EVOSTC ANNUAL PROJECT REPORT

Recipients of funds from the *Exxon Valdez* Oil Spill Trustee Council must submit an annual project report in the following format by **Sept. 1 of each fiscal year** for which project funding is received (with the exception of the final funding year in which a final report must be submitted). **Please help ensure that continued support for your project will not be delayed by submitting your report by Sept. 1. Timely receipt of your report allows more time for court notice and transfer, report review and timely release of the following year's funds.**

Satisfactory review of the annual report is necessary for continuation of multi-year projects. Failure to submit an annual report by **Sept. 1** of each year, or unsatisfactory review of an annual report, will result in withholding of additional project funds and may result in cancellation of the project or denial of funding for future projects. **PLEASE NOTE:** Significant changes in a project's objectives, methods, schedule, or budget require submittal of a new proposal that will be subject to the standard process of proposal submittal, technical review, and Trustee Council approval.

Project Number: 10100132-I

Project Title: PWS Herring Survey: Herring Disease Program (HDP)

PI Name: Paul Hershberger

Time period covered: Oct. 1, 2011 – Sept. 1, 2012

Date of Report: ..... August 29, 2011

Report prepared by: ..... Paul Hershberger

Work Performed: Summarize work performed during the reporting period, including any results available to date and their relationship to the original project objectives. Explain deviations from the original project objectives, procedural or statistical methods, study area or schedule. Also describe any known problems or unusual developments, and whether and how they have been or can be overcome. Include any other significant information pertinent to the project.

This project represents an integration of PWS herring disease efforts with other herring efforts that are coordinated through the PWSSC. Additionally, this project is a logical extension and expansion of the previous project PWS herring Disease Program (#070819).

Field Surveillances of Infection and Disease Prevalence:

Prince William Sound juvenile herring

Location	Collection date	Length (mm)	n	*Ichthyophonus*	VEN	VHSV
Simpson Bay	Oct 13, 2011	YOY	43	ND	ND	0%
Simpson Bay	Nov 15, 2011	61	60	ND	ND	0%
Whale Bay	Nov 20, 2011	60	60	0%	ND	0%
Simpson Bay	Dec 13, 2011	60	60	0%	ND	0%
Simpson Bay	Jan. 11 2012	57	60	0%	0%	0%
Simpson Bay	April 2012	ND	30	3%	ND	0%

Prince William Sound adult herring

Location	Collection date	Length (mm)	n	*Ichthyophonus*	VEN	VHSV
Port Gravina	Nov 21, 2011	205	30	63%	3%	0%
Port Gravina	Nov 22, 2011	157	30	13%	0%	0%
Port Gravina	Mar 28, 2012	218	60	42%	0%	0%
Port Gravina	Mar 31, 2012	215	60	40%	0%	0%
Fidalgo Bay	April 2, 2012	231	60	35%	0%	0%

Sitka Sound pre-spawn adult herring

Location	Collection Date	Length (mm)	n	*Ichthyophonus* *	VEN	VHSV
N. Kasiana Island	April 3, 2012	232	60	20%	0%	0%
St. John Bay	April 4, 2012	214	60	32%	0%	0%
Breakwater north of Sitka	April 4, 2012	226	60	10%	0%	0%

* Note: During 2012, for the first time since we started sampling herring from Sitka, we observed some extremely heavy infection intensities with *Ichthyophonus*. Note the heavy *Ichthyophonus* infection (creamy discoloration) in the heart of the herring below:

Other samples:
- 15 fish adult herring (mean size = 240mm), demonstrating odd lethargic behavior were subsampled from tight surface aggregations near Glacier Island Pass, off Irish cove (PWS) on February 8, 2012; none tested positive for VHSV by cell culture. Samples were provided by John Moran (Auke Bay Labs).
- 60 adult herring (mean fork length = 176mm) were collected by jigging from Lynn Canal (Tee Harbor) on June 8, 2012. None tested positive for *Ichthyophonus*. Samples were provided by JJ Vollenweider and Fletcher Sewall (Auke Bay Labs).

