use in a strand-specific reverse transcription reaction. VHSv-IVb F2 primer and a primer (R#) corresponding to the tag sequence were used for PCR amplification and detection.

2. We successfully extracted, reverse transcribed, and PCR amplified RNA from several fish species: white perch (*Morone americana*), smallmouth bass (*Micropterus dolomieui*), round goby (*Neogobius melanostomus*), muskellunge (*Esox masquinongy*) and yellow perch (*Perca flavescens*). We additionally extracted, reverse transcribed using gene-specific reverse transcription, and PCR amplified RNA from muskellunge (*Esox masquinongy*) and cell culture pellets from epithelioma papulosum cyprini (EPC) infected cell lines.

3. *In vitro* transcription was used to generate RNA fragments of the plus and minus strand of VHSv-IVb to use as controls for the development of the active test. Specificity and sensitivity experiments are underway along with optimization of the strand-specific reverse transcription and PCR conditions.

I. Scientific Meetings

**Research Presentations and Posters(*) at National, Local, and International Conferences** that acknowledge our grant support: **Genetic Detection and Geographic Analysis of Great Lakes Fish Infection by Viral Hemorrhagic Septicemia Virus (VHSv)**

1. **International Association of Great Lakes Research** annual conference in Duluth, MN (May 2011): “A Rapid Genetic Test for the VHS Fish Virus and Viral Load from Laboratory Challenge Experiments” by Lindsey Pierce, James Willey, Mohamed Faisal, Robert Kim, and Carol Stepien (platform presentation by Pierce).
3. **Ohio Department of Agriculture Aquatic Animal Health** meeting in Reynoldsburg, OH (April 2011): “Genetic Detection and Geographic Analysis of Great Lakes Fish Infection by Viral Hemorrhagic Septicemia Virus (VHSv)” by Carol Stepien, James Willey, Douglas Leaman, and Jonathan Bossenbroek (platform presentation by Palsule and Pierce).
4. 2nd **Annual Midwest Graduate Symposium** annual conference in Toledo, OH (March 2011): “A Rapid Genetic Test for the VHS Fish Virus and Viral Load from
Lindsey Pierce, Erin Crawford, Vrushalee Palsule, James Willey, Mohamed Faisal, Robert Kim and Carol Stepien (platform presentation by Pierce). Won best student paper award.

5. **Ohio Fish and Wildlife Managers Association** annual conference in Columbus, OH (February 2011): “Results of unique RT-PCR test for detecting VHS viral infections in fish” by Lindsey Pierce, James Willey, Erin Crawford, Douglas Leaman and Carol Stepien (platform presentation by Pierce).

6. **Lake Erie – Inland Waters Annual Research Review** in Columbus, OH (January 2011): “A rapid genetic test for the VHS fish virus and viral load from laboratory challenge experiments” by Lindsey Pierce, James Willey, Mohamed Faisal, Robert Kim and Carol Stepien (platform presentation by Pierce).

7. **Lake Erie Fishery Genetics and Stock Structure Workshop for Managers** held at the Lake Erie Center in Oregon, OH for Ohio and Michigan Lake Erie federal and state managers (December 2010): “Unique RT-PCR Test with Internal Controls to Detect, Quantify and Distinguish Active VHSv in Fish” by Lindsey Pierce, James Willey, Erin Crawford, Douglas Leaman and Carol Stepien (platform presentation by Pierce).

8. **American Fisheries Society** annual conference in Pittsburg, PA (September 2010): “Unique RT-PCR test for replicating viral hemorrhagic septicemia virus, with internal controls” by Lindsey Pierce, James Willey, Erin Crawford, Douglas Leaman and Carol Stepien (platform presentation by Pierce). Selected as one of 20 to compete for best student paper award. Won 2nd place.

9. **International Conference on Aquatic Invasive Species** annual conference in San Diego, CA (August 2010): “Unique RT-PCR test for replicating viral hemorrhagic septicemia virus, with internal controls” by Carol Stepien, Lindsey Pierce, James Willey, Erin Crawford, and Douglas Leaman (platform presentation by Stepien).


