Exxon Valdez Oil Spill
Restoration Project Annual Report

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

Section I: Field Survey of Diseases in Prince William Sound Herring
Section II: Controlled Field and Laboratory Studies of VHSV and *Ichthyophonus* in Pacific Herring
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Restoration Project 97162
Annual Report

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

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

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Study History: Between 1993-94 there was an unexplained disappearance of approximately 110K tons of spawning herring in Prince William Sound (PWS). An emergency project (94320S) was proposed by the Alaska Department of Fish and Game and funded in April 1994 by the *Exxon Valdez* Trustee Council to investigate the cause of the massive herring loss. As a result it was found that VHS, a viral disease previously unreported from Pacific herring, was present in some surviving herring. In 1994 VHSV was present in <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 initiated a second study. A detailed work plan was written which covered: 1) Field surveys in PWS; 2) Controlled experimental studies and 3) Physiological studies. The three components of the study were designed to interact and supply information to each other in order to answer questions regarding infection, pathogenicity and recovery prospects of Prince William Sound herring.

Abstract: In spring '97 VHSV prevalence was significantly greater in fish from PWS (15%) than from Sitka (0.8%). The prevalence of *Ichthyophonus* continued to decrease within the population as a whole due to recruitment of younger fish with lower rates of infection. The spawn-on-kelp study demonstrated that with one exception, newly captured fish did not have detectable levels of VHS virus, but by 2-4 days post-capture 15%-30% had significant levels of virus in their tissues, and by 6-8 days the prevalence dropped to 0-7.5%. No evidence for oil-related immunosuppression was evident after exposure of both wild and lab-reared herring to both oil and VHS virus. Fish of all ages developed a protective acquired immunity following infection and recovery from VHS. Between 1995 and 1997 only one on 121 herring balls of 0-year fish was observed to be resistant to VHS. Of over 1,000 fish sampled during this period, only one fish was found to have an active VHS infection, and it came from the resistant school. Adult herring exposed to varying concentrations of oil for 4 and 22 days showed no difference in swimming stamina, however there was a significant increase in mortality of oil-exposed fish following strenuous exercise.

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

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

Citation:

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Executive Summary (97162)

Introduction

In 1993 the Prince William Sound herring population declined over 80% and viral hemorrhagic septicemia virus (VHSV) was isolated from 5% of the survivors. This was the first report of this viral pathogen from wild Pacific herring, and it has subsequently been isolated from bait fish in Puget Sound and herring collected in the vicinity of a diesel fuel spill in Prince Rupert, B.C. In 1992 herring held in the roe-on-kelp fishery in Prince William Sound were observe to have hemorrhages on the skin, fin bases and mouth - the fish swam erratically and did not spawn properly. Although no virus isolations were attempted, it was noted that these lesions closely resembled the lesions observed in confirmed cases of VHS in wild herring the following year. Since VHS had not previously been reported in Pacific herring, this suggested the possibility that VHS was responsible for the heavy losses observed in 1993-'94. In 1995, a study was initiated to study the implications of disease factors on herring populations. This project was a collaborative effort among four separate groups: Alaska Department of Fish and Game, U.C. Davis, U. of Washington and Simon Fraser University. These groups approached the disease problem in herring from several aspects: 1) By conducting annual on-site surveys of Prince William and Sitka Sound herring it was possible to follow changes in annual prevalence of VHS and *Ichthyophonus* in each age class, and to compare PWS herring, with Sitka Sound herring where no previous oil contamination occurred and no losses of herring were apparent. 2) By conducting controlled experimental disease studies on laboratory-reared and wild herring it was demonstrated that VHS virus was unequivocally pathogenic for nonimmune herring, causing sever mortality in juvenile fish, that net pen confinement resulted in increased disease transmission, that VHS virus could survive for up to 6 hours in sea water, and that herring recovered from VHS were solidly immune to reinfection. 3) By measuring biochemical and physiological changes in diseased and healthy herring it was demonstrated that hematological changes occurred in oiled fish that could potentially compromise their resistance to infection and that swimming stamina was affected.

Objectives

This study consisted of three distinct components with interrelated objectives.

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

Experimental studies (II):
1) Determine the means of transmission from infected to uninfected fish.
2) Determine the prevalence of VHSV in wild Puget Sound herring of different age classes.
3) Evaluate serologic techniques for field monitoring herring populations for VHS
4) Evaluate in vitro culture as a technique for field monitoring herring for *Ichthyophonus* prevalence
5) Determine the immune status of wild herring that survive an epizootic of VHS.

