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 95320S
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.

G.D. Marty & D.E. Hinton
Dept. of Anatomy, Physiology, and Cell Biology
School of Veterinary Medicine, University of California
Davis, CA 95616

R.M. Kocan & M.L. Landolt
School of Fisheries
Box 355100
University of Washington
Seattle, WA 98195

J.R. Winton
National Biological Service
7500 Sandpoint Way NE
Seattle, WA 98115

C.J. Kennedy & A.P. Farrell
Simon Fraser University
Dept. of Biological Science
Burnaby, B.C. V5A 1S6

for:

Alaska Department of Fish and Game
Habitat and Restoration Division
333 Raspberry Road
Anchorage, Alaska 99518

April 1996

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Restoration Project 95320S
Annual Report

Study History: In 1993 there was a sudden and unexplained disappearance of approximately 60% (80K tons) of spawning herring in Prince William Sound, and the following year another 30K tons disappeared. An emergency project (94320 S) was authorized under emergency conditions in April 1994, and was not a part of a work plan. This emergency project was funded 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 which also exhibited external ulcers and cutaneous hemorrhaging. 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 (ADF&G) issued an RFP to study these declines in herring and to determine the involvement of the two above mentioned diseases. A detailed work plan was written as three components: 1) Field surveys (University of California, Davis); 2) Controlled laboratory infections (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: Field studies begun in 1993-1994 with the examination of herring from Prince William Sound (PWS) continued in 1995 in both Sitka Sound (SS) and PWS. Severe focal skin reddening or ulcers were >7 times more prevalent in spawning fish from PWS than from SS in 1995, less than its prevalence in 1994. The prevalence of *I. hoferi* was the same in PWS in 1995 as in 1994 and did not differ from SS in 1995. No VHSV was isolated from spawning fish from PWS or SS in 1995 but was isolated from >6% of pre-spawning PWS fish. External examinations concluded that spawning PWS herring were healthier in 1995 than in 1994, but those examined from SS in 1995 were in worse condition. Laboratory studies commenced to determine if VHSV and *Ichthyophonus* were pathogenic for Pacific herring by exposing laboratory reared specific pathogen-free (SPF) juvenile fish to pure cultures of both. Despite rapid death following exposure, they did not demonstrate the ulcers and extensive cutaneous hemorrhaging frequently observed in wild herring. *I. hoferi* was cultured for transmission and pathogenicity studies. Studies with SPF fish continue to determine the pathogenicity of this organism and its effect on herring recovery in PWS.

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

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

Citation:

Marty, G.D., D.E. Hinton, R.M. Kocan, M.L. Landolt, J.R. Winton, C.J. Kennedy, and A.P. Farrell. 1996. Investigations of disease factors affecting declines of Pacific herring populations in Prince William Sound, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 95320S), Alaska Department of Fish and Game, Habitat and Restoration Division, Anchorage, Alaska.

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Executive Summary

Overview of Disease Sections I, II, & III.

Restoration project 95320-S consists of three separate but interrelated components which are headed by Dr. Gary Marty of the University of California, Davis.(Component I), Dr. Richard Kocan, University of Washington (Component II) and Dr. Christopher Kennedy of Simon Fraser University, British Columbia (Component III). In depth summaries of each component can be found at the beginning of each section of this Annual Report.

Component I involves field surveys of morbidity and mortality in herring in Prince William Sound (PWS) and Sitka Sound (SS). Data generated from these studies gives managers the ability to track disease effects and recovery, thus enabling them to make more informed management decisions. This information also gives other investigators data on how natural infections and epizootic behave under natural conditions, thus allowing them to design meaningful controlled pathogenicity studies.

This study began in 1993-1994 with the examination of herring from PWS only. In 1995 herring were examined from Sitka and Prince William Sound and it was found that severe focal skin reddening or ulcers were more 7 times more prevalent in spawning fish from PWS than from SS. However, the prevalence of these conditions in PWS in 1995 was less than that found in 1994. The prevalence of I. hoferi was the same in PWS in 1995 as in 1994 and was not different from what was seen in SS in 1995. No VHSV was isolated from spawning fish from PWS or SS in 1995 but it was isolated from >6% of pre spawning fish from PWS. It was concluded that based on external examination, spawning PWS herring were healthier in 1995 than in 1994, but were in worse condition than spawning fish examined from SS in 1995. (A more detailed summary of this study will appear as an addendum).

Component II is establishing the pathogenicity of VHSV & *Ichthyophonus hoferi* in wild and laboratory-reared pathogen-free herring under controlled conditions, as well as documenting the influence of stress factors on the course and outcome of diseases in herring. This is accomplished by infecting fish with known levels of pathogens and comparing the data with that obtained from PWS and other habitat utilized by Pacific herring. By superimposing physical, chemical and biological stresses on fish infected with various pathogens, it is possible to determine the role played by stressors on the course of disease in both immune and non-immune individuals.

This project began in 1995 and focused mainly on the growth and husbandry of Pacific herring in the laboratory. It was necessary to establish a pathogen-free population of herring in order to evaluate the pathogenicity of individual organisms. The year was also dedicated to establishing *Ichthyophonus* cultures in the laboratory and evaluating methods for its cultivation and transfer to wild herring. Both of these objectives were met, and studies began on the exposure of these fish to various concentrations of VHSV as well as *I. hoferi*. Koch's Postulates were fulfilled, establishing with certainty that VHSV was a primary pathogen of juvenile herring and that it was capable of causing massive mortality in these young fish. Studies on juvenile wild fish were also undertaken and it was found that wild herring in Puget Sound were already infected with VHSV by the time they were 5 months old, and that capture and confinement of these fish resulted in massive epizootics killing 60% of the population. The survivors however, were solidly immune to re infection by VHSV, even at concentrations of virus in excess of 100 times the minimum lethal dose. *I. hoferi*, on the other hand, showed equivocal results when injected into wild herring of all ages, but was definitely lethal to pathogen-free juvenile herring.

Component III uses blood and tissues obtained from fish examined in Components I and II. These samples are used to determine hematological parameters for healthy and diseased herring as well as the status of physiological changes observed in infected fish relative to their uninfected counterparts. Measurements such as hematocrit (red blood cell levels), leucocrit (white blood cell levels), enzymes related to tissue damage and reproductive hormones are being investigated to determine if they could be used as biomarkers for identifying disease conditions in the field.

Because Component III depends heavily on Component II for specific pathogen-free herring, its major activity began late in 1995 when experiments were initiated with SPF herring which had reached 6 months of age. However, during the Summer of 1995 samples were collected from wild 5-month-old herring that had just suffered an epizootic of VHS as well as wild herring that had been fed *Ichthyophonus*. These blood samples showed that fish became extremely anemic, and the anemia persisted for several months after mortality ceased and virus could no longer be isolated from surviving individuals. Since none of the fish showed any signs of infection by *Ichthyophonus*, it was concluded that the observed response was to the VHSV. In addition, changes in differential white blood cell counts and elevated leucocrits (white blood cell volume) following the epizootics, indicated that the fish were responding with a cellular immune response. Most of the fish surviving the epizootic were observed to have bright green livers. Although it is not know if this condition is related to the VHS virus or some other parasite resent in the wild fish, it was presumably due to stasis of bile flow.

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 Spring 1995

Restoration Project 95320S
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.

Gary D. Marty[1]
Corrine R. Davis[1]
Ellen F. Freiberg[1]
David E. Hinton[1]
Theodore R. Meyers[2]
John Wilcock[3]

[1]Department of Anatomy, Physiology, and Cell Biology
School of Veterinary Medicine, University of California
Davis, CA 95616

Alaska Department of Fish and Game
Commercial Fisheries Management and Development Division
[2]P.O. Box 25526
Juneau, AK 99802
[3]P.O. Box 669Cordova, AK 99574

April 1996

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 Spring 1995

Restoration Project 95320S
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, Disease Impacts on PWS Herring Populations, the subject of this annual 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 bacteriology, virology, hematology, and histopathology, viral hemorrhagic septicemia virus and the fungus *Ichthyophonus hoferi* contributed most to population decline in 1994. Study was expanded in 1995 to include examination of prespawning fish and fish from a reference site, Sitka Sound. In 1995, moderate or severe focal skin reddening or ulcers were more prevalent in spawning fish from Prince William Sound (2.8%) than in spawning fish from Sitka Sound (1.3%), but lesion prevalence at both sites was less than in spawning fish from Prince William Sound in 1994 (8.4%). *Ichthyophonus* prevalence in Prince William Sound spawning fish in 1995 (29%) was the same as in 1994 and no different from Sitka Sound in 1995 (26%). At both sites in 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 Sound, 31% vs. 22%). Also, viral hemorrhagic septicemia virus was not isolated from any spawning fish in Prince William Sound or Sitka Sound, but VHSV was isolated from 6.2% of prespawning fish from Prince William Sound.

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:

Marty, G.D., C.R. Davis, T.R. Meyers, J. Wilcock, E.F. Freiberg, and D.E. Hinton. 1996. 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 spring 1995, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 95320S), University of California, Davis, California.

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Executive Summary

Introduction

Before the 1993 spawning season, 120,000 tons of Pacific herring (*Clupea pallasi*) were forecast to arrive on the spawning grounds of Prince William Sound (PWS); only 20,000 tons appeared. Viral hemorrhagic septicemia virus (VHSV), but no other significant pathogens, was isolated from those herring. In 1994, when only 17,000 tons of an expected 27,000 tons of herring appeared on the spawning grounds, this study was initiated to investigate the cause of herring population decline. Given the history of VHSV isolation in 1993, the study was designed to investigate the role of infectious disease in the PWS Pacific herring population, with primary emphasis on the role of VHSV. Study of spawning fish in PWS in 1994 revealed two significant diseases: 1) 5% of the fish had VHSV, and the virus was commonly associated with external lesions described by biologists and fishers in 1993; and 2) 29% of the fish were infected with the fungus *Ichthyophonus hoferi*, and the fungus was associated with severe internal lesions (external lesions were less common).

We concluded that disease was significantly contributing to population decline, but background disease prevalence and the role of reproductive stage were unknown. Therefore, field study was expanded in 1995 to include prespawning samples and samples from a reference site, Sitka Sound (SS). Like PWS, the Pacific herring population in SS has a dominant 1988 year class, but the SS population continues to support commercial and subsistence fishing. Although a recently closed pulp mill that contributed an unknown amount of pollution into SS, there was no history of a large oil spill. Prince William Sound Pacific herring fisheries were severely curtailed in 1993, and were never opened in 1994, 1995, or 1996. This section reports the findings from field disease studies in 1995. Laboratory study was initiated to explore details of VHSV and *Ichthyophonus* infections under controlled conditions; results from the laboratory component are reported in sections II and III.

Objectives

Field study had three objectives: 1) determine the relation among VHSV, *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 VHSV more severe during a given reproductive stage? Does a history of previous oil exposure correlate with prevalence and severity of disease?]; and 3) determine the impact of disease on population size and structure of herring [Are fish of a particular year class more likely to be diseased than other year classes?].

Methods

To determine disease status, Pacific herring were sampled in 3 groups: 1) 240 spawning fish from SS (March 25-29, 1995); 2) 80 immature or prespawning fish from PWS (April 11,

1995); and 3) 180 fish with ripe gonads (spawning) from PWS (April 24-27, 1995). Because of administrative delays in the Request for Proposal process used for this project, funding was approved too late to obtain prespawning samples as proposed in SS. All 500 fish were subjected to complete necropsy, which included weight, standard length, and scoring of external lesions. Samples were taken for histopathology (12 organs), virus isolation (head kidney and spleen), age determination (from scales), and plasma chemistry analysis (total protein, albumin, osmolality, cholesterol, glucose, total bilirubin, 5 enzymes, and 5 electrolytes). Also, white blood cell differential counts were made from blood smears, and smears were examined for the only other known herring virus, viral erythrocytic necrosis (all were negative). For histopathology, tissues were coded for blind study, and lesions were ranked on a four-point scale as none (0), mild (1), moderate (2), or severe (3). In all fish with severe external lesions, kidney was cultured for bacteria (all were negative). As a measure of immune function, an assay for Pacific herring immunoglobulin (IgM) was developed and used on plasma samples from 476 fish. To determine significance, results were analyzed statistically. Parasites or other pathogens that were not statistically associated with lesions or alterations in blood values were not considered pathologically significant.

Results

Moderate or severe focal skin reddening or ulcers were more prevalent in spawning fish from PWS (2.8%) than in spawning fish from SS (1.3%), but prevalence of these lesions in 1995 was less than in spawning fish from PWS in 1994 (8.4%). For internal lesions, *Ichthyophonus* prevalence in PWS spawning fish (29%) was the same as in 1994 and no different from the *Ichthyophonus* prevalence in spawning fish from SS (26%). At both sites in 1995, prevalence of *Ichthyophonus* among all fish was higher in 7-year-old fish than in 2- and 3-year-old fish (PWS, 35% vs. 9.6%; SS, 31% vs. 22%), but age differences were significant only for PWS fish. Viral hemorrhagic septicemia virus was not isolated from any spawning fish in PWS or SS, but VHSV was isolated from 6.2% of prespawning fish from PWS.

Prevalence of 4 subtle inflammatory lesions was significantly greater (chi-square test, $P \leq 0.05$) in spawning fish from PWS than SS: 1) white blood cells around vessels in skeletal muscle (PWS = 77%, SS =65 %); 2) foci of white blood cells in the liver (PWS = 81%, SS = 49%); 3) foci of white blood cells in the heart (PWS = 32%, SS = 24%); and 4) foci of white blood cells in the stomach (PWS = 13%, SS = 1.7%).

Parasite prevalence in spawning fish from PWS and SS was significantly different for 3 parasites in 1995: 1) testicular coccidian *Eimeria sardinae* (PWS = 85%, SS = 66%); 2) renal intraductal protozoan (PWS = 11%, SS = 3.8%); and 3) branchial *Epitheliocystis*, (PWS = 15%, SS = 25%). Differences in prevalence of other common parasites were not significantly different: 1) peritoneal larval Anisakidae (PWS = 100%, SS = 99%); 2) unclassified intestinal coccidian (PWS = 95%, SS = 91%); 3) hepatic coccidian *Goussia clupearum* (PWS = 73%, SS = 71%); 4) gall bladder myxosporean *Ceratomyxa auerbachi* (PWS = 39%, SS = 32%); 4) renal intraductal

myxosporean *Ortholinea orientalis* (PWS = 29%, SS = 20%); 6) gastric trematodes (PWS = 12%, SS = 10%); and 7) branchial monogenetic trematodes (PWS and SS = 11%).

Discussion

After the first year of study in 1994, we considered whether the oil spill could have been linked to disease outbreak 4 years later. Fish that were hatched or were yearlings in 1989 at the time of the spill (1988 and 1989 year classes) might have incurred permanent damage to their ability to fight disease (i.e., irreversible immunosuppression), so we examined the association of lesions with age. Several lesions were significantly associated with age (e.g., pigmented macrophage aggregates), but nearly all these lesions were more severe in older fish (i.e., fish hatched before 1988). Also, among VHSV, *Ichthyophonus*, and 10 other common parasites, none were more prevalent in the 1988 and 1989 year classes than in the entire sampled population. Therefore, the weight of evidence suggested that the disease outbreak in PWS was not a result of permanent immune suppression caused by hydrocarbon exposure when fish were larvae or yearlings. In 1995, prevalence of *Ichthyophonus* in PWS was significantly higher in the 1988 year class. However, because *Ichthyophonus* prevalence was also relatively higher in the 1988 year class in SS, increased *Ichthyophonus* prevalence in these fish was more likely a function of advanced age and not directly related to previous oil exposure.

None of the diseases in Pacific herring in PWS were unique. In our previous studies of Pacific herring in PWS and Auke Bay, Alaska (1989-1993), *Ichthyophonus* was present, but its prevalence was never more than 15%. Published studies of acute population declines in Atlantic herring (*Clupea harengus*) reported that *Ichthyophonus* was the primary cause. In those studies, *Ichthyophonus* prevalence was ≥25%. The first studies of VHSV in Pacific herring in PWS (1993) were done on samples pooled from several fish, so prevalence could not be determined. Since then, VHSV has been isolated from Pacific herring sampled elsewhere in Alaska, British Columbia, and Washington. Only one other report of disease-associated population decline of Pacific herring has been published; the cause was not determined, but clinical and pathological findings were more similar to our findings associated with VHSV than with *Ichthyophonus*. We established that VHSV and *Ichthyophonus* were associated with several lesions in 1994, and their role as a primary invaders has been confirmed on disease-free fish reared in the laboratory (see section II, this report).

The weight of evidence implicates VHSV as the major cause of disease and mortality in PWS Pacific herring in 1993. By 1994, both VHSV and *Ichthyophonus* were important. And in 1995, the role of VHSV was decreasing (e.g., no virus was isolated from spawning fish) while *Ichthyophonus* continued to be a major cause of disease. Based on external examination, spawning PWS Pacific herring were in better condition in 1995 than in 1994, but PWS spawning fish were in worse condition than SS spawning fish. By comparison, *Ichthyophonus* prevalence was similar among all three sample groups. The significance of VHSV in prespawning PWS samples is unknown, but prespawning samples were collected from PWS and SS in 1996 to better understand the dynamics of VHSV before spawning. For *Ichthyophonus*, high prevalence in

spawning fish is reason for concern for both the PWS and SS populations. However, because *Ichthyophonus* cases were concentrated among 7-year-old fish, and 2- and 3-year-old fish were infected at historically endemic levels, the *Ichthyophonus* outbreak may also be subsiding. If younger fish remain relatively free of *Ichthyophonus*, disease is less likely to impair their continued recruitment into the fishery in 1996 and 1997. As with VHSV, the second year of sampling in SS is critical for determining the role of *Ichthyophonus* in population size in PWS and SS. Also, ongoing laboratory studies with *Ichthyophonus* and VHSV (University of Washington and Simon Fraser University) will begin to determine methods of transmission, routes of infection, incubation period, and other variables that might provide clues to how these diseases can be managed in the future.

Conclusions

Disease was probably the primary force driving population decline in 1993, 1994, and 1995, but its role in 1995 was primarily limited to older fish. Preliminary evidence from analysis of fall 1995 samples indicates that the proportion of 7-year-olds in the Pacific herring populations of both PWS and SS decreased by up to 80% between spring and fall samples. This provides evidence that the high rate of *Ichthyophonus* infection in older fish was significant. No other variables—food availability, predation, water temperature, currents, or recruitment—were needed to explain the significant decline in the 1988 year class. These other variables may be more important during population recovery, but if young fish cannot escape disease transmission, disease will continue to limit recovery. Pacific herring populations in PWS were not healthy in 1994 or 1995, and until Pacific herring populations recover, continued study of herring disease is recommended.

Introduction

When the *Exxon Valdez* oil spill occurred in March, 1989, the biomass of spawning Pacific herring in PWS 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 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. In Press). 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, its role in population decline could not be determined. By 1994, spawning biomass declined to the lowest level ($1.5x10^7$ 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 (SS). The Pacific herring population in SS 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 SS. 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 2 different sites. At the reference site (SS), 240 fish in spawning condition were captured by purse seine or cast net (March 25 - 29, 1995), transported to a heated garage in Sitka, and subjected to complete necropsy. In Prince William Sound (PWS), fish in 2 different spawning stages were captured and subjected to complete necropsy: 1) 80 fish with immature or unripe gonads (prespawning) were sampled from Zaikof Bay on Montague Island on April 11,1995, on board the *Auklet*; and 2) 180 fish with ripe gonads (spawning) were sampled from Rocky Bay on Montague Island from April 24 through 27, 1995, on board the *R/V Montague*. All PWS fish were captured using purse seines. Because of

administrative delays in the Request for Proposal process used for this project, funding was approved too late to obtain prespawning samples as proposed in SS.

Each fish was assigned a necropsy number, 95HER-1 through 95HER-500, in order of necropsy. After capture in SS, fish were held in plastic containers filled with about 60 L of seawater (7 fish per container) for no more than 4 hours before necropsy. After capture in PWS, fish were held in plastic containers filled with about 300 L of seawater for no more than 3.5 hours before necropsy. 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. 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 chilled on ice for analysis by Med Veterinary Laboratory, Concord, California, within 72 h of sampling. A 100-µL plasma aliquot from each fish was frozen separately for IgM analysis; details of assay development are described elsewhere (p. I-10 below). Too little plasma was collected from 24 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 in a Monarch-plus analyzer from Instrumentation Laboratories (IL®) that was calibrated and run at a stabilized 25° C. Plasma was analyzed for total protein (biuret method), albumin (bromocresol green method), and CO_2 (enzymatic method); IL® substrates were used to analyze calcium, cholesterol, glucose, phosphorus, total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine phosphokinase (CPK); Sigma® substrates were used to analyze gamma glutamyltransferase (GGT); ion selective electrodes were used to analyze sodium, potassium, and chloride.

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; all were negative). Also, differential leukocyte counts were done by counting approximately 100 white blood cells in randomly selected fields (mean = 48 fields per slide).

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

We had proposed to split the 180-fish PWS spawning sample into 160 randomly selected fish and 20 fish selected based on gross lesions. Because only 3 fish were found with sufficiently severe lesions to meet the special selection criteria, 177 spawning fish were selected at random from PWS. At both SS and PWS, nearly all fish in the spawning sample had gonads in spawning condition. Some of the fish in SS were actively spawning when captured, whereas fish in Rocky bay, PWS, did not start active spawning until the last day of sampling. Among fish in the PWS prespawning sample, 44 of 80 (55%) were reproductively immature. The proposal called for sampling only mature fish, but to maintain a random selection process, immature fish were also

necropsied. Samples from these immature fish were useful for determining the distribution of disease in young Pacific herring.

Histopathology

Tissues from 500 herring were sent to the Aquatic Toxicology Laboratory, University of California, Davis, and randomly assigned a histopathology number (95H5-1 through 95H5-500) 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 SS vs. PWS) were calculated.

IgM detection in Plasma: ELISA Development and Assay

In April 1995, prespawning Pacific herring from PWS were caught in purse seines and held in 300 L salt water for a maximum of 2 hours. Fish were anesthetized in tricaine methane sulfonate (Finquel®) and blood was collected from the caudal vein into heparinized syringes. Samples from 100 fish were pooled into 50-mL plastic tubes and centrifuged at 3000 rpm for 10 minutes. Plasma was harvested and held at −20° C for transport to the laboratory, then stored at −70° C until processed.

Heparinized pooled plasma was thawed and centrifuged at 4° C at 10,000 *g* for 30 minutes to remove precipitated fibrin. The supernatant was placed in Spectra-por 2 molecularporous membrane tubing and suspended in 4 L of 5 mM Tris dialysis buffer, pH 7.4 at 4° C. The dialysis solution was changed every 12 hours for 4 days. The precipitate (euglobulin fraction) was collected via centrifugation at 4° C. The pellet was washed twice in 5 mM Tris buffer and resuspended in 0.1 M Tris HCL plus 1 M NaCl, pH 8.6. The solution was desalted (Sephadex PD10 column). Protein content was determined via the Lowry (Folin:Ciocalteu) method.

The euglobulin fraction was analyzed by sodium dodecyl-sulfonate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and nonreduced conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) and stained with Coomassie blue. Membranes were also incubated with specific polyclonal rabbit anti-herring euglobulin antibodies (described below). Internal sequence analysis

was carried out on the heavy chain portion after SDS-PAGE and electrophoretic transfer to PVDF membranes (Protein Structure Laboratory, UC Davis).

The euglobulin fraction was deglycosylated using a modification of a previously described protocol (Mattes and Steiner 1978). Euglobulin (2.5 mg) was eluted in 0.04 M sodium acetate buffer, pH 5.4, 0.1 M NaCl using a Sephadex PD-10 column. An equal volume of 20 mM sodium periodate was added to the eluent and incubated at room temperature for one hour. Glycerol was added to 50% total volume and the mixture was dialyzed, using Spectra-por 2 tubing, against 4 L PBS, pH 7.4, overnight at 4° C. The tubing content was centrifuged at 14,000 *g* for 2 minutes. The supernatant was collected, divided into aliquots and stored at −20 or −70 ° C. Protein concentration was determined via Lowry (Folin-Ciocalteau) method.

A single male New Zealand White rabbit received an initial inoculation of 400 µg deglycosylated euglobulin in complete Freund's adjuvant. A second inoculation of 400 µg deglycosylated euglobulin in incomplete Freund's adjuvant was administered 4 weeks later. Titered by ELISA, rabbit antibodies to the herring euglobulin were detected at a dilution of 1:51,200 in rabbit serum collected 10 days after the second inoculation. Polyclonal rabbit immunoglobulin was purified by methods previously described (Mckinney and Parkinson 1987). A portion of the purified antibody was biotinylated with NHS-LC-Biotin according to manufacturer instructions (Pierce).

The ELISA was optimized using serial dilutions of rabbit-anti herring immunoglobulin run on wells coated with 1 or 4 µg/mL euglobulin and streptavidin at 1:1000 or 1:2000. With an optimal rabbit immunoglobulin dilution of 1:2500 (1.2 µg/mL) and streptavidin dilution of 1:1000, serial dilutions of euglobulin were run against several dilutions of biotinylated antibody. From these data, the linear portion of the standard curve was determined and a single concentration (2.5 µg/mL) of euglobulin was then run against serial dilutions of biotinylated antibody to determine the optimal dilution.

To determine IgM concentrations in plasma samples, 96-well immunoassay plates (Falcon®, Pro-bind™) were coated with 50 µL/well rabbit anti-herring euglobulin antibody diluted 1:2500 (1µg/mL) in 50 mM bicarbonate/carbonate buffer, pH 9.6 and incubated overnight at 4° C or 2 hours at 37° C. Following incubation, plates were washed 5 times with Tween TBS (TTBS: 50 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl and 1 mL Tween-20 per L) and shaken vigorously to remove excess fluid. Wells were blocked with 5% nonfat dry milk in TTBS and incubated at 37° C for 60 minutes. Plates were then washed 5 times. Fifty (50) µL of test sample were added to each well and incubated at 37° C for 60 minutes. Plates were again washed 5 times, followed by addition of 50 µL/well of biotinylated rabbit anti-herring euglobulin antibody diluted 1:800 in blocking buffer. After a 60 minute incubation at 37° C, the plates were again washed, followed by addition of 50 µL/well peroxidase-streptavidin diluted 1:1000 in blocking buffer. After a 30 minute incubation at 37° C, plates were washed 9 times. A TMB substrate solution (100 µL/well) was added and incubated 20 to 40 minutes at 37°C. The reaction was stopped with addition of 50 µL/well 1 M H_2SO_4. Each plate contained reference blanks and

standard curves in triplicate. The standard curve was constructed using serial dilutions of euglobulin of known protein concentration. Plates were read at 450 nm on a Ceres 900 Hdi plate reader (Bio-Tek) and interpreted with Kineti Calc™ version 2.12 software using end point curvilinear regression.