Laboratory Rearing of Specific Pathogen-Free Herring:
For the ninth consecutive year, we were successful at rearing specific pathogen-free (SPF), immunologically naïve Pacific herring in the laboratory at the USGS - Marrowstone Marine Field Station. Naturally deposited herring eggs were collected from adult herring spawning locations in Puget Sound, WA (Quilcene Bay and Cherry Point). We currently maintain 3 age classes of SPF herring at the Marrowstone Marine Field Station, including age 0 (N ~ 10,000), age 1 (N ~ 2,400), age 2 (N ~ 750); these fish continue to be utilized as test animals for empirical studies and for development of disease forecasting tools.

Laboratory Studies:

I. Disseminative stage of *Ichthyophonus* Identified:

Small amoeboid cells, believed to be the infectious stage of *Ichthyophonus*, were observed in the bolus (stomach contents) and tunica propria (stomach wall) of Pacific staghorn sculpins (*Leptocottus armatus*) and rainbow trout (*Oncorhynchus mykiss*) shortly after they ingested *Ichthyophonus*-infected tissues**.** By 24-48 hrs post-exposure the parasite morphed from the classically reported multi-nucleate thick walled schizonts to two distinct cell types; a larger multinucleate amoeboid cell surrounded by a narrow translucent zone and a smaller spherical cell surrounded by a "halo" and resembling a small schizont. Both cell types also appeared in the tunica propria, indicating that they had recently penetrated the columnar epithelium of the stomach. No *Ichthyophonus* pseudo-hyphae ("germination tubes") were observed in the bolus or penetrating the stomach wall. Simultaneously, *Ichthyophonus* was isolated in vitro from aortic blood, which was consistently positive from 6 -144 hrs post-exposure, then only intermittently for the next four wks. Small PAS-positive cells observed in blood cultures grew into colonies consisting of non-septate tubules (pseudo-hyphae) terminating in multinucleated knob-like apices similar to those seen in organ explant cultures. Organ explants were culture-positive every day, however typical *Ichthyophonus* schizonts were not observed histologically until 20-25 days post-exposure. From 20 to 60 days p.e. schizont diameter increased from $\leq$ 25µm to $\geq$ 82µm. Based on the data presented here, a life cycle within the piscivorous host is proposed.

