Exxon Valdez Oil Spill
Restoration Project Annual Report

Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound

Sections:

I. Field Survey of Diseases in Prince William Sound Herring
II. Laboratory Challenge of Pacific Herring With and Without Stressors
III. Survival, Performance and Reproduction in Pacific Herring

Restoration Project 96162
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill Trustee Council restoration program for the purpose of assessing project progress. Peer review comments have not been addressed in this annual report.

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April 1997

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Study History: In 1993 there was a sudden and unexplained disappearance of approximately 60% (80K tons) of spawning herring in Prince William Sound, with another 30K tons missing in 1994. An emergency project (94320 S) was authorized April 1994 to investigate the circumstances surrounding the massive disappearance and it found that VHS, a viral disease heretofore unreported from Pacific herring was present in a number of surviving herring. In 1994 VHSV was present in less than 6% of the fish examined, however the prevalence of *Ichthyophonus hoferi*, a fungal pathogen of fish increased from 5% to 29%. As a result of these findings, the Alaska Department of Fish and Game issued an RFP to study the herring declines and to determine the involvement of the two pathogens. A detailed work plan was written as three components: 1) Field surveys (University of California, Davis); 2) Controlled laboratory exposures (University of Washington, Seattle) and Physiological studies (Simon Fraser University, British Columbia). The three components of the study were designed to interact and supply information to each other in order to answer the questions regarding infection, pathogenicity and long-term recovery prospects of Prince William Sound herring.

Abstract: From 1994 to 1996, prevalence of virus decreased annually from PWS (4.7%, 1.9%, 0.0%) and virus was not isolated from Sitka Sound herring. Ichthyophonus prevalence continued to be high in PWS from 1994 through 1996 (29%, 29%, 25%) as well as Sitka Sound (26% for '95, 21% for '96). Laboratory studies confirm that VHSV was highly pathogenic for nonimmune Pacific herring, causing almost 100% mortlality after 1 hr exposure to $\geq$ 100 PFU/ml. Histologic examination of tissues revealed lesions similar to those seen in wild herring suffering from VHS, but with minimal cutaneous hemorrhating compared with wild fish, indicating that hemorrhaging maya be due to the combined effects of VHSV and other microorganisms. Wild Puget Sound herring ranging in age from 0-year to 3+ had no detectable VHS virus; the same fish held in net pens or seawater tanks in the laboratory began shedding virus as early as 24 hours post capture; virus could be isolated from their tissues from 24 hr to 8 days post capture. The only significant mortality observed in wild herring was in the 0-year fish which exhibited over 50% mortality within 3 wks of capture. Less than 1% of older fish died of VHSV.

Key Words: *Clupea pallasi*, *Exxon Valdez* oil spill, hematology, herring, *Ichthyophonus*, morbidity, mortality, Prince William Sound, Viral Hemorrhagic Septicemia Virus (VHSV).

Project Data: (will be addressed in the final report)

Citation:

Marty, G.D., D.E. Hinton, R.M. Kocan, J.R. Winton, C.J. Kennedy, and A.P. Farrell. 1997. Investigations of disease factors affecting declines of Pacific herring populations in Prince William Sound, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 96162), Alaska Department of Fish and Game, Habitat and Restoration Division, Anchorage, Alaska.

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Executive Summary (96162)

Introduction

In 1993 the Prince William Sound herring population declined over 80% and viral hemorrhagic septicemia virus (VHSV) was isolated from a portion of the survivors. This was the first report of this viral pathogen from wild Pacific herring, and it has subsequently been isolated from bait fish in Puget Sound and herring collected in the vicinity of a diesel fuel spill in Prince Rupert, B.C. In 1992 herring being held in the roe-on-kelp fishery in Prince William Sound were observe to have hemorrhages on the skin, fin bases and mouth. The fish swam erratically and did not spawn properly. Although no virus isolations were attempted, it was noted that these lesions closely resembled the lesions observed in confirmed cases of VHS in wild herring the following year. Since VHS had not previously been reported in Pacific herring, this suggested the possibility that VHS was responsible for the heavy losses observed in 1993-'94. However, because wild fish are infected with numerous potential pathogens, extensive studies were necessary to produce conclusive evidence that VHS was responsible or even capable of causing morbidity or mortality in herring.

In 1994 an additional 30% reduction in herring biomass occurred and the returning herring were found to have an unexpectedly high prevalence (29%) of *Ichthyophonus*, a pathogenic fungus suspected to be the cause of earlier declines in Atlantic herring. Because there was no unequivocal proof that *I. hoferi* was actually responsible for the massive Atlantic herring die-offs, or was pathogenic to Pacific herring, studies were initiated to evaluate its pathogenicity and the natural history of this organism in wild and specific pathogen-free (SPF) laboratory-reared herring.

Objectives

This study consists of three distinct components with interrelated objectives.

Component I (field):
1) Determining the relationships among pathogens, visible and microscopic lesions, plasma chemistry and immune status.
2) Determine the role of age and reproductive state on the severity of disease
3) Determine the impact of disease on the age structure of PWS herring.
4) Determine the role of the spawn-on-kelp pound fishery on VHSV expression.

Component II (experimental):
1) Establish the pathogenicity of VHSV for Pacific herring (eg. fulfill Koch's Postulates).
2) Describe the pathobiology of the virus for specific pathogen-free (SPF) and wild herring.
3) Determine the means of transmission from infected to uninfected fish.
4) Determine the prevalence of VHSV in wild Puget Sound herring of different age classes.
5) Evaluate the effect of capture and captivity on the course of VHS in wild herring.
6) Determine the immune status of wild herring that survive an epizootic of VHS while in captivity.

Component III (biochemical/physiological):
1) Determine baseline levels of biochemical and immunologic parameters for Pacific herring
2) Determine relevant assays for the analysis of immunological and biochemical fitness in herring
3) Determine the effects of oil exposure on herring health and disease resistance
4) Determine the biochemical changes (eg. biomarkers) in herring associated with VHSV and Ichthyophonus infections.

Methods

Field studies consisted of sampling adult Pacific herring from Prince William Sound and Sitka Sound and determining their white blood cell differential counts (Component III), histopathology,

plasma chemistries, IgM levels and virus isolations. Tissues from fish held in SOK pounds were also examined for the presence of VHS virus at the time of release from the pounds.

Experimental studies relied on specific-pathogen-free (SPF), laboratory-reared herring. When fish reached approximately 5 months of age, they were exposed to various levels of water-borne virus while housed in flowing filtered seawater. Exposures lasted for 1 hr and the studies was terminated at 14 days. Natural transmission studies were carried out by exposing SPF herring to wild herring known to have been exposed to VHSV during an epizootic.

Wild herring were captured from Puget Sound and age classes of 0-year, 1+, 2+ and 3+ were assayed for the presence of VHSV at the time of capture and at regular intervals for the first 30 days post capture. Immunity was determined by exposing surviving herring to 10 - 100 times the known lethal dose of VHS virus for 1 hr. Data was compared with that obtained form SPF fish which had no immunity to the virus.

Ichthyophonus was studied in SPF herring by injecting 8-month-old fish IP with 1,000 spores and maintaining them in flowing sterile seawater for 60 days during which time observations were made on mortality, lesions, growth and behavior.

Physiological studies consisted of sampling blood from herring and determining the biochemical changes associated with exposure to oil, VHSV and Ichthyophonus. Fish were also placed into a swimming channel and evaluated for their stamina under various flow rates. Data was related to the condition of the fish at the beginning of the study and evaluated as to the potential for the fish to survive under wild conditions.

Results

In 1996 spawning samples, moderate or severe focal skin reddening or ulcers were more prevalent in PWS than in Sitka sound and were not different from prevalences observed in 1995. Ichthyophonus prevalence in PWS was nearly the same as in 1994 and 1995, and not different from the Ichthyophonus prevalence in spawners from Sitka. The 1988 year class had the highest prevalence of Ichthyophonus in both 1995 and 1996. VHSV was not isolated from any free-ranging herring in PWS or SS in fall 1995 or spring 1996, but 21% of 38 fish sampled from the SOK pound fishery in Craig, AK in 1996 were positive for VHSV.

Viral hemorrhagic septicemia virus (VHSV) was conclusively shown to be capable of causing disease and extensive mortality in nonimmune juvenile Pacific herring. Although tissue virus titers exceeded 1 million virus particles per gram of tissue, external signs of disease was limited to 1-2 mm hemorrhagic areas on the lower jaw, isthmus and around the eye. Virus was first detected in tissues of SPF herring 48 h post-exposure and peaked at 96 h. New virus was observed 48 h post-exposure with maximum shedding occurring on days 4-5 post exposure, just prior to peak mortality. Histopathologic examination of VHSV infected SPF herring revealed primarily multifocal coagulative necrosis of liver hepatocytes, diffuse necrosis of the kidney interstitial hematopoietic tissues, and diffuse necrosis of the spleen, epidermis and subcutis.

No virus was isolated from any wild fish at the time of capture. However, 2-3 weeks post-capture approximately 60% of the 0-year herring died with massive hemorrhages of the skin, fins and mouth. Juveniles (1+ and 2+) as well as adults (3+) were also negative for VHSV when initially captured, but began showing virus by 24-48 hours post-capture. Mortality was significantly less (< 10%) in older fish. Virus was detected in 10%, 33% and 10% of live fish on days 2, 8, and 11 respectively, but undetectable by day 21 post capture. Surviving herring exposed to $1X10^3$ to $1X10^6$ $PFU*ml^{-1}$ for 1 hour 6-8 weeks post-capture exhibited no mortality in any age class and no virus could be isolated from tissues of these fish 10 days post-exposure. When infected wild herring

were placed in flowing seawater tanks with SPF herring, the SPF herring became infected and cultured positive for VHSV, with mortality occurring between 7 and 14 days post-exposure.

Ichthyophonus: Laboratory-reared SPF herring injected IP with ca 1,000 *Ichthyophonus* spores began dying by 11 days post exposure and had visible lesions on the heart, liver and spleen and on the skin. By 56 days post exposure 90% of the fish were dead. *Ichthyophonus* was cultured from all but one of the fish which died or presented with lesions. Infected tissues from these herring were cultured then injected IP into coast range sculpins (*Cottus aleuticus*), all of which became infected and/or died by 14 days post exposure. Infected sculpin tissues were fed to other sculpins which also became infected and died from *Ichthyophonus*. No control sculpins were found to be infected with *Ichthyophonus*.

Three year classes of wild herring (0-year, 1+ and 3+) were captured from Puget Sound and examined for the presence of *Ichthyophonus* . External skin lesions were observed in 6%, 5% and 4% of the three groups respectively while 6% 23% and 52% of each group cultured positive for *Ichthyophonus*. There was no significant difference in mortality between the infected and uninfected individuals within age classes.

No significant effects were seen in measured biochemical parameters in adult fish following 6 days exposure to oil. However, juvenile herring exhibited classical stress response by hypersecretion of corticosteroids, hyperlacticemia and hyperglycemia by 24 hours post oil-exposure. They returned to normal by 96 hours. Significant alterations in both white blood cell populations and in the phagocytic activity of macrophages were noted although not in a dose-dependent manner.

Exposure to oil-water-dispersions (OWD) affected antibody titers in herring. In juvenile herring swimming performance was reduced in the two highest OWD concentrations, thus showing that OWD affected the recovery of aspects of herring biochemistry which is typically altered during exercise.

Discussion

Because VHSV was not isolated from any herring from PWS in fall 1995 and spring 1996, it appears that the disease outbreak caused by VHSV in PWS is resolving. However, data from the Craig pound fishery which indicates that wild herring carry VHSV suggests that the virus is either present at high levels in the population as a latent infection, or it is rapidly spread from a few carrier fish under conditions of capture and confinement.

Controlled laboratory studies confirmed the pathogenicity of VHSV for nonimmune herring, thus establishing it as a possible cause of the extensive losses that occurred in Prince William Sound in 1993. The course of the disease is very rapid, with new virus being shed by 48 hours post-exposure, peak tissue virus occurring by 72 hours and mortality peaking by 6-8 days post-exposure. Transmission occurs by virus being shed into the water column, thus exposing susceptible fish.

Although no VHSV could be isolated from wild-caught herring, they appear to be infected with VHSV by the time they are 5-6 months-old. All age classes had detectable virus within 24-48 hours post-capture, with the most sever mortality occurring in the 0-year fish. By 3-4 weeks post-capture virus was no longer detectable and the fish were solidly resistant to challenge infection, demonstrating that herring surviving an epizootic are solidly immune to reinfection. It is not clear however if the fish have cleared the virus or are carrying latent infections that can be activated under stress conditions at a later time.

Ichthyophonus prevalence was lower in 3- and 4-year-old fish, indicating that it will probably not severely affect recovery of the PWS population. This, in combination with the absence of active

VHSV infections and the observation that PWS fish were healthier in 1996 than in 1995, suggests that the population may be recovering.

Controlled experiments using SPF laboratory-reared herring, has shown *Ichthyophonus* to be a pathogen for herring, capable of causing nearly 100% mortality in nonimmune individuals. Wild herring were found to be infected 2-4 months post-metamorphosis with a prevalence of 6%, while the highest prevalence (52%) was found in adult spawners. There was no evidence that the organism affected the health or survival of wild fish, but different environmental conditions and levels of infection could result in a significant level of morbidity and mortality. The most reliable method for detecting infection by *Ichthyophonus* was by in vitro culture.

Based on feeding studies, carnivorous fish are potentially at risk of becoming infected by eating infected herring.

Conclusions and Recommendations

Both VHSV and *Ichthyophonus* are capable of causing morbidity and mortality in non-immune Pacific herring, thus making it possible that the severe losses of herring in Prince William Sound in 1993-'94 was the result of infection by one or both of these organisms. However, since pathologic findings in Pacific herring from PWS in 1996 were essentially consistent with a healthy population, it is suggested that the resource be upgraded from "not recovering" to "possibly recovering". If the population increases continue without overt signs of disease, it could be possible to change this to "recovering" by next year.

Apparently wild herring are infected with both VHSV and *Ichthyophonus* during their first year of life and apparently carry them without consequence until exposed to some environmental stress. Just what triggers the rapid growth and disease caused by these pathogens in nature is not clearly understood at this time. However, any "stress" condition that affects the immune system could be the trigger; such as confinement, exposure to toxic substances, malnutrition or a combination of these. These stresses should be closely monitored and the fish associated with them examined regularly for signs of disease or increased infection rate.

Exxon Valdez Oil Spill
Restoration Project Annual Report

Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound

Section I: Causes of Morbidity in Pacific Herring from Sitka Sound and Prince William Sound, Alaska, during Fall 1995 and Spring 1996

Restoration Project 96162
Section I - Field Component
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill Trustee Council restoration program for the purpose of assessing project progress. Peer review comments have not been addressed in this annual report.

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Investigations of Disease Factors Affecting Declines of
Pacific Herring Populations in Prince William Sound

Section I: Causes of Morbidity in Pacific Herring from Sitka Sound
and Prince William Sound, Alaska, during Fall 1995 and Spring 1996

Restoration Project 96162
Annual Report

Study History: The project effort was initiated under Restoration Project 94320S. An annual report was issued in 1995 by Marty, G.D., E.F. Freiberg, T.R. Meyers, J. Wilcock, C.R. Davis, T.B. Farver, and D.E. Hinton, under the title *Ichthyophonus hoferi*, viral hemorrhagic septicemia virus, and other causes of morbidity in Pacific herring spawning in Prince William Sound in 1994. The project effort was continued under Restoration Project 95320S, and an annual report was issued in 1996 by Marty, G.D., C.R. Davis, E.F. Freiberg, D.E. Hinton, T.R. Meyers, and J. Wilcock, under the title Causes of Morbidity in Pacific Herring from Sitka Sound and Prince William Sound, Alaska, in Spring 1995. The project effort was continued under Restoration Project 96162, the subject of this report.

Abstract: Pacific herring (*Clupea pallasi*) populations in Prince William Sound declined from an estimated 9.9×10^7 kg in 1992 to about 1.5×10^7 kg in 1994. Based on complete virology, hematology, and histopathology, viral hemorrhagic septicemia virus contributed most to population decline in 1994, and the fungus *Ichthyophonus hoferi* was also important. Study was expanded in 1995 to include fish from a reference site, Sitka Sound. From 1994 to 1996, prevalence of virus decreased in annual samples from Prince William Sound (4.7% → 1.9% →0.0%) and virus was never isolated in fish from Sitka Sound. *Ichthyophonus* prevalence was high at both sites, but the trend indicates decreasing prevalence in Prince William Sound (29% → 29% → 25%) and Sitka Sound (26% in 1995, 22% in 1996). At both sites in 1995 and 1996, prevalence of *Ichthyophonus* among all fish was higher in the 1988 year class than in 2- and 3-year-old fish. Decreasing disease prevalence and increasing population are evidence that the Pacific herring population in Prince William Sound is recovering. In a preliminary study of the spawn-on-kelp pound fishery in Craig, Alaska, 21% of nonrandomly selected Pacific herring were positive for virus on the day fish were released from the pounds.

Key Words: *Clupea pallasi*, disease, *Exxon Valdez* oil spill, histopathology, *Ichthyophonus hoferi*, morbidity, Pacific herring, plasma chemistries, Prince William Sound, Sitka Sound, viral hemorrhagic septicemia virus (VHSV).

Citation:

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Executive Summary

Introduction

The estimated spawning biomass of Pacific herring (*Clupea pallasi*) in Prince William Sound, Alaska, decreased precipitously from over 100,000 tons in 1992 to less than 20,000 tons in 1994. In 1993, Ted Meyers (ADFG) isolated viral hemorrhagic septicemia virus and no other significant pathogens from Pacific herring in Prince William Sound. Prince William Sound Pacific herring fisheries were severely curtailed in 1993, and were never opened in 1994 or 1995. The population began to recover in 1996, and a small bait fishery was opened in November of 1996. All other Pacific herring fisheries were opened in Prince William Sound in April 1997.

In 1994, 233 Pacific herring were sampled from Prince William Sound: 29% had the disseminated fungus *Ichthyophonus hoferi*, and viral hemorrhagic septicemia virus was isolated from 5% of the fish. In 1995 and 1996, the study included fish from a reference site, Sitka Sound, in which the herring fishery was strong and there was no history of a large oil spill. This report describes the major findings in Pacific herring from Sitka Sound and Prince William Sound in fall 1995 and spring 1996. Results from laboratory study exploring details of viral hemorrhagic septicemia virus and *Ichthyophonus* infections under controlled conditions are reported in sections II and III. Laboratory study identified crowding stress as a risk factor in expression of viral hemorrhagic septicemia virus. Because fish are often crowded during the spawn-on-kelp pound fishery, we also report findings from a preliminary study of virus prevalence in the spawn-on-kelp pound fishery near Craig, Alaska.

Objectives

Field study had four objectives: 1) determine the relation among viral hemorrhagic septicemia virus, *Ichthyophonus*, macroscopic and microscopic lesions, plasma chemistries, and immune status; 2) determine the role of reproductive stage on the general health of herring [Are lesions and viral hemorrhagic septicemia virus more severe during a given reproductive stage? Does a history of previous oil exposure correlate with prevalence and severity of disease?]; 3) determine the impact of disease on population size and structure [Are fish of a particular year class more likely to be diseased than other year classes?]; and 4) determine the role of spawn-on-kelp pound fisheries on expression of viral hemorrhagic septicemia virus.

Methods

Adult Pacific herring were sampled and subjected to complete necropsy in November 1995 (30 fish from Sitka, 130 fish from Prince William Sound), March 1996 (240 spawning fish from Sitka), and April 1996 (80 prespawning and 180 spawning fish from Prince William Sound). Analysis on all fish included gross examination, white blood cell differential counts (done in Chris

Kennedy's laboratory, Simon Fraser University), histopathology on 10 organs, plasma chemistries, IgM levels, and culture of head kidney and spleen for virus isolation (done in Ted Meyers' laboratory, ADFG). In all fish with severe external lesions, kidney was cultured for bacteria (all were negative).

To determine if viral hemorrhagic septicemia virus was being expressed by Pacific herring being released from pound fisheries, Pacific herring were also examined from spawn-on-kelp pound fisheries near Craig, Alaska (Prince-of-Wales Island in SE Alaska) in April, 1996. As fish were being released from pounds (4-5 days after capture), 38 fish were sampled for virus isolation from about 800 fish that were screened for gross lesions. Fish were also weighed, measured (standard length), and examined for gonad fullness and gross lesions indicative of infection with the fungus *Ichthyophonus hoferi*.

Results

In 1996 spawning samples, moderate or severe focal skin reddening (or ulcers) were more prevalent in Prince William Sound (2.8%) than in Sitka (0.8%), and prevalence of these lesions was no different than in spawning fish in 1995 (Prince William Sound, 2.8%; Sitka, 0.4%). Among internal lesions, *Ichthyophonus* prevalence in Prince William Sound spawning fish (25%) was nearly the same as in 1994 and 1995 (29%) and no different from the *Ichthyophonus* prevalence in spawning fish from Sitka (26% in 1995, 22% in 1996). At both sites in spring 1995, prevalence of *Ichthyophonus* among all fish was higher in 7-year-old fish than in 2- and 3-year-old fish (Prince William Sound, 35% vs. 9.6%; Sitka, 31% vs. 22%). In spring 1996, this trend continued: *Ichthyophonus* prevalence was higher in 8-year-old fish than in 3- and 4-year-old fish (Prince William Sound, 26% vs. 14%; Sitka, 38% vs. 19%). Viral hemorrhagic septicemia virus was not isolated from any fish in Prince William Sound or Sitka in fall 1995 or spring 1996. By comparison, 21% of the 38 fish sampled from the spawn-on-kelp pound fishery (Craig, Alaska, 1996) were positive for viral hemorrhagic septicemia virus.

In 1995, prevalence of several subtle inflammatory lesions was greater in spawning fish from Prince William Sound than Sitka. By comparison in 1996, inflammatory lesion prevalences in spawning fish were not different, and values at both sites were most similar to those of Sitka in 1995. For example, focal parenchymal leukocytes in the stomach wall were more common in Prince William Sound fish in 1995 (Prince William Sound = 13%, Sitka = 1.7%) but not in 1996 (Prince William Sound = 2.2%, Sitka = 2.1%).

In 1995, parasite prevalence in spawning fish was often greater in fish from Prince William Sound than Sitka. In 1996, the only parasites more common in Prince William Sound fish were trematodes in the intestine (Prince William Sound = 12%, Sitka = 1.7%); however, trematodes in the stomach were more common in Sitka fish (Prince William Sound = 14%, Sitka = 22%). Branchial *Epitheliocystis* prevalence in 1995 was greater in Sitka than in Prince William Sound; the trend was the same in 1996 (Sitka = 21%, Prince William Sound = 12%), but differences were

no longer statistically significant. For most parasites in 1996 spawning fish, prevalences were significantly not different between sites: 1) intestinal coccidian (Prince William Sound = 93%, Sitka = 92%); 2) hepatic coccidian *Goussia clupearum* (Prince William Sound = 81%, Sitka = 79%); 3) testicular coccidian *Eimeria sardinae* (Prince William Sound = 76%, Sitka = 78%); 4) gall bladder myxosporean *Ceratomyxa auerbachi* (Prince William Sound = 26%, Sitka = 23%); 5) renal intraductal myxosporean *Ortholinea orientalis* (Prince William Sound = 19%, Sitka = 21%); 6) renal intraductal protozoan (Prince William Sound = 9.5%, Sitka = 7.9%); and 7) branchial monogenetic trematodes (Prince William Sound = 1.7%, Sitka = 2.1%).

Several differences in Prince William Sound fish from fall 1995 and spring 1996 were consistent with minimal feeding during the winter. First, prevalence of several gastrointestinal parasites decreased from fall to spring; e.g., gastric cestodes (fall = 9.2%, spring = 1.7%). Second, mild inflammation in the liver--consistent with a functional immune system and/or food material and bacteria in the digestive tract--decreased from fall to spring; e.g., pericholangial eosinophilic granular leukocytes (fall = 16%, spring = 4.6%). And third, energy stores decreased from fall to spring. For example, 55% of the fall samples had liver cells with none or only mild depletion of energy stores (glycogen depletion), whereas 100% of the spring samples had liver cells with moderate or severe glycogen depletion.

Discussion

Because viral hemorrhagic septicemia virus was not isolated from any fish in fall 1995 and spring 1996, the outbreak of viral hemorrhagic septicemia virus in Prince William Sound seems to have resolved. However, results from the spawn-on-kelp fishery near Craig, Alaska, provide evidence that up to 20% of Pacific herring carry the virus in a latent or noninfectious form. The virus begins replicating after periods of prolonged stress that can occur within pounds. Therefore, re-establishment of the Prince William Sound pound fishery in 1997 may result in active expression of viral hemorrhagic septicemia virus in pounded fish, but significance to the unpounded population is unknown. The dynamics of viral hemorrhagic septicemia virus in pound fisheries will be defined through dedicated study of the spawn-on-kelp fishery in Prince William Sound in 1997.

The continued high prevalence of *Ichthyophonus* in spawning fish in Prince William Sound is a concern, but *Ichthyophonus* probably will not severely inhibit population recovery. In 1996, *Ichthyophonus* prevalence was similar at both study sites, and prevalence was slightly lower than in 1995. Also, *Ichthyophonus* prevalence at both sites was lower in 3- and 4-year-old fish than in older fish.

External examination provided strong evidence that Prince William Sound fish were healthier in 1996 than in 1995, and that in 1996 the overall health of Prince William Sound fish was about the same as Sitka fish. Internal examination provided similar evidence. In 1995, prevalence of many inflammatory lesions and parasites was often greater in spawning Prince William Sound fish than

in fish from Sitka. By spring 1996, parasite load and inflammatory lesions in Prince William Sound fish were more like Sitka fish. Differences in lesions and physiologic changes between fall- and spring-samples fish were as expected based on limited feeding by overwintering Pacific herring.

Conclusions and Recommendations

Pathologic findings in Pacific herring from Prince William Sound in 1996 were essentially consistent with a healthy population. Concurrent studies by ADFG found that the Prince William Sound population is increasing and able to support a small fishery. Therefore, we recommend that the status of Pacific herring in Prince William Sound be upgraded from "not recovering" to "recovering." Note, however, that it would be premature to consider the population "recovered," because a large year class has not yet recruited into the fishery. Trustee Council sponsored research through the SEA program has documented a large 1994 year class (E.D. Brown, personal communication). Also, our Pacific herring disease samples from October 1996 included 67% 2-year-old fish (= 1994 year class) compared with only 28% 2-year-old fish in November 1995 samples. Also, our PWS samples from spring 1997 included 35% 3-year-olds, considerably higher than in 1996, when 3-year-olds comprised only 15% of the total sample.

Spring samples in 1997 were the first to document the extent of recovery of the potentially large 1994 year class into the spawning population. Assuming that recovery continues uneventfully, final samples for this project will be collected during spawning in 1998: when the 1994 year class will be 4 years old. While it might be tempting at that time to upgrade status of Pacific herring to "recovered," it would still be premature. Pacific herring do not fully recruit into the fishery until 5 years old. The last large year class (1988) was part of a near-record population during its fourth year in 1992, yet the population crashed this year class reached its 5th year in 1993.

Introduction

When the *Exxon Valdez* oil spill occurred in March 1989, the biomass of spawning Pacific herring in **Prince William Sound (PWS)**, Alaska, was the highest in 20 years of reliable estimates (about 10×10^7 kg; Figure 1), and the population remained near record levels through 1992. Pacific herring in PWS first spawn when 3 or 4 years old. They rarely live more than 12 years, and abundant year classes recruit into the fishery about once every 4 years. In 1993, recruitment from the 1988 year class was expected to be excellent; therefore, fisheries biologists predicted a record spawning biomass of 11×10^7 kg before the spawning season (Figure 1). However, when the 1993 spawning season commenced, only 17% of the expected biomass appeared, fish were lethargic, and many had external hemorrhages. Hence, PWS Pacific herring fisheries were severely curtailed in 1993, and were never opened in 1994, 1995, or spring 1996. In PWS, Pacific herring normally support 5 commercial fisheries, with an average annual ex-vessel value of $8.3 million. Roe fisheries, the most valuable, are harvested in April just before spawning.

Toxicants such as crude oil cause more severe damage in younger fish, particularly larvae (McKim 1985); therefore long-term effects of the oil spill were thought most likely to occur in the 1988 and 1989 year classes which entered the spawning population in 1992 and 1993. Indeed, preliminary study of 4-year-old PWS Pacific herring in 1992 revealed less reproductive success in fish spawning in previously oiled sites than in unoiled sites, and fish with poor reproductive success had more severe microscopic lesions (Kocan et al. 1996). In 1993, the North American strain of **viral hemorrhagic septicemia virus (VHSV)** was isolated from pooled samples of Pacific herring from PWS, but no other significant pathogens were isolated (Meyers et al. 1994). Because VHSV had not previously been isolated from Pacific herring in Alaska, its role in population decline could not be determined. By 1994, spawning biomass declined to the lowest level (1.5×10^7 kg) recorded in 20 years of reliable estimates.

This study was initiated in 1994 to determine the cause of morbidity in PWS Pacific herring. Study included thorough necropsy, virology, bacteriology, hematology, and histopathology linked to traditional age-weight-length analysis. Our primary hypothesis was that VHSV was the most important cause of mortality, but the study was designed to diagnose other potential pathogens. We confirmed that VHSV was a significant cause of morbidity, and we also found that the fungus, *Ichthyophonus hoferi*, was important. Ten other parasites each affected more than 10% of the sampled population, but their role in population decline probably was minimal. Also, prevalence of most parasites was independent of age. We concluded that disease was significantly contributing to population decline, but background disease prevalence and the role of reproductive stage were unknown.

Study was expanded in 1995 to include prespawning samples and samples from a reference site (Sitka Sound). The Pacific herring population in Sitka supports commercial and subsistence fishing, and there is no history of a large oil spill. In PWS in 1995, VHSV was a less important pathogen, but *Ichthyophonus* continued to be significant. We also found that *Ichthyophonus* was

a significant pathogen in Sitka. Laboratory study was initiated to explore details of VHSV and *Ichthyophonus* infections under controlled conditions. This section reports the findings from field disease studies in 1995; results from the laboratory component are reported in sections II and III.

Methods

Necropsy

Pacific herring were captured in November 1995, and March or April 1996, at 2 different sites. At the reference site (Sitka), 30 fish were captured by purse seine on November 8, 1995, and 240 fish in spawning condition were captured by purse seine or cast net during March 23 - 27, 1996. Sitka fish were transported to a heated garage and subjected to complete necropsy. In **Prince William Sound (PWS)**, fish were sampled by purse seine at subjected to complete necropsy on board contracted vessels on site as follows: 1) 130 fish, November 13 and 14, 1995 (40 fish from Zaikof Bay, Montague Island, and 90 fish from the eastern side of Green Island); 2) 80 prespawning fish, April 7 and 8, 1996 (20 fish from Zaikof Bay, and 60 fish from Rocky Bay); and 3) 180 spawning fish, April 19 - 21, 1996 (all from Rocky Bay).

Each fish was assigned a unique necropsy number: 95HER-501 through 95HER-660 for fall-sampled fish, and 96HER-1 through 96HER-500 for spring-sampled fish. After capture, fish were held in plastic fish totes filled with about 300 L of seawater for no more than 4 hours before necropsy. The only exception was the fall-sampled fish in Sitka; contracted fishers caught only one set of fish during the entire trip, so fish were held up to 10 hours to sample as many as possible. In groups of 2, herring were anesthetized in tricaine methane sulfonate (Finquel®), weighed and measured (standard length), and a scale was removed for age determination. Several diagnostic procedures were done on each fish:

1) external lesions were scored as none (0), mild (1), moderate (2), or severe (3). After lesions were scored, a summary "external lesion score" was determined for each fish. The external lesion score was the most severe score for fin base reddening, caudal fin reddening, focal skin reddening, or diffuse skin reddening. External lesions "iris reddening" and "caudal fin fraying" were not used for determination of external lesion score. For spring samples, gonadal fullness was estimated and scored as 3 (75-100% full), 2 (50-74% full), 1 (25-49% full), or 0 (0-25% full).

2) about 1.5 mL of blood was drawn from the caudal vein into 3-mL syringes that contained 0.1 mL of lithium heparin (1,000 IU/mL); a capillary tube was filled and centrifuged (5500 × *g* for 5 min) for determination of **packed cell volume** (PCV), a blood smear was made and air-dried, and remaining blood was centrifuged (13,600 × *g* for 5 min) and plasma was immediately frozen for later analysis by the clinical chemistry laboratory at the Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis. A 100-µL plasma aliquot from each fish was frozen separately for IgM analysis;

details of assay development were described in last year's report (Marty et al. 1996). Too little plasma was collected from 31 fish for IgM analysis; plasma chemistry analysis was given highest priority for analysis.

Osmolality was analyzed on a Micro Osmometer Model 3MO-plus from Advanced Instruments (Norwood, MA) using 20 µL of sample. All other analyses were done using about 200 µL of sample. Electrolytes (sodium, potassium, chloride, total CO_2) were analyzed using ion selective electrodes on a Beckman Instruments EL-ISE electrolyte analyzer. Enzymes **alkaline phosphatase** (ALP), **alanine aminotransferase** (ALT), **aspartate aminotransferase** (AST), and **creatine phosphokinase** (CPK) were analyzed at 27°C on a Cobas Mira Analyzer (Roche) using Sigma® Chemical substrates. Total Protein (Biuret method), albumin (bromcresol green method) and calcium were analyzed on a Dacos Analyzer (Coulter Electronics) at 37°C using reagents by Trace America®. Lactate, phosphate, cholesterol, total bilirubin, and glucose were analyzed on a Dacos Analyzer at 37°C using Sigma® Chemical substrates.

Blood smears were sent to the laboratory of Chris Kennedy, Simon Fraser University, where they were stained with Diff-Quik (Dade Diagnostics, Inc., Aquada, Puerto Rico) and 30 1000×-fields were examined for cytoplasmic inclusions of viral erythrocytic necrosis (VEN). Also, differential leukocyte counts were done by counting approximately 100 white blood cells in randomly selected fields.

3) for virus isolation, head kidney and spleen from each fish were pooled in a plastic bag and shipped on ice to the Alaska Department of Fish and Game Fish Pathology Laboratory in Juneau, Alaska; skin lesions, if present, were sampled and bagged separately for individual virus assay. Propagation of 1 cell line (EPC), media formulation, and tissue preparation for cell line inoculation was as described by Meyers et al. (1994). Propagation of a second cell line (PHE, a herring cell line) was the same except that the media used did not contain tryptose phosphate broth.

4) for histopathology, samples of gill, liver, gonad, spleen, trunk kidney, gastrointestinal tract, heart, skin, skeletal muscle, and brain were fixed in 10% neutral buffered formalin;

5) bacterial isolation was attempted from herring with severe external lesions; kidney tissues were aseptically inoculated onto trypticase soy agar (TSA) and marine agar and plates were incubated at 23° C for at least 5 days (all were negative);

6) a touch preparation of kidney was air-dried, stained with Dipp-Kwik ® (Differential Staining Solution Set, American Histology Reagent Company, Lodi, CA), and examined for pansporoblasts of the myxosporean *Ortholinea orientalis*; extent of infestation was scored as for external lesions;

7) liver and gonads were weighed;

8) herring worms (Anisakidae) in the peritoneal cavity were counted;

9) archived samples (frozen at -80° C) from each fish included liver (0.1 - 0.2 g, in 1.5-mL plastic vials), and a wedge of epaxial skeletal muscle from just anterior to the dorsal fin (also in a 1.5-mL plastic vials);

10) during the spring, fish with gross lesions consistent with *Ichthyophonus* infection (5 from PWS and 5 from Sitka) were further sampled: affected organs were minced with a clean razor blade, transferred to tissue culture media at 4°C. Tissues were delivered to Dr. Richard Kocan at the University of Washington for further study.

At both Sitka and PWS, nearly all fish in the spawning sample had gonads in spawning condition. Prespawning samples from PWS in 1995 included a large proportion of immature (juvenile) fish, but 1996 PWS prespawning samples had only 4 of 80 (5%) fish that were reproductively immature. Because the PWS prespawning and spawning samples were similar except for spawning status (see Table 1), their numbers were combined for most statistical comparisons.

Histopathology

Tissues from 160 (fall) and 500 (spring) herring were sent to the Aquatic Toxicology Laboratory, University of California, Davis, and randomly assigned a histopathology number (95H21-1 through 95H21-160, and 96H3-1 through 95H3-500) for blind study. Pieces of skin/skeletal muscle and gill were postfixed in Bouin's for 24 h and then returned to 10% neutral buffered formalin. Tissues were processed routinely into paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Tissues from each organ were read in ascending numerical order using the random histopathology number. In most cases, all tissues from one organ were read before tissues from the next organ were started. Lesions were scored using a four-point scale as none (0), mild (1), moderate (2), or severe (3). For quality control, autolysis and artifact in each organ were scored on the same four-point scale. Ranking of lesions was often based on the number of structures (e.g., *Ichthyophonus* resting spores) per 100× field; the 100× field was examined through a 10× objective lens and a 10× ocular lens on an Olympus binocular light microscope. After all organs were examined and lesions scored, data were rearranged by necropsy number and basic statistics (e.g., prevalence in Sitka vs. PWS) were calculated.

Statistical Analysis

Analysis involved two major hypotheses: 1) fish with lesions were different from fish without lesions; and 2) fish from PWS were different from Sitka. Because only 30 fish were captures from Sitka during November 1995, those fish were not subjected to statistical analysis. In most cases, lesions with a score of none (0) were used as controls for determining significance of lesions. The association of categorical variables (e.g., none, mild, moderate, and severe) with continuous variables (e.g., CPK values) was determined using one-way analysis of variance (one-way ANOVA). For example, the CPK values for fish with a liver *Ichthyophonus* score of zero

were compared to livers with mild, moderate, and severe *Ichthyophonus*. When necessary, categories were combined to ensure that each group had at least 8 fish. Also, some values were ln transformed before analysis; % data were arcsine square root transformed before analysis. In most cases, data were retransformed to the geometric mean and the first-order Taylor series was used to estimate the standard error of retransformed geometric means. Category-specific means and standard errors were calculated for each continuous variable and compared using Tukey's Studentized range method. Levene's test was used to evaluate the homogeneity of variance assumption for the ANOVA. Analyses were run separately for spawning fish from Sitka and PWS.

Analysis of variance with two grouping factors was used to evaluate the relationship between continuous variables (e.g., CPK) and independent variables site, gender, and site-gender interactions. Analyses were run separately for spawning fish from Sitka and PWS.

The association between 2 selected categorical variables (e.g., caudal fin fraying versus scores for hepatic focal necrosis) was evaluated using chi-square methods for categorical data analysis; comparisons were considered valid only if individual expected cell frequencies were >1. Odds ratios were calculated for standard (2×2) 2-way contingency tables only. Spring data from Sitka and PWS were combined for this analysis. Fall data from PWS were analyzed separately.

To measure the strength of the linear relationship between 2 continuous variables, the correlation coefficient r was calculated separately for spawning fish from PWS and Sitka. Multiple regression analysis was used in 1994 to examine the relationships between selected dependent variables (e.g., plasma albumin) and associated variables (e.g., focal skin reddening, splenic congestion, and VHSV). However, determining significance of potentially important interactions requires large numbers of fish; therefore, additional multiple regression analyses are not a part of this annual report. Instead, we anticipate that multiple regressions will be used frequently in the final report (with $n > 2{,}000$) to characterize more fully variations in plasma chemistry values.

To determine if certain age classes of fish were more likely to be infected by certain parasites, the association of fish age with common parasites was evaluated using the chi-square test for homogeneity. Fish were separated by site. Fall-sampled fish from PWS were grouped into 4 categories for analysis: 2-year-olds, 3-year-olds, 4- through 6-year-olds, and ≥7-year-olds. Spring-sampled fish from Sitka and PWS were each grouped into the same year classes (now one year older) for analysis: 3-year-olds, 4-year-olds, 5- through 7-year-olds, and ≥8-year-olds. Regardless of severity of infestation, fish with a given parasite were classified as positive, and fish without the parasite were classified as negative.

For all analyses, comparisons were considered significant when $P \leq 0.050$ and highly significant when $P \leq 0.010$. Use of the term "prevalence" refers to the sample prevalence.

Spawn-on-Kelp Pound Fishery

Pacific herring (*Clupea pallasi*) were examined in spawn-on-kelp pound fishery near Craig, Alaska (Prince-of-Wales Island in SE Alaska) on April 9-11, 1996. Most of the pounds had many dead fish at the bottom of the nets, but few of the remaining living fish had significant gross lesions. As fish were being released from pounds (4-5 days after fish had been capture and put into the pounds), 38 fish were selected for virus isolation from about 800 fish that were screened for gross lesions. Tissues for virus isolation from individual fish were placed in separate plastic bags. For each fish, brain was submitted separately from the kidney-spleen pool. Virus isolation was done in ADFG's Fish Pathology Laboratory in Juneau using the same methods as regular field studies. Fish were also weighed, measured (standard length), and examined for gonad fullness and gross lesions indicative of *Ichthyophonus* infection. In addition to the 800 fish screened for virus isolation, 100 fish from 4 different pounds were selected at random, screened for gross lesions, and returned to the ocean. The primary objective of this preliminary study was to determine if VHSV was being expressed by Pacific herring being released from pound fisheries.

Results

Note on the contents of the results section: the annual report for project 94320-S (Marty et al. 1995) contains detailed descriptions and micrographs of most of the significant lesions in Pacific herring. Those descriptions are not repeated in this report, but will be included in the final synthesis report. This report concentrates on significant differences in lesions, necropsy findings, and plasma chemistry values in samples from fall 1995 and spring 1996. Variables considered included spawning stage, site of capture, season and year of capture, with special emphasis on organisms and lesions likely to result in population level effects.

Revisions from 1994 Study (94320S)

In 1994, two Pacific herring had gross and histologic lesions suggestive of infection with the lymphocystis virus. Grossly, affected fish had one or two spherical, white foci, each about 2 mm in diameter. One focus was in the cranial part of the peritoneal cavity; the other focus was in the intestinal mesenteries. Histologically, each white focus was composed of a single hypertrophic fibroblast. The affected fibroblast had a multilayered, 12-μm-thick, hyaline capsule, with abundant granular basophilic cytoplasm, and a large nucleus (500 μm in diameter) with vacuolated and marginated chromatin (Figure 2). The infected fibroblast was not associated with any inflammatory cells. To determine the contents of the expanded cytoplasm, 6-μm-thick paraffin sections were sent to Dr. Theodore Meyers in Juneau, Alaska, where sections of the cell were processed for transmission electron microscopy as previously described (Meyers et al. 1990). Ultrastructurally, the cytoplasm contained abundant icosahedral viral particles, each about 200 nm in diameter, with an electron-dense viroplasm. The ultrastructural features of the virus are characteristic of lymphocystis virus (Figure 2).

Necropsy Findings and External Gross Lesions - Fall 1995 and Spring 1996 Samples

For spring 1996 samples, spawning fish from PWS were significantly older than other sample groups (Table 1). Significant differences in most other morphometric necropsy variables were consistent with age differences among the sample groups. Testis weight was significantly greater in prespawning fish than in spawning fish, but ovary weights were not significantly different. Significantly greater hold time for Sitka-sampled fish reflected the extra time it took to transport Sitka fish to a garage before necropsy (necropsy was done on a ship at the capture site in PWS). Hold time never exceeded 3.8 hr at either site. Unlike spring 1995 samples, fin base reddening was not significantly associated with increased hold time in 1996 samples.

Overall prevalence of external lesions was less in spawning fish from PWS than from Sitka, but prevalence of ulcers (=severe focal skin reddening) was greater in fish from PWS than from Sitka. The summary external lesion score was moderate or severe in 33% of Sitka fish, but only 5.0% of prespawning PWS fish and 18% of spawning PWS fish had moderate or severe external lesions. By comparison, ulcers occurred in 1.5% of all PWS samples, but none of the Sitka samples had ulcers. Sample size from Sitka in fall 1995 was too small for statistical comparisons, but prevalence of external lesions (31%) and ulcers (3%) in PWS samples from fall 1995 was higher than spring 1996 PWS samples.

The relation between external lesions and plasma chemistry values was often highly significant, but significant variables in Sitka and PWS fish were rarely the same (Table 2). A few plasma chemistry values were consistently related to external lesions when spring samples from PWS and Sitka were compared; examples include: 1) increased caudal fin reddening vs. increased sodium, chloride, and calcium; and 2) increased iris reddening vs. increased globulin and CPK.

External lesions were significantly associated with several gross and microscopic lesions (Table 3). Significant associations for both fall and spring samples included only 2 comparisons: 1) increased caudal fin reddening vs. increased caudal fin fraying, and 2) increased fin base reddening vs. increased diffuse skin reddening. No microscopic lesions were significantly associated with external lesions in both fall and spring samples.

Opercular copepods were not scored before 1996 because they were not noticed. However, in 1996 the copepods were relatively common in the dorsal part of the medial side of the operculum. The copepod body was about 1 to 1.5 mm long, and paired egg sacs were up to 2 mm long. There were never more than 2 copepods per opercula. In spring PWS samples, 59% of prespawning fish and 37% of spawning fish had opercular copepods, and the copepods were more common in younger fish (Table 2). Prevalence of opercular copepods in spring 1997 samples was similar: Sitka spawning (48%), PWS prespawning (57%), and PWS spawning (47%).

Microscopic Lesions - 1995 fall and 1996 spring Samples

Ichthyophonus

Overall prevalence of *Ichthyophonus* was slightly less in spawning samples from PWS in 1996 (25%, 45 of 180) than in spawning samples from 1994 (29%, 62 of 212) and 1995 (29%, 52 of 180). By comparison, *Ichthyophonus* prevalence in fall 1995 PWS samples (19%, 25 of 130) and prespawning 1996 samples (13%, 10 of 80) was significantly less than in other samples since 1994. *Ichthyophonus* prevalence in 1996 Sitka samples (22%, 53 of 240) was slightly less than 1995 Sitka samples (26%, 62 of 240) but the same as all 1996 PWS samples (21%, 55 of 260). In 1994, *Ichthyophonus* prevalence among PWS age groups was not significantly different, but in 1995, *Ichthyophonus* was significantly more frequent in the 1988 year class (35%) than in the 1992 and 1993 year classes (9.6%). This difference continued in 1996 samples from PWS, where the 1988 year class made up a disproportionally high proportion of the *Ichthyophonus* cases (chi-square test, $P = 0.050$; Figure 3). The 1988 year class also made up a disproportionally high proportion of the *Ichthyophonus* cases in Sitka in 1995 and 1996 (Figure 4), and difference were significant in 1996 (chi-square test, $P = 0.004$).