The purified euglobulin fraction consisted primarily of a protein with a molecular weight of approximately 800 kD. Under reducing conditions, this protein broke down into a 70,000 kD heavy chain and 3 light chains in the 20-23 kD range (characterization in progress). Amino acid sequencing from the N-terminal end was not possible due to blocking; however, internal sequences had significant sequence homology with Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), tarpon (*Elops* sp.), and sheep Ig heavy chain constant regions.

The intraplate coefficient of variation for the ELISA (same plate, same day, same sample: 5 standard dilutions run in triplicate on a total of 60 plates) ranged from 0.26% to 36.31% (mean = 10.90%). The interplate coefficient of variation (different plate, different day, same sample: 9 samples run in triplicate on 3 different days) ranged from 3.66% to 32.60% (mean = 16.09%). The lower limit of detection for the ELISA was 0.039 µg IgM/mL. The test can be performed in 4-6 hours.

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 SS. Because unripe prespawning fish were not captured in Sitka, and prespawning samples from PWS had many immature fish not present in spawning samples, statistical analysis usually focused on comparing spawning fish from SS (n = 240) and PWS (n = 180). 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 SS 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 SS 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. Data from SS and PWS were combined for this analysis.

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 SS. 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. Fish from SS were grouped into three categories for analysis: 2 or 3 years old, 4 to 6 years old, or ≥7 years old. Fish from PWS were grouped into 4 categories for analysis: 2 or 3 years old, 4 to 6 years old, 7 years old, or ≥8 years old. 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 $P \leq 0.05$ and highly significant when $P \leq 0.01$. Use of the term "prevalence" refers to the sample prevalence.

Results

Note on the contents the results section in this report: 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 1995. Variables considered included spawning stage, site of capture, and year of capture, with special emphasis on organisms and lesions likely to result in population level effects.

Revisions from 1994 Study (94320S)

In the 1994 annual synthesis report, the intestinal trematode category incorrectly included intestinal cestodes. Also, the testicular coccidian *Eimeria sardinae* was not scored. Slides were re-examined and scores corrected. Among 211 intestines examined, corrected scores for intestinal trematodes include 204 none, 6 mild, and 0 moderate or severe (overall prevalence = 2.9%); corrected scores for intestinal cestodes include 205 none, 1 mild, 4 moderate, and 0 severe

(overall prevalence = 2.4%). Among 102 testes that were reexamined, coccidians were scored as 44 none, 52 mild, 6 moderate, and 0 severe (overall prevalence in males = 57%). Statistical analysis was not redone for this report, but will be part of the final synthesis report.

Necropsy Findings and External Gross Lesions - 1995 Samples

Difference in mean age of the 3 sample groups were highly significant (Table 1). Prespawning samples from PWS included 55% immature fish, whereas immature fish were rare in spawning samples from PWS and SS. Spawning fish from PWS had relatively more 7-yr-olds than did the SS samples, so mean age of spawning PWS fish was greater that SS fish (Table 1). Significant differences in other morphometric necropsy variables were consistent with age differences between the sample groups. Hold time was significantly longer for fish from SS because of the extra time it took to transport SS fish to a garage before necropsy (necropsy was done on a ship at the capture site in PWS). Hold time never exceeded 4.1 hr at either site. Fin base reddening was significantly associated with increased hold time and plasma CO_2, particularly in PWS samples.

Prevalence of external lesions was greater in spawning fish from PWS than in spawning fish from SS. The summary external lesion score was moderate or severe in 46 of 180 (26%) of PWS fish but only 16 of 240 (6.7%) SS fish. Further, focal skin reddening in spawning fish was moderate or severe in 2.8% of spawning PWS fish but only 1.1% of SS fish. By comparison, 8.3% of spawning fish from PWS in 1994 had moderate or severe focal skin reddening.

The relation between external lesions and plasma chemistry values was often highly significant, but significant variables in SS and PWS fish were never the same (Table 2). However, a few plasma chemistry values were consistently related to external lesions when spawning samples from PWS in 1994 were compared to spawning samples from SS in 1995; examples include: 1) increased caudal fin fraying vs. increased osmolality and calcium; and 2) increased iris reddening vs. increased calcium, osmolality, potassium, and phosphorus.

External lesions, especially caudal fin fraying, were significantly associated with several gross and microscopic lesions (Table 3). Interestingly, increased focal skin reddening was not associated with any external lesions but was significantly associated with several *Ichthyophonus* scores and with the intestinal coccidian (*Goussia* sp?). As scores for external lesions increased, scores for associated lesions usually increased. Two exceptions were (1) lower hepatic lipidosis scores were were associated with higher scores for caudal fin fraying, and (2) lower scores for the intestinal coccidian (*Goussia* sp?) were associated with higher scores for diffuse skin reddening. Also, scores for diffuse skin reddening were significantly lower when gonads were full; that is, scores for diffuse skin reddening tended to increase as spawning progressed.

Microscopic Lesions - 1995 Samples

Ichthyophonus

Overall prevalence of *Ichthyophonus* was the same in spawning samples from PWS in 1994 (29%, 62 of 212) and 1995 (29%, 52 of 180), and prevalence of *Ichthyophonus* in 1995 SS samples (26%, 62 of 240) was not significantly different. In 1994, *Ichthyophonus* prevalence among 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%); therefore, the 1988 year class made up a disproportionally high proportion of the *Ichthyophonus* in PWS in 1995 (Figure 2). Interestingly, the 1988 year class also made up a disproportionally high proportion of the *Ichthyophonus* cases in SS in 1995 (Figure 3), but difference were not significant.

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 were nearly the same as reported last year (Marty et al. 1995). The 1 exception was scoring of *Ichthyophonus* in the stomach, where 2 scores were given: 1) ICH - the standard *Ichthyophonus* as described in 1994 required that organisms were in the sections before a score was given; and 2) ICH+ - includes all standard ICH scores, but also includes scores for stomachs that contained characteristic ~200-µm-diameter rims of inactive fibroblasts and mature collagen, but no *Ichthyophonus* organisms. Differences between ICH and ICH+ scores in SS were highly significant (10.4 vs. 18.8%, chi-square test, 2×2 contingency table), whereas differences between ICH and ICH+ scores in PWS spawning fish were not significant (15.6 vs. 18.9%).

Although the overall *Ichthyophonus* prevalence in spawning fish was 29% in PWS and 26% in SS, in no single organ was *Ichthyophonus* prevalence > 22% in PWS or > 17% in SS (Figure 4). As in 1994, the skin and skeletal muscle had the highest prevalence of mild *Ichthyophonus* cases, whereas cases in the heart and kidney were more likely to be severe (Figure 4). A sum-*Ichthyophonus* (sumICH) score was calculated for each fish by adding the individual *Ichthyophonus* scores from all 10 organs for that particular fish as described in the 1994 report (Marty et al. 1995). In 1995, the highest sumICH scores were 23 for PWS and 19 for SS.

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 SS *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.

VHSV

Only 5 fish were positive for VHSV, all from prespawning samples in PWS. None of the 180 spawning samples from PWS or the 240 spawning samples from SS were positive for VHSV. The 5 positive fish included a 3-yr-old immature female, 3- and 4-yr-old mature females, a 4-yr-old mature male, and a 7-yr-old mature male. Interestingly, mature fish comprised only 45% of the prespawning samples but were 80% of the VHSV+ cases among prespawning samples. All 5 VHSV+ cases were cultured on the EPC cell line, whereas only one case was also positive using the PHE cell line. Among external lesions, all 5 VHSV+ fish had mild to severe caudal fin fraying, but only 2 fish had caudal fin reddening (fish #s 241 and 288), only 1 fish had fin base reddening (mild in fish #288), only 1 fish had focal skin reddening (mild in fish #241), and no VHSV+ fish had diffuse skin reddening. Hence, 3 VHSV+ fish had no external lesions other than mild caudal fin fraying.

Because only 5 fish were VHSV+, statistical analysis against plasma chemistries was not done. However, true mean IgM values for VHSV+ fish (416 mg/mL) were less than true mean IgM values for other PWS prespawning fish (460 mg/mL), and considerably less than true mean values for spawning fish from SS (719 mg/mL) and PWS (975 mg/mL). Also, lactate levels–an indication of metabolic acidosis–were greater in VHSV+ fish than in other PWS prespawning fish (92 vs. 61 mmol/dL), even though CO_2 levels–an indication of respiratory acidosis–were less in VHSV+ fish than in other PWS prespawning fish (5.2 vs. 6.2 mmol/L).

Gender-associated Lesions

Site differences in oocyte morphology were minimal (Table 4), but comparisons were made difficult by the severity of artifact in ripe eggs. Unripe eggs in prespawning fish sectioned well, whereas ripe eggs often were lost during processing or sectioning; hence, more unripe than ripe eggs were retained on each section and available to be counted. Ruptured and atretic follicle were rare in ovaries from all samples groups (Table 4).

Gonads had several differences in lesion prevalence. As in 1994, lesions more frequent in ovaries included hyalinization of vessel walls and pigmented macrophage aggregates, and prevalence of both lesions in 1995 (about 40%; Table 2) was slightly less than in 1994 (about 60%). In 1994, granulomatous inflammation was more common in testes than in ovaries, but neither occurred at greater than 9% prevalence. By comparison, granulomatous inflammation in 1995 was more common than in 1994, more common in ovaries than testes, prevalence was greater in fish from PWS than SS, and prevalence among males was greater in prespawning mature fish than in spawning fish (Table 2). As in 1994, *Ichthyophonus* was rare in either gonad (Table 2, Figure 4).

Gender differences spawning fish were significant for several nongonadal lesions (Table 5). Prevalence of *Ichthyophonus* in heart, liver, and skeletal muscle was significantly greater in females than males. Gall bladder myxosporeans (*Ceratomyxa auerbachi*) were significantly more

frequent in females (as they were in 1994). Females were more likely than males to have hepatic lipidoses or severe depletion of pancreatic zymogen granules, but females were less likely to have hepatic glycogen depletion. Livers were not stained specifically for glycogen or lipid, so some lipid vacuoles in females might have been mistakenly classified as glycogen. Males had a significantly greater frequency of vacuolated proximal renal tubular epithelial cells.

In addition to these lesions, females were heavier, longer, and had greater gonad and liver weights than males (Table 7). Gender differences were significant for several plasma chemistries and hematology variables (Table 7). Females had significantly lower albumin, calcium, cholesterol, CO_2, glucose, packed cell volume, potassium, sodium, % thrombocytes, and total protein. Females had significantly higher ALP and % thrombocytes. Gender differences were not significant for other plasma chemistries.

Iris reddening

Most lesions significantly associated with iris reddening were more prevalent in fish with mild or moderate iris reddening than in fish with no iris reddening (Table 5). For example, caudal fin fraying and renal congestion were significantly more frequent in fish with mild or moderate iris reddening. By comparison, splenic congestion was more likely in fish with no iris reddening (Table 5).

Intraperitoneal Herring Worms (Anisakidae)

All 240 Pacific herring sampled from PWS contained larval parasites of the family Anisakidae within their peritoneal cavities, and 236 of 238 examined from SS had intraperitoneal Anisakidae. 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). Unlike in 1994, gender differences in numbers of herring worms were were not significant. But as in 1994, increasing severity of several lesions were significantly associated with increased numbers of herring worms (Table 6). For example, increased numbers of herring worms were associated with increased severity of gastric eosinophilic leukocytes, gastric serositis, and gastric trematodes. By comparison, decreased numbers of herring worms were associated with increased severity of hepatic pigmented macrophage aggregates and focal, intimal, hyperplasia of intestinal arterioles. Middle-aged fish tended to have more peritoneal Anisakidae, and age differences were significant.

Other Lesions and Potential Pathogens

No significant bacterial pathogens were isolated, and none of the blood smears had evidence of VEN. Spawning Pacific herring had 11 other common parasites, most of which were

associated with few lesions. These parasites, roughly in descending order of prevalence (spawning fish), included:

1) intestinal coccidian *Goussia*? sp. (PWS = 95%, SS = 91%);
2) hepatic coccidian *Goussia* [*Eimeria*] *clupearum* (PWS = 73%, SS = 71%);
3) testicular coccidian *Eimeria sardinae* (PWS = 85%, SS = 66%);
4) gall bladder myxosporean *Ceratomyxa auerbachi* (PWS = 39%, SS = 32%)
5) renal intraductal myxosporean *Ortholinea orientalis* (PWS = 29%, SS = 20%);
6) branchial *Epitheliocystis* (PWS = 15%, SS = 25%);
7) renal intraductal protozoan (PWS = 11%, SS = 3.8%);
8) gastric trematodes (PWS = 12%, SS = 10%);
9) branchial monogenetic trematodes (PWS and SS = 11%);
10) intestinal trematodes, e.g., *Lecithaster gibbosus* (PWS = 8.9%, SS = 2.1%); and
11) intestinal cestodes, e.g., *Nybelinia surmenicola* (PWS = 3.3%, SS = 2.5%).

Site differences in parasite prevalence were statistically significant (chi-square test) only for the testicular coccidian, renal intraductal protozoan, and branchial *Epitheliocystis*. Branchial ciliated protozoa (e.g., *Trichodina*) were rare in 1995 spawning samples from both PWS (1.3%) and SS (1.1%) compared to 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 fish had moderate infestation (i.e., >15 organisms per 400× field), and infestation was not associated with alterations in plasma chemistry values. By comparison, in PWS in 1995, 12% of the spawning fish and 31% of the prespawning fish had moderate infestations (Table 2); prevalence of moderate infestations in SS in 1995 was only 2%. Severity of intestinal coccidians in 1995 was significantly related to greater CPK and AST values in PWS fish but not in SS fish.

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. Increased lesion scores were significantly related to decreased age (Table 2), and for SS fish only, increased lesion scores were associated with decreased plasma glucose levels.

As in 1994, diagnosis of the renal intraductal myxosporean *Ortholinea orientalis* was less sensitive by histopathology. This difference was particularly obvious in spawning fish from PWS, where the touch preparation prevalence (28%) was significantly higher than the prevalence determined by histopathology (7%). Overall prevalence was significantly greater in spawning fish from PWS in 1995 than in 1994 (29 vs. 19%, chi-square test). Also, as histopathology scores for *Ortholinea orientalis* in spawning PWS fish increased, values for PCV, neutrophils, and basophils significantly increased (Table 2). Plasma chemistry values for other methods of diagnosis and for SS were either not significant or significant trends were nonlinear.

Prevalence of an unidentified protozoan (or myxosporean?) in the archinephric duct (kidney) was significantly higher in spawning fish from PWS than SS, but prevalence in PWS spawning fish in 1994 and 1995 was not significantly different. Infestation was significantly related to changes in plasma chemistries in SS but not in PWS (Table 2).

The gall bladder sometimes contained large numbers of the myxosporean *Ceratomyxa auerbachi*. Prevalence among 1995 spawning fish from PWS and SS were not significantly different, but 1995 prevalence in PWS (39%) was significantly greater than 1994 prevalence (19%). The gall bladder is small (3 - 5 mm in diameter) and difficult to include in all sections, but a higher frequency of gall bladders were examined in 1995 (97%) than in 1994 (66%). Hence, the increased prevalence in 1995 may have resulted from better sampling in 1995. Severe infestations sometimes had mild mononuclear inflammation in the lamina propria, but infestations were not significantly associated with changes in plasma chemistries.

Prevalence of 4 subtle inflammatory lesions was significantly greater (chi-square test, $P \leq 0.05$) in spawning fish from PWS than SS: 1) perivascular leukocytes in skeletal muscle (PWS = 77%, SS =65 %); 2) focal parenchymal leukocytes in the liver (PWS = 81%, SS = 49%); 3) focal parenchymal leukocytes in the heart (PWS = 32%, SS = 24%); and 4) foci of white blood cells in the submucosa and muscularis of the stomach (PWS = 13%, SS = 1.7%).

Age-associated Changes

The most consistent age-related change, as in 1994, 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). External lesion scores that significantly increased with age included fin base reddening (PWS only) and iris reddening (SS only). Microscopic lesion scores that were significantly related to increased age included (Table 2): 1) gill lamellar telangiectasis; 2) renal tubular epithelial vacuolation (SS only); 3) hepatic pericholangial leukocytes (PWS only); 4) hepatocellular lipidosis (SS only); 5) perivascular leukocytes in skeletal muscle connective tissue (SS only); 6) focal, intimal arteriolar hyperplasia in intestinal cecal vessels; and 7) splenic ellipsoid hyalinization (both sites). Microscopic lesion scores that were significantly related to decreased age included: 1) focal parenchymal leukocytes in the heart (PWS only); 2) intestinal cecal Anisakidae (SS only); 3) interstitial renal congestion (SS only); and 4) splenic vascular congestion (SS only).

Increased severity scores for several parasites were significantly related to decreased age among spawning fish in 1995 (ANOVA): 1) intestinal trematodes (PWS only; prevalence was too low in SS for statistical analysis); 2) hepatic coccidian *Goussia clupearum* (both sites); 3) branchial *Epitheliocystis* (PWS only); and 4) testicular coccidian *Eimeria sardinae* (both sites). By comparison, severity scores for common parasites *Ichthyophonus* and *Ortholinea orientalis* were not significantly related to age.

Comparing age-related prevalence of common parasites using the chi-square test for homogeneity sometimes produced results markedly different than those produced through one-way ANOVA of severity scores. The best example is with the hepatic coccidian *Eimeria sardinae*, in which decreasing age was significantly related to severity score (ANOVA), but prevalence was unrelated to age (chi-square test, $P > 0.40$). Histopathology prevalence for the renal intraductal myxosporean *Ortholinea orientalis* was significantly associated with age in SS but not PWS (chi-square test), but age-related differences were not significant for either site using ANOVA. Overall prevalences of *Ichthyophonus* (Figure 3) and branchial *Epitheliocystis* (Figure 5) were significantly associated with age in PWS but not SS. Prevalence of the testicular coccidian *Eimeria sardinae* was significantly associated with age at both sites and with both analysis techniques (Figure 6), where prevalence was generally highest in the youngest mature fish but was lower in immature fish and 7-year-old fish. In 1994, the gall bladder myxosporean *Ceratomyxa auerbachi* was significantly more common in older fish and the renal intraductal protozoan (or myxosporean?) was significantly more common in younger fish; however, neither parasite had significant age-related differences in prevalence in 1995 (both sites).

Several values for plasma chemistries, weights, and length were significantly related to age (Table 8). Values that significantly increased with age at both sites included IgM, all weights (body, gonad, and liver), and length. Only CPK significantly decreased with age at both sites. 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 (Table 2). For example, increased frequency of neutrophils was significantly related to *Ichthyophonus* scores in PWS samples from the brain, heart, intestine, liver, spleen, stomach (only the ICH+ lesion score), and skin/skeletal muscle. By comparison, in SS samples, neutrophil frequency was increased with *Ichthyophonus* only in skin/skeletal muscle, but basophil frequency was increased with *Ichthyophonus* scores in gill, liver, and stomach (both ICH and ICH+ lesion scores). Basophil frequency was never significantly increased with *Ichthyophonus* in PWS samples. Increased frequency of neutrophils was significantly related to increased scores for branchial hematopoietic cells in PWS but not SS samples (Table 2).

Lymphocyte frequency significantly increased with scores for several lesions in in PWS but not in SS (Table 2); examples include: 1) hepatic coccidian *Eimeria sardinae*; 2) intestinal foreign body granuloma; 3) intestinal mesenteric steatitis; and 4) focal parenchymal leukocytes in the liver. Note that the frequency of lymphocytes was significantly less in samples from SS than in samples from PWS, regardless of gender (Table 7). Splenic granulomatous inflammation was the only lesion for which lymphocyte frequency significantly increased in SS samples. Lymphocyte

frequency significantly decreased for both PWS and SS samples for only 2 lesions: interstitial renal congestion and pancreatic zymogen granule depletion.

Significant changes in thrombocyte frequency were uncommon, and when they occurred, they were usually matched by an opposite change in frequency of another white blood cell. For example, in SS samples with intestinal steatitis, thrombocyte frequency significantly decreased while frequency of lymphocytes, neutrophils, basophils, and eosinophils significantly increased. Eosinophil frequency did significantly change for any lesions other than intestinal steatitis. Only 3 monocytes were identified among all 500 smears examined; therefore, statistical analysis was not useful for comparing monocyte frequency.

Plasma chemistries

As hold time increased, increases in plasma potassium were highly significant at both sites (Table 9). The increase in plasma CO_2 with increased hold time was more highly correlated in PWS than in SS (Table 9), but CO_2 in SS samples was significantly higher than PWS samples, regardless of hold time (Table 7). Increased plasma lactate and decreased plasma glucose were significantly correlated with hold time for samples from SS but not PWS, and SS lactate levels were significantly higher than PWS lactate levels regardless of hold time (Tables 7 and 9). Changes in several other plasma chemistries were not as significant in relation to hold time ($|r| < 0.25$). A complicating factor was that SS fish had commenced spawning whereas PWS fish had not. Therefore, many of the marginally significant changes might have been related to spawning condition rather than hold time.

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 1). Values for AST were significantly greater in samples from SS than from PWS (Table 7). Variability of ALP was intermediate, but several lesions were significantly related to increased ALP values (e.g., intestinal trematodes and gastric *Ichthyophonus*, both in PWS samples; Table 2). Values for ALP were significantly greater in females than males, and significantly greater in samples from SS than from PWS (Table 7). Variability of ALT and GGT was minimal and measured values, except for one fish, were never greater than 18 U/L.

Albumin and total protein were unusually low when compared to published values for other species (McDonald and Milligan 1992), but 1995 values were comparable to 1994 values, except that 1995 values for albumin were higher than in 1994. Both albumin and total protein were significantly higher in males than females, but IgM levels were not significantly different by gender (Table 7). Albumin and total protein were significantly higher in SS than in PWS, but IgM levels in samples from PWS were significantly higher than samples from SS (Table 7).

Annual Trends in Spawning Biomass and Pathogen Prevalence

Sample prevalence of *Ichthyophonus* in this study was 1.5 to 10 times that of years before 1994 (Table 10). When only scores for liver, kidney, and spleen are considered, *Ichthyophonus* prevalence in 1994 and 1995 (23 and 24%) was no longer significantly different from sample prevalence in 1989 and 1990 (13 and 15%), but was significantly higher than all other samples (chi-square test, 2×2 contingency tables). By comparison, prevalence of *Goussia clupearum* has remained fairly constant between 41 and 63% for most years, but 1995 prevalence in SS and PWS was the highest recorded. Based only on histopathology, the prevalence of *Ortholinea orientalis* prevalence seemed to be higher in 1991 than in 1994 or 1995 (Table 9). The slight increase in *Ortholinea orientalis* prevalence in spawning fish from PWS in this study (28%) was probably at least partly due to increased efficiency of diagnosis when touch preparations were examined; prevalence data before 1994 were derived from histopathology only. Prevalence of VHSV was lower in spawning fish from PWS in 1995 than in 1994, but prevalence was not determined before 1994 because appropriate tissues were not examined.

Discussion

Note on the contents the discussion section in this report: The annual report for project 94320-S (Marty et al. 1995) contains 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 1995. Variables, considered included spawning stage, 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 1995 (Figure 1). However, *Ichthyophonus* seems to have been the cause of unexpected high mortality of 7-year-old fish in both PWS and SS 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. Preliminary evidence from fall 1995 samples indicates that the population of 7-year-olds (1988 year class) in both SS and PWS was significantly lower than in spring 1995, and the prevalence of *Ichthyophonus* in the remaining fish was only about 60% of spring levels. Because no other pathogens were significantly more common in older fish, *Ichthyophonus* seems the most likely cause of differential mortality in these 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 along the Scandinavian coasts was attributed to *Ichthyophonus* (Lang 1992). 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.

Among fish infected with *Ichthyophonus* in PWS and SS, the general pattern and distribution of lesions were very similar except in the stomach. The stomachs of fish from SS were much more likely to contain empty foreign-body type granulomas (i.e., no organisms in the granulomas; scored as ICH+). Although the stomach ICH+ prevalence was identical for PWS and SS (19%), prevalence of stomachs with *Ichthyophonus* was 16% in PWS but only 10% in SS. This may indicate that SS fish were better able to mount an effective inflammatory reaction against *Ichthyophonus*. Alternatively, the granulomas may have been caused by another organism or foreign substance not affecting PWS fish.

Ichthyophonus infections in both sites in 1995 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. Infection with *Ichthyophonus* in 1995 was less commonly related to changes in plasma CPK than in 1994, particularly in PWS samples, although AST was about equally effective both years. Both enzymes were consistently good markers of *Ichthyophonus* in 1995 samples from SS. The role of CPK in *Ichthyophonus* infections will be further defined by laboratory determination of CPK isozymes as part of this project (96162; section III, biochemistry and physiology) during the next fiscal year.

VHSV

The lack of VHSV in spawning samples from both PWS and SS 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 relatively stable population size from 1994 to 1995. Significance of the 5 prespawning VHSV isolates is unknown. Because prespawning fish were difficult to catch, the sample may not have been representative of the entire population. Schools of fish observed on sonar were mostly too deep for the purse seine, so the net seemed to only "skim" fish off the top of the school. Alternatively, it may be that a small fraction of Pacific herring normally express VHSV just before final gonadal maturation. Unpublished observations of VHSV isolates from other Alaskan waters by T.R. Meyers support this hypothesis. Analysis of prespawning samples captured in 1996 should provide more evidence for the role of VHSV in prespawning samples. [In PWS prespawning 1996 samples, 50% were sampled at night, after the fish rose towards the surface; hence, the samples seemed more representative of the schools from which they were caught.]

The biggest difference between VHSV-infected fish in 1995 and 1994 was the lack of external lesions in VHSV+ fish in 1995. Among spawning fish with VHSV in 1994, 9 of 11 had mild to severe fin base reddening, but in 1995 prespawning samples, only 1 of 5 had mild fin base reddening and none were moderate or severe. It may be that VHSV commonly causes external lesions only during spawning, a period of great physiologic stress; at other times, external signs of disease may be minimal.

The reason for increased prevalence of several subtle inflammatory lesions (e.g., focal parenchymal leukocytes in the liver) in fish from PWS than from SS is unknown. These foci may be remnants of VHSV-induced damage. For example, hepatic focal necrosis was associated with VHSV in 1994, and leukocytes would normally be part of the healing process in recovered fish. Alternatively, these foci of leukocytes may be normal in wild fish, and decreased frequency of leukocytes in SS may have reflected their active spawning status (i.e., a result of stress-induced leukocytopenia during spawning). As evidence, the frequency of neutrophils and basophils in blood smears from SS significantly decreased as gonadal fullness decreased (Table 2). 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.

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*. But in 1995, VHSV+ fish had few external lesions, whereas focal skin reddening was significantly associated with *Ichthyophonus* infection. The lists of internal lesions associated with various external lesions (Table 3) provide evidence that external lesions were useful indicators of poor health, but their use for more specific diagnoses seems limited.