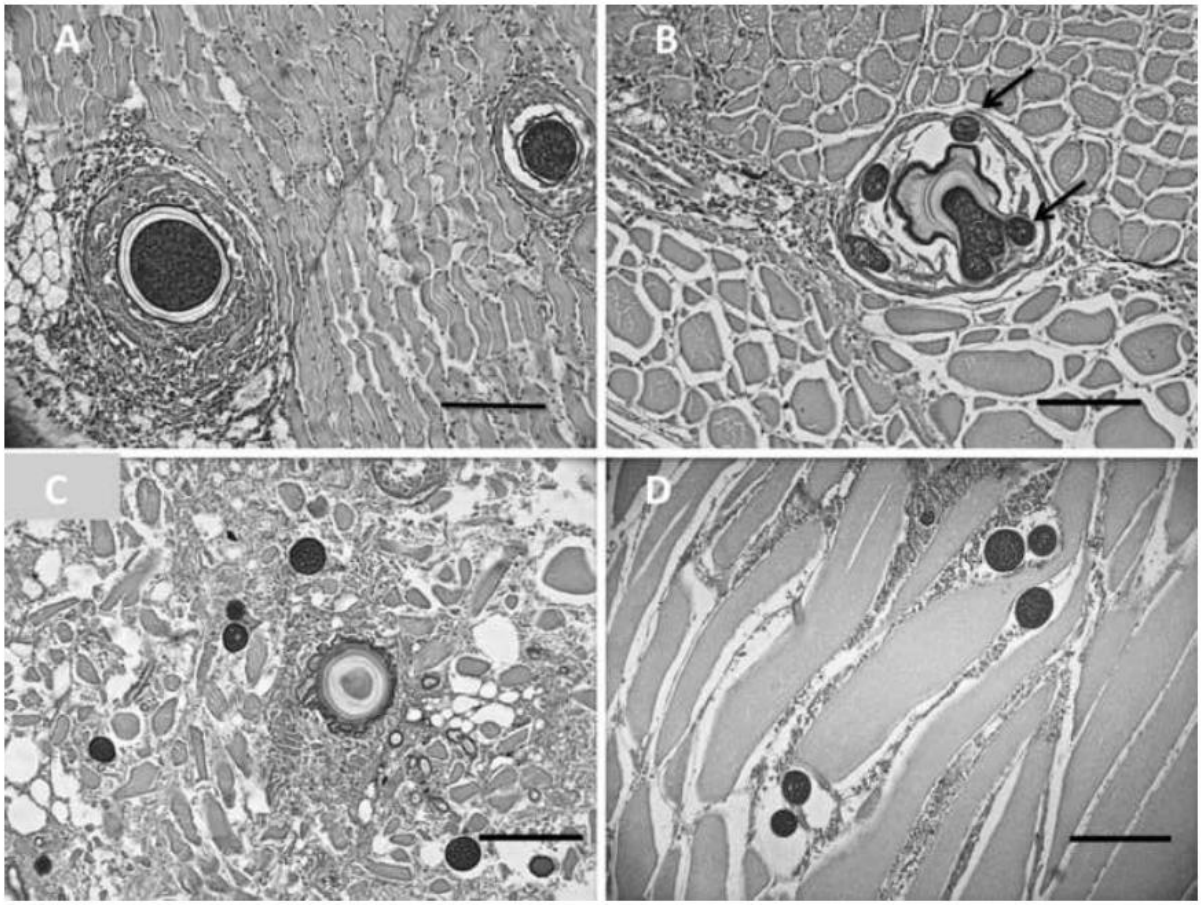

Figure 1. Transformation of *Ichthyophonus* schizonts in the stomach (bolus) of Pacific staghorn sculpins following ingestion of infected herring tissue. (A) Normal multinuclear spherical schizonts surrounded by a granuloma (0hr - infected homogenate). (B) Small amoeboid cells (arrows) budding off of parent schizont (48h post consumption). (C) Empty schizont surrounded by dispersing amoeboid cells (48hr post-consumption). (D) Amoeboid cells dispersed throughout digesting herring muscle (48 - 96h post consumption). (Bar = 50µm) Stain = Periodic acid-Schiff (PAS) reagent.

II. Inability to demonstrate fish-to-fish transmission of *Ichthyophonus* between sympatric Pacific herring:

Forage fishes are a likely source of *Ichthyophonus* infection for higher trophic level predators; however the processes which maintain *I. hoferi* in herring populations are not well understood. Lack of an identified intermediate host has led to the convenient hypothesis that the parasite can be maintained within populations of schooling fishes by direct fish-to-fish transmission. To test this hypothesis we established *I. hoferi* infections in Age-1 and young-of-year (YOY) Pacific herring via intraperitoneal (IP) injection and cohabitated these donors with naïve conspecifics (sentinels) in the laboratory. IP injections established infection in 75 to 84% of donor herring and this exposure led to clinical disease and mortality in the YOY cohort. However, after cohabitation for 113 d no infections were established in naïve sentinels. These negative data do not preclude the possibility of direct horizontal transmission, but they do suggest that other transmission processes are responsible for maintaining *Ichthyophonus* in wild populations of Pacific herring.

Table 1. Summary of Pacific herring, *Clupea pallasii*, stocked into 113 d cohabitation experiments. Age-1 (477 d) and Young-of-the-Year (YOY) (156 d) donors were exposed to *I. hoferi* by IP injection. Prevalence tank was terminated at 23 d, to estimate infection prevalence that resulted from initial IP exposure.

Treatment	No. of fish stocked		No. of mortalities		No. of survivors		No. of fish infected	
	Donors	Sentinels	Age-1	YOY	Age-1	YOY	Age-1	YOY
Trial 1: Age-1 donors/YOY sentinels								
Ichthyophonus	49[a]	194	0	19	49	175	37	0
PBS (control)	50	196	0	15	50[b]	181[c]	0	0
Trial 2: YOY donors/YOY sentinels								
Ichthyophonus	100	193	na	44	na	249[d]	na	84
PBS (control)	100	198	na	27	na	271[e]	na	0
Prevalence estimate (YOY)								
Ichthyophonus	102	0	na	13	na	89	na	92[f]

[a]Initially 50 fish were stocked, but 1 fish that jumped from the tank was discarded; [b]A subsample of Age-1 control survivors (n = 39) were assayed for *Ichthyophonus hoferi* infection; [c]A subsample of YOY control survivors (n = 73) were assayed for *I. hoferi* infection; [d]Five of these 249 cultures were unreadable, see 'Results'; [e]A subsample of YOY control survivors (n = 77) were assayed for *I. hoferi* infection; [f]Infection prevalence was 90.2% with a confidence interval of 83 to 95%

