Final Report

"Fishing" for Cytokines and Immune Molecules to Better Understand Pallid Sturgeon Health

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INTRODUCTION

Iridoviral infection has hindered pallid sturgeon restoration efforts during the last decade. This is primarily the result of decreased numbers of stockable fish and approved stocking sites. During September of 2006, an iridovirus outbreak occurred at the USFWS– Bozeman Fish Technology Center (BFTC) that affected endangered pallid sturgeon and their close relatives the shovelnose sturgeon. In an attempt to better understand the host immune response to the virus specific to this outbreak, samples were collected and archived for anticipated future research. The archived tissue samples included spleen samples for mRNA analysis; pectoral fins for electron microscopy; cardiac muscle, liver, fin and barbels for histopathology; and blood smears. The archived samples specifically utilized for this investigation were the spleen samples for mRNA analysis, and fixed fins and cardiac tissue for histological analysis. Analysis of these samples has benefited the restoration efforts by clarifying aspects of sturgeon anatomy and physiology associated with iridoviral infection, innate and cell-mediated immunity, hematology and viral pathogenicity and overall sturgeon health.

The overall goal of this project was to evaluate hematological, innate and adaptive immunological parameters that provide a better understanding of iridovirus infection in pallid sturgeon. To accomplish this goal, we 1) identified cytokines and iridovirus induced immune related molecules in sturgeon, and optimized quantitative methods to evaluate their expression; 2) determined the association of stress, the immune response (including immune-associated gene expression) and iridovirus pathogenicity in sturgeon and 3) compiled data obtained from objectives 1 and 2 and performed statistical analysis to determine potential correlations between heterophil function assays, immune gene expression assays and histopathology.

A previous, unpublished characterization of sturgeon blood cells did not evaluate functional properties of leukocytes (Jenkins 2003). The immunological study of sturgeon leukocytes (WAPA 2006) revealed that the white cell function in sturgeon is uniquely different from that of modern teleosts. For instance, white blood cells functionally characterized as neutrophils were cytochemically similar to heterophils, a white blood cell found in birds. Thus, a sturgeon's immune system is notably different than that of modern boney fishes. This observation emphasizes that a sound understanding of

sturgeon immunological health can only be achieved by specifically studying sturgeon immune responses.
From a management perspective, this work has lead to the development of valuable tools that can be used during routine health exams and future outbreaks. For instance, in most vertebrates specific immune related molecules are produced in a predictable sequence during a virus outbreak. Thus, discovery and characterization of such molecules in sturgeon could lead to diagnostic tools that would identify the early, peak or recovery stage of a virus epizootic. An adult could be tested prior to spawning for specific immune molecules to determine immune function and stress level. The scope of this project fulfills the needs of the 10 Year Strategic Plan of I.1.c., pertains to items 7.3, 7.5-7.8 (Quist et al 2004) and recommendation 41 of Webb et al 2004. The Army Corp of Engineers funded this project for $25,000 in July of 2007.