Focal skin reddening and diffuse skin reddening are distinct in their pathogenesis. In 1994, both lesions were clumped into focal skin reddening. In 1995, the lesions were separated. Although diffuse skin reddening was relatively uncommon in 1995 samples, affecting about half as many fish as focal skin reddening, the lesions significantly associated with diffuse skin reddening were different from focal skin reddening in all but one case. Focal skin reddening was most often associated with *Ichthyophonus*, whereas diffuse skin reddening was more a marker of spawning stage and infection with coccidians. Removal of diffuse skin reddening from the focal skin reddening score may have been all that was needed to show that *Ichthyophonus* was significantly related to severity of focal skin reddening. The second year of study at both sites (1996) will test the validity of these associations.

As in 1994, mild iris reddening seems to be the normal condition in Pacific herring in spawning condition. Of 10 significantly associated lesions in 1995, 7 were more severe when iris reddening was scored as none than when iris reddening was scored as mild or moderate. There were too few moderate cases to determine if moderate lesions might also be significantly different

from mild lesions. Many of the lesions associated with changes in iris reddening were closely associated with the cardiovascular system.

Other Potential Pathogens

The best candidate for significant pathogens among the other common parasites was the unidentified intestinal coccidian (*Goussia*? sp.), particularly in fish from PWS. Both herring worms (Anisakidae) and the intestinal coccidian occurred in more than 90% of the fish, but only the intestinal coccidian was significantly related to increased plasma chemistries (e.g., CPK and AST) as lesion scores increased. Numbers of herring worms increased with severity of several lesions, but the inflammatory reaction was usually minimal. Fish with moderate cases of the intestinal coccidian were more likely to have severe depletion of pancreatic zymogen granules, indicating that affected fish were in relatively poor condition. However, the causal relation between the intestinal coccidian and poor condition is unknown. Do intestinal coccidians cause poor condition, or are they allowed to multiply when fish are in poor condition? Answering these questions would require controlled laboratory study. Preliminary evidence from fall 1995 samples provides evidence that severity of infestation with the intestinal coccidian decreased over the summer; overall prevalence dropped to about 30% and no fish had moderate lesions. Lack of inflammation against these parasites is further evidence that their contribution to population decline is minimal. As a secondary candidate for significant pathogen, infection with the renal intraductal myxosporean *Ortholinea orientalis* also was related to changes in plasma chemistries, and prevalence in 1995 was slightly greater than in 1994.

Age- and Gender-associated Lesions

Age-related differences in parasite prevalence changed from 1994 to 1995. In 1994, the gall bladder myxosporean *Ceratomyxa auerbachi* was more common in older fish, and the renal intraductal protozoan (or myxosporean) was more common in younger fish, but no parasites were significantly more common in the dominant 1988 year class. By 1995, the gall bladder myxosporean and renal intraductal protozoan were no longer associated with age, but *Ichthyophonus* and the testicular coccidian *Eimeria sardinae* were significantly related to age. *Ichthyophonus* was more common in older fish. However, because the trend in SS was towards higher *Ichthyophonus* prevalence in older fish, age-related differences in *Ichthyophonus* were attributed to ageing changes rather than to latent effects from the oil spill.

Plasma Chemistries

Plasma chemistries were highly sensitive markers of several lesions, reproductive status, and other variables (e.g., age and weight). Because several plasma chemistry changes are significantly related to the process of spawning, many of the significant difference attributed to site of capture probably resulted from slight differences in spawning stage of fish from SS (some had started spawning) and PWS (none had started spawning before sampling). Samples from both sites in 1996 included actively spawning fish and spawned out fish, so site-related differences

in plasma chemistries might not be as distinct in 1996. The significant site-related differences in lactate and CO_2 provided information that was used to alter 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 movement while fish are held from capture to necropsy. The effect of this change on lactate and CO_2 levels will be included in next year's report.

Conclusions

Disease was probably the primary force driving Pacific herring population decline since 1993. No other variables–food availability, predation, water temperature, currents, or recruitment–were needed to explain this significant decline, although these variables may have contributed to conditions that were favorable for initiation of a disease epidemic. This conclusion is based on integration of results from this project, literature review including Meyers et al. (1994), plus information from biologists, fishers, and preliminary laboratory study where both VHSV and *Ichthyophonus* killed Pacific herring in the absence of other diseases. 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 less important as a cause of mortality. If *Ichthyophonus* prevalence continues to decrease, as indicated by fall 1995 samples, and young herring in PWS remain relatively free of disease, then disease should no longer limit population recovery. Continued monitoring of disease in PWS and SS 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, 1995. Note that all spawning fish from both sites were sexually mature, but only 45% (36 of 80) of the PWS "prespawning" fish were sexually mature. Also, gonads from all fish were in ripe, but some fish from Sitka Sound were partly or completely spawned out, whereas no fish from Prince William Sound had spawned before being sampled. 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 ($P \leq 0.05$). 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	79	3.0^{A}	0.2	<0.001*
	PWS	spawning	180	6.6^{C}	0.1	
	SS	spawning	239	5.1^{B}	0.1	
standard length (mm)	PWS	prespawning	80	170.0^{A}	3.1	<0.001*
	PWS	spawning	180	219.4^{C}	1.3	
	SS	spawning	240	201.8^{B}	1.2	
body weight (g)	PWS	prespawning	80	62.0^{A}	4.5	<0.001*
	PWS	spawning	180	142.4^{C}	2.8	
	SS	spawning	240	122.2^{B}	2.6	
liver weight (g)	PWS	prespawning	80	0.7^{A}	0.1	<0.001*
	PWS	spawning	166	1.1^{C}	0	
	SS	spawning	237	1.0^{B}	0	
ovary weight (g)	PWS	prespawning	23	16.4^{A}	3.4	<0.001*
	PWS	spawning	85	35.8^{C}	3.9	
	SS	spawning	92	26.8^{B}	2.8	
testis weight (g)	PWS	prespawning	13	13.8^{A}	3.8	<0.001*
	PWS	spawning	94	28.1^{C}	2.9	
	SS	spawning	147	19.3^{B}	1.6	
hold time (min)	PWS	prespawning	80	94.5^{A}	4.5	<0.001
	PWS	spawning	180	95.9^{A}	3.1	
	SS	spawning	240	151.7^{B}	2.9	

Variable	Site	Spawning status	# examined	Mean	±SE	ANOVA *P* value
IgM (mg/mL)[a]	PWS	prespawning	57	413.6[A]	51.2	<0.001*
	PWS	spawning	177	814.8[C]	72.0	
	SS	spawning	239	637.8[B]	40.0	
packed cell volume (%)[b]	PWS	prespawning	80	35.3[A]	1.3	<0.001*
	PWS	spawning	179	45.3[B]	0.6	
	SS	spawning	223	44.7[B]	0.8	
peritoneal cavity - number of herring worms (Anisakidae)	PWS	prespawning	80	13.5	1.0	NS[c]
	PWS	spawning	180	14.5	0.7	
	SS	spawning	238	13.4	0.5	
gills - number of 0.5-mm-diameter white foci	PWS	prespawning	76	0.6	0.3	NS
	PWS	spawning	179	0.5	0.1	
	SS	spawning	232	0.5	0.1	

[a]Values for IgM 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.

[b]Values for packed cell volume 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.

[c]NS = not significant.

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
fin base reddening	prespawning-PWS	79	78.5	16.5	3.8	1.3	21.5	ND
	spawning-PWS	180	46.7	41.7	8.9	2.8	53.3	↑- age (0.004), hold time (<0.001), CO_2 (0.001), bilirubin (0.027), neutrophils (0.005) ↓- phosphorus (0.002), cholesterol (0.014), total protein (0.001), albumin (0.003) NT- potassium (0.045)
	spawning-SS	240	63.3	32.1	3.3	1.3	36.7	NT- hold time (0.020), calcium (0.025), CO_2 (0.030)
iris reddening	prespawning-PWS	79	72.2	27.8	0.0	0.0	27.8	ND
	spawning-PWS	180	30.0	67.8	2.2	0.0	70.0	↓- ALP (0.031), PCV (0.030)
	spawning-SS	240	30.8	68.3	0.8	0.0	69.2	↑- age (0.001), sodium (<0.001*), potassium (0.001*), phosphorus (<0.001*), calcium (<0.001), lactate (<0.001*), osmolality (<0.001) ↓- glucose (0.012)
skin reddening, diffuse	prespawning-PWS	80	97.5	2.5	0.0	0.0	2.5	ND
	spawning-PWS	180	88.9	8.9	1.7	0.6	11.1	↑- hold time (<0.001), potassium (0.006), CO_2 (0.017) ↓- phosphorus (0.014)
	spawning-SS	240	92.1	7.1	0.8	0.0	7.9	↑- total protein (0.018), PCV (0.002)
skin reddening, focal	prespawning-PWS	80	68.8	23.8	0.0	7.5	31.3	ND
	spawning-PWS	180	79.4	17.8	1.7	1.1	20.6	↑- $\log_e$CPK (0.003), $\log_e$AST (0.006*)
	spawning-SS	240	71.3	27.5	0.8	0.4	28.8	none

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 March 1995, or from Prince William Sound (PWS) during April 1995. Lesions were scored as none (0), mild (1), moderate (2), or severe (3). Note that all spawning fish from both sites were sexually mature, but only 45% (36 of 80) of the PWS "prespawning" fish were sexually mature. Also, gonads from all spawning fish were ripe. Some spawning fish from Sitka Sound were partly or completely spawned out, whereas no fish from Prince William Sound had spawned before being sampled. Age, hold time, and blood values were compared for groups of spawning fish based on lesion scores using one-way analysis of variance and Tukey's multiple comparison procedure. Significant trends ($P \leq 0.05$) 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 or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
External gross lesions:								
caudal fin fraying	prespawning-PWS	80	12.5	73.8	12.5	1.3	87.5	ND[a]
	spawning-PWS	180	5.6	57.2	33.3	3.9	94.4	↓- glucose (0.019), cholesterol (0.008) NT- PCV (0.027), IgM (0.014)
	spawning-SS	240	10.4	82.5	7.1	0.0	89.6	↑- sodium (0.002), calcium (<0.001*), osmolality (0.028)
caudal fin reddening	prespawning-PWS	80	51.3	45.0	3.8	0.0	48.8	ND
	spawning-PWS	180	35.6	46.1	18.3	0.0	64.4	↓-cholesterol (0.006), lactate (0.031), total protein (0.011), albumin (0.005)
	spawning-SS	240	47.9	50.4	1.7	0.0	52.1	none

[a]ND = not done

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Other Gross findings:								
gonad fullness	prespawning-PWS	36	0.0	0.0	2.8	97.2	100	ND
	spawning-PWS	176	0.0	0.6	0.0	99.4	100	ND
	spawning-SS	238	13.0	7.1	15.5	64.3	87.0	↑- chloride (<0.001*), glucose (<0.001), total protein (0.001), PCV (0.001) ↓- sodium (0.002), phosphorus (0.001), osmolality (0.002*), neutrophils (<0.001) NT- age (0.048), ALP (0.024), basophils (0.041), log_eAST (0.037)
Brain microscopic lesions:								
Ichthyophonus	prespawning-PWS	80	97.5	1.3	1.3	0.0	2.5	ND
	spawning-PWS	180	88.9	11.1	0.0	0.0	11.1	↑- neutrophils (0.003*), log_eAST (0.036), IgM (<0.001)
	spawning-SS	240	92.5	7.1	0.4	0.0	7.5	↑- ALP (0.029), total protein (0.024), log_eCPK (0.005), log_eAST (<0.001), IgM (<0.001)
meningeal eosinophilic granular leukocytes	prespawning-PWS	80	11.3	67.5	21.3	0.0	88.8	ND
	spawning-PWS	180	11.1	61.7	26.7	0.6	88.9	NT- potassium (0.003), PCV (0.047)
	spawning-SS	240	13.8	57.9	26.3	2.1	86.3	↑- osmolality (0.002*) ↓- log_eCPK (0.011) NT- PCV (0.044)
meningoencephalitis	prespawning-PWS	80	95.0	3.8	1.3	0.0	5.0	ND
	spawning-PWS	180	92.8	7.2	0.0	0.0	7.2	none
	spawning-SS	240	96.7	3.3	0.0	0.0	3.3	↑- ALP (0.054), CO_2 (0.044), PCV (0.051), IgM (0.036)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Gall bladder microscopic lesions:								
myxosporean (*Ceratomyxa auerbachi*)	prespawning-PWS	72	81.9	13.9	4.2	0.0	18.1	ND
	spawning-PWS	175	60.6	30.3	9.1	0.0	39.4	NT- total protein (0.039)
	spawning-SS	216	67.6	23.6	8.3	0.5	32.4	none
Gill microscopic lesions:								
ciliated protozoa (e.g., *Trichodina* spp.)	prespawning-PWS	80	98.8	1.3	0.0	0.0	1.3	ND
	spawning-PWS	180	98.9	1.1	0.0	0.0	1.1	ND
	spawning-SS	240	98.8	1.3	0.0	0.0	1.3	ND
Epitheliocystis	prespawning-PWS	80	67.5	32.5	0.0	0.0	32.5	ND
	spawning-PWS	180	85.0	14.4	0.6	0.0	15.0	↓- age (0.023)
	spawning-SS	240	75.0	22.9	2.1	0.0	25.0	↑- $\log_e$CPK (0.026), $\log_e$AST (0.032)
foreign body granuloma	prespawning-PWS	80	92.5	6.3	1.3	0.0	7.5	ND
	spawning-PWS	180	94.4	5.6	0.0	0.0	5.6	none
	spawning-SS	240	94.2	5.8	0.0	0.0	5.8	none
gill arch inflammation or hematopoiesis	prespawning-PWS	80	0.0	98.8	1.3	0.0	100.0	ND
	spawning-PWS	180	0.0	95.0	5.0	0.0	100.0	↑- neutrophils (0.006), IgM (0.005)
	spawning-SS	240	0.8	93.8	5.4	0.0	99.2	↓- cholesterol (0.016), PCV (0.050)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ichthyophonus	prespawning-PWS	80	96.3	1.3	0.0	2.5	3.8	ND
	spawning-PWS	180	85.6	10.6	3.3	0.6	14.4	↑- basophils (0.003*), $\log_e$AST (0.020), IgM (<0.001) ↓- CO_2 (0.023)
	spawning-SS	240	89.2	7.9	2.9	0.0	10.8	↑- total protein (0.054), basophils (0.025), $\log_e$CPK (0.045), $\log_e$AST (<0.001), IgM (<0.001) ↓- glucose (0.014)
lamellar hyperplasia	prespawning-PWS	80	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	180	99.4	0.6	0.0	0.0	0.6	ND
	spawning-SS	240	99.6	0.4	0.0	0.0	0.4	ND
lamellar telangiectasis	prespawning-PWS	80	93.8	6.3	0.0	0.0	6.3	ND
	spawning-PWS	180	77.2	21.7	1.1	0.0	22.8	↑- age (0.022) ↓- lactate (0.001*)
	spawning-SS	240	84.6	12.9	2.5	0.0	15.4	↑- glucose (0.030)
monogenetic trematodes (e.g., *Gyrodactylus* spp.)	prespawning-PWS	80	98.8	1.3	0.0	0.0	1.3	ND
	spawning-PWS	180	89.4	10.6	0.0	0.0	10.6	↓- potassium (0.039)
	spawning-SS	240	89.2	10.8	0.0	0.0	10.8	↑- IgM (0.020)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Gonad - female microscopic lesions:								
eosinophilic granular leukocytes	immature prespawning-PWS	25	8.0	24.0	16.0	52.0	92.0	ND
	mature prespawning-PWS	24	54.2	37.5	0.0	8.3	45.8	ND
	spawning-PWS	85	40.0	43.5	8.2	8.2	60.0	↓- cholesterol (0.020), total protein (0.035), albumin (0.021), PCV (0.050)
	spawning-SS	94	42.6	40.4	6.4	10.6	57.4	none
granulomatous inflammation	immature prespawning-PWS	25	8.0	68.0	12.0	12.0	92.0	ND
	mature prespawning-PWS	24	25.0	70.8	4.2	0.0	75.0	ND
	spawning-PWS	85	31.8	65.9	2.4	0.0	68.2	↓- neutrophils (0.003*)
	spawning-SS	93	43.0	53.8	1.1	1.1	55.9	↓- bilirubin (0.020)
hyalinization of vessel walls	immature prespawning-PWS	25	96.0	4.0	0.0	0.0	4.0	ND
	mature prespawning-PWS	24	87.5	8.3	4.2	0.0	12.5	ND
	spawning-PWS	85	58.8	31.8	9.4	0.0	41.2	↓- ALP (0.039) NT- age (0.009)
	spawning-SS	94	53.2	38.3	8.5	0.0	46.8	NT- age (0.008*)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ichthyophonus	immature prespawning-PWS	24	95.8	4.2	0.0	0.0	4.2	ND
	mature prespawning-PWS	23	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	84	97.6	2.4	0.0	0.0	2.4	ND
	spawning-SS	93	100.0	0.0	0.0	0.0	0.0	ND
macrophage aggregates (pigmented)	immature prespawning-PWS	25	76.0	16.0	4.0	4.0	24.0	ND
	mature prespawning-PWS	24	87.5	12.5	0.0	0.0	12.5	ND
	spawning-PWS	85	60.0	40.0	0.0	0.0	40.0	none
	spawning-SS	94	73.4	26.6	0.0	0.0	26.6	↑- age (0.001*), osmolality (0.010*) ↓- $\log_e$CPK (0.030)
Gonad - male microscopic lesions:								
Eimeria sardinae	immature prespawning-PWS	15	93.3	6.7	0.0	0.0	6.7	ND
	mature prespawning-PWS	12	16.7	66.7	8.3	8.3	83.3	ND
	spawning-PWS	94	14.9	68.1	14.9	2.1	85.1	↑- lymphocytes (0.021) ↓- age (<0.001) NT- thrombocytes (0.034), neutrophils (0.022)
	spawning-SS	145	34.5	60.0	5.5	0.0	65.5	↓- age (<0.001), lactate (0.026), total protein (0.013), albumin (0.001) NT- ALP (0.040), sodium (0.036), phosphorus (0.028), calcium (0.032), cholesterol (0.013)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
eosinophilic granular leukocytes	immature prespawning-PWS	15	6.7	20.0	0.0	73.3	93.3	ND
	mature prespawning-PWS	12	50.0	50.0	0.0	0.0	50.0	ND
	spawning-PWS	94	84.0	13.8	0.0	2.1	16.0	↑- calcium (0.022), lymphocytes (0.041)
	spawning-SS	146	59.6	26.7	4.8	8.9	40.4	none
granulomatous inflammation	immature prespawning-PWS	14	57.1	21.4	14.3	7.1	42.9	ND
	mature prespawning-PWS	12	75.0	25.0	0.0	0.0	25.0	ND
	spawning-PWS	94	88.3	11.7	0.0	0.0	11.7	none
	spawning-SS	146	95.9	4.1	0.0	0.0	4.1	ND
hyalinization of vessel walls	prespawning-PWS							
	- immature	15	100.0	0.0	0.0	0.0	0.0	ND
	- mature	12	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	94	100.0	0.0	0.0	0.0	0.0	ND
	spawning-SS	145	100.0	0.0	0.0	0.0	0.0	ND
Ichthyophonus	prespawning-PWS							
	- immature	15	100.0	0.0	0.0	0.0	0.0	ND
	- mature	12	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	94	100.0	0.0	0.0	0.0	0.0	ND
	spawning-SS	146	97.3	2.7	0.0	0.0	2.7	ND

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
macrophage aggregates (pigmented)	immature prespawning-PWS	15	93.3	6.7	0.0	0.0	6.7	ND
	mature prespawning-PWS	12	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	94	100.0	0.0	0.0	0.0	0.0	ND
	spawning-SS	146	99.3	0.7	0.0	0.0	0.7	ND
spermatocyte numbers (3 = abundant)	immature prespawning-PWS	14	100.0	0.0	0.0	0.0	0.0	ND
	mature prespawning-PWS	12	0.0	0.0	0.0	100.0	100.0	ND
	spawning-PWS	94	0.0	0.0	1.1	98.9	100.0	ND
	spawning-SS	146	1.4	6.2	17.8	74.7	98.6	↑- glucose (<0.001) ↓- sodium (0.001*), phosphorus (0.013), osmolality (0.005*), neutrophils (<0.001) NT- ALP (0.014), chloride (0.048)
Heart microscopic lesions:								
atrial phagocyte hypertrophy	prespawning-PWS	80	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	179	100.0	0.0	0.0	0.0	0.0	ND
	spawning-SS	240	100.0	0.0	0.0	0.0	0.0	ND
epicarditis	prespawning-PWS	80	38.8	61.3	0.0	0.0	61.3	ND
	spawning-PWS	179	34.6	60.9	3.9	0.6	65.4	↑- IgM (0.021) ↓- glucose (0.023), cholesterol (0.030)
	spawning-SS	240	43.8	53.8	2.1	0.4	56.3	↓- cholesterol (0.013)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ichthyophonus	prespawning-PWS	80	93.8	1.3	0.0	5.0	6.3	ND
	spawning-PWS	179	77.7	7.3	7.8	7.3	22.3	↑- neutrophils (0.001*), IgM (<0.001)
	spawning-SS	240	82.9	6.7	4.6	5.8	17.1	↑- $\log_e$CPK (0.007), $\log_e$AST (<0.001), IgM (<0.001) NT- PCV (0.035), thrombocytes (0.031), lymphocytes (0.011)
leukocytes, focal, parenchymal	prespawning-PWS	80	68.8	31.3	0.0	0.0	31.3	ND
	spawning-PWS	179	67.6	31.8	0.6	0.0	32.4	↓- age (0.017), $\log_e$ALT (0.031)
	spawning-SS	240	76.3	23.8	0.0	0.0	23.8	↓- lactate (0.025), neutrophils (0.012)
mineralization, myocardial	prespawning-PWS	80	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	179	100.0	0.0	0.0	0.0	0.0	ND
	spawning-SS	240	100.0	0.0	0.0	0.0	0.0	ND
thrombosis	prespawning-PWS	80	98.8	1.3	0.0	0.0	1.3	ND
	spawning-PWS	179	92.2	7.8	0.0	0.0	7.8	↑- lactate (0.047)
	spawning-SS	240	95.4	3.8	0.8	0.0	4.6	none
Intestine and intestinal cecae, microscopic lesions:								
Anisakidae	prespawning-PWS	80	17.5	58.8	11.3	12.5	82.5	ND
	spawning-PWS	180	17.2	54.4	18.3	10.0	82.8	none
	spawning-SS	240	22.1	52.1	19.2	6.7	77.9	↓- age (<0.000*) NT- glucose (0.043), thrombocytes (0.051)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
arteriolar hyperplasia, focal, intimal	prespawning-PWS	80	72.5	27.5	0.0	0.0	27.5	ND
	spawning-PWS	180	48.3	45.6	6.1	0.0	51.7	↓- phosphorus (0.022)
	spawning-SS	240	57.1	41.7	1.3	0.0	42.9	↑- age (0.053) ↓- bilirubin (0.040)
cestodes	prespawning-PWS	80	86.3	3.8	10.0	0.0	13.8	ND
	spawning-PWS	180	96.7	2.8	0.6	0.0	3.3	ND
	spawning-SS	240	97.5	1.7	0.8	0.0	2.5	ND
coccidian, intraepithelial (*Goussia*? sp.)	prespawning-PWS	80	3.8	65.0	31.3	0.0	96.3	ND
	spawning-PWS	180	5.0	82.8	12.2	0.0	95.0	↑- $\log_e$CPK (0.037), $\log_e$ALT (0.021)
	spawning-SS	240	8.8	89.6	1.7	0.0	91.3	none
eosinophilic granular leukocytes, submucosal	prespawning-PWS	80	0.0	92.5	7.5	0.0	100.0	ND
	spawning-PWS	180	1.7	96.1	2.2	0.0	98.3	ND
	spawning-SS	240	2.1	95.0	2.9	0.0	97.9	ND
foreign body granuloma	prespawning-PWS	80	72.5	27.5	0.0	0.0	27.5	ND
	spawning-PWS	180	55.6	43.9	0.6	0.0	44.4	↑- neutrophils (0.047) ↓- PCV (0.052), lymphocytes (0.025)
	spawning-SS	240	65.0	35.0	0.0	0.0	35.0	↑- neutrophils (0.003)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ichthyophonus	prespawning-PWS	80	93.8	3.8	2.5	0.0	6.3	ND
	spawning-PWS	180	88.3	11.7	0.0	0.0	11.7	↑- glucose (0.031), total protein (0.037), neutrophils (0.009*), IgM (<0.001)
	spawning-SS	240	91.7	8.3	0.0	0.0	8.3	↑- $\log_e$CPK (0.029), $\log_e$AST (0.004), IgM (0.002) ↓- calcium (0.040), lactate (0.031)
steatitis	prespawning-PWS	80	5.0	88.8	6.3	0.0	95.0	ND
	spawning-PWS	180	0.0	94.4	5.6	0.0	100.0	↑- potassium (0.024) ↓- ALP (0.034)
	spawning-SS	240	0.0	94.6	5.4	0.0	100.0	↑- lymphocytes (0.012), neutrophils (0.045), basophils (0.040), eosinophils (0.020), $\log_e$CPK (0.010), $\log_e$AST (0.001), IgM (0.001) ↓- cholesterol (0.030), thrombocytes (<0.001)
trematodes (e.g., *Lecithaster gibbosus*), cecal	prespawning-PWS	80	81.3	18.8	0.0	0.0	18.8	ND
	spawning-PWS	180	91.1	8.3	0.6	0.0	8.9	↑- ALP (0.026), lactate (0.038) ↓- age (0.001)
	spawning-SS	240	97.9	2.1	0.0	0.0	2.1	ND