All organs contained *Ichthyophonus* (Table 2), and the multinucleate resting spore stage was the most common form. Morphology of *Ichthyophonus* and the host reaction were similar to those reported in infections in Atlantic herring (*Clupea harengus*) (Daniel 1933, Sindermann 1970). Scoring, histologic features, and differential diagnoses in Pacific herring were essentially the same as reported previously (Marty et al. 1995, Marty et al. 1996).

Although the overall *Ichthyophonus* prevalence in 1996 spawning fish was 21% in PWS and 22% in Sitka, in no single organ was *Ichthyophonus* prevalence > 18% (Figures 5 and 6). Organ *Ichthyophonus* prevalence in 1996 was similar to 1994 and 1995; heart, liver, kidney, spleen, and skeletal muscle were the most commonly affected organs. As in previous years, cases in the heart and kidney were more likely to be moderate or severe (Figures 5 and 6). A sum-*Ichthyophonus* (sumICH) score was calculated for each fish by adding the individual *Ichthyophonus* scores from all 10 organs for that particular fish (Marty et al. 1995). In 1996, the highest sumICH scores were 23 for PWS and 24 for Sitka.

Association of *Ichthyophonus* scores with plasma chemistries was variable (Table 2). In 1994, increased AST and CPK values were significantly associated with increased *Ichthyophonus* scores in every organ (univariate ANOVA). In 1995, the association of *Ichthyophonus* scores with AST and CPK was less distinct: AST and CPK significantly increased with most Sitka *Ichthyophonus* scores, but only AST significantly increased with most PWS *Ichthyophonus* scores. Increased IgM levels were significantly associated with increased *Ichthyophonus* scores in all organs analyzed, and differences were highly significant. In 1996, the association of *Ichthyophonus* scores with AST and IgM was about the same as in 1995, but CPK was significant only for intestinal *Ichthyophonus* in the intestine. New in 1996, severity of

Ichthyophonus infection was consistently related to increased ALT and decreased PCV (Table 2). In fall 1995 samples from PWS, *Ichthyophonus* infection was consistently related to increased IgM, ALT, AST, and CPK, and with decreased PCV.

VHSV

All of the field-collected samples from fall 1995 and spring 1996 were negative for VHSV.

Gender-associated Lesions

Site differences in oocyte morphology were minimal in fall 1995 samples (Table 4). Because of the severity of artifact in ripe eggs, and lack of differences in spring 1995 fish, oocyte morphometry was not done on spring 1996 fish in favor of semiquantitative lesion scores. Using this scoring system, atresia of mature oocytes was more frequent in fish from PWS than from Sitka, and atresia was more frequent in fall-sampled fish than in spring-sampled fish (Table 2).

Gonads had several differences in lesion prevalence. As in 1994 and 1995, lesions more frequent in ovaries included hyalinization of vessel walls and pigmented macrophage aggregates. Prevalence of pigmented macrophage aggregates in 1995 and 1996 (about 40%; Table 2) was slightly less than in 1994 (about 60%). A big difference in 1996 was the lack of hyalinization of vessel walls in ovaries in spawning samples from PWS (1994, 61%; 1995, 41%; 1996, 5.8%) and Sitka (1995, 47%; 1996, 3.3%).

Granulomatous inflammation in the gonads continues to be highly variable. Prevalence in ovaries ranged from 1.8% in spawning PWS females in 1994 to 92% in immature prespawning fish in 1995. Prevalence of granulomatous inflammation in testes ranged from 2.0% in spawning fish from Sitka and PWS in 1996 to 43% in immature prespawning fish from PWS in 1995. As in 1994 and 1995, *Ichthyophonus* was rare in either gonad in fall 1995 and spring 1996 samples (Table 2, Figures 5 and 6).

Gender differences within fall 1995 and spring 1996 were significant for several nongonadal lesions (Table 5). In 1995, prevalence of *Ichthyophonus* in heart, liver, and skeletal muscle was significantly greater in females than males; in 1996, however, gender differences in *Ichthyophonus* prevalence were no longer significant. Gall bladder myxosporeans (*Ceratomyxa auerbachi*) were significantly more prevalent in females in spring 1996 (as they were in spring 1994 and spring 1995) but not in fall 1995. In the spring, females were significantly more likely than males to have hepatic lipidosis, and males were significantly more likely to have hepatocellular glycogen depletion, but differences were not significant in the fall (Table 5). In spring 1996 samples, males were more likely to have vacuolated renal tubular epithelial cells or dilated renal tubules, but differences were not significant in fall samples. Hepatocellular single cell necrosis and focal hepatic necrosis were unusually high in spring 1996 samples, and both lesions were significantly more likely in females than in males (Tables 2 and 5).

Several weights and plasma chemistry values were significantly different, and differences changed from fall 1995 to spring 1996. Gonad weight in fall 1995 samples was greater in males than in females, but by spring 1996, gonad weight was greater in females than in males (Table 7); differences in each season were highly significant. Liver weight was greater in females during both fall and spring (Table 7). In fall 1995 samples, females had significantly lower lactate, PCV, and % monocytes than males, but females had significantly greater globulin, calcium, and glucose. In spring 1996 samples, females had significantly lower albumin, cholesterol, CO_2, glucose, lactate, % neutrophils, PCV, potassium, sodium, and total protein. Females had significantly higher ALP, AST, CPK, and % thrombocytes. Interestingly, in fall 1995 samples the only significant enzyme difference was for ALP, which was lower in females. Gender differences in spring values for electrolytes and proteins were similar in 1994 and 1995, but the highly significant gender differences in AST and CPK levels were unique to 1996 samples.

Iris reddening

Some lesions significantly associated with iris reddening were more prevalent in fish with moderate iris reddening than in fish with mild or no iris reddening (Table 6). Examples include intestinal foreign body granuloma in fall 1995 PWS fish and hepatic *Ichthyophonus* in spring 1996 fish. In contrast, the intestinal intraepithelial coccidian (*Goussia* sp.?) was never found in spring 1996 fish with moderate iris reddening. Splenic congestion was significantly more likely in spring 1996 fish with no iris reddening; the same pattern occurred in spring 1994 and spring 1995, but differences were not significant in fall 1995 samples.

Intraperitoneal Herring Worms (Anisakidae)

All 600 Pacific herring sampled from Sitka and PWS (fall 1995 and spring 1996) had larval parasites of the family Anisakidae within their peritoneal cavities. No attempt was made to differentiate species (e.g., *Anisakis* vs. *Contracecum*), and parasite morphology and inflammatory response were consistent with previous descriptions (Hauck and May 1977).

Other Lesions and Potential Pathogens

No significant bacterial pathogens were isolated. Two blood smears, both from Sitka, had erythrocyte inclusions characteristic of VEN. The first fish, a 3-year-old female sampled on November 9, 1995, had a mild VEN inclusion score and a PCV of 41%. The second fish, a 4-year-old female sampled on March 23, 1996, had a severe VEN inclusion score and a PCV of 32%. By comparison, the mean PCV for Sitka fish in fall 1995 and spring 1996 was 44%.

Pacific herring have 11 other common parasites, most of which were associated with few lesions. These parasites in spring fish, roughly in descending order of prevalence, include:

1) intraepithelial intestinal coccidian *Goussia*? sp.
   1995: PWS = 95%, Sitka = 91%;
   1996: PWS = 94%, Sitka = 92%;

2) hepatic coccidian *Goussia* [*Eimeria*] *clupearum*
   1995: PWS = 73%, Sitka = 71%;
   1996: PWS = 80%, Sitka = 79%;
3) testicular coccidian *Eimeria sardinae*
   1995: PWS = 85%, Sitka = 66%
   1996: PWS = 74%, Sitka = 81%;
4) gall bladder myxosporean *Ceratomyxa auerbachi*
   1995: PWS = 39%, Sitka = 32%
   1996: PWS = 29%, Sitka = 23%;
5) renal intraductal myxosporean *Ortholinea orientalis*
   1995: PWS = 29%, Sitka = 20%
   1996: PWS = 14%, Sitka = 21%;
6) branchial *Epitheliocystis*
   1995: PWS = 15%, Sitka = 25%
   1996: PWS = 17%, Sitka = 21%;
7) renal intraductal protozoan
   1995: PWS = 11%, Sitka = 3.8%
   1996: PWS = 9%, Sitka = 8%;
8) gastric trematodes
   1995: PWS = 12%, Sitka = 10%
   1996: PWS = 15%, Sitka = 22%;
9) branchial monogenetic trematodes
   1995: PWS and Sitka = 11%
   1996: PWS = 1.5%, Sitka = 2.1%;
10) intestinal trematodes, e.g., *Lecithaster gibbosus*
   1995: PWS = 8.9%, Sitka = 2.1%
   1996: PWS = 14%, Sitka = 1.7%; and
11) intestinal cestodes, e.g., *Nybelinia surmenicola*
   1995: PWS = 3.3%, Sitka = 2.5%
   1996: PWS = 1.5%, Sitka = 3.8%.

In 1995, site differences in parasite prevalence were significant (chi-square test) for the testicular coccidian, renal intraductal protozoan, and branchial *Epitheliocystis*. In 1996, by comparison, the only significant site difference in parasite prevalence was with the intestinal trematodes. Branchial ciliated protozoa (e.g., *Trichodina*) were rare in 1995 and 1996 spawning samples from both PWS (1995 = 1.3%; 1996 = 0.4%) and Sitka (1995 = 1.1%; 1996 = 0.0%). *Trichodina* was much more common in spawning samples from PWS in 1994 (12%).

Intestinal coccidians were common in small numbers throughout the intestine, including the intestinal cecae. In 1994, only 1% of the PWS spawning fish had moderate infestation (i.e., >15 organisms per 400× field), and infestation was not associated with alterations in plasma chemistry values. In spring 1995, overall prevalence was not different in PWS and Sitka. However, 31% of the PWS prespawning fish and 12% of the PWS spawning fish and had

moderate infestations, compared to only 2% of Sitka fish with moderate infestations. In spring 1996, overall prevalence was the same at each site, but fish with moderate infestations were again more prevalent in PWS than in Sitka (10.4% vs. 1.7%). Severity of intestinal coccidians in 1995 was significantly related to greater CPK and AST values in PWS fish but not in Sitka fish. In spring 1996, intestinal coccidians were not associated with alterations in plasma chemistries (Table 2).

Morphologic features and distribution of the hepatic coccidian were very similar to descriptions of *Goussia clupearum* in Atlantic and Pacific herring (Morrison and Hawkins 1984, Marty et al. 1995). Despite the relatively large volume of hepatic parenchyma displaced by the parasites in severe cases, inflammation was minimal. For 1996 Sitka fish only, increased lesion scores were associated with decreased plasma cholesterol levels, but this difference was not consistent with 1995 findings, where increased lesion scores were associated with decreased plasma glucose levels.

As in 1994 and 1995, diagnosis of the renal intraductal myxosporean *Ortholinea orientalis* was less sensitive by histopathology. This difference was particularly obvious in 1996 spawning fish from Sitka, where the touch preparation prevalence (22%) was significantly higher than the prevalence determined by histopathology (11%). Overall prevalence was significantly greater in spawning fish from PWS in 1995 than in 1994 (29 vs. 19%, chi-square test). In 1995, as histopathology scores for *Ortholinea orientalis* in spawning PWS fish increased, values for PCV, neutrophils, and basophils significantly increased, but these trends were no longer significant in 1996 (Table 2). Plasma chemistry values for other methods of diagnosis and for Sitka were either not significant or significant trends were nonlinear.

The gall bladder sometimes contained large numbers of the myxosporean *Ceratomyxa auerbachi*. PWS prevalence in 1996 (21%) was significantly less than in spring 1995 (39%) but not different from 1994 prevalence (19%). The gall bladder is small (3 - 5 mm in diameter) and difficult to include in all sections. A higher frequency of gall bladders were examined in 1996 (97%) and 1995 (97%) than in 1994 (66%), but the increased prevalence in 1995 cannot be explained by differences in sample frequency. Severe infestations sometimes had mild mononuclear inflammation in the lamina propria. Infestations were significantly associated with increased total protein and albumin levels in spring 1996 PWS fish, but not in spring Sitka samples for any 1995 samples.

In 1995, prevalence of 4 subtle inflammatory lesions was significantly greater in spawning fish from PWS than from Sitka, but differences were no longer significant in 1996, and levels in spawning fish from both PWS and Sitka in 1996 were more like Sitka in 1995:

1) perivascular leukocytes in skeletal muscle
   1995: PWS = 77%, Sitka = 65 %,
   1996: PWS = 92%, Sitka = 92%;
2) focal parenchymal leukocytes in the liver
   1995: PWS = 81%, Sitka = 49%,

1996: PWS = 37%, Sitka = 35%;

3) focal parenchymal leukocytes in the heart

1995: PWS = 32%, Sitka = 24%,

1996: PWS = 11%, Sitka = 17%;

4) foci of leukocytes in the submucosa and muscularis of the stomach

1995: PWS = 13%, Sitka = 1.7%,

1996: PWS = 2.2%, Sitka = 2.1%.

Age-associated Changes

The most consistent age-related change (as in 1994 and 1995) was increased severity of pigmented macrophage aggregates in older fish. Indeed, age-related changes were significant in all organs in which pigmented macrophage aggregates were scored: exocrine pancreas, liver, ovary, spleen, and trunk kidney (Table 2). Among external lesions, fin base reddening (fall 1995 PWS only) was the only one that significantly increased with age. Microscopic lesion scores that were significantly related to increased age at both PWS and Sitka included: 1) foreign body granulomas in the intestine; 2) renal tubular dilation; and 3) splenic ellipsoid hyalinization or hypertrophy (Table 2). At both PWS and Sitka in 1996, no microscopic lesion scores significantly decreased as age increased.

Decreased severity scores for several parasites were significantly related to increased age in spring 1996 (ANOVA): 1) opercular copepods (scored only in PWS); 2) branchial *Epitheliocystis* (PWS only); 3) testicular coccidian *Eimeria sardinae* (Sitka only); and 4) intestinal trematodes (PWS only; prevalence was too low in Sitka for statistical analysis). By comparison, severity scores for other parasites increased with age in spring 1996: 1) gall bladder myxosporean (*Ceratomyxa auerbachi*; Sitka only; and 2) branchial *Ichthyophonus* (Sitka only). *Ortholinea orientalis* was not significantly related to age in spring 1996 samples. In fall 1995 samples from PWS, severity scores for *Ichthyophonus* were significantly related to increased age in several organs.

Comparing age-related prevalence of common parasites using the chi-square test for homogeneity produced results very similar to one-way ANOVA of severity scores, except that the chi-square test identified two significant relations not tested with ANOVA: 1) increasing severity of the sum-*Ichthyophonus* score was significantly related to age in spring 1996 fish from Sitka ($P = 0.004$) and PWS ($P = 0.050$); and 2) gill trematodes were more common in younger fish in 1996 samples from PWS ($P = 0.009$) but age differences were not significant in 1996 samples from Sitka.

Several values for plasma chemistries, weights, and length were significantly related to age (Table 8). Values that significantly increased with age at both sites and both seasons included albumin, globulin, total protein, all weights (body, gonad, and liver), and length. AST was high in young fish, decreased in middle aged fish, and then increased again in older fish. No plasma chemistry values significantly decreased with age in all three sample groups. Hematology values

did not consistently change with age at both sites, although among spawning fish from PWS, % lymphocytes decreased with age as % neutrophils increased.

Leukocyte Differential Counts

Although interpretation of leukocyte differential counts is limited without knowledge of the total white blood cell count, the values provide useful information for generating hypotheses that can be further examined with laboratory study, particularly in Section III of this project (C. Kennedy, Simon Fraser University). Several lesions were significantly related to changes in the frequency of various leukocytes, but significant relationships were often inconsistent between samples (Table 2). For example, in spring 1995 samples, increased frequency of neutrophils was significantly related to *Ichthyophonus* scores in PWS samples from several organs, but differences were not significant in spring 1996 samples. In contrast, severity of *Ichthyophonus* scores was significantly related to decreased neutrophil frequency in fall 1995 samples from PWS. Also, gonadal macrophage aggregates were related to increased neutrophil frequency in 1996 Sitka samples but to decreased neutrophil frequency in 1996 PWS samples.

Lymphocyte frequency significantly increased with scores for several lesions in 1996 samples from PWS (Table 2); examples include: 1) intestinal mesenteric steatitis; 2) gastric trematodes; and 3) focal parenchymal leukocytes in liver and stomach. As scores for pancreatic zymogen granule depletion and renal interstitial hematopoiesis increased, lymphocyte frequency decreased. Note that the frequency of lymphocytes was significantly less in samples from Sitka than in samples from PWS, regardless of gender or season (Table 7).

Although thrombocyte frequency was significantly greater in spring 1996 Sitka samples, significant changes in thrombocyte frequency were more common in PWS samples. In spring 1996 PWS samples, thrombocyte frequency decreased as scores increased for several lesions: 1) caudal fin fraying; 2) *Ichthyophonus* in multiple organs; 3) gastric focal parenchymal leukocytes; 4) renal interstitial hematopoietic cells; and 5) splenic ellipsoid hypertrophy. Thrombocyte frequency increased in PWS as scores increased only for gastric foreign body granulomas. In contrast, thrombocyte frequency increased with scores for renal interstitial hematopoietic cells in fall 1995 PWS samples.

Eosinophil frequency increased for only 3 lesions (Table 2): 1) caudal fin reddening (fall 1995 PWS); 2) gall bladder submucosal eosinophilic granular leukocytes (spring 1996 PWS); and 3) intestinal Anisakidae (spring 1996 Sitka). Monocytes were rare, and the only significant trends were inconsistent. Infection with the intestinal intraepithelial coccidian (*Goussia*? sp.) was related to increased monocyte frequency in fall 1995 PWS samples and to decreased monocyte frequency in spring 1996 Sitka samples.

Plasma chemistries

Plasma chemistry values and peritoneal Anisakidae numbers were significantly correlated with several general indicators of fish health (Table 9). Among enzymes, AST and CPK values were most variable, and differences in several lesion scores could be discerned on the basis of AST and CPK (Table 2). Values for AST were significantly greater in samples from Sitka than from PWS (Table 7), but values for CPK were higher in PWS than in Sitka. Values for ALP were significantly greater in females than males, and significantly greater in samples from Sitka than from PWS (Table 7); these differences have been consistent in all samples since spring 1995.

Holding fish for up to 4 hours as they awaited necropsy resulted in several changes in plasma chemistry values. As hold time increased, plasma potassium and CO_2 increased, but lactate and PCV decreased (Table 9). For spring 1996 samples, the increase in plasma CO_2 with increased hold time was more highly correlated in PWS than in Sitka (Table 9), but CO_2 in Sitka samples was significantly higher than PWS samples, regardless of hold time (Table 7); the same pattern occurred in spring 1995 samples. Lactate levels in 1996 PWS samples were significantly higher than 1995 Sitka samples; in spring 1995, lactate was greater in Sitka than in PWS. Fall 1995 fish had several highly significant changes in relation to hold time that were not significant in spring 1996 samples: calcium, chloride, and sodium levels increased, but phosphorus levels decreased. Changes in other plasma chemistries were not as significant in relation to hold time ($|r| < 0.25$).

Albumin and total protein were unusually low when compared to published values for other species (McDonald and Milligan 1992), but spring 1996 values were comparable to spring 1994 and 1995 values. Fall 1995 total protein levels were about 1 g/dL greater than spring values from either site. Both albumin and total protein were significantly higher in males than females in spring 1995 and 1996 samples, but differences in fall 1995 were not significant. IgM levels were not significantly different by gender in either fall or spring samples (Table7).

Annual Trends in Spawning Biomass and Pathogen Prevalence

Sample prevalence of *Ichthyophonus* decreased from fall to spring 1995, and the decrease was maintained through spring 1996 (Table 10). *Ichthyophonus* prevalence in spring 1996 samples was still higher than samples taken before 1994, but differences from prevalence in 1989 and 1990 were not significant when only liver, kidney, and spleen infections were used for comparison (chi-square test, 2×2 contingency tables). Prevalence of *Goussia clupearum* was fairly constant between 41 and 63% for most years before 1995, but prevalence increased in 1995 and again in 1996, when prevalence in Sitka and PWS was the highest recorded (Tables 2 and 10). Based only on histopathology, the prevalence of *Ortholinea orientalis* prevalence seemed to be higher in 1991 than in 1994 or 1995, but prevalence increased in fall 1995 and spring 1996 (Table 10).

Spawn-on-Kelp Pound Fishery

Of 38 fish sampled from the spawn-on-kelp pound fishery near Craig, 21% were positive for VHSV. Pooled spleen and head kidney from each of the 8 positive fish were positive for virus.

Brain was positive in 2 of the 8 fish, but brain was never positive when other tissues were negative. Four of the 8 VHSV+ fish were from 17 fish sampled from one pound, and the other 4 positive fish were from 21 fish sampled from 2 other pounds. The summary gross score was greater in selected fish (mean = 1.5) than in the random fish (mean = 0.4), but among selected fish, gross scores were not effected by VHSV status (Table 11). Among the 38 selected fish, VHSV status did not seem to affect any measured or scored variables. Three of 38 selected fish had gross signs of infection with the fungus *Ichthyophonus hoferi*, but all fish with signs of *Ichthyophonus* were negative for VHSV.

Discussion

Note on the contents the discussion section in this report: The annual reports for project 94320-S (Marty et al. 1995) and 95320-S (Marty et al. 1996) contain detailed discussion, including historical perspective, on most of the significant lesions and plasma chemistry changes in Pacific herring. That discussion is not repeated in this annual report, but will be included in the final synthesis report. This report concentrates on significant differences in lesions, necropsy findings, and plasma chemistry values in samples from fall 1995 and spring 1996. Variables, considered included spawning stage, season, site of capture, and year of capture, with special emphasis on organisms and lesions likely to result in population level effects.

Ichthyophonus hoferi

In Pacific herring from PWS, the large increase in prevalence of *Ichthyophonus* in 1994 was not associated with an unusual population decline between 1994 and 1996 (Figure 1). However, *Ichthyophonus* seems to have been the cause of unexpected high mortality of 7-year-old fish in both PWS and Sitka during the summer of 1995. The main difference in *Ichthyophonus* epidemiology between 1994 and spring 1995 was the age distribution of the fungus. In 1994, all age groups were infected in equal proportions. But in spring 1995, prevalence of *Ichthyophonus* was significantly greater in older fish. This trend towards higher prevalence in older fish continued into spring 1996 samples. The gall bladder myxosporean (*Ceratomyxa auerbachi*) was related to increasing age, but only for Sitka fish. No other pathogens were significantly more common in older fish, and *Ichthyophonus* seems the most likely cause of differential mortality in older fish. It may be that *Ichthyophonus* takes several months to years to cause mortality after a Pacific herring is infected, and mortality may require interaction with other variables such as ageing, predation, or other parasites.

In Atlantic herring populations as recently as 1991, major population decline in the North Sea was attributed to *Ichthyophonus* (Lang 1992). Mathematical analysis of population trends following this outbreak revealed that the use of *Ichthyophonus* prevalence significantly improved the fit of the catch and survey observations to the conventional assessment model (Patterson 1996). Because *Ichthyophonus* seems to cause mortality more readily in Atlantic herring than in Pacific herring, the role of *Ichthyophonus* in population decline may be less in Pacific herring

than in Atlantic herring. To clarify details of the pathogenesis, continued study is proposed as part of the laboratory component of this project, to include infectivity studies with *Ichthyophonus* cultured from the Atlantic ocean. Also, use of *Ichthyophonus* prevalence in the age-structured assessment model has not yet been attempted with Pacific herring, but a proposal for this work was submitted to the United States National Science Foundation, Division of Biological Oceanography, on Feb. 15, 1997 (G.D. Marty, principal investigator).

Among fish infected with *Ichthyophonus* in PWS and Sitka in 1996, the general pattern and distribution of lesions were very similar. In spring 1995, the stomachs of fish from Sitka were much more likely than PWS fish to contain empty foreign-body type granulomas (i.e., no organisms in the granulomas; scored as ICH+). In spring 1996 samples, site differences in the stomach ICH+ score were no longer significant. Differences in 1995 were evidence that Sitka fish may have been better able to mount an effective inflammatory reaction against *Ichthyophonus*, but 1996 results show that these differences were not consistent over 2 years.

Ichthyophonus infections in both sites in 1995 and 1996 were significantly related to increased IgM levels–consistent with the chronic nature of the disease. Further definition of the IgM response would require development of an ELISA specific for anti-*Ichthyophonus* antibodies, but development is beyond the current scope of the project. Infection with *Ichthyophonus* in 1995 and 1996 was less commonly related to changes in plasma CPK than in 1994, particularly in PWS samples, although AST was about equally effective all 3 years. The role of CPK in *Ichthyophonus* infections could not be further defined because isozymes could not be consistently separated using commercial tests that had been developed for use in mammalian plasma (C.J. Kennedy, personal communication).

VHSV

The lack of VHSV in spawning samples from both PWS and Sitka clearly demonstrates that Alaskan populations of Pacific herring can spawn without expressing significant quantities of VHSV. Also, the role of VHSV in PWS population decline is decreasing–consistent with the increasing population size from 1995 to 1996. The last time VHSV+ fish were sampled was in prespawning samples from 1995. Lack of VHSV+ fish in prespawning samples from 1996 provide evidence that prespawning samples can also be free of diagnosable levels of VHSV.

The reason for increased prevalence of hepatic necrosis (both hepatocellular single cell necrosis and coagulative focal necrosis) in fish from PWS is unknown. These lesions were related to VHSV infection in 1994, but in 1996 might have resulted from stress during spawning.

In spring 1995, several subtle inflammatory lesions were more prevalent in fish from PWS than from Sitka. Lack of these differences in 1996 samples is consistent with the hypothesis that these foci were a marker of previous VHSV infection. Increased numbers of leukocytes as part of the healing process would be more likely in 1995 than in 1996 fish. Alternatively, foci of leukocytes may be normal in wild fish, and decreased frequency of leukocytes in Sitka may have reflected

their active spawning status (i.e., a result of stress-induced leukocytopenia during spawning). Note that "spawning" 1995 fish from PWS had ripe gonads, but they had not yet spawned, whereas many "spawning" fish sampled in 1996 from PWS had already spawned. In support of this alternative hypothesis, the frequency of focal parenchymal leukocytes in the liver and heart decreased in spring 1996 PWS from prespawning to spawning samples. Confirmation of one of these hypotheses probably will require examination of fish in various stages of the disease, including recovery, after known exposure to VHSV in the laboratory.

Spawn-on-Kelp Pound Fishery

We have clearly shown that fish released from the 1996 spawn-on-kelp pound fisheries near Craig, Alaska, were actively expressing VHSV. The true prevalence of VHSV in released fish is unknown, but probably was between 1 and 21%. The low estimate assumes that all 760 fish not sampled for VHSV were negative for VHSV, and high estimate assumes that VHSV prevalence was the same in the 760 fish not sampled as in the 38 fish that were sampled. Three fish among the 38 selected fish had an overall external lesion score of zero, and one of these was positive for VHSV. This is evidence that Pacific herring can express VHSV without external signs of disease. We estimate that the actual prevalence of VHSV was in the range of 10 to 15%. Results from this study provide evidence that brain was not more useful for diagnosis than the spleen and head kidney samples that are used now on a routine basis.

VHSV prevalence of 10 to 15% in released fish could be a serious threat to the fishery. This concern is based on our knowledge of the virus from field and laboratory studies:

1) Naturally spawning Pacific herring populations have very low prevalence of VHSV. In 1995, all 240 spawning samples from Sitka Sound and all 180 spawning samples from Prince William Sound were negative for VHSV; in 1996, all 660 samples from PWS and Sitka were negative for VHSV.
2) A sample VHSV prevalence of 5% (11 of 233 spawning samples from PWS in 1994) was associated with severe gross lesions and continued population decline in PWS;
3) Laboratory studies by Dick Kocan (Univ. of Washington) and Jim Winton (U.S. National Biological Service) have shown that VHSV is readily shed and transmitted in water (section II, this report). Mortality from VHSV often occurs in a bimodal fashion: fish originally infected with the virus develop disease (VHS) and die; fish exposed to the virus from the first wave of infection develop VHS and die about a week later. This process could potentially continue for several weeks to months in schooling fish in the wild (i.e., through bioamplification).
4) Studies from the NMFS Auke Bay Laboratory have shown that Pacific herring in spawning condition can be held for several weeks without expressing VHSV. When this hold period was followed by low level hydrocarbon exposure, VHSV expression was significantly increased (Carls et al. 1997).

The effect of releasing several tons VHSV+ fish from spawn-on-kelp pounds to mix with unpounded fish is unknown and may be highly variable. In some years, exposure to infected fish

may effectively vaccinate large numbers of Pacific herring, thereby preventing a VHSV epidemic. In other years (1992-1993?), exposure to infected fish released from pounds may start an epidemic that results in severe mortality and population decline. Alternatively, the number of fish released from pounds may have no effect on the course of an epidemic in the population. Dedicated study of spawn-on-kelp pounds in PWS in 1997 (97162), coupled with continued laboratory investigation, may begin to answer some of these important questions.

External Lesions and Iris Reddening

External lesions are too nonspecific to be consistently related to any single cause. As evidence, in 1994 the external lesion fin base reddening was significantly associated with VHSV, and no external lesions were significantly associated with *Ichthyophonus*. In 1995, VHSV+ fish had few external lesions, whereas focal skin reddening was significantly associated with *Ichthyophonus* infection. In 1996, external lesions were no longer associated with *Ichthyophonus* infection. In last year's report, we hypothesized that separation of diffuse skin reddening from the focal skin reddening score (done in 1995 but not 1994) may have been all that was needed to show that *Ichthyophonus* was significantly related to severity of focal skin reddening. Results from spring 1996 refute this hypothesis. The lists of internal lesions associated with various external lesions (Table 3) provide evidence that external lesions are useful indicators of population health, but they cannot be used for more specific diagnoses.

Other Potential Pathogens

The best candidate for significant pathogens among the other common parasites in 1995 was the unidentified intestinal coccidian (*Goussia*? sp.), particularly in fish from PWS. In fall 1995 and spring 1996, however, infection with the intestinal coccidian was not related to any changes in plasma chemistries. Therefore, effects of infection with the intestinal coccidian are not consistent. As a secondary candidate for a significant pathogen, infection with the renal intraductal myxosporean *Ortholinea orientalis* was related to decreased protein levels in fall 1995 samples from PWS. Although differences were not highly significant, decreased protein levels could potentially inhibit overwinter survival. *Ortholinea orientalis* infection was not related to any plasma chemistry changes in spring 1996 samples, but analysis of fall 1996 samples may provide further evidence of the effect of *Ortholinea orientalis* on fall fish.

Plasma Chemistries

Plasma chemistries were highly sensitive to changes in several lesions, reproductive status, and other variables (e.g., age and weight). The significant site-related differences in lactate and CO_2 from spring 1995 samples provided information that was used to improve fish holding techniques as early as fall 1995 samples. Fish for sampling are now held in about 300 L of seawater (in large totes used by fish processors) to allow for maximum swimming space while fish are held from capture to necropsy. These changes minimized some of the site differences between Sitka and PWS samples.

Conclusions

Disease was probably the primary force driving Pacific herring population decline in 1993 and 1994. No other variables–food availability, predation, water temperature, currents, or recruitment–were needed to explain this significant decline; however, these variables may have contributed to conditions favorable for initiation of the epidemic. The conclusion that disease was significant is based on integration of results from this project, literature review including Meyers et al. (1994), plus information from biologists, fishers, and laboratory study where both VHSV and *Ichthyophonus* killed Pacific herring in the absence of other diseases (section II, this report). Among the 2 significant diseases, VHSV was most important in 1993. By 1994, *Ichthyophonus* prevalence had increased, possibly as a result of VHSV-induced immunosuppression, so that *Ichthyophonus* and VHSV were of about equal importance. By 1995, VHSV prevalence had decreased to where it was a less important cause of mortality, and by 1996, VHSV was not an important cause of mortality. If *Ichthyophonus* prevalence continues to decrease, as indicated by fall 1995 and spring 1996 samples, and young Pacific herring in PWS remain relatively free of disease, then disease should no longer limit population recovery. Opening of all Pacific herring fisheries in PWS in 1997 is evidence of population recovery consistent with decreased disease prevalence. Continued monitoring of disease in PWS and Sitka will increase our knowledge of how disease interacts with pelagic schooling fish like Pacific herring, and further study will serve to document population recovery or, alternatively, identify reasons that recovery fails to occur.

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Table 1. Mean values for continuous necropsy variables in Pacific herring sampled from Sitka Sound (SS) and Prince William Sound (PWS) in March and April, 1996. Group means within each variable were compared using one-way analysis of variance and Tukey's multiple comparison procedure; groups with the same letter are not significantly different ($P \leq 0.050$). Comparisons in which Levene's test for equality of variance was significant ($P \leq 0.05$) are marked (*).

Variable	Site	Spawning status	# examined	Mean	±SE	ANOVA *P* value
Age (yrs)	PWS	prespawning	80	5.2^{B}	0.3	<0.001*
	PWS	spawning	180	6.1^{A}	0.2	
	SS	spawning	240	4.6^{B}	0.1	
standard length (mm)	PWS	prespawning	80	209.6^{A}	2.2	0.047
	PWS	spawning	180	$204.3^{A,B}$	1.3	
	SS	spawning	240	204.0^{B}	1.2	
body weight (g)	PWS	prespawning	80	117.9^{A}	4.5	<0.001*
	PWS	spawning	180	125.5^{A}	2.8	
	SS	spawning	240	106.5^{B}	2.1	
liver weight (g)	PWS	prespawning	63	1.1^{A}	0.1	<0.001*
	PWS	spawning	180	1.1^{A}	0.0	
	SS	spawning	240	0.9^{B}	0.0	
ovary weight (g)[a]	PWS	prespawning	36	22.5	2.2	NS[b]
	PWS	spawning	69	23.6	1.9	
	SS	spawning	92	22.5	1.4	
testis weight (g)[a]	PWS	prespawning	40	23.8^{A}	1.7	<0.001
	PWS	spawning	111	12.9^{B}	1.1	
	SS	spawning	148	13.6^{B}	0.9	
hold time (min)	PWS	prespawning	80	94.5^{B}	4.1	<0.001
	PWS	spawning	180	100.8^{B}	3.1	
	SS	spawning	240	139.1^{A}	2.6	
IgM (mg/mL)[c]	PWS	prespawning	77	649	99.7	NS
	PWS	spawning	152	610	78.0	
	SS	spawning	240	662	54.8	

Variable	Site	Spawning status	# examined	Mean	±SE	ANOVA *P* value
PCV (%)[d]	PWS	prespawning	78	41.9[B]	1.2	0.016
	PWS	spawning	180	42.3[A,B]	0.8	
	SS	spawning	240	43.5[A]	0.6	
peritoneal cavity - number of herring worms (Anisakidae)[c]	PWS	prespawning	80	14.7[A]	2.0	0.002
	PWS	spawning	179	11.6[B]	1.1	
	SS	spawning	240	14.2[A]	1.2	
gills - number of 0.5-mm-diameter white foci	PWS	prespawning	79	0.3[B]	0.1	<0.001*
	PWS	spawning	174	0.2[B]	0.0	
	SS	spawning	235	0.6[A]	0.1	

[a]Juvenile fish were not used for comparisons of ovary and testis weight.
[b]NS = not significant.
[c]Values for IgM and Anisakidae were compared after natural log transformation; values listed here are retransformed to the geometric means and the first-order Taylor series approximation of the standard error.
[d]Values for PCV were arcsine square root transformed for statistical analysis; mean values listed here are retransformed, and the first-order Taylor series was used as an approximation of the standard error of the mean.

Table 2. Lesion severity (% of fish classified in each lesion score) and lesion prevalence (% of sample having lesion score >0) in Pacific herring sampled from Sitka Sound (SS), Alaska, during November 1995 (F95) or March 1996 (S96), or from Prince William Sound (PWS) during November 1995 (F95) or April 1996 (S96). Lesions were scored as none (0), mild (1), moderate (2), or severe (3). Age, hold time, and blood values were compared for groups based on lesion scores using one-way analysis of variance and Tukey's multiple comparison procedure. Significant trends ($P \leq 0.050$) were based on rank order of mean responses for fish groups classified by lesion scores. Compared to fish with the lowest lesion score, mean response for the fish group with the highest lesion score was significantly higher (↑), lower (↓), or there was no significant trend (NT) in the rank order. For comparisons in which Levene's test for equality of variance was significant (*), only ANOVA comparisons with $P \leq 0.010$ are shown.

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
External gross lesions:								
caudal fin fraying	F95-SS all	30	0.0	53.3	46.7	0.0	100.0	ND[a]
	F95-PWS all	130	0.0	86.2	12.3	1.5	100.0	↓- total protein (0.019), globulin (0.005)
	S96-SS spawning	240	5.8	66.7	24.6	2.9	94.2	↑- calcium (<0.001*) NT- lnIgM (0.035)
	S96-PWS all	260	11.2	73.8	14.6	0.4	88.8	↓- total protein (0.001), albumin (0.002), globulin (0.001), cholesterol (<0.001), glucose (0.030) NT- chloride (0.024), lactate (0.044), % thrombocytes (0.029)
	S96-PWS prespawn	80	20.0	70.0	10.0	0.0	80.0	ND
	S96-PWS spawning	180	7.2	75.6	16.7	0.6	92.8	ND
caudal fin reddening	F95-SS all	30	0.0	80.0	20.0	0.0	100.0	ND
	F95-PWS all	130	16.2	71.5	11.5	0.8	83.8	↑- % eosinophils (0.004*) NT- % basophils (0.009)