MATERIAL & METHODS

Sample collection – Clinically infected shovelnose sturgeon were defined as those exhibiting erratic swimming patterns, petechial hemorrhages and the presence of iridovirus infected (eosinophilic) cells in histological analysis. Subclinical fish were defined as those lacking the clinical and histological signs, but positive for iridovirus via PCR (MacConnell, personal communication). The clinical fish were reared in 22 °C water. The subclinical sturgeon were at 16°C. Experimental fish were quickly netted from tanks, euthanized with an overdose (500 mg mL-1) of MS-222. Sturgeon head kidney tissue was aseptically dissected from 80 fish via ventro-lateral incision, pooled (10 fish) and suspended in Hank's balanced salt solution (HBSS) without calcium, magnesium and phenol red. Pectoral fins, internal organs, and barbels were placed in tissue cassettes, preserved in Davidson's fixative for 48 hours and transferred to 70% ethanol. Spleens were collected from iridovirus asymptomatic and symptomatic shovelnose sturgeon, placed in RNA Later®, and frozen at -80ºC for RNA extraction.
Functional Assays – Kidney tissue was macerated, centrifuged, and separated via a discontinuous gradient to obtain a concentration of heterophils. The heterophils were counted on a hemocytometer and diluted to 20 x 10^6 cells/ml for each sample (Palic et al 2005a).
The QuantiChrom™ Alkaline Phosphatase Assay Kit was used to quantify the degranulation of sturgeon heterophils. Ninety six well plates were used. Samples were run in duplicate. Stimulated wells consisted of 75 µl of HBSS with calcium and magnesium without phenol red, 50 µl calcium ionophore (5 µg/mL final concentration) and 25 µl of sample. Non-stimulated wells consisted of 125 µl of HBSS with calcium and magnesium without phenol red, and 25 µl of sample. The 100 % control wells were 25 µl of sample and 125 µl of Cetyl trimethylammonium bromide (CTAB). Plates were incubated in the dark at room temperature for 20 min, cooled on ice for 2 min and centrifuged at 600 g for 5 min. Fifty µl of sample was added to a new 96 well plate. A working solution of 200 µl assay buffer, 5 µl Mg Acetate, and 2 µl of pNPP (*p*-Nitrophenyl Phosphate) liquid substrate was added to each sample and control well. Water and tartazine were added to 2 blank wells each as assay standards. Plates were read immediately and at 4 min on the BioTek Optical Density reader at 405 nm. Percent degranulation was calculated (Palic et al 2005b).

The Heterophil extracelluar traps (HET's) assay was conducted on 20 x 10^6 cells/ml to determine the release of extracellular DNA. Working solution of SYTOX green consisted of 4 µl of SYTOX green and 996 µl of HBSS with calcium and magnesium without phenol red. A 96 well plate was used to run this assay. Six wells were designated for each stimulated sample consisting of 25 µl of cells, 75 µl of HBSS with calcium and magnesium without phenol red and 50 µl of calcium ionophore. Six wells for each non-stimulated sample consisted of 25 µl of cells and 125 µl of HBSS with calcium and magnesium without phenol red. The plate was incubated for 60 min at room temperature and centrifuged for 5 min at 600 g. Supernatant was poured off and 50 µl of SYTOX green working solution was added to each well. The plate was incubated in the dark for 10 min. The plate was centrifuged for 5 min at 600 g, supernatant was poured off and 100 µl of HBSS with calcium and magnesium without phenol red was added to each well. The plate was spun for 5 min at 600 g, supernatant was poured off and 200 µl of HBSS with calcium and magnesium without phenol red was added to each well. The plate was read on the Perkin Elmer Spectrofluorometer at excitation wavelength of 488, emission 527 and cutoff at 515. Arbitrary fluorescence units (AFU) were calculated (Palic et al 2007).

Molecular Analysis - Spleens from 62 individual fish with clinical or sub-clinical iridoviral infections were processed for RNA extraction. Isolation of total RNA was achieved using RNAeasy® (Qiagen) spin columns according to the manufacturer supplied protocol and pooled to produce a single RNA stock for cDNA library construction. The quality of the RNA sample was evaluated and then shipped to Agencourt for standard cDNA library construction. A cDNA library consisting of approximately 2 x 10^7 clones was produced of which 1795 were screened and sequenced. The resulting nucleotide sequences were entered into the non-redundant protein sequence database using the Blastx program (NCBI). Transcripts were assigned a putative identity based on the best Blastx match of known identity.

Histology of Pectoral Fins & Cardiac Muscle - Pectoral fins were processed through a series of graded ethanol, embedded in parafinn wax, sectioned at 5um and stained with hemotoxylin and eosin. Sections were evaluated at 100 to 400 power magnification. Pectoral fins from 40 clinical and 40 subclinical shovelnose sturgeon were evaluated for virus infected cells and total mature mucus cells were counted. Twenty fins from clinical pallid sturgeon were evaluated by the same method.

Six hearts from iridovirus infected pallid sturgeon and 5 hearts from infected shovelnose sturgeon were selected for the lymphoid tissue quantification. Hearts were measured with a digital caliper, injected with blue elastomer in 3 locations as spatial markers and cut into ten equidistant 7- 8 µm sections for histological analysis. Sections were stained with H&E, and digitally captured using Q-cam Imaging software and Leica MZ16A stereoscope. The lymphoid tissue surface area reconstruction and the ratio of lymphoid tissue and cardiac muscle were used to quantify lymphoid tissue in sturgeon specimens.