III. Viral tropism and pathology associated with viral hemorrhagic septicemia in larval and juvenile Pacific herring:

We described the progression of VHS in young Pacific herring. The tissue and cellular tropisms of VHSV in larval and juvenile herring were investigated with immunohistochemistry, transmission electron microscopy, and viral titer. In larval herring, early viral tropism for epithelial tissues (6d post-exposure) was indicated by foci of epidermal hyperplasia that contained heavy concentrations of virus. This was followed by a cellular tropism for fibroblasts within the fin bases and the dermis, but expanded to cells of the kidney, liver, pancreas, gastrointestinal tract and meninges in the brain. Among wild juvenile herring that underwent a VHS epizootic in the laboratory, the disease was characterized by acute and chronic stages of mortality. Acute stage mortalities were characterized by systemic infections in tissues including the submucosa of the gastrointestinal tract, spleen, kidney, liver, and meninges. The disease then transitioned into a chronic stage that was characterized by the appearance of neurological signs including erratic and corkscrew swimming, darkening of the dorsal skin, and reduced mortality. During the chronic stage viral persistence occurred in nervous tissues including meninges and brain parenchymal cells and in one case in peripheral nerves, while virus was mostly cleared from the other tissues. The results demonstrate the varying VHSV tropisms dependent on the timing of infection and the importance of neural tissues for the persistence and perpetuation of chronic infections in Pacific herring.

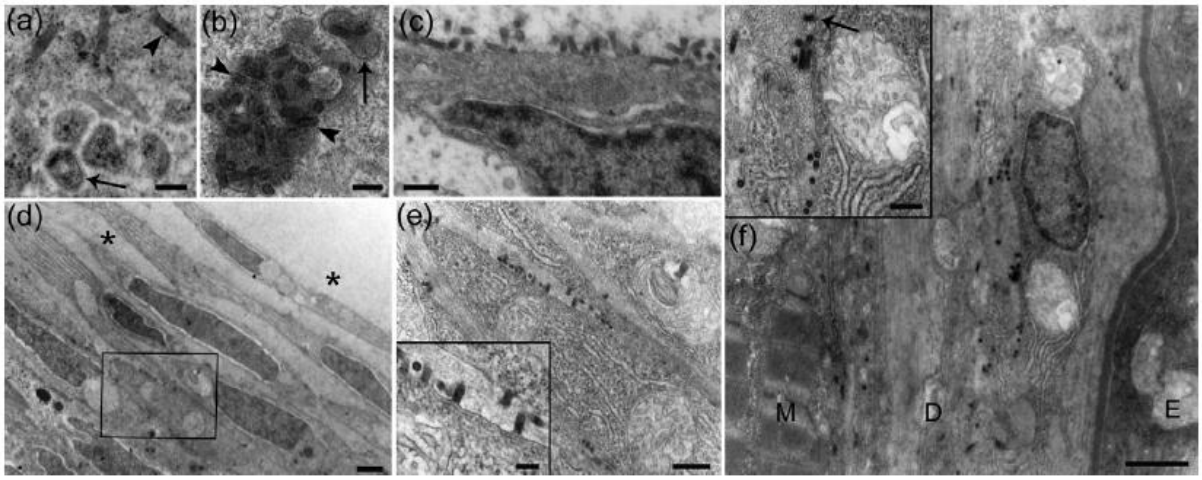

Figure 2. Transmission electron microscopy demonstrating VHSV within cells of larvae 9d post-immersion with virus. (a) VHSV (arrowheads) within the cell cytoplasm associated with membrane-bound structures decorated with surface projections (arrows) (bar=250nm). (b) VHSV surrounded with electron dense material (arrowheads) within the cell cytoplasm associated with a membrane-bound, surface decorated structure (arrow) which contains virions. (bar=250nm). (c) An endothelial cell with virions associated with the plasma membrane of the cell (bar=500nm). (d) Fibroblasts surrounded by extracellular matrix (*) adjacent to cartilage near the fin base (bar=2µm), (e) higher magnification of boxed area from (d) showing an elongated fibroblast with abundant RER within the cytoplasm and surrounded with VHS virions, inset demonstrates that virions are in continuation with the plasma membrane (bar=1µm, inset bar=250nm). (f) VHSV (arrows) within the dermis (D) and extending into the muscle layer (M), no virus is observed within the epidermis (E). Virus is associated with a dermal fibroblast, inset demonstrates virus in close association or fused (arrow) with the plasma membrane (bar=2µm, inset bar=500nm).