[a]ND = not done

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-SS spawning	240	17.1	55.8	24.2	2.9	82.9	↑- sodium (0.005), chloride (0.008*), calcium (<0.001*) NT- osmolality (0.017), lnCPK (0.048)
	S96-PWS all	260	39.2	51.5	8.1	1.2	60.8	↑- sodium (<0.001), chloride (<0.001*), calcium (0.001), osmolality (0.021), ↓- total protein (<0.001), albumin (<0.001), globulin (<0.001), cholesterol (<0.001), glucose (0.006), % thrombocytes (0.005)
	S96-PWS prespawn	80	55.0	41.2	2.5	1.2	45.0	ND
	S96-PWS spawning	180	32.2	56.1	10.6	1.1	67.8	ND
fin base reddening	F95-SS all	30	10.0	63.3	23.3	3.3	90.0	ND
	F95-PWS all	130	43.1	40.8	8.5	7.7	56.9	↑- Age (<0.001*), hold time (0.027), ↓- phosphate (0.001) NT- potassium (0.005), CO2 (0.001*)
	S96-SS spawning	240	32.5	55.8	7.5	4.2	67.5	NT- chloride (0.047), calcium (0.037)
	S96-PWS all	260	51.2	43.5	4.2	1.2	48.8	↑- chloride (0.001*), % neutrophils (0.001*) ↓- glucose (0.008) NT- sodium (0.004*), total protein (0.035), globulin (0.027)
	S96-PWS prespawn	80	65.0	32.5	2.5	0.0	35.0	ND
	S96-PWS spawning	180	45.0	48.3	5.0	1.7	55.0	ND
iris reddening	F95-SS all	30	6.7	90.0	3.3	0.0	93.3	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	F95-PWS all	130	36.9	56.2	6.9	0.0	63.1	↑- sodium (<0.001), lactate (<0.001), osmolality (<0.001) ↓- CO2 (0.026) NT- chloride (0.004), phosphate (0.012)
	S96-SS spawning	240	34.6	62.5	2.9	0.0	65.4	↑- potassium (0.021), globulin (0.037), lnCPK (0.006)
	S96-PWS all	260	31.2	66.9	1.9	0.0	68.8	↑- total protein (0.001), albumin (0.007), globulin (<0.001), ALP (0.001), cholesterol (0.035), glucose (<0.001*), lnCPK (0.016) ↓- bilirubin (0.006)
	S96-PWS prespawn	80	37.5	61.2	1.2	0.0	62.5	ND
	S96-PWS spawning	180	28.3	69.4	2.2	0.0	71.7	ND
opercular copepod	F95-SS all			not scored				ND
	F95-PWS all			not scored				ND
	S96-SS spawning	44	0.0	100.0	0.0	0.0	100.0	ND (not all fish were scored)
	S96-PWS all	253	56.5	43.5	0.0	0.0	43.5	↑- potassium (0.006), phosphate (0.012) ↓- Age (<0.001*), CO2 (0.004)
	S96-PWS prespawn	73	41.1	58.9	0.0	0.0	58.9	ND
	S96-PWS spawning	180	62.8	37.2	0.0	0.0	37.2	ND
skin reddening, diffuse	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	129	79.8	15.5	3.9	0.8	20.2	↑- total protein (0.014), albumin (0.003) ↓- bilirubin (0.037)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-SS spawning	240	80.4	12.9	5.4	1.2	19.6	↓- Globulin (0.046) NT- PCV (0.002)
	S96-PWS all	260	94.6	4.6	0.8	0.0	5.4	↑- hold time (0.009), lnALT (0.014) ↓- total protein (0.011), albumin (0.019), globulin (0.011), cholesterol (0.007), lactate (0.011), glucose (0.044)
	S96-PWS prespawn	80	97.5	2.5	0.0	0.0	2.5	ND
	S96-PWS spawning	180	93.3	5.6	1.1	0.0	6.7	ND
skin reddening, focal	F95-SS all	30	70.0	26.7	3.3	0.0	30.0	ND
	F95-PWS all	130	47.7	46.2	3.1	3.1	52.3	↓- cholesterol (0.001)
	S96-SS spawning	240	57.5	41.7	0.8	0.0	42.5	↑- sodium (0.016), chloride (0.001), calcium (0.002), osmolality (<0.001) ↓- bilirubin (0.019)
	S96-PWS all	260	80.0	18.1	0.4	1.5	20.0	↑-chloride (0.012) ↓- total protein (<0.001), albumin (0.002), globulin (<0.001), ALP (0.001), cholesterol (<0.001), lactate (0.029), glucose (0.002), lnIgM (0.012)
	S96-PWS prespawn	80	80.0	20.0	0.0	0.0	20.0	ND
	S96-PWS spawning	180	80.0	17.2	0.6	2.2	20.0	ND
Other Gross findings:								
gonad fullness	F95-SS all		not scored (all full)					ND
	F95-PWS all		not scored (all full)					ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-SS spawning	239	16.3	6.7	13.4	63.6	83.7	↑- PCV (0.002), Total protein (0.001), Globulin (<0.001), bilirubin (<0.001), CO2 (<0.001), glucose (<0.001), lnCPK (<0.001) ↓- sodium (<0.001), chloride (<0.001), ALP (0.015), osmolality (<0.001), % neutrophils (<0.001) NT- cholesterol (0.004), % basophils (0.005*), lnAST (0.021)
	S96-PWS all	256	21.1	4.7	9.0	65.2	78.9	↑- PCV (0.033), total protein (<0.001), albumin (<0.001), globulin (<0.001), cholesterol (<0.001*), lactate (0.028), glucose (<0.001*), % lymphocytes (<0.001*), lnCPK (<0.001*) ↓- sodium (<0.001*), chloride (<0.001*), % neutrophils (<0.001*) NT- hold time (0.047), ALP (0.020), calcium (<0.001*), lnIgM (0.031)
	S96-PWS prespawn	76	0.0	0.0	0.0	100.0	100.0	ND
	S96-PWS spawning	180	30.0	6.7	12.8	50.6	70.0	ND
Brain microscopic lesions:								
Ichthyophonus	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	95.0	4.6	0.0	0.0	4.6	ND (too few responses)
	S96-SS spawning	240	91.2	7.5	1.2	0.0	8.8	↑- lnALT (0.003*), lnAST (0.002), lnIgM (<0.001) ↓- PCV (0.040)
	S96-PWS all	260	91.5	6.9	1.5	0.0	8.5	↑- lnALT (0.005), lnAST (<0.001) ↓- PCV (021), % thrombocytes (0.048)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS prespawn	80	93.8	5.0	1.3	0.0	6.3	ND
	S96-PWS spawning	180	90.6	7.8	1.7	0.0	9.4	ND
meningeal eosinophilic granular leukocytes	F95-SS all	30	3.3	43.3	53.3	0.0	96.7	ND
	F95-PWS all	130	0.0	43.1	56.2	0.7	100.0	↑- lnAnisakidae (0.016)
	S96-SS spawning	240	7.9	62.9	29.2	0.0	92.1	↑- hold time (0.025) NT- bilirubin (0.031), lnAST (0.006)
	S96-PWS all	260	6.2	54.2	39.6	0.0	93.8	↑- lnAnisakidae (0.005), lnCPK (0.025)
	S96-PWS prespawn	80	5.0	52.5	42.5	0.0	95.0	ND
	S96-PWS spawning	180	6.7	55.0	38.3	0.0	93.3	ND
meningoencephalitis	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND
	F95-PWS all	130	93.1	6.9	0.0	0.0	6.9	none
	S96-SS spawning	240	93.1	6.9	0.0	0.0	6.9	none
	S96-PWS all	260	97.3	2.7	0.0	0.0	2.7	ND (too few responses)
	S96-PWS prespawn	80	97.5	2.5	0.0	0.0	2.5	ND
	S96-PWS spawning	180	97.2	2.8	0.0	0.0	2.8	ND
Gall bladder microscopic lesions:								
eosinophils, submucosal	F95-SS all			not scored				ND
	F95-PWS all			not scored				ND
	S96-SS spawning	233	54.5	41.2	4.3	0.0	45.5	none
	S96-PWS all	253	49.8	46.6	3.6	0.0	50.2	↑- % eosinophils (0.008*), lnALT (0.030) NT- hold time (0.032)
	S96-PWS prespawn	78	59.0	39.7	1.3	0.0	41.0	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS spawning	175	45.7	49.7	4.6	0.0	54.3	ND
myxosporean (*Ceratomyxa auerbachi*)	F95-SS all	23	91.3	8.7	0.0	0.0	8.7	ND
	F95-PWS all	125	79.2	16.0	4.0	0.8	20.8	none
	S96-SS spawning	231	76.6	21.2	2.2	0.0	23.4	↑- Age (<0.001*), total protein (0.017), albumin (0.005)
	S96-PWS all	251	70.9	20.3	8.4	0.4	29.1	↓- calcium (0.003)
	S96-PWS prespawn	78	64.1	25.6	9.0	1.3	35.9	ND
	S96-PWS spawning	173	74.0	17.9	8.1	0.0	26.0	ND
Gill microscopic lesions:								
ciliated protozoa (e.g., *Trichodina* spp.)	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S96-SS spawning	240	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S96-PWS all	260	99.6	0.4	0.0	0.0	0.4	ND (too few responses)
	S96-PWS prespawn	80	100.0	0.0	0.0	0.0	0.0	ND
	S96-PWS spawning	180	99.4	0.6	0.0	0.0	0.6	ND
Epitheliocystis	F95-SS all	30	90.0	10.0	0.0	0.0	10.0	ND
	F95-PWS all	130	94.6	5.4	0.0	0.0	5.4	ND (too few responses)
	S96-SS spawning	240	78.8	19.2	1.7	0.4	21.2	↑- lnAnisakidae (0.013) ↓- lnALT (0.011)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS all	260	83.5	16.2	0.0	0.4	16.5	↑- lnAST (0.050) ↓- Age (<0.001*), sodium (0.004), chloride (0.014), total protein (0.018), albumin (0.034), globulin (0.018), CO2 (0.001), calcium (0.011), % neutrophils (0.033)
	S96-PWS prespawn	80	72.5	27.5	0.0	0.0	27.5	ND
	S96-PWS spawning	180	88.3	11.1	0.0	0.6	11.7	ND
foreign body granuloma	F95-SS all	30	90.0	10.0	0.0	0.0	10.0	ND
	F95-PWS all	130	92.3	7.7	0.0	0.0	7.7	none
	S96-SS spawning	240	90.8	9.2	0.0	0.0	9.2	none
	S96-PWS all	260	93.1	6.5	0.4	0.0	6.9	↑- lactate (0.022)
	S96-PWS prespawn	80	91.2	7.5	1.2	0.0	8.8	ND
	S96-PWS spawning	180	93.9	6.1	0.0	0.0	6.1	ND
gill arch inflammation or hematopoiesis	F95-SS all	30	0.0	96.7	3.3	0.0	100.0	ND
	F95-PWS all	130	0.0	96.9	3.1	0.0	100.0	ND (too few responses)
	S96-SS spawning	240	0.4	92.1	7.5	0.0	99.6	none
	S96-PWS all	260	0.0	90.0	10.0	0.0	100.0	↑- lnALT (0.005), lnAST (0.033) ↓- PCV (0.006), total protein (0.005), albumin (0.026), globulin (0.003), ALP (0.033), cholesterol (<0.001), lactate (0.013)
	S96-PWS prespawn	80	0.0	92.5	7.5	0.0	100.0	ND
	S96-PWS spawning	180	0.0	88.9	11.1	0.0	100.0	ND
Ichthyophonus	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	F95-PWS all	130	94.6	2.3	2.3	0.8	5.4	ND (too few responses)
	S96-SS spawning	240	91.2	6.2	1.2	1.2	8.8	↑- Age (0.047), sodium (0.036), osmolality (0.006), lnALT (0.009*), lnAST (0.009) ↓- PCV (0.006), bilirubin (0.012)
	S96-PWS all	260	88.8	5.8	3.8	1.5	11.2	↑- lnALT (0.007), lnAST (0.002) ↓- PCV (0.038), cholesterol (0.009) NT- total protein (0.002), albumin (0.002), globulin (0.004), ALP (0.024), lnIgM (0.002)
	S96-PWS prespawn	80	92.5	3.8	1.2	2.5	7.5	ND
	S96-PWS spawning	180	87.2	6.7	5.0	1.1	12.8	ND
lamellar hyperplasia	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND
	F95-PWS all	130	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S96-SS spawning	240	98.8	0.8	0.4	0.0	1.2	ND (too few responses)
	S96-PWS all	260	98.1	1.9	0.0	0.0	1.9	ND (too few responses)
	S96-PWS prespawn	80	97.5	2.5	0.0	0.0	2.5	ND
	S96-PWS spawning	180	98.3	1.7	0.0	0.0	1.7	ND
lamellar telangiectasis	F95-SS all	30	70.0	23.3	6.7	0.0	30.0	ND
	F95-PWS all	130	88.5	10.0	1.5	0.0	11.5	↑- bilirubin (0.022)
	S96-SS spawning	240	94.6	4.2	1.2	0.0	5.4	↑- sodium (0.008) ↓- globulin (0.005), lnCPK (0.044)
	S96-PWS all	260	95.4	4.2	0.4	0.0	4.6	none
	S96-PWS prespawn	80	95.0	5.0	0.0	0.0	5.0	ND
	S96-PWS spawning	180	95.6	3.9	0.6	0.0	4.4	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
monogenetic trematodes (e.g., *Gyrodactylus* spp.)	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND
	F95-PWS all	130	98.5	1.5	0.0	0.0	1.5	ND (too few responses)
	S96-SS spawning	240	97.9	2.1	0.0	0.0	2.1	ND (too few responses)
	S96-PWS all	260	98.5	1.5	0.0	0.0	1.5	ND (too few responses)
	S96-PWS prespawn	80	98.8	1.2	0.0	0.0	1.2	ND
	S96-PWS spawning	180	98.3	1.7	0.0	0.0	1.7	ND
Gonad - female microscopic lesions:								
eosinophilic granular leukocytes	F95-SS all	14	0.0	64.3	35.7	0.0	100.0	ND
	F95-PWS all	63	4.8	76.2	15.9	3.2	95.2	↑- lnIgM (0.048) ↓- % yolked eggs (0.035)
	S96-SS spawning	92	26.1	56.5	16.3	1.1	73.9	NT- ALP (0.037)
	S96-PWS all	105	39.0	54.3	6.7	0.0	61.0	↑- PCV (0.022) ↓- hold time (0.050)
	S96-PWS prespawn	36	44.4	47.2	8.3	0.0	55.6	ND
	S96-PWS spawning	69	36.2	58.0	5.8	0.0	63.8	ND
granulomatous inflammation	F95-SS all	14	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	63	96.8	3.2	0.0	0.0	3.2	ND (too few responses)
	S96-SS spawning	92	82.6	17.4	0.0	0.0	17.4	none
	S96-PWS all	105	82.9	16.2	0.0	1.0	17.1	↑- sodium (0.035), chloride (0.019), osmolality (0.048) ↓- total protein (0.036), albumin (0.030), globulin (0.047)
	S96-PWS prespawn	36	86.1	11.1	0.0	2.8	13.9	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS spawning	69	81.2	18.8	0.0	0.0	18.8	ND
hyalinization of vessel walls	F95-SS all	14	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	63	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S96-SS spawning	92	96.7	3.3	0.0	0.0	3.3	none
	S96-PWS all	105	95.2	4.8	0.0	0.0	4.8	none
	S96-PWS prespawn	36	97.2	2.8	0.0	0.0	2.8	ND
	S96-PWS spawning	69	94.2	5.8	0.0	0.0	5.8	ND
Ichthyophonus	F95-SS all	14	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	63	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S96-SS spawning	92	98.9	1.1	0.0	0.0	1.1	ND (too few responses)
	S96-PWS all	105	98.1	1.9	0.0	0.0	1.9	ND (too few responses)
	S96-PWS prespawn	36	100.0	0.0	0.0	0.0	0.0	ND
	S96-PWS spawning	69	97.1	2.9	0.0	0.0	2.9	ND
macrophage aggregates (pigmented)	F95-SS all	14	42.9	57.1	0.0	0.0	57.1	ND
	F95-PWS all	63	22.2	76.2	1.6	0.0	77.8	none
	S96-SS spawning	92	70.7	29.3	0.0	0.0	29.3	↑- Age (<0.001*), total protein (0.019), globulin (0.008), CO2 (0.035) ↓- % neutrophils (0.043)
	S96-PWS all	105	58.1	41.9	0.0	0.0	41.9	↑- Age (<0.001), cholesterol (0.045), CO2 (0.014), % neutrophils (0.020) ↓- bilirubin (0.007), lnAST (0.009)
	S96-PWS prespawn	36	63.9	36.1	0.0	0.0	36.1	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-PWS spawning	69	55.1	44.9	0.0	0.0	44.9	ND
oocyte atresia, mature follicles	F95-SS all	14	42.9	42.9	7.1	7.1	57.1	ND
	F95-PWS all	63	17.5	46.0	34.9	1.6	82.5	none
	S96-SS spawning	92	90.2	7.6	1.1	1.1	9.8	↑- sodium (0.030), chloride (0.015), % neutrophils (0.029) ↓- globulin (0.019), cholesterol (0.007), glucose (0.025), lnCPK (0.004)
	S96-PWS all	105	78.1	20.0	1.0	1.0	21.9	↑- chloride (0.044) ↓- cholesterol (0.035)
	S96-PWS prespawn	36	75.0	25.0	0.0	0.0	25.0	ND
	S96-PWS spawning	69	79.7	17.4	1.4	1.4	20.3	ND
oocyte rupture, mature follicles	F95-SS all	82	98.8	1.2	0.0	0.0	1.2	ND
	F95-PWS all	114	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S96-SS spawning	92	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S96-PWS all	105	99.0	1.0	0.0	0.0	1.0	ND (too few responses)
	S96-PWS prespawn	36	100.0	0.0	0.0	0.0	0.0	ND
	S96-PWS spawning	69	98.6	1.4	0.0	0.0	1.4	ND
Gonad - male microscopic lesions:								
Eimeria sardinae	F95-SS all	16	50.0	37.5	12.5	0.0	50.0	ND
	F95-PWS all	65	38.5	60.0	1.5	0.0	61.5	↑- chloride (0.040), CO2 (0.005) ↓- total protein (0.034), globulin (0.036), bilirubin (0.001), cholesterol (0.037)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-SS spawning	148	18.9	74.3	5.4	1.4	81.1	↓- Age (<0.001*) NT- bilirubin (0.048), lnIgM (0.037)
	S96-PWS all	151	26.5	68.9	4.6	0.0	73.5	↑- total protein (<0.001), albumin (0.001), globulin (<0.001), bilirubin (0.035), ALP (0.031), cholesterol (<0.001), lactate (0.003), glucose (<0.001), lnCPK (<0.001*), lnAST (0.006), lnIgM (0.050) ↓- chloride (<0.001*), % neutrophils (0.005)
	S96-PWS prespawn	40	2.5	90.0	7.5	0.0	97.5	ND
	S96-PWS spawning	111	35.1	61.3	3.6	0.0	64.9	ND
eosinophilic granular leukocytes	F95-SS all	16	18.8	75.0	6.3	0.0	81.3	ND
	F95-PWS all	65	9.2	78.5	10.8	1.5	90.8	↑- chloride (0.032), osmolality (0.037)
	S96-SS spawning	148	52.7	45.3	2.0	0.0	47.3	↑- chloride (0.021), % neutrophils (0.015) ↓- bilirubin (0.024), lnCPK (0.002)
	S96-PWS all	151	49.0	43.7	6.6	0.7	51.0	↑- % neutrophils (0.001), % basophils (<0.001*) ↓- glucose (<0.001*) NT- phosphorus (0.016)
	S96-PWS prespawn	40	65.0	35.0	0.0	0.0	35.0	ND
	S96-PWS spawning	111	43.2	46.8	9.0	0.9	56.8	ND
granulomatous inflammation	F95-SS all	16	93.8	6.3	0.0	0.0	6.3	ND
	F95-PWS all	65	93.8	6.2	0.0	0.0	6.2	none
	S96-SS spawning	148	98.0	2.0	0.0	0.0	2.0	ND (too few responses)
	S96-PWS all	151	98.0	2.0	0.0	0.0	2.0	ND (too few responses)
	S96-PWS prespawn	40	95.0	5.0	0.0	0.0	5.0	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS spawning	111	99.1	0.9	0.0	0.0	0.9	ND
Ichthyophonus	F95-SS all	16	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	65	98.5	1.5	0.0	0.0	1.5	ND (too few responses)
	S96-SS spawning	148	98.6	0.7	0.7	0.0	1.4	ND (too few responses)
	S96-PWS all	151	95.4	4.6	0.0	0.0	4.6	none
	S96-PWS prespawn	40	100.0	0.0	0.0	0.0	0.0	ND
	S96-PWS spawning	111	93.7	6.3	0.0	0.0	6.3	ND
macrophage aggregates (pigmented)	F95-SS all	16	93.8	6.3	0.0	0.0	6.3	ND
	F95-PWS all	65	98.5	1.5	0.0	0.0	1.5	ND (too few responses)
	S96-SS spawning	148	98.0	2.0	0.0	0.0	2.0	ND (too few responses)
	S96-PWS all	151	96.7	3.3	0.0	0.0	3.3	none
	S96-PWS prespawn	40	95.0	5.0	0.0	0.0	5.0	ND
	S96-PWS spawning	111	97.3	2.7	0.0	0.0	2.7	ND
spermatocyte numbers (3 = abundant)	F95-SS all	16	0.0	18.8	81.3	0.0	100.0	ND
	F95-PWS all	65	3.1	27.7	64.6	4.6	96.9	↑- total protein (0.016), albumin (0.009)
	S96-SS spawning	148	8.1	5.4	17.6	68.9	91.9	↑- Total protein (<0.001), albumin (0.032), globulin (<0.001), cholesterol (0.005), CO2 (0.001), glucose (<0.001*), lnCPK (<0.001) NT- sodium (<0.001*), chloride (<0.001), bilirubin (0.004), % lymphocytes (0.011), % neutrophils (0.050)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS all	151	13.9	9.3	9.9	66.9	86.1	↑- total protein (<0.001), albumin (<0.001), globulin (<0.001), cholesterol (<0.001), glucose (<0.001*), lnCPK (<0.001) ↓- sodium (0.001*), chloride (<0.001*) NT- % neutrophils (0.001), % basophils (0.006*), lnIgM (0.032)
	S96-PWS prespawn	40	0.0	0.0	0.0	100.0	100.0	ND
	S96-PWS spawning	111	18.9	12.6	13.5	55.0	81.1	ND
Heart microscopic lesions:								
epicarditis	F95-SS all	30	6.7	90.0	3.3	0.0	93.3	ND
	F95-PWS all	130	14.6	78.5	6.9	0.0	85.4	↑- lnCPK (0.001), lnAST (0.008) NT- lnALT (0.047)
	S96-SS spawning	240	13.8	82.1	3.3	0.8	86.2	NT- chloride (0.013), osmolality (0.043), lnAnisakidae (0.021)
	S96-PWS all	260	15.4	75.4	8.5	0.8	84.6	NT- lnIgM (0.017)
	S96-PWS prespawn	80	21.2	73.8	5.0	0.0	78.8	ND
	S96-PWS spawning	180	12.8	76.1	10.0	1.1	87.2	ND
Ichthyophonus	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	87.7	5.4	3.1	3.8	12.3	↑- Age (<0.001*), total protein (0.026), albumin (0.045), globulin (0.047), ALP (0.031), % basophils (0.009), lnCPK (<0.001), lnAST (<0.001), lnALT (0.033), lnIgM (<0.001) ↓- PCV (0.006), bilirubin (<0.001), cholesterol (0.003)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-SS spawning	240	82.1	5.4	7.1	5.4	17.9	↓- PCV (0.011) NT- Age (0.033), total protein (0.047), albumin (0.031), lnCPK (0.036), lnALT (0.002), lnAST (0.001), lnIgM (0.014)
	S96-PWS all	260	82.3	5.8	4.6	7.3	17.7	↑- lnALT (<0.001), lnAST (<0.001), lnIgM (0.001) ↓- PCV (0.035) NT- osmolality (0.026)
	S96-PWS prespawn	80	88.8	6.2	3.8	1.2	11.2	ND
	S96-PWS spawning	180	79.4	5.6	5.0	10.0	20.6	ND
leukocytes, focal, parenchymal	F95-SS all	30	76.7	23.3	0.0	0.0	23.3	ND
	F95-PWS all	130	43.8	53.8	2.3	0.0	56.2	none
	S96-SS spawning	240	82.9	17.1	0.0	0.0	17.1	none
	S96-PWS all	260	86.5	13.5	0.0	0.0	13.5	↑- phosphate (0.023) ↓- % neutrophils (0.002)
	S96-PWS prespawn	80	80.0	20.0	0.0	0.0	20.0	ND
	S96-PWS spawning	180	89.4	10.6	0.0	0.0	10.6	ND
thrombosis	F95-SS all	30	86.7	13.3	0.0	0.0	13.3	ND
	F95-PWS all	130	89.2	10.8	0.0	0.0	10.8	↑- sodium (0.006*), chloride (0.004*), osmolality (0.008)
	S96-SS spawning	240	81.2	15.4	1.7	1.7	18.8	NT- bilirubin (0.021)
	S96-PWS all	260	76.9	18.8	3.5	0.8	23.1	↑- lnAST (0.011), lnIgM (0.003) NT- cholesterol (0.025)
	S96-PWS prespawn	80	88.8	8.8	2.5	0.0	11.2	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS spawning	180	71.7	23.3	3.9	1.1	28.3	ND
Intestine and intestinal cecae, microscopic lesions:								
Anisakidae	F95-SS all	30	6.7	70.0	10.0	13.3	93.3	ND
	F95-PWS all	130	10.0	60.0	23.1	6.9	90.0	↑- lnAnisakidae (<0.001) ↓- lnALT (0.002) NT- PCV (0.009*), cholesterol (0.003)
	S96-SS spawning	240	22.5	57.9	14.2	5.4	77.5	↑- % eosinophils (0.006*), lnAnisakidae (<0.001) NT- globulin (0.042), cholesterol (0.036)
	S96-PWS all	260	13.5	63.1	18.8	4.6	86.5	↑- lnAnisakidae (0.006) NT- hold time (0.050)
	S96-PWS prespawn	80	13.8	63.8	17.5	5.0	86.2	ND
	S96-PWS spawning	180	13.3	62.8	19.4	4.4	86.7	ND
arteriolar hyperplasia, focal, intimal	F95-SS all	30	66.7	33.3	0.0	0.0	33.3	ND
	F95-PWS all	130	44.6	52.3	3.1	0.0	55.4	↓- PCV (0.008)
	S96-SS spawning	240	51.2	45.4	3.3	0.0	48.8	NT- albumin (0.016)
	S96-PWS all	260	44.6	52.7	2.7	0.0	55.4	none
	S96-PWS prespawn	80	41.2	56.2	2.5	0.0	58.8	ND
	S96-PWS spawning	180	46.1	51.1	2.8	0.0	53.9	ND
cestodes	F95-SS all	30	73.3	10.0	16.7	0.0	26.7	ND
	F95-PWS all	130	90.8	7.7	1.5	0.0	9.2	↑- potassium (0.039) ↓- albumin (0.010), lnALT (0.006)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-SS spawning	240	96.2	0.8	2.9	0.0	3.8	↑- total protein (0.005), globulin (0.002), cholesterol (0.015), lactate (0.001*), calcium (0.008*)
	S96-PWS all	260	98.5	0.4	1.2	0.0	1.5	ND (too few responses)
	S96-PWS prespawn	80	98.8	0.0	1.2	0.0	1.2	ND
	S96-PWS spawning	180	98.3	0.6	1.1	0.0	1.7	ND
coccidian, intraepithelial (*Goussia*? sp.)	F95-SS all	30	53.3	46.7	0.0	0.0	46.7	ND
	F95-PWS all	130	73.8	26.2	0.0	0.0	26.2	↑- %monocytes (<0.001*) ↓- %yolked eggs (0.002*)
	S96-SS spawning	240	7.9	90.4	1.7	0.0	92.1	↓- % monocytes(0.003*)
	S96-PWS all	260	6.5	83.1	10.4	0.0	93.5	none
	S96-PWS prespawn	80	6.2	80.0	13.8	0.0	93.8	ND
	S96-PWS spawning	180	6.7	84.4	8.9	0.0	93.3	ND
eosinophilic granular leukocytes, submucosal	F95-SS all	30	0.0	100.0	0.0	0.0	100.0	ND
	F95-PWS all	130	0.0	93.1	6.9	0.0	100.0	↑- chloride (0.010), calcium (0.005*)
	S96-SS spawning	240	3.3	93.8	2.9	0.0	96.7	none
	S96-PWS all	260	2.3	92.3	5.4	0.0	97.7	↑- glucose (0.002)
	S96-PWS prespawn	80	3.8	90.0	6.2	0.0	96.2	ND
	S96-PWS spawning	180	1.7	93.3	5.0	0.0	98.3	ND
foreign body granuloma	F95-SS all	30	56.7	43.3	0.0	0.0	43.3	ND
	F95-PWS all	130	61.5	38.5	0.0	0.0	38.5	↑- total protein (0.003), albumin (0.008), globulin (0.009), lnALT (0.008), lnIgM (0.017)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-SS spawning	240	81.7	18.3	0.0	0.0	18.3	↑- Age (<0.001*), total protein (0.032), albumin (0.006)
	S96-PWS all	260	74.2	25.8	0.0	0.0	25.8	↑- Age (<0.001), CO2 (0.046) ↓- phosphate (0.004), osmolality (0.046)
	S96-PWS prespawn	80	78.8	21.2	0.0	0.0	21.2	ND
	S96-PWS spawning	180	72.2	27.8	0.0	0.0	27.8	ND
Ichthyophonus	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	95.4	3.8	0.8	0.0	4.6	ND (too few responses)
	S96-SS spawning	240	90.4	9.2	0.4	0.0	9.6	↑- lnCPK (0.038), lnALT (0.003), lnAST (<0.001), lnIgM (0.013) ↓- PCV (0.004)
	S96-PWS all	260	90.0	9.2	0.8	0.0	10.0	↑- % neutrophils (0.035), % basophils (0.002*), lnALT (0.007), lnAST (0.001), lnIgM (0.001) ↓- PCV (0.007), % thrombocytes (0.031)
	S96-PWS prespawn	80	91.2	8.8	0.0	0.0	8.8	ND
	S96-PWS spawning	180	89.4	9.4	1.1	0.0	10.6	ND
steatitis	F95-SS all	30	93.3	6.7	0.0	0.0	6.7	ND
	F95-PWS all	130	75.4	24.6	0.0	0.0	24.6	↑- sodium (0.004), albumin (0.026), phosphate (0.023), osmolality (0.007), % lymphocytes (0.029), lnCPK (0.022), lnALT (0.029), lnIgM (0.015) ↓- bilirubin (0.032), glucose (0.001), % neutrophils (0.002)
	S96-SS spawning	240	2.9	95.8	1.2	0.0	97.1	none

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS all	260	1.9	95.8	2.3	0.0	98.1	none
	S96-PWS prespawn	80	0.0	98.8	1.2	0.0	100.0	ND
	S96-PWS spawning	180	2.8	94.4	2.8	0.0	97.2	ND
trematodes (e.g., *Lecithaster gibbosus*), cecal	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	99.2	0.8	0.0	0.0	0.8	ND (too few responses)
	S96-SS spawning	240	98.3	1.7	0.0	0.0	1.7	ND (too few responses)
	S96-PWS all	260	86.5	13.5	0.0	0.0	13.5	↓- Age (0.008*)
	S96-PWS prespawn	80	83.8	16.2	0.0	0.0	16.2	ND
	S96-PWS spawning	180	87.8	12.2	0.0	0.0	12.2	ND
Kidney (trunk)microscopic lesions:								
congestion, interstitial, vascular	F95-SS all	30	93.3	6.7	0.0	0.0	6.7	ND
	F95-PWS all	130	89.2	9.2	1.5	0.0	10.8	↑- PCV (0.044)
	S96-SS spawning	240	96.7	3.3	0.0	0.0	3.3	↑- bilirubin (<0.001*), lnALT (0.050) ↓- albumin (0.014), osmolality (0.015)
	S96-PWS all	259	96.5	3.1	0.4	0.0	3.5	none
	S96-PWS prespawn	80	92.5	6.2	1.2	0.0	7.5	ND
	S96-PWS spawning	179	98.3	1.7	0.0	0.0	1.7	ND
granulomatous inflammation	F95-SS all	30	76.7	23.3	0.0	0.0	23.3	ND
	F95-PWS all	130	73.8	23.8	2.3	0.0	26.2	none
	S96-SS spawning	240	85.4	13.8	0.8	0.0	14.6	↓- hold time (0.014)
	S96-PWS all	259	79.9	20.1	0.0	0.0	20.1	↑- lactate (0.049)
	S96-PWS prespawn	80	82.5	17.5	0.0	0.0	17.5	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS spawning	179	78.8	21.2	0.0	0.0	21.2	ND
hematopoietic cells (relative area)	F95-SS all	30	26.7	70.0	3.3	0.0	73.3	ND
	F95-PWS all	130	2.3	80.0	17.7	0.0	97.7	↑- sodium (0.044), total protein (0.008), globulin (0.004), calcium (0.045), osmolality (0.013), % thrombocytes (0.004), % basophils (0.001*), lnAST (0.035), lnIgM (<0.001) ↓- CO2 (0.001), % lymphocytes (0.001)
	S96-SS spawning	240	10.8	80.0	8.8	0.4	89.2	↑- lnCPK (0.019) NT- lnAST (0.027)
	S96-PWS all	259	8.5	76.8	14.7	0.0	91.5	↓- % thrombocytes (0.002) NT- potassium (0.040), globulin (0.049), cholesterol (0.026), lnCPK (0.014), lnIgM (0.008)
	S96-PWS prespawn	80	3.8	76.2	20.0	0.0	96.2	ND
	S96-PWS spawning	179	10.6	77.1	12.3	0.0	89.4	ND
Ichthyophonus	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	90.8	3.8	2.3	3.1	9.2	↑- total protein (0.034), albumin (0.021), % basophils (0.010), lnCPK (<0.001), lnALT (<0.001), lnIgM (0.001) ↓- PCV (0.004), bilirubin (0.003), cholesterol (0.004), % neutrophils (0.005)
	S96-SS spawning	240	82.1	8.8	5.0	4.2	17.9	↓- PCV (0.003) NT- total protein (0.032), albumin (0.022), lnAnisakidae (0.036), lnALT (0.024), lnAST (0.016), lnIgM (0.012)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS all	259	84.6	5.0	5.4	5.0	15.4	↑- lnALT (<0.001), lnAST (<0.001), lnIgM (0.002) ↓- % thrombocytes (0.032) NT- Age (0.028), PCV (0.004), albumin (0.041), cholesterol (0.034), % lymphocytes (0.019)
	S96-PWS prespawn	80	91.2	2.5	3.8	2.5	8.8	ND
	S96-PWS spawning	179	81.6	6.1	6.1	6.1	18.4	ND
intratubular mineral, with associated tubular hyperplasia	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND
	F95-PWS all	130	98.5	1.5	0.0	0.0	1.5	ND (too few responses)
	S96-SS spawning	240	92.1	6.7	1.2	0.0	7.9	↑- lnAST (0.018)
	S96-PWS all	259	95.8	4.2	0.0	0.0	4.2	↑- calcium (0.038)
	S96-PWS prespawn	80	95.0	5.0	0.0	0.0	5.0	ND
	S96-PWS spawning	179	96.1	3.9	0.0	0.0	3.9	ND
intraductal protozoan	F95-SS all	29	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	129	94.6	4.7	0.8	0.0	5.4	ND (too few responses)
	S96-SS spawning	240	92.1	7.1	0.4	0.4	7.9	none
	S96-PWS all	259	90.7	8.1	1.2	0.0	9.3	↓- globulin (0.050), lnCPK (0.047)
	S96-PWS prespawn	80	91.2	8.8	0.0	0.0	8.8	ND
	S96-PWS spawning	179	90.5	7.8	1.7	0.0	9.5	ND
macrophage aggregates, pigmented	F95-SS all	30	10.0	56.7	26.7	6.7	90.0	ND
	F95-PWS all	130	6.2	67.7	20.8	5.4	93.8	↑- Age (<0.001*) NT- lnALT (0.023)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-SS spawning	240	2.1	62.5	28.8	6.7	97.9	↑- Age (<0.001*), lnIgM (<0.001) NT- lnAnisakidae (0.015)
	S96-PWS all	259	0.4	49.8	40.9	8.9	99.6	↑- Age (<0.001), CO2 (0.004), glucose (0.022) ↓- phosphate (<0.001) NT- calcium (0.006), osmolality (0.013), lnIgM (0.036)
	S96-PWS prespawn	80	0.0	58.8	36.2	5.0	100.0	ND
	S96-PWS spawning	179	0.6	45.8	43.0	10.6	99.4	ND
Ortholinea orientalis (intraductal myxosporean), histopathology	F95-SS all	29	82.8	13.8	3.4	0.0	17.2	ND
	F95-PWS all	130	87.7	2.3	6.2	3.8	12.3	↓- total protein (0.027), albumin (0.043), cholesterol (0.018)
	S96-SS spawning	240	88.3	7.1	2.5	2.1	11.7	NT- %monocytes (0.002*)
	S96-PWS all	259	89.2	3.1	3.9	3.9	10.8	↑- potassium (0.003*) NT- PCV (0.047), ALP (0.021)
	S96-PWS prespawn	80	85.0	1.2	7.5	6.2	15.0	ND
	S96-PWS spawning	179	91.1	3.9	2.2	2.8	8.9	ND
Ortholinea orientalis (intraductal myxosporean), kidney touch preparation	F95-SS all	30	93.3	6.7	0.0	0.0	6.7	ND
	F95-PWS all	130	83.8	10.0	3.1	3.1	16.2	NT- % lymphocytes (0.037)
	S96-SS spawning	240	73.8	13.8	4.2	8.3	26.2	none
	S96-PWS all	225	80.4	10.7	3.1	5.8	19.6	↑- lactate (0.028) NT- bilirubin (0.025)
	S96-PWS prespawn	80	80.0	8.8	3.8	7.5	20.0	ND
	S96-PWS spawning	145	80.7	11.7	2.8	4.8	19.3	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ortholinea orientalis (intraductal myxosporean); sum of both techniques	F95-SS all	30	80.0	13.3	6.7	0.0	20.0	ND
	F95-PWS all	130	81.5	5.4	2.3	10.8	18.5	none
	S96-SS spawning	240	72.5	12.1	4.2	5.0	21.2	none
	S96-PWS all	260	80.4	7.7	2.3	4.2	14.2	none
	S96-PWS prespawn	80	77.5	5.0	1.2	7.5	13.8	ND
	S96-PWS spawning	180	81.7	8.9	2.8	2.8	14.4	ND
tubular dilation (of lumen)	F95-SS all	30	66.7	33.3	0.0	0.0	33.3	ND
	F95-PWS all	130	97.7	2.3	0.0	0.0	2.3	ND (too few responses)
	S96-SS spawning	240	91.2	8.3	0.4	0.0	8.8	↑- Age (0.004*) ↓- lnCPK (0.009*), lnALT (0.019), lnAST (0.012)
	S96-PWS all	259	86.9	12.7	0.4	0.0	13.1	↑- age (0.004), sodium (<0.001), chloride (<0.001), calcium (0.014), % neutrophils (<0.001*) ↓- glucose (0.015), % thrombocytes (0.024), % lymphocytes (0.021)
	S96-PWS prespawn	80	98.8	1.2	0.0	0.0	1.2	ND
	S96-PWS spawning	179	81.6	17.9	0.6	0.0	18.4	ND
tubular epithelial vacuolation	F95-SS all	30	66.7	30.0	3.3	0.0	33.3	ND
	F95-PWS all	130	90.8	9.2	0.0	0.0	9.2	↑- Age (0.046), total protein (0.010), albumin (0.009), globulin (0.033), cholesterol (0.030), lnIgM (0.030) ↓- potassium (0.041), phosphate (0.011)
	S96-SS spawning	240	84.2	15.8	0.0	0.0	15.8	none

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS all	259	61.8	36.7	1.5	0.0	38.2	↑- Age (0.003), sodium (0.004), total protein (0.037), globulin (0.038),cholesterol (0.001), CO2 (0.008), glucose (0.001) ↓- phosphate (<0.001), lnCPK (0.002), lnAST (0.012)
	S96-PWS prespawn	80	57.5	41.2	1.2	0.0	42.5	ND
	S96-PWS spawning	179	63.7	34.6	1.7	0.0	36.3	ND
Liver microscopic lesions:								
cholangitis or biliary hyperplasia	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	96.2	1.5	2.3	0.0	3.8	ND (too few responses)
	S96-SS spawning	240	97.1	2.5	0.4	0.0	2.9	ND (too few responses)
	S96-PWS all	260	94.2	5.4	0.4	0.0	5.8	↑- ALP (0.047) ↓- lnALT (0.025)
	S96-PWS prespawn	80	90.0	10.0	0.0	0.0	10.0	ND
	S96-PWS spawning	180	96.1	3.3	0.6	0.0	3.9	ND
coccidiosis (*Goussia* [*Eimeria*] *clupearum*)	F95-SS all	30	33.3	53.3	10.0	3.3	66.7	ND
	F95-PWS all	130	36.9	35.4	18.5	9.2	63.1	NT- osmolality (0.047)
	S96-SS spawning	240	20.8	43.8	21.7	13.8	79.2	↑- cholesterol (0.024)
	S96-PWS all	260	20.0	50.0	21.5	8.5	80.0	NT- PCV (0.027)
	S96-PWS prespawn	80	21.2	51.2	18.8	8.8	78.8	ND
	S96-PWS spawning	180	19.4	49.4	22.8	8.3	80.6	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
eosinophilic granular leukocytes, perivascular	F95-SS all	30	0.0	80.0	20.0	0.0	100.0	ND
	F95-PWS all	130	0.8	54.6	43.8	0.8	99.2	none
	S96-SS spawning	240	2.5	75.0	22.5	0.0	97.5	none
	S96-PWS all	260	2.3	68.8	28.8	0.0	97.7	↓- PCV (0.022)
	S96-PWS prespawn	80	1.2	73.8	25.0	0.0	98.8	ND
	S96-PWS spawning	180	2.8	66.7	30.6	0.0	97.2	ND
glycogen depletion, hepatocellular	F95-SS all	30	0.0	46.7	53.3	0.0	100.0	ND
	F95-PWS all	130	14.6	40.0	38.5	6.9	85.4	↑- Age (0.003*), lnCPK (0.018) ↓- cholesterol (0.001), CO2 (0.018), glucose (0.006) NT- PCV (<0.001), ALP (0.010), lnAST (0.036)
	S96-SS spawning	240	0.0	0.0	1.7	98.3	100.0	ND (too few responses)
	S96-PWS all	260	0.0	0.0	4.6	95.4	100.0	↑- sodium (0.031), lnIgM (0.049) ↓- Age (0.050), lnCPK (0.008)
	S96-PWS prespawn	80	0.0	0.0	3.8	96.2	100.0	ND
	S96-PWS spawning	180	0.0	0.0	5.0	95.0	100.0	ND
granulomatous inflammation	F95-SS all	30	76.7	23.3	0.0	0.0	23.3	ND
	F95-PWS all	130	63.8	34.6	1.5	0.0	36.2	none
	S96-SS spawning	240	85.4	13.8	0.8	0.0	14.6	↑- ALP (0.008), lnAST (0.041) ↓- lnIgM (0.014)
	S96-PWS all	260	84.2	15.0	0.0	0.8	15.8	none
	S96-PWS prespawn	80	83.8	15.0	0.0	1.2	16.2	ND
	S96-PWS spawning	180	84.4	15.0	0.0	0.6	15.6	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ichthyophonus	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND
	F95-PWS all	130	91.5	6.2	0.8	1.5	8.5	↑- Age (0.007*), sodium (0.009), chloride (0.031), osmolality (0.045), % basophils (0.004), lnCPK (<0.001), lnAST (<0.001), lnALT (0.036), lnIgM (0.001) ↓- PCV (<0.001), bilirubin (0.001), cholesterol (0.005), % neutrophils (0.049)
	S96-SS spawning	240	83.3	10.0	5.0	1.7	16.7	↑- lnALT (0.006*), lnAST (0.002) ↓- PCV (0.002) NT- Age (<0.001*), total protein (0.004), albumin (0.002), globulin (0.028), lnIgM (0.010)
	S96-PWS all	260	82.7	10.4	5.0	1.9	17.3	↑- lnALT (0.002), lnAST (<0.001), lnIgM (0.002) ↓- PCV (0.015) NT- osmolality (0.015)
	S96-PWS prespawn	80	87.5	10.0	0.0	2.5	12.5	ND
	S96-PWS spawning	180	80.6	10.6	7.2	1.7	19.4	ND
leukocytes, focal, parenchymal	F95-SS all	30	46.7	53.3	0.0	0.0	53.3	ND
	F95-PWS all	130	14.6	82.3	3.1	0.0	85.4	none
	S96-SS spawning	240	65.0	35.0	0.0	0.0	35.0	↑- bilirubin (0.019) ↓- % neutrophils (0.041)
	S96-PWS all	260	55.4	44.6	0.0	0.0	44.6	↑- PCV (0.037), cholesterol (0.046), glucose (0.022), osmolality (0.025), % lymphocytes (0.003) ↓- Age (0.006)
	S96-PWS prespawn	80	38.8	61.2	0.0	0.0	61.2	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-PWS spawning	180	62.8	37.2	0.0	0.0	37.2	ND
leukocytes, pericholangial	F95-SS all	30	90.0	10.0	0.0	0.0	10.0	ND
	F95-PWS all	130	83.8	14.6	1.5	0.0	16.2	none
	S96-SS spawning	240	98.3	1.7	0.0	0.0	1.7	ND (too few responses)
	S96-PWS all	260	95.4	4.6	0.0	0.0	4.6	↓- bilirubin (0.016), lnCPK (0.043)
	S96-PWS prespawn	80	96.2	3.8	0.0	0.0	3.8	ND
	S96-PWS spawning	180	95.0	5.0	0.0	0.0	5.0	ND
lipidosis, hepatocellular	F95-SS all	30	83.3	13.3	3.3	0.0	16.7	ND
	F95-PWS all	130	46.9	43.8	8.5	0.8	53.1	NT- potassium (0.017)
	S96-SS spawning	240	83.3	14.2	2.5	0.0	16.7	↑- bilirubin (0.014), lnCPK (0.008*) ↓- sodium (<0.001), chloride (0.003), lactate (0.025), osmolality (0.006)
	S96-PWS all	260	84.6	13.8	0.8	0.8	15.4	↑- ALP (0.003), phosphate (0.048) ↓- hold time (0.005), CO2 (0.015), % neutrophils (0.001)
	S96-PWS prespawn	80	80.0	18.8	1.2	0.0	20.0	ND
	S96-PWS spawning	180	86.7	11.7	0.6	1.1	13.3	ND
macrophage aggregates, pigmented	F95-SS all	30	10.0	70.0	16.7	3.3	90.0	ND
	F95-PWS all	130	3.8	77.7	14.6	3.8	96.2	↑- Age (<0.001*), sodium (0.017), albumin (0.003), osmolality (<0.001), % yolked eggs (0.050), lnIgM (0.031) ↓- bilirubin (0.007*), glucose (0.015)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-SS spawning	240	0.8	60.8	26.2	12.1	99.2	↑- Age (<0.001*), lnIgM (<0.001) ↓- bilirubin (0.006), phosphate (0.048) NT- sodium (0.006), chloride (0.009)
	S96-PWS all	260	0.0	48.5	34.6	16.9	100.0	↑- Age (<0.001), CO2 (0.006), lnIgM (0.039) ↓- phosphate (0.002), osmolality (0.019)
	S96-PWS prespawn	80	0.0	53.8	32.5	13.8	100.0	ND
	S96-PWS spawning	180	0.0	46.1	35.6	18.3	100.0	ND
megalocytosis, hepatocellular	F95-SS all	30	90.0	10.0	0.0	0.0	10.0	ND
	F95-PWS all	130	90.8	6.9	2.3	0.0	9.2	↑- PCV (0.007) ↓- sodium (0.037), ALP (0.048), lactate (0.017), osmolality (0.010)
	S96-SS spawning	240	97.1	2.9	0.0	0.0	2.9	ND (too few responses)
	S96-PWS all	260	96.2	3.5	0.0	0.4	3.8	none
	S96-PWS prespawn	80	96.2	2.5	0.0	1.2	3.8	ND
	S96-PWS spawning	180	96.1	3.9	0.0	0.0	3.9	ND
necrosis, hepatocellular, focal	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND
	F95-PWS all	130	99.2	0.8	0.0	0.0	0.8	ND (too few responses)
	S96-SS spawning	240	97.1	2.5	0.4	0.0	2.9	ND (too few responses)
	S96-PWS all	260	97.7	0.8	0.8	0.8	2.3	ND (too few responses)
	S96-PWS prespawn	80	97.5	0.0	2.5	0.0	2.5	ND
	S96-PWS spawning	180	97.8	1.1	0.0	1.1	2.2	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
necrosis, hepatocellular, single cell	F95-SS all	30	86.7	13.3	0.0	0.0	13.3	ND
	F95-PWS all	130	77.7	21.5	0.8	0.0	22.3	none
	S96-SS spawning	240	90.4	9.2	0.4	0.0	9.6	↑- lnCPK (0.014), lnAST (0.001) ↓- sodium (0.026), cholesterol (<0.001)
	S96-PWS all	260	92.3	7.3	0.4	0.0	7.7	↑- lnIgM (0.050) ↓- sodium (<0.001), chloride (0.010), cholesterol (0.022), glucose (0.043), % neutrophils (0.004*)
	S96-PWS prespawn	80	88.8	10.0	1.2	0.0	11.2	ND
	S96-PWS spawning	180	93.9	6.1	0.0	0.0	6.1	ND
Pancreas, exocrine, microscopic lesions:								
macrophage aggregates, pigmented	F95-SS all	30	83.3	16.7	0.0	0.0	16.7	ND
	F95-PWS all	130	73.8	26.2	0.0	0.0	26.2	↑- Age (0.003*), sodium (0.001*), lactate (0.035), osmolality (0.002*), lnALT (0.040) ↓- lnAnisakidae (0.037)
	S96-SS spawning	240	57.5	42.5	0.0	0.0	42.5	↑- Age (<0.001*) ↓- phosphate (0.048)
	S96-PWS all	260	48.1	51.9	0.0	0.0	51.9	↑- Age (<0.001*), total protein (0.045), albumin (0.024), CO2 (0.001), % neutrophils (0.041) ↓- PCV (0.008), phosphate (0.001), osmolality (0.044), lnAnisakidae (0.006)
	S96-PWS prespawn	80	56.2	43.8	0.0	0.0	43.8	ND
	S96-PWS spawning	180	44.4	55.6	0.0	0.0	55.6	ND
zymogen granule depletion	F95-SS all	30	3.3	50.0	43.3	3.3	96.7	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	F95-PWS all	130	0.0	43.1	53.8	3.1	100.0	↑- Age (0.001*), albumin (0.009), lactate (0.044), lnCPK (0.035) ↓- glucose (0.014)
	S96-SS spawning	240	0.0	1.7	49.2	49.2	100.0	↑- sodium (0.004), osmolality (0.011) ↓- hold time (0.019), bilirubin (0.007), cholesterol (0.039), CO2 (0.041), glucose (0.003), % lymphocytes (0.001), lnCPK (0.042)
	S96-PWS all	260	0.0	0.4	40.4	59.2	100.0	↑- Age (0.008), chloride (0.040) ↓- PCV (<0.001), total protein (0.007*), albumin (0.050), globulin (0.004*), bilirubin (0.015), cholesterol (0.001), glucose (<0.001), % lymphocytes (0.047), lnCPK (<0.001*)
	S96-PWS prespawn	80	0.0	0.0	45.0	55.0	100.0	ND
	S96-PWS spawning	180	0.0	0.6	38.3	61.1	100.0	ND
Skin and skeletal muscle, microscopic lesions:								
arteriolar hyperplasia, focal, intimal	F95-SS all	30	53.3	46.7	0.0	0.0	46.7	ND
	F95-PWS all	130	60.8	39.2	0.0	0.0	39.2	↓- lnAST (0.018)
	S96-SS spawning	240	72.1	27.9	0.0	0.0	27.9	↑- total protein (0.046)
	S96-PWS all	260	75.4	24.6	0.0	0.0	24.6	↑- lnIgM (0.040) ↓- cholesterol (0.045)
	S96-PWS prespawn	80	77.5	22.5	0.0	0.0	22.5	ND
	S96-PWS spawning	180	74.4	25.6	0.0	0.0	25.6	ND
Ichthyophonus	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	F95-PWS all	130	90.8	5.4	1.5	2.3	9.2	↑- ALP (0.022), % basophils (0.001), lnCPK (<0.001), lnAST (<0.001), lnIgM (0.005) ↓- PCV (<0.001), bilirubin (<0.001), cholesterol (0.001), % neutrophils (0.039)
	S96-SS spawning	240	85.4	7.5	5.8	1.2	14.6	↑- lnALT (0.004), lnAST (<0.001) ↓- PCV (<0.001), cholesterol (0.035) NT- Age (0.007*), lnIgM (0.041)
	S96-PWS all	260	85.0	6.5	5.4	3.1	15.0	↑- % basophils (0.001*), lnALT (<0.001), lnAST (<0.001) NT- total protein (0.033), albumin (0.013), osmolality (0.045), % thrombocytes (0.045)
	S96-PWS prespawn	80	91.2	5.0	2.5	1.2	8.8	ND
	S96-PWS spawning	180	82.2	7.2	6.7	3.9	17.8	ND
leukocytes, perivascular	F95-SS all	30	33.3	66.7	0.0	0.0	66.7	ND
	F95-PWS all	130	26.9	73.1	0.0	0.0	73.1	none
	S96-SS spawning	240	8.3	91.7	0.0	0.0	91.7	none
	S96-PWS all	260	9.2	90.4	0.4	0.0	90.8	none
	S96-PWS prespawn	80	11.2	88.8	0.0	0.0	88.8	ND
	S96-PWS spawning	180	8.3	91.1	0.6	0.0	91.7	ND
myodegeneration or myonecrosis	F95-SS all	30	96.7	3.3	0.0	0.0	3.3	ND
	F95-PWS all	130	96.9	1.5	1.5	0.0	3.1	ND (too few responses)
	S96-SS spawning	240	99.6	0.4	0.0	0.0	0.4	ND (too few responses)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S96-PWS all	260	97.7	2.3	0.0	0.0	2.3	↑- % basophils (0.002*), lnALT (<0.001), lnAST (<0.001) ↓- PCV (0.048), cholesterol (0.005)
	S96-PWS prespawn	80	97.5	2.5	0.0	0.0	2.5	ND
	S96-PWS spawning	180	97.8	2.2	0.0	0.0	2.2	ND
myositis	F95-SS all	30	90.0	10.0	0.0	0.0	10.0	ND
	F95-PWS all	130	85.4	14.6	0.0	0.0	14.6	none
	S96-SS spawning	240	91.2	8.3	0.4	0.0	8.8	↑- chloride (0.003), lnALT (0.004), lnAST (<0.001) ↓- bilirubin (0.041), cholesterol (0.014)
	S96-PWS all	260	92.7	7.3	0.0	0.0	7.3	none
	S96-PWS prespawn	80	92.5	7.5	0.0	0.0	7.5	ND
	S96-PWS spawning	180	92.8	7.2	0.0	0.0	7.2	ND
Spleen microscopic lesions:								
arteriolar hyperplasia, focal, intimal	F95-SS all	30	86.7	10.0	3.3	0.0	13.3	ND
	F95-PWS all	130	83.1	16.9	0.0	0.0	16.9	↑- Age (0.001*), lnIgM (0.002) ↓- bilirubin (0.035), glucose (0.027)
	S96-SS spawning	239	66.9	32.2	0.8	0.0	33.1	none
	S96-PWS all	257	63.8	35.0	1.2	0.0	36.2	↑- sodium (0.010) ↓- % lymphocytes (0.012)
	S96-PWS prespawn	79	74.7	24.1	1.3	0.0	25.3	ND
	S96-PWS spawning	178	59.0	39.9	1.1	0.0	41.0	ND
congestion, vascular	F95-SS all	30	46.7	23.3	16.7	13.3	53.3	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	F95-PWS all	130	12.3	37.7	20.8	29.2	87.7	↓- sodium (0.001), lactate (<0.001), osmolality (<0.001) NT- albumin (0.022), CO2 (0.013), phosphate (<0.001), lnCPK (0.025)
	S96-SS spawning	239	29.3	49.8	14.6	6.3	70.7	↓- sodium (<0.001), osmolality (0.001) NT- Age (0.007*), ALP (0.018), lactate (<0.001), lnIgM (0.028)
	S96-PWS all	257	40.5	35.8	10.5	13.2	59.5	↑- hold time (0.005) ↓- Age (<0.001*), total protein (0.001), albumin (0.004), globulin (0.001), ALP (0.002) NT- bilirubin (0.045), CO2 (0.049), lactate (0.027), osmolality (0.043)
	S96-PWS prespawn	79	43.0	22.8	8.9	25.3	57.0	ND
	S96-PWS spawning	178	39.3	41.6	11.2	7.9	60.7	ND
ellipsoid hyalinization or hypertrophy	F95-SS all	30	20.0	76.7	3.3	0.0	80.0	ND
	F95-PWS all	130	3.8	80.8	15.4	0.0	96.2	↑- Age (<0.001*), total protein (0.028), albumin (0.024), lnIgM (0.018)
	S96-SS spawning	239	3.8	40.6	55.2	0.4	96.2	↑- Age (<0.001) ↓- bilirubin (<0.001*)
	S96-PWS all	257	1.9	28.8	67.3	1.9	98.1	↑- Age (<0.001*), sodium (0.043), CO2 (0.041), % neutrophils (0.004) ↓- % thrombocytes (0.041), lnAST (0.001)
	S96-PWS prespawn	79	5.1	36.7	58.2	0.0	94.9	ND
	S96-PWS spawning	178	0.6	25.3	71.3	2.8	99.4	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
granulomatous inflammation	F95-SS all	30	93.3	6.7	0.0	0.0	6.7	ND
	F95-PWS all	130	91.5	6.9	0.8	0.8	8.5	↓- potassium (0.020)
	S96-SS spawning	239	94.1	5.4	0.4	0.0	5.9	none
	S96-PWS all	257	94.9	4.7	0.0	0.4	5.1	none
	S96-PWS prespawn	79	97.5	2.5	0.0	0.0	2.5	ND
	S96-PWS spawning	178	93.8	5.6	0.0	0.6	6.2	ND
Ichthyophonus	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	89.2	5.4	1.5	3.8	10.8	↑- Age (<0.001*), Total protein (0.013), albumin (0.023), globulin (0.029), ALP (0.003), osmolality (0.043), % basophils (<0.001), lnCPK (<0.001), lnAST (<0.001), lnALT (0.012), lnIgM (<0.001) ↓- PCV (<0.001), bilirubin (<0.001), cholesterol (<0.001)
	S96-SS spawning	239	84.5	7.9	3.3	4.2	15.5	↑- lnALT (0.026), lnAST (<0.001) ↓- cholesterol (0.046) NT- PCV (0.038), total protein (0.020), globulin (0.034)
	S96-PWS all	257	83.7	7.4	3.1	5.8	16.3	↑- lnALT (<0.001), lnAST (<0.001) NT- PCV (0.001), albumin (0.042), cholesterol (0.045), osmolality (0.045), lnIgM (0.004)
	S96-PWS prespawn	79	89.9	3.8	3.8	2.5	10.1	ND
	S96-PWS spawning	178	80.9	9.0	2.8	7.3	19.1	ND
macrophage aggregates, pigmented	F95-SS all	30	30.0	43.3	23.3	3.3	70.0	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	F95-PWS all	130	18.5	45.4	30.8	5.4	81.5	↑- Age (<0.001*), Total protein (0.015), albumin (0.002), lactate (0.021), lnALT (0.021), lnIgM (0.017) ↓- bilirubin (0.041) NT- PCV (0.017), %yolked eggs (0.037)
	S96-SS spawning	239	13.0	33.5	38.5	15.1	87.0	↑- Age (<0.001*), CO2 (0.027), lnIGM (0.001*) ↓- phosphate (<0.001) NT- lnALT (0.021)
	S96-PWS all	257	3.1	23.3	48.6	24.9	96. 9	↑- Age (<0.001*) ↓- phosphate (0.003), lnALT (0.020), lnCPK (0.003*), lnAST (0.003) NT- CO2 (0.004), calcium (0.015)
	S96-PWS prespawn	79	6.3	24.1	50.6	19.0	93.7	ND
	S96-PWS spawning	178	1.7	23.0	47.8	27.5	98.3	ND
Stomach microscopic lesions:								
foreign body granuloma	F95-SS all	30	76.7	23.3	0.0	0.0	23.3	ND
	F95-PWS all	130	79.2	20.8	0.0	0.0	20.8	↑- hold time (0.002), % neutrophils (0.004*)
	S96-SS spawning	240	73.3	26.7	0.0	0.0	26.7	none
	S96-PWS all	260	78.1	21.9	0.0	0.0	21.9	↑- Age (0.001), total protein (0.043), albumin (0.008), % thrombocytes (0.015) ↓- sodium (0.043), chloride (0.032)
	S96-PWS prespawn	80	72.5	27.5	0.0	0.0	27.5	ND
	S96-PWS spawning	180	80.6	19.4	0.0	0.0	19.4	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
gastritis, submucosal (formerly = eosinophilic granular leukocytes, submucosal)	F95-SS all	30	0.0	86.7	13.3	0.0	100.0	ND
	F95-PWS all	130	0.0	46.9	53.1	0.0	100.0	↑- potassium (0.021)
	S96-SS spawning	240	3.3	90.4	6.2	0.0	96.7	NT- total protein (0.024), globulin (0.041), cholesterol (0.035)
	S96-PWS all	260	0.8	87.3	11.9	0.0	99.2	↑- lnALT (0.028) ↓- PCV (0.008*), cholesterol (0.015)
	S96-PWS prespawn	80	0.0	87.5	12.5	0.0	100.0	ND
	S96-PWS spawning	180	1.1	87.2	11.7	0.0	98.9	ND
Ichthyophonus (includes only cases with organisms)	F95-SS all	30	100.0	0.0	0.0	0.0	0.0	ND
	F95-PWS all	130	90.0	9.2	0.0	0.8	10.0	↑- lnCPK (0.002), lnIgM (0.039) ↓- bilirubin (0.021), cholesterol (0.038), % neutrophils (0.023)
	S96-SS spawning	240	90.8	6.7	1.2	1.2	9.2	↑- total protein (0.044), globulin (0.026), lnAST (0.004), lnIgM (0.001)
	S96-PWS all	260	89.6	6.9	3.1	0.4	10.4	NT- PCV (0.025), total protein (0.041), albumin (0.027), osmolality (0.041), lnALT (0.007), lnAST (0.002), lnIgM (<0.001)
	S96-PWS prespawn	80	90.0	8.8	1.2	0.0	10.0	ND
	S96-PWS spawning	180	89.4	6.1	3.9	0.6	10.6	ND
Ichthyophonus+ (includes cases with characteristic inflammation, but no organisms)	F95-SS all	30	93.3	6.7	0.0	0.0	6.7	ND
	F95-PWS all	130	88.5	10.0	0.8	0.8	11.5	↑- % basophils (0.043), lnCPK (0.001), lnALT (0.037) ↓- bilirubin (0.018), cholesterol (0.017), % neutrophils (0.005)