Statistical Analysis - Data were analyzed by descriptive statistics, One Way Analysis of Variance and the Mann-Whitney Rank Sum Test. The software SigmaStat Version 3.11.0 (SYSTAT Software, Inc. 2004) was used for analysis. Where differences are stated to be significant, a level of $P < 0.05$ is implied. All assumptions of the statistical tests were achieved.

RESULTS

Functional Assays - There were no significant differences in the alkaline phosphatase degranulation response. The subclinical fish yielded 32-99% degranulation and the clinicals ranged from 25-82%. The heterophil extracellular traps release results revealed no significant differences between the two groups, with values ranging from 1.0 to 2.2 AFU for the subclinical and 1.3 to 2.0 AFU for the clinical group.

Molecular Assays – The spleen cDNA library developed from shovel nose sturgeon clinically or subclinically infected with iridovirus consist of approximately 2 x 10^7 clones with inserts approaching 700 base pairs. Of these clones 1920 colonies were picked and plated for sequencing yielding 1795 sequences. A total of 240 sequences (including redundancy) were identified that were associated with immune function. They include, but were not limited to: cytokine and chemokine receptors, B-cell receptors, T-cell receptors, cluster of differentiation molecules (CD markers), proteins associated with innate immunity (complement [C1q and C3], nephrosin), interferon inducible proteins (induced by viral infection), MHC II, lectins, functional proteins of monocytes and granulocytes, and a number of intracellular proteins associated with immune receptor transduction signaling (Table 1).

Table 1. Summary of immune relate genes identified in spleen DNA library.

Putative gene	BlastX identity	# of clones
Major basic protein	34%	17
Major histocompatibility complex II invariant chain	50%	15
Lectin	43%	14
Complement component 1, q subcomponent, alpha polypeptide	45%	8
Ferritin M	75%	7
Major histocompatibility complex II β-chain	41%	7
Immediate early response-2	44%	5
Allograft inflammatory factor 1	68%	4
Inhibitor of nuclear factor kappa B alpha	63%	4
Leukocyte elastase inhibitor	64%	4
Nephrosin	61%	4
Complement component 1, q B-chain precursor	45%	2
C-type lectin	49%	2
Integrin alpha 2b (CD41)	56%	2
B lymphocyte adapter protein Bam32	69%	1
Chemokine (C-X-C motif) receptor 4 (CXCR4)	66%	1
Class I helical cytokine receptor number 28	31%	1
Complement component 4 binding protein, alpha	29%	1
C-type lectin superfamily member 13	25%	1
C-type lectin superfamily member 9	42%	1
CXCR2	59%	1

Fas-activated serine/threonine kinase	47%	1
GATA transcription factor	39%	1
HLA-B-associated transcript 1A (BAT1)	98%	1
Ikaros	89%	1
Integrin alpha V (CD51)	60%	1
Integrin beta-2 (CD18)	61%	1
LBP/BPI-1	42%	1
Leukocyte receptor cluster member 2	77%	1
Leukotriene A4 hydrolase	81%	1
Leukotriene C4 synthase	68%	1
Lymphocyte-specific protein tyrosine kinase	76%	1
Lysozyme C-type	64%	1
Major histocompatibility complex class II alpha	76%	1
Mast cell immunoreceptor signal transducer isoform 2	43%	1
Mast cell-like procarboxypeptidase B	58%	1
Matrix metaloprotease-9	68%	1
Myeloid cell leukemia sequence 1b-like	100%	1
Neutrophil cytosolic factor 2	69%	1
NF-E2 inducible megakaryocyte specific protein	55%	1
p67phox	73%	1
Variable lymphocyte receptor B diversity region	68%	1
Immunoglobulin heavy chain	89%	31
Immunoglobulin kappa light, F class	82%	19
Receptor activated c kinase	97%	18
Activated blocked unfolded protein response	20%	16
Natural killer cell activator	38%	8
Natural killer cell enhancing factor	85%	3
Galectin-9	50%	2
Zeta-chain (TCR) associated protein kinase	76%	1
V-set and immunoglobulin domain containing 4 (VSIG4)	35%	1
VHSV-induced protein	38%	1
Tumor necrosis factor receptor superfamily member 14 (HVEM)	44%	1
Tumor necrosis factor ligand superfamily member 14 (LIGHT)	40%	1
T cell receptor gamma chain	33%	1
T cell receptor beta chain	42%	1
Nuclear factor kappa B binding protein	79%	1
Notchless protein homolog 1	77%	1
NFAT activation molecule 1	28%	1
Neural cell adhesion molecule 2 (NK cell)	33%	1