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Kidney (trunk) microscopic lesions:								
congestion, interstitial, vascular	prespawning-PWS	79	93.7	5.1	1.3	0.0	6.3	ND
	spawning-PWS	180	91.1	8.3	0.6	0.0	8.9	↑- phosphorus (<0.001*), $\log_e$CPK (0.001), $\log_e$AST (<0.001), $\log_e$ALT (0.015) ↓- chloride (0.002*), glucose (<0.001), cholesterol (0.045), lymphocytes (0.018)
	spawning-SS	240	79.6	20.0	0.0	0.4	20.4	↑- phosphorus (0.009) ↓- age (0.014), chloride (0.006), glucose (0.009), lymphocytes (0.014)
granulomatous inflammation	prespawning-PWS	79	83.5	16.5	0.0	0.0	16.5	ND
	spawning-PWS	180	77.8	20.0	1.7	0.6	22.2	none
	spawning-SS	240	82.1	15.8	2.1	0.0	17.9	↑- cholesterol (0.004), total protein (0.009), albumin (0.051)
hematopoietic cells (relative area)	prespawning-PWS	79	3.8	73.4	22.8	0.0	96.2	ND
	spawning-PWS	180	2.8	78.3	18.3	0.6	97.2	↑- neutrophils (0.001*)
	spawning-SS	240	9.6	76.7	13.8	0.0	90.4	↑- IgM (<0.001) ↓- hold time (0.023)
Ichthyophonus	prespawning-PWS	79	94.9	1.3	0.0	3.8	5.1	ND
	spawning-PWS	180	81.1	8.3	6.1	4.4	18.9	↑- IgM (<0.001) NT- lymphocytes (0.035), neutrophils (0.004*)
	spawning-SS	240	82.9	7.5	5.4	4.2	17.1	↑- $\log_e$CPK (0.013), $\log_e$AST (<0.001), IgM (<0.000) ↓- PCV (0.003) NT- total protein (0.002*)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
intratubular mineral, with associated tubular hyperplasia	prespawning-PWS	79	97.5	2.5	0.0	0.0	2.5	ND
	spawning-PWS	180	97.2	2.8	0.0	0.0	2.8	ND
	spawning-SS	240	97.1	2.1	0.8	0.0	2.9	ND
intraductal protozoan	prespawning-PWS	79	92.4	7.6	0.0	0.0	7.6	ND
	spawning-PWS	180	88.9	10.6	0.6	0.0	11.1	none
	spawning-SS	240	96.3	3.8	0.0	0.0	3.8	↑- glucose (0.051), $\log_e$ALT (0.047) ↓- neutrophils (0.018)
macrophage aggregates, pigmented	prespawning-PWS	79	10.1	57.0	29.1	3.8	89.9	ND
	spawning-PWS	180	1.1	28.9	48.3	21.7	98.9	↑- age (<0.001*), neutrophils (0.001)
	spawning-SS	240	5.8	43.3	41.3	9.6	94.2	↑- age (<0.001*), glucose (0.035) ↓- $\log_e$CPK (<0.001), $\log_e$AST (0.001) NT- lactate (0.047), total protein (0.034), albumin (0.049), IgM (<0.001)
Ortholinea orientalis (intraductal myxosporean), histopathology	prespawning-PWS	79	86.1	10.1	0.0	3.8	13.9	ND
	spawning-PWS	180	92.8	4.4	2.8	0.0	7.2	↑- PCV (0.048), neutrophils (0.049), basophils (0.036) ↓- potassium (0.020)
	spawning-SS	240	96.3	2.5	1.3	0.0	3.8	none
Ortholinea orientalis (intraductal myxosporean), kidney touch preparation	prespawning-PWS	79	75.9	8.9	3.8	11.4	24.1	ND
	spawning-PWS	180	72.2	16.7	4.4	6.7	27.8	↓- total protein (0.019), albumin (0.019) NT- neutrophils (0.030), $\log_e$ALT (0.042)
	spawning-SS	240	81.3	13.3	2.5	2.9	18.8	NT- potassium (0.026)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ortholinea orientalis (intraductal myxosporean); sum of both techniques	prespawning-PWS	80	71.3	11.3	2.5	15.0	28.8	ND
	spawning-PWS	180	71.1	15.6	5.6	7.8	28.9	NT- cholesterol (0.026), albumin (0.039)
	spawning-SS	240	79.6	14.2	2.9	3.3	20.4	NT- ALP (0.040), bilirubin (0.037), basophils (0.009)
tubular dilation (of lumen)	prespawning-PWS	79	93.7	5.1	1.3	0.0	6.3	ND
	spawning-PWS	180	96.7	3.3	0.0	0.0	3.3	ND
	spawning-SS	240	89.6	9.2	1.3	0.0	10.4	↑- hold time (0.024), sodium (0.002), potassium (0.002), phosphorus (0.002), neutrophils (0.001), $\log_e$ALT (0.049) ↓- chloride (0.003*), PCV (0.010)
tubular epithelial vacuolation	prespawning-PWS	79	88.6	11.4	0.0	0.0	11.4	ND
	spawning-PWS	180	73.9	26.1	0.0	0.0	26.1	↑- glucose (0.020), cholesterol (0.011) ↓- phosphorus (<0.001)
	spawning-SS	240	59.6	39.2	1.3	0.0	40.4	↑- age (0.046)
Liver microscopic lesions:								
cholangitis or biliary hyperplasia	prespawning-PWS	80	97.5	1.3	1.3	0.0	2.5	ND
	spawning-PWS	180	92.2	7.8	0.0	0.0	7.8	↑- hold time (0.005)
	spawning-SS	240	92.9	7.1	0.0	0.0	7.1	↑- calcium (0.047), cholesterol (0.028)
coccidiosis (*Goussia* [*Eimeria*] *clupearum*)	prespawning-PWS	80	32.5	27.5	18.8	21.3	67.5	ND
	spawning-PWS	180	29.4	40.6	18.9	11.1	70.6	↓- age (0.002) NT- total protein (0.028)
	spawning-SS	240	27.5	41.3	15.8	15.4	72.5	↓- age (0.040), glucose (0.007) NT- cholesterol (0.052), neutrophils (0.054)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
eosinophilic granular leukocytes, perivascular	prespawning-PWS	80	23.8	73.8	2.5	0.0	76.3	ND
	spawning-PWS	180	6.7	74.4	18.9	0.0	93.3	none
	spawning-SS	240	7.5	80.0	12.5	0.0	92.5	↑- ALP (0.005), IgM (0.003*) ↓- hold time (0.010), potassium (0.041) NT- calcium (0.021), lymphocytes (0.028), $\log_e$ALT (0.034)
glycogen depletion, hepatocellular	prespawning-PWS	80	0.0	0.0	0.0	100.0	100.0	ND
	spawning-PWS	180	0.0	0.0	3.3	96.7	100.0	ND
	spawning-SS	240	0.0	0.0	2.5	97.5	100.0	ND
granulomatous inflammation	prespawning-PWS	80	76.3	21.3	2.5	0.0	23.8	ND
	spawning-PWS	180	58.3	38.9	1.7	1.1	41.7	↑- ALP (0.005), total protein (0.036) ↓- CO_2 (0.047)
	spawning-SS	240	66.3	31.7	1.7	0.4	33.8	↑- total protein (0.027), IgM (0.013)
Ichthyophonus	prespawning-PWS	80	90.0	6.3	1.3	2.5	10.0	ND
	spawning-PWS	180	78.9	11.1	7.2	2.8	21.1	↑- neutrophils (0.011), $\log_e$AST (0.026), IgM (<0.001) NT- phosphorus (0.025), bilirubin (0.032)
	spawning-SS	240	83.3	9.2	4.2	3.3	16.7	↑- basophils (0.010), $\log_e$AST (0.004), IgM (<0.001) ↓- thrombocytes (0.013)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
leukocytes, focal, parenchymal	prespawning-PWS	80	25.0	72.5	2.5	0.0	75.0	ND
	spawning-PWS	180	18.9	78.9	2.2	0.0	81.1	↑- chloride (0.001*), calcium (<0.001), glucose (<0.001*), cholesterol (0.001), lactate (<0.001), total protein (<0.001), albumin (<0.001), osmolality (0.005), PCV (0.004), lymphocytes (<0.001) ↓- thrombocytes (0.008), $\log_e$AST (0.008)
	spawning-SS	240	51.3	48.3	0.0	0.4	48.8	none
leukocytes, pericholangial	prespawning-PWS	80	76.3	22.5	1.3	0.0	23.8	ND
	spawning-PWS	180	68.9	29.4	1.7	0.0	31.1	↑- age (0.023) ↓- potassium (0.008)
	spawning-SS	240	71.3	27.1	1.7	0.0	28.8	none
lipidosis, hepatocellular	prespawning-PWS	80	86.3	10.0	2.5	1.3	13.8	ND
	spawning-PWS	180	93.3	6.7	0.0	0.0	6.7	↑- total protein (0.009), albumin (0.012) ↓- neutrophils (0.035)
	spawning-SS	240	60.0	29.2	9.6	1.3	40.0	↑- age (<0.001), hold time (<0.001), ALP (0.027), sodium (<0.001), potassium (<0.001*), phosphorus (<0.001*), calcium (0.026), lactate (<0.001), osmolality (<0.001) ↓- thrombocytes (0.038), IgM (0.031)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
macrophage aggregates, pigmented	prespawning-PWS	80	6.3	61.3	25.0	7.5	93.8	ND
	spawning-PWS	180	0.0	33.3	38.3	28.3	100.0	↑- age (<0.001), neutrophils (0.004) NT- glucose (0.032), cholesterol (0.004)
	spawning-SS	240	2.5	52.1	29.6	15.8	97.5	↑- age (<0.001*), neutrophils (0.016), IgM (<0.001) NT- glucose (<0.001), total protein (0.012), $\log_e$CPK (0.011), $\log_e$AST (<0.001)
necrosis, hepatocellular, focal	prespawning-PWS	80	100.0	0.0	0.0	0.0	0.0	ND
	spawning-PWS	180	100.0	0.0	0.0	0.0	0.0	ND
	spawning-SS	240	99.2	0.8	0.0	0.0	0.8	ND
necrosis, hepatocellular, single cell	prespawning-PWS	80	93.8	6.3	0.0	0.0	6.3	ND
	spawning-PWS	180	96.7	2.2	0.6	0.6	3.3	ND
	spawning-SS	240	97.5	2.5	0.0	0.0	2.5	ND
Pancreas, exocrine, microscopic lesions:								
macrophage aggregates, pigmented	prespawning-PWS	80	71.3	28.8	0.0	0.0	28.8	ND
	spawning-PWS	180	38.9	60.6	0.6	0.0	61.1	↑- age (0.002*), PCV (0.017), IgM (0.019)
	spawning-SS	240	44.6	55.4	0.0	0.0	55.4	↑- age (0.000) ↓- $\log_e$CPK (0.017), $\log_e$AST (0.001)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
zymogen granule depletion	prespawning-PWS	80	0.0	3.8	57.5	38.8	100.0	ND
	spawning-PWS	180	0.0	0.6	46.1	53.3	100.0	↑- phosphorus (<0.001), thrombocytes (<0.001), log_eAST (0.004*) ↓- chloride (0.009), calcium (0.006), glucose (<0.001), cholesterol (<0.001), lactate (0.001), total protein (<0.001), albumin (<0.001), osmolality (0.003), PCV (0.043), lymphocytes (<0.001*)
	spawning-SS	240	0.0	0.4	35.4	64.2	100.0	↑- neutrophils (0.038) ↓- chloride (0.001), glucose (<0.001), cholesterol (0.002), total protein (0.002), lymphocytes (0.044)
Skin and skeletal muscle, microscopic lesions:								
arteriolar hyperplasia, focal, intimal	prespawning-PWS	80	63.8	36.3	0.0	0.0	36.3	ND
	spawning-PWS	180	52.2	47.8	0.0	0.0	47.8	↑- log_eAST (0.034) ↓- PCV (0.007)
	spawning-SS	240	53.3	46.7	0.0	0.0	46.7	↑- lactate (0.024), albumin (0.041), neutrophils (0.024) ↓- thrombocytes (0.034)
Ichthyophonus	prespawning-PWS	80	92.5	2.5	2.5	2.5	7.5	ND
	spawning-PWS	180	82.8	11.1	5.0	1.1	17.2	↑- neutrophils (0.007), lymphocytes (0.026), IgM (<0.001)
	spawning-SS	240	84.2	11.7	3.8	0.4	15.8	↑- log_eAST (<0.001), IgM (<0.001) NT- log_eCPK (0.031)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
leukocytes, perivascular	prespawning-PWS	80	36.3	63.8	0.0	0.0	63.8	ND
	spawning-PWS	180	22.8	77.2	0.0	0.0	77.2	↑- sodium (0.017) ↓- cholesterol (<0.001), total protein (0.050)
	spawning-SS	240	34.6	65.4	0.0	0.0	65.4	↑- age (0.001)
myodegeneration or myonecrosis	prespawning-PWS	80	98.8	0.0	1.3	0.0	1.3	ND
	spawning-PWS	180	95.0	3.3	1.7	0.0	5.0	↑- $\log_e$CPK (0.003) ↓- potassium (0.051), glucose (0.018)
	spawning-SS	240	96.3	3.3	0.4	0.0	3.8	↑- $\log_e$ALT (0.016)
myositis	prespawning-PWS	80	85.0	12.5	1.3	1.3	15.0	ND
	spawning-PWS	180	92.2	7.2	0.6	0.0	7.8	↑- $\log_e$CPK (0.050), $\log_e$AST (0.030) ↓- sodium (<0.001*), osmolality (0.015)
	spawning-SS	240	96.7	3.3	0.0	0.0	3.3	↑- basophils (0.004*) ↓- thrombocytes (0.010)
Spleen microscopic lesions:								
arteriolar hyperplasia, focal, intimal	prespawning-PWS	79	83.5	11.4	0.0	0.0	11.4	ND
	spawning-PWS	180	81.7	17.8	0.0	0.0	17.8	none
	spawning-SS	235	82.6	17.4	0.0	0.0	17.4	↓- hold time (0.001), potassium (0.048)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
congestion, vascular	prespawning-PWS	79	1.3	24.1	21.5	53.2	98.7	ND
	spawning-PWS	180	2.8	71.7	18.3	7.2	97.2	↓- albumin (0.023), osmolality (0.036), IgM (0.026) NT- hold time (0.015), chloride (0.017), CO_2 (0.022), glucose (0.051), lactate (0.018), $\log_e$AST (0.042)
	spawning-SS	235	23.0	58.7	13.2	5.1	77.0	↓- age (0.004*), sodium (<0.001), phosphorus (<0.001), calcium (<0.001*), lactate (<0.001), osmolality (0.001) NT- potassium (0.024)
ellipsoid hyalinization or hypertrophy	prespawning-PWS	79	24.1	72.2	3.8	0.0	75.9	ND
	spawning-PWS	180	2.2	75.6	22.2	0.0	97.8	↑- age (0.018), ALP (0.046) ↓- $\log_e$CPK (0.001), $\log_e$AST (0.010)
	spawning-SS	235	5.5	82.1	12.3	0.0	94.5	↑- age (0.002*), chloride (0.016)
granulomatous inflammation	prespawning-PWS	79	97.5	2.5	0.0	0.0	2.5	ND
	spawning-PWS	180	92.8	6.1	0.6	0.6	7.2	↑- neutrophils (0.038), basophils (0.015) ↓- calcium (0.024), glucose (0.021), lactate (0.013), lymphocytes (0.006)
	spawning-SS	235	94.9	4.3	0.4	0.4	5.1	↑- lymphocytes (0.031) ↓- hold time (0.007*), calcium (0.013)

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ichthyophonus	prespawning-PWS	79	92.4	3.8	2.5	1.3	7.6	ND
	spawning-PWS	180	82.2	7.2	7.2	3.3	17.8	↑- neutrophils (<0.001*), log_eAST (0.005), IgM (<0.001) NT- hold time (0.037), CO_2 (0.032), log_eCPK (0.018)
	spawning-SS	235	85.1	8.5	3.8	2.6	14.9	↑- log_eAST (0.001), IgM (<0.001) NT- total protein (0.017)
macrophage aggregates, pigmented	prespawning-PWS	79	15.2	40.5	32.9	11.4	84.8	ND
	spawning-PWS	180	0.6	15.6	48.3	35.6	99.4	↑- age (<0.001), neutrophils (0.018), IgM (0.043) ↓- log_eCPK (0.013) NT- glucose (0.045), cholesterol (0.054), osmolality (0.048)
	spawning-SS	235	14.0	30.6	31.9	23.4	86.0	↑- age (<0.001), glucose (0.044), neutrophils (0.010), IgM (<0.001) ↓- log_eCPK (0.003), log_eAST (<0.001) NT- osmolality (0.017)
Stomach microscopic lesions:								
eosinophilic granular leukocytes, submucosal	prespawning-PWS	80	0.0	76.3	23.8	0.0	100.0	ND
	spawning-PWS	180	0.0	76.1	23.9	0.0	100.0	↓- glucose (0.012), cholesterol (0.001), total protein (0.007), albumin (0.017)
	spawning-SS	240	0.4	88.3	11.3	0.0	99.6	↑- log_eALT (0.034), IgM (0.016) ↓- neutrophils (0.045)
foreign body granuloma	prespawning-PWS	80	88.8	11.3	0.0	0.0	11.3	ND
	spawning-PWS	180	85.0	15.0	0.0	0.0	15.0	none
	spawning-SS	240	85.4	14.2	0.4	0.0	14.6	none

Organ - lesion or tissue type	Spawning status - site	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
Ichthyophonus (includes only cases with organisms)	prespawning-PWS	80	95.0	3.8	1.3	0.0	5.0	ND
	spawning-PWS	180	84.4	11.7	3.9	0.0	15.6	↑- total protein (0.008), albumin (0.042), IgM (<0.001) ↓- hold time (0.048), CO_2 (0.033)
	spawning-SS	240	89.6	8.8	1.7	0.0	10.4	↑- ALP (0.041), basophils (0.016), $\log_e$AST (0.019), IgM (0.007)
Ichthyophonus+ (includes cases with characteristic inflammation, but no organisms)	prespawning-PWS	80	93.8	5.0	1.3	0.0	6.3	ND
	spawning-PWS	180	81.1	15.0	3.9	0.0	18.9	↑- total protein (0.040), neutrophils (0.024), IgM (<0.001) ↓- hold time (0.035), CO_2 (0.051)
	spawning-SS	240	81.3	17.1	1.7	0.0	18.8	↑- basophils (0.019), $\log_e$AST (0.035), IgM (0.007)
leukocytes, focal, parenchymal	prespawning-PWS	80	98.8	1.3	0.0	0.0	1.3	ND
	spawning-PWS	180	86.7	12.2	1.1	0.0	13.3	↑- PCV (0.007) ↓- CO_2 (0.040)
	spawning-SS	240	98.3	1.7	0.0	0.0	1.7	ND
serositis	prespawning-PWS	80	47.5	52.5	0.0	0.0	52.5	ND
	spawning-PWS	180	42.2	57.8	0.0	0.0	57.8	none
	spawning-SS	240	45.4	54.6	0.0	0.0	54.6	↓- PCV (0.053), lymphocytes (0.053)
trematodes, intraluminal (e.g., Hemiuridae)	prespawning-PWS	80	83.8	16.3	0.0	0.0	16.3	ND
	spawning-PWS	180	88.3	11.7	0.0	0.0	11.7	none
	spawning-SS	240	89.6	10.0	0.4	0.0	10.4	↑- glucose (0.038) ↓- age (0.027), neutrophils (0.008)

Table 3. Other lesions associated with external lesions in Pacific herring sampled from Prince William Sound and Sitka Sound, Alaska, during spawning, 1995. Chi-square test for association (n = 420 for most comparisons). 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		↑ fin base reddening		↑ focal skin reddening		↑ diffuse skin reddening	
Associated lesion	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
caudal fin fraying			↑	<0.001	↑	<0.001*				
caudal fin reddening	↑	<0.001			↑	<0.001*			↑	<0.001
fin base reddening	↑	<0.001*	↑	<0.001*					↑	<0.001*
diffuse skin reddening			↑	<0.001	↑	<0.001*				
iris reddening	NL	0.014								
eggs in stomach	NL	0.005	NL	0.021						
brain *Ichthyophonus*	↑	0.016					↑	0.004*		
branchial *Ichthyophonus*	↑	0.022					↑	0.006*		
cardiac epicarditis									↑	0.034
cardiac *Ichthyophonus*							↑	<0.001*		
cardiac thrombosis			NL	0.032						

	↑ caudal fin fraying		↑ caudal fin reddening		↑ fin base reddening		↑ focal skin reddening		↑ diffuse skin reddening	
Associated lesion	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
gastric focal parenchymal leukocytes	↑	0.007	↑	0.001						
gastric trematodiasis									↓	0.022
gastric serositis					↑	0.008	↑	0.036		
gastritis, submucosal			↑	0.007						
gonadal fullness	↑	0.049							↓	0.005
gonadal granulomatous inflammation									↑	0.028
gonadal pigmented macrophage aggregates			↑	0.030						
hepatic coccidiosis (*Goussia clupearum*)	NL	0.045							NL	0.008
hepatic eosinophilic granular leukocytes	↑	0.053	↑	0.019						
hepatic focal/multifocal parenchymal leukocytes	NL	0.032								
hepatic *Ichthyophonus*			↑	0.127			↑	<0.001*		
hepatic lipidosis	↓	0.011								

	↑ caudal fin fraying		↑ caudal fin reddening		↑ fin base reddening		↑ focal skin reddening		↑ diffuse skin reddening	
Associated lesion	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
hepatic pericholangial leukocytes	↑	0.023	NL	0.017						
intestinal Anisakidae			↑	0.036						
intestinal coccidian (*Goussia*? sp.)							NL	<0.001*	↓	0.023
intestinal foreign body granulomas					↑	0.007				
intestinal mesenteric steatitis	↑	0.009								
renal congestion			NL	0.039						
renal hematopoietic cells	↑	0.020								
renal *Ichthyophonus*							↑	0.001*		
renal pigmented macrophage aggregates	NL	0.001								
splenic *Ichthyophonus*	↑	0.046								
skeletal muscle, perivascular leukocytes	↑	0.031					↑	0.030		
testicular coccidian (*Eimeria sardinae*)									NL	0.045

Table 4. Oocyte morphology in mature female Pacific herring sampled from Sitka Sound (SS) and Prince William Sound (PWS) in March and April, 1995. Note that all spawning fish from both sites were sexually mature, but only 45% (36 of 80) of the PWS "prespawning" fish were sexually mature.

Variable	Spawning status-site	# of fish	Mean #	±SE
oocyte atresia - mature follicles	prespawning-PWS	24	0.0	0.5
	spawning-PWS	85	0.1	0.7
	spawning-SS	94	0.0	0.0
ruptured follicles	prespawning-PWS	24	0.0	0.0
	spawning-PWS	85	0.0	0.5
	spawning-SS	94	0.0	0.0
yolked oocytes	prespawning-PWS	24	66.2	5.4
	spawning-PWS	85	14.9	4.5
	spawning-SS	94	13.6	4.4
nonyolked oocytes	prespawning-PWS	24	66.8	5.2
	spawning-PWS	85	87.1	8.3
	spawning-SS	94	116.6	8.7
% yolked oocytes	prespawning-PWS	24	49.9	4.0
	spawning-PWS	85	15.7	3.6
	spawning-SS	94	15.8	4.6

Table 5. Lesion frequency (%) within variables of gender and iris reddening in Pacific herring sampled from Prince William Sound and Sitka Sound, Alaska, during spawning, 1995. 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.

Variable and lesion	Lesion score	Frequency		χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio
Gender		Female (n ≈ 179)	Male (n ≈ 240)			
brain eosinophilic granular leukocytes	0	13	13	0.003		
	1	68	53		ND[b]	
	2+3	20	34			
cardiac *Ichthyophonus*	0	75	85	0.001		
	1	10	5		ND	
	2	10	3			
	3	4	8			
epicarditis	0	30	48	0.001		
	1	66	50		ND	
	2+3	4	3			
gall bladder myxosporeans (*Ceratomyxa auerbachi*)	0	54	72	<0.001		
	1	31	23		ND	
	2+3	14	5			
gonadal granulomas (or focal granulomatous inflammation)	0	37	93	<0.001		
	1	63	7		22	12, 39

Variable and lesion	Lesion score	Frequency		χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio
Gender (continued)		Female (n ≈ 179)	Male (n ≈ 240)			
gonadal eosinophilic granular leukocytes	0	41	69	<0.001		
	1	42	22		ND	
	2	7	3			
	3	9	6			
gonadal pigmented macrophage aggregates	0	67	100	<0.001		
	1	33	0		118	16, 859
gonadal hyalinized vessel walls	0	56	100	<0.001		
	1	35	0		ND	
	2	9	0			
hepatic glycogen depletion	2	7	0	<0.001		
	3	93	100		0	none
hepatic *Ichthyophonus*	0	77	85	0.012		
	1	16	6		ND	
	2	5	6			
	3	3	3			
hepatic lipidosis	0	68	79	0.016		
	1	22	18		ND	
	2+3	9	4			
intestinal foreign body granuloma	0	51	68	0.001		
	1+2	49	32		2.0	1.3, 3.0

Variable and lesion	Lesion score	Frequency		χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio
Gender (continued)		Female (n ≈ 179)	Male (n ≈ 240)			
pancreatic zymogen granule depletion	1+2	24	53	<0.001		
	3	76	48		3.5	2.3, 5.4
renal proximal tubular epithelial vacuolation	0	74	60	0.002		
	1+2	26	40		0.51	0.33, 0.78
skeletal muscle *Ichthyophonus*	0	80	86	0.053		
	1	16	8		ND	
	2+3	4	6			
splenic ellipsoid hyalinization	0	4	4	0.002		
	1	72	85		ND	
	2	24	11			

Variable and lesion	Lesion score	Frequency		χ^2 *P*-value	Odds ratio[a]	95% Confidence interval for odds ratio
Iris reddening		Mild/Moderate (n ≈ 290)	None (n ≈ 128)			
caudal fin fraying	0	6	14	0.014		
	1	75	65		ND	
	2+3	20	21			
gastric eosinophilic granular leukocytes	0+1	80	90	0.018		
	2	20	10		2.1	1.1, 4.1
gonadal hyalinized vessel walls	0	78	89	0.023		
	1	18	9		ND	
	2	4	2			
hepatic pigmented macrophage aggregates	0+1	41	55	0.042		
	2	36	28		ND	
	3	23	17			
hepatic lipidosis	0	71	83	0.028		
	1	23	13		ND	
	2+3	7	5			
intestinal mesenteric Anisakidae	0	21	17	0.014		
	1	57	45		ND	
	2	16	26			
	3	7	12			
renal congestion	0	82	90	0.046		
	1+2+3	18	10		1.9	1.0, 3.7

Variable and lesion	Lesion score	Frequency		χ^2 *P*-value	Qdds ratio[a]	95% Confidence interval for odds ratio
Iris reddening (continued)		Mild/ Moderate (n ≈ 290)	None (n ≈ 128)			
renal intratubular mineral	0	98	95	0.033		
	1+2	2	5		0.30	0.094, 0.97
skeletal muscle arteriolar hyperplasia, focal, intimal	0	50	60	0.047		
	1	50	40		1.5	1.0, 2.3
splenic congestion	0	18	6	<0.001		
	1	67	59		ND	
	2	13	21			
	3	3	13			

[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, females were 118 times more likely to have pigmented gonadal macrophage aggregates than were males, fish with mild/moderate iris reddening were 1.9 times more likely to have renal congestion than were fish with no iris reddening.