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (P-value)
	S96-SS spawning	240	88.3	9.2	1.2	1.2	11.7	↑- globulin (0.037), lnALT (0.034), lnAST (0.014), lnIgM (0.009)
	S96-PWS all	260	86.5	10.0	3.1	0.4	13.5	NT- Age (0.050), PCV (0.031), albumin (0.020)
	S96-PWS prespawn	80	86.2	12.5	1.2	0.0	13.8	ND
	S96-PWS spawning	180	86.7	8.9	3.9	0.6	13.3	ND
leukocytes, focal, parenchymal	F95-SS all	30	83.3	13.3	3.3	0.0	16.7	ND
	F95-PWS all	130	80.0	18.5	1.5	0.0	20.0	none
	S96-SS spawning	240	97.9	2.1	0.0	0.0	2.1	ND (too few responses)
	S96-PWS all	260	96.9	3.1	0.0	0.0	3.1	↑- PCV (0.032), phosphate (0.016), % lymphocytes (0.035) ↓- % thrombocytes (0.008)
	S96-PWS prespawn	80	95.0	5.0	0.0	0.0	5.0	ND
	S96-PWS spawning	180	97.8	2.2	0.0	0.0	2.2	ND
serositis	F95-SS all	30	63.3	36.7	0.0	0.0	36.7	ND
	F95-PWS all	130	17.7	80.8	1.5	0.0	82.3	none
	S96-SS spawning	240	69.2	30.8	0.0	0.0	30.8	↑- total protein (0.010), albumin (0.011), globulin (0.026), calcium (0.019), % neutrophils(0.013), lnIgM (0.026)
	S96-PWS all	260	60.0	39.6	0.4	0.0	40.0	none
	S96-PWS prespawn	80	60.0	38.8	1.2	0.0	40.0	ND
	S96-PWS spawning	180	60.0	40.0	0.0	0.0	40.0	ND

Organ - lesion	Date-Site-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
trematodes, intraluminal (e.g., Hemiuridae)	F95-SS all	30	53.3	36.7	10.0	0.0	46.7	ND
	F95-PWS all	130	64.6	33.1	2.3	0.0	35.4	↑- PCV (0.022), % basophils (0.044) ↓- glucose (0.050)
	S96-SS spawning	240	77.9	19.6	2.5	0.0	22.1	↑- glucose (0.017), lnCPK (0.049) ↓- chloride (0.043), % neutrophils(0.046)
	S96-PWS all	260	84.6	15.0	0.4	0.0	15.4	↑- % lymphocytes (0.050) ↓- sodium (<0.001), chloride (<0.001)
	S96-PWS prespawn	80	81.2	17.5	1.2	0.0	18.8	ND
	S96-PWS spawning	180	86.1	13.9	0.0	0.0	13.9	ND

Table 3. Other lesions associated with external lesions in Pacific herring sampled in November 1995 (fall, Prince William Sound only, n = 130) and in spring 1996 (Prince William Sound and Sitka Sound, n = 500). Chi-square test for association. For lesions with minimum expected cell frequency <1 (*), only chi-square tests with $P \leq 0.010$ are included. Trends in the associated lesion scores were classified in comparison to an increase in the given external lesion score. As the external lesion score increased, the associated lesion score either increased (↑), decreased (↓), or changes in the associated lesion score were not linear (NL; e.g., as scores for the external lesion increased, associated lesion scores initially increased and then later decreased). Lesions not listed were not significant.

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
caudal fin fraying	fall			↑	<0.001						
	spring			↑	<0.001*			NT	0.005*	↑	<0.001
caudal fin reddening	fall	↑	<0.001								
	spring	↑	<0.001*			↑	<0.001*	↑	<0.001*	↑	<0.001
diffuse skin reddening	fall							↑	<0.001		
	spring			↑	<0.001*			↑	<0.001*	↑	<0.001
fin base reddening	fall					↑	<0.001				
	spring	↑	0.005*	↑	<0.001*	↑	<0.001*			↑	<0.001
focal skin reddening	spring	↑	<0.001	↑	<0.001*	↑	0.001	↑	<0.001		
eggs in stomach	spring			↑	<0.001*	↑	0.005	↑	<0.001	↑	0.001
brain meningoencephalitis	fall			↓	0.042						
branchial lamellar telangiectasis	spring					↑	<0.001*				

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
gall bladder *Ceratomyxa auerbachi*	spring			↓	0.025						
gastric focal parenchymal leukocytes	fall					↑	0.040				
gastric foreign body granuloma	fall	↑	0.041								
gastric trematodiasis	fall					↓	0.020				
gastritis, submucosal	spring									↓	0.019
gonadal fullness	spring			↓	0.007*						
gonadal *Ichthyophonus*	spring	↑	<0.001*								
hepatic Anisakidae	fall			↓	0.042						
	spring			NT	0.049						
hepatic granulomatous inflammation	fall							↑	0.035		
hepatic *Ichthyophonus*	spring									NT	0.011
intestinal Anisakidae	spring									NT	0.031
intestinal cestodes	spring			↑	0.007						
intestinal foreign body granulomas	fall	↓	0.010					↑	0.020		
	spring					NT	0.013				

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
intestinal mesenteric steatitis	fall					↓	0.029				
intestinal trematodes	spring									↓	0.030
opercular copepod	spring			↑	0.037					↑	0.017
renal intratubular mineral	spring	↑	0.001*								
renal *Ortholinea orientalis*	fall					↓	0.032				
	spring							NT	0.009*	NT	0.046
renal tubular epithelial vacuolation	spring			NT	0.025			NT	0.007	↓	0.014
splenic congestion	fall	↓	0.045								
splenic granulomatous inflammation	fall					↑	0.029	↑	<0.001*		
splenic macrophage aggregates	spring							NT	0.033	↓	0.021

Table 4. Oocyte morphology in adult female Pacific herring sampled from Sitka Sound (n = 14) and Prince William Sound (n = 63) in November, 1995.

Variable	Site	Mean #[a]	SE
ruptured follicles	Sitka Sound	0.0	0.0
	Prince William Sound	0.0	0.0
yolked oocytes	Sitka Sound	54.2	5.9
	Prince William Sound	54.5	3.8
nonyolked oocytes	Sitka Sound	80.2	14.5
	Prince William Sound	68.0	6.2
% yolked eggs	Sitka Sound	43.9	3.9
	Prince William Sound	47.7	1.7

[a]Counted using a binocular microscope; number per 40× objective field.

Table 5. Lesion frequency (%) within gender in Pacific herring sampled in November 1995, from Prince William Sound (PWS) and in March and April 1996, from PWS and Sitka Sound. Lesions were scored as none (0), mild (1), moderate (2), or severe (3). Chi-square test for homogeneity. Lesions not listed were not significant. For some lesions, sum of individual frequencies within a category is different from 100% due to rounding differences.

Sample date and lesion	Lesion score	Frequency (%)		χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio
Fall 1995 (PWS only)		Female (n ≈ 57)	Male (n ≈ 64)			
gonadal pigmented macrophage aggregates	0	23	98	<0.001		
	1	77	2		213	1690, 27
hepatic glycogen depletion	0	25	6	0.031		
	1	32	45		ND[b]	
	2	35	42			
	3	9	6			
hepatic granulomatous inflammation	0	74	56	0.046		
	1+2	26	44		0.46	0.99, 0.21
hepatic pigmented macrophage aggregates	0+1	93	69	0.001		
	2+3	4	31		0.17	0.52, 0.05
intestinal mesenteric steatitis	0	88	61	0.001		
	1	12	39		.22	0.56, 0.09
pancreatic zymogen granule depletion	1+2	51	30	0.017		
	3	49	70		0.41	0.86, 0.19
splenic congestion	0	5	20	0.002		
	1	28	44		ND	
	2	21	19			
	3	46	11			

Sample date and lesion	Lesion score	Frequency (%)		χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio
Spring 1996 (Sitka and PWS)		Female (n ≈ 197)	Male (n ≈298)			
cardiac focal parenchymal leukocytes	0	78	89	<0.001		
	1	22	11		2.4	3.9, 1.5
gall bladder *Ceratomyxa auerbachi*	0	67	78	0.020		
	1	26	17		ND	
	2+3	7	4			
gastric trematodes	0	76	85	0.010		
	1+2	24	15		1.82	2.87, 1.15
gill trematode	0	100	97	0.014		
	1	0	3		0.00	ND
gonadal fullness	0	15	21	<0.001		
	1	1	9		ND	
	2	3	17			
	3	82	53			
gonadal eosinophilic granular leukocytes	0	33	51	<0.001		
	1	55	44		ND	
	2+3	12	5			
gonadal granulomatous inflammation	0	83	98	<0.001		
	1+2+3	17	2		10.2	24.8, 4.19
gonadal pigmented macrophage aggregates	0	64	97	<0.001		
	1	36	3		20.5	43.8, 9.6
hepatic focal necrosis	0	95	99	0.006		
	1+2+3	5	1		5.28	19.4, 1.4

Sample date and lesion	Lesion score	Frequency (%)		χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio
Spring 1996 (Sitka and PWS)		Female (n ≈ 197)	Male (n ≈298)			
hepatic *Ichthyophonus*	0	82	83	0.015		
	1	13	9		ND	
	2	2	7			
	3	3	1			
hepatic lipidosis	0	73	91	<0.001		
	1	22	9		ND	
	2+3	5	0			
hepatic single cell necrosis	0	80	99	<0.001		
	1+2	20	1		18.2	52, 6.4
iris reddening	0	28	35	<0.001		
	1	66	64		ND	
	2	6	1			
renal intratubular mineral	0	91	96	0.012		
	1+2	9	4		2.62	5.68, 1.2
renal tubular epithelial vacuolation	0	85	64	<0.001		
	1+2	15	36		0.31	0.49, 0.19
renal tubular dilatation	0	96	84	<0.001		
	1+2	4	16		0.23	0.49, 0.10
splenic congestion	0	41	32	<0.001		
	1	46	40		ND	
	2	6	17			
	3	7	11			

Sample date and lesion	Lesion score	Frequency (%)	χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio

[a]Odds ratio is defined as the ratio of the odds of a fish being at one level of a condition (e.g., having a scorable lesion) as opposed to being at another level of a condition (e.g. having no lesion) for one category of a variable (e.g., female) to the corresponding odds for the other category of the variable (e.g. male). For example, fall females were 213 times more likely to have pigmented gonadal macrophage aggregates than were males.

[b]ND = not done; odds ratios were not calculated for lesions with more than 2 groups.

Table 6. Lesion frequency (%) within iris reddening (IR) in Pacific herring sampled in November 1995, from Prince William Sound (PWS) and in March and April 1996, from PWS and Sitka Sound. Lesions were scored as none (0), mild (1), moderate (2), or severe (3). Chi-square test for homogeneity. Lesions not listed were not significant. For some lesions, sum of individual frequencies within a category is different from 100% due to rounding differences.

Sample date and lesion	Lesion score	Lesion score frequency (%)			χ^2 *P*-value
Fall 1995 (PWS only)		IR moderate (n = 9)	IR mild (n = 73)	IR none (n = 48)	
intestinal foreign body granuloma	0	22	62	69	0.031
	1	78	33	31	
pancreatic pigmented macrophage aggregates	0	89	63	88	0.006
	1	11	37	13	
renal interstitial hematopoietic cells	0+1	56	81	90	0.043
	2	44	19	10	
Spring 1996 (Sitka and PWS)		IR moderate (n≈12)	IR mild (n ≈ 323)	IR none (n ≈ 164)	
gill lamellar telangiectasis	0	75	96	95	0.004
	1	25	4	5	
gonad fullness	0	8	14	30	<0.001
	1	0	4	10	
	2	0	11	11	
	3	92	71	49	
gonadal pigmented macrophage aggregates	0	50	82	90	<0.001
	1	50	18	10	

Sample date and lesion	Lesion score	Lesion score frequency (%)			χ^2 *P*-value
Spring 1996 (Sitka and PWS)		IR moderate (n≈12)	IR mild (n ≈ 323)	IR none (n ≈ 164)	
hepatic *Ichthyophonus*	0	75	83	84	0.010
	1	8	10	10	
	2	0	5	5	
	3	17	2	1	
intestinal intraepithelial coccidian (*Goussia*? sp.)	0	25	5	10	0.009
	1	75	90	81	
	2	0	5	9	
renal tubular dilatation	0	92	93	82	0.001
	1+2	8	7	18	
splenic congestion	0	75	37	28	0.005
	1	25	43	42	
	2	0	12	15	
	3	0	8	15	

Table 7a. Mean plasma chemistry and hematology values in adult Pacific herring sampled from Prince William Sound, Alaska, in November 1995. Analysis of variance. Sample size varied slightly for some variables, but usually included 64 males and 57 females.

Variable	Males		Females		significance[a]
	mean	SE	mean	SE	
Age	3.8	0.2	3.6	0.3	NS
Length (mm)	206.1	2.1	206.7	2.3	NS
Body weight (g)	121.0	4.6	120.0	4.6	NS
Gonad weight (g)	12.5	1.0	5.0	0.4	***
Liver weight (g)	0.9	0.0	1.9	0.1	***
Hold time (min)	103.9	5.3	117.1	5.6	NS
SumICH	1.4	0.6	1.0	0.4	NS
PCV (%)[c]	46.2	0.7	44.2	0.7	*
Albumin (g/dL)	0.9	0.0	0.9	0.0	NS
Globulin (g/dL)	2.5	0.0	2.7	0.0	**
$\log_e$ IgM (mg/mL)[b]	877.7	74.1	745.9	67.5	NS
Total protein (g/dL)	3.4	0.1	3.5	0.1	NS
ALP (U/L)	23.8	1.6	31.2	1.7	**
$\log_e$ ALT (U/L)[b]	23.8	1.6	31.2	1.7	NS
$\log_e$ AST (U/L)[b]	471.0	43.0	517.2	71.7	NS
$\log_e$ CPK (U/L)[b]	1764.1	276.9	1605.1	330.0	NS
Calcium (mg/dL)	13.1	0.2	14.1	0.3	**
Chloride (mmol/L)	159.3	1.0	159.4	0.8	NS
Cholesterol (mg/dL)	397.3	11.0	370.0	10.5	NS
CO_2 (mmol/L)	8.7	0.3	9.0	0.2	NS
Glucose (mg/dL)	103.2	2.4	117.5	2.2	***
Lactate (mmol/dL)	8.0	0.4	6.7	0.5	*
Osmolality (mOsm/kg)	415.7	3.0	409.3	2.5	NS
Phosphorus (mg/dL)	6.7	0.2	6.3	0.2	NS

Variable	Males		Females		significance[a]
	mean	SE	mean	SE	
Potassium (mmol/L)	2.3	0.1	2.3	0.1	NS
Sodium (mmol/L)	185.0	1.4	182.2	1.1	NS
Total bilirubin (mg/dL)	0.2	0.0	0.2	0.0	NS
Basophils (%)[c]	0.9	0.18	0.7	0.14	NS
Eosinophils (%)[c]	0.3	0.08	0.3	0.11	NS
Lymphocytes (%)[c]	43.3	1.4	48.2	1.7	NS
Monocytes (%)[c]	0.11	0.07	0.23	0.07	*
Neutrophils (%)[c]	8.8	0.8	7.2	0.7	NS
Thrombocytes (%)[c]	46.7	1.4	43.4	1.7	NS

[a]Significance is designated as $P > 0.050$ (NS), $P \leq 0.050$ (*), $P \leq 0.010$ (**), or $P \leq 0.001$ (***).

[b]Values for liver weight, IgM, ALT, AST, and CPK were ln transformed for analysis of variance, but true means and standard errors of actual % values are reported here.

[c]All % values were arcsin square root transformed for analysis; however, true means and standard errors of actual % values are reported here.

Table 7b. Mean plasma chemistry and hematology values in males and females sampled from Sitka Sound and Prince William Sound Alaska, during spawning in 1996. Analysis of variance. Sample size varied slightly for some variables, but usually was as follows: Sitka Sound males (n = 148), Sitka Sound females (n = 92), Prince William Sound males (n = 151), Prince William Sound females (n = 105).

	Sitka Sound				Prince William Sound						
	Males		Females		Males		Females		Significance[a]		
Variable	mean	SE	mean	SE	mean	SE	mean	SE	site	sex	site*sex
Age	4.6	0.1	4.6	0.2	5.9	0.2	5.8	0.2	***	NS	NS
Length (mm)	204.5	1.4	203.1	1.9	207.1	1.4	205.2	2.0	NS	NS	NS
Body weight (g)	103.9	2.6	110.5	3.7	122.9	2.8	125.9	4.2	***	NS	NS
Gonad weight (g)	13.6	0.9	22.5	1.4	15.8	1.0	23.2	1.5	NS	***	NS
$\log_e$ Liver weight (g)[b]	0.8	0.0	1.1	0.0	1.0	0.0	1.4	0.1	***	***	NS
Hold time (min)	134.6	3.4	146.3	4.0	102.4	3.2	93.8	3.9	***	NS	**
SumICH	1.9	0.4	1.9	0.5	2.0	0.4	2.2	0.5	NS	NS	NS
PCV (%)[c]	43.9	0.4	42.9	0.5	42.8	0.4	41.5	0.5	**	*	NS
Albumin (g/dL)	0.6	0.0	0.5	0.0	0.7	0.0	0.6	0.0	***	***	*
Globulin (g/dL)	1.9	0.0	1.8	0.0	2.0	0.0	1.8	0.0	NS	***	NS
$\log_e$ IgM (mg/mL)[b]	810.4	42.2	782.7	52.4	821.3	59.6	842.2	76.5	NS	NS	NS
Total protein (g/dL)	2.5	0.0	2.3	0.0	2.6	0.0	2.3	0.1	NS	***	NS
ALP (U/L)	34.8	0.9	38.8	1.3	32.4	0.7	35.0	0.9	**	***	NS
$\log_e$ ALT (U/L)[b]	2.3	0.2	3.1	0.3	2.5	0.2	3.4	0.4	NS	NS	NS
$\log_e$ AST (U/L)[b]	334.5	8.8	445.3	13.6	326.4	12.2	416.0	17.1	*	***	NS

	Sitka Sound				Prince William Sound						
	Males		Females		Males		Females		Significance[a]		
Variable	mean	SE	mean	SE	mean	SE	mean	SE	site	sex	site*sex
$\log_e$ CPK (U/L)[b]	643.3	55.7	1394.4	170.8	1049.2	110.2	1525.7	186.1	**	***	NS
Calcium (mg/dL)	11.1	0.1	11.3	0.2	11.0	0.1	10.9	0.1	*	NS	NS
Chloride (mmol/L)	167.1	0.8	164.4	0.8	175.1	0.7	169.8	0.8	***	***	NS
Cholesterol (mg/dL)	280.1	4.7	194.8	3.9	290.5	5.8	215.0	5.7	**	***	NS
CO_2 (mmol/L)	8.8	0.1	8.4	0.2	7.4	0.2	6.6	0.2	***	**	NS
Glucose (mg/dL)	115.1	2.9	98.8	2.0	113.4	3.5	95.3	2.5	NS	***	NS
Lactate (mmol/dL)	6.0	0.3	4.3	0.3	7.5	0.3	6.2	0.3	***	***	NS
Osmolality (mOsm/kg)	409.7	1.4	401.0	1.9	417.3	1.4	410.5	2.1	***	***	NS
Phosphorus (mg/dL)	4.9	0.1	5.0	0.1	4.1	0.1	4.9	0.1	***	***	**
Potassium (mmol/L)	2.6	0.0	2.6	0.1	2.2	0.1	2.0	0.1	***	NS	NS
Sodium (mmol/L)	192.4	0.8	186.1	1.0	196.1	0.6	188.7	0.7	***	***	NS
Total bilirubin (mg/dL)	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	NS	*	NS
Basophils (%)[c]	0.7	0.1	0.4	0.1	0.4	0.1	0.7	0.2	NS	NS	*
Eosinophils (%)[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	NS	NS
Lymphocytes (%)[c]	16.0	1.0	16.5	1.1	23.0	1.2	24.2	1.1	***	NS	NS
Monocytes (%)[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	NS	NS
Neutrophils (%)[c]	11.8	0.9	7.1	0.8	13.8	1.0	7.2	0.8	NS	***	NS

	Sitka Sound				Prince William Sound						
	Males		Females		Males		Females		Significance[a]		
Variable	mean	SE	mean	SE	mean	SE	mean	SE	site	sex	site*sex
Thrombocytes (%)[c]	71.4	1.2	76.0	1.4	62.7	1.3	67.8	1.4	***	***	NS

[a]Significance is designated as $P > 0.050$ (NS), $P \leq 0.050$ (*), $P \leq 0.010$ (**), or $P \leq 0.001$ (***).

[b]Values for liver weight, IgM, ALT, AST, and CPK were ln transformed for analysis of variance, but true means and standard errors of actual % values are reported here.

[c]All % values were arcsin square root transformed for analysis; however, true means and standard errors of actual % values are reported here.

Table 8. Significantly different values (ANOVA, $P \leq 0.05$) based on year class (age). Pacific herring were sampled in the fall of 1995 (F95) from Prince William Sound (PWS), and in the spring of 1996 (S96) from PWS and Sitka Sound (SS), Alaska. For comparisons in which Levene's test for equality of variance was significant (*), only comparisons with $P \leq 0.010$ are shown. Plasma chemistries, hematology variables, and weights and lengths not shown were not significant (NS).

		Year class = 1993 PWS-F95, n = 36 SS-S96, n = 75 PWS-S96, n = 40		Year class = 1992 PWS-F95, n = 57 SS-S96, n = 102 PWS-S96, n = 78		Year classes = 1989 - 1991 PWS-F95, n = 20 SS-S96, n = 15 PWS-S96, n = 50		Year classes = 1984 - 1988 PWS-F95, n = 17 SS-S96, n = 48 PWS-S96, n = 91		
Variable	Site-Date	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P*-value
albumin (g/dL)	PWS-F95	0.68	0.03	0.86	0.03	0.92	0.03	1.09	0.05	<0.001
	SS - S96	0.52	0.01	0.57	0.01	0.55	0.03	0.63	0.02	<0.001
	PWS-S96	0.55	0.02	0.56	0.02	0.64	0.02	0.68	0.02	<0.001
ALT[a] (U/L)	PWS-F95	2.9	0.82	3.5	0.88	5.3	1.6	4.9	2.4	0.047
	SS - S96									NS
	PWS-S96									NS
AST[a] (U/L)	PWS-F95	510	82	367	74	270	169	513	178	0.023
	SS - S96	390	25	347	25	310	34	347	30	0.025
	PWS-S96	434	44	355	27	279	62	301	24	<0.001
cholesterol (mg/dL)	PWS-F95									NS
	SS - S96									NS
	PWS-S96	249	11.3	243	7.1	258	11.9	277	8.4	0.023
CO_2 (mmol/L)	PWS-F95									NS
	SS - S96	8.1	0.2	8.7	0.2	8.5	0.5	9.3	0.3	0.001
	PWS-S96	6.0	0.3	6.5	0.2	7.4	0.3	7.7	0.22	<0.001

Variable	Site-Date	Year class = 1993 PWS-F95, n = 36 SS-S96, n = 75 PWS-S96, n = 40 Mean	SE	Year class = 1992 PWS-F95, n = 57 SS-S96, n = 102 PWS-S96, n = 78 Mean	SE	Year classes = 1989 - 1991 PWS-F95, n = 20 SS-S96, n = 15 PWS-S96, n = 50 Mean	SE	Year classes = 1984 - 1988 PWS-F95, n = 17 SS-S96, n = 48 PWS-S96, n = 91 Mean	SE	*P*-value
CPK[a] (mmol/L)	PWS-F95									NS
	SS - S96	387	119	507	108	465	322	855	224	0.004*
	PWS-S96									NS
globulin (g/dL)	PWS-F95	2.4	0.05	2.5	0.05	2.6	0.06	2.8	0.08	0.002
	SS - S96	1.8	0.03	1.8	0.03	1.8	0.06	2.0	0.03	<0.001
	PWS-S96	1.7	0.04	1.7	0.04	1.9	0.06	2.0	0.04	<0.001
glucose (mg/dL)	PWS-F95									NS
	SS - S96	99	2.6	108	2.9	112	6.8	125	5.6	<0.001
	PWS-S96	91	3.3	101	3.8	110	4.9	114	4.38	0.007*
IgM[a] (mmol/L)	PWS-F95	478	89	624	102	935	190	1098	199	<0.001
	SS - S96	491	65	694	94	847	217	886	120	<0.001
	PWS-S96									NS
Lactate (mmol/dL)	PWS-F95									NS
	SS - S96	4.6	0.3	5.5	0.3	7.2	0.8	5.5	0.4	0.026
	PWS-S96									NS
osmolality (mOsm/kg)	PWS-F95	405	3.9	411	2.5	413	3.7	430	5.5	0.001
	SS - S96									NS
	PWS-S96									NS

Variable	Site-Date	Year class = 1993 PWS-F95, n = 36 SS-S96, n = 75 PWS-S96, n = 40 Mean	SE	Year class = 1992 PWS-F95, n = 57 SS-S96, n = 102 PWS-S96, n = 78 Mean	SE	Year classes = 1989 - 1991 PWS-F95, n = 20 SS-S96, n = 15 PWS-S96, n = 50 Mean	SE	Year classes = 1984 - 1988 PWS-F95, n = 17 SS-S96, n = 48 PWS-S96, n = 91 Mean	SE	*P*-value
phosphate (mg/dL)	PWS-F95									NS
	SS - S96	5.5	0.1	4.7	0.1	4.9	0.3	4.5	0.1	<0.001
	PWS-S96									NS
potassium (mmol/L)	PWS-F95									NS
	SS - S96									NS
	PWS-S96	2.4	0.10	2.2	0.09	2.0	0.08	1.9	0.06	<0.001
total bilirubin (mg/dL)	PWS-F95	0.26	0.02	0.24	0.01	0.22	0.02	0.17	0.02	0.007
	SS - S96	0.12	0.02	0.09	0.01	0.1	0.02	0.06	0.01	0.006
	PWS-S96									NS
total protein (g/dL)	PWS-F95	3.1	0.07	3.4	0.07	3.5	0.08	3.9	0.13	<0.001
	SS - S96	2.3	0.04	2.4	0.04	2.4	0.07	2.6	0.05	<0.001
	PWS-S96	2.3	0.06	2.3	0.05	2.5	0.08	2.7	0.06	<0.001
Hematology										
basophils[b] (%)	PWS-F95	0.45	0.31	0.21	0.16	0.08	0.14	0.83	0.64	0.028
	SS - S96									NS
	PWS-S96									NS
lymphocytes[b] (%)	PWS-F95									NS
	SS - S96									NS
	PWS-S96	27	5.0	21	2.7	25.4	3.4	20	2.5	0.018

Variable	Site-Date	Year class = 1993 PWS-F95, n = 36 SS-S96, n = 75 PWS-S96, n = 40 Mean	SE	Year class = 1992 PWS-F95, n = 57 SS-S96, n = 102 PWS-S96, n = 78 Mean	SE	Year classes = 1989 - 1991 PWS-F95, n = 20 SS-S96, n = 15 PWS-S96, n = 50 Mean	SE	Year classes = 1984 - 1988 PWS-F95, n = 17 SS-S96, n = 48 PWS-S96, n = 91 Mean	SE	*P*-value
neutrophils[b] (%)	PWS-F95									NS
	SS - S96									NS
	PWS-S96	6.8	2.6	6.6	1.9	10.4	2.7	10.6	2.4	0.022
Weight and Length										
body weight (g)	PWS-F95	86.8	1.8	111.4	2.4	136.3	6.4	183.8	6.2	<0.001*
	SS - S96	78.7	1.3	101.5	1.9	122.8	5.2	155.3	3.4	<0.001*
	PWS-S96	76.3	2.07	101.6	2.3	131.8	3.7	158.4	2.9	<0.001*
gonad weight (g)	PWS-F95	4.2	0.47	7.3	0.63	12.2	1.8	17.4	2.3	<0.001*
	SS - S96	10.3	0.74	15.4	0.91	19.6	3.9	30.1	2.4	<0.001*
	PWS-S96	10.0	0.96	13.9	1.06	22.2	1.9	25.0	1.8	<0.001*
liver weight (g)	PWS-F95	1.0	0.07	1.2	0.07	1.3	0.17	2.2	0.21	<0.001*
	SS - S96	0.68	0.04	0.84	0.03	0.94	0.05	1.3	0.04	<0.001
	PWS-S96	0.63	0.04	1.0	0.08	1.1	0.05	1.5	0.06	<0.001
standard length (mm)	PWS-F95	188.8	1.2	203.5	1.2	214.4	2.6	234.5	2.6	<0.001
	SS - S96	187.1	0.9	202.8	1.2	216.7	2.0	228.9	1.2	<0.001*
	PWS-S96	181.9	1.8	195.9	1.1	213.2	1.7	221.7	1.2	<0.001

[a]Values were ln transformed for statistical analysis; values shown are geometric means and first-order Taylor series approximation of standard errors.

[b]Percent values were arcsine square root transformed for statistical analysis; values shown are re-transformed means and first-order Taylor series approximation of standard errors.

Table 9. Linear correlations (r) of age (yr), body weight and gonad weight (g), standard length (mm), hold time (min), sum-*Ichthyophonus* (sumICH) scores, albumin (g/dL), lnIgM, and blood values in Pacific herring sampled from Prince William Sound (PWS) in fall 1995 (F95), and PWS and Sitka Sound (SS), Alaska, during spring 1996 (S96). Significant correlations are denoted for $P < 0.050$ (*) and $P < 0.010$ (**). Values for PCV and white blood cells were arcsine square root transformed for analysis.

Variable	Site - date	Age	Body weight	Standard Length	Gonad weight	Hold time	sumICH	Albumin	lnIgM
Body weight	PWS - F95	0.822**							
	SS - S96	0.818**							
	PWS - S96	0.813**							
Length	PWS - F95	0.812**	0.971**						
	SS - S96	0.806**	0.928**						
	PWS - S96	0.803**	0.879**						
Gonad weight	PWS - F95	0.572**	0.761**	0.703**					
	SS - S96	0.540**	0.795**	0.562**					
	PWS - S96	0.430**	0.721**	0.549**					
Hold time	PWS - F95	0.112	0.082	0.087	-0.018				
	SS - S96	-0.042	0.044	0.003	0.120				
	PWS - S96	-0.020	-0.070	-0.077	-0.075				
SumICH	PWS - F95	0.178*	0.010	0.022	-0.082	-0.079			
	SS - S96	0.157*	0.083	0.135*	-0.021	-0.062			
	PWS - S96	0.124*	0.053	0.082	-0.069	0.068			
Albumin	PWS - F95	0.507**	0.651**	0.645**	0.517**	-0.095	0.089		
	SS - S96	0.279**	0.269**	0.312**	0.101	-0.111	0.127*		
	PWS - S96	0.322**	0.469**	0.417**	0.345**	-0.207**	0.108		
lnIgM	PWS - F95	0.357**	0.414**	0.377**	0.403**	-0.180*	0.255**	0.416**	
	SS - S96	0.312**	0.296**	0.306**	0.248**	-0.163*	0.195**	0.314**	
	PWS - S96	0.156*	0.129	0.175**	0.137*	-0.072	0.216**	0.235**	

Variable	Site - date	Age	Body weight	Standard Length	Gonad weight	Hold time	sumICH	Albumin	lnIgM
Liver weight	PWS - F95	0.590**	0.603**	0.628**	0.009	0.081	0.122	0.391**	0.159
	SS - S96	0.564**	0.694**	0.668**	0.531**	0.093	0.091	0.300**	0.190**
	PWS - S96	0.485**	0.473**	0.512**	0.227**	-0.041	0.210**	0.106	0.067
PCV	PWS - F95	-0.039	0.098	0.100	0.192*	-0.234**	-0.305**	0.269**	0.061
	SS - S96	-0.036	0.011	-0.060	0.109	-0.051	-0.219**	0.173**	0.089
	PWS - S96	-0.093	-0.021	-0.096	0.091	-0.154*	-0.165**	0.315**	0.087
Total protein	PWS - F95	0.412**	0.589**	0.558**	0.393**	-0.121	0.065	0.828**	0.478**
	SS - S96	0.262**	0.303**	0.271**	0.249**	-0.117	0.125	0.824**	0.330**
	PWS - S96	0.315**	0.479**	0.385**	0.424**	-0.207**	0.096	0.941**	0.286**
lnAST	PWS - F95	0.073	-0.014	-0.046	-0.076	-0.056	0.650**	0.154	0.253**
	SS - S96	-0.114	-0.073	-0.108	0.048	0.011	0.271**	0.038	0.068
	PWS - S96	-0.232**	-0.212**	-0.238**	-0.099	-0.077	0.279**	0.074	0.125
ALP	PWS - F95	0.126	0.167	0.133	-0.070	0.135*	0.079	0.028	0.204*
	SS - S96	0.027	0.026	0.069	-0.073	-0.059	0.093	0.389**	0.129*
	PWS - S96	0.113	0.279**	0.154*	0.250**	-0.222**	-0.045	0.451**	0.064
lnALT	PWS - F95	0.193*	0.256**	0.226**	0.089	-0.127	0.247**	0.328**	0.244*
	SS - S96	-0.164*	-0.151*	-0.121	-0.110	-0.058	0.223**	0.004	-0.036
	PWS - S96	-0.141*	-0.116	-0.094	-0.096	0.033	0.289**	0.075	0.158*
lnCPK	PWS - F95	0.078	-0.046	-0.046	-0.094	-0.016	0.575**	0.075	0.091
	SS - S96	0.213**	0.331**	0.194**	0.490**	-0.004	0.090	0.290**	0.146*
	PWS - S96	-0.040	0.104	-0.009	0.299**	-0.206**	0.126*	0.439**	0.178**
Calcium	PWS - F95	-0.136	-0.197*	-0.189*	-0.228**	0.299**	0.034	-0.041	-0.057
	SS - S96	0.142*	-0.157*	-0.158*	-0.129*	0.157*	0.004	0.304**	-0.126
	PWS - S96	-0.134*	-0.163**	-0.260**	-0.262**	-0.038	-0.007	0.278**	-0.068
Chloride	PWS - F95	0.027	-0.082	-0.085	-0.022	0.381**	0.164	-0.257*	-0.106
	SS - S96	0.103	-0.024	0.117	-0.292**	-0.014	0.114	0.108	-0.119
	PWS - S96	-0.078	-0.238**	-0.128*	-0.504**	0.064	0.005	-0.293**	-0.252**

Variable	Site - date	Age	Body weight	Standard Length	Gonad weight	Hold time	sumICH	Albumin	lnIgM
Cholesterol	PWS - F95	0.063	0.285**	0.244**	0.294**	-0.236**	-0.313**	0.433**	0.226**
	SS - S96	0.101	0.137*	0.129*	0.043	-0.087	-0.137*	0.386**	0.083
	PWS - S96	0.136*	0.286**	0.236**	0.274**	-0.153*	-0.122*	0.724**	0.119
CO_2	PWS - F95	-0.042	-0.027	-0.029	-0.101	0.255**	-0.050	-0.218*	-0.054
	SS - S96	0.240**	0.418**	0.366**	0.382**	0.132*	-0.014	0.211**	0.183**
	PWS - S96	0.278**	0.302**	0.209**	0.210**	0.442**	0.089	0.107	-0.021
Glucose	PWS - F95	-0.054	0.047	0.047	-0.162	-0.121	-0.062	0.170	0.124
	SS - S96	0.280**	0.405**	0.327**	0.394**	-0.033	-0.057	0.330**	0.223**
	PWS - S96	0.198**	0.340**	0.216**	0.489**	-0.073	-0.094	0.386**	0.118
Lactate	PWS - F95	0.220*	0.280**	0.274**	0.269**	-0.124	0.069	0.418**	0.156
	SS - S96	0.093	0.022	0.066	-0.122	-0.245**	-0.058	0.178**	0.107
	PWS - S96	-0.091	0.005	0.059	0.088	-0.289**	-0.095	0.269**	0.122
Osmolality	PWS - F95	0.367**	0.378**	0.349**	0.397**	0.195*	0.144	0.411**	0.230**
	SS - S96	0.079	-0.005	0.102	-0.239**	-0.072	0.083	0.306**	-0.038
	PWS - S96	-0.179**	-0.167*	-0.139*	-0.131*	-0.211**	-0.161**	0.153*	0.036
Phosphorus	PWS - F95	-0.048	-0.112	-0.100	-0.101	-0.299**	0.103	0.222*	-0.002
	SS - S96	-0.238**	-0.239**	-0.287**	-0.138*	0.022	-0.126	0.110	-0.094
	PWS - S96	-0.342**	-0.231**	-0.285**	-0.044	-0.364**	-0.041	-0.049	-0.029
Potassium	PWS - F95	-0.042	-0.135	-0.133	-0.085	-0.006	0.009	-0.206*	-0.145
	SS - S96	-0.066	-0.144*	-0.104	-0.183**	0.165**	0.017	-0.189**	-0.105
	PWS - S96	-0.248**	-0.281**	-0.236**	-0.217**	0.409**	-0.006	-0.324**	-0.123
Sodium	PWS - F95	0.133	0.074	0.070	0.135	0.308**	0.164	0.055	-0.026
	SS - S96	0.089	-0.083	0.077	-0.401**	-0.054	0.045	0.169**	-0.057
	PWS - S96	0.006	-0.112	-0.015	-0.397**	-0.074	-0.046	-0.054	-0.098
Total bilirubin	PWS - F95	-0.312**	-0.236**	-0.266**	-0.184*	-0.131	-0.309**	-0.186*	-0.191*
	SS - S96	-0.190**	-0.130*	-0.225**	0.079	0.027	-0.136*	-0.206**	-0.045
	PWS - S96	-0.062	-0.062	0.021	-0.122	0.036	0.015	0.228**	0.109

Variable	Site - date	Age	Body weight	Standard Length	Gonad weight	Hold time	sumICH	Albumin	lnIgM
%	PWS - F95	0.098	0.022	-0.014	-0.036	0.022	0.226**	0.085	0.051
Basophils	SS - S96	-0.093	-0.162*	-0.087	-0.224**	0.019	0.022	0.073	0.056
	PWS - S96	-0.071	-0.124*	-0.118	-0.100	0.107	0.166**	-0.076	-0.126
%	PWS - F95	0.051	0.104	0.126	0.113	-0.020	-0.068	0.013	0.045
Eosinophils	SS - S96	-0.043	-0.033	-0.024	-0.042	-0.146	-0.007	0.107	0.001
	PWS - S96	0.089	0.135*	0.107	0.085	0.028	-0.079	0.067	-0.087
%	PWS - F95	0.021	0.074	0.055	0.177*	-0.052	0.027	0.060	0.114
Lymphocytes	SS - S96	-0.066	0.049	-0.021	0.126	0.167*	-0.015	-0.185*	0.033
	PWS - S96	-0.111	-0.058	-0.023	0.135*	-0.090	0.074	0.019	0.062
%	PWS - F95	-0.097	-0.012	-0.009	0.041	0.096	0.038	-0.095	-0.114
Monocytes	SS - S96	-0.075	-0.173*	-0.222**	-0.116	-0.069	-0.073	-0.041	-0.019
	PWS - S96	-0.013	-0.006	-0.017	0.068	-0.127*	-0.024	0.002	0.139*
%	PWS - F95	-0.032	-0.117	-0.110	-0.144	0.288**	-0.173*	-0.238**	-0.096
Neutrophils	SS - S96	0.012	-0.207**	-0.098	-0.403**	0.069	0.095	0.150*	-0.078
	PWS - S96	0.141*	0.094	0.124*	-0.233**	0.045	0.083	0.084	-0.048
%	PWS - F95	-0.009	-0.024	-0.011	-0.109	-0.095	0.026	0.056	-0.070
Thrombo-	SS - S96	0.038	0.097	0.074	0.188*	-0.172*	-0.088	0.035	0.032
cytes	PWS - S96	-0.006	-0.013	-0.062	0.078	0.029	-0.116	-0.066	0.004
# Anisakidae	PWS - F95	-0.100	-0.133	-0.106	-0.131	0.067	-0.079	-0.063	0.080
	SS - S96	-0.346**	-0.291**	-0.228**	-0.217**	-0.005	-0.030	-0.022	0.084
	PWS - S96	-0.209**	-0.177**	-0.077	-0.094	-0.017	-0.016	-0.107	0.042

Table 10. Sample prevalence (%) of parasites and virus in adult Pacific herring in Prince William Sound, Alaska, 1989-1996.