Interleukin enhancer binding factor 2 (ILF2)	97%	1
Interferon regulatory factor 8 (IRF8)	46%	1
Interferon regulatory factor 1 (IRF2)	65%	1
Interferon inducible guanylate-binding protein 2	67%	1
Interferon induced protein 35 (IFI35)	29%	1
Interferon induced N-myc (and STAT) interactor	41%	1
Immune induced protein, defense protein-1	54%	1
Immediate early response-3	49%	1

The untrimmed single pass forward sequences and their corresponding putative identities are included in Appendices 1 and 2 respectively. They include approximately 115 sequences (including redundancy) that are generally expressed at consistent levels in most cell types and are commonly used as house-keeping genes for quantitative molecular analyses. An additional 10 sequences (including redundancy) were also identified that are associated with stress response. In addition, a number of unidentified, hypothetical proteins that may be associated with immune function were identified. Further research is necessary to elucidate their identity and association with the immune response. A number of primer sets have been developed for quantitative analysis of selected immune related molecules identified in this library including the cytokine receptor herpes virus entry mediator and its cognate ligand LIGHT.

Histological Assays - Histological evaluation of 40 fins each from clinical and subclinical iridovirus infected shovelnose sturgeon revealed a statistically significant difference between the groups by the Mann-Whitney Rank Sum Test ($P < 0.001$). There was no infected epithelial cells observed in the subclinical group were as 0 to 403 infected epithelial cells were observed in the clinical group The median number of enlarged eosinophilic squamous epithelial cells was 12 in the clinically infected fish (Fig. 1).

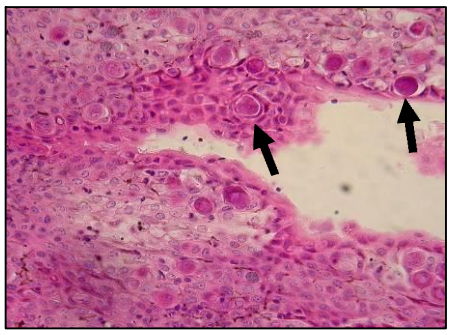
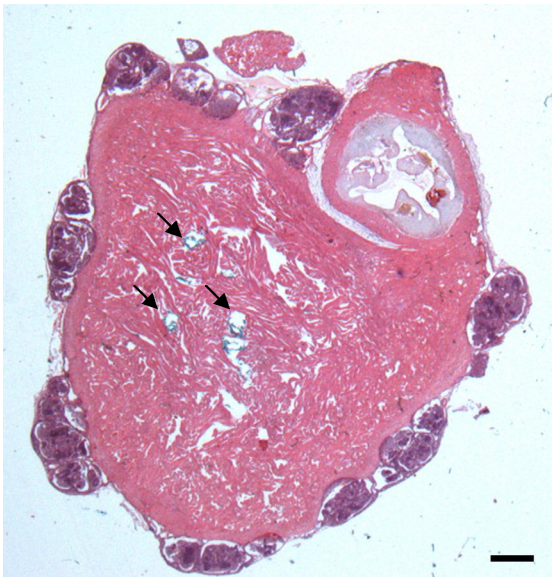


Figure 1. Enlarged eosinophilic squamous epithelial tissue infected with sturgeon iridovirus (arrows).

The clinical iridovirus infected pallid sturgeon had an average of 141 infected epithelial cells and a median of 102.