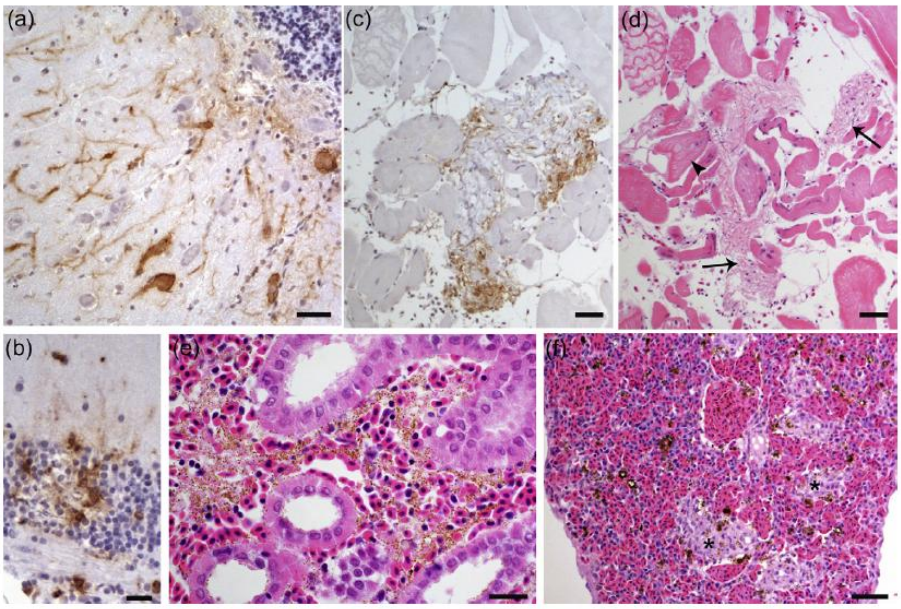

Figure 3. Herring juveniles sampled during the late part of a VHS outbreak with IHC staining for VHSV and routine H&E staining. (a) A heavy VHSV infection within the brain tissue, in which neuron cell bodies and processes are heavily immuno-stained (bar=20µm). (b) Brain tissue with immuno-positive cells, which in this case are found within the granule cell layer (bar=10µm). (c,d) A fish with immuno-positive staining for VHSV within a peripheral nerve, the adjacent H&E stained section shows degeneration of the nerve (arrows) and some of the adjacent muscle (arrowheads) (bars=20µm). (e) Kidney tissue stained with H&E showing loss of hematopoietic tissue, which has been replaced by hemorrhage and acid hematin (bar=10µm). (f) Spleen tissue stained with H&E showing a reduced amount of white blood cells, hemorrhaging with islands of parenchyma (*), and acid hematin (bar=20µm).

IV. Efficacy of a DNA glycoprotein vaccine against viral hemorrhagic septicemia (VHS) for Pacific herring *Clupea pallasii*:

A DNA vaccine containing the glycoprotein gene of the North American strain (genogroup IVa) of viral hemorrhagic septicemia virus (VHSV) was effective at protecting Pacific herring against viral hemorrhagic septicemia (VHS). The vaccine was protective at 6 weeks (29.4% relative percent survival (RPS) and 15 weeks (43.5% RPS) post vaccination; further, an analogous DNA vaccine for infectious hematopoietic necrosis virus (IHNV), a closely-related rhabdovirus, did not confer cross protection against VHS in Pacific herring. The results provide useful insights into the immunological mechanisms of adaptive immunity against VHS in herring.