Sample Date	n	*Goussia clupearum*	*Ichthyophonus hoferi*[a]	*Ortholinea orientalis*[b]	Viral hemorrhagic septicemia virus
1989 April[c]	40	63	13	TNE[d]	TNE
1990 October[c]	99	60	15	6.1	TNE
1991 April[c]	59	54	5.1	17	TNE
1991 October[c]	48	54	2.1	15	TNE
1992 April[e]	105	53	5.7	3.1	TNE
1993 April[f]	79	41	5.1	4.3	2 of 3 5-fish pools
1994 April	212	61	24 (29)	5.7 (19)	4.7
1995 April (spawning)	180	73	23 (29)	7.2 (29)	0.0
1995 November	130	63	13 (19)	12 (19)	0.0
1996 April	260	80	19 (21)	11 (14)	0.0

[a]Prevalence in liver, kidney, and spleen for all samples except April 1989, where only liver and spleen were examined. Note that more organs were examined after 1993, and those results are in parentheses.

[b]Prevalence values for *Ortholinea orientalis* are for histopathology; values that include examination of touch preparations of kidney are included in parentheses.

[c]unpubl. data from G.D. Marty, M. S. Okihiro, and D. E. Hinton

[d]TNE = Tissue not examined

[e](Kocan et al. 1996)

[f](Meyers et al. 1994) and unpubl. data from T.R. Meyers

Table 11. Viral hemorrhagic septicemia (VHSV), gender, length, weight, and gross findings in Pacific herring sampled from spawn-on-kelp pounds in Craig, Alaska, in April, 1996. Gross findings were scored as none (0), mild (1), moderate (2), or severe (3).

	nonrandom samples				random samples	
VHSV status	+		-		ND[a]	
#	8		30		100	
# males	2		9		ND	
# females	6		21		ND	
Measurement	mean	SE	mean	SE	mean	SE
standard length (mm)	195.9	7.7	200.6	4.5	ND	ND
wet body weight (g)	103.1	16.0	108.7	8.2	ND	ND
External Gross Findings						
caudal fin fraying	1.4	0.2	1.3	0.1	1.0	0.0
caudal fin reddening	0.8	0.3	0.7	0.1	0.3	0.1
fin base reddening	0.6	0.2	1.1	0.2	0.1	0.0
iris reddening	1.9	0.2	1.7	0.1	0.5	0.1
skin reddening, focal	1.5	0.3	1.2	0.1	0.3	0.1
skin reddening, diffuse	0.0	0.0	0.3	0.1	0.0	0.0
summary gross score	1.5	0.3	1.5	0.2	0.4	0.1
Internal Gross Findings						
gonadal fullness	1.8	0.5	1.5	0.3	ND	ND
Ichthyophonus	0.0	0.0	0.2	0.1	ND	ND

[a]ND = not done

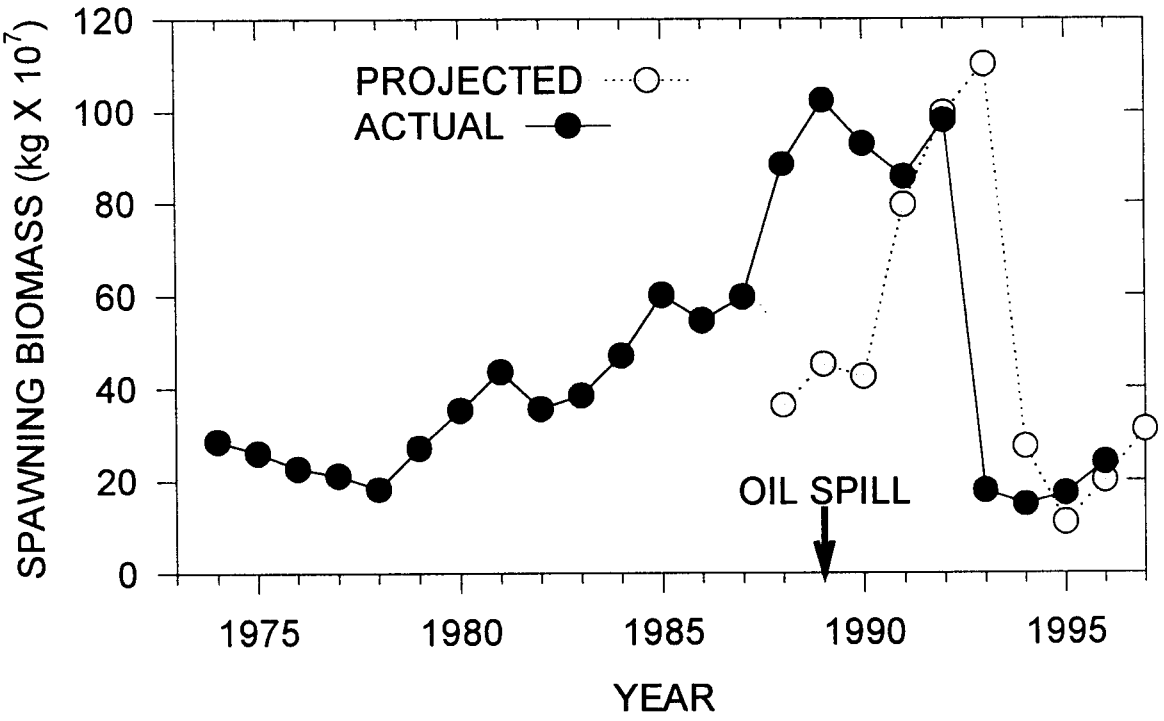

Figure 1. Biomass estimates of mature Pacific herring in Prince William Sound, Alaska. Unexploited spawning biomass projected in the year before spawning (PROJECTED) and calculated after spawning (ACTUAL) using the age-structure assessment model. Estimates were made by Fritz Funk, Alaska Department of Fish and Games, Juneau, Alaska; unpubl. data.

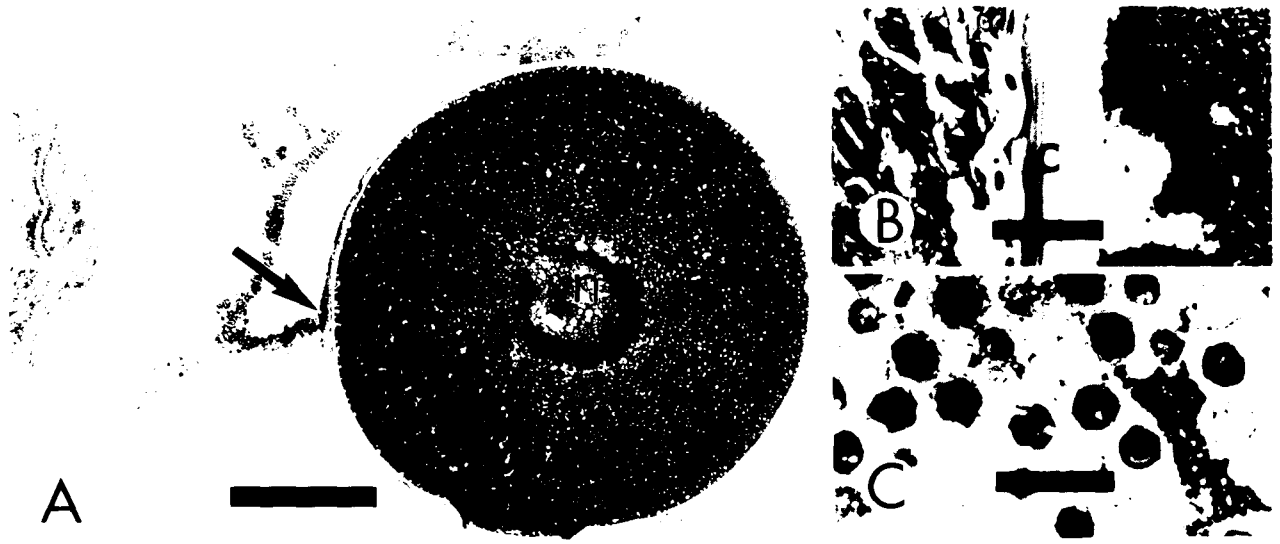

Figure 2. Lymphocystis virus in a spawning Pacific herring from Prince William Sound, Alaska, 1994. A - intestine (left) and hypertrophied fibroblast expanding intestinal mesenteries (arrow); note hypertrophic fibroblast nucleus (n); arrow margin of hypertrophic fibroblast and area shown in detail in B; bar length 600 µm. B - multilayered hyaline cell membrane (c) of hypertrophied fibroblast; atrophic exocrine pancreas is on the left and the expanded granular basophilic cytoplasm is on the right; bar length 25 µm. C - transmission electron micrograph of the cytoplasm of the hypertrophic fibroblast; note icosahedral viral particles; bar length 0.5 µm.

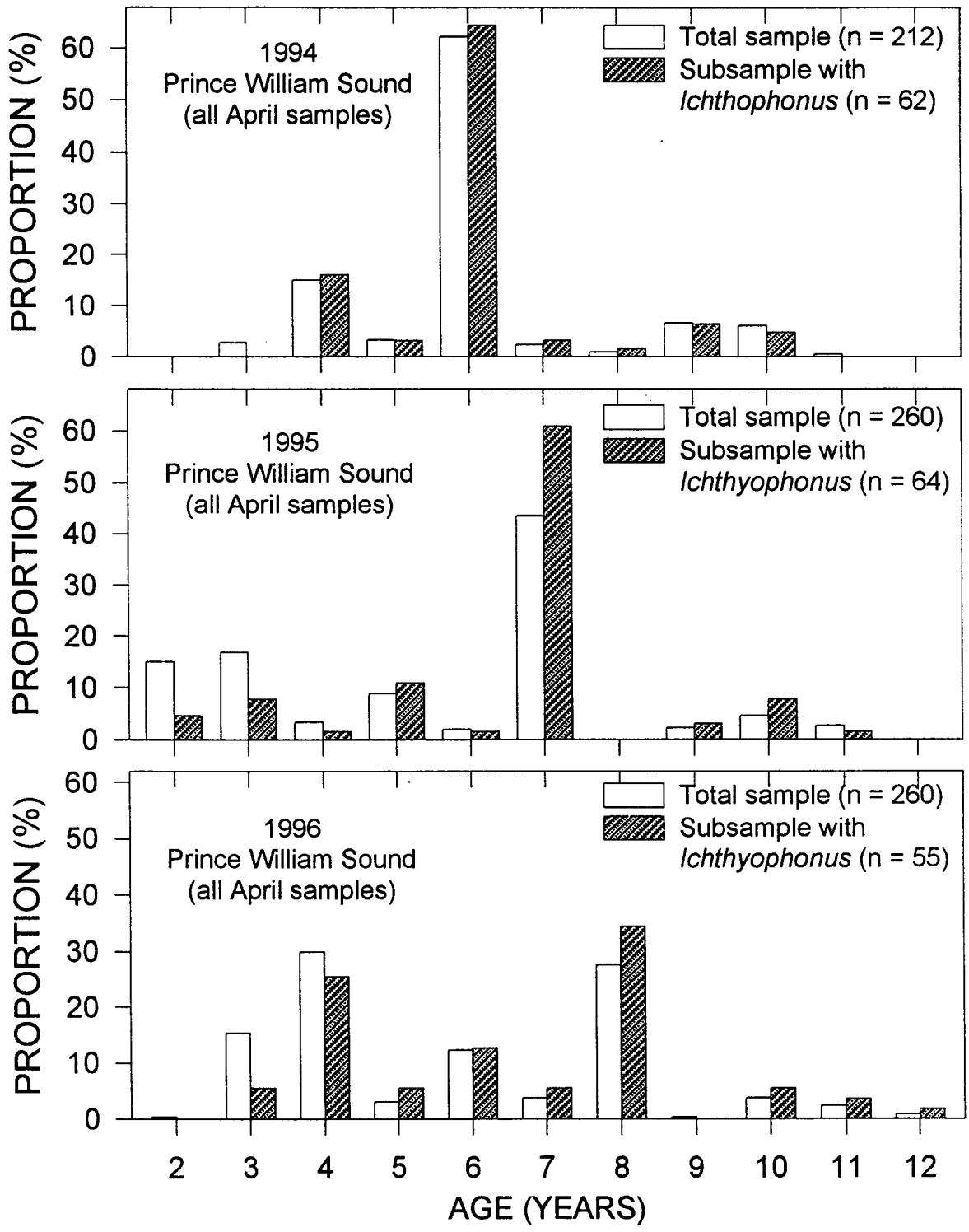

Figure 3. Age distribution of spawning Pacific herring in Prince William Sound, Alaska, that had *Ichthyophonus* compared with the age distribution of fish that were examined for *Ichthyophonus*; 1994, 1995, and 1996.

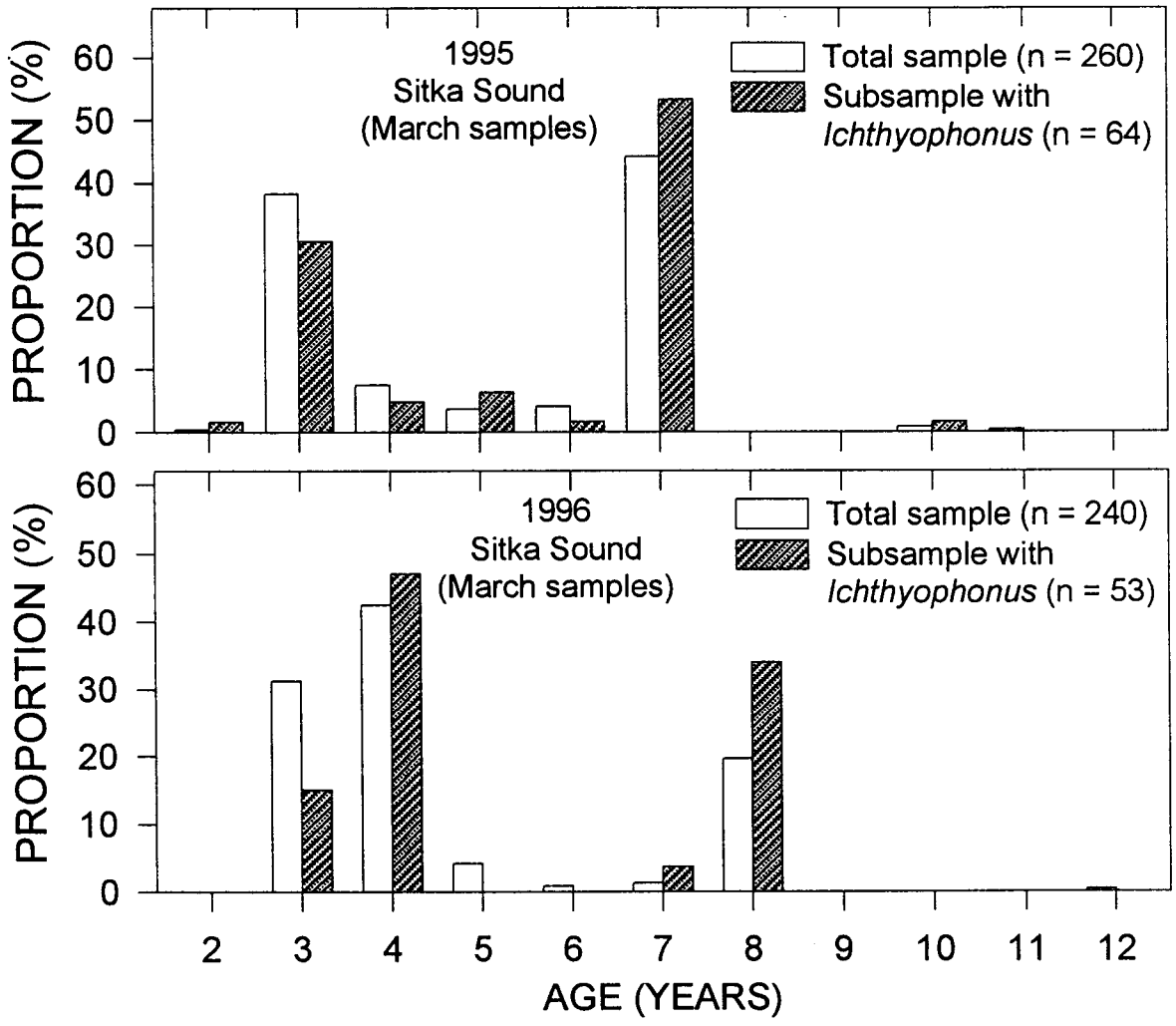

Figure 4. Age distribution of spawning Pacific herring in Sitka Sound, Alaska, that had *Ichthyophonus* compared with the age distribution of fish that were examined for *Ichthyophonus*; 1995, and 1996.

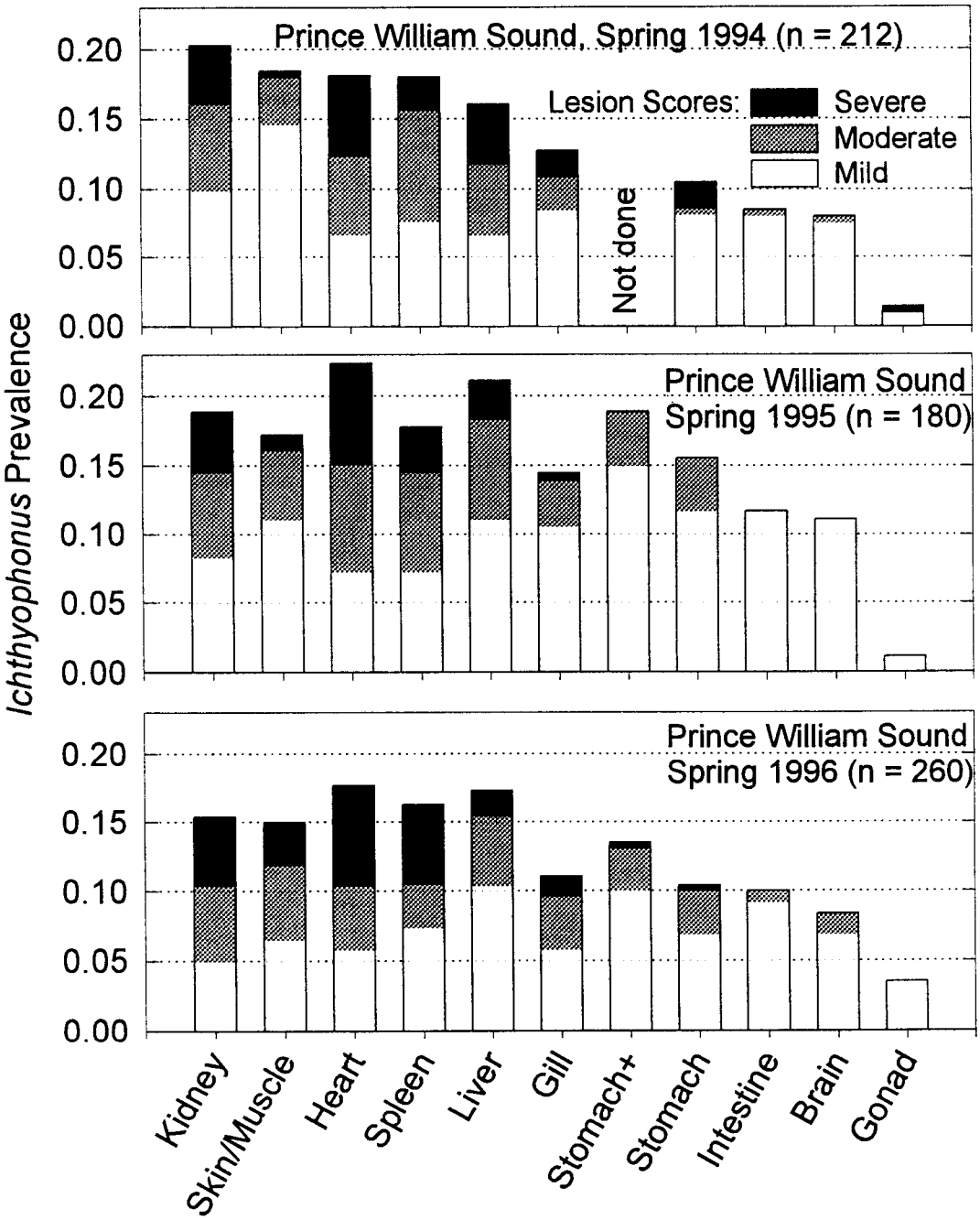

Figure 5. Sample prevalence of *Ichthyophonus* lesion scores in various organs of mature Pacific herring sampled from Prince William Sound in 1994, 1995, and 1996. Lesions were scored as none (0), mild (1), moderate (2), or severe (3).

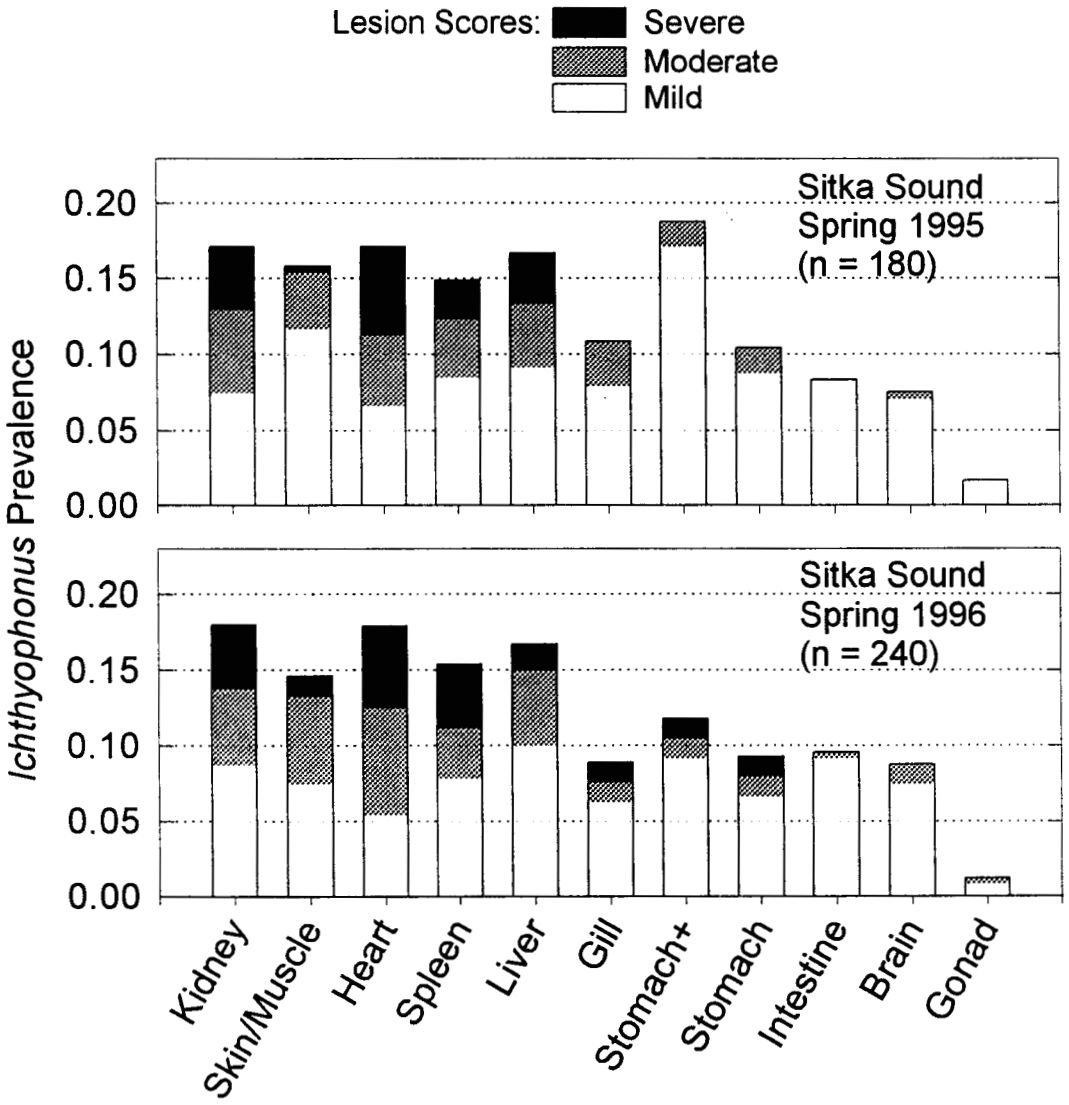

Figure 6. Sample prevalence of *Ichthyophonus* lesion scores in various organs of mature Pacific herring sampled from Sitka Sound in 1995 and 1996. Lesions were scored as none (0), mild (1), moderate (2), or severe (3).

Exxon Valdez Oil Spill
Restoration Project Annual Report

Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound

Section II. Laboratory Challenge of Pacific Herring With and Without Stressors

Restoration Project 96162
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill Trustee Council restoration program for the purpose of assessing project progress. Peer review comments have not been addressed in this annual report.

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April 1997

Investigations of Disease Factors Affecting Declines
of Pacific Herring Populations in Prince William Sound

Section II: Laboratory Challenge of Pacific Herring With and Without Stressors

Restoration Project 96162
Annual Report

Study History: Restoration project 95320S was initiated in response to an RFP issued by Alaska Department of Fish and Game. The study was intended to determine the relationship of diseases on the declines in Prince William Sound herring populations since 1993. A detailed work plan was written as three components: 1) Field component (University of California, Davis); 2) Controlled infection component (University of Washington & National Biological Service, Seattle) and 3) Physiology component (Simon Fraser University, B.C.). The report covers the period of March through September, 1995; however, the study is ongoing.

Abstract: Pacific herring were artificially spawned and their offspring reared in filtered, ultraviolet (U.V.) sterilized seawater at the NBS Marrowstone Island field station in Nordland, Washington. After 6 months fish had reached a length of 4.5 - 6.0 cm. Overall, about 42.3% of the successfully hatched eggs survived with a linear growth rate of 0.33 mm * d^{-1} for the first 90 days post hatch. No viral hemorrhagic septicemia virus (VHSV), *Ichthyophonus* or other pathogen was observed in these fish by either *in vitro* culture or histologic examination of tissues. Natural transmission studies showed that specific pathogen-free (SPF) lab-reared herring are susceptible to VHSV via direct exposure to VHSV contaminated water and naturally infected herring. Exposure of rainbow trout to the same strain of VHSV demonstrated that they were also susceptible to infection and showed signs of disease similar to those seen in juvenile herring. *Ichthyophonus* -infected tissues from Prince William Sound (PWS) herring were cultured in tissue culture medium, and have been successfully maintained in vitro and in vivo in herring, English sole and freshwater sculpin. Unlike the *Ichthyophonus* infecting North Atlantic herring, the PWS strain does not readily infect trout, suggesting that it is a genetically distinct strain. Mortality studies on wild and lab-reared herring are currently underway to determine if *Ichthyophonus* is lethal for Pacific herring. Wild 0-year herring were collected from several locations around Puget Sound and assayed for the presence of VHS virus. No VHS virus was detectable within 6 h of capture, but after 7 - 14 d in captivity the fish began dying, exhibiting typical hemorrhagic signs of VHS as well as actively shedding virus (These wild fish transmitted the virus to SPF fish, resulting in hemorrhage and mortality). After 4 weeks in captivity the wild fish no longer showed signs of VHS virus infection, indicating that the epizootic ran its course. When newly captured 0-year herring were distributed into tanks in densities ranging from 0.5 to 2.0 fish * gal^{-1}, they exhibited what appeared to be density dependant cumulative mortality, ranging from 20% to 70% within three weeks. Studies designed to determine the cause(s) for this density dependant mortality are underway.

Key Words: *Clupea pallasi*, *Exxon Valdez* oil spill, *Ichthyophonus*, morbidity, mortality, Prince William Sound, Viral Hemorrhagic Septicemia Virus (VHSV).

Project Data: (will be addressed in final report)

Citation:

Kocan, R.M., and J.R. Winton 1997. Investigations of disease factors affecting declines of Pacific herring populations in Prince William Sound. II. Laboratory challenge of Pacific herring with and without stressors, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 96162), Alaska Department of Fish and Game, Habitat and Restoration Division, Anchorage, Alaska.

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Executive Summary

Introduction

In 1993 the Prince William Sound herring population declined over 80% and viral hemorrhagic septicemia virus (VHSV) was isolated from a portion of the survivors. This was the first report of this viral pathogen from wild Pacific herring, and it has subsequently been isolated from bait fish in Puget Sound and herring collected in the vicinity of a diesel fuel spill in Prince Rupert, B.C. In 1992 herring being held in the roe-on-kelp fishery in Prince William Sound were observe to have hemorrhages on the skin, fin bases and mouth. The fish swam erratically and did not spawn properly. Although no virus isolations were attempted, it was noted that these lesions closely resembled the lesions observed in confirmed cases of VHS in wild herring the following year. Since VHS had not previously been reported in Pacific herring, this suggested the possibility that VHS was responsible for the heavy losses observed in 1993-'94. However, because wild fish are infected with numerous potential pathogens, extensive studies were necessary to produce conclusive evidence that VHS was responsible or even capable of causing morbidity or mortality in herring.

In 1994 an additional 30% reduction in herring biomass occurred and the returning herring were found to have an unexpectedly high prevalence (29%) of *Ichthyophonus*, a pathogenic fungus suspected to be the cause of earlier declines in Atlantic herring. Because there was no unequivocal proof that *I. hoferi* was actually responsible for the massive Atlantic herring die-offs, or was pathogenic to Pacific herring, studies were initiated to evaluate its pathogenicity and the natural history of this organism in wild and specific pathogen-free (SPF) laboratory-reared herring.

Objectives

1) Establish the pathogenicity of VHSV for Pacific herring (eg. fulfill Koch's Postulates).
2) Describe the pathobiology of the virus for specific pathogen-free (SPF) and wild herring.
3) Determine the means of transmission from infected to uninfected fish.
4) Determine the prevalence of VHSV in wild Puget Sound herring of different age classes.
5) Evaluate the effect of capture and captivity on the course of VHS in wild herring.
6) Determine the immune status of wild herring to VHS.

Methods

Specific-pathogen-free (SPF) herring were reared from egg to 2 years old in filtered UV-sterilized natural seawater. Larvae were fed oyster trochophores, marine rotifers, brine shrimp larvae and pelletized trout chow. When fish reached approximately 5 months of age, studies on their susceptibility to VHSV began.

Groups of 10 fish were exposed to various levels of water-borne virus while housed in 30L tanks of flowing filtered seawater. Exposures lasted for 1 hr, after which the fish were maintained in flowing seawater until they died or the study was terminated, usually at 14 days. Exposure virus was originally obtained from wild Puget Sound herring and grown in vitro in EPC cells, then frozen at -70°C until used. Records on time-to-death, percent mortality, gross and microscopic lesions and behavior were recorded daily.

Natural transmission studies were carried out by exposing 10 SPF herring to wild herring known to have been exposed to VHSV during an epizootic while being held in the laboratory. Data similar to that taken for laboratory exposures to VHSV was collected on both SPF fish and the wild infected fish. One exposure group consisted of 3 replicates of 10 fish each exposed to a single wild fish, while a second exposure group of 3 replicates was exposed to 3 wild fish.

Wild herring were captured from Puget Sound by net and transported in oxygenated tanks to the Marrowstone Island laboratory where they were housed in 70 gal tanks of natural flowing

seawater. Age classes of 0-year, 1+, 2+ and 3+ were assayed for the presence of VHSV at the time of capture and at regular intervals for the first 30 days post capture. Records on mortality, morbidity, lesions and abnormal behavior were recorded. Tissues from 30 - 100 fish from each group were macerated, filter sterilized and examined by plaque assay on EPC cells in MEM-10 medium. The virus titers were recorded as plaque forming units (PFU) per gram of tissue ($PFU*g^{-1}$). Water titers were expressed as
$PFU*ml^{-1}$.

Immunity was determined by exposing surviving herring to 10 - 100 times the known lethal dose of VHS virus ($1X10^3$ - $1X10^4$ $PFU*ml^{-1}$) for 1 hr then quantitating tissue virus load by plaque assay for 10 - 14 days post-exposure. Data was compared with that obtained form SPF fish which had no immunity to the virus.

Ichthyophonus was studied in SPF herring by injecting 10 8-month-old fish IP with 1,000 spores. Fish were maintained in flowing sterile seawater for 60 days during which time observations were made on mortality, lesions, growth and behavior. Lesions included skin erosion visible as small holes in the skin, *Ichthyophonus* on heart, liver or spleen, and histologic lesions. Tissues from all fish were also cultured in L-15 medium plus 5% fetal bovine serum, to determine the overall ability of correctly diagnosing the organism by visual, histologic and cultural methods.

Results

Laboratory-reared herring: Viral hemorrhagic septicemia virus (VHSV) was conclusively shown to be capable of causing disease and extensive mortality in nonimmune juvenile Pacific herring. Specific pathogen-free (SPF) herring were reared from eggs in the laboratory in filtered UV-sterilized natural seawater, then exposed to waterborne VHSV for 1 h when 5-9 months old. Fish exposed to 5-7 X 10^2 PFU began dying 7 days post-exposure with peak mortality occurring on days 10-11. Mortality began 5 days post-exposure and peaked on day 7 in fish exposed to $\geq$ 3 X 10^4 $PFU*ml^{-1}$. No mortality was observed in SPF herring exposed to VHSV concentrations of < 2 X 10^2 $PFU*ml^{-1}$ and no virus could be isolated from their tissues. External signs of disease was limited to 1-2 mm hemorrhagic areas on the lower jaw, isthmus and around the eye. Only 4 of 130 infected fish exhibited extensive cutaneous hemorrhaging.

Virus was first detected in tissues of experimentally infected SPF herring 48 h post-exposure and peaked at 96 h at 6 X 10^6 $PFU*g^{-1}$. Fish began shedding new virus 48 h post-exposure with maximum shedding occurring on days 4-5 post exposure, just prior to peak mortality. Histopathologic examination of tissues from moribund fish 2 to 8 days post-exposure revealed primarily: 1) multifocal coagulative necrosis of liver hepatocytes, 2) diffuse necrosis of the kidney interstitial hematopoietic tissues, and 3) diffuse necrosis of the spleen, epidermis and subcutis.

VHSV in wild herring: Three age classes of wild herring were captured in Puget Sound in 1995 and 1996 and held in flowing seawater tanks at the Marrowstone Island Field Station (Nordland, WA). Immediately upon capture a subsample of fish was frozen at -70°C until assayed for virus. The remaining fish were held at various densities ranging from 5 to 300 fish per tank for up to 5 months. During this time dead fish and subsamples of live fish were collected and frozen for later virus assay. Fish were fed frozen brine shrimp and krill during the time they were held in captivity.

VHSV transmission: When infected wild herring were placed in 30L flowing seawater tanks with 10 SPF herring, the SPF herring became infected and cultured positive for VHSV, with mortality occurring between 7 and 14 days post-exposure.

No virus was isolated from any wild fish at the time of capture. However, 2-3 weeks post-capture approximately 60% of the 0-year herring died with massive hemorrhages of the skin, fins and mouth. Plaque assays on EPC cells revealed that > 90% of the dead fish had > 1 X 10^6

PFU*gm^{-1} tissue at the time of death. Ninety percent of the live fish sampled from the same tanks carried slightly lower titers of virus from 5 to 14 days post-capture, then virus titers declined until they were undetectable by 4 weeks post-capture. Juveniles (1+ and 2+) as well as adults (3+) were also negative for VHSV when initially captured, but began showing virus by 24-48 hours post-capture. Mortality was significantly less (< 10%) in older fish. Virus was detected in 10%, 33% and 10% of live fish on days 2, 8, and 11 respectively, but undetectable by day 21 post capture. Surviving herring exposed to 1X10^3 to 1X10^6 PFU*ml^{-1} for 1 hour 6-8 weeks post-capture exhibited no mortality in any age class and no virus could be isolated from tissues of these fish 10 days post-exposure.

Ichthyophonus: Laboratory-reared SPF herring injected IP with ca 1,000 *Ichthyophonus* spores began dying by 11 days post exposure and had visible lesions on the heart, liver and spleen. Skin lesions (small holes in the epidermis) were detectable after 36 days, as were spores in the musculature under the skin. By 56 days post exposure 90% of the fish were dead. *Ichthyophonus* was cultured in MEM-10 from all but one of the fish which died or presented with lesions. Infected tissues from these herring were cultured then injected IP into coast range sculpins (*Cottus aleuticus*), all of which became infected and/or died by 14 days post exposure. Infected sculpin tissues were fed to these fish which also became infected and cultured positive for *Ichthyophonus*. No control sculpins were found to be infected with *Ichthyophonus*.

Three year classes of wild herring (0-year, 1+ and 3+) were captured from Puget Sound between June 1995 and February 1997 and examined for the presence of *Ichthyophonus* by gross examination and in vitro culture of heart, liver and spleen. External skin lesions were observed in 6%, 5% and 4% of the three groups respectively while 6% 23% and 52% of each group cultured positive for *Ichthyophonus*. There was no significant difference in weight or length between infected and uninfected fish within each age class, and when the fish were held in captivity for up to 90 days post-capture, there was no significant difference in mortality between the infected and uninfected individuals within age classes.

Discussion

Koch's Postulates have been fulfilled for VHSV, confirming the organism's pathogenicity for nonimmune herring and establishing it as a possible cause of the extensive losses that occurred in Prince William Sound in 1993. The course of the disease is very rapid, with new virus being shed by 48 hours post-exposure, peak tissue virus occurring by 72 hours and mortality peaking by 6-8 days post-exposure. Transmission occurs by virus being shed into the water column, thus exposing susceptible fish.

Although no VHSV could be isolated from wild-caught herring, they appear to be carrying VHSV by the time they are 5-6 months-old. All age classes had detectable virus within 24-48 hours post-capture, with the most sever mortality occurring in the 0-year fish. By 3-4 weeks post-capture virus was no longer detectable in tissues and the fish were solidly resistant to challenge infection with > 10^3 PFU*ml^{-1} water. Previous studies with nonimmune herring demonstrated the minimum lethal dose of virus to be ca 5 X 10^2 PFU*ml^{-1} for 1 hour. Consequently it has been demonstrated that infected herring that survive an epizootic are solidly immune to reinfection. It is not clear however if the fish have cleared the virus or are carrying latent infections that can be activated under stress conditions at a later time.

Using pathogen-free laboratory-reared herring, Koch's Postulates were fulfilled and *Ichthyophonus* was shown to be a pathogen for lab-reared herring, capable of causing nearly 100% mortality. The earliest external signs of disease were skin lesions, appearing as small holes in the skin, through which the parasite presumably escapes from the host. Gross and microscopic lesions of the heart, liver, spleen, muscle and skin preceded the appearance of the skin lesions. Culture of tissues however, appears to be the most rapid and accurate method for detecting this organism.

Wild herring were found to be infected by 2-4 months post-metamorphosis with a prevalence of 6%, while the highest prevalence (52%) was found in adult spawners. There was no evidence that the organism affected the health or survival of wild fish, but different environmental conditions and levels of infection could result in a significant level of morbidity and mortality.

Based on the sculpin feeding studies, carnivorous fish are potentially at risk of becoming infected by eating infected herring.

Conclusions

Both VHSV and *Ichthyophonus* are capable of causing morbidity and mortality in non-immune Pacific herring, thus making it possible that the severe losses of herring in Prince William Sound in 1993-'94 was the result of infection by one or both of these organisms. Wild herring are infected with both of these pathogens during their first year of life and apparently carry them without consequence until exposed to some environmental stress. Just what triggers the rapid proliferation of these organisms and the diseases they cause in nature is not clearly understood at this time. Any "stress" condition that affects the immune system could be the trigger; such as confinement, exposure to toxic substances, malnutrition or a combination of these.

Acknowledgments

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A. Pathogenicity of the North American Strain of Viral Hemorrhagic Septicemia Virus for Laboratory-reared Pathogen-free Herring

Introduction

Viral hemorrhagic septicemia (VHS) is generally considered to be a disease of cultured rainbow trout (*Oncorhynchus mykiss*) in Europe (Wolf 1988); however in 1989, the causative rhabdovirus, viral hemorrhagic septicemia virus (VHSV), was identified in North America during routine examinations of returning adult chinook (*O. tshawytscha*) and coho (*O. kisutch*) salmon in Washington State (Winton et al. 1991). In 1990 and 1991, the virus was recovered from Pacific cod (*Gadus macrocephalus*) from Prince William Sound, Alaska (Meyers et al. 1992) suggesting the presence of a marine reservoir for the virus. Beginning in 1993, VHSV has been regularly isolated from stocks of Pacific herring (Clupea pallasi) collected from Prince William Sound and other locations in Alaska, as well as from British Columbia, Canada, and Puget Sound, Washington (Meyers et al. 1994; Meyers and Winton 1995) extending the range of the virus along the Pacific coast of North America from Washington to Alaska. On several occasions, high titers of virus were recovered from herring associated with suspicious lesions and unusual mortality (Meyers and Winton 1995) indicating that the virus could be a significant pathogen for this important species; however, Koch's Postulates (Stanier et al. 1963) had not been fulfilled.

The isolation of VHSV from Pacific herring in Prince William Sound in 1993 coincided with the disappearance of 83% of the predicted biomass of 134,133 metric tons of herring and with the appearance of hemorrhagic lesions on many of the surviving fish. Simultaneously, the sac-roe seine fishery harvest, predicted to be 15,586 metric tons, was a total failure (Donaldson et al. 1995). Although no dead fish were observed at the time of the mass disappearance, herring that did return to Prince William Sound appeared lethargic and up to 43% of the fish had skin ulcers and/or external hemorrhagic lesions (Meyers et al. 1994). While VHSV was isolated from some of these affected fish, no dead herring were available for pathologic examination and it was not possible to determine whether VHSV contributed to the loss of adult spawning herring in 1993.

Following the massive decline in herring biomass, the Alaska Department of Fish and Game initiated an extensive field survey of spawning herring in Prince William Sound in 1994 in an attempt to identify the cause of the 1993 population decline. In that survey, 4.7% of 233 fish were found to be infected with VHSV and 29% were infected with *Ichthyophonus hoferi*; however, more than 10 additional parasitic species were identified (Marty et al. 1995) making it difficult to identify which organism(s), if any, might be responsible for the 1993 population decline. The objectives of the study reported here were: 1) to produce specific-pathogen-free (SPF) Pacific herring in the laboratory, 2) to expose SPF herring to an isolate of the North American strain of VHSV in order to fulfill Koch's Postulates, and 3) to determine the pathogenicity of VHSV for this commercially important marine fish species.