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

Table 6. Number of intraperitoneal herring worms (Anisakidae) in categories based on age or lesion scores in Pacific herring sampled from Prince William Sound (PWS) and Sitka Sound (SS), Alaska, during spawning, 1995. One-way analysis of variance (ANOVA) and Tukey's multiple-comparison procedure. Means were ln transformed for statistical analysis; values shown are geometric means and first-order Taylor series approximation of standard errors. If Levene's test for equality of variances was significant (*), only comparisons with $P \leq 0.010$ are listed. Within rows, geometric means with a superscript in common were not significantly different ($P > 0.05$); lesions not shown were not significant.

Variable - site	Category based on lesion score									*P*-value for ANOVA
	A			B			C			
	Mean	SE	n	Mean	SE	n	Mean	SE	n	
	age = 2, 3			age = 4, 5, 6			age = 7			
age - PWS	$12.4^{A,B}$	4.0	19	15.2^{A}	3.6	31	10.8^{B}	1.4	105	0.044
age - SS	12.7^{A}	1.4	92	16.1^{A}	3.6	36	8.9^{B}	1.0	104	<0.001
gastric eosinophilic granular leukocytes - PWS	mild			moderate			NA[a]			
	11.1^{A}	1.3	137	13.9^{A}	2.6	43				0.052
gastric serositis - PWS	none			mild			NA			
	10.3^{B}	1.6	76	12.9^{A}	1.6	104				0.027
gastric trematodes - PWS	none			mild			NA			
	11.3^{B}	1.2	159	15.3^{A}	4.4	21				0.050
hepatic pigmented macrophage aggregates - SS	none/mild			moderate			severe			
	12.3^{A}	1.3	129	10.0^{A}	1.6	70	10.2^{A}	2.2	37	0.048
hepatic lipidosis - SS	none			mild/moderate/severe			NA			
	12.0^{A}	1.2	141	10.1^{B}	1.3	95				0.039

Variable - site	Category based on lesion score									*P*-value for ANOVA
	A			B			C			
	Mean	SE	n	Mean	SE	n	Mean	SE	n	
intestinal arteriolar hyperplasia, focal, intimal - SS	12.3[A]	none 1.3	134	9.9[B]	mild/moderate 1.3	102		NA		0.009
intestinal mesenteric Anisakidae - PWS	8.2[C]	none 1.5	31	11.4[B,C]	mild 1.6	98	13.5[A,B]	moderate 2.5	33	
- SS	8.6[C]	1.5	52	10.3[C]	1.0	122	14.8[B]	2.5	46	
intestinal mesenteric Anisakidae -PWS (cont.)	19.9[A]	severe 4.8	18							PWS <0.001
- SS (cont.)	23.5[A]	4.7	16							SS <0.001
meningitis - SS	11.0[B]	none 0.9	229	21.3[A]	mild 9.6	7		NA		0.006
renal congestion - SS	10.7[B]	none 1.0	188	13.6[A]	mild/severe 2.1	48		NA		0.019
skeletal muscle, perivascular leukocytes - PWS	9.1[B]	none 2.0	41	12.7[A]	mild 1.3	139		NA		0.005

[a]NA = not applicable (i.e., only 2 categories were used)

Table 7. Mean plasma chemistry and hematology values in males and females sampled from Sitka Sound and Prince William Sound, Alaska during spawning in 1995. Analysis of variance. Note that gonads from all fish were in spawning condition; some fish from Sitka Sound were partly or completely spawned out, whereas no fish from Prince William Sound had spawned before being sampled.

		Sitka Sound				Prince William Sound						
		Males		Females		Males		Females		Significance[a]		
Variable	age	mean	SE	mean	SE	mean	SE	mean	SE	site	sex	site*sex
Age	all[b]	5.0	0.2	5.3	0.2	6.5	0.2	6.8	0.2	***	NS	NS
	7[c]	7.0	0.0	7.0	0.0	7.0	0.0	7.0	0.0	NS	NS	NS
Length (mm)	all	200.1	1.5	204.5	1.9	216.4	1.8	222.7	1.7	***	***	NS
	7	215.0	1.2	217.0	1.5	222.4	1.4	226.7	1.3	***	*	NS
Body weight (g)	all	116.7	3.2	130.6	4.3	135.0	3.9	150.4	3.8	***	***	NS
	7	149.9	3.4	157.2	4.2	146.5	3.4	159.8	3.8	NS	**	NS
Gonad weight (g)	all	19.2	1.1	26.7	1.8	28.1	1.1	35.8	1.2	***	***	NS
	7	27.6	1.8	33.2	2.7	32.2	1.0	38.5	1.3	**	***	NS
Liver weight (g)	all	0.8	0.0	1.2	0.0	1.0	0.0	1.2	0.0	***	***	*
	7	1.1	0.0	1.4	0.0	1.1	0.0	1.3	0.0	**	***	*
Hold time (min)	all	154.0	3.7	148.1	4.6	96.9	4.6	95.0	4.2	***	NS	NS
	7	147.4	5.7	145.6	6.4	95.1	6.6	98.5	5.5	***	NS	NS
SumICH	all	1.7	0.4	1.9	0.4	2.1	0.5	2.5	0.5	NS	NS	NS
	7	1.8	0.6	2.1	0.6	2.9	0.8	2.9	0.7	NS	NS	NS
PCV (%)[d]	all	45.4	0.5	43.5	0.7	46.2	0.5	44.4	0.4	NS	***	NS
	7	45.7	0.7	43.8	1.0	46.6	0.6	44.4	0.5	NS	**	NS
Albumin (g/dL)	all	1.1	0.0	0.9	0.0	1.0	0.0	0.9	0.0	*	***	NS
	7	1.1	0.0	0.9	0.0	1.0	0.0	0.9	0.0	*	***	NS

		Sitka Sound				Prince William Sound						
		Males		Females		Males		Females		Significance[a]		
Variable	age	mean	SE	mean	SE	mean	SE	mean	SE	site	sex	site*sex
$\log_e$ IgM (mg/mL)[e]	all	751.0	29.2	668.8	39.5	999.7	64.5	947.3	78.3	***	NS	NS
	7	831.4	48.5	753.1	59.5	1198.1	99.5	1095.3	112.3	***	NS	NS
Total protein (g/dL)	all	2.5	0.0	2.3	0.1	2.5	0.0	2.1	0.1	*	***	NS
	7	2.8	0.1	2.4	0.1	2.6	0.1	2.2	0.1	*	***	NS
ALP (U/L)	all	52.0	1.2	58.4	1.9	46.6	1.1	50.1	1.4	***	***	NS
	7	53.7	1.6	58.8	2.7	47.3	1.5	51.1	1.8	**	*	NS
ALT (U/L)	all	4.2	0.2	4.7	0.3	4.3	0.2	4.6	0.5	NS	NS	NS
	7	4.0	0.4	4.1	0.5	4.6	0.3	3.9	0.4	NS	NS	NS
AST (U/L)	all	568.9	22.3	717.2	42.8	279.6	24.3	288.7	39.2	***	*	*
	7	493.9	36.7	644.9	57.7	291.6	33.2	257.9	48.0	***	NS	NS
$\log_e$ CPK (U/L)[e]	all	1074.3	81.2	1260.5	174.7	1285.1	130.2	1487.8	276.3	NS	NS	NS
	7	878.9	119.1	1141.1	281.3	1283.0	169.2	1068.0	197.6	NS	NS	NS
GGT (U/L)	all	0.9	0.1	0.7	0.1	1.2	0.1	0.8	0.1	NS	*	NS
	7	1.0	0.1	0.6	0.1	1.5	0.2	0.7	0.1	*	***	NS
Calcium (mg/dL)	all	12.2	0.1	12.0	0.2	11.5	0.1	11.2	0.1	***	*	NS
	7	12.4	0.2	11.8	0.3	11.6	0.1	11.3	0.1	***	*	NS
Chloride (mmol/L)	all	143.2	1.3	141.5	1.3	135.8	3.4	140.3	3.4	NS	NS	NS
	7	144.8	2.6	139.6	1.7	128.9	4.7	143.1	4.1	NS	NS	**
Cholesterol (mg/dL)	all	273.5	5.7	200.5	5.1	289.8	7.6	217.1	7.0	*	***	NS
	7	286.2	10.1	201.5	7.3	297.8	10.5	227.8	8.6	*	***	NS

		Sitka Sound				Prince William Sound						
		Males		Females		Males		Females		Significance[a]		
Variable	age	mean	SE	mean	SE	mean	SE	mean	SE	site	sex	site*sex
$\log_e CO_2$ (mmol/L)[e]	all	13.2	0.3	13.6	0.8	8.4	0.3	7.9	0.3	***	NS	NS
	7	13.7	0.5	13.4	1.1	8.3	0.3	8.2	0.3	***	NS	NS
Glucose (mg/dL)	all	105.8	3.5	91.6	3.4	129.9	4.1	91.3	2.6	***	***	***
	7	124.2	5.3	101.2	4.6	136.9	5.2	95.6	3.2	NS	***	*
Lactate (mmol/dL)	all	96.0	3.8	93.3	4.5	73.8	3.2	68.1	2.7	***	NS	NS
	7	95.4	6.2	97.5	6.8	77.6	4.1	72.4	3.4	***	NS	NS
Osmolality	all	423.4	3.5	420.3	5.0	418.5	1.5	410.6	1.6	*	NS	NS
(mOsm/kg)	7	425.8	5.1	425.6	5.8	419.3	1.8	413.9	1.8	*	NS	NS
Phosphorus	all	7.4	0.2	7.4	0.3	4.8	0.2	5.6	0.1	***	*	NS
(mg/dL)	7	7.6	0.4	7.7	0.4	4.8	0.2	5.5	0.2	***	NS	NS
Potassium (mmol/L)	all	2.4	0.1	2.4	0.1	1.7	0.1	1.8	0.1	***	NS	NS
	7	2.2	0.1	2.3	0.1	1.7	0.1	1.9	0.1	***	NS	NS
Sodium (mmol/L)	all	209.7	1.2	207.7	1.6	191.1	0.6	184.3	2.2	***	**	NS
	7	209.3	2.0	207.6	1.8	192.0	0.8	183.5	3.4	***	*	NS
Total bilirubin	all	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	**	NS	NS
(mg/dL)	7	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	NS	NS	*
Basophils (%)	all	1.0	0.1	0.8	0.1	0.5	0.1	0.4	0.1	***	NS	NS
	7	1.0	0.2	0.7	0.1	0.4	0.1	0.6	0.1	***	NS	NS
Eosinophils (%)	all	0.6	0.1	0.4	0.1	0.5	0.1	0.6	0.1	NS	NS	NS
	7	0.6	0.2	0.4	0.1	0.4	0.1	0.6	0.1	NS	NS	NS

		Sitka Sound				Prince William Sound						
		Males		Females		Males		Females		Significance[a]		
Variable	age	mean	SE	mean	SE	mean	SE	mean	SE	site	sex	site*sex
Lymphocytes (%)	all	15.9	0.8	16.0	0.9	27.4	1.2	21.0	1.0	***	**	***
	7	15.5	1.3	16.1	1.2	25.8	1.5	19.9	1.2	***	NS	**
Monocytes (%)	all	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	NS	NS
	7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	NS	NS
Neutrophils (%)	all	9.4	0.7	6.7	0.5	9.1	0.7	7.7	0.7	NS	**	NS
	7	10.4	1.2	7.4	0.8	9.4	1.0	8.3	0.9	NS	*	NS
Thrombocytes (%)	all	73.1	1.0	76.1	1.0	62.4	1.3	70.3	1.2	***	***	*
	7	72.5	1.7	75.4	1.4	63.9	1.6	70.6	1.7	***	**	NS

[a]Significance is designated as $P > 0.05$ (NS), $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***).

[b]Sample size of all ages varies slightly for some variables, but usually was as follows: Sitka Sound males (n = 146), Sitka Sound females (n = 94), Prince William Sound males (n = 94), Prince William Sound females (n = 86).

[c]Sample size of 7-yr-olds varies slightly for some variables, but usually was as follows: Sitka Sound males (n = 56), Sitka Sound females (n = 50), Prince William Sound males (n = 50), Prince William Sound females (n = 55).

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

[e]Values for CO_2 and CPK were ln transformed for analysis of variance, but true means and standard errors of actual % values are reported here.

Table 8. Significantly different values (ANOVA, $P \leq 0.05$) based on age (yrs). Pacific herring were sampled during spawning in Prince William Sound (PWS) and Sitka Sound (SS), Alaska, 1995. Note that gonads from all fish were in spawning condition; some fish from Sitka Sound were partly or completely spawned out, whereas no fish from Prince William Sound had spawned before being sampled. 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.

	Age = 2, 3 (PWS, n = 19) (SS, n = 93)		Age = 4, 5, 6 (PWS, n = 31) (SS, n = 37)		Age = 7 (PWS, n = 105) (SS, n = 106)		
Variable	Mean	SE	Mean	SE	Mean	SE	*P*-value
Plasma chemistry							
albumin (g/dL) - SS	0.93	0.02	1.02	0.03	1.05	0.02	0.002
AST[a] (U/L) - SS	634.6	63.5	547.3	80.7	495.2	49.6	0.003
calcium (mmol/L) - SS	11.87	0.20	12.79	0.27	12.09	0.18	0.038
cholesterol (mg/dL) - SS	233.2	6.2	266.1	11.9	245.9	7.5	0.053
CPK[a] (mmol/L) - PWS	1449.5	598.2	835.5	313.1	746.2	139.7	0.026
CPK[a] (mmol/L) - SS	967.8	169.7	702.7	199.6	638.4	113.2	0.004
glucose (mg/dL) - SS	86.5	3.8	98.2	6.8	113.3	3.7	<0.001
IgM[a] (mmol/L) - PWS	422.4	102.8	686.1	113.5	967.8	106.4	<0.001
IgM[a] (mmol/L) - SS	552.8	56.4	669.8	97.5	709.8	65.5	0.001
osmolality (mOsm/kg) - SS	413.3	5.2	433.0	7.4	425.7	3.8	0.041
total protein (g/dL) - SS	2.27	0.05	2.51	0.10	2.60	0.05	<0.001

	Age = 2, 3 (PWS, n = 19) (SS, n = 93)		Age = 4, 5, 6 (PWS, n = 31) (SS, n = 37)		Age = 7 (PWS, n = 105) (SS, n = 106)		
Variable	Mean	SE	Mean	SE	Mean	SE	*P*-value
Hematology							
lymphocytes[b] (%) - PWS	33.1	4.6	27.0	4.3	21.9	1.9	<0.001
neutrophils[b] (%) - PWS	2.83	1.4	6.71	1.9	7.96	1.2	<0.001
Weight and Length							
body weight (g) - PWS	75.5	2.7	120.8	3.5	153.5	2.7	<0.001*
body weight (g) - SS	85.8	1.8	119.3	5.3	153.3	2.7	<0.001*
gonad weight (g) - PWS	12.6	0.8	25.5	1.4	35.5	0.9	<0.001*
gonad weight (g) - SS	12.9	0.7	21.6	2.3	30.2	1.6	<0.001*
liver weight (g) - PWS	0.55	0.03	0.89	0.05	1.19	0.03	<0.001*
liver weight (g) - SS	0.65	0.03	0.86	0.05	1.25	0.03	<0.001*
length (mm) - PWS	183.2	1.6	210.5	1.5	224.6	1.0	<0.001
length (mm) - SS	185.1	1.2	201.1	2.4	215.9	1.0	<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), $\log_e$IgM, and blood values in Pacific herring sampled from Prince William Sound (PWS) and Sitka Sound (SS), Alaska, during spawning, 1995. Note that gonads from all fish were in spawning condition; some fish from Sitka Sound were partly or completely spawned out, whereas no fish from Prince William Sound had spawned before being sampled. Significant correlations ($P < 0.05$) are denoted (*); sample size varies from 170 to 180 for PWS and 220 to 240 for SS. Values for PCV and white blood cells were arcsine square root transformed for analysis.

Variable - site	Age	Body weight	Length	Gonad weight	Hold time	sumICH	Albumin	$\log_e$IgM
Body weight - PWS	0.717*							
Body weight - SS	0.796*							
Length - PWS	0.795*	0.897						
Length - SS	0.786*	0.906*						
Gonad weight - PWS	0.601*	0.833*	0.779*					
Gonad weight - SS	0.510*	0.832*	0.659*					
Hold time - PWS	-0.008	0.002	-0.008	0.059				
Hold time - SS	-0.145*	-0.090	-0.100	-0.023				
SumICH - PWS	0.088	0.011	0.038	-0.055	-0.096			
SumICH - SS	0.029	-0.008	0.030	-0.000	-0.007			
Albumin - PWS	0.134	0.277*	0.198*	0.156*	-0.299*	0.063		
Albumin - SS	0.236*	0.274*	0.264*	0.116	-0.054	0.082		
IgM - PWS	0.264*	0.210*	0.262*	0.140	-0.167*	0.367*	0.362*	
IgM - SS	0.227*	0.174*	0.206*	0.081	-0.116	0.335*	0.247*	
Liver weight - PWS	0.656*	0.766*	0.769*	0.675	-0.008	0.151	0.138	0.106
Liver weight - SS	0.732*	0.832*	0.782*	0.640*	-0.162*	0.084	0.189*	0.142*

Variable - site	Age	Body weight	Length	Gonad weight	Hold time	sumICH	Albumin	$\log_e$IgM
PCV - PWS	-0.067	-0.051	-0.065	-0.086	-0.262*	-0.068	0.386*	0.195*
PCV - SS	0.108	0.239*	0.155*	0.258*	-0.061	-0.166*	0.250*	-0.051
Total protein - PWS	0.053	0.201*	0.115	0.092	-0.356*	0.132	0.924*	0.361*
Total protein - SS	0.281*	0.424*	0.356*	0.357*	-0.050	0.132*	0.692*	0.221*
$\log_e$ AST - PWS	-0.062	-0.202*	-0.166*	-0.226*	-0.099	0.197*	-0.174*	0.067
$\log_e$ AST - SS	-0.208*	-0.209*	-0.206*	-0.151*	0.141	0.282*	0.021	0.081
ALP - PWS	0.082	0.202*	0.183*	0.218*	-0.239*	0.079	0.331*	0.196*
ALP - SS	0.106	0.115	0.087	-0.008	-0.038	0.117	0.412*	0.136*
$\log_e$ ALT - PWS	-0.022	-0.075	-0.044	-0.040	-0.007	0.089	-0.067	0.146
$\log_e$ ALT - SS	-0.120	-0.135*	-0.158*	-0.078	0.169*	-0.082	0.004	-0.083
$\log_e$ CPK - PWS	-0.126	-0.2404	-0.205*	-0.256*	-0.213*	0.110	-0.101	0.003
$\log_e$ CPK - SS	-0.170*	-0.221*	-0.201*	-0.176*	-0.002	0.183*	0.009	-0.039
GGT - PWS	0.198*	0.146*	0.176*	0.072	-0.101	0.031	0.076	0.215*
GGT - SS	0.009	0.015	0.011	-0.030	-0.138*	0.159*	0.154*	0.217*
Calcium -PWS	-0.033	0.056	-0.043	-0.013	-0.036	0.064	0.521*	0.241*
Calcium - SS	0.051	0.091	0.099	0.104	0.209*	0.027	0.298*	-0.024
Chloride -PWS	-0.034	0.034	0.024	0.053	0.046	-0.086	0.110	-0.046
Chloride -SS	0.064	0.178*	0.163*	0.232	-0.135*	-0.018	0.042	0.043
Cholesterol -PWS	0.050	0.175*	0.079	0.074	-0.230*	-0.080	0.814*	0.182*
Cholesterol -SS	0.118	0.204*	0.162*	0.089	-0.047	-0.079	0.565*	0.179*
CO_2 - PWS	0.005	0.003	0.039	0.015	0.566*	-0.130	-0.229*	-0.041
CO_2 - SS	0.004	-0.008	0.019	-0.059	0.227*	0.024	0.168*	0.035

Variable - site	Age	Body weight	Length	Gonad weight	Hold time	sumICH	Albumin	$\log_e$IgM
Glucose -PWS	0.014	0.188*	0.095	0.079	-0.107	0.041	0.625*	0.105
Glucose - SS	0.306*	0.466*	0.371*	0.441*	-0.278*	-0.123	0.292*	0.142*
Lactate - PWS	0.000	0.178*	0.055	0.171*	0.144	0.037	0.345*	0.070
Lactate - SS	0.066	0.138*	0.117	0.133*	0.270*	-0.055	0.286*	-0.022
Osmolality - PWS	0.061	0.169*	0.143	0.137	-0.070	-0.015	0.381*	0.163*
Osmolality - SS	0.127	0.050	0.143*	-0.098	0.192*	-0.003	-0.009	-0.026
Phosphorus -PWS	-0.021	-0.070	-0.039	-0.039	-0.471*	0.084	0.032	0.144
Phosphorus -SS	0.060	-0.021	0.033	-0.139*	0.180*	-0.036	0.144*	-0.020
Potassium -PWS	-0.101	0.008	-0.032	0.048	0.661*	-0.070	-0.212*	-0.199*
Potassium -SS	-0.148*	-0.179*	-0.159*	-0.161*	0.608*	-0.035	-0.107	-0.121
Sodium - PWS	-0.003	-0.049	-0.020	0.044	0.151*	0.057	-0.063	-0.023
Sodium - SS	0.033	-0.037	0.094	-0.166*	0.275*	-0.013	-0.007	-0.078
Total bilirubin - PWS	-0.006	-0.004	0.017	0.059	0.117	-0.146*	0.088	-0.150*
Total bilirubin - SS	-0.136*	-0.143*	-0.048	-0.125	0.260*	-0.064	-0.077	-0.020
Thrombocytes - PWS	0.152	0.185	0.192*	0.206*	-0.104	-0.114	-0.134	-0.016
Thrombocytes - SS	-0.102	-0.000	-0.055	0.101	-0.065	-0.105	0.043	0.035
Lymphocytes - PWS	-0.337*	-0.240*	-0.304*	-0.272*	0.150*	-0.046	0.171*	-0.199*
Lymphocytes - SS	0.039	0.083	0.068	0.119	-0.075	0.060	-0.075	-0.115
Neutrophils - PWS	0.328*	0.142	0.226*	0.140	-0.044	0.244*	0.012	0.330*
Neutrophils - SS	0.111	-0.113	-0.016	-0.325*	0.144*	0.044	0.020	0.073
Basophils - PWS	-0.015	-0.099	-0.053	-0.161*	-0.039	0.165*	-0.108	0.034
Basophils - SS	0.014	0.028	0.036	0.047	0.152*	0.122	0.100	0.160*

Variable - site	Age	Body weight	Length	Gonad weight	Hold time	sumICH	Albumin	$\log_e$IgM
Eosinophils - PWS	-0.058	-0.079	-0.033	-0.028	0.051	-0.054	-0.047	0.071
Eosinophils - SS	-0.046	-0.112	-0.117	-0.087	0.159*	0.080	-0.098	0.012
Monocytes - PWS	0.020	0.029	0.045	0.001	-0.033	-0.050	0.098	-0.027
Monocytes - SS	-0.071	-0.042	-0.045	-0.058	0.040	-0.027	0.029	-0.114
# Anisakidae - PWS	-0.102	-0.145	-0.075	-0.166*	0.073	-0.002	-0.205*	-0.007
# Anisakidae - SS	-0.247*	-0.178*	-0.200*	-0.126	0.047	-0.103	-0.035	0.006

Table 10. Sample prevalence (%) of parasites and virus in adult Pacific herring in Prince William Sound, Alaska, 1989-1995.

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

[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 in 1994 and 1995, and those results are in parentheses.