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

Methods

Field studies consisted of sampling adult Pacific herring from Prince William Sound and Sitka Sound and determining their white blood cell differential counts (Component III), histopathology, plasma chemistries, IgM levels and virus isolations. Tissues from fish held in SOK pounds were examined for the presence of VHS virus during confinement and at the time of release from the pounds, and water from the pounds was sampled for the presence of virus.

Experimental studies relied on specific-pathogen-free (SPF), laboratory-reared herring as well as captive and wild free-ranging herring. Laboratory-reared fish were exposed to virus every 6 months until they reached 2-years-old to establish a pattern of natural immunity development and were exposed to oil to determine if immunosuppression occurred. Wild herring captured from Puget Sound were assayed for the presence of VHSV at the time of capture and at regular intervals for the first 30 days post capture. Immunity was determined by exposing surviving herring to 10 - 100 times the known lethal dose of VHS virus for 1 hr and by examining the virus neutralizing capability of plasma from pre and post-infected fish. Serologic data was compared with that obtained form SPF fish which had no immunity to the virus.

Ichthyophonus was studied in wild herring by culturing tissues from different age classes and from the same age-class over a period of a year. Changes in prevalence were noted in terms of increasing age and difference in geographic location of the fish. A comparison of visual examination, histologic examination and in vitro culture was made to determine the most sensitive and economic technique for monitoring I*chthyophonus* in the field.

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

Results

Field sampling: In spring '97, virus prevalence was significantly greater in fish from PWS (15%) than Sitka (0.8%), but it was higher than in any previous year sampled. No virus has been observed in fall samples. VHSV was more common in females, younger fish and fish with external lesions, however the external lesions in spring samples were not as well correlated with virus prevalence as in past years.

The prevalence of Ichthyophonus continued to decrease with much of the decrease due to recruitment of younger fish with lower rates of infection. The prevalence of Ichthyophonus in the '88 year-class has not changed in four years (eg 30, 35, 26, & 30%). In 1997 the 9-year-old class had the highest prevalence of Ichthyophonus (30%), with 4 and 5 year-olds at 20% and 3-year-olds at 4.3%.

In addition the VHS and Ichthyophonus epitheliocystis in the gills and trematodes in the intestine appeared in significant numbers of fish.

The spawn-on-kelp study demonstrated that with one exception, newly captured fish did not have detectable levels of VHS virus, but by 2-4 days post-capture 15-30% had significant levels of virus in their tissues, and by 6-8 days the prevalence dropped to 0 - 7.5%. Of 40 wild spawning fish captured just outside the pounds, none were positive for VHSV.

Controlled experimental studies: No evidence could be found to support the hypothesis that exposure to oil resulted in increased susceptibility to VHS in wild or laboratory-reared herring. It was demonstrated however, that herring have little natural immunity to VHS until they are 2-years-old, when they develop a clear natural resistance to the virus. Conversely, fish of all ages that survived an initial exposure to VHS virus developed an acquired protective immunity that could be demonstrated by plaque neutralization. Titers ranged from 1/80 dilutions to as high as 1/640 dilutions. Control fish dying with VHS did not develop a detectable neutralizing antibody.

Between 1995 and 1997 no evidence of VHSV could be found in wild free-ranging Puget Sound herring of any age at the time of capture. However, once in captivity, an epizootic occurred which was highly lethal to 0-year fish and produced about 15% mortality in older fish.

In only one case, a single infected 0-year wild herring was detected in a school captured in September of 1997. The remainder of the school did not experience an epizootic once in captivity, but rather was solidly immune to experimental reinfection by VHS virus.

Studies on the survival of VHS virus in sea water clearly showed that virus could survive for up to 2 hours in raw, filtered or oiled sea water. In the first study a loss of 20% was observed after 1 hour and 40% after 2 hours. The second study resulted in a loss of about 60% during the first hour and a loss of 90% by 6 hours.

Biochemical/physiologic studies: Oil-water dispersions of North Slope crude oil was not acutely lethal to adult herring although it did initiate a classical biochemical stress response consisting of hypersecretion of corticosteroids, hyperlacticemia, hyperglycemia and ionic disturbances. By 72 hours post exposure, these values returned to normal and remained at baseline for 22 days, indicating a transient response to oil exposure.