Objectives

1) Establish the pathogenicity of VHSV for Pacific herring (eg. fulfill Koch's Postulates).
2) Describe the pathobiology of the virus for specific pathogen-free (SPF) and wild herring.
3) Determine the means of transmission from infected to uninfected fish.

Methods

Production of specific pathogen-free herring.
Seawater for hatching and rearing SPF herring was pumped from 15 m below mean low water off the north end of Marrowstone Island in northern Puget Sound, Washington where ambient water temperatures during the year typically range from 8-13°C. The water was initially filtered through

a 20-40 m non-hydrous silicon dioxide filter medium (Jacuzzi, Little Rock, AR), followed by a 30 m spun polyester cartridge filter, and finally through a 16 m canister filter (Aquatic Eco-systems, Apopka, FL). After filtration, the water was passed through two, in-line, 8 watt, ultraviolet (UV) light sterilization units at 20 L/min (Aquatic Eco-Systems). The filters were back-flushed for 1 h every 48 h and the UV bulbs changed every 6 months when they reached 50% of their effective life. To evaluate the relative effectiveness of the UV sterilization, six tanks of larvae were reared in UV-sterilized seawater and two tanks of larvae in filtered seawater without UV sterilization. Larval survival and growth were used as endpoints for the effects of UV sterilization on larval rearing water.

Artificial spawning of adult herring was carried out using methods described by Kocan (1996). Briefly, sexually mature herring were captured by purse seine on May 10, 1995 near Cherry Point in northern Puget Sound. Eggs were stripped from six females, pooled and distributed onto 12 x 30 cm egg racks made of nylon netting. When the eggs were firmly attached to the netting, pooled sperm from six males was poured over the eggs and the mixture allowed to incubate at 10°C for 30 min. The eggs were rinsed in sterile seawater and placed into plastic boxes containing sterile, aerated seawater for transport to the laboratory.

In the laboratory, the egg racks were suspended in 250 L tanks of ambient-temperature, sand-filtered, UV-sterilized, flowing seawater for incubation and grow-out of the larvae. The egg racks were removed 3 d following the peak hatch and the flow rate in the tanks was adjusted to 2 $L*min^{-1}$ for the first 30 d post-hatching, then increased to 4 L/min. Larvae were given a mixture of rotifers (*Brachionus* sp), larval brine shrimp (*Artemia*), dry larval fish food, and algae paste for the first 90 d post-hatch. Rotifers and brine shrimp were soaked in an omega-3 fatty acid supplement (Super Selco, Aquaculture Supply, Dade City, FL) prior to feeding. Frozen brine shrimp and dry trout chow were introduced at 4 months and fed continuously for the remainder of the study.

Virus propagation and assay.
The isolate of viral hemorrhagic septicemia virus used in this study was recovered in 1993 from Pacific herring held in net pens at a bait shop in south-central Puget Sound. The chinook salmon embryo (CHSE-214) cell line (Lannan et al. 1984) and the epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) were grown in minimum essential medium supplemented with 10% fetal bovine serum (MEM-10) as described by Batts et al. (1993). The master stock of virus had been passed less than 5 times in cell culture before being frozen at -70°C. Working stocks of virus were grown at 15°C as needed.

The titers of infectious virus in cell culture fluid, aquarium water, or homogenates of herring tissue were determined by plaque assay using monolayer cultures of CHSE-214 or EPC cells pretreated with polyethylene glycol as described by Batts and Winton (1989). Virus titers were expressed as plaque-forming units (PFU) per milliliter of fluid or per gram of tissue. For titration of virus in herring, the head and tail were removed and the entire mid-portion of the fish homogenized in minimum essential medium. Serial dilutions of the homogenates were plated on monolayers of EPC cells.
Exposure of herring to virus. - Replicate groups of SPF herring were transferred from grow-out tanks to 40 L aquaria (8-10 fish/aquarium depending upon size), provided with ambient-temperature, sand-filtered, UV-sterilized, seawater at a flow rate of 0.5 L/min, and allowed to acclimate for 24 h. For virus challenge, the water flow was turned off and the volume in the aquaria reduced to 3 L. Dilutions of stock virus in MEM-10 or equivalent volumes of MEM-10 as controls were added to the water (2-3 aquaria/dose) to provide replicate low, medium, and high challenge levels (approximately 10^2, 10^4, and 10^6 $PFU*ml^{-1}$, respectively) of freshly grown VHSV. At the end of the 1 h waterborne challenge, the water flow was resumed for the remainder of each study and fish were then observed twice daily for 21 d. Experimental fish were fed twice

daily with frozen brine shrimp and krill and dead fish were removed and frozen at -70°C for later virus assay.

Susceptibility of SPF herring to VHSV
Virus susceptibility tests were conducted when the SPF herring were 5, 9 and 13 months of age. Percent mortality, mean day to death, gross external signs of disease, weight of fish, and virus concentration in visceral tissues were determined or recorded. The first challenge was conducted in November, 1995 when the juvenile fish were 5 months of age, or approximately 2.0 g in weight. Virus concentrations used for the first waterborne challenge were: $10^{2.5}$ PFU*ml^{-1}, $10^{4.5}$ PFU*ml^{-1},, and $10^{6.5}$ PFU*ml^{-1}, The second challenge was conducted when the fish were 9 months of age, or approximately 3.7 g. Virus concentrations used for the second challenge were: $10^{1.5}$ PFU/mL, $10^{3.5}$ PFU*ml^{-1}, and $10^{5.5}$ PFU*ml^{-1}. The third challenge was conducted when the fish were 13 months of age, or approximately 5.0 g. Virus concentrations for the third challenge were: $10^{2.3}$ PFU*ml^{-1}, $10^{4.3}$ PFU*ml^{-1}, and $10^{6.3}$ PFU*ml^{-1}.

Virus shedding by VHSV-infected herring.
Virus shedding rates were determined by placing 10 SPF herring into each of two 40 L aquaria, exposing them to $10^{6.7}$ PFU*ml^{-1} VHSV for 1 h, then sampling the water for virus during the next 5 d. Immediately following the exposure period, the water was turned on at 0.5 L*min^{-1} and 1.0 mL water samples were taken hourly for the next 3 h. At 24 h post-exposure, and at the same time each day for the next 5 d, the water was turned off and a 1.0 mL water sample was taken immediately and then hourly for 3 h. At the end of the 3 h period, the water was turned on again until the next day. Water samples were placed directly into 1 mL of 2X MEM-10 and frozen at -70°C until assayed. This made it possible to monitor both the level and the rate of virus shedding.

Histopathology and replication of virus
Pathological changes in tissues and replication of VHSV over time were examined using 6 month old, juvenile SPF herring. Following exposure to VHSV at low (101.5 PFU/mL), medium ($10^{3.5}$ PFU*ml^{-1}) or high ($10^{5.5}$ PFU*ml^{-1}) doses of virus for 1 h, live or moribund fish were collected from replicate aquaria at 2, 4, 6 and 8 days post-exposure and processed for histology and for virus titration.

For histology, fish were euthanized in MS-222, their abdominal cavities opened, and the whole fish preserved in Bouin's fixative. After 48 h tissues were transferred to 70% ethanol until processed. Paraffin sections 2-3 m thick were stained in hematoxylin and eosin. Histological sections from all fish groups were coded and examined blind. Tissues selected for examination included: liver, kidney, spleen, gastrointestinal tract (stomach, pyloric caeca, intestine, rectum), pancreas, skeletal muscle, gills and a longitudinal portion of the head. The head sections contained esophagus, head kidney, heart, buccal cavity, epidermis, subcutis and areolar connective tissue, bone, cartilage, brain, spinal cord, thyroid, thymus, and sensory tissues of the eye and olfactory epithelium.

For titration of virus, live or moribund fish were collected and frozen at -70°C. At the end of the experiment, the fish were thawed, the mid-portion of individual fish was homogenized in minimum essential medium, and homogenates were plated on monolayers of EPC cells.

Results

Production of SPF herring

Peak hatching of fish occurred 12 d post-fertilization with water temperatures ranging from 11.2°C to 13.0°C during incubation. The mean length of newly hatched larvae was 8.54 + 0.29 mm and increased linearly to 90 d when the fry were 28.98 + 1.65 mm (Figure 1). By 40 weeks, the mean length for the fish was >100 mm.

Larval survival in the six UV-treated tanks was greater than 90% through 90 d post-hatching. The two tanks receiving only filtered seawater experienced a gradual die-off until all larvae were dead by 50 d, even though the larvae appeared healthy, ate readily and grew at the same rate as fish in the UV-treated tanks during this period. When the larvae in the UV-treated tanks were more than 90 d of age, 30 larvae were transferred to the non-UV treated tanks and observed for another 90 d. No additional mortality occurred in the non-UV treated tanks during the next 9 months.

Susceptibility of SPF herring to VHSV

Typically, mortality began 4-6 days post-exposure and peaked about day 7 in groups of herring exposed to high doses (105.5-6.5 PFU$*$ml^{-1}) of VHSV regardless of the age of the fish (Table 1). Fish exposed to low levels of VHSV began dying 6-12 days post-exposure with peak mortality occurring on days 10-11 (Table 1). Tissues from individual fish that died during the study often had a mean virus concentration in excess of 106 PFU/g while fish surviving to the end of the 21 d experiment had low or undetectable levels of VHSV in their tissues. Mortality and pathology were not observed in control fish or in some groups of SPF herring exposed to the lowest levels of VHSV, and no virus could be isolated from their tissues. Both the onset of mortality and mean-day-to-death showed a dose-response; however, once infected, the total mortality in the groups and the virus titers in infected fish were generally similar regardless of the initial challenge dose (Table 1). Little, if any, effect of increased size upon resistance was noted and the fish remained highly susceptible to infection at more than 1 year of age. Reisolation of VHSV from the fish completed Koch's postulates.

Virus shedding by VHSV-infected herring

Following waterborne exposure to $10^{6.7}$ PFU$*$ml^{-1} VHSV for 1 h, the water flow was resumed and the virus titer in the aquaria declined to below detectable levels (5 PFU$*$ml^{-1}). No virus was found in any sample collected at 24 h post-infection (Figure 2), but infected herring began shedding detectable levels of new virus by 48 h post-exposure, coinciding with the first appearance of virus in the tissues (Table 2). By 72 h post-exposure, the fish were shedding sufficient virus to produce titers of 102 PFU$*$ml^{-1} of flowing aquarium water and, when the water flow was turned off, the titer rose to more than 103 PFU$*$ml^{-1} during the 3 h sampling period. Virus shedding peaked on days 4 and 5 post-exposure at levels of $10^{2.5}$ PFU$*$ml^{-1} of flowing water and the first mortalities were observed on day 5. On both day 4 and day 5, virus titers in the 40 L aquaria increased from $10^{2.5}$ to $10^{3.5}$ PFU$*$ml^{-1} during the 3 h period, indicating that, on average, the 10 fish were shedding more than $10^{6.7}$ PFU/fish$*$h^{-1}.

Histopathology and replication of virus

Gross external examination of experimentally infected herring showed minimal hemorrhaging in fish that died from VHSV or were confirmed to be infected. Hemorrhaging of the body wall and fin-base reddening were observed in only two fish, while small 1-2 mm areas of hemorrhage were observed on the lower jaw, mouth and eyes of the remaining 130 infected SPF fish in this experiment.

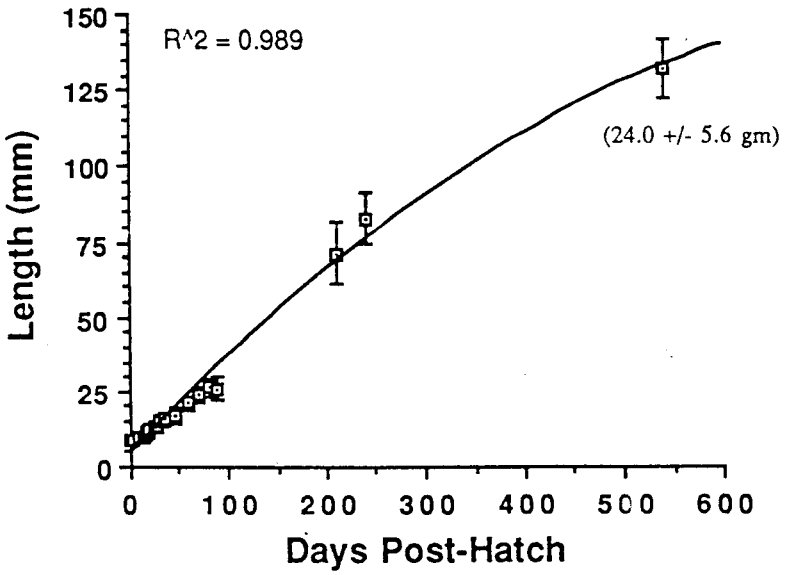

Figure 1. Growth rate of specific-pathogen-free (SPF) Pacific herring reared at the Marrowstone Island Field Station (USGS-BRD) during the first 1.5 years post-hatching. Approximately 100 fish have survived to over 2-years-old (April, 1997).

Microscopic examination of SPF fish exposed to virus revealed lesions in tissues of both groups of herring exposed to the high dose of VHSV, but in only one of the two groups exposed to the medium dose. No lesions were observed in the low dose or control groups of fish (Table 3). Histopathologic changes consisted of: (1) moderate to severe multifocal coagulative necrosis of liver hepatocytes; (2) moderate diffuse necrosis of the spleen ; (3) infrequent multifocal necrosis of acinar cells in the pancreas ; mild to moderate diffuse necrosis of kidney interstitial hematopoietic tissues including occasional glomeruli or tubules and mild to moderate diffuse necrosis of the epidermis and subcutis. Lesions in one fish also included diffuse pyknosis and karyorrhexis of granular cells in the stratum granulosum and lamina propria of the intestine with similar changes in the mucosal epithelium accompanied by some sloughing in the intestine, pyloric caeca and rectum.

Virus was first detected in the tissues of one high dose fish on day 2 post-exposure while tissues from all fish in medium and high dose tanks were positive for virus on days 4 and 6 post-exposure. By day 8 post-exposure, one fish exposed to a medium dose still had detectable virus while the other fish was below the detection limit. The sole surviving high dose fish had detectable virus on day 8. No fish mortality occurred in controls or low dose tanks and no virus was detected in tissues from these fish at the end of the study (Table 2).

Table 1. Mortality in specific-pathogen-free Pacific herring challenged with a North American strain of viral hemorrhagic septicemia virus.

Challenge Dose	Percent Mortality	Initial Mortality [1]	Mean Day to Death	Mean Virus Titer [2] Mortality	Survivors
Challenged at 5 months of age (2.0 g average weight)					
Control (MEM-10)					
1	0	-[3]	-	-	BDL[4]
2	0	-	-	-	BDL
3	0	-	-	-	BDL
Low ($10^{2.5}$PFU*ml^{-1})					
1	0	-	-	-	BDL
2	90	6	10.2	6.0	BDL
3	100	7	10.0	6.7	-
Medium ($10^{4.5}$PFU*ml^{-1})					
1	90	5	7.4	6.1	4.8
2	100	4	7.3	6.1	-
3	-[5]	-	-	-	-
High ($10^{6.5}$PFU*ml^{-1})					
1	100	4	5.4	6.1	-
2	100	4	5.9	6.1	-
3	100	6	6.3	6.2	-
Challenged at 9 months of age (3.7 g average weight)					
Control (MEM-10)					
1	0	-	-	-	BDL
2	0	-	-	-	BDL
Low ($10^{1.5}$PFU*ml^{-1})					
1	70	8	12.1	6.2	3.6
2	80	12	13.5	6.7	5.5
Medium ($10^{3.5}$PFU*ml^{-1})					
1	70	7	9.7	6.6	4.2
2	90	7	11.1	6.6	BDL
High ($10^{5.5}$PFU*ml^{-1})					
1	80	6	8.9	6.7	4.5
2	90	6	8.1	6.7	3.9

(Table 1 cont'd)

Challenged at 13 months of age (5.0 g average weight)

Control (MEM-10)					
1	0	-	-	-	-
2	50	1	5.5	BDL	-
Low ($10^{2.3}$PFU*ml^{-1})					
1	63	8	11.8	4.8	BDL
2	75	12	14.3	5.0	3.3
Medium ($10^{4.3}$PFU*ml^{-1})					
1	75	5	6.0	5.3	3.4
2	100	6	7.8	4.0	-
High ($10^{6.3}$PFU*ml^{-1})					
1	87	5	6.9	4.4	BDL
2	100	4	5.6	5.0	-

1 Days post-exposure
2 Expressed as the geometric mean of log10PFU/g titers of positive fish
3 No data or no sample available
4 Below detection limit (<102.6PFU/g) of the plaque assay
5 Replicate lost

Discussion

Pathogen-free juvenile Pacific herring were highly susceptible to infection by the North American strain of VHSV delivered by waterborne exposure. Infected fish exhibited mortality that approached 100% by 7-10 d post-exposure, shed large amounts of free virus by 3 d post-exposure, and sustained extreme cellular damage to liver, spleen, kidney and skin. High levels of virus were reisolated from affected fish and identified as VHSV, thus fulfilling Koch's Postulates and confirming the North American strain of VHSV was the cause of disease and mortality in these juvenile Pacific herring.

All groups of fish exposed to the medium and high levels of VHSV ($10^{3.5\text{-}6.5}$ PFU*ml^{-1}) for 1 h became infected and sustained high mortality (70-100%), while infected fish were found in 6 of 9 tanks exposed to lower concentrations of the virus ($10^{1.5\text{-}2.5}$ PFU*ml^{-1}). Based on these and other unpublished studies in our laboratory, it appeared that the minimum dose of VHSV for infection of juvenile herring by waterborne exposure for 1 h was in the range of $10^{1.5\text{-}2.0}$ PFU*ml^{-1}.

Virus could be isolated from tissues of fish exposed to a high dose ($10^{6.5}$ PFU*ml^{-1}) of VHSV by 2 d post-exposure and in fish exposed to a medium ($10^{4.5}$) dose of virus by 4 d post-exposure. By the second week post-exposure, tissue concentrations of virus began to drop dramatically in surviving fish, indicating that the fish were beginning to clear the virus. Elimination of the virus was also observed among survivors of outbreaks of VHS in wild-caught, naturally-infected

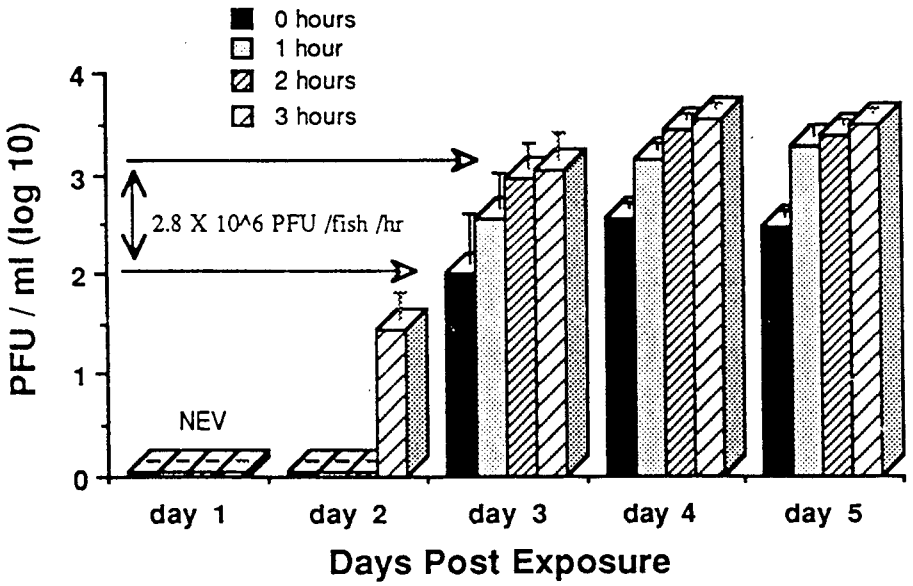

Figure 2. Mean concentrations of the North American strain of viral hemorrhagic septicemia virus shed by specific-pathogen-free Pacific herring. Fish were infected via a 1 h water-borne exposure to $10^{6.7}$ PFU*ml^{-1} of virus and water samples were collected from replicate aquaria at each of three hourly intervals during 5 d post-infection. NEV = no evidence of virus

herring held for several months in the laboratory and fish surviving either natural or artificial infection were strongly protected against reinfection (data not shown).

The first fish mortality occurred at day 4-6 post-exposure in the highest virus exposure groups with a mean-day-to-death of 5-9 days, while in the lowest exposure groups, the initial mortality began on day 6-12 with a mean-day-to-death of 10-14 days. Two hypotheses might explain this difference: 1) a low initial exposure dose infected all fish, but required a longer incubation period for the virus to produce enough tissue damage to result in mortality, or 2) only a few fish were initially infected in the low dose tanks, and these fish shed sufficient virus to initiate a subsequent round of infections in the remaining fish. The observations that some low-dose tanks produced no infected fish and that infected fish shed high levels of VHSV (in excess of that used to challenge the low and medium groups) support the latter hypothesis. Also, when fish at the low dose challenge did become infected, the ultimate percent mortality and the virus titer in dead fish became comparable to groups of fish infected at higher challenge doses.

Virus shedding by the 10 SPF herring in each of two 40 L aquaria greatly exceeded our expectations. Infected herring shed detectable levels of new virus by 48 hours post-exposure, coinciding with the first appearance of virus in the tissues. By 72 h post-exposure, virus shedding was sufficient to produce titers greater than 10^2 PFU*ml^{-1} in the aquarium in spite of the 80 min turnover rate of the water. Virus shedding peaked on days 4 and 5 when, on average, the 10 herring in each aquarium were shedding VHSV at a rate of $10^{6.7}$ PFU/fish*h^{-1}. It was important to note that during the peak period, the amount of virus shed by the 10 infected fish in 40 L aquaria was sufficient to exceed the challenge levels used to induce high mortality in this species,

suggesting that in nature, large natural epizootics could be sustained even at low densities of juvenile fish.

Table 2. Viral titers in visceral tissues of Pacific herring following challenge with a N. A. strain of viral hemorrhagic septicemia virus. Values expressed as the geometric mean of titers of positive fish (log_{10} PFU*gm^{-1})

Challenge Dose	Day post-exposure 2	4	6	8
Control (MEM-10)				
1	BDL(1)	BDL	BDL	BDL
2	BDL	BDL	BDL	BDL
Low ($10^{1.5}$PFU*ml^{-1})				
1	BDL	BDL	BDL	BDL
2	BDL	BDL	BDL	BDL
Medium ($10^{3.5}$PFU*ml^{-1})				
1	BDL	NS (2)	6.1	7.7
2	BDL	5.5	7.7	BDL
High ($10^{5.5}$PFU*ml^{-1})				
1	4.6	6.0	6.9	NS
2	BDL	6.1	2.6	6.3

1) Below detection limit of the plaque assay (<$10^{2.6}$PFU*gm^{-1})
2) No sample

The minimal cutaneous hemorrhaging observed in experimentally infected SPF herring was in contrast to descriptions of naturally infected fish reported in the literature (Meyers et al. 1994; Marty et al. 1995) and observations of VHSV-infected herring from Puget Sound, Washington (unpublished data). Extensive hemorrhaging of the fin bases, skin, jaws and eyes was common in wild fish infected with VHSV. However, the minimal hemorrhaging observed in SPF fish from our study suggests that the extensive hemorrhaging in wild herring may result from the interaction of multiple pathogens and possible environmental stressors, or from a more chronic form of the disease occurring in the older fish typically obtained from the wild.

Histologic lesions in the mid and high dose fish were progressive, becoming visible and/or most severe in the fish surviving 6-8 d post exposure. Internal target tissues of viral infection, based on frequency and severity of lesions were (in descending order): liver, kidney, and spleen (Table 3) with pancreas variably affected. Epidermis and subcutis were also consistently targeted and probably were primary sites of virus replication during initial infection. Although clearly not due to postmortem change, the gut lesions observed in one fish were inconsistent and questionable regarding their significance to VHSV infection.

Table 3. Presence of histologic lesions in selected tissues of SPF Pacific herring following waterborne exposure to a North American strain of VHS virus.

Challenge Dose	Presence of lesions[1] Liver	Kidney	Spleen	Pancreas	Epidermis	Subcutis
Control (MEM-10)	-	-	-	-	-	-
Low ($10^{1.5}$PFU*ml^{-1}) All samples	-	-	-	-	-	-
Medium ($10^{3.5}$PFU*ml^{-1})						
Day 2	-	-	-	-	±	-
Day 4	-	±	-	-	-	-
Day 6	+	+	+	-	+	+
High ($10^{5.5}$PFU*ml^{-1})						
Day 4	+	+	±	-	+	-
Day 6	+	+	+	+	+	+
Day 8	+	+	NS [2]	-	+	+

1 (-) = no visible lesion; (±) = possible lesions; (+) = visible lesions.
2 No sample

The lesions in these VHSV-infected SPF juvenile herring were somewhat typical of lesions seen in salmonids infected with the European strain of VHSV where major degenerative changes and necrosis occur in the liver, kidney and spleen (Liversidge and Munro 1978, Wolf 1988). However, significant hemorrhaging caused by the endotheliotropic nature of the virus was not apparent in these juvenile fish, unlike the extensive hemorrhaging typical of European VHSV in rainbow trout and for North American VHSV in wild juvenile and adult Pacific herring (Meyers et al. 1994). Although the tissues targeted by VHSV in juvenile SPF herring were also affected in wild, adult herring, the lesions were different. Skin hemorrhages with occasional ulceration were prominent in the adult herring from Prince William Sound and, although some focal hepatic necrosis was evident (Marty et al. 1995), other associated microscopic lesions of the liver, kidney and spleen were of a chronic and proliferative nature (Meyers et al. 1994).

Interestingly, these Pacific herring were quite resistant to the related fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), a major pathogen of salmonids. Only 1 of 20 herring died at the highest IHNV challenge level ($10^{6.4}$ PFU*ml^{-1}) where trout or salmon would be expected to sustain nearly 100% mortality (data not shown). Although many of the surviving herring were infected, the IHNV titers in their tissues were relatively low indicating the fish could serve as carriers for the virus in nature. In contrast, Pacific salmon are relatively resistant to the North American strain of VHSV (Winton et al. 1991).

B. Net Pen Studies on VHSV Expression

Objectives

To determine the effect of capture and confinement on the course of VHS in Pacific herring

Methods

Pilot studies were initiated in late May (1996) on herring in the Puget Sound roe-on-kelp fishery to determine if fish expressed or shed VHSV following capture and introduction into pens. Fish from a commercial R-O-K operation were sampled at the time of capture and again when released from the pens. Fish were introduced at the rate of 2,000, 3,000 and 21,500 lb on days 1, 2 and 3 respectively. Samples were collected from the last group captured (day 3) prior to introduction into the pounds, and again on the day of release (day 4). No attempt was made to distinguish among the three introduction groups at the time of release.

Results

None of the 30 fish sampled prior to introduction into the pounds had detectable virus, however 12% of the fish examined at the time of release from the pens were positive for VHSV (Fig. 3). It is not known if the fish samples at the time of release were from any one collection day or were a composit of all three groups. Bedause of the 10-fold higher number of fish introduced into the pens on day 3, it is highly likely that most of the fish sampled on release were from this group.

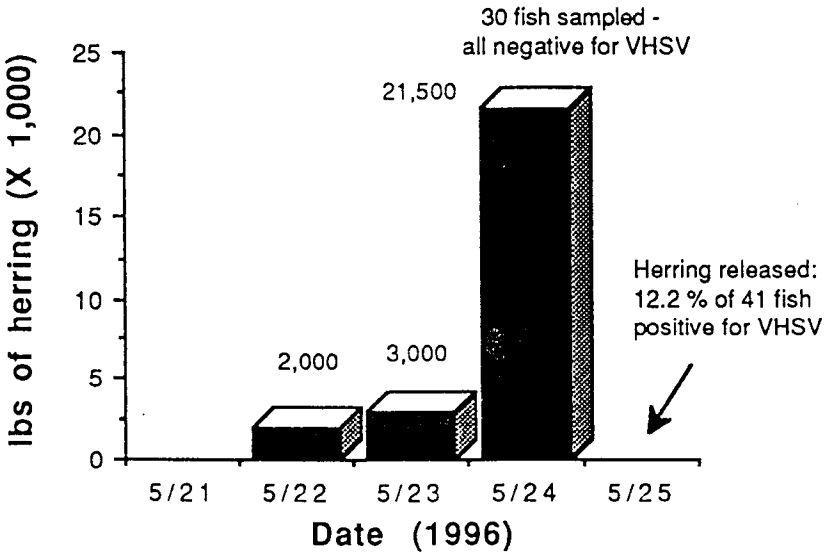

Figure 3. Tonnage of herring introduced into spawn-on-kelp pound over a 3 day period in Puget Sound in May, 1996. No evidence of VHSV was found in fish prior to placment into pens but by 20-48 hours later (at release) 12.2% were found to be infected by plaque assay.

Discussion

There was a significnat increase in prevalence of detectable virus in herring placed into a net pen as a part of the roe-on-kelp fishery. Based on a 30 fish pre-pound sample there was no evidence of VHSV in Puget Sound herring at the time of capture, but over 12% of 42 fish were positive for the virus at the time of release. The initial 30 fish sample was capable of detecting a 10% or greater prevalence of the virus, so it is possible that fewer than 10% of the sample was infected and therefor undetectable with this sample size. However, the 12% prevalence in the second sample indicated a significant increase in the number of fish with active infections. It is not clear at this time if there are a large proportion of fish carrying latent infections that become active after capture, or if just a few infected fish in the population are infected and these infect the remaining fish by releasing virus into the confined space of the pens.

C. Pathogenicity of *Ichthyophonus* for Lab-reared and Wild Pacific Herring

Objectives

1) Confirm Koch's Postulates
2) Establish if *Ichthyophonus* is capable of causing mortality in Pacific herring
3) Describe the natural history of *Ichthyophonus* in wild herring of various ages
4) Determine the prevalence rate of Ichthyophonus in different age classes of wild herring

Methods

Ichthyophonus hoferi was initially isolated from wild Pacific herring from Prince William Sound (PWS), AK. Infected heart, liver and spleen were placed in minimal essential medium containing 10% fetal bovine serum (MEM-10) and cultured at 12°C. A culture of *I. hoferi* obtained from a laboratory in Denmark was also maintained for comparative purposes. The Danish strain was originally isolated from Atlantic salmon and has been in culture for over 2 years. The large vegetative spores from the two isolates were used for experimental infections in specific-pathogen free (SPF) fish.

Within a 1-7 days after infected tissues are placed in culture, the organism appears primarily as branched "hyphae" which ultimately produce and release microscopic spores from the terminal ends of the hyphae. After several weeks in culture, the organism is present primarily as large spherical multicellular spores visible to the naked eye. These appear to be identical to the stages described by Marty (1995) in histologic sections of tissues from infected PWS herring.

Laboratory herring

Experimental SPF herring were reared from egg to 1.5 years-old in 10 µm filtered - UV sterilized flowing seawater. When the SPF herring reached 6 months-old and confirmed disease-free, they were experimentally exposed by injecting 1,000 spores per fish of either PWS or the Danish strains of *Ichthyophonus* on 21 December, 1995.

Experimental groups consisted of: 1) 10 sham-injected controls, 2) 8 fish injected with the Danish strain, and 3) 10 fish injected with the PWS strain. Fish were observed and mortality recorded for 6 weeks, at which time the study was terminated. When experimental SPF herring died, they were examined grossly for external and internal lesions and their heart, liver and spleen placed into MEM-10 for culture.

Wild herring

Wild 0-year herring were collected from Puget Sound in August, 1996by dip-netting 300-400 fish from "herring balls" located near the Marrostone Island Field Station. Netted fish were placed in a cooler, gassed with pure oxygen and transported to the laboratory where they were examined for the presence of *Ichthyophonus* by visual, microscopic and culture techniques.

Wild 18 to 24-month-old Puget Sound herring were obtained from a local bait dealer within 24-48 hours post-capture and transferred from the net pens to the laboratory where they were maintained in 700L flowing seawater tanks and examined for the presence of *Ichthyophonus hoferi.* by visual, microscopic and culture techniques. Transport time was less than 20 minutes and fish were supplemented with pure oxygen during the transport. Wild herring were also collected by Evelyn Brown and Malcolm McEwen as a part of the SEA juvenile herring study. Tissues from the PWS fish were removed on ship-board and placed into cutlure tubes containing MEM-10 with antibiotics. Tissues from 5 fish were placed into each tube, then they were shipped back to the University of Washington for incubation and evaluation. No data on external or internal lesions were tanken from the PWS fish, but this project is proposed for April 1997.

Ichthyophonus prevalence in wild herring was evaluated by three methods. First, each fish was observed for external lesions that appeared as small dark spots on the skin, presumably caused by the parasite eroding the epithelium. Second, after the skin was examined, each fish was opened and the heart, liver and spleen examined for the presence of whitish lesions caused by actively growing *Ichthyophonus*. Third, pieces of heart, liver and spleen were minced and placed into MEM-10 culture medium and maintained at 12°C for 14 days to detect infections not observed grossly.

Non herring studies
Rainbow trout (*O. mykiss*), English sole (*Parophrys vetulus*) and coast range sculpins (*Cottus aleuticus*) and a saltwater sculpin (*Cottus sp)* were injected with cultured spores originally isolated from infected PWS herring. Fish were maintained in the laboratory until they began dying, then they were sacraficed, their tissues visually examined, then cultured in vitro. Tissue from infected sculpins was fed to 6 additional sculpins to determine if transmission is possible via eating infected tissue. Horizontal (fish-to-fish) transmission was attempted by placing 3 infected coastrange sculpins in the same tank with 3 uninfected sculpins. These were observed for 14 days then necropsied and cultured to determine if transmission occurred between infected and uninfected fish.

Results

Laboratory-reared herring:
Specific-Pathogen-Free herring were successfully raised in the laboratory from egg to 2-years-old. Once the fish metamorphosed at 90 days post-hatch, mortality was minimal (< 5%) and no fish were diagnosed to be infected with any known pathogen.

The data from experimental exposure of SPF herring to *Ichthyophonus* spores are summarized in Table 4. Only one fish from the control group died of unknown causes and no fish died in the Danish group. Herring exposed to the PWS isolate of *Ichthyophonus* exhibited significant mortality following exposure, and 80 percent (8/10) were positive for *Ichthyophonus* at by the end of the study. No evidence of infection could be found in the first fish to die (7 days) nor in one of the two surviving fish at the end of the study (56 days). The first fish to die was negative by all methods of examination. The first fish confirmed positive for *Ichthyophonus* died 14 days post-exposure, and mortalities resulting from infection continued through 56 days post-exposure when the study was terminated and the last two surviving herring were sacrificed.

Table 4. Experimental exposure of SPF herring to two strains of *Ichthyophonus* by IP injection.

	day post exposure	dead (-) live(+)	mm	gm	skin lesions	visceral lesions	histopathology heart	liver	skin
Controls									
C1	41	-	86	5.4	-	-	-	-	-
C2	56	+	80	5.7	-	-	-	-	-
C3	56	+	73	4.6	-	-	-	-	-
C4	56	+	77	5.1	-	-	-	-	-
C5	56	+	95	8.0	-	-	-	-	-
		mean =	82.2	5.76	0%	0%			
		stdev =	8.58	1.32					
Danish									
D1	56	+	93	6.7	+	-	-	-	-
D2	56	+	91	7.1	-	-	-	-	-
D3	56	+	70	3.9	-	-	-	-	-
D4	56	+	83	7.0	+	-	-	1 spore	-
D5	56	+	87	5.8	-	-	-	-	-
D6	56	+	87	5.9	-	-	-	-	-
D7	56	+	84	6.0	-	-	-	-	-
		mean =	85.0	6.1	20%	0%			
		stdev =	7.51	1.09					
PWS									
P1	7	-	87	5.7	-	-	-	-	-
P2	11	-	72	3.4	-	+	no tissue	+	++
P3	18	-	78	4.1	-	+	+++	++	+++
P4	33	-	79	3.0	-	+	+++	+	+++
P5	36	-	87	6.3	+	+	+++	+	+++
P6	36	-	83	5.1	+	+	+++	+	++
P7	46	-	86	6.2	+	+	+++	+	++
P8	55	-	66	3.1	+	+	no tissue	++	++
P9	56	+	74	4.2	+	+	+++	+++	++
P10(a)	56	+	76	4.1	-	-	-	-	-
		mean =	78.8	4.52	50%	80%			
		stdev =	7.04	1.24					

a) Two surviving fish at 56 days were sacraficed
b) - : no spores observed
+: light infection
++: moderate infection
+++: heavy infection

No visible nor histologic evidence of infection was detected in any of the control fish. Skin lesions were observed in 2/7 fish exposed to the Danish strain and only a single spore was observed in a histologic section of liver from one of the fish with skin lesions. In contrast, in the

fish exposed to the PWS strain the first gross (viseral) and histopathologic lesions were apparent 14 days post-exposure while the first visible skin lesions did not appear until 36 days post-exposure. Both skin and visceral lesions were still visible when the study was terminated 56 days post-exposure.

Culture of tissues revealed no evidence of *Ichthyophonus* in the control group, but 7 of 7 of the Danish group and 8 of 10 of the PWS group cultured positive for the organism. The length and weight of the controls and Danish group were not significantly different from each other, but the PWS group was significantly smaller than the other two groups (P = 0.05; t-Test) by the end of the study period.

Wild herring:
Wild 0-year, 1+ and 3+ herring were collected from Puget Sound and 2+ fish were collected from PWS. The PS fish were examined for the presence of *Ichthyophonus* by external examination, internal examination and in vitro culture, while the PWS fish were only examined by in vitro culture.

The prevalence of *Ichthyophonus* appeared to increase with age in Puget Sound fish, with a low of 6% in 0-year fish to a high of 52% in 3+ spawners. PWS 1+ herring showed a minimum prevalence of 1.7% and a high of 23.1% (Table 5). Because the PWS fish were examined in pools of 5 fish per culture, these are minimum values and will be reevaluated in April 1997.

Table 5. *Ichthyophonus* survey of wild herring from Puget and Prince William Sound (1996)

	N	length (mm)	skin lesion (%)	visceral lesions (%)	culture (% positive)
Puget Sound					
0 - year	100	79 (± 4.7)	6	0	6
1+ year	100	152 (± 8.3)	5	0	23
3+ year	100	185 (± 10.9)	4	5	52
Prince William Sound [a]					
1-2 year-olds					
Whale Bay (July)	60	ND	ND	ND	1.7 - 8.3 [b]
Whale Bay (Aug)	60	ND	ND	ND	3.3 - 16.7
Simpson Bay	60	ND	ND	ND	4.6 - 23.1
Nellie Juan	60	ND	ND	ND	3.3 - 16.7

(a) Eaglet, Zaikof, Hidden and Wells Bay were negative by culture
(b) Minimum - maximum prevalence estimate
ND Data not collected

Non herring studies:
Rainbow trout were resistant to infection by IP injection of up to 10,000 spores. None of the 12 fish died and no *Ichthyophonus* was cultured from their hearts, livers or spleens. The one English sole died within 30 days of exposure and cultured positive for *Ichthyophonus*. The one saltwater scupin survived for 60 days when it was sacrificed and cultured positive for *Ichthyophonus*. The 6 coastrange sculpins injected with 1,000 spores began dying 14 days post-exposure and were all postive by culture. The 6 coastrange sculpins fed infected tissues began dying by 14 days post-exposure and all cultured positive for *Ichthyophonus*.

Discussion

Laboratory-reared herring
Ichthyophonus , originally isolated from diseased PWS herring was grown in pure culture and injected into nonimmune herring where it produced the disease observed in nature. The organism was subsequently reisolated from the experimentally infected fish and once again grown in culture, thus fulfilling Koch's Postulates for this organism.

The organism is apparently a pathogen for nonimmune herring capable of causing sever mortality. The difference in pathogenicity between the PWS and Danish strains of *Ichthyophonus* could be the result of strain differences or more likely due to attenuation of the Danish strain after prolonged time in culture.

The use of in vitro culture appears to be the most sensitive of the methods used to detect *Ichthyophonus*. Only in vitro culture revealed 100% infection by the Danish strain even in the absence of mortality, gross lesions and histologic lesions. All fish that showed evidence of infection by gross or microscopic examination also cultured positive for the organism resulting in a correpondence between conventional methods and in vitro culture.

The appearance of minute skin lesions in SPF herring 36 days post-exposure indicates that the organism migrates from the internal organs to the musculature just under the skin where it somehow erodes the epithelium, causing a small hole to appear. Prior to the appearance of the small holes in the skin (lesions), large cysts can be found just under the skin causing small bumps remeniscent of the "sandpaper skin" described by Sinderman (1990).

SPF fish infected with *Ichthyophonus* either lose weight or do not gain weight at the same rate as uninfected fish or fish infected with a nonpathogenic strain of the organism. Weight relationships between infected and uninfected wild herring are currently being investigated under 97162.

Wild herring
Wild Puget Sound herring become infected with *Ichthyophonus* soon after metamorphosis (3-4 months post-hatch) with the prevalence increasing with age. There is no obvious difference in the health of infected wild fish relative to noninfected fish, but closer scrutiny could reveal subtle differences that might render them less competative with their noninfected cohorts. It is also not clear if infected fish ultimately clear the organisms from their body or if they carry the parasite for life.

The most sensitive method for identifying *Ichthyophonus* is in vitro culture of heart, liver and spleen. This method consistantly detected the most infected fish and always corresponded with both the external skin lesions and visible internal organ lesions. When comparing different age classes, the number of fish with skin lesions remained constant with age while the actual number

of infected fish increased (Table 2A). Thus, skin lesions were a more accurate measure of actual infection rate in the youngest fish and became less predictive as the fish aged. Gross lesions on the heart, liver and spleen appeared to become more evident as the fish aged, but this may be a function of a greater survace area to observe on the larger organs rather than in increase in the actual number of infected organs.

Non herring studies
English sole, coast range sculpins and a single staghorn sculpin all became infected with *Ichthyophonus* following IP injection of cultured spores, indicating that these species could act as hosts to this pathogen if they were exposed via a suitable route. Rainbow trout however, were resistant to up to 10,000 spores. Coastrange sculpins fed infected tissues also became infected and began dying 14 days post exposure, confirming that *Ichthyophonus* could be transmitted to carnivorous fish by eating infected herring. Although no studies have yet been conducted, it is possible that herring act as a source of infection for other species that feed on them. How herring become infected however, is still not clear. Because they are not carnivorous it is unlikely that they become infected by eating other fish. It is possible however that an invertebrate becomes infected or carries a stage of the parasite, thus making it possible for herring to become exposed by eating the invertebrate.

D. Oil generator studies

Objective

To determine the amount of oil that could be solubilized into seawater from an oil-contaminated substrate colonized by normal marine microbes.

Methods

Cintered glass collars like those used in biological filters were used as a substrate for oil and marine microbes. 100 grams of collars equaled 2,000 ft^2 of surface area, with each piece weighing 1.32 ($\pm$.04) g dry and 2.22 ($\pm$.04) g when oiled. Five-year old Exxon Valdez oil was used to soak the collars for 1 hr, after which the collars were air dried on a screen for 24 hours prior to being placed into the generator. A 2 inch PVC pipe was fitted with an inlet and outlet through which filtered seawater could be pumped. A 24 inch-long 2 inch diamater PVC pipe was filled with 72 oiled collars, giving 64.8 g of oil available for exposure. This apparatus (described in FY95 annual report) was used to generate a natural level of weathered crude oil into 10 g tanks containing either wild or SPF 9-month-old herring. Water was run over the oiled substrate at 0.5 $L*min^{-1}$ for 24 hr prior to beginning the exposures, and water samples were collected for 25 days after exposures began. Water samples were collected into acid cleaned, glass stoppered 1L bottles and were analyzed by Analytical Resources, Inc. (Seattle, WA). The study was terminated and the fish examined for alterations in blood values (see Kennedy, FY95-96 annual reports) at 25 days.

Results

Background seawater levels of oil were below detection limits (<0.03 ppm) by HPLC-FID methodology. By 24 hours after the water was turned on, levels rose to 0.07 ppm then again fell to below detection limits by 10 days after water flow began. Oil levels began to rise again after 15 days and by 25 days the levels rose to 0.52 ppm and continued to rise until the water was turned off at 6 weeks. Figure 4 summarizes the date for values from background through 25 days. This study was repeated three times with essentially the same results. At the end of the exposues it was apparent that the oiled collars were heavely colonized by marine microbes, but no attempt was made at this time to identify the organisms.

Discussion

After initially falling to undetectable levels by 10 days, whole crude oil levels unexpectedly began to rise after about 2 weeks exposue of oiled substrate to seawater, and ultimately reached levels over 0.52 ppm. This is probably the result of microbial metabolism of the oil following colonization of the oiled substrate by marine microbes. An article by Middaugh et al. (1996) supports this contention by demonstrating that microbial colonization of oiled substrate by bacteria isolated from PWS increased the amount of soluble oil in a closed seawater system as well as its toxicity to silveside embryos. This situation undoubtedly occurs in natural marine ecosystems where residual oil occurs. If fish or invertebrate embryos are present in the are they are potentially at risk of being exposed to the increased toxicity of the weathered crude oil.