Sturgeon hearts and adjacent lymphoid tissue were sectioned and subjected to imaging and quantification analysis (Figure 2). The Adobe CS3 imaging software was used to perform analysis of the lymphoid tissue surface based on different staining properties of lymphoid vs. cardiac muscle tissue. After initial analysis, it was necessary to adjust the tissue preparation technique to produce thicker sections (>10 µm) and to include tissue markers in order to provide better orientation of the lymphoid tissue for 3D analysis. Therefore, optimization of the sectioning procedure and 3D analysis of the sturgeon heart-associated lymphoid tissue is ongoing, but the analysis of the lymphoid tissue to cardiovascular tissue surface ratios has been completed and lymphoid tissue quantified in shovelnose and pallid sturgeon. Lymphoid tissue surrounding the cardiac muscle was evaluated in fish in different stages of infection (ie. clinical vs. subclinical). It has been observed that lymphoid tissue surface ratios are significantly higher in larger fish (36% vs 10% in smaller fish), but no significant correlation was observed with the infection status.

Figure 2. Sturgeon heart surrounded by lymphoid tissue. Differences in coloration allowed for software capturing of tissue surface ratios on tissue sections. Arrows indicate blue elastomer injection sites for 3D orientation. Scale bar = 250 µm.



DISCUSSION & CONCLUSIONS

Due to the spontaneous 2006 iridovirus outbreak at the BFTC and the foresight to collect histology, blood chemistry, and immunological samples for further investigation, numerous tools were developed to monitor sturgeon health. The functional assays, AP and HET's, did not show significance between the clinical and subclincal fish; however, they were only sampled at time 0 and did not undergo stress. Potentially, stress for 24 hours and sampled may have revealed a difference in immunological activity due to a prior pallid sturgeon stress study that was performed. The results from that study revealed that stressed pallid sturgeon had a statistical significant decrease in AP and HET's activity (Palic et. al. unpublished data). In addition, due to the size of the young

of the year shovelnose sturgeon sampled, 10 fish had to be pooled for each sample. Therefore, the pooling of so many fish probably contributed to the variability in the functional assay results due to the variability in infection levels revealed in the histological analysis. Future studies should include a controlled stress of iridovirus positive fish versus non stressed virus positive fish for 24h to determine if our hypothesis is correct.

Development of nucleotide expression databases is a critical step required to better understand disease processes in organisms using contemporary technologies. Unfortunately the development of such databases is costly and therefore do not exist for most non-mammalian model species. To our knowledge, this is the first cDNA library available for shovelnose sturgeon. Given that shovelnose sturgeon are used as surrogates for the endangered pallid sturgeon, and there is very little difference in protein encoding gene sequence between these species, we anticipate that this library can be used to develop molecular diagnostic tools for both species. This statement is also based on sequencing results of pallid sturgeon DNA (and cDNA) using PCR primers developed from this shovelnose sturgeon cDNA library.

Host-pathogen interactions are complex and lead to a number of different outcomes often either in the favor of the host or pathogen. Death is one possible outcome of such interactions, and is an end-point of dire significance in the case of endangered species restoration programs. Disease prevention is a critical aspect of hatchery-based restoration programs. Due to the dependence of gametes from wild fish for sturgeon propagation, the possibility of introducing iridovirus into hatchery reared populations is a constant concern. Selective breeding efforts to develop virus resistant populations of sturgeon is not an option as maintaining genetic diversity is a paramount goal of hatchery-based population augmentation. Providing specific disease resistance via vaccination or non-specific disease resistance by bolstering immune function with immunostimulants is a realistic alternative. Successful disease prevention measures as these, however, require an understanding of the host immune response. The current research presented here provides a number of tools and techniques necessary to assess the immune response in pallid and shovelnose sturgeon thus providing basic science to support such future efforts.

While we originally intended to develop a subtractive cDNA library, this was not possible due to a low number of samples from virus-free control fish. This is an unfortunate reflection of this opportunistic sample collection that was by no means a controlled virus exposure study. In any case, the immune related genes identified here were likely upregulated by iridovirus infection. If they were not, it is improbable that they would be represented in the library in the first place. Future experimental work is necessary to confirm this assumption. Given the phylogenetic relationship of sturgeon (Chondrostei) to teleosts, further characterization of these expressed sequence tags are likely to compliment comparative immunological efforts. Identification of molecules associated with cell maintenance, signaling, and stress and understanding their correlation with clinical pathology and histopathology of the disease is essential for the development of rapid, molecular tools for future investigation of immune system-virus interactions.