Adult fish exposed to oil for 28 days, then transferred to clean sea water showed significant alterations in white blood cell populations, phagocytic and respiratory burst activity of macrophages and lysozyme activity, which all occurred in a dose-dependent manner.

Adult herring exposed to varying concentrations of oil for 4 and 22 days showed no difference in swimming ability (eg stamina), however there was a significant increase in mortality of oil-exposed fish following strenuous exercise.

Conclusions and recommendations:

It appears that although the Prince William Sound herring population is increasing, there is still a residual of VHS activity within the population, evidenced by a higher than expected prevalence in PWS, that may be affecting the recovery rate. Continued surveillance at this time is recommended to determine what effect this activity will have on the long-term recovery of the resource.

Data from experimental studies show clearly that herring develop both a natural age-related resistance and an acquired immunity to VHS. How long this resistance lasts or how it might be compromised is unknown. Although no evidence for increased infection or mortality could be found following oil exposure, physiologic studies with oil indicate that the fish may be immunocompromised, at least at the cellular level.

Juvenile herring in the 0-year age class appear to be very susceptible to high mortality when exposed to VHS virus. This results in heavy losses of these young fish under captive conditions, and may represent a situation which goes unnoticed in wild fish because of the difficulty in tracking these

populations. If heavy losses do occur and go unnoticed in 0-year herring, this may explain the dramatic differences observed in egg biomass and predicted spawner biomass of this age class.

The observation that VHS virus can survive for up to 6 hours in natural sea water supports the hypothesis that water-born transmission may be responsible for the high prevalence rates of VHS infection observed in the spawn-on-kelp fishery. A few infected individuals shed virus into the water under crowed conditions and susceptible fish become infected.

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

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

Exxon Valdez Oil Spill
Restoration Project Annual Report

Causes of Disease in Pacific Herring from Prince William Sound, Alaska, during Fall 1996 and Spring 1997

Restoration Project 97162
Section I - Field Component
Annual Report

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

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Causes of Morbidity in Pacific Herring from Prince William Sound, Alaska, during Fall 1996 and Spring 1997

Restoration Project 97162
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 as the field component of Restoration Project 95320S, and an annual report was issued in 1996 by Marty, G.D., C.R. Davis, E.F. Freiberg, D.E. Hinton, T.R. Meyers, and J. Wilcock, under the title Causes of Morbidity in Pacific Herring from Sitka Sound and Prince William Sound, Alaska, in Spring 1995. The project effort was continued as the field component of Restoration Project 96162, and an annual report was issued in 1997 by Marty, G.D., C.R. Davis, E.F. Freiberg, T.R. Meyers, G. Carpenter, and D.E. Hinton, under the title Causes of Morbidity in Pacific Herring from Sitka Sound and Prince William Sound, Alaska, during Fall 1995 and Spring 1996. The project effort was continued as the field component of Restoration Project 97162, the subject of this report. The bulk of the annual report for project 94320S has been published as Marty, G. D., E. F. Freiberg, T. R. Meyers, J. Wilcock, T. B. Farver, and D. E. Hinton. 1998. Viral hemorrhagic septicemia virus, *Ichthyophonus hoferi*, and other causes of morbidity in Pacific herring *Clupea pallasi* spawning in Prince William Sound, Alaska, USA. Dis. Aquat. Org. 32:15-40. Some of results from projects 95320S and 96162 have been submitted for review (we are now responding to reviewer comments): Davis, C. R., G. D. Marty, M. A. Adkison, E. F. Freiberg, and R. P. Hedrick. In review. Association of plasma IgM with body size, histopathologic changes, and plasma chemistries in adult Pacific herring *Clupea pallasi*. Dis. Aquat. Org.