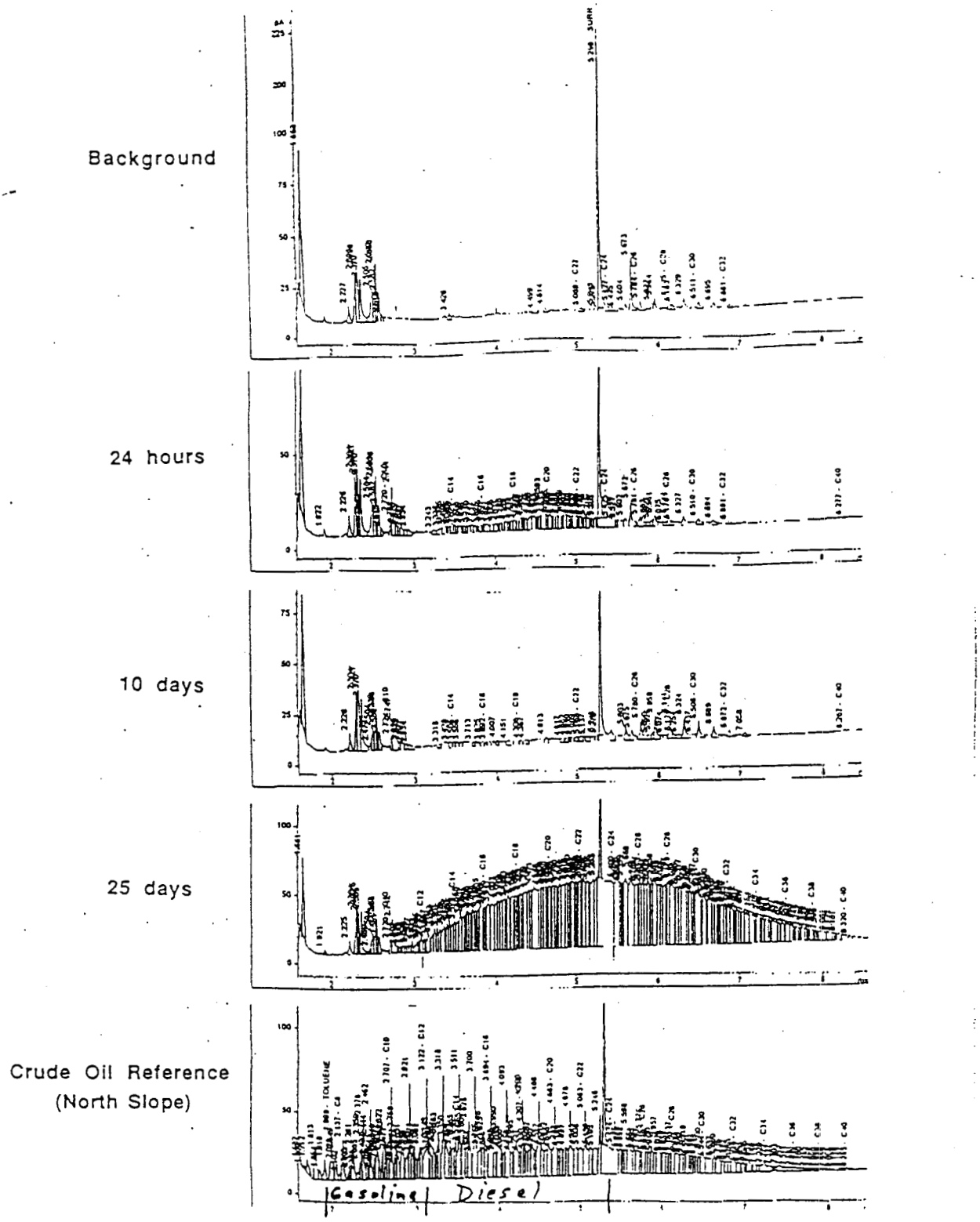

Figure 4. Crude oil profiles in natural seawater that had been passed over oiled substrate for 25 days. Background < 0.03 ppm; 24 hr = 0.07 ppm; 10 days < 0.03 ppm; 25 days = 0.52 ppm. Compared with the crude oil reference, the primary missing component after 25 days was the LMW (gasoline) range hydrocarbons which were lost during the weathering process. The large component of hydrocarbons with MW > C24 were presumably the result of microbial metabolism and solubilization.

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Exxon Valdez Oil Spill
Restoration Project Annual Report

Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound

Section III. Survival, performance and reproduction in the Pacific herring, *Clupea harengus pallasi*: Effects of environmental contamination, viral hemorrhagic septicemia virus and *Ichthyophonus hoferi*

Restoration Project 96162
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill Trustee Council restoration program for the purpose of assessing project progress. Peer review comments have not been addressed in this annual report.

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April 1997

Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound

Section III. Survival, Performance and Reproduction in the Pacific Herring, *Clupea harengus pallasi*: Effects of Environmental Contamination, Viral Hemorrhagic Septicemia Virus and *Ichthyophonus hoferi*

Restoration Project 96162
Annual Report

Study History: The project effort was initiated under Restoration Project 95320S in response to a request for proposals to investigate disease factors affecting Pacific herring decline in Prince William Sound. The proposal is a joint effort of the University of Washington, Simon Fraser University, University of California at Davis and Alaska Department of Fish & Game.

Abstract: This study continues to document cause-effect relationships for oil, Viral Hemorrhagic Septicemia Virus (VHSV) and Ichthyophonus hoferi (ITP) on herring survival, performance and reproduction in order to determine their role in population declines in Prince William Sound. Exposure of juvenile herring to an oil-water dispersion (OWD) of crude oil resulted in increased acute mortality and a classical biochemical 'stress' response. Exposure to an OWD resulted in modifications in the immune system including changes in white blood cell populations, macrophage phagocytosis and antibody production to bacterial pathogens. Overall disease resistance was affected by OWD exposure, however, fish exposed to the highest concentration exhibited the most resistance to a pathogen in disease challenge experiments. Swimming performance and recovery from swimming exercise were inhibited by exposure to OWD. Results collected from herring exposed to VHSV and ITP indicate that infection by these pathogens also alters herring hematology and immunology. These results begin to explain possible roles of oil, VHSV and ITP in herring population declines. An increased understanding the responses of herring to these stressors has important implications to herring management strategies and herring fishery practices.

Key Words: *Clupea harengus pallasi, Exxon Valdez* oil spill, fitness, herring, *Ichthyophonus hoferi*, oil, Viral Hemorrhagic Septicemia Virus (VHSV).

Project Data: *Description of data-* Several sets of data were gathered by laboratory experiments and include: effects of oil exposure, Viral Hemorrhagic Septicemia Virus and Ichthyophonus hoferi on juvenile and adult Pacific herring biochemistry, immunology and disease resistance, and swimming performance and exercise recovery. Format- Data regarding experimental data are stored in Microsoft Excel and text files in MS Word 5.0. *Custodian* - Contact Dr. Chris Kennedy at the Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada, V5A 1S6. Phone: (604) 291-5640, fax: (604) 291-3496 or email at: ckennedy@sfu.ca). *Availability* - Copies of all data and text in annual reports are available for the cost of duplication. Reprints of any manuscripts will also be available when published.

Citation:

Kennedy, C.J., and A.P. Farrell. 1997. Investigations of disease factors affecting declines of Pacific herring populations in Prince William Sound. Section III. Survival, performance and reproduction in the Pacific herring, *Clupea harengus pallasi*: Effects of environmental contamination, viral hemorrhagic septicemia virus and *Ichthyophonus hoferi*, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 96162), Simon Fraser University, Burnaby, British Columbia, Canada.

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Executive Summary

Due to the prevalence of Viral Hemorrhagic Septicemia Virus (VHSV) and *Ichthyophonus hoferi* (ITP) in spawning herring sampled from Prince William Sound (PWS), these two pathogens have been considered to be likely the cause of morbidity of herring in PWS. However, from the information that had existed prior to the start of this project in 1995, there had been no definitive evidence on whether VHSV, ITP or oil exposure through the *Exxon Valdez* oil spill, or some combination of these stressors had caused a decline in herring populations. The longterm objective of this study is to document cause-effect relationships for oil, VHSV and ITP on herring fitness or 'health". The categories of fitness chosen for this study include herring survival, blood biochemistry, performance in terms of the immune system and swimming performance, and reproduction. These studies used both laboratory-raised specific-pathogen-free herring raised from eggs collected from PWS and wild caught juvenile and adult herring. Small but significant differences existed between SPF and wild caught fish in some aspects of hematology, information which was useful in the planning of future experiments and in data interpretation. The disease state of wild fish was also determined. Wild juveniles were negative in all tests for both VHSV and ITP. Adult herring were also negative for presence of VHSV, however, 30% of adult fish were positive for the presence of ITP. Experiments to determine the effects of an oil-water dispersion (OWD) of North Slope crude oil on herring biochemistry were performed with both juvenile and adult herring. No significant effects were seen in measured biochemical parameters in adult fish following six days exposure to OWD. However, in juvenile herring, a classical 'stress' response was seen in fish exposed to OWD for 24 h which included a hypersecretion of corticosteroids, hyperlacticemia and a hyperglycemia. By 96 h of exposure to OWD, all of these parameters measured had returned to preexposure values indicating that the stress response was transient and may in part explain the lack of a measured response in adult fish after 6 days. Immunological parameters were also measured in adult fish exposed to OWD for 6 days. Significant alterations in both white blood cell populations and in the phagocytic activity of macrophages were noted although not in a dose-dependent manner. When juvenile herring were exposed to OWD chronically for 22 days, significant alterations were only seen in macrophage phagocytosis. The ultimate test of the immune system is the disease challenge test. These juvenile fish were subjected to a challenge with the marine bacteria *Vibrio anguillarum* following a chronic exposure to OWD. The state of the immune system in these fish was also monitored and it was found that OWD affected antibody titers to *Vibrio*. Curiously, however, fish exposed to the highest concentration of OWD, were the least susceptible to *Vibrio* and showed the lowest mortalities. Juvenile herring were exposed to varying concentrations of OWD to determine effects on swimming performance. In these experiments, significant mortalities (15%) were found in the highest OWD concentration, indicating a higher acute toxicity of OWD than previously reported. Following a 96 h exposure to OWD, swimming ability was reduced in the two highest OWD concentrations. Due to high mortalities in fish which were swum in these experiments, the effects of OWD exposure on exercise recovery were determined. It was shown that OWD affected the recovery of aspects of herring biochemistry which is typically altered during exercise. Prior experiments in which juvenile herring were exposed to either VHSV or ITP indicated that infection with

either of these pathogens can alter aspects of herring hematology and the immune system including hematocrit, leucocrit and differential white blood cell counts. These results begin to explain the possible roles of oil, VHSV and ITP in herring population declines. Understanding how herring respond to these stressors and their recovery from exposure has important implications to herring management strategies and herring fisheries practices and will aid in the recovery of the resource as well as in successful monitoring of fish health in the future.

Introduction

In 1993, less than half of the expected 5 year old 1988 year class of Pacific herring, *Clupea harengus pallasi*, returned to spawn in Prince William Sound. Approximately 15 to 43% of the returning fish were observed to have external lesions including ulcerations and hemorrhaging beneath the skin. Meyers et al. (1993) reported isolation of a rhabdovirus, identified as the North American strain of viral hemorrhagic septicemia virus (VHSV), by serum neutralization and cDNA probe methods. VHSV has now been isolated from herring over a wide geographical area spanning the USA/Canada International boundary. It has been suggested that VHSV may be indigenous to Pacific herring throughout Alaska and possibly the Pacific Northwest (Meyers et al. 1993). Therefore, the role of VHSV in the population decline of the herring populations in Prince William Sound remains unclear. One suggestion is that mortality may occur during these epizootics from progressive ulcerating skin lesions resulting in possible osmoregulatory failure and/or entry points for other pathogens (Meyers et al. 1993). These authors suggest that the virus may manifest its effects following stress from various factors including viral erythrocytic necrosis virus (VENV), spawning, commercial fishing or nutritional deficiency through lack of forage. More recent studies have indicated that VHSV was present in about 5% of herring tested in 1994, but lesions associated with *Ichthyophonus hoferi* (ITP) infection were present in about 29% of herring sampled and is suggested as the major cause of herring morbidity between the 1992 and 1993 spawning seasons (Marty et al. 1994).

It is possible that stress due to anthropogenic contamination, i.e. the *Exxon Valdez* oil spill, either directly through water and sediment exposure or indirectly *via* the food chain affected fish health or performance leading to the observed high mortalities and infection rates in surviving fish. Other studies have shown that stress from exposure to polycyclic aromatic hydrocarbons (PAHs), toxic compounds found in crude oil, reduce reproductive capacity and impair immunological responses of fish, resulting in reduced survival or fitness (Garrett, 1993). It has been shown that VHSV expression in carrier fish appears to be enhanced under stress of exposure to oil (Meyers, unpublished report). Furthermore, it is suggested that even if VHSV is not the primary pathogen, the high level of ITP incidence is indicative of a much weaker immune system in the herring. In addition, the extent of ITP infection and tissues infected (heart, skeletal muscle and brain) suggest life threatening effects (Freiberg and Farver, 1995;Marty et al., 1994). At this time, field and laboratory data confirm that a) oil exposure in 1989 could affect juvenile survival; b) VHSV prevalence was high in 1993 and 1994; c) ITP prevalence was high in 1994; d) females from previously oiled sites produced fewer live larvae.

Given the present information base, it is not clear whether VHSV, ITP , or oil exposure, or some combination of these stressors contributed to a decline in herring survival, performance or reproductive fitness. Moreover, survivors of oil exposure, VHSV or ITP infection may continue to experience a reduction in fitness which may have consequences for continued longterm survival and reproduction. Understanding the responses of herring to these stressors has important implications to herring management strategies and herring fisheries practices.

Objectives

The longterm objectives of Section III of this project are to document cause-effect and interactive relationships for oil, VHSV and ITP on herring survival, performance and reproduction and to establish the effects of important abiotic modifiers such as density and temperature on herring responses to these stressors.

The overall hypothesis being tested in this project is:

'The exposure of herring to VHSV, ITP or oil or combinations of these parameters reduces herring fitness in one or more of the following categories: 1) immunology, 2) biochemistry, 3) performance, and 4) reproduction.'

The specific objectives outlined for 1996 were as follows:

1. To supply analytical support for Section I (the field component: Dr. G. Marty) of this research project.

2. To determine relevant and appropriate assays for the analysis of immunological and biochemical fitness in herring and to develop protocols which are highly successful in the laboratory and to begin to assess them for field applicability.

3. To determine baseline levels of biochemical and immunological parameters in wild and specific-pathogen-free Pacific herring.

4. To determine the effects of oil exposure on the biochemistry of Pacific herring.

5. To determine the effects of oil exposure on the immune system and disease resistance of Pacific herring.

6. To determine the effects of oil exposure on the swimming performance of Pacific herring.

7. To determine the extent of immunological and biochemical modification and recovery in Pacific herring following an infection with VHSV.

8. To determine the extent of immunological and biochemical modification and recovery in Pacific herring following an infection with ITP.

Methods

General

1) Fish

Specific-pathogen-free (SPF) young of the year were successfully raised by Dr. Kocan (Section II of this report) and were available for some of the experiments planned in 1996. Due to the small size and limited number of SPF fish, Dr. Kocan's group and ours had decided to also use wild caught Pacific herring with known disease status with respect to VHSV and ITP. Adult Pacific herring were caught in Barkley Sound, Vancouver Island, by purse seine by the Department of Fisheries and Oceans, Canada, and the British Broadcasting Corporation in the Spring of 1996 and donated for our experiments. Juvenile young of the year were caught in Barkely Sound by beach seine in the summer of 1996. Both adults and juveniles were transported to the laboratory without using nets and held at least two weeks until any experiment was performed. Disease status was determined for both VHSV and ITP in wild adult and juvenile fish. Virology was performed on the whole body of herring at the Marrowstone Facility by Dr. Kocan's group. Virology was also performed on juveniles using the whole body and pooled liver, spleen, pyloric cecae and gill of adult herring by Dorothee Keiser of the Department of Fisheries and Oceans, Pacific Biological Station, Naniamo, BC. These assays are performed at no charge to this project due to that institutions interest in VHSV in wild Pacific herring populations. ITP prevalence was performed by histopathology of liver and heart of herring and by procedures recommended by Dr. Kocan of Section II which included grinding heart and liver (pooled for each fish) in a petri dish or test-tube in cell culture medium (L-15) supplemented with 2-10% FBS (fetal bovine serum) and either gentamicin or penicillin/streptomycin combination. Petri dishes were incubated at 14.5°C and observed after 1 to 2 weeks for spores or hyphae characteristic of ITP.

2) Chemical and stressor exposure durations

Depending upon the question asked for each experiment, varying times were used to dose herring to oil. Dose times ranged from 24 h acute exposures to 3 week chronic exposures to more fully determine possible effects on herring fitness.

3) Chemical analysis

In order to definitively determine if any of the stressors (oil, VHSV or ITP) causes alterations in herring fitness, it is imperative to be able to quantitate the doses of each stressors applied to a fish. Methods developed by Dr. Kocan in Section II were used to dose fish with sublethal levels of either VHSV or ITP. Dosing of herring with oil in 1996 was performed using the dosing apparatus or 'oil generators' which had been selected for this research project and were developed by Carls et al. (unpublished method and analysis data). Essentially, this apparatus consists of a 15 cm diameter X 80 cm tall polyvinyl chloride plastic cylinder containing ceramic beads which have been soaked in North Slope Crude oil. Water upwells through the cylinder and over the oil soaked beads and flows into the bottom of an individual treatment tank

containing herring. A trap inside the generator prevents slick overflow. Appropriate levels of hydrocarbons are generated through the apparatus by varying the amount of beads in each column. Hydrocarbon analysis using this method has been documented by Carls et al. (unpublished) using Alaska North Slope Crude oil with polycyclic aromatic hydrocarbon (PAH) concentrations in the range of 10 to 100 ppb at the start of water flow to 0.3 to 30 ppb 16 days following the initiation of water flow. Our preliminary analysis includes both total fluorescence analysis coupled with gas chromatography and FID detection. Samples are in the process of being analyzed by GC-FID at Simon Fraser University at no charge to the project. Total hydrocarbon concentrations are given in the results as control (no detectable hydrocarbons), low (7-12 ppb), medium (26-49 ppb) and high (78-120 ppb) concentrations at the beginning of the experiments.

3) Biochemical assays

Biochemical parameters assayed in experiments to integrate with field studies (Section I of this annual report) included: plasma glucose, lactate, albumin, protein, alkaline phosphatase activity, electrolytes including chloride and sodium according standard techniques by Sigma Chemical Co. (St. Louis, MO.). Biochemical parameters associated with a typical 'stress response' were measured in some experiments and included: plasma cortisol, lactate, glucose according to Kennedy et al. (1995). Biochemical parameters which were measured as indicators of exercise recovery included: plasma lactate, plasma chloride and sodium according to Graham et al. (1982).

4) Immunological assays

Immunological assays include both hematological and immunological parameters. Hematocrit (% packed red blood cells) and leucocrit (% packed white blood cells) were measured according to Kennedy et al. (1995).

Differential white blood cells were performed as follows: Smears were stained with Diff-Quik (Dade Diagnostics, Inc., Aquada, Puerto Rico), using the recommended protocol on the product package. Smears were examined microscopically at 1000X oil immersion magnification. Approximately 100 white blood cells were counted from the randomly selected fields. The number of fields examined varied with the smear, however, on average, 48 fields per slide were counted. The number of red blood cells in each field were not counted but were similar: with approximately 150-175 red blood cells per field. White blood cells were differentiated into six cell types; thrombocytes, lymphocytes, neutrophils, basophils, eosinophils and monocytes. Identification of each type was based on morphology and staining characteristics (Ainsworth 1992: Sherburne 1973). Red blood cells from each smear were examined for viral erythrocytic necrosis (VEN) .

Macrophage and their phagocytic activities were extensively modified for use with herring from Secombes (1990) and are briefly as follows: Macrophage isolation begins with the aseptic removal of the head kidney and placed in tissue cell culture medium. Herring macrophages have viability only in L-15 medium. Tissue is then macerated

by grinding through a fine mesh screen and the tissue homogenate are resuspended in cell culture medium. Cell suspensions are then centrifuged on discontinuous Percoll density gradients. The density that has been successful for obtaining a macrophage band for herring is 1.075. This corresponds to 53% Percoll (53 ml Percoll:10ml: 1.5M NaCl: 37 ml ddH20). Cell suspensions were then spun for 25 min at 4C at 400 g and bands were collected with Pasteur pipettes. Cells are washed with PBS and resuspended in cell culture media. Cell viability was determined by using 0.4% trypan blue and a hemocytometer to count the number of viable macrophages (95% viability ideal for assay).

The developed phagocytosis assay was as follows: yeast was used for assays with adult fish and bacteria was used for juveniles which may be more "realistic" but is harder to quantify due to small size of particles. Yeast/bacteria suspensions are autoclaved with formalin to produce a formalin-killed solution. The suspension is washed with PBS and then resuspended in PBS/L-15/Hanks. PBS was used for both the adult yeast assay and the juvenile *Vibrio* assay. The suspension was then opsonized by incubation for 30 min at 28C in the presence of pooled herring serum. An aliquot of macrophage suspension is placed on a glass slide and allowed to incubate for 90 min in a moist chamber. The slide is then gently rinsed with PBS and yeast/bacteria suspension is added to the slide and the slide again incubates for 90 min. The slide is washed again with PBS and stained using Diff Quik. 100 macrophages in random fields were counted and scored as to number of yeast/bacteria ingested. For yeast the categories are 0 - 4 plus. With bacteria it is a proportion of macrophages containing any internalized bacteria vs those with no bacteria as numbers of ingested bacteria per macrophage is very labour intensive.

The lysozyme assay is based on the lysis of the lysozyme-sensitive , Gram positive bacterium *Micrococcus lysodeikticus* (Stolen et al. 1993). Agarose gel containing *M. lysodeikticus*, is prepared in petri plates. Wells are punched in the gel and serum/plasma samples are dispensed into the wells and lysis is measured as clearance zones in the gel surrounding wells. Plates are incubated in a moist chamber overnight (17 - 20 hours) at room temperature. Standards or hen egg white lysozyme (HEWL) are simultaneously run. The concentration of lysozyme in samples is determined from a standard curve calculated from the clearance zones of HEWL standards. Lysozyme activity is reported in U/ml (units of activity per ml) based upon the activity of HEWL.

5) Swimming performance

The swim-test apparatus used to swim adult herring is a swim chamber as described in Nikl and Farrell (1993). Briefly, the apparatus consists of a 2,470-L ovoid, fiberglass raceway tank equipped with two variable-output propulsion motors. Two test chambers are used to house the fish inside the raceway. A series of straightening vanes, screens and contraction cones were placed upstream of the chambers to correct for rotational disturbances, smoothing the velocity profile within the enclosed cylindrical testing chambers. Water velocity is controlled by regulating voltage output to the propulsion motors. A portable current meter was used to determine water velocity within the test chambers at various voltages. Swim trials

using juveniles were performed by a modified swim chamber. Briefly, the small size of young of the year herring necessitated a smaller (1 m in length, 25 cm diameter) swim chamber which was enclosed rather than open as in the larger version.

Following exposure to one of the stressors, fish were transferred to a swim chamber without using nets and allowed to acclimate to the chamber for at least 2 hours before a test began. Critical swimming speed (U_{crit}) was measured according to Brett (1964). The initial velocity was selected for each group of fish, and the speed was increased in increments at 15-min intervals until all fish had fatigued. Fatigued fish were individually removed from the test chamber and time to exhaustion, fish fork length and weight were recorded. U_{crit} was calculated using the method of Brett (1964). A fish was considered exhausted when it rested against the rear grid and did not respond to mechanical stimulation.

6) Statistical analysis

Values are reported as means ± standard error. All data were analyzed by analysis of variance (ANOVA) and were considered significant at $p<0.05$. Percent data were arcsine transformed before statistical analysis.

Objective 1: To supply analytical support for Section I (the field component:Dr. G. Marty) of this research project.

Blood smears from 660 Pacific herring sampled in October and March/April 1996 in Prince William Sound and Sitka Sound were received from Dr. G. Marty of the University of California at Davis. Smears were stained and analyzed for differential white blood cell counts as described in the section on immunological techniques previously. White blood cells were differentiated into six cell types; thrombocytes, lymphocytes, neutrophils, basophils, eosinophils and monocytes. Red blood cells from each smear were examined for viral erythrocytic necrosis.

Due the strong statistical relationship between CPK and lesions in herring (Marty unpublished), frozen plasma samples from 100 Pacific herring sampled March and April 1996 in Prince William sound were received from Dr. G. Marty for CPK analysis. Plasma samples were unfrozen and kept at 0°C. Electrophoretic analysis of plasma was performed according to Sigma Chemical Co. (Mississauga, Ont.) to identify and calculate the relative proportions of CPK1, CPK2, CPK3, brain, cardiac and skeletal muscle isozymes, respectively.

Objective 2: To develop relevant and appropriate immunological and biochemical assays in herring

The assessment of biochemical and immunological 'health' in fish is of paramount importance to the recovery and continued monitoring of the herring population in PWS. In these studies, blood and tissues from adult and juvenile herring were routinely sampled and assayed for various biochemical and immunological parameters to be used in subsequent tests and for recommendation for use in biomonitoring projects when this project is completed. A wide range of standard

measurements was tested in herring to determine their suitability for use in this species.

Objective 3: To determine baseline levels of immunological parameters in wild and SPF herring.

SPF herring and wild herring from Puget Sound, WA at approximately 1 year old were used in these experiments to determine if differences existed in baseline biochemical and immunological parameters. These two groups of fish were also exposed to an oil-water dispersion (OWD) as described above using oil 'generators' to determine if differences exist between the effects of oil on herring. Fish were exposed to the OWD for either 2 or 3 weeks. Fish were sacrificed and blood sampled from the caudal vasculature. Selected immunological parameters were measured as described above.

Objective 4: To determine the effects of oil exposure on the biochemistry of Pacific herring.

Adult Pacific herring were exposed to control, low, medium and high concentrations of an OWD as described above for 6 days. Fish were sacrificed and sampled via the caudal vasculature. Fish were analyzed for selected biochemical parameters as described above.

Juvenile herring were exposed to control, low, medium and high concentrations of an OWD as described above for 24 h and 96. Blood was analyzed for biochemical parameters which have been shown to be good indicators of 'stress' in fish at several times during exposure to OWD up to 96 h as described previously.

Objective 5: To examine any alterations in the immune system and disease resistance of Pacific herring following oil exposure.

Juvenile herring were exposed for 22 days to control, low, medium and high concentrations of an OWD as described previously. Fish were sacrificed and blood sampled via the caudal vasculature and head kidney dissected. Various immunological parameters were performed as previously described.

Following exposure of another group of juvenile herring to the same concentrations of OWD, fish were static bath challenged with the marine pathogen *Vibrio anguillarum* to determine oil effects on overall disease resistance. The pathogen for disease challenge experiments *Vibrio* was grown in the laboratory according to Henning (personal communication). Disease challenges were of one hour duration with a dose of 6.5×10^{12} cfu/ml. Fish were then transferred to tanks supplied with uncontaminated flowing seawater. Fish were monitored for 6 weeks post challenge for mortalities. All dead fish were assayed to determine if the cause of death was *Vibrio*. To determine if a fish had died from *Vibrio*, isolation of *Vibrio* was determined by Gram stain, colony morphology, motility test, oxidase reaction, sensitivity to 0/129 and novobiocin discs and agglutination of specific latex bead antibodies. Plasma antibody titers to *Vibrio* were evaluated at 6 weeks post-challenge via a plate microdilution agglutination technique (Stolen et al. 1993).

Objective 6. To determine the effects of oil exposure on the swimming performance of Pacific herring.

Juvenile herring were acutely exposed to three concentrations of an OWD for either 24 or 96 hours as described previously. Upon completion of an exposure period, fish were examined for 1) acute mortality, 2) swimming performance and 3) their recovery from exercise. To determine the acute toxicity of OWD to herring, exposure and control tanks were monitored for mortalities for up to 96 hours. Following an exposure of herring to either 24 or 96h of OWD, fish were transferred to modified swim chambers and the U_{crit} determined. U_{crit} is defined as the maximal prolonged swimming speed of a fish. A second group of fish were exposed to OWD and then subject to a bout of 'burst' swimming. These fish were subsequently sampled for the following biochemical parameters (as described previously) which are indicators of exercise recovery: plasma lactate, hematocrit, plasma [Cl^-] and [Na^+].

Objective 7. To determine the extent of immunological and biochemical modification in Pacific herring following an infection with VHSV.

Wild 1 year old herring were exposed to VHSV as described in Section II (Dr. Kocan's group). Fish were sampled before VHSV exposure and 2 weeks post infection. Blood was collected via the caudal vasculature and immunological tests performed and blood frozen for biochemical analysis in 1997 as described previously.

Objective 8. To determine the extent of immunological and biochemical modification and recovery in Pacific herring following an infection with ITP.

The disease state of adult wild herring were determined for ITP before use in these experiments because no experiments were planned for SPF herring in 1996 by Dr, Kocan's group in Section II in which biochemical and immunological examination could occur. Sublethal doses of ITP have been worked out in Section II for use in herring. However, the high incidence of ITP in wild Pacific herring precluded their use in ITP exposure experiments. Therefore, due to the unsuitability of fish in FY 1996, these experiments were postponed until FY 1997. Preliminary experiments with ITP exposure were performed, although not scheduled for in 1995, and reported in the annual report in 1996 and briefly here.

Results

Objective 1: To supply analytical support for Section I (the field component:Dr. G. Marty) of this research project.

Statistical analysis and reporting of differential white blood cell counts and presence of viral erythrocytic necrosis are given in Section I (Field studies-Dr. Marty) of this annual report for both fall and spring samples from Sitka Sound and Prince William Sound. CPK analysis proved again unsuccessful possibly due to the instability of CPK in field plasma samples during blood chemistry analysis at the University of California laboratory (Section I of the project). Substantial differences may also exist

in herring CPK isoforms which preclude their separation and quantitation by standard techniques.

Objective 2: To develop relevant and appropriate immunological and biochemical assays in herring

The following parameters were found to be useful and successful measures of biochemical parameters and are now in use in the examination of the effects of stressors on herring biochemical fitness: plasma cortisol, glucose, lactate, albumin, protein, alkaline phosphatase activity, electrolytes including chloride and sodium. Immunological assays which are successful in herring are: hematocrit, leucocrit, differential white blood cell counts, IgM levels, macrophage phagocytic activity, lysozyme activity, antibody production to specific pathogens and disease challenge using the marine pathogen *Vibrio anguillarum*.

Objective 3: To determine baseline levels of immunological parameters in wild and SPF herring.

SPF herring and wild herring from Puget Sound, WA at approximately 1 year old were used in these experiments to determine if differences existed in baseline values and post oil-exposure values of immunological parameters. The results of this experiment indicate that there may be minor differences between SPF fish and wild fish in baseline levels of some hematological parameters (Figure 1 and 2), for example, the hematocrit values for SPF fish were significantly higher than wild fish. This experiment also indicates that the responses of SPF and wild fish to stressors may be different in magnitude, for example, there is a significant decrease in hematocrit in SPF fish exposed to oil, whereas this reduction occurs in wild fish but to a lesser extent. This data has important ramifications for experiment planning, data comparisons and choice of controls in Sections II and III of this project.

Figure 1. Baseline hematocrit values in SPF and wild herring (controls) and values 2 or 3 weeks following exposure to the OWD of oil. Significant differences were seen at $p<0.05$ between SPF and wild herring. Significant effects on hematocrit (*) were seen between SPF control and oiled fish. No significant effect of oil on wild herring hematocrit was noted at $p<0.05$.

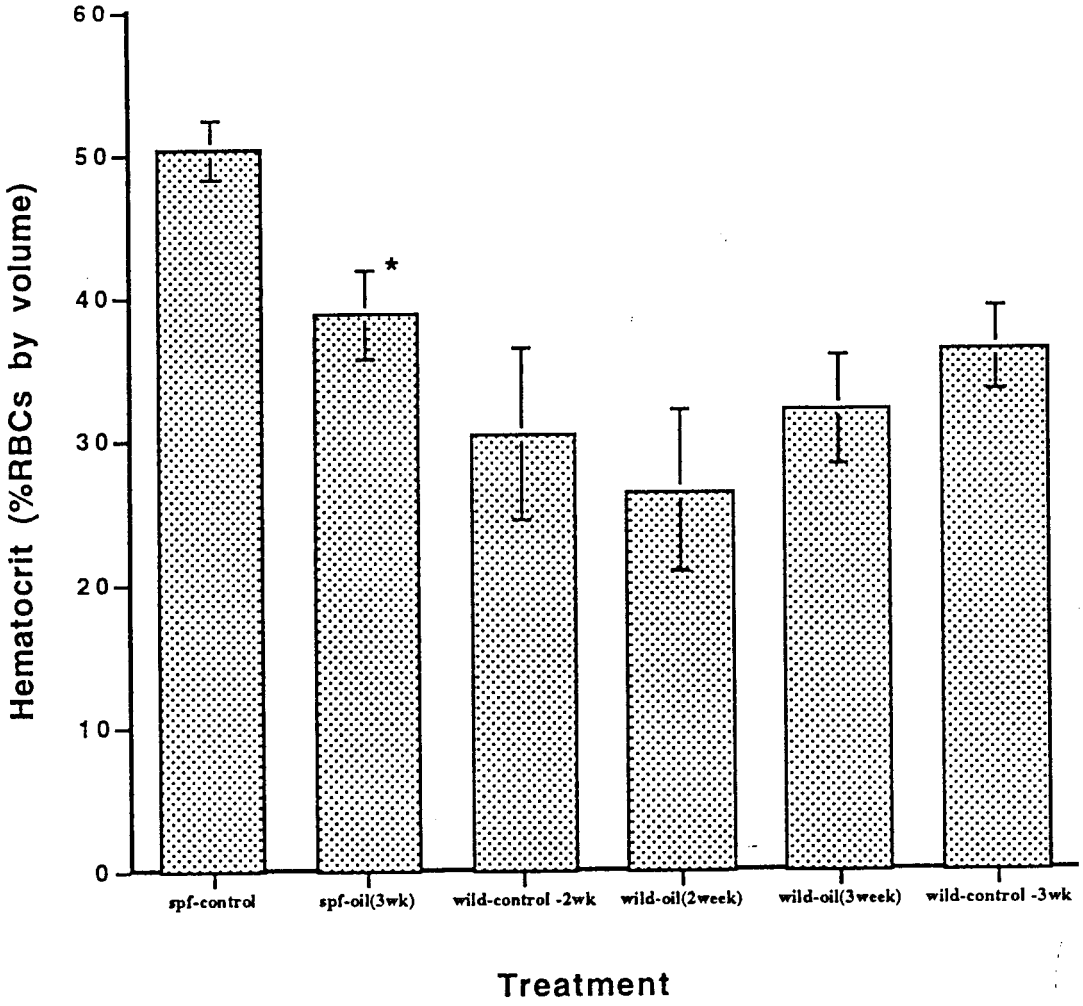
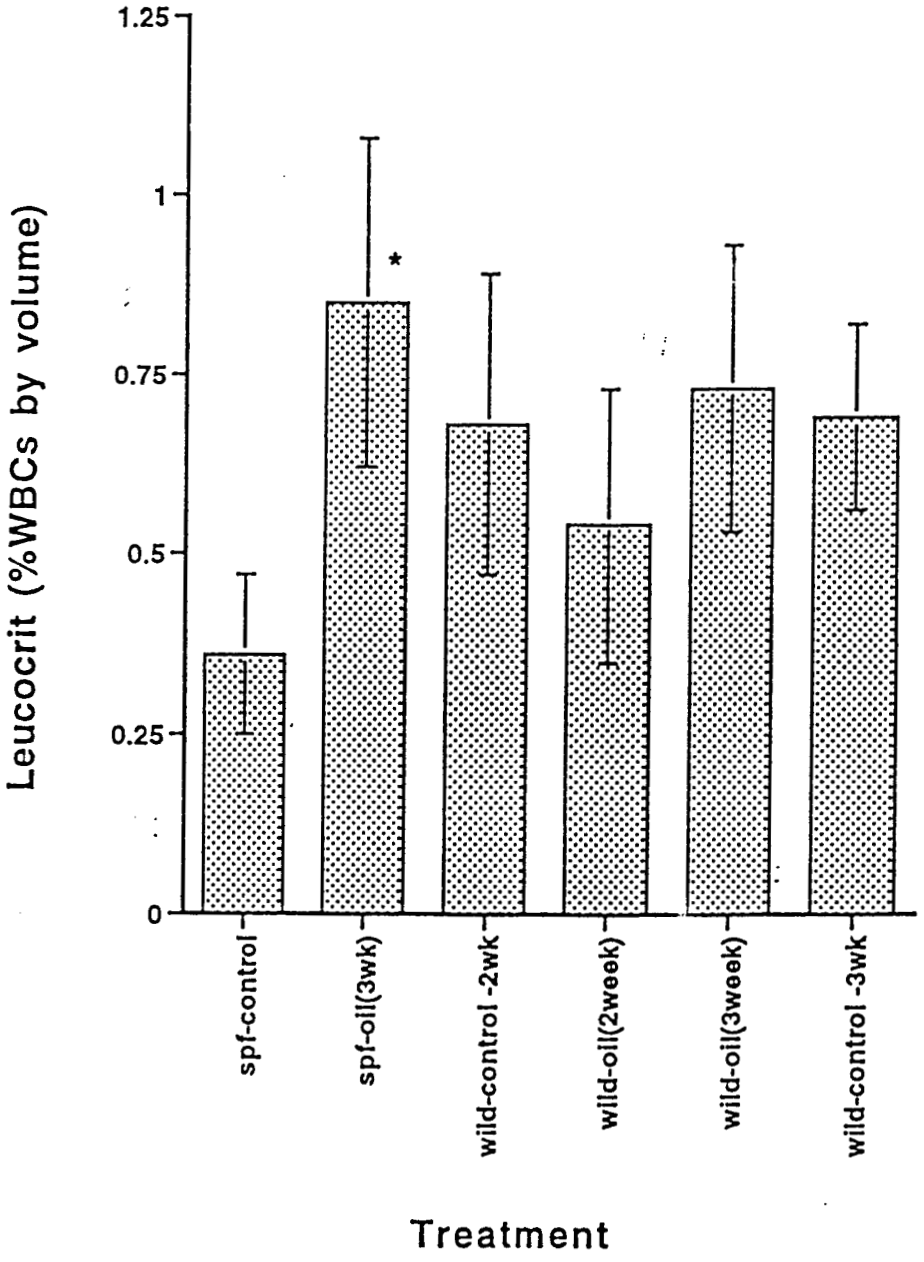


Figure 2. Baseline leucocrit values in SPF and wild herring (controls) and values 2 or 3 weeks following exposure to the OWD of oil. No significant differences were seen at $p<0.05$ between SPF and wild herring. Significant effects on leucocrit (*) were seen between SPF control and oiled fish. No significant effect of oil on wild herring hematocrit was noted at $p<0.05$.



The disease status of wild juvenile and adult herring was also determined. VHSV was isolated from 1/30 adult fish and 0/20 juvenile fish. ITP prevalence in adult fish was 10/30 fish tested, and 0/30 juvenile fish.

Objective 4: To determine the effects of oil exposure on the biochemistry of Pacific herring.

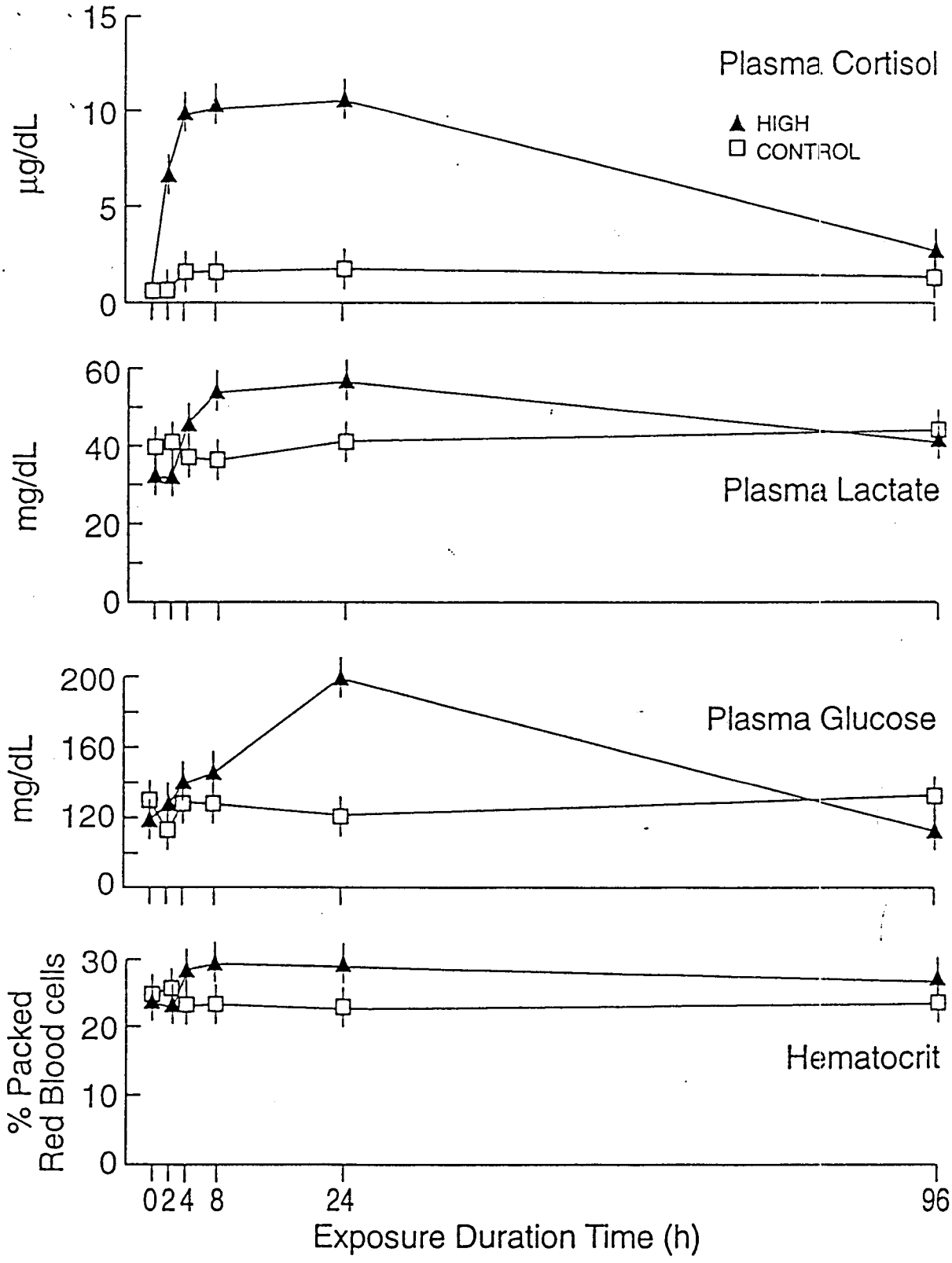
Adult Pacific herring were exposed to control, low, medium and high concentrations of an OWD to determine effects on herring biochemistry. Biochemical measures are given in Table 3. No significant differences were seen in the biochemical parameters between oiled and control fish.

Table 1. Various biochemical and hematological parameters in adult herring exposed to varying concentrations of OWD for 6 days. Values are means±SE for 6 fish. No significant differences were seen between control and oiled fish at $p<0.05$.

Parameter	Control	Low OWD	Medium OWD	High OWD
Glucose (mg/dL)	110.44±11.5	90.65±15.6	120.12±2.4	101.43±16.9
Lactate (mg/dL)	51.4±18.5	50.2±13.4	43.1±23.6	55.4±15.1
Albumin (mg/dL)	8.4±1.1	8.7±0.8	9.2±0.6	9.2±1.2
Protein (mg/dL)	9.1±1.0	9.3±0.6	10.7±0.7	10.5±1.1
Alkaline phosphatase (U/ml)	18.0±2.8	20.5±1.6	19.3±4.2	20.7±4.3
Hematocrit	29.76±1.65	28.58±2.65	34.68±1.32	34.32±2.85
Leucocrit	0.50±0.13	0.46±0.13	0.25±0.05	0.49±0.12
Liver wt (gm)	0.82±0.07	0.96±0.23	1.15±0.18	0.87±0.13
Body wt (gm)	73.42±9.59	84.36±7.11	85.43±7.41	72.52±8.64
Liver:body ratio	0.011	0.011	0.013	0.012

Juvenile herring exposed to OWD for 24 and 96 h showed different responses than adults exposed for 6 days. Due to the limited blood that can be taken from juvenile herring, parameters which have been shown to indicate a classical 'stress' response were chosen. Figure 3 shows the time course of changes in plasma cortisol, lactate, glucose and hematocrit. Significant initial increases occur in fish exposed to OWD, however, values appear to return to pre exposure level by 96 h, even with continued exposure.

Figure 3. Effects of varying concentrations of an OWD of oil on the plasma cortisol, lactate, glucose and hematocrit in herring before and during 2, 4, 8, 24 and 96 h exposures. Values are means±SE of three sets of pooled blood (3 fish each set). Values at 96 h are not significantly different at $p<0.05$.



Objective 5: To determine the effects of oil exposure on the immune system and disease resistance of Pacific herring.

Adult Pacific herring were exposed to control, low, medium and high concentrations of an OWD to determine effects on herring immunology. Hematological measures are given in Table 2. There were no significant differences between control and oiled fish at $p<0.05$ in either leucocrit or hematocrit. Differential white blood cell counts and significant differences between control and oiled fish are seen in Table 3. Phagocytic macrophage activity is seen in Table 4. Lysozyme activity in control and oiled fish are seen in Table 5. Significant differences were only seen in macrophage activity although the results were not dose-dependent.

Table 2. Hematocrit and leucocrit values in adult herring exposed for 6 days to OWD. Values are means±SE for 6 fish. No significant differences at $p<0.05$ were noted.

OWD Dose	Hematocrit (%)	Leucocrit (%)
Control	29.76±1.65	0.50±0.13
Low	28.58±2.65	0.46±0.13
Medium	34.68±1.32	0.25±0.05
High	34.32±2.85	0.49±0.12

Table 3. Differential white blood cell counts in adult herring exposed for 6 days to OWD. Values are means±SE for 6 fish. Significant differences at $p<0.05$ are denoted *.