[b]Prevalence values for *Ortholinea orientalis* are for histopathology. Note that touch preparations of kidney were examined in 1994 and 1995, and those results are included in the overall prevalence values 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. In Press)

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

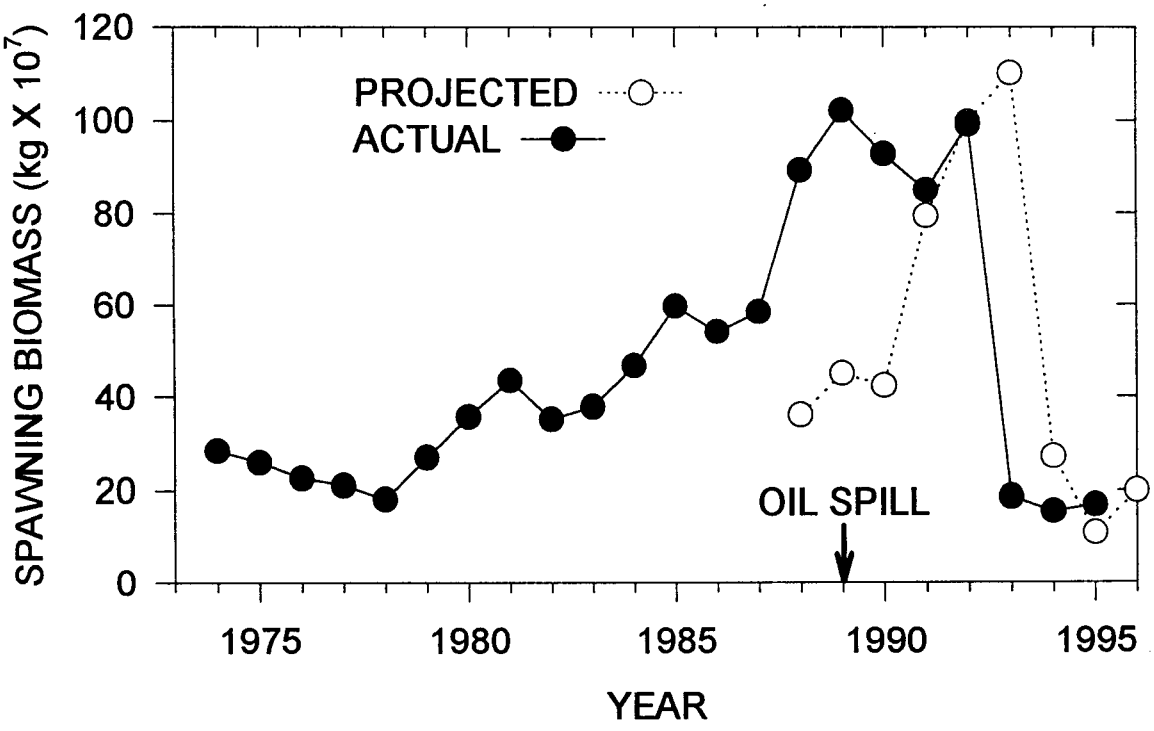

Figure 1. Biomass estimates of mature Pacific herring in Prince William Sound, Alaska. Unexploited spawning biomass projected in the year before spawning (PROJECTED) and calculated after spawning (ACTUAL) using the age-structure assessment model. Estimates were made by Fritz Funk, Alaska Department of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

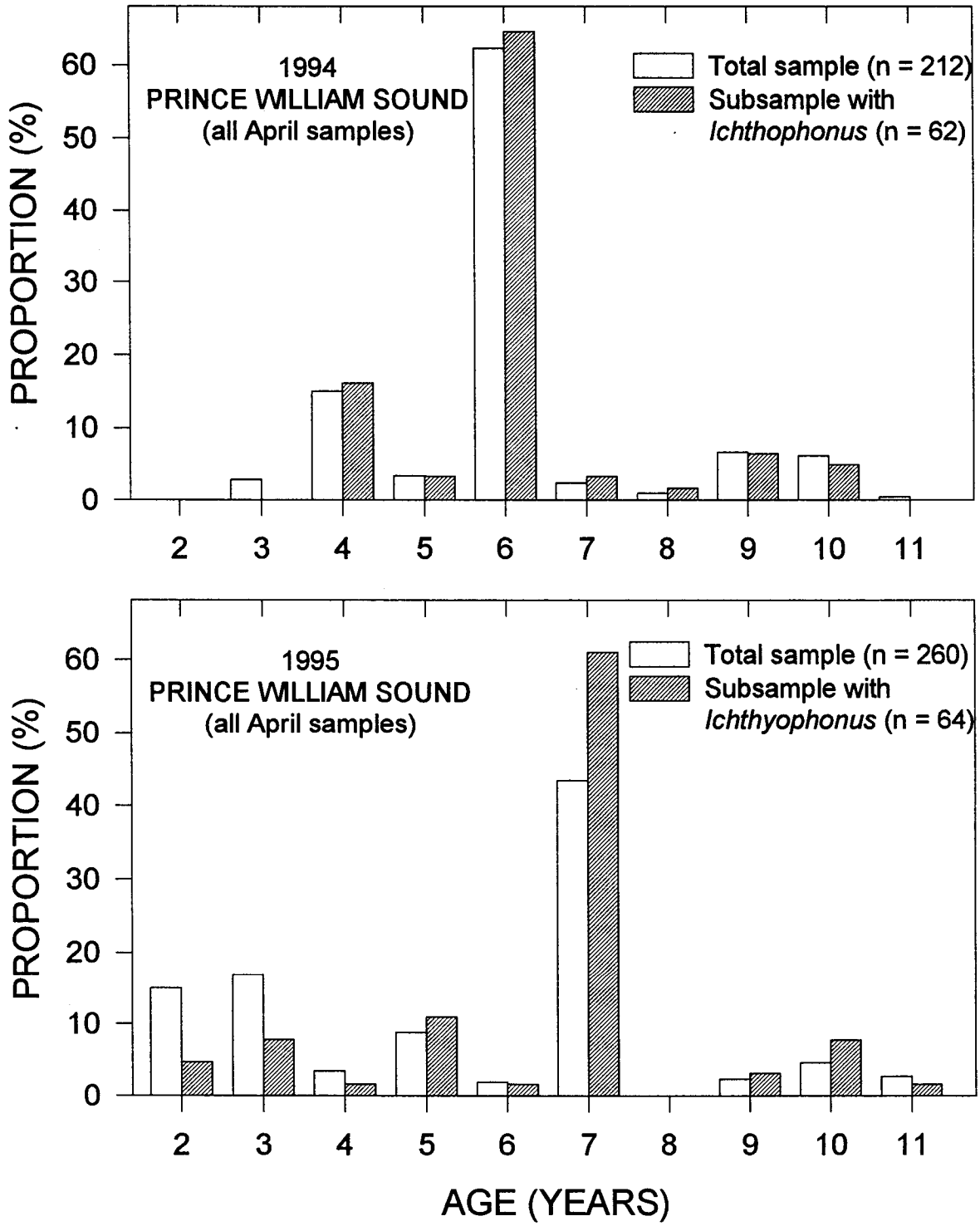

Figure 2. Age distribution of spawning Pacific herring in Prince William Sound, Alaska, that had *Ichthyophonus* compared with the age distribution of fish that were examined for *Ichthyophonus*. Top - Pacific herring sampled during April, 1994. Bottom - Pacific herring sampled during April, 1995.

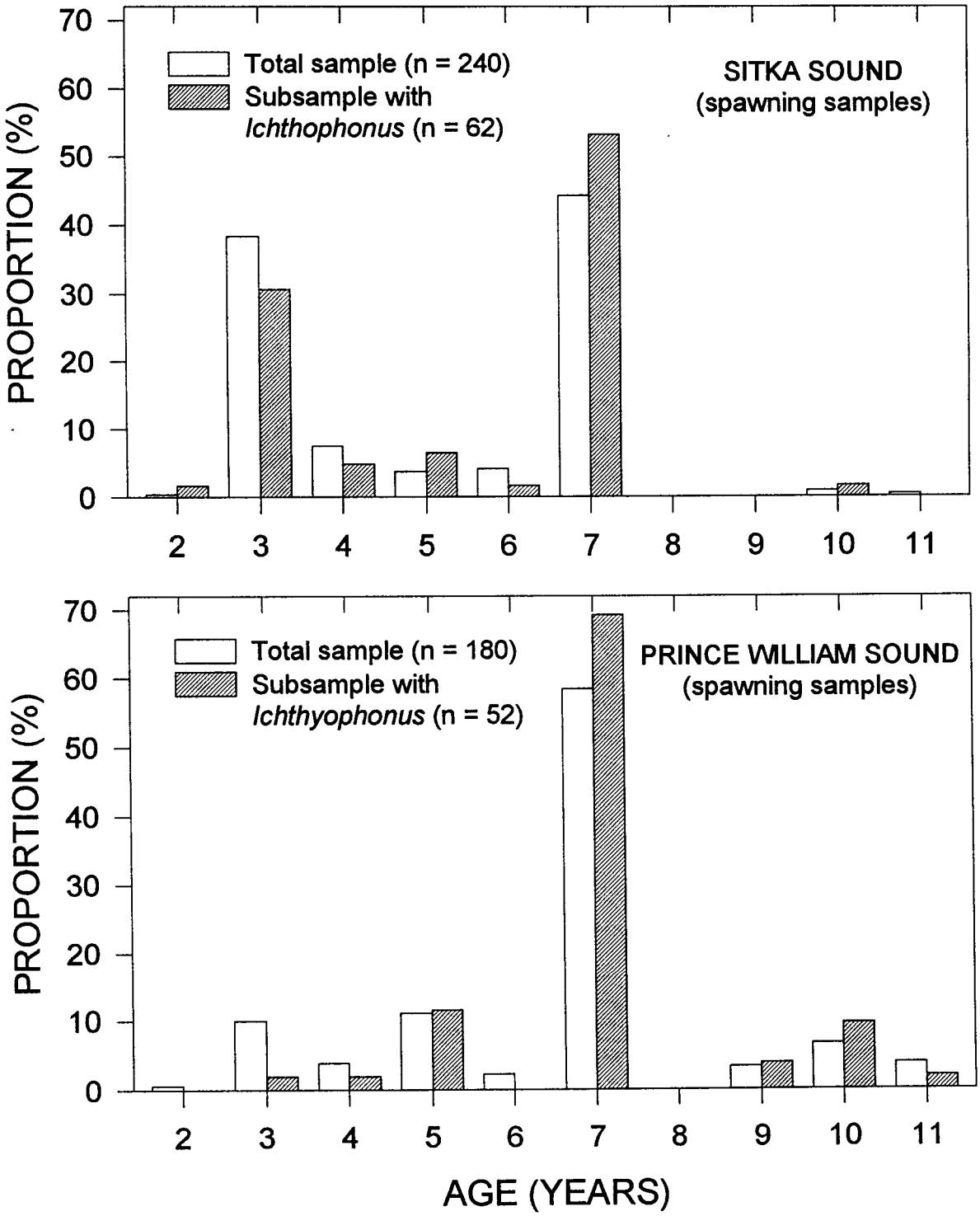

Figure 3. Age distribution of spawning Pacific herring that had *Ichthyophonus* compared with the age distribution of fish that were examined for *Ichthyophonus*. Top - Pacific herring sampled from Sitka Sound, Alaska, during March, 1995. Bottom - Pacific herring sampled from Prince William Sound, Alaska, during April, 1995.

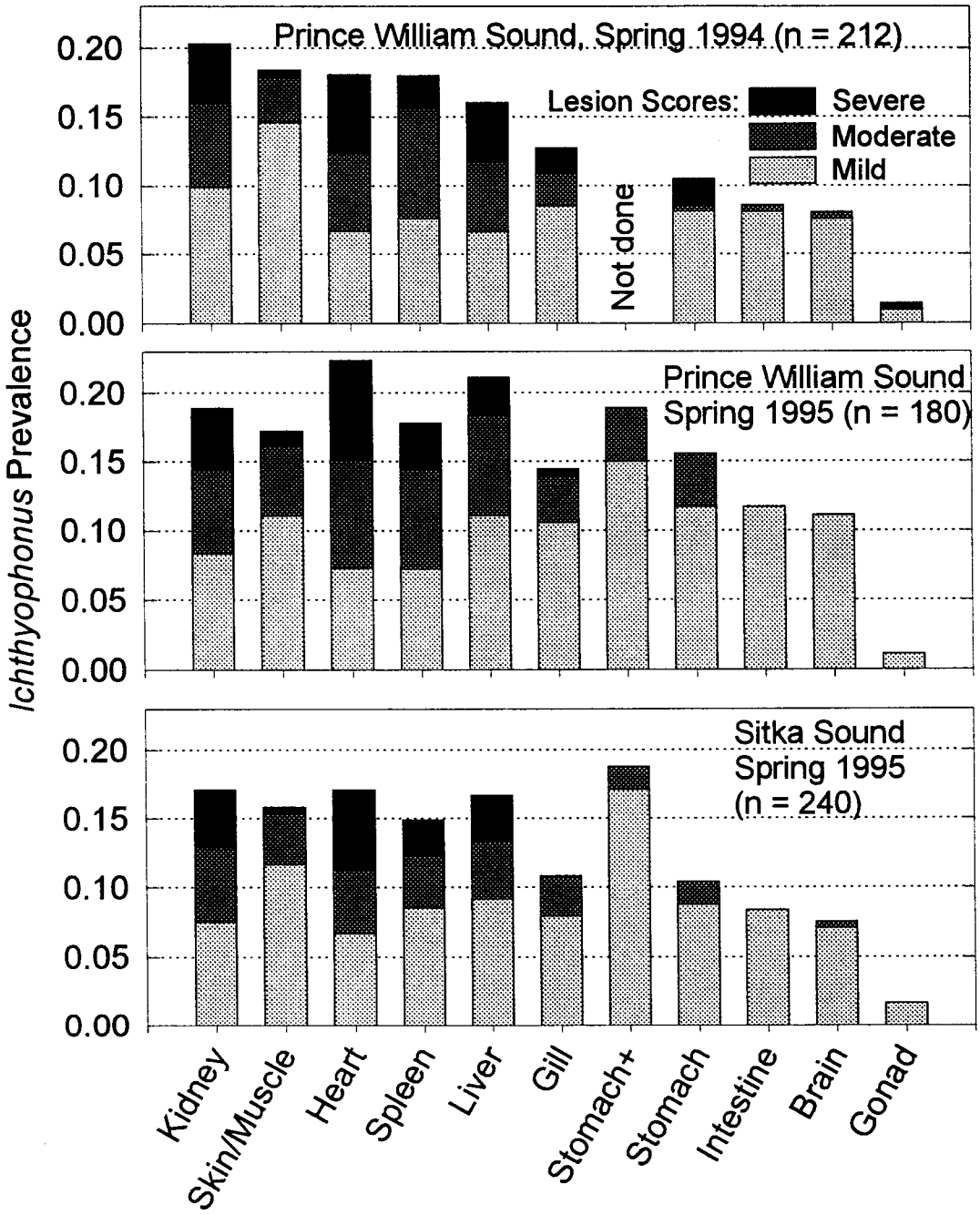

Figure 4. Sample prevalence of *Ichthyophonus* lesion scores in various organs of mature Pacific herring sampled from Prince William Sound in 1994 and 1995, and Sitka Sound in 1995. Lesions were scored as none (0), mild (1), moderate (2), or severe (3).

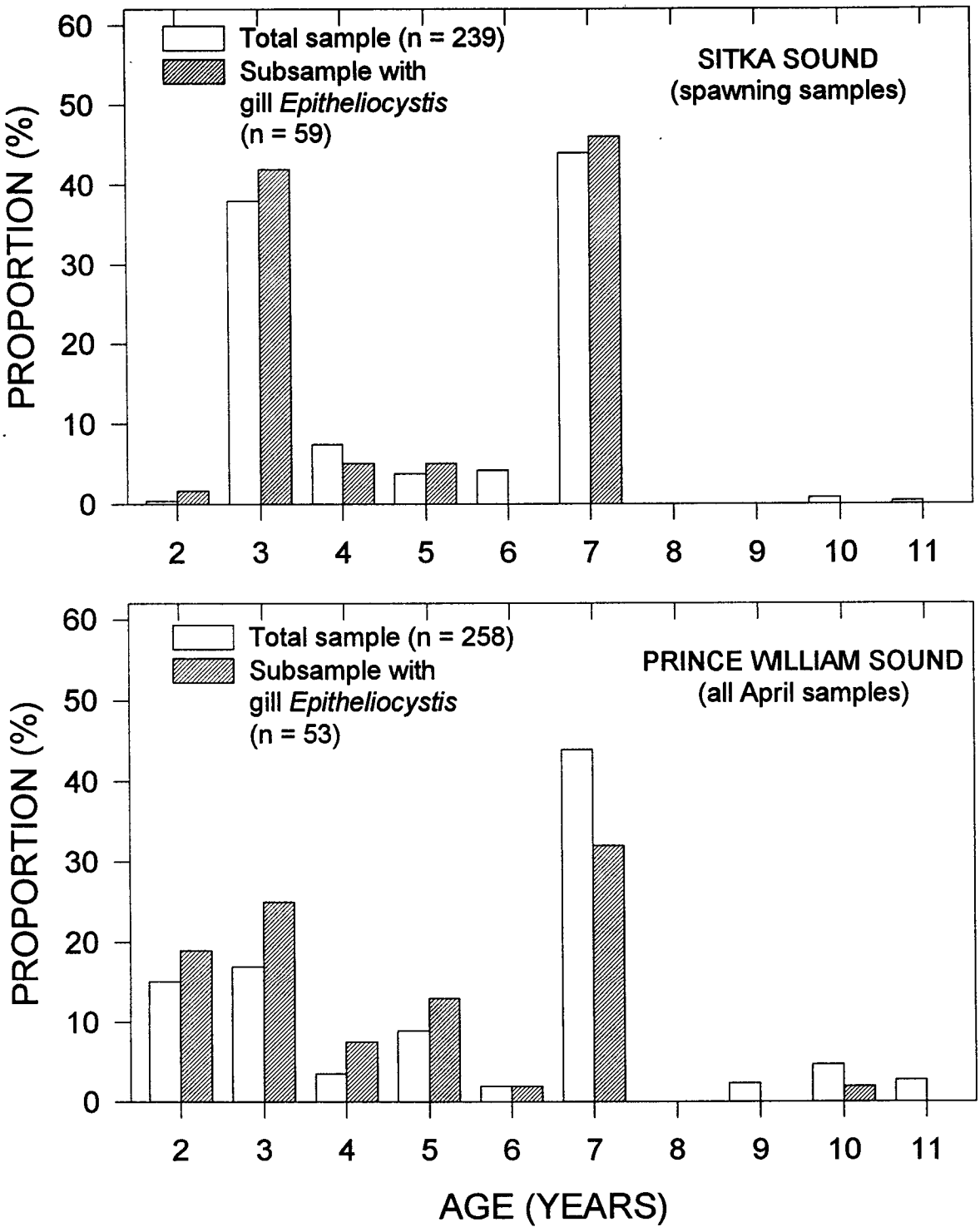

Figure 5. Age distribution of spawning Pacific herring in 1995 that had branchial *Epitheliocystis* compared with the age distribution of fish that were examined for branchial *Epitheliocystis*. Top - Samples from Sitka Sound, Alaska. Bottom - Samples from Prince William Sound, Alaska.

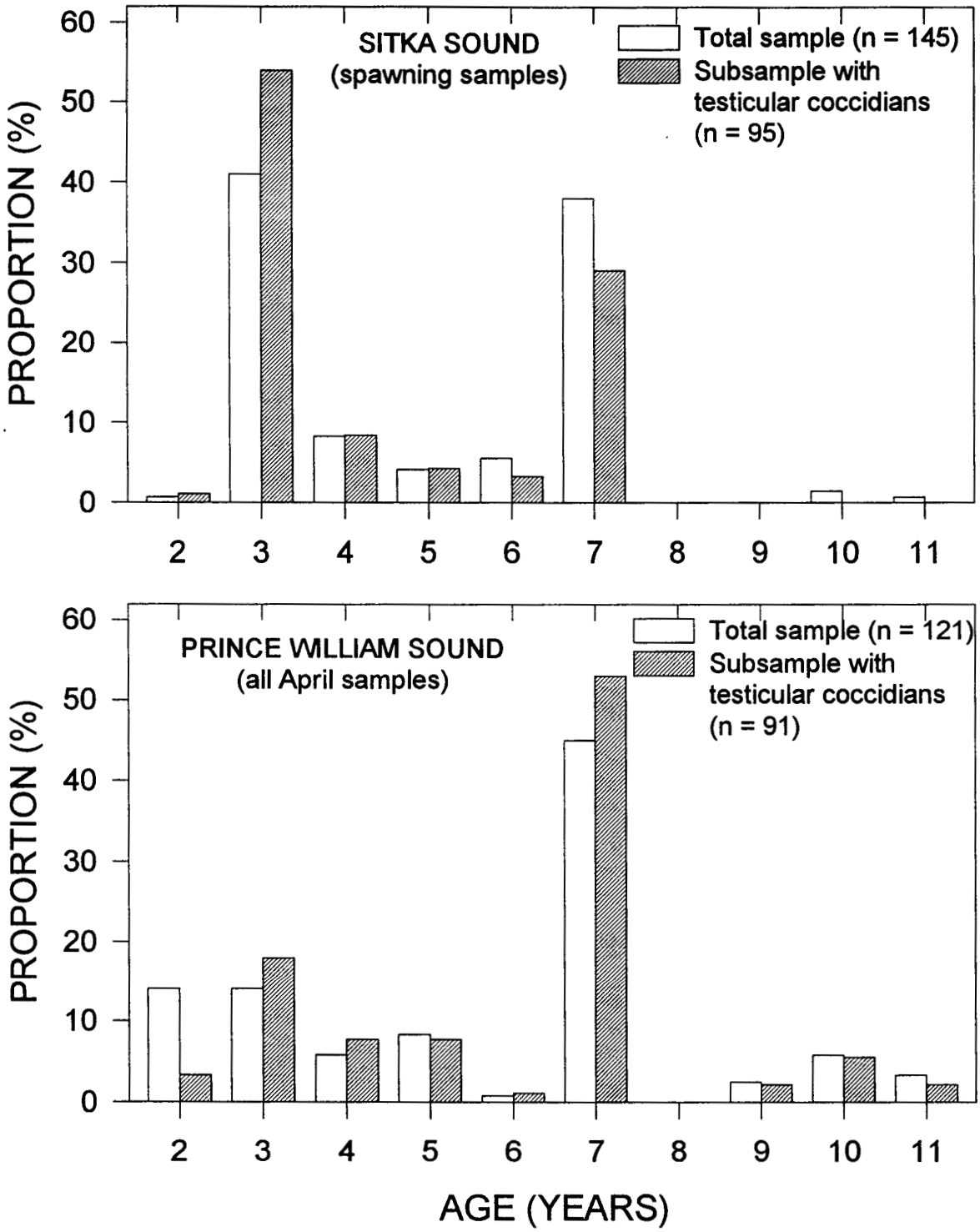

Figure 6. Age distribution of male Pacific herring in 1995 that had testicular coccidians (*Eimeria sardinae*) compared with the age distribution of fish that were examined for testicular coccidians. Top - Samples from Sitka Sound, Alaska. Bottom - Samples from Prince William Sound, Alaska.

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 95320S
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.

R.M. Kocan
M.L. Landolt

School of Fisheries
Box 355100
University of Washington
Seattle, WA 98195

J.R. Winton

National Biological Service
7500 Sandpoint Way NE
Seattle, WA 98115

April 1996

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 95320S
Annual Report

Study History: Restoration Project 95320S was initiated in response to an RFP issued by Alaska Department of Fish and Game and was intended to determine the relationship of diseases on the declines in Prince William Sound herring populations since 1993. A three component work plan was prepared: 1) Field component (University of California, Davis); 2) Controlled infection component (University of Washington, 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: Viral hemorrhagic septicemia virus (VHSV) was conclusively shown to be capable of causing disease and extensive mortality in nonimmune juvenile Pacific herring. SPF herring began dying 7 days post-exposure with peak mortality occurring on days 10-11. No mortality was observed in SPF herring exposed to VHSV concentrations of $< 2 \times 10^2$ PFU$*$ml^{-1} and no virus could be isolated from their tissues. Virus was first detected in tissues of experimentally infected SPF herring 48 h post-exposure and peaked at 96 h. 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 moribund fish 2 to 8 days post-exposure revealed: 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. No virus was isolated from any wild herring 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 revealed > 90% of the dead fish had $> 1 \times 10^6$ PFU$*$gm^{-1} tissue. Ninety percent of the live fish sampled from the same tanks carried slightly lower titers of virus from 2 to 14 days post-capture, then virus titers declined until they were undetectable by 4 weeks post-capture. Mortality was significantly less (< 10%) in older fish. Surviving herring exposed to $1X10^3$ to $1X10^6$ PFU$*$ml^{-1} for 1 hour 6-8 weeks post-capture exhibited no mortalities in any age class and no virus could be isolated from tissues of these fish 10 days post-exposure. 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 (spores and small holes) were detectable by 36 days, as were spores in the musculature under the skin. By 56 days post exposure 90% of the fish were dead. Infected tissues from these herring were cultured then injected IP into coastrange 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 cultured positive for *Ichthyophonus*. No control sculpins were found to be naturally infected. 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 8% 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.

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

Citation:

Kocan, R.M., M.L. Landolt, and J.R. Winton. 1996. 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 Annual Report (Restoration Project 95320S), University of Washington, Seattle, Washington.

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Executive Summary

Introduction

In 1993, four years after the Exxon Valdez oil spill in Prince William Sound, there was a dramatic and unexpected loss of Pacific herring. The spawning biomass declined from an expected 120K tons to less than 30K tons. This decline continued into 1994 when less than 20K tons of spawning adults returned to Prince William Sound. A study designed to evaluate the health of the returning adults in 1993 and 1994 revealed that there was an unexpected appearance of the agent which causes viral hemorrhagic septicemia (VHS) in 1993 and a significant increase in prevalence of Ichthyophonus hoferi, as suspected pathogen of herring in 1994. Because of these findings a disease study was initiated with the objective of clarifying the role of these pathogens in the decline of Pacific herring in Prince William Sound.

Objectives

Laboratory rearing of Pacific herring

- Obtain and hatch herring eggs
- Rear herring larvae for experimental use

Verification of disease-free laboratory herring

- Histopathology
- In vitro culture of *Ichthyophonus*
- In vitro culture of VHSV

Challenge without stressors

- Challenge lab-reared herring with VHSV
- Challenge lab-reared herring with *Ichthyophonus*
- Assay experimental fish for VHSV & *Ichthyophonus*

Continuing '95 projects ('95 -> '96)

Methods

Specific Pathogen-free (SPF) herring: Pacific herring were artificially spawned and their eggs incubated in filtered UV-sterilized seawater. The newly hatched larvae were initially fed oyster trochophores and rotifers, followed by brine shrimp nauplei treated with Selco@ and finally frozen adult brine shrimp, commercial trout chow and frozen krill. The growth rate and disease status of the larvae and juveniles was monitored daily, as was the water quality in which they were rearing. When the fish reached 90 days old, they were used for controlled disease studies.

Challenge without stressors: Pathogen-free 5-month-old herring were challenged with both VHS virus (VHSV) and *I. hoferi* to determine the pathogenicity of these organisms in the absence of physical or chemical stressors. Increasing concentrations of VHSV ($7X10^2$; $7X10^4$; $7X10^6$ PFU) was added to aquarium water for 1 hour, after which the water was turned on and the fish allowed to develop the disease without further experimental exposure.

I. hoferi was injected intraperitoneally into herring, rainbow trout and Japanese medaka to determine its host range and growth characteristics.

Naturally infected herring: Wild 0-year Puget Sound herring were netted from herring balls from August through October and brought into the laboratory for study. Fish were initially housed at about 200 fish per 70 gallon tank. Subsequent catches were distributed into tanks in densities ranging from 5 fish per tank to 85 fish per tank in order to study the effects of density as well as capture stress. These fish were used for both VHS and *Ichthyophonus* studies.

Fish-to-fish transmission: Wild 0-year herring which survived an epizootic of VHS but still showing signs of disease (eg. hemorrhaging) were introduced into tanks of SPF herring. The SPF fish were observed for signs of disease for three weeks, dead fish were collected for viral assay, and at the end of the study all fish were assayed for the presence of VHSV.

Density as a stressor: Wild 0-year herring were distributed into 70 gallon tanks at densities ranging from 5 fish/tank to 85 fish/tank to determine if density has an effect on the natural epizootics observed in these fish after they were brought into the laboratory. Fish were monitored for mortality and dead fish assayed for VHSV. At the termination of the study all survivors were also assayed for virus.

VHSV in rainbow trout with & without pristane
The rainbow trout (*Oncorhynchus mykiss*) was examined as a possible surrogate laboratory species for studying the effect of VHS. In conjunction with studies on infectivity and pathogenicity, some fish were exposed to pristane, a branched alkane known to have immunosuppressant effects in mammals. The rationale being that crude oil contains numerous molecules other than polycyclic aromatic hydrocarbons that may affect the health of aquatic organisms following an oil spill such as occurred in 1989 in Prince William Sound. Pristane is a naturally occurring compound in some marine organisms as well as a component of crude oil. Considering these relationships, it was felt that a preliminary look at its effects on the immune system of fish was in order.

Four groups of 30 fish each were used to examine the infectivity and pathogenicity of VHS virus and the influence of pristane on the course of infection. The fish were 6-months-old with mean lengths and weights at the start of the study of 13.7 mm and 99.1 gm. The exposed groups were exposed to 1X10^6 PFU$*$ml^{-1} for one hour, while the controls consisted of unexposed controls, culture medium alone (MEM) and pristane plus MEM.

Results

Specific Pathogen-free (SPF) herring: The mean growth rate for SPF herring for the first 90 days was 0.33 mm* d-1. By 6 months post-hatch they were 4.5-6.0 cm in length. Periodic histologic examination of tissues as well as in vitro culture of specific organs revealed neither VHSV or I. hoferi. No other pathogens could be identified and the fish were considered to be "pathogen-free" and suitable for use in controlled disease studies.