The histology slides revealed that within a tank there is high variability between each fish. Some fish that were infected with virus did not show clinical signs of infection. Furthermore, the onset of clinical signs was first seen in shovelnose sturgeon reared at

22°C and then subsequently seen in pallid sturgeon and shovelnose sturgeon reared at 16°C.
The analysis of heart associated lymphoid tissue revealed no significant correlation with the infection status. It appears that lymphoid tissue associated with the heart is strongly size/age dependant. In quantification of the lymphoid tissue, shovelnose sturgeon had an average of 36% lymphoid tissue, while pallid sturgeon had a much lower average of 10%. This distribution appears to be correlated to the size of the heart, indirectly associating the quantity of lymphoid tissue with the age of the fish. It is likely that full three-dimensional analysis will confirm this observation based on surface ratios. It is known that in younger fish, including sturgeon, the thymus is very well developed; therefore it may be that in younger sturgeon there is less need for abundant heart associated lymphoid tissue. It may be also possible that lymphoid tissue takes over the role of processing of the immune system stimuli in older fish, while thymus is the primary processing organ in younger fish. To answer the posed questions it would be necessary to perform the challenge experiment with control and infected groups of sturgeon that have completed their development and/or are similar in size.

Management applications - It is expected that routine blood smear collection and subsequent analysis can be of most use to direct hatchery application, involving minimal training of personnel and material expenses (investment in staining kits, glass slides, and microscope). Hatchery personnel can be trained for routine blood sampling, preparation of slides, counting and recognizing normal and abnormal counts. Furthermore, sampling for histology and PCR can be utilized in the hatcheries as well, increasing turnover of data and supplementing standard fish health examinations. Rapid detection of fish health status changes is of increased value and can help management decisions and at the same time reduce potential for loss of fish while waiting for the results of the conventional analysis.
The cDNA library will be deposited on a public database as a sturgeon management tool and used to enable the evaluation of immune and general sturgeon health. The health evaluation could possibly be accomplished in a minimally invasive, non-lethal sampling using whole blood. Routine health monitoring using such an approach could facilitate health assessments in hatchery populations of sturgeon. Similarly, these same tools could be used to indicate the effectiveness of vaccination or other immune enhancement strategies. Given that more than just immune related genes were identified, this library will provide the tools to address other questions regarding sturgeon health, nutritional condition and general physiology. A number of small nucleotide polymorphisms (SNPs) have been identified in this library and will be used for population genetics applications in collaboration with Tim King (USGS, Leetown Science Center, Aquatic Ecology Branch).

Study Results Presented:
1. Molecular Microbiology and Biological Applications. NRP Research Committee Meeting. Bavarian Inn, May 2008, Shepherdstown, WV.

2.Preliminary exploration of the host response to natural iridovirus: immune parameters and molecular identification of immune-related molecules. American Fisheries Society - Fish Health Section. July 2008, PEI, Canada

3. Effects of acute stress and iridovirus infection on innate and cell mediated immunity of pallid and shovelnose sturgeon. International Conference on Fish Diseases and Fish Immunology. September 2008, Reykjavik, Iceland

4. Molecular Methods as a Complimentary Tool to Support Fish Health Research. Strategic Planning Evaluation of the Genetics, Genomics, and Molecular Biology Programs at Leetown Science Center, October 2008, Leetown, WV

Publications in Progress:

1. Mortality and pathology in sturgeon species *Scaphirhynchus albus* and *Scaphirhynchus platorynchus* associated with a spontaneous iridovirus outbreak under tank culture conditions.
2. Identification of the herpes virus entry mediator receptor (HVEM-R) and its cognate ligand (LIGHT) in shovelnose and pallid sturgeon.
3. Regulation of immune related molecules in shovelnose sturgeon infected with iridovirus.
4. The role of nephrosin in sturgeon immunity.

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