13. **Sigma Xi** Scientific Honorary Society at the University of Toledo (October 2009): “Viral Hemorrhagic Septicemia (VHS) Immersion Challenge in Juvenile Muskellunge Using StaRT-PCR: A Quantification Study” by Lindsey Pierce, Carol Stepien, James Willey, and Erin Crawford (platform presentation by Pierce).

Controls for Detecting the VHS Fish Virus” by Carol Stepien, Lindsey Pierce, James Willey, and Erin Crawford (Poster presented by Stepien).

15. **Ohio Fish and Wildlife Management Conference**, Columbus, OH (February 2009): “Developing a Rapid Molecular Assay with Internal Controls for Detecting the VHS Fish Virus” by Lindsey Pierce, James Willey, Erin Crawford, and Carol Stepien (Poster presented by Pierce).

VI. Publications  (* ARS project team members*)

Publications:


Sepulveda-Villet, O.J.* and Stepien, C.A.* Fine-scale population genetic structure of Lake Erie yellow perch *Perca flavescens*: Any relation to management units? *Canadian Journal of Fisheries and Aquatic Sciences* (in re-review)


Newsletters:


Abstracts:

Pierce, L.R., Willey, J, Faisal, M., Kim, R., Stepien, C.A. 2011. A Rapid Genetic Test for the VHS Fish Virus and Viral Load from Laboratory Challenge Experiments. International Association of Great Lakes Research, Duluth, MN.

Sieracki, J., Bossenbroek, J. 2011. Modeling the Spread of Viral Hemorrhagic Septicemia Virus (VHSv) by Within Great Lakes Shipping. International Association of Great Lakes Research, Duluth, MN.

Pierce, L.R., Palsule, V., Willey, J., Faisal, M., Kim, R., Stepien, C.A. 2011. A Rapid Genetic Test for the VHS Fish Virus and Viral Load from Laboratory Challenge Experiments. 2nd Annual Midwest Graduate Symposium, Toledo, OH.

Pierce, L.R., Willey, J., Crawford, E., Leaman, D., Stepien, C.A. Results of a unique RT-PCR test for detecting VHS viral infections in fish. Ohio Fish and Wildlife Managers Association, Columbus, OH.


Pierce, L.R., Willey, J.M., Crawford, E.L., Leaman, D., Stepien, C.A. 2010. Unique RT-PCR Test with Internal Controls to Detect, Quantify and Distinguish Active VHSv in Fish. Lake Erie Fishery Genetics and Stock Structure Workshop for Managers, Toledo, OH.


Pierce, L.R., Willey, J.M., Crawford, E.L., Leaman, D., Stepien, C.A. 2011. Results of unique RT-PCR test for detecting VHS viral infections in fish. Ohio Fish and Wildlife Managers Association annual meeting, Columbus, OH.


Pierce, L.R., Crawford, E.L., Willey, J.C., Stepien, C.A. 2009. Developing a Rapid Molecular Assay with Internal Controls for Detecting the VHS Fish Virus. Ohio Fish and Wildlife Managers Association Conference, Columbus, Ohio.

V. Awards

Lindsey Pierce
- Best Woman in STEMM award: $100 at the 2nd Annual Midwest Graduate Symposium conference
- 2nd Best Oral Presentation at the 2nd Annual Midwest Graduate Symposium conference
- National Science Foundation Doctoral Dissertation Improvement Grant (NSF-DDIG): $15,000 to study VHSv phylogenetics (under C. Stepien, P.I.)
- American Fisheries Society (AFS) nominee for best paper to compete for best student presentation (one of only 20)
- American Fisheries Society (AFS) best presentation award (honorable mention)
- International Association for Great Lakes Research Scholarship award: $2,000
- Sigma Xi Scientific Honorary Society best student poster award

VI. Planned Workshop and Conference Dissemination Output

A. Website: VHS website for the project was developed by Ph.D. candidate Lindsey Pierce at the Lake Erie Center stating our current work and progress at http://www.utoledo.edu/as/lec/research/glgl/VHS/VHS_main.html. The website is updated as needed.

B. VHS Session: Dr. Stepien plans to co-host a special VHS session at a future International Association of Great Lakes Research (IAGLR) or American Fisheries Society (AFS) conference

C. We hope to send Adam Pore, an MS student supported by the grant, to a conference to present his data.