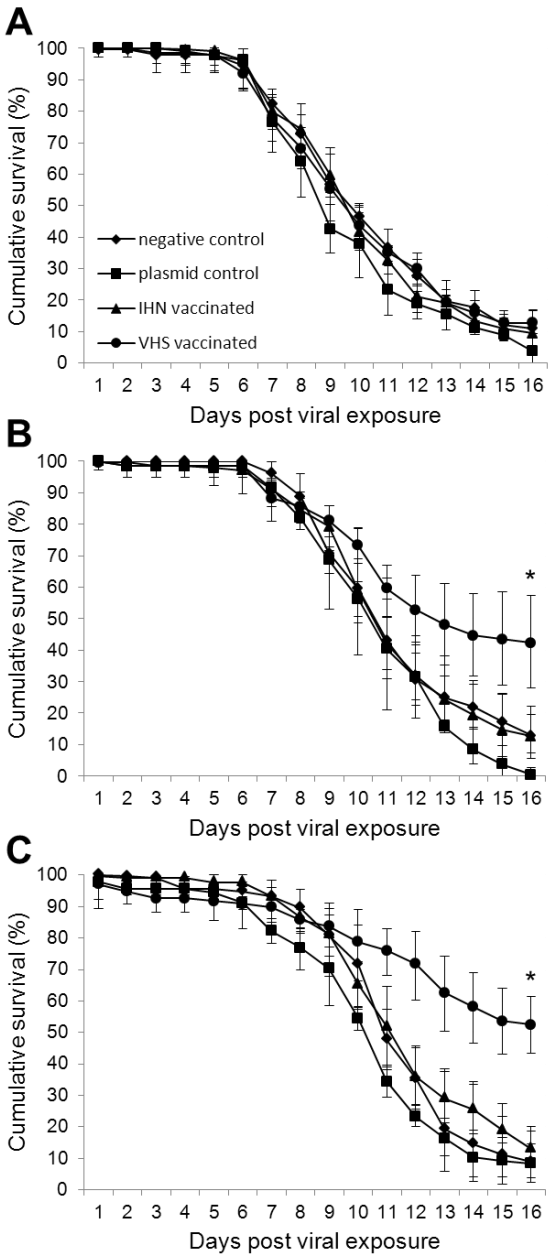

Figure 4. Susceptibility of VHS vaccinated Pacific herring *Clupea pallasii* to VHS virus. (A) Challenged 6 days post vaccination (p.v.). (B) Challenged 6 weeks p.v. (C) Challenged 15 weeks p.v. Error bars represent SD. All data points represent back-transformed percentages corresponding to the means of the arcsine-transformed proportions from triplicate tanks. *$P < 0.05$.

V. Production and Characterization of Monoclonal Antibodies to IgM of Pacific Herring: We report the identification of the gene encoding the immunoglobulin mu (IgM) heavy chain constant and variable regions, as well as the development and characterization of monoclonal antibodies (MAbs) that specifically react with Pacific herring IgM. Pacific herring immunoglobulin was purified and consisted of heavy and light chains of approximately 80 and 25 kDa. Three hybridoma clones were initially identified by ELISA as reactive with purified immunoglobulin but only one clone was able to detect an 80 kDa protein in Pacific and Atlantic herring (*C. harengus*) whole plasma by denaturing western blot. However, all three MAbs were able to precipitate an 80 kDa protein from Pacific herring and LCMS sequencing of peptide fragments derived from this protein matched the predicted amino acid sequence of the cloned, heavy chain gene. In addition, two of the MAbs stained cells within the putative lymphocyte gates for the spleen, anterior kidney and posterior kidney but were not reactive for myeloid/granulocyte gates, which is consistent with these Mabs reacting with surface IgM^+ B-cells. To our knowledge, this is the first report of IgM-related gene sequences and anti-IgM monoclonal antibodies from any member of the family *Clupeidae.* The antibodies produced in this study are critical for achieving our long-term goal of conducting serological surveillance to assess pathogen exposure in natural populations of Pacific herring.