OWD DOSE	Thrombocytes (%)	Lymphocytes (%)	Neutrophils (%)
Control	17.36±1.27*	62.38±5.31	20.26±5.59
Low	34.03±6.70*	49.96±5.81	16.01±3.48
Medium	23.13±2.96	51.17±3.76	25.69±3.73
High	26.11±3.53	46.56±6.58	27.33±6.51

Table 4. Ingestion of 0 to 4 yeast cells by isolated macrophages from adult herring exposed for 6 days to OWD. Values are means±SE for 6 fish. Significant differences at $p<0.05$ are denoted *.

OWD dose	0 cells	1 cell	2 cells	3 cells	4+ cells
Control	9.63±2.10	30.25±3.90	16.38±2.24	11.88±1.55	31.75±3.20
Low	5.00±1.49	31.63±3.96	22.63±2.10	16.25±0.96	24.50±5.26
Medium	10.43±3.90*	37.14±2.42	24.14±1.79	12.57±2.13	17.00±4.54*
High	4.38±0.71	27.38±3.16	25.00±2.23	16.00±1.73	27.25±3.74

Table 5. Lysozyme activity of adult herring plasma from fish exposed for 6 days to OWD. Values are means±SE for 6 fish. No significant differences at $p<0.05$ were noted between control and oiled fish.

OWD Dose	Lysozyme activity (U/ml)
Control	31.21± 8.19
Low	18.57± 5.40
Medium	45.05±12.52
High	66.08±23.62

Juvenile herring were exposed for 22 days to control, low, medium and high concentrations of an OWD to determine the effects of longterm exposure on immunological parameters and resistance to infection by *Vibrio anguillarum* as a disease challenge. Figures 4 and 5 show the effects of OWD exposure on leucocrit and hematocrit.

Figure 4. Leucocrit values in juvenile herring exposed to varying concentrations of an OWD of oil. Values are means±SE of ten fish. No significant differences were seen at $p<0.05$.

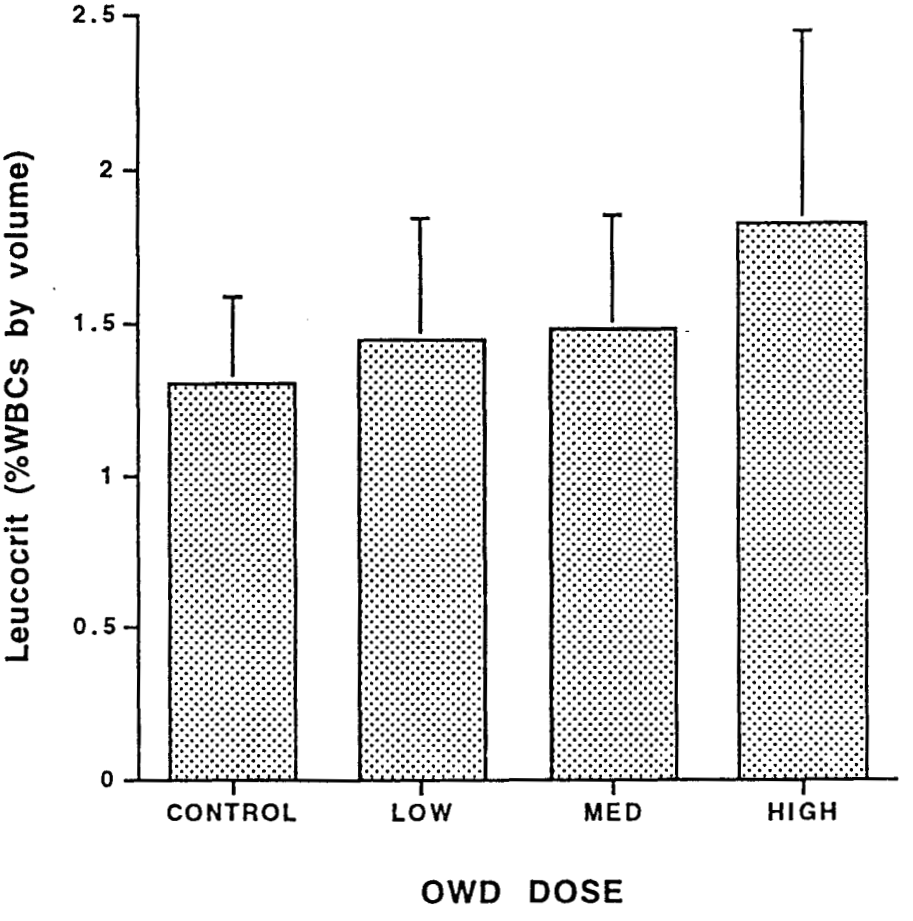
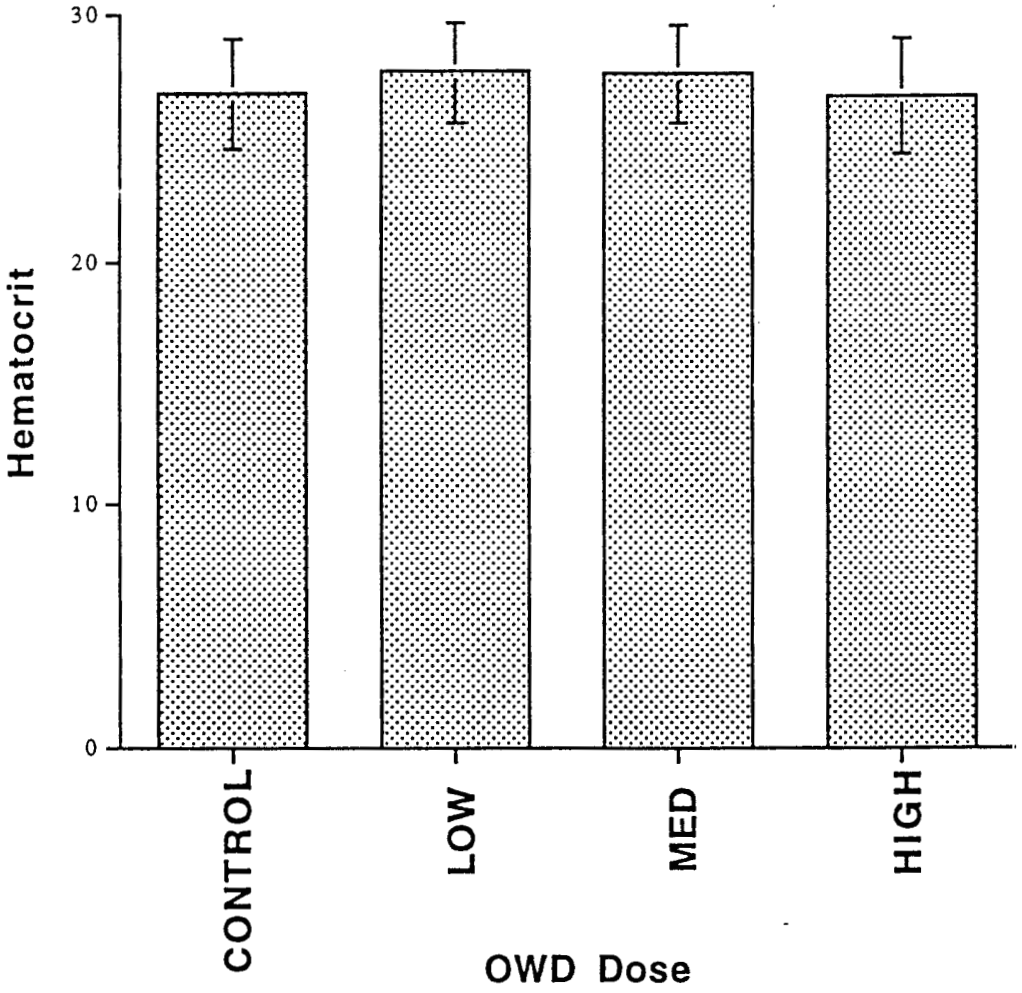


Figure 5. Hematocrit values in juvenile herring exposed to varying concentrations of an OWD of oil. Values are means±SE of ten fish. No significant differences were seen at $p<0.05$.



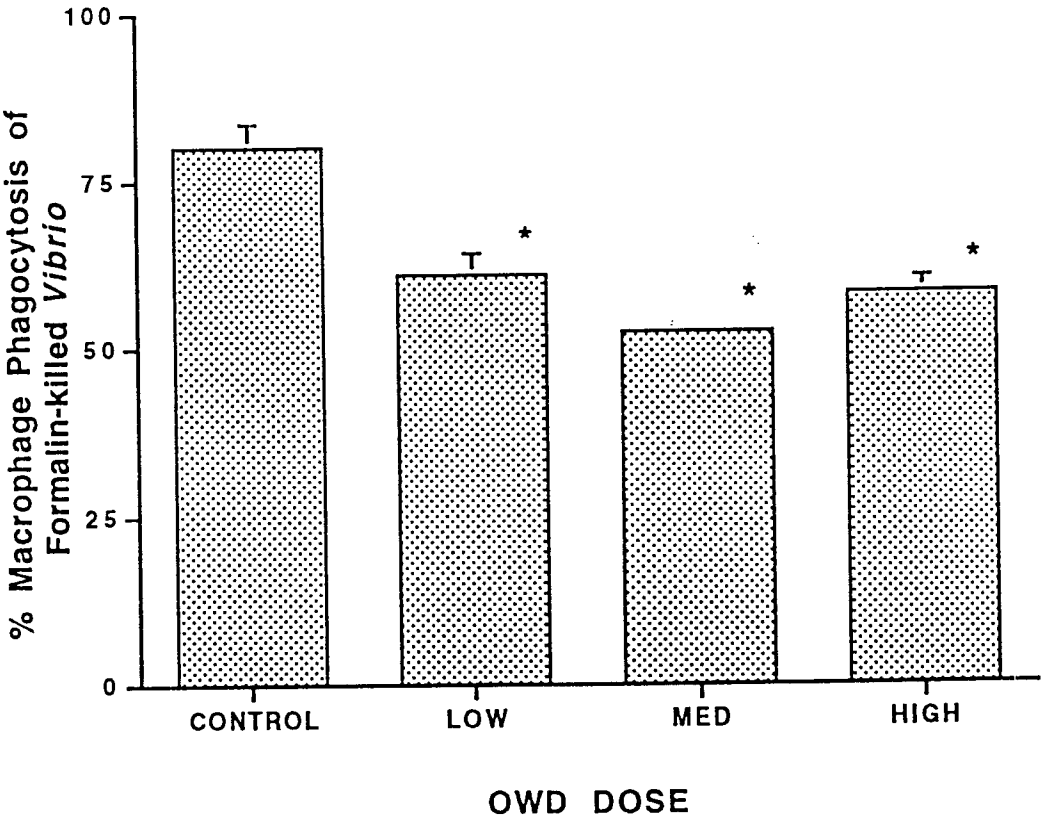
Differential white blood cell counts in juvenile herring exposed to OWD for 22 days are given below in Table 6.

Table 6. Differential white blood cell counts for juvenile herring exposed to OWD for 22 days. Values are means±SE for ten fish. No significant differences were noted between control and oiled fish at $p<0.05$.

OWD dose	Thromb. (%)	Lymph. (%)	Neutroph. (%)	Mono. (%)
Control	17.39±3.57	66.02±5.10	16.03±4.40	0.57±0.28
Low	12.13±2.12	69.36±4.93	18.44±4.00	0.07±0.07
Medium	16.69±3.34	60.07±5.35	23.17±4.58	0.07±0.07
High	19.69±2.53	60.10±4.49	19.63±3.98	0.58±0.21

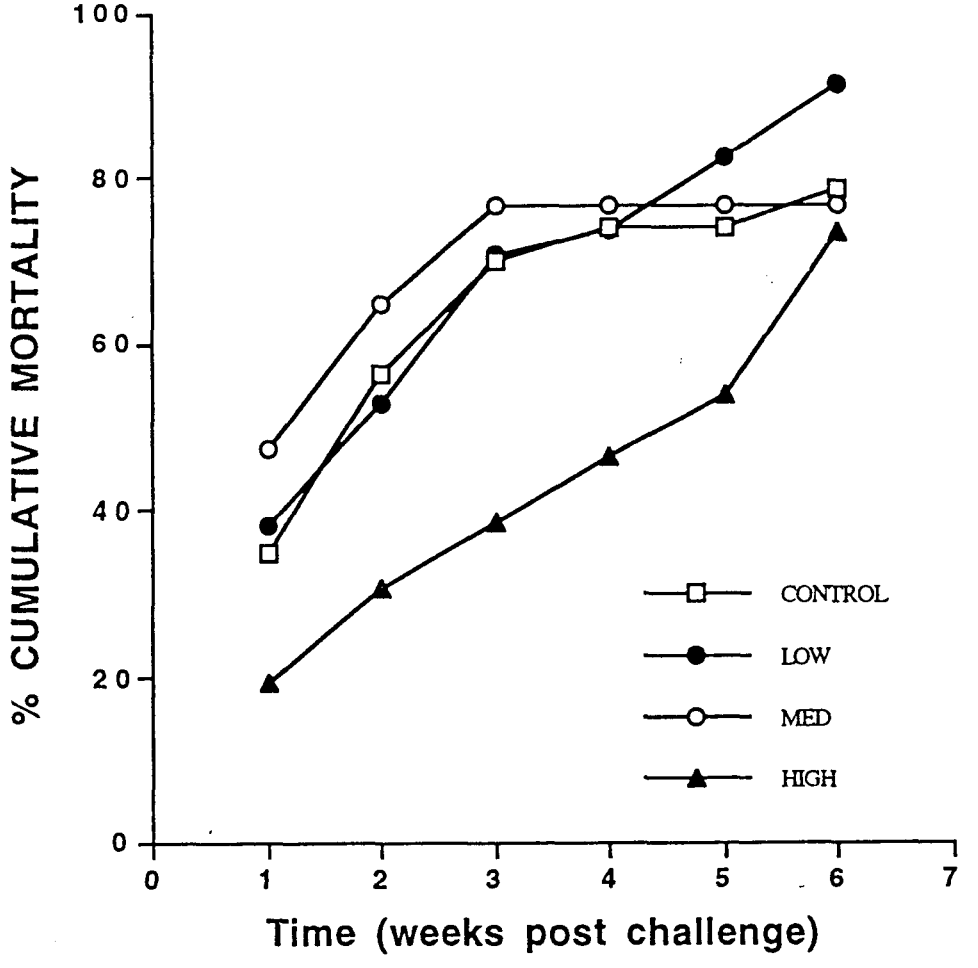
Macrophage phagocytic activity is shown in Figure 6.

Figure 6. Macrophage phagocytosis in juvenile herring exposed to varying concentrations of an OWD of oil. Values are means±SE of ten fish. Significant differences seen at $p<0.05$ are denoted by *.



The results of the disease challenge are shown in Figure 7. This figure shows cumulative mortality in herring exposed to *Vibrio anguillarum* following an exposure to varying concentrations of OWD for 22 days. The lowest mortalities through 6 weeks occurred in the highest OWD concentration.

Figure 7. The cumulative percent mortality in juvenile herring exposed to varying concentrations of an OWD of oil and the marine pathogen *Vibrio anguillarum*. Values are means of duplicate tanks. Significant differences were seen at $p<0.05$ in the high concentration.



The effects of OWD exposure and a secondary infection with *Vibrio anguillarum* on hematocrit and leucocrit in surviving fish are shown in Figure 8 and 9. Analysis of antibodies to Vibrio 6 weeks post challenge are shown in Figure 10. Control fish had the highest titers of antibodies to Vibrio when compared to OWD-exposed fish.

Figure 8. Leucocrit values in juvenile herring exposed to varying concentrations of an OWD of oil and the marine pathogen *Vibrio anguillarum* 6 weeks post infection. Values are means ±SE of ten fish. Significant differences seen at $p<0.05$ are denoted with an *.

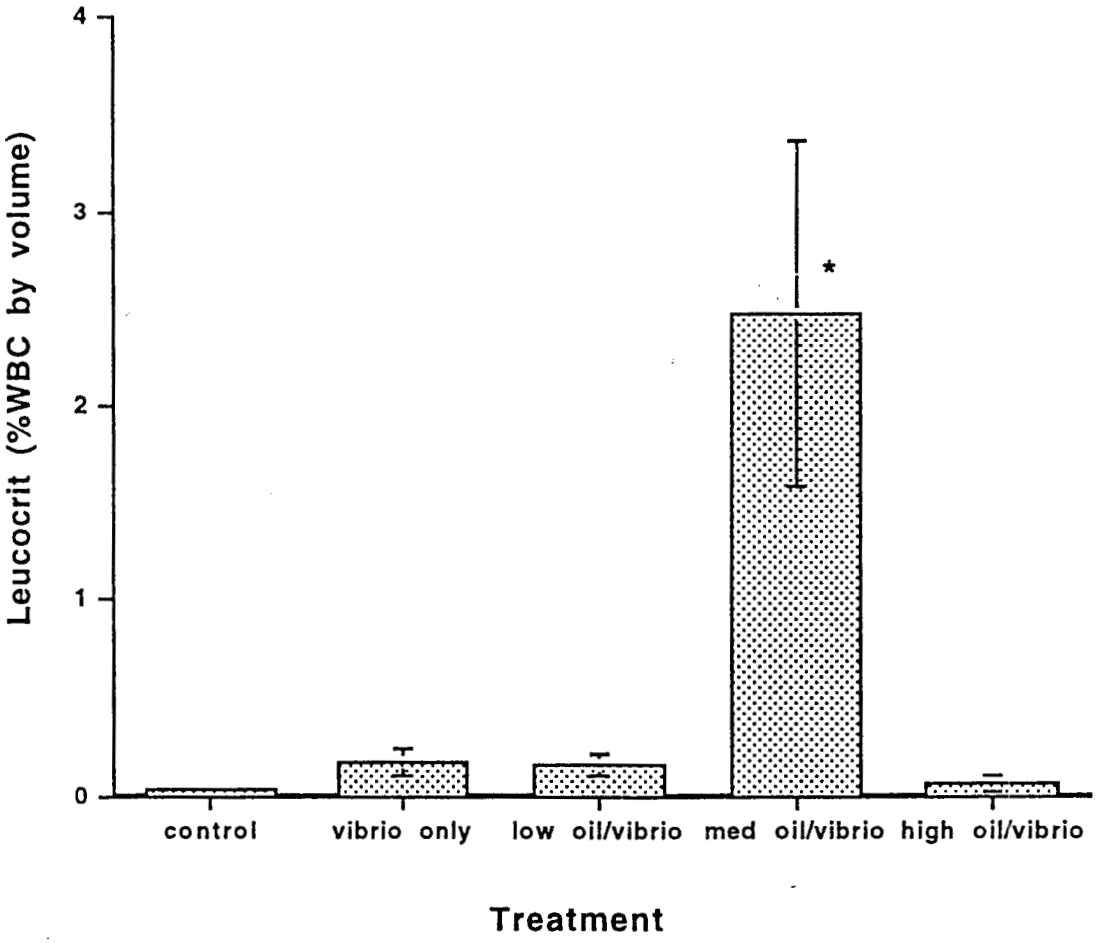


Figure 9. Hematocrit values in juvenile herring exposed to varying concentrations of an OWD of oil and the marine pathogen *Vibrio anguillarum* 6 weeks post infection. Values are means ±SE of ten fish. No significant differences seen at $p<0.05$.

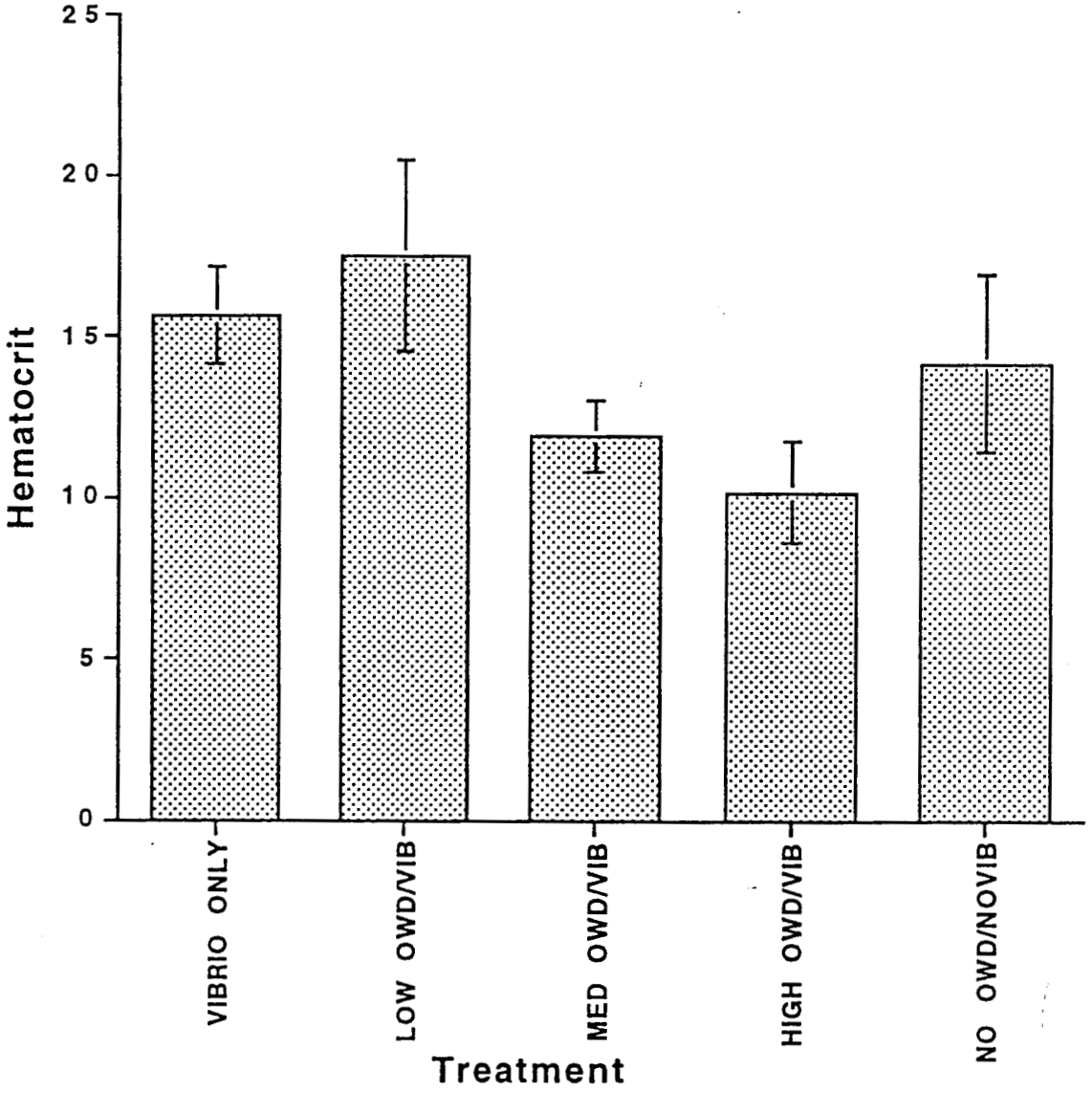
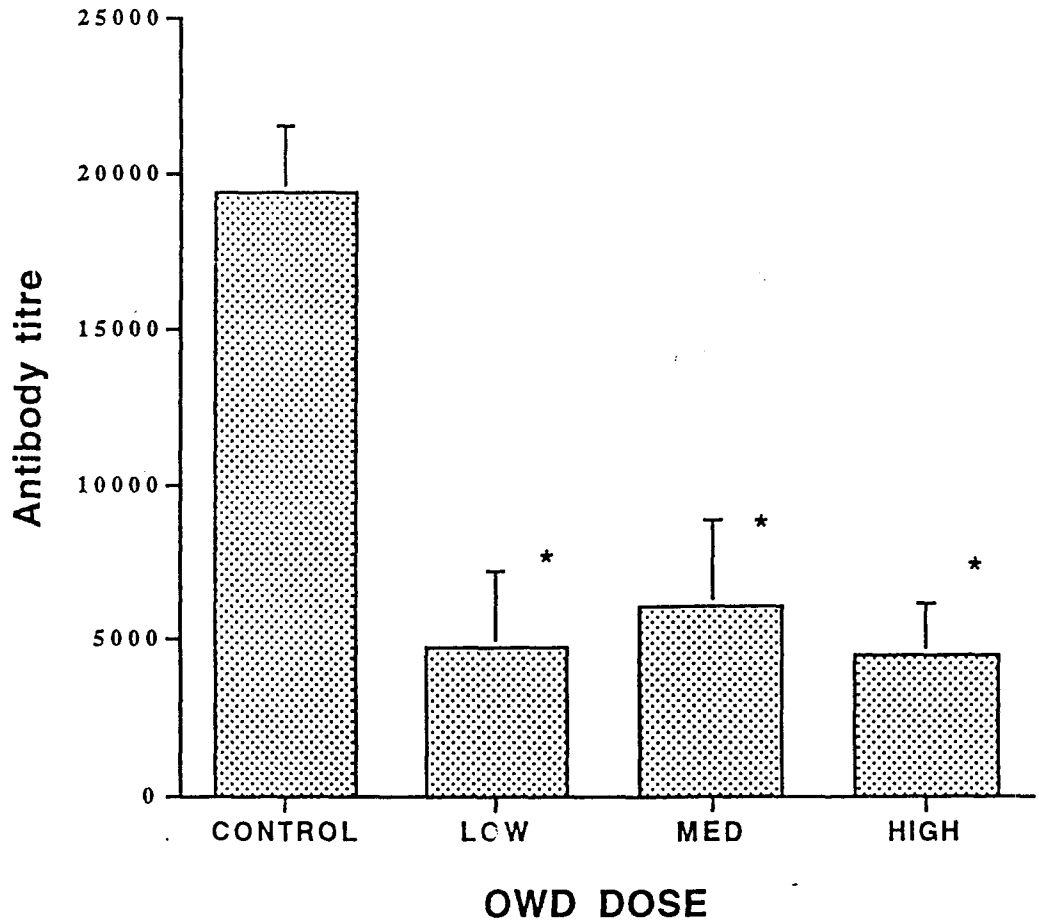


Figure 10. Antibody titers in juvenile herring exposed to varying concentrations of an OWD of oil and the marine pathogen *Vibrio anguillarum* 6 weeks post infection. Values are means ±SE of ten fish. Significant differences seen at $p<0.05$ are denoted with an *.



Objective 6. To determine the effects of oil exposure on the swimming performance of Pacific herring.

Juvenile herring were exposed to OWD to determine effects on swimming performance and recovery of fish from exercise. OWD in this experiment resulted in significant mortalities which are shown in Figure 11. The highest percentage of herring died in the highest OWD concentration. The effects of sublethal OWD exposure on herring swimming performance as measure by critical swimming speed (Ucrit) can be seen in Figure 12. Significant effects on fish swimming were seen only in the medium and high OWD concentrations at 96 h. Due to the high mortalities in fish which had been forced to swim, a separate experiment was set up to determine OWD effects on the recovery of herring from 'burst's swimming'. Figure 13 shows the effects of exercise on hematocrit, plasma lactate and [Cl-] and [Na+]. Exposure of

fish to high doses of OWD caused more disturbance in most parameters measured and appeared to inhibit a return of these values to 'normal' following exercise.

Figure 11. Percent mortality of juvenile herring exposed to varying concentrations of an OWD of oil. Values are means of replicate tanks. Total number of fish in each tank at start of an experiment was approximately 100.

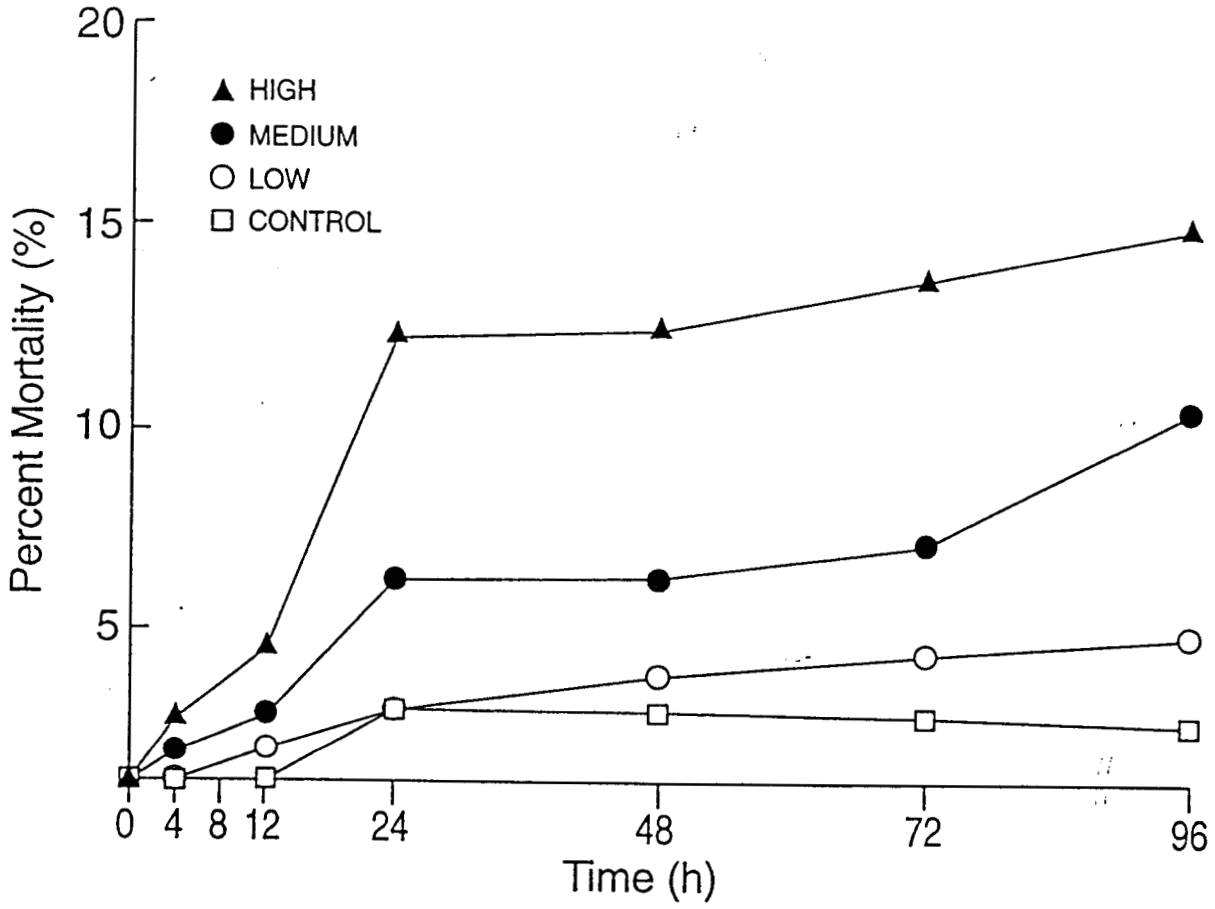


Figure 12. Effects of varying concentrations of an OWD of oil on the critical swimming speed of juvenile herring following a 24 or 96 h exposure. Values are means±SE of three fish. Significant differences from control fish at $p<0.05$ are denoted by *.

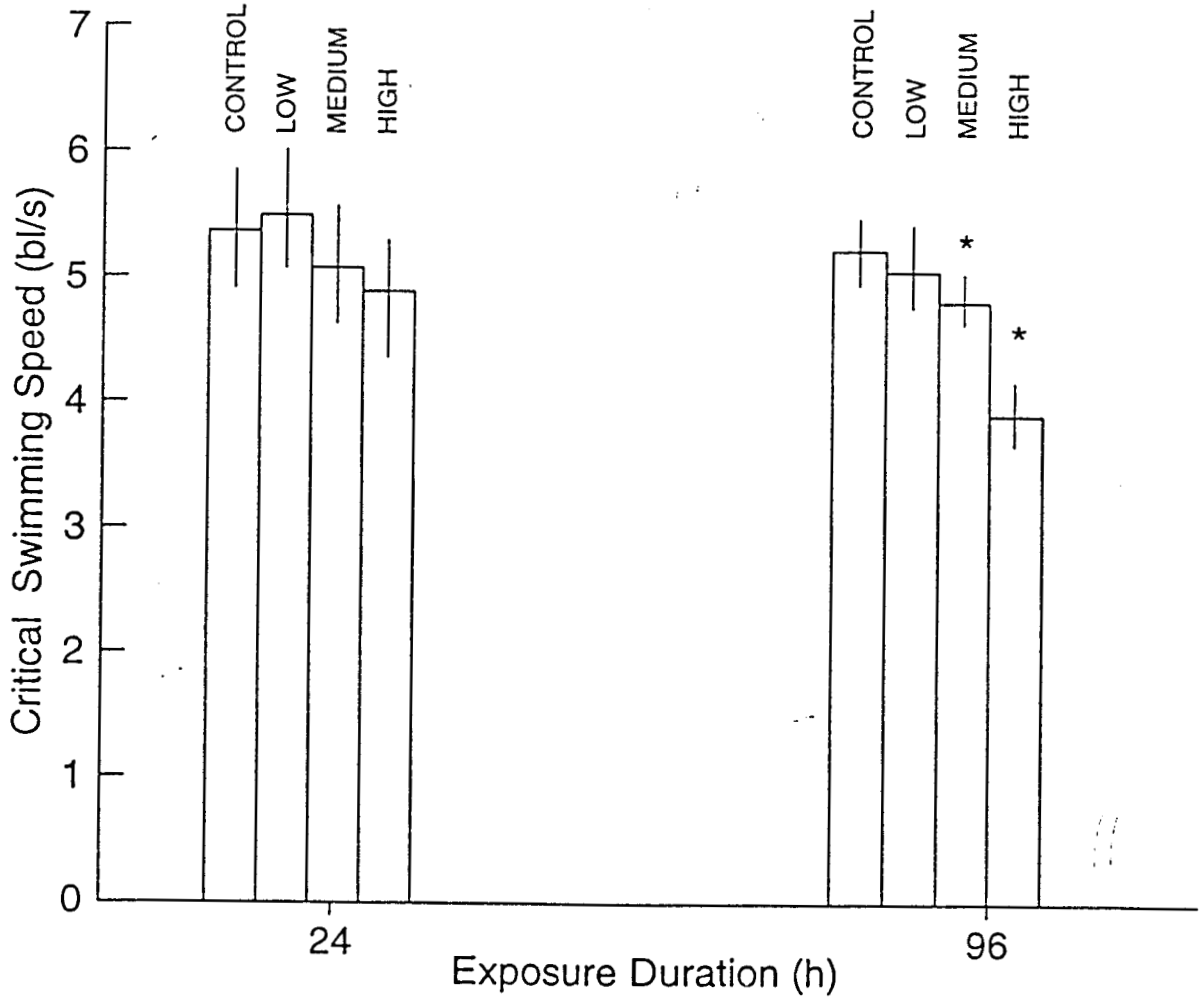
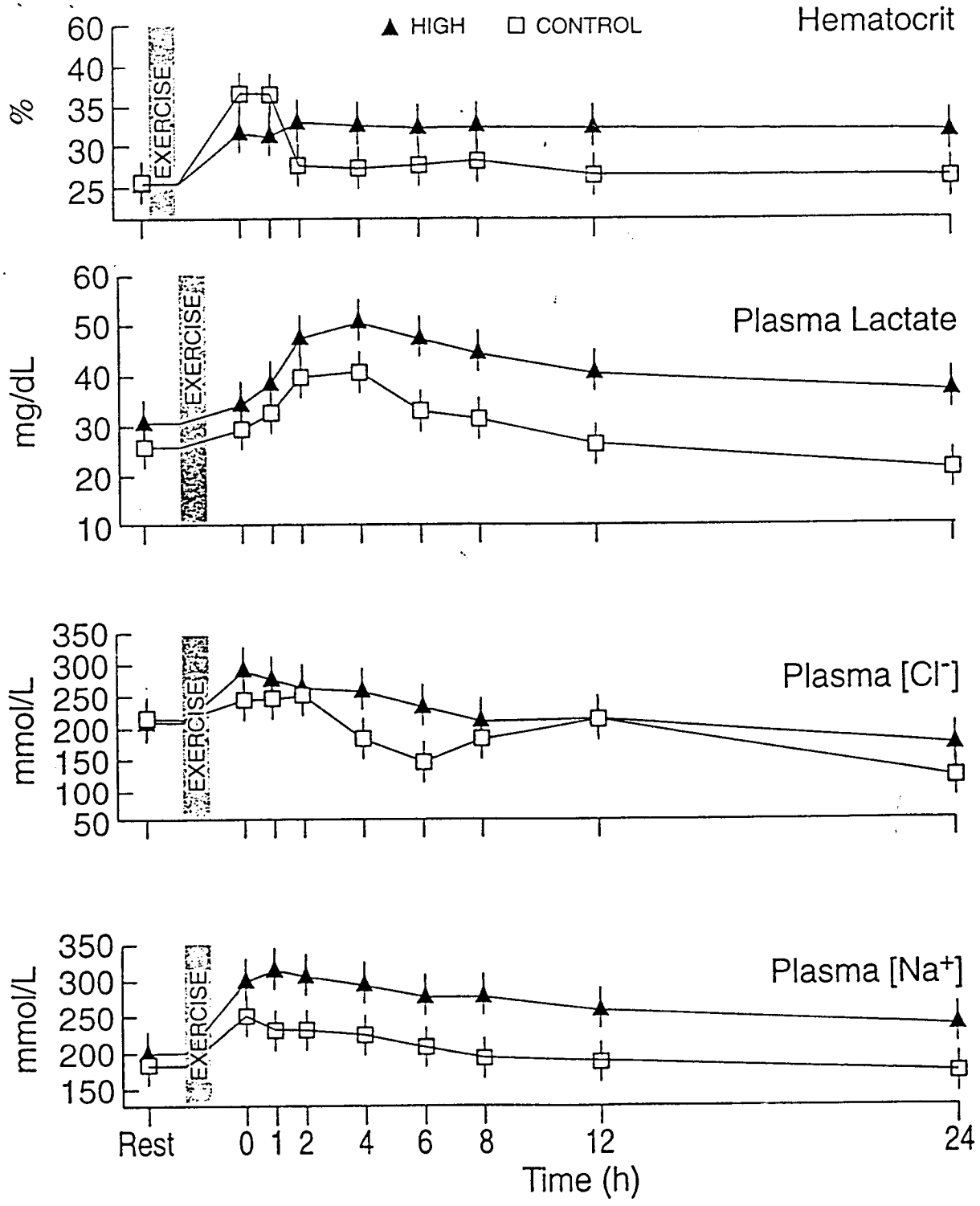


Figure 13. Effects of varying concentrations of an OWD of oil on juvenile herring hematocrit, plasma lactate, plasma chloride and sodium concentrations following 6 minutes of "burst' swimming. Values are means±SE of three fish. Significant differences from control fish at $p<0.05$ 24 h following exercise is found in hematocrit, plasma lactate and plasma [Na^+].



Objective 7. To determine the extent of immunological and biochemical modification in Pacific herring following an infection with VHSV.

Herring exposed to VHSV were sampled for changes in immunological and biochemical changes as described previously to determine its effects and to determine how long it took fish to recover from a VHSV infection. Due to the large data base and samples collected in 1996, these data have yet to be analyzed statistically and so conclusions regarding the experiment would be premature. The results will be included in the 1997 annual report.

Objective 8. To determine the extent of immunological and biochemical modification and recovery in Pacific herring following an infection with ITP.

The disease state of adult wild herring were determined for ITP and were 10/30 fish sampled. Therefore, this proposed experiment for 1996 was rescheduled when fish were available in the fall of 1997. However, results from these scheduled experiments with ITP exposure were performed in preliminary 1995 experiments already and reported in the annual report for 1996.

Discussion

Support services for Section I of the research (field studies) were successful. Statistical analysis and conclusions regarding the results of the differential white blood cell counts and presence/absence of viral erythrocytic necrosis from both Sitka Sound and Prince William Sound are discussed in Section I (field study-Dr. Marty) of this annual report. Considerable effort was placed into the separation and identification of creatine phosphokinase isozymes in herring plasma. This data would have identified the likely mechanism of action of ITP infection and had potential as a biomonitoring tool in field sampling. However, CPK isozymes could not be resolved with standard techniques used for both mammals and rainbow trout in our laboratory. It is possible that the freeze/thaw regimes employed in plasma chemistry analysis (the first priority) resulted in the degradation of this enzyme. It is also possible that this enzyme in herring cannot be separated by standard techniques which have not been previously employed in a marine species. Due to these results, the CPK isoform analysis has been removed from the list of parameters to be measured in 1997 and proposed 1998 field samples.

The size and availability of SPF herring has in some circumstances necessitated the use of wild herring in Sections II (Dr. Kocan) and III of this project. Adult and juvenile wild herring caught at Barkely Sound on Vancouver Island have shown to be negative with respect to the VHSV virus, although the adult population shows low ITP prevalence. Therefore, studies with ITP will only occur with juvenile fish. Our pilot study comparing several aspects of herring hematology in these two groups of fish show that they are similar and respond similarly to oil exposure. This data is useful in that a 'backup' pool of fish exist if SPF fish are limited. Proper controls will ensure that all experiments and data gathered using wild fish will be relevant to the goals of the project.

Our studies on the effects of oil on herring biochemistry are interesting in that they illustrate that age may be an important factor in the toxicity of oil to herring. Adult herring were found to be negligibly affected in terms of their biochemistry when dosed for 6 days with oil. However, similar studies with juveniles shows a typical 'stress' response in that fish exposed to oil exhibited increases in hematocrit, plasma cortisol, plasma lactate and plasma glucose. It has been suggested that only those stimuli that cause 'fright, discomfort or pain' are capable of eliciting this response (Thomas 1990). Corticosteroid hormone release after exposure to stressors often triggers a variety of biochemical and physiological responses called secondary stress responses. Typical secondary stress responses elicited by increases in plasma cortisol levels include hyperglycemia, depletion of tissue glycogen reserves, catabolism of muscle protein and altered blood levels of protein and cholesterol. Of these, sublethal exposure to oil evoked hyperlacticemia and hyperglycemia. These values returned to preexposure values by 96 h, a response typical of many pollutants as the fish begins to compensate biochemically to the stressor. This may be one explanation for the lack of a measured response in adult fish exposed for 6 days. It is possible that the biochemical alterations elicited by oil had returned to normal by the sampling period. However, it should be noted that even if these parameters are returned to normal, other tertiary effects on herring fitness could become evident with longer sublethal exposures. Clearly, the sublethal exposure duration could have direct implication in the selection of biochemical parameters used as indicators of aquatic contamination and should be the focus of further research. Recommendations for biochemical parameters (and their relevant time frames) that have potential as biomonitoring tools of population recovery will be made in the final report once all data has been synthesized.

It is known that components of oil have the potential to be immunosuppresive. Experiments with juvenile herring exposed to oil show that sublethal concentrations can have effects on the immune system. A high natural variability in several parameters between fish necessitates high sample sizes to ensure proper statistical analysis. Exposures were longterm in nature in these experiments to better mimic conditions which may have occurred during the EVOS. Several important aspects of the herring immune system were affected by oil exposure and included alterations in the population of circulating white blood cells, the ability of macrophages in phagocytosis foreign particles, the activity of lysozyme and the ability of fish to mount an immune response to a model pathogen. These results seem significant biologically, however, when fish were exposed to oil and subsequently challenged with a bacterial pathogen, the results indicated that the fish dosed with the highest concentration of oil suffered the fewest mortalities of all groups including fish which had not been exposed to oil at all. The disease challenge is the definitive test of the immune system and is a test of the functionality of the whole integrated system. It is unclear at this time why this group survived the challenge better than the other groups when parts of the immune system measured were impaired. Further challenges are planned in 1997 and 1998 with *Vibrio* and other potential pathogens which should shed light on this finding.

Results from the swimming experiments have revealed important information on the effects of oil exposure on the survival and fitness of herring. At very low

concentrations (<100 ppb), significant mortalities occurred in herring indicating that oil can be acutely toxic to juvenile herring at concentrations lower than previously reported in the literature. These results indicate that exposure of juveniles to low concentrations following the spill may have resulted in direct mortality and contributed to population declines. Furthermore, at sublethal concentrations of oil, ecologically significant effects were seen in juveniles with respect to their ability to swim. Appropriate swimming performance is paramount to herring as it is imperative in foraging for food, escaping predators, migration etc. Low concentrations of hydrocarbons were found to reduce swimming performance in these fish and increased mortalities in fish which underwent strenuous exercise. Due to these increased mortalities in swum fish, it was decided to investigate the effects of oil on the ability of fish to recover from exercise. Significant alterations in biochemistry are known to occur during 'burst' swimming in fish, changes which return to preexercise values shortly after. An inhibition of exercise recovery as seen in this study would severely inhibit a fish's ability to exercise repeatedly, adding to the reduction in fitness seen in a reduced swimming performance. Reductions in swimming performance may have also contributed to increased fish mortality following the spill. Future studies are focused on the effects of VHSV and ITP on fish swimming and the ability of fish to recover from these stressors and oil exposure, information which is important in determining the overall health of fish.

The examination of the effects of the two pathogens on aspects of herring fitness have indicated that both VHSV and ITP have effects on the immune systems and biochemistry of herring. These results are not surprising, as infection often results in such changes. It is important, however, to determine the time course of these changes and to determine when fish have recovered from VHSV or ITP infections. These studies are began in 1996 and continue in 1997.

Conclusions

The results of this study indicate that exposure of herring to oil, Viral Hemorrhagic Septicemia Virus or *Ichthyophonus hoferi* can alter various aspects of herring fitness. Significant alterations in biochemistry, immunology and swimming performance were noted in fish exposed to an oil-water dispersion of crude oil, however, it appears that age-dependent factors may modify responses as does exposure time and duration of responses to the stressor. It was also shown that Viral hemorrhagic Septicemia Virus and *Ichthyophonus hoferi* can alter aspects of herring hematology and immunology. These results begin to explain the possible roles of oil, VHSV and ITP in herring population declines. Data gathered in this study details information which will be useful to an informed herring management plan and herring fishery practices.

Acknowledgments

The authors wish to acknowledge the work of Dr. Richard Kocan (author of Section II of this report) for his efforts at obtaining a SPF stock of PWS herring and wild Washington State herring which are essential for the successful completion of this project. We would like to also thank him for the maintenance of fish stocks and access to both VHSV and ITP.

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