Challenge without stressors: SPF herring proved to be highly susceptible to VHSV when infected via water-borne exposure. By two weeks post exposure 100% (29/29) mortality occurred in fish exposed to $7X10^6$ PFU, 95% mortality in those exposed to $7X10^6$ PFU (18/19) and 62% (16/26) exposed to $7X10^2$ PFU. Fish dying during this period had > 6 logs of virus / gm tissue regardless of the level of initial exposure. Eight survivors of the

low dose were below detection limits while one was just above the detection limit (eg. 2.9 PFU). The single survivor of the medium dose had just 4.8 PFU per gram tissue.

This study fulfilled Koch's Postulates for VHSV as a pathogen of Pacific herring, indicating that it can cause mass mortality in susceptible populations of this species.

Naturally infected herring: Upon initial capture wild 0-year herring showed no signs of infection by VHSV (0/30; 0/30). By the second week in captivity in densities of 200 fish / 70 gallon tank, massive mortalities had occurred, reaching over 50% by week three. The disease prevalence in both live and dead individuals was 100% at this time, but by 4 weeks in captivity the number of individuals with detectable infections had returned to "0". When these fish were challenged with VHSV at $3X10^6$ PFU for one hour they were solidly immune, showing no sighs of mortality or disease.

When wild herring were injected with *Ichthyophonus* they developed active infections, but because both exposed and unexposed fish died at the same rate, it was not possible to show a cause-and-effect relationship between herring mortality and infection by *Ichthyophonus*. Studies are planned to infect SPF herring with this organism to eliminate the complication of multiple natural infections which might cloud the issue of *Ichthyophonus*' capability to cause mortality in this species. Neither the rainbow trout nor Japanese medaka were susceptible to infection by injection of *Ichthyophonus*. Other species (English sole, fresh water sculpin, tide pool sculpin) are being examined to determine the host range of this organism.

Fish-to-fish transmission: Individual wild herring with signs of VHS were capable of transmitting the disease via water to pathogen-free herring. Mortality was 27% in the SPF fish by week 3 with 27% of the survivors testing positive for the virus by the third week.

Density as a stressor: There appeared to be a density related mortality in wild herring captured in September, but not in those captured in October, 1995. The VHSV infection rate was the same regardless of tank density (11% - 17%). The intensity of infection (PFU/gm tissue) was somewhat higher in the October fish, but both groups averaged over 5 PFU/gm tissue. The titer per gram tissue did however, show a distinct decrease from 8 PFU/gm (log10) during the first week in captivity to 0 PFU by the fourth week.

VHSV in rainbow trout with & without pristane
After 3 weeks, mortality was observed only in the VHSV exposed groups with no significant difference between pristane and non-pristane treated groups. Pristane alone had no effect on survival. Fish dying during the first two weeks following exposure were uniformly positive for VHSV, while those dying during the third week were negative even though they showed signs of disease and severe anemia. This is similar to what was observed in wild Pacific herring which experienced an epizootic of VHS following capture and confinement.

Discussion & Conclusions

Pacific herring can be artificially spawned and reared in the laboratory under disease-free conditions and for long enough periods to conduct meaningful studies on herring pathogens. Although they are not as easy to rear under culture conditions as some other species, the techniques for growing this species are well established and they can be cultured successfully if adequate manpower and facilities are dedicated to the effort. Trying to unravel the pathogenesis of specific organisms in wild species in virtually impossible because of the complication of superimposed and uncontrolled infections by other

organisms. SPF herring have allowed us to study one organism at a time (one variable) as well as multiple organisms all under our control. This will allow us to confirm the pathogenicity of both VHSV and *Ichthyophonus hoferi* and establish their role in the massive losses of herring which occurred in Prince William Sound in 1993-1994.

VHS is capable of causing mass mortality in both wild and laboratory reared pathogen-free Pacific herring. The organism appears to be transmitted via water from infected to susceptible fish with an incubation period ranging from 4 to 10 days. Once infected, the virus is amplified in herring tissues and can reach levels of $> 10^6$ PFU / gram tissue. Survival of an epizootic appears to confer a solid immunity to individual fish, even when challenged with extremely high levels of virus.

Wild herring appear to be carrying VHSV by the time they are 5-months-old and are very susceptible to epizootics of VHS when captured or crowded in captivity. They also appear to become solidly immune once they recover from an infection.

Objectives

Laboratory rearing of Pacific herring

Obtain and hatch herring eggs

Rear herring larvae for experimental use

Verification of disease-free laboratory herring

Histopathology

In vitro culture of *Ichthyophonus*

In vitro culture of VHSV

Challenge without stressors

Challenge lab-reared herring with VHSV

Challenge lab-reared herring with *Ichthyophonus*

Assay experimental fish for VHSV & *Ichthyophonus*

Methods

Laboratory rearing of Pacific herring

Obtain and hatch herring eggs

Herring eggs were obtained from Prince William Sound (PWS) in April and from Puget Sound (PS) in May, 1995. Actively spawning herring were captured by gill net, the eggs removed from 8 to 10 females, pooled and broadcast onto a 300 cm^2 piece of nytex netting at a density of 3-5 eggs * cm^{-2}. Once the adherent eggs were securely stuck to the netting, milt from 3-5 males was pooled in sterile seawater and poured over the eggs. Fertilization and transport was in pathogen-free seawater made from aged tap water and sea salts. In the laboratory, each nytex net containing approximately 1,200 eggs was placed into a 70 gal flow-through tank supplied with filtered natural seawater from Admiralty Inlet (Puget Sound) and sterilized with ultraviolet (u.v.) light. Eggs remained in the tanks throughout incubation, hatching and subsequent rearing.

Rear herring larvae for experimental use

Pacific herring larvae were continuously maintained on filtered-u.v. sterilized natural seawater. Larvae were initially fed trochophores (oyster), rotifers (*Brachionus* sp) and *Artemia* larvae. The rotifers and brine shrimp were treated with Super Selco@ for 8 hours prior to feeding in order to maintain adequate levels of omega-3 fatty acids in the larval diet. Trochophores were discontinued after two weeks and rotifers after 12 weeks. At three

months the fry were introduced to frozen brine shrimp and commercial trout chow (1-2 mm) and have been continuously maintained on these food items. During the first 90 days post-hatch, larval growth data was collected every 5-7 days, then monthly thereafter. The entire collection was catalogued and deposited in the University of Washington larval fish collection for future study and teaching purposes.

Verification of disease-free laboratory herring

Histopathology

When the fish were 6-months-old, a subset of 25 randomly selected individuals was taken from 8 different tanks and were fixed in 10% neutral buffered formalin for histologic examination. The organs were examined primarily for the presence of *I. hoferi* and pathologic damage associated with VHSV infection. However, other common herring pathogens were also looked for.

In vitro culture of *Ichthyophonus*

Heart, liver, kidney and spleen were removed from 50 SPF herring at 6-months-old, examined microscopically (wet prep) and cultured in MEM-10. Cultures were microscopically examined weekly for 3 months for the presence of *I. hoferi*. Control cultures were prepared from fish experimentally infected by I.P. injection with spores previously cultured from PWS herring tissues.

In vitro culture of VHSV

A random sample of 6-month-old SPF herring were assayed for the presence of VHSV by culturing the homogenized viscera in the presence of EPC cells and examining the cultures for the presence of plaques. Positive controls consisted of cultures infected with a known amount of virus. Infections were quantitated by comparing the number of plaques produced from each fish.

Disease challenge without stressors

Challenge lab-reared herring with VHS

SPF herring fry were divided into triplicate groups of 10 fish each and exposed for 3 h to increasing concentrations of VHS virus ($6.7X10^2$; $6.7X\ 10^4$ & $6.7X\ 10^6$ $pfu*ml^{-1}$ plus negative controls). After 1 h the water was turned on and the fish were maintained in flowing filtered seawater for 2 weeks, during which time they were observed for visible hemorrhage, mortality, and behavioral changes. As fish died their condition was recorded and they were frozen at -70°C until assayed for virus. The study was terminated after 21 days.

Challenge lab-reared herring with *Ichthyophonus*

Wild herring, rainbow trout and Japanese medaka (*Orizias latipes*) were exposed to up to 10,000 *Ichthyophonus* cells by either intraperitoneal injection (I.P.) or orally (per os).

SPF herring were also exposed to *I. hoferi* by injecting 1,000 spores intraperitoneally into 10 80-90 mm 7-month-old fish. Controls consisted of 10 fish injected with only culture medium (MEM) and 10 fish injected with 1,000 spores of the North Atlantic strain of *I. hoferi* which had been in culture for ~ 2 years. Each group was maintained in a 70 g flow-

through tank and observed for mortality and abnormal behavior. Dead fish were removed daily and preserved in 10% Formalin for later evaluation. After 6 weeks the study was terminated and the remaining fish either fixed in 10% Formalin for histopathology or cultured for the presence of *I. hoferi* .

VHSV in rainbow trout with & without pristane

VHSV in rainbow trout with & without pristane

The rainbow trout (*Oncorhynchus mykiss*) was examined as a possible surrogate laboratory species for studying the effect of VHS. In conjunction with studies on infectivity and pathogenicity, some fish were exposed to pristane, a branched alkane known to have immunosuppressant effects in mammals. The rationale being that crude oil contains numerous molecules other than polycyclic aromatic hydrocarbons that may affect the health of aquatic organisms following an oil spill such as occurred in 1989 in Prince William Sound. Pristane is a naturally occurring compound in some marine organisms as well as a component of crude oil. Considering these relationships, it was felt that a preliminary look at its effects on the immune system of fish was in order.

Four groups of 30 fish each were used to examine the infectivity and pathogenicity of VHS virus and the influence of pristane on the course of these infections. The fish were 6-months-old with mean lengths and weights at the start of the study of 13.7 mm and 99.1 gm. The exposed groups were exposed to 1X10^6 PFU$*$ml^{-1} for one hour, while the unexposed groups consisted of unexposed controls, culture medium alone (MEM) and pristane plus MEM.

Survey of wild PS herring for VHSV infection

Survey of wild PS herring for VHSV infection

Wild 5-to-6-month-old herring were netted from "herring balls" in Discovery Bay (WA), returned to the laboratory in tanks gassed with pure O_2 and distributed into 70 gal flowing, unfiltered seawater tanks at densities of 100-200 fish per tank. A subset of 60 fish was immediately removed and frozen at -70°C for later virus assay. The remainder of the fish were maintained for 30 days, during which they were observed for mortality, external lesions and behavioral aberrations. Dead fish were collected several times per day and frozen at -70°C until assayed for virus.

Hematologic and biochemical evaluation of wild VHS survivors

A sub sample of fish surviving beyond 30 days was samples for hematologic and biochemical changes associated with VHSV infection (See Section III: Kennedy-Simon Fraser University).

Determine VHSV transmission rate for wild herring

Three weeks after capture, wild herring surviving an epizootic of VHS were placed into tanks containing SPF herring to determine if they were capable of transmitting the virus via water. Each of 3 tanks contained 10 SPF herring and either 1 or 3 wild VHS survivors and controls consisted of three tanks containing only SPF herring.

Results

Laboratory rearing of Pacific herring

Obtain and hatch herring eggs

Eggs obtained from PWS hatched 14-16 d post fertilization and began actively feeding on rotifers (*Brachionus* sp) and *Artemia* nauplei one week later. They survived for 30 days but died when a valve was inadvertently opened and stagnant seawater containing hydrogen sulfide (H_2S) entered the tanks. The problem was corrected and PS eggs hatched normally one month later and are still surviving at this writing (February 1996).

Rear herring larvae for experimental use

The mean growth rate for the first three months post hatching was 0.33 mm * day^{-1} (Figure 1). By 6 months post-hatch the fish reached 4.5-6.0 cm in length and by 8 months were 85 - 100 cm. Water temperature increased 2°C, from 11.2 to 13.2°C between early June and August (Figure 2).

Verification of disease-free laboratory herring

Histopathology

No evidence of VHSV or *Ichthyophonus* infection was detected by histologic examination of tissues from 6-month-old larvae. An unexplained cartilage growth was observed on the lip of about 10% of the fry after 3 months, but did not affect growth or feeding behavior. The growth was surgically removed from a subset of 10 anesthetized fish with no apparent long-term affect on the fish's survival or growth. These fish have survived for >30 d and appear indistinguishable form unaffected cohorts.

In vitro culture of *Ichthyophonus*

No *Ichthyophonus* was detected in tissues of > 60 6-month-old SPF fish cultured in MEM-10 tissue culture medium for up to 90 days. Infected herring could be detected by culture in every case but only 70-80% of the experimentally infected fish could be detected by examining wet-preps using a limited amount of tissue.

In vitro culture of VHSV

No VHS virus was detected in assays carried out on >90 SPF fry at 6 months of age. Idiopathic mortality in SPF fish was relatively low (< 1%) during the first 6 months post hatching, and those that did die showed no signs of VHSV or other recognized herring pathogens.

Disease challenge without stressors

Challenge lab-reared herring with VHSV

Mortalities began 4 days post exposure and continued until the study was terminated at 14 days. During the first week mortalities were clearly dose related (Figure 3a) while during the second week the remaining fish died (Figure 3b), presumably as a result of being

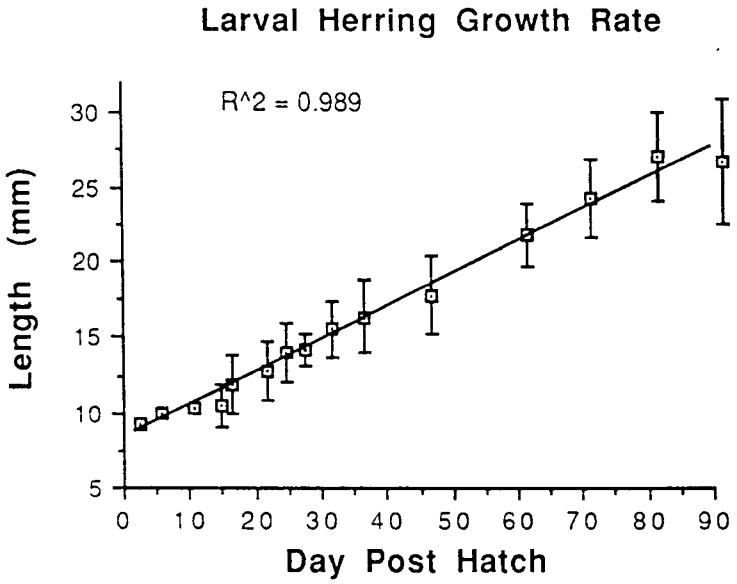

Figure 1. Three month growth data for Pacific herring larvae reared in captivity in filtered - U.V. sterilized natural seawater

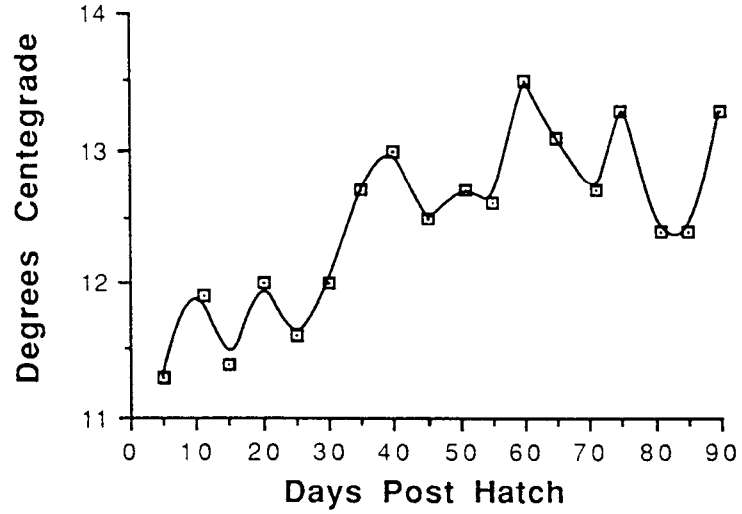

Figure 2. Temperature range of herring larval seawater from May 27 through August 20, 1995

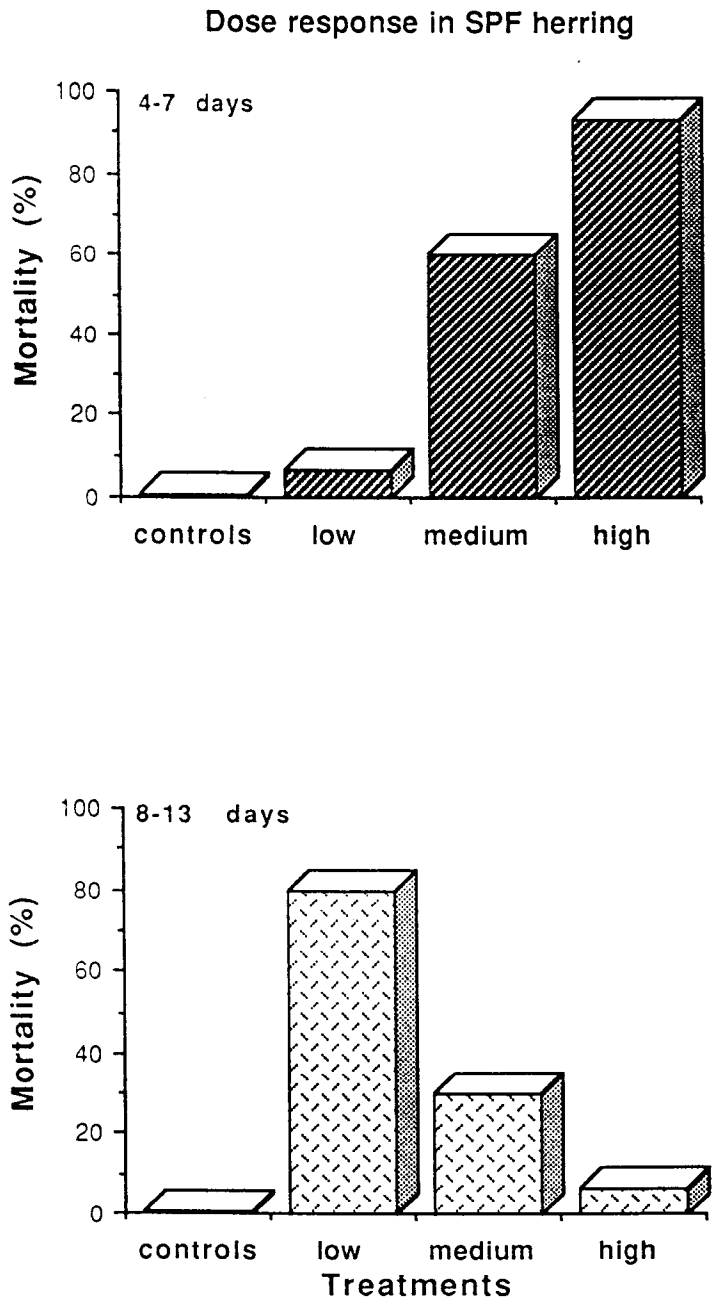

Figure 3. Mortality dose response for VHS virus in specific pathogen-free (SPF) laboratory reared herring one week following exposure (top panel), and secondary mortality (bottom panel) resulting from exposure to virus shed by herring dying following initial exposure.

exposed to virus shed by fish dying during the first week. Virus assays of dead fish showed that all fish from the high and medium level exposures were infected at the time of death, while only 1 of 2 dead fish from the low exposure groups was infected (Table I). Among the surviving fish at the end of the study, only one from the medium exposure group was infected, and at a level 100 times lower than that found in the dead fish (Table II).

Challenge of herring with *Ichthyophonus*

Herring was the only species that could be infected, and then only by I.P. injection of cultured cells. (Table III). The heart, spleen, liver and kidney were the most frequently infected organs and *I.hoferi* cells could be seen grossly or under 40X magnification on the surface of the organs (Figure 4). It appeared that following death of the host, the *I. hoferi* cells began to grow "hyphae" which contained microscopic "spores" at their tips. Individuals examined immediately after death contained primarily large resting spores.

SPF herring injected with the PWS strain of I. hoferi exhibited an 80% mortality rate by 6 weeks post exposure. The viscera contained grossly visible spores of I.hoferi in 50% of the fish. Tissues are presently being processed for histopathologic confirmation of infection.

Table I. Number of SPF herring infected with VHSV at the time of death. Individual fish assayed following dissection (head & tail removed), homogenization, and dilution.

Challenge Group ($PFU*ml^{-1}$)	tank number	# infected / total examined	mean log10 $PFU*gm^{-1}$
Low	7	7/9	6.0[a]
($7X10^2$)	35	9/9	6.7
	12	0/8	--
Medium	5	10/10	6.1
($7X10^4$)	20	9/9	6.1
High	9	9/9	6.2
($7X10^6$)	14	10/10	6.1
	37	10/10	6.1

(a) Lower detection limit = 2.6 $PFU * gm^{-1}$

Table II. Number of surviving SPF herring infected with VHSV 14 days post exposure. Two 4-fish pools (when available) were assayed following dissection (head & tail removed), homogenization, and dilution.

Challenge Group ($PFU*ml^{-1}$)	tank number	# of fish examined	mean log10 $PFU*ml^{-1}$
Controls	2	8	BDL[a]
(uninfected)	28	8	BDL
	33	8	BDL
Low	7	8	BDL
($7X10^2$)	12	1	2.9
	35	0	--
Medium ($7X10^4$)	20	1	4.8
High ($7X10^6$)		No survivors	

(a) Below detection limit (2.6 $PFU * ml^{-1}$)

Table III. Experimental Exposure of Pacific herring, rainbow trout and Japanese medaka with cultured *Ichthyophonus* cells

Species	Expt. #	Route of exposure	Incubation period	Infected (%)
Herring	1	I.P.	25d	4/5 (80)
	2	I.P.	18d	4/4 (100)
	3	per os	18d	0/8 (0)
	4	per os	21d	0/20 (0)
Rainbow	1	I.P.	61d	0/6 (0)
trout		per os	61d	1/6 (17)
	2	I.P.	28d	0/4 (0)
	3	I.P.	42d	0/20 (0)
		per os	42d	0/20 (0)
	4	I.P.		0/4 (0)
Medaka	1	I.P.	71d	0/2 (0)
	2	I.P.	137d	0/3 (0)
		per os	157d	0/3 (0)
	3	per os	60d	0/6 (0)
	4	per os	20d	0/3 (0)

VHSV in rainbow trout with & without pristane

VHSV in rainbow trout with & without pristane

After 3 weeks, mortality was observed only in the VHSV exposed groups with no significant difference between pristane and non-pristane treated groups (Table IV). Pristane alone had no effect on survival. Fish dying during the first two weeks following exposure were uniformly positive for VHSV, while those dying during the third week were negative even though they showed signs of disease and severe anemia (See Section III; Physiology). This is similar to what was observed in wild Pacific herring which experienced an epizootic of VHS following capture and confinement.

Table IV. Mortality and infection in rainbow trout exposed to VHSV and pristane

	N	morts	%	VHSV +/-
Controls	30	0	0	-
MEM (blank)	30	0	0	-
VHSV	30	12	40	+(a)
Pristane + VHSV	30	7	23	+(a)

(a) week 1 & 2 morts positive for VHSV; week 3 morts negative for VHSV but showed severe anemia

Tasks carried over: FY95 -> FY96

Survey of wild PS herring for VHSV infection

No VHS virus was detected in >60 fish sampled immediately after capture. However, heavy mortality began during the first week after capture and continued for about 30 days post capture. By the second week in captivity virus was isolated from 100% (10/10) of the fish sampled (Table IV). Mortality began to wain by the third week but fish continued to exhibit external signs of hemorrhaging and were lethargic. Both dead and surviving fish exhibited obvious hemorrhaging around the jaw, eyes, skin and fins. During week three post capture some hemorrhaging fish were shown to be capable of transmitting the VHSV to SPF fish, which also died with skin hemorrhages (see Task 95/96-2).

Table V. VHSV isolated from 5-month-old wild herring (no treatment)

Status	time in captivity (days)	number examined	% infected
Pre-crisis	0	30	0
Crisis (die-off)	10-21	10	100
Post-crisis (mortality ends)	>30	30	0

Hematologic and biochemical evaluation of wild VHS survivors

Blood samples were collected for hematology and biochemical evaluation (see Section III: Kennedy -Simon Fraser University).

Determine VHSV transmission rate for wild herring

SPF fish exposed to 3 wild fish suffered both mortality and infection by VHSV 3 weeks post exposure, while those exposed to only one wild fish exhibited neither mortality nor infection by VHSV (Table V).

Table VI. Mortality in SPF herring exposed to wild herring which had survived an epizootic of VHS.(a)

	% mortality	% VHS positive dead fish	% VHS positive survivors
Controls	0	--(b)	0
1 Wild fish(c)	0	--	0
3 Wild fish(c)	26.7	87.5	27.2

(a) N = 30; (3 tanks / treatment) X (10 fish / tank)
(b) No mortalities
(c) Wild fish selected during week 4 of epizootic

Determine the effect of stressors on VHS in Pacific herring.

Density was considered a factor for the transmission of VHS virus to susceptible herring. To determine the effect of density, 0-year herring (5-6 months old) were captured by dip net, transported to the Marrowstone Island laboratory and immediately distributed into triplicate 50 gal tanks at 5, 10, 25, 45 and 65 fish * $tank^{-1}$. Significant mortality occurred during the first week of confinement and continued for about 2 weeks, after which mortalities declined and fish numbers per tank remained stable after 4 weeks (Figure 4). Dead fish were collected twice daily, frozen at -70°C and assayed for VHS virus. Infection rates for all groups ranged from 10% -17%, with no obvious density effect. Survivors showing signs of hemorrhage were used for natural transmission studies to SPF herring.

Between August and September wild herring increased 50% in weight and 7.2% in length (1.81 gm and 5.7 mm respectively). The older fish did not exhibit the same density dependant mortality seen in the first group (Figure 5) but did show an obvious decline in virus titer (Figure 6). No virus could be detected in the fish by the fourth week post-capture.

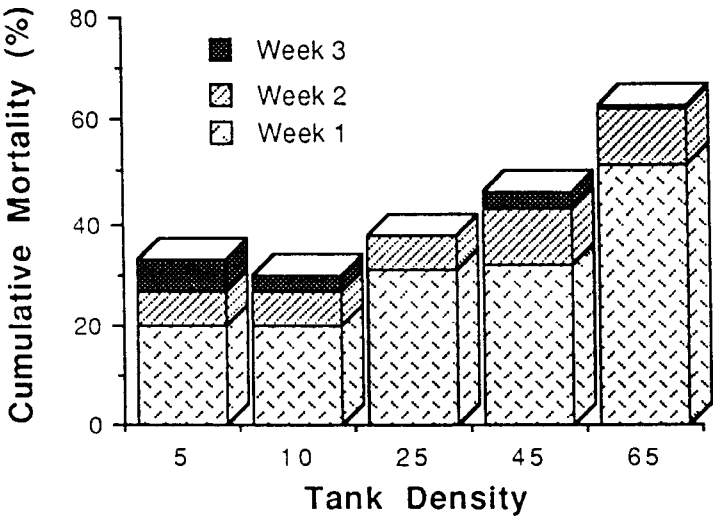

Figure 4. Mortality in wild 0-year herring captured 15 August 1995 from Pt. Wilson, WA. Mean wgt = 3.6 +/- 0.74 gm; mean length = 78.9 +/- 4.71 mm.