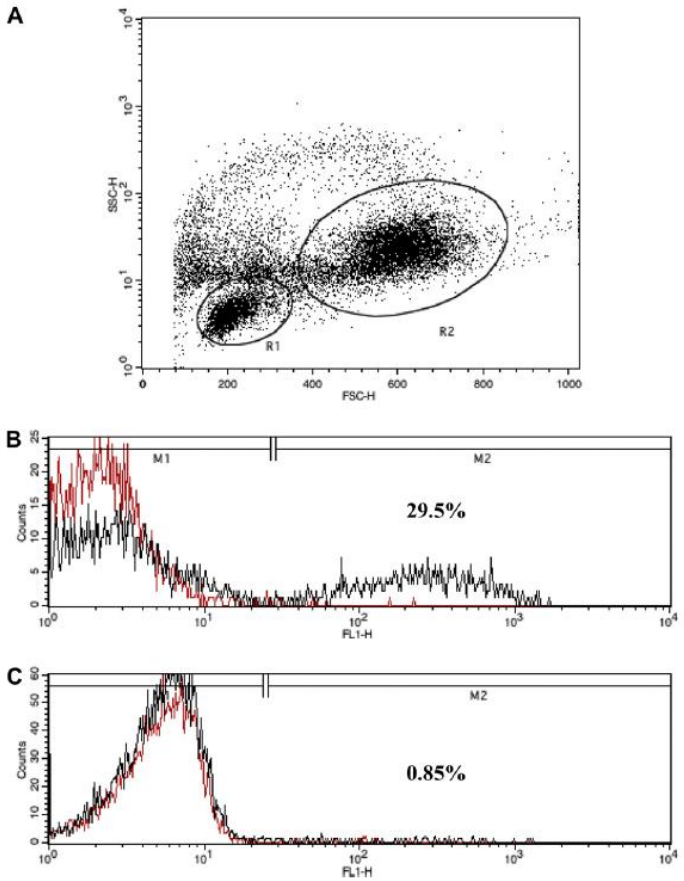

Figure 5. Flow cytometry analysis of IgM positive cells in anterior kidney of Pacific herring. Single-cell suspensions were stained with anti-herring IgM MAb IA10.2 (Black) or an irrelevant, isotype matched (Red). (A) The gate for lymphocytes was defined as R1 and the gate for myeloid cells and granulocytes was defined as R2. Propidium iodide was used to remove dead cells from the analysis. Histograms are based upon the frequency of negative – M1 – and positive –M2 – staining cells in the (B) R1 and (C) R2 gates. Percentages indicate percentage of positive staining cells after subtraction of background (red).

Future Work: Summarize work to be performed during the upcoming year, if different from the original proposal. Describe any proposed changes in objectives, procedural or statistical methods, study area or schedule. ***NOTE:*** *Significant changes in a project's objectives, methods, schedule or budget require submittal of a new proposal subject to the standard process of proposal submittal, technical review and Trustee Council approval.*

No changes to the original work plan are anticipated during FY 2013.

Coordination/Collaboration: Describe efforts undertaken during the reporting period to achieve the coordination and collaboration provisions of the proposal, if applicable.

The field components of this project relied heavily on collaboration with local and state collaborations. Herring were collected by Jennifer Todd (PWSSC), Steve Moffitt Dr. Rich Brenner (ADF&G – Cordova), Eric Coonradt (ADF&G: Sitka), JJ Vollenweider, Fletcher Sewall, John Moran, Ron Heintz, and Jeep Rice (Ted Stevens Marine Science Center in Juneau), Virology / parasitology samples for field surveillances were processed by the ADF&G Fish Pathology Laboratory in Juneau and the USGS, Marrowstone Marine Field Station.

Community Involvement/TEK & Resource Management Applications: Describe efforts undertaken during the reporting period to achieve the community involvement/TEK and resource management application provisions of the proposal, if applicable.

Five student interns and two post docs were partially supported by this project during FY'12.

Information Transfer: List (a) publications produced during the reporting period, (b) conference and workshop presentations and attendance during the reporting period, and (c) data and/or information products developed during the reporting period. ***NOTE:*** *Lack of compliance with the Trustee Council's data policy and/or the project's data management plan will result in withholding of additional project funds, cancellation of the project, or denial of funding for future projects.*

Publications

Kocan, R, S. LaPatra, P. Hershberger. *Accepted*. Developmental stages of *Ichthyophonus* occurring throughout the host infection cycle. J. Parasitology.

Lovy, J., N.L. Lewis, P.K. Hershberger, W. Bennett, K.A. Garver. *In Press*. Viral tropism and pathology associated with viral hemorrhagic septicemia in larval and juvenile Pacific herring from British Columbia. Veterinary Microbiology.

Purcell, M.K., E.S. Bromage, J. Silva, J.D. Hansen, S.M. Badil, J.C. Woodson, P.K. Hershberger. 2012. Production and characterization of monoclonal antibodies to IgM of Pacific herring (*Clupea pallasii*). Fish and Shellfish Immunology 33: 552-558.