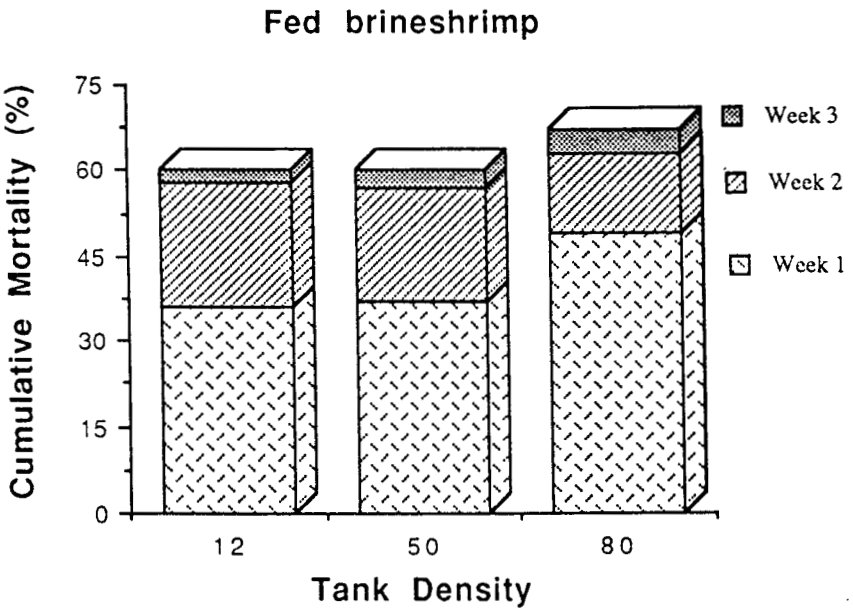

Figure 5. Density dependant mortality in wild 0-year herring captured 13 September 1995 from Discovery Bay, WA. Wgt = 5.41 +/- 0.65 gm; Length = 84.6 +/- 3.54 mm.

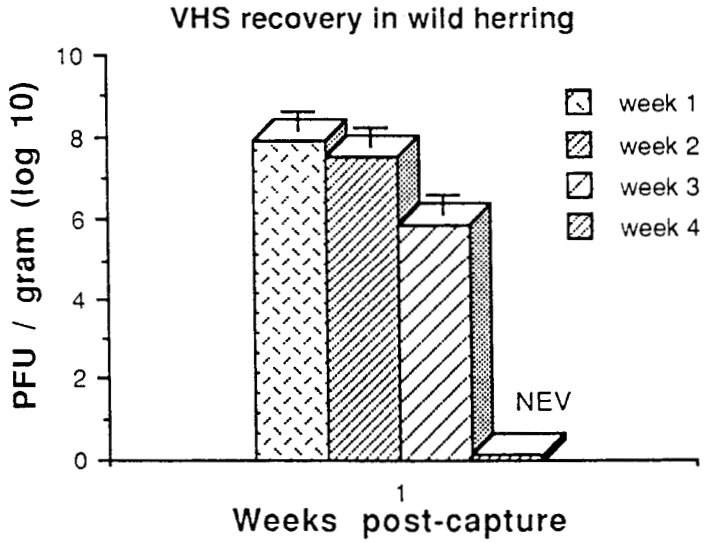

Figure 6. VHS virus tissue concentration in wild 0-year herring during a 3-week epizootic following capture (herring source; WDF, Discovery Bay; 13 Sept. '95).

Determine pathogenicity of *Ichthyophonus* for herring

At this point the natural route of transmission for *I. hoferi* in Pacific herring is not known. Oral transmission has not been successful using organisms isolated from herring tissues or cultured cells, but transmission by I.P. injection has been successful. The stage of this organism that is released from the herring host and the infective stage are not known at this time. It appears that the tissue phase of the *Ichthyophonus* organism is not the natural infective stage for herring, but rather a second biological host is required for rapid and efficient transmission of the agent.

Conclusions:

1. Pacific herring can be successfully reared disease-free in the laboratory in sufficient numbers to be used for experimental purposes.

2. Transmission of VHS virus is direct from fish-to-fish via water.

3. Non-immune juvenile Pacific herring are extremely sensitive to the VHS virus.

4. 0-year Puget Sound herring are infected with VHS virus before they are 6-months-old.

5. Artificial crowding of wild herring produces a VHS epizootic which can result in > 60% mortality.

6. Rainbow trout become infected with VHSV and exhibit morbidity and mortality similar to that seen in juvenile Pacific herring.

7. The natural route of infection of *Ichthyophonus* is not known, but the pathogenicity of the tissue stages has been demonstrated by artificial transmission via injection.

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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 95320S
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.

Dr. Christopher J. Kennedy
Dr. Anthony P. Farrell

Department of Biological Sciences
Simon Fraser University
Burnaby, B.C., Canada. V5A 1S6

April 1996

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 95320S
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 was a joint effort of the University of Washington, Simon Fraser University and University of California at Davis.

Abstract: It has been demonstrated that exposure of fish to the stressors viral hemorrhagic septicemia virus (VHSV), the hydrocarbon pristane and *Ichthyophonus hoferi* (ITP) can significantly alter their hematological and immunological status, specifically hematocrit, leucocrit and differential white blood cell counts. Multiple stressors (VHSV and pristane) appear to act synergistically in their effects on fish. Baseline levels of hematological and immunological parameters in herring which have survived a natural epizootic of VHSV are reported. Higher fish stocking densities can modulate the immunological status in fish following a natural epizootic of VHSV, results which have important management implications. No effects of ITP on these parameters were seen in herring, 3 or 9 weeks following exposure. These preliminary results may, in part, begin to explain the trends in prevalence of VHSV and ITP in herring caught in Prince William Sound following the *Exxon Valdez* oil spill.

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

Citation:

Kennedy, C.J., and A.P. Farrell. 1996. 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 95320S), Simon Fraser University, Burnaby, British Columbia, Canada.

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; c) ITP prevalence was high in 1994; d) females from previously oiled sites produced fewer live larvae.

Objectives

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. The effects of environmental modulators such as stocking density on the responses of herring to these stressors has important implications to herring management strategies.

The longterm objectives of this section of the 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 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 for 1995 were as follows:

1) To develop and build an exposure system for the delivery of oil to herring holding aquaria. To build a swim tunnel apparatus for examining the swimming performance of juvenile herring.

2) To supply analytical support for Section I (the field component) of this research project. Blood smears from 500 herring sampled from Prince William Sound were to be analyzed for differential white blood cell counts and erythrocytes scored for inclusion bodies. Plasma samples from these fish were to be analyzed to determine the relative proportions of creatine phosphokinase (CPK) isozymes present.

3) Disease-free young of the year from PWS raised by Dr. Kocan's group were not available for experiments for Section III in 1995 due to their small size. Testing with these fish has begun in 1996. Therefore, test sampling and analysis of several hematological, immunological and biochemical parameters were performed in wild caught Pacific herring from Washington State and a surrogate species, the rainbow trout, before testing in 1996 on disease-free Prince William Sound herring began.

Methods

Objective 1: Apparatus development.

Dosing of herring with oil is scheduled and proceeding in 1996. The dosing apparatus or 'oil generators' which had been selected for this research project are those developed by Carls et al. (unpublished). Essentially, this apparatus consists of a 15 cm diameter X 80 cm tall polyvinyl chloride plastic cylinder containing either rock or ceramic beads. Water upwells through the cylinder and flows into the bottom of the individual treatment tank. Appropriate levels of hydrocarbons are generated through the apparatus as indicated by hydrocarbon analysis documented by Carls et al. (unpublished) using Alaska North Slope Crude oil. A trap inside the generator prevents slick overflow.

Experiments on the effects of stressors on herring swimming performance have been started in 1996. The swim-test apparatus assembled is 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.

Objective 2: Field study support.

Blood smears from 500 Pacific herring sampled March and April 1995 in Prince William Sound were received from Dr. G. Marty of the University of California at Davis. 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 form each smear were examined for viral erythrocytic necrosis (VEN) .

Due the strong statistical relationship between CPK and lesions in herring (Marty unpublished), frozen plasma samples from 100 Pacific herring sampled March and April 1995 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 3: Herring and surrogate species testing and technique development

Disease-free young of the year from PWS were not available in 1995 for fitness experiments due to their small size. At 5-6 g these juveniles will be suitable for sublethal toxicological testing and disease challenges in 1996. Although not scheduled for 1995, several preliminary experiments were performed.

1) Hematological and immunological status of herring following ITP exposure

0-year herring were captured and transported to the Marrowstone Island laboratory where they underwent a natural VHSV epizootic (see Section II-Dr.

Kocan for details on fish capture and holding). Fish were then dosed with both low and high doses of ITP spores *per os* as described in Section II (laboratory study-Dr. Kocan). Fish were sampled 3 and 9 weeks following ITP exposure. Blood was removed by severing the caudal peduncle. Hematocrit (% volume of packed red blood cells), leucocrit (% volume of packed white blood cells) and differential white blood cell counts were examined. Methods for these measurements are summarized in Stolen et al. (1992).

2)Rainbow trout challenge with VHSV and pristane

Previous studies have successfully used rainbow trout of this size range in determining the sublethal toxicity of several natural wood products and antisapstain chemicals (Johansen et al. 1995; Kennedy et al. 1995). In this section, rainbow trout were used as a surrogate species to determine the effects of pristane (a hydrocarbon and immunosuppresive agent in mammalian systems), VHSV and a combination of these factors on trout immunocompetence. Trout were exposed to pristane, VHSV or combination according to procedures outlined in Section II (Laboratory studies-Dr. Kocan). Fish were sampled 21 days following exposure. Blood was removed by severing the caudal peduncle. Several immunological indicators including hematocrit (% volume of packed red blood cells), leucocrit (% volume of packed white blood cells), differential white blood cell counts (as described earlier), phagocyte activity using the nitroblue tetrazolium assay and glass adherent phagocytes and lysozyme assay using the lysoplate method were examined. Methods for these measurements are summarized in Stolen et al. (1992).

3) Density effects on hematological and immunological status of herring following a VHSV epizootic

0-year herring were captured and transported to the Marrowstone Island laboratory and distributed into triplicate tanks at varying densities as described in Section II (laboratory studies-Dr. Kocan). Significant mortalities occurred during the first week of confinement (VHSV epizootic) and continued for about 2 weeks, after which mortalities ceased and fish numbers in each tank remained stable. Fish were sampled 8 weeks following the start of the experiment and blood sampled by severing the caudal peduncle. Several immunological indicators including hematocrit (% volume of packed red blood cells), leucocrit (% volume of packed white blood cells), differential white blood cell counts (as described earlier), phagocyte activity using the nitroblue tetrazolium assay and glass adherent phagocytes and lysozyme assay using the lysoplate method were examined (Stolen et al. 1992).

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 arcsin transformed before statistical analysis.

Results

Objective 1: Apparatus development.

A schematic of the dosing apparatus is shown in Figure 1. Fifteen of the units have been built for the oil exposure studies. A schematic of the swim raceway apparatus is shown in Figure 2. One unit has been built for the swimming performance studies.

Objective 2: Field study support.

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. CPK analysis proved unsuccessful because of the improper handling (several thaw and freeze episodes) of field plasma samples during blood chemistry analysis at the University of California laboratory (Section I of the project).

Objective 3: Experiments with herring and rainbow trout

1) Hematological and immunological status of herring following ITP exposure

Hematocrit and leucocrit values for herring treated with varying doses of ITP spores for 3 or 9 weeks can be seen in Table 1. There were no significant differences for leucocrit, hematocrit or white blood cell differential counts between control fish and those treated with low or high oral doses of ITP spores, 3 or 9 weeks post-exposure. Observation of blood cells indicated that large intracytoplasmic, eosinophilic-staining inclusion bodies were found in the erythrocytes of 70.4% of herring exposed to ITP. These inclusion bodies resemble those associated with viral erythrocytic necrosis virus (VEN). The red blood cells are degenerate as displayed by cytoplasmic vacuolization, karyorrhexis and nuclear pyknosis. These cells are generally round with a moderate degree of basophilia, immature cells and mitotic figures. These observations are consistent with those of researchers who have demonstrated the presence of VEN by transmission electron microscopy. Further investigation needs to be done to determine if VEN is present, and to what extent it may be affecting the herring populations ability to combat VHSV or ITP. It is likely that several pathogens are simultaneously contributing to the increased mortality, especially if the population is immunocompromised.

Figure 1. Schematic diagram of an 'Oil Generator' designed after Carls et al. (unpublished) for dosing of herring to oil in single and multiple stressor experiments.

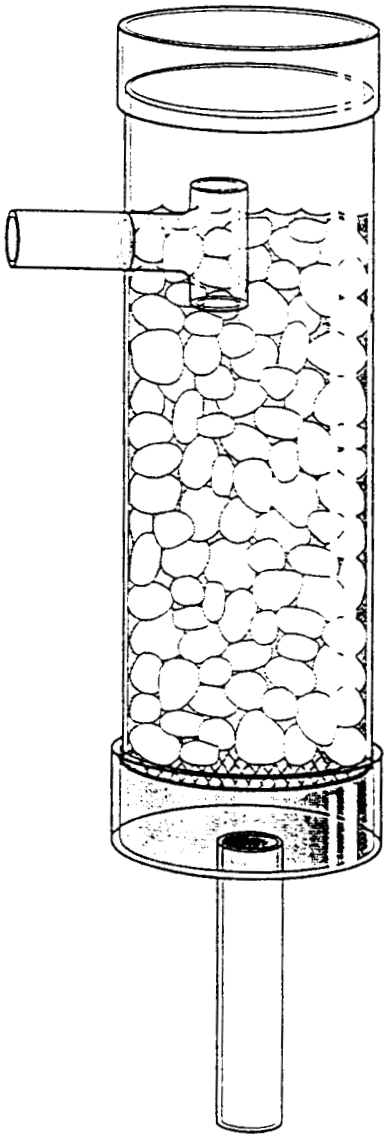

Figure 2. Schematic diagram of the swimming raceway to be used in physiological fitness testing of herring following exposure to single or multiple stressors.

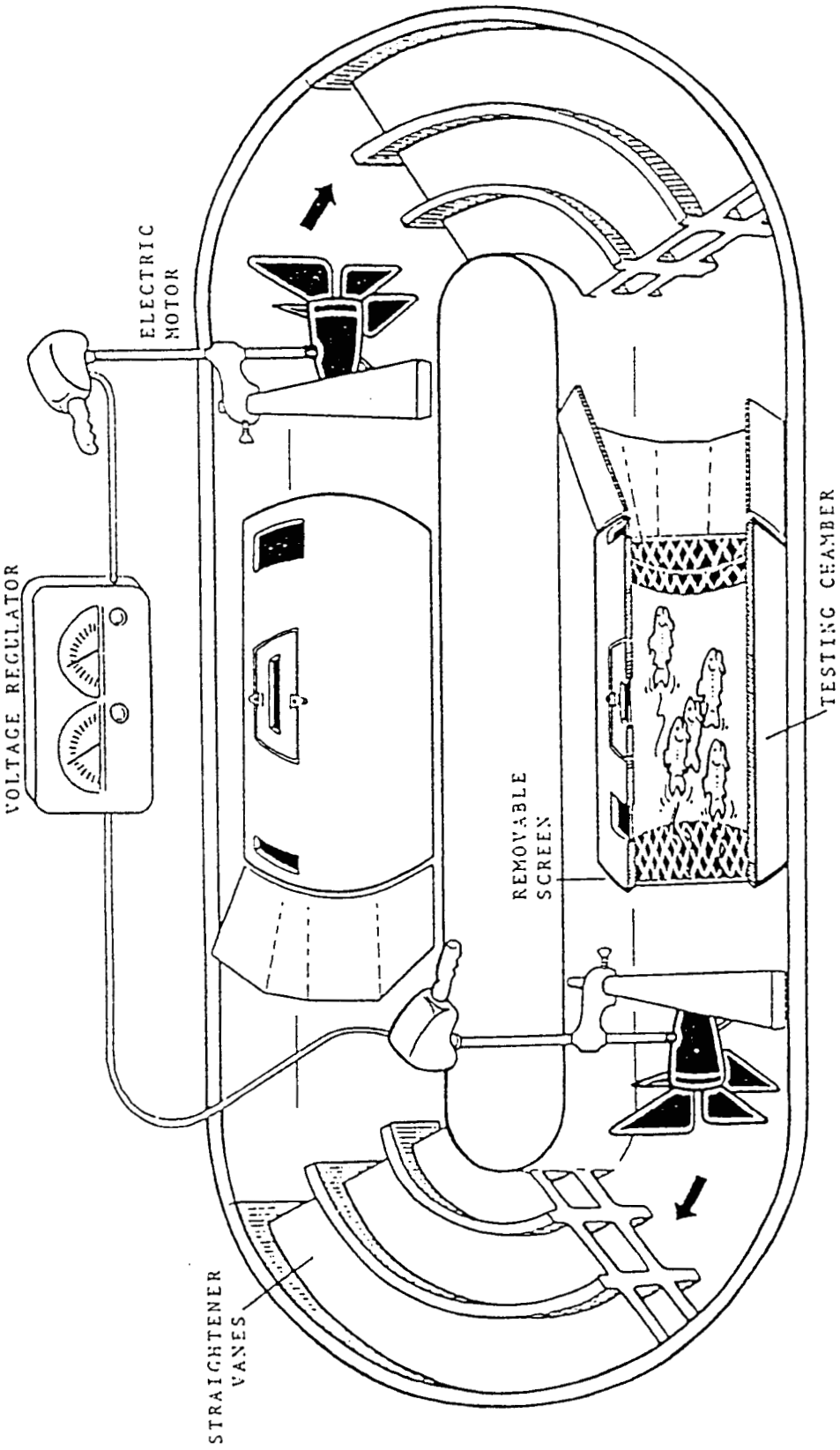

Table 1. The effects of ITP exposure on hematological parameters in wild Pacific herring which had undergone a natural epizootic of VHSV. Samples were taken from fish at 3 and 9 weeks following ITP exposure. Values are means±standard errors. There were no significant differences in measured parameters between ITP-exposed and control fish at a significance level of $p<0.05$.

ITP concentration	Duration (weeks post exposure)	Hematocrit (%)	Leucocrit (%)
0	3	17.1±3.1	0.29±0.10
0	9	24.5±4.9	0.44±0.10
Low	3	14.1±1.8	0.48±0.19
Low	9	24.5±3.0	0.35±0.09
High	3	22.2±3.0	0.51±0.20
High	9	36.1±2.7	0.44±0.11

Table 2. The effects of ITP exposure on differential white blood cell counts in wild Pacific herring which had undergone a natural epizootic of VHSV. Samples were taken from fish at 3 weeks following ITP exposure. Values are means±standard errors. There were no significant differences in cell counts between control and exposed groups at a significance level of $p<0.05$.

ITP Conc.	Lymphocytes (%)	Macrophages (%)	Neutrophils (%)	Thrombocytes (%)	Monocytes (%)	Eosinophils (%)
Control	64.1±4.3	1.8±0.5	6.7±1.9	26.7±5.2	0.3±0.2	0.4±0.4
Low	74.6±2.9	0.8±0.3	1.9±0.5	22.0±3.2	0.7±0.4	0.1±0.1
High	65.1±5.2	0.1±0.1	6.3±2.3	28.0±5.4	0.0±0.0	0.0±0.0

2)Rainbow trout challenge with VHSV and pristane

Hematological and immunological parameters in trout exposed to pristane, VHSV or a combination of both factors are shown in Table 2. Trout exposed to pristane only showed significant reductions in leucocrit values when compared to control fish. Trout exposed to VHSV only showed significant reduced values in both hematocrit and leucocrit values when compared to control fish. When trout were exposed to both pristane and VHSV, significant reductions were

seen in both hematocrit and leucocrit values. VHSV and pristane, when administered together, appeared to act synergistically in their effects on these parameters. No statistically significant effect was seen in lysozyme activity with any treatment. White blood cell differential counts indicated that trout exposed to pristane and VHSV had a leucopenia with a concurrent neutrophilia and lymphocytopenia (Table 4).

Table 3. The effects of pristane and VHSV exposure on hematological and immunological parameters in juvenile rainbow trout. Values are means±standard errors. The * denotes a significant difference between fish exposed to Minimum Essential Medium (MEM, a balanced saline solution as control) and either VHSV or pristane, or combination, at a significance level of $p < 0.05$.

Treatment	Hematocrit (%)	Leucocrit (%)	Lysozyme Activity (Units/ml plasma)
MEM	45.2±1.8	1.26±0.10	9.34±2.18
Pristane	41.8±0.8	0.66±0.10 *	7.66±1.93
VHSV	25.3±6.7 *	0.65±0.34 *	11.49±1.87
VHSV/Pristane	22.4±4.9 *	0.32±0.01 *	11.81±1.9

Table 4. The effects of pristane and VHSV exposure on differential white blood cell counts in juvenile rainbow trout. Values are means±standard errors. The * denotes a significant differences between control and exposed fish at a significance level of $p<0.05$.

Treatment	Lymphocytes (%)	Macrophages (%)	Neutrophils (%)	Thrombocytes (%)	Monocytes (%)
MEM	90.4±1.4	2.5±1.0	4.1±1.0	0.5±0.3	2.5±0.9
PRIS/ MEM	88.9±1.7	2.6±0.7	6.4±1.5	1.2±0.5	0.9±0.4
PRIS/ VHS	53.6±9.2 *	6.3±1.6	29.2±8.8 *	0.5±0.4	1.3±0.6
VHS	88.2±2.2	3.4±0.7	7.1±1.7	0.0±0.0	1.3±0.4

3) Density effects on hematological and immunological status of herring following a VHSV epizootic

Hematocrit and leucocrit values for herring which underwent a natural VHSV epizootic under various density conditions can be seen in Table 5. Significant reductions in hematocrit were seen in fish at fish kept at densities of 80 fish per tank compared to fish at a density of 12 or 50 per tank. Significant reductions in leucocrit were seen in fish kept at densities of 50 and 80 fish per tank compared to fish at a density of 12 per tank. Lysozyme activity appeared to be unaffected by fish stocking density. These values represent baseline data for fish which had undergone a natural epizootic of VHSV and cannot be compared to preinfected fish where no data is available. Phagocytic activity data have not been analyzed statistically and will be reported in the next annual report. 100% of fish in these experiments showed large intracytoplasmic, eosinophilic-staining inclusion bodies in the erythrocytes as seen in the herring exposed to ITP. Again, no conclusions regarding this observation can be made and needs to be investigated further.

Table 5. The effects of fish stocking density on hematological and immunological parameters in wild Pacific herring which had undergone a natural epizootic of VHSV. Values are means±standard errors. The * denotes a significant difference from fish stocked at 12/tank at a significance level of $p < 0.05$.

Stocking Density (Fish per tank)	Hematocrit (%)	Leucocrit (%)	Lysozyme Activity (Units/ml plasma)
12	44.1±2.6	3.2±1.1	4.3±6.0
50	39.7±4.9	1.5±0.1 *	10.4±6.0
80	28.0±3.9 *	1.3±0.1 *	12.2±6.2

Table 6. The effects of fish stocking density on differential white blood cell counts in wild Pacific herring which had undergone a natural epizootic of VHSV. There were no significant differences in differential white blood cell counts between fish stocked at higher densities compared to the lowest density at a significance level of $p<0.05$.

Stocking Density (Fish/tank)	Lymphocytes (%)	Neutrophils (%)	Macrophages (%)	Monocytes (%)	Thrombocytes (%)	Eosino. (%)
12	59.9±6.3	18.5±6.1	1.1±0.5	0.1±0.1	19.8±3.8	0.0±0.0
50	42.3±4.3	9.4±2.7	0.7±0.5	0.0±0	47.9±4.7	0.1±0.1
80	35.±4.0	1.0±0.0	0.1±0.1	0.0±0.0	59.4±4.1	0.0±0.0

Discussion

The results for research in this section of this annual report are preliminary and supportive of research which has begun in 1996. No experiments with disease-free Prince William Sound juvenile herring were planned or performed due to the small size of herring at this stage. However, preliminary studies were performed with rainbow trout and wild caught Pacific herring from Washington State to assess the feasibility of planned herring experiments taking place in 1996.

The majority of the work performed in 1995 was allocated to apparatus development and assembly. Both oil generators and swim raceway are built and functioning and have been transported to the Marrowstone Island Field Station in order to begin experiments with PWS herring raised by Dr. Kocan. The probability of success of 1996-98 planned experiments has been increased dramatically by the successful raising of disease-free PWS juvenile herring at Marrowstone Island by Dr. Kocan.

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, examination for viral erythrocytic necrosis and creatine phosphokinase isozymes in field sampled herring are discussed in Section I (field study-Dr. Marty) of this annual report.

Results utilizing wild caught Pacific herring and rainbow trout have yielded important information for the success of the research in 1996-1998. The techniques utilized for the measure of hematological and immunological parameters have proven successful for use in herring and will be used for the experiments in 1996-1998. The preliminary study with rainbow trout

indicated that exposure of fish to the alkane pristane, a known immunosuppressant in mammalian systems, causes significant alterations in immune system parameters. Exposure of trout to VHSV also altered components of the immune system in trout which may have implications for secondary infections in surviving fish. Pristane and VHSV exposure together appeared to act synergistically on the immune system. Results also indicate that abiotic factors may modify the hematological and immunological responses of herring to stressors such as VHSV. ITP exposure *via* the oral route did not cause any significant alterations in herring hematological or immunological parameters. It is unclear at this time whether fish were infected with ITP by the experimental exposure route. Moreover, effects of ITP infection may occur later in the sampling period and so conclusions regarding the effects of ITP are premature. The information obtained from these studies indicates that the research approach in this project using multiple stressors in combination (oil, VHSV and ITP) may yield important results regarding cause and effect relationships between the stressors and Pacific herring decline in PWS.

Conclusions

The preliminary studies performed in 1995 provide significant support to the proposed research plan. It has been demonstrated that exposure of juvenile trout to the stressors VHSV and pristane, a hydrocarbon, can alter their hematological and immunological status. This is an important first step in evaluating any link between oil exposure and altered immunocompetence in fish. Furthermore, two of the stressors combined acted synergistically on some of the measured parameters in trout. Baseline levels of hematological and immunological parameters in herring which have survived a natural epizootic of VHSV are reported. Higher fish stocking densities can modulate the immunological status in fish following such an epizootic, results which have important management implications. No effects of ITP on these parameters were seen in herring, 3 or 9 weeks following exposure. These preliminary results may, in part, begin to explain the trends in prevalence of VHSV and ITP in herring caught in PWS following the oil spill.

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 exposure of herring and trout to pristane, VHSV and ITP.

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