Hart, L.M., N. Lorenzen, S.E. LaPatra, C.A. Grady, S.E. Roon, J. O'Reilly, J.L. Gregg, P.K. Hershberger. *In Press* Efficacy of a glycoprotein DNA vaccine against viral hemorrhagic septicemia (VHS) in Pacific herring *Clupea pallasii*. Journal of Fish Diseases.

Glenn, J.A., E.J. Emmenegger, C. M. Conway, J. R. Winton, C.A. Grady, J.L. Gregg, S.E. Roon, P.K. Hershberger. 2012. Kinetics of viral load and erythrocytic inclusion body formation in Pacific herring artificially infected with erythrocytic necrosis virus. Journal of Aquatic Animal Health 195-200.

Gregg, J.L., C.A. Grady, C.S. Friedman, P.K. Hershberger. 2012. Inability to demonstrate fish-to-fish transmission of *Ichthyophonus* from laboratory-infected Pacific herring *Clupea pallasii* to naïve conspecifics. Diseases of Aquatic Organisms 99: 139-144.

Beaulaurier, J., N. Bickford, J.L. Gregg, C.A. Grady, A. Gannam, J.R. Winton, P.K. Hershberger. 2012. Susceptibility of Pacific herring *Clupea pallasii* to viral hemorrhagic septicemia (VHS) is influenced by diet. Journal of Aquatic Animal Health 24: 43-48.

Hansen, J.D., J.C. Woodson, P.K. Hershberger, C. Grady, J.L Gregg, M.K. Purcell. 2012. Induction of anti-viral genes during acute infection with *Viral hemorrhagic septicemia virus* (VHSV) in Pacific herring *(Clupea pallasii*). Fish and Shellfish Immunology 32: 259-267.

Scientific Presentations

Gregg, J., F. Morado, C. Dykstra, B. Failor, C. Friedman, B. Harris, M. Purcell, P. Hershberger. January 16-20, 2012. Poster. Prevalence and phylogenetic analysis of the parasite Ichthyophonus from several marine fish hosts in the NE Pacific. Alaska Marine Science Symposium.

Hershberger, P.K., J.L. Gregg, S. Moffitt, R. Brenner, K. Garver, J. Lovy, J.J. Vollenweider, T.R. Meyers. January 16-20, 2012. Poster. Epizootiological observations from five years of Ichthyophonus monitoring in Pacific herring populations. Alaska Marine Science Symposium. (Presented)

Vollenweider, J.J., R. Heintz, A. Sreenivasan, P. Hershberger, J. Gregg. January 16-20, 2012. Poster. Overwinter bioenergetics of Pacific herring (*Clupea pallasii*). Alaska Marine Science Symposium.

Jovy, J., K.A. Garver, P.K. Hershberger. October 25-27, 2011. Platform. Pathogens of Pacific herring in the Strait of Georgia and the impacts of viral hemorrhagic septicemia. 2011 Salish Sea Research Conference. Vancouver, Canada.

Other Presentations

Guest Lectures: Friday Harbor Laboratories, University of Washington: Biol 533 (Ecology of Infectious Marine Diseases)

"Environmental Perturbations and Infectious Diseases of Marine Fishes"

"What Can We Do about Diseases in Wild Marine Fishes?"

Guest Lecture: School of Aquatic and Fishery Sciences, University of Washington: FISH 404.

"The Ecology of Diseases in Pacific Herring?"

Budget: Explain any differences and/or problems between actual and budgeted expenditures, including any substantial changes in the allocation of funds among line items on the budget form. Also provide any new information regarding matching funds or funds from non-EVOS sources for the project. ***NOTE**: Any request for an increased or supplemental budget must be submitted as a new proposal that will be subject to the standard process of proposal submittal, technical review, and Trustee Council approval.*

Budget expenditures are proceeding as per projections; no problems are anticipated.

We can accept your annual report as a digital file (Microsoft Word or WordPerfect), with all figures and tables embedded. Acrobat Portable Document Format (PDF) files (version 4.x or later) are also acceptable; please do not lock PDF files or include digital signatures.

Please submit reports electronically in ProjectView or by email to catherine.boerner@alaska.gov. Also, please be sure to post your annual report on your own website, if you have one.

We appreciate your prompt submission of your annual report and thank you for your participation.