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. Viral hemorrhagic septicemia virus probably contributed most to population decline in 1994, but the fungus *Ichthyophonus hoferi* was also important. After viral prevalence steadily decreased from 1994 to 1996 (4.7% → 1.9% → 0.0%), viral prevalence unexpectedly increased to 15% in spring 1997. Virus was never isolated from fish sampled annually in the fall (1995-1997). In spring samples, the prevalence of *Ichthyophonus* decreased from 29% in 1994 and 1995, to 25% in 1996, and 18% in 1997, but *Ichthyophonus* prevalence in the 1988 year class remained fairly constant from 1994 through 1997 (30, 35, 26, and 30%, respectively). The unexpected 1997 increase in viral prevalence in Prince William Sound has not been associated with severe population decline; however, increased viral expression may impair population recovery. Study of viral hemorrhagic septicemia in the spawn-on-kelp pound fishery in Prince William Sound revealed that viral

prevalence peaked one to four days after fish were captured (peak viral prevalence = 15 - 28%), and viral expression was not related to fish mortality or fish density.

Key Words: *Clupea pallasi*, disease, *Exxon Valdez*, histopathology, *Ichthyophonus hoferi*, Pacific herring, plasma chemistries, Prince William Sound, spawn-on-kelp pound fishery, viral hemorrhagic septicemia virus (VHSV).

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

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Executive Summary

Introduction

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

In 1994, 233 Pacific herring were sampled from Prince William Sound: 29% had the disseminated fungus *Ichthyophonus hoferi*, and viral hemorrhagic septicemia virus was isolated from 5% of the fish. In 1995 and 1996, the study included fish from a reference site, Sitka Sound, in which the herring fishery was strong and there was no history of a large oil spill. This report describes the major findings in Pacific herring from Prince William Sound in fall 1996 and spring 1997. Results from laboratory study exploring details of viral hemorrhagic septicemia virus and *Ichthyophonus* infections under controlled conditions are reported in sections II and III. Preliminary study from the spawn-on-kelp fishery in Craig, Alaska (1996) indicated that pounded fish were more likely to express viral hemorrhagic septicemia virus than wild-caught fish. When the spawn-on-kelp pound fishery was reopened in Prince William Sound in 1997, the effects of the pound fishery on expression of viral hemorrhagic septicemia virus was studied, and significant findings are reported herein.

Objectives

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

Methods

Adult Pacific herring from Prince William Sound were sampled at random and subjected to complete necropsy in October 1996 (160 fish), late March 1997 (80 prespawning fish), and April 1997 (180 fish in spawning condition). Analysis on all fish included gross examination, white blood cell differential counts (done in Chris Kennedy's laboratory, Simon Fraser University),

histopathology on 10 organs, plasma chemistries, IgM levels, and culture of head kidney and spleen for virus isolation (done in Ted Meyers' laboratory, ADFG). At the reference site, Sitka Sound, 250 Pacific herring were sampled in March 1997 and subjected to gross necropsy, virology, and bacteriology; tissues for histopathology and blood analysis were collected, but analysis was not done. In all fish with severe external lesions, kidney was cultured for bacteria (all were negative).

To study the spawn-on-kelp fishery in Prince William Sound, 40 Pacific herring were sampled daily from each of three pounds, for up to 8 days. Other samples were also analyzed for virus: 1) two 40-fish samples of naturally spawning fish were captured from areas around the pounds; 2) one 40-fish sample was selected from recently dead fish in one pound; and 3) water within the pounds was sampled daily at slack tide (dissolved oxygen concentration was also determined). Fish were weighed, measured, aged (by counting scale annuli), examined for external lesions, and tested for viral hemorrhagic septicemia virus. Fish were also examined for gender and gross lesions consistent with *Ichthyophonus hoferi* infection.

Results

In spring 1997 samples, virus prevalence was significantly greater in fish from Prince William Sound (15%) than Sitka (0.8%), and virus prevalence in Prince William Sound was significantly greater than in any other spring sampled (1994, 4.7%; 1995, 1.8%; 1996, 0.0%). Virus has never been isolated from fish sampled in the fall (1995, 1996, and 1997). In spring 1997, viral hemorrhagic septicemia virus was more common in females, younger fish, and fish with external lesions. Unlike previous years, prevalence of ulcers was not well-correlated with virus prevalence in spring 1997 samples. However, virus prevalence in 1997 was significantly associated with several microscopic lesions: death of liver cells (hepatocellular coagulative necrosis), decreased numbers of immature blood cells in the kidney (hematopoietic cell hypoplasia), inflammation in the stomach (lymphocytic gastritis), and an absence of white blood cells (leukocytes) in tissues of the heart and liver (evidence of immunosuppression, or a decreased ability to fight off disease).

The prevalence of *Ichthyophonus* continued to decrease. In spring 1997 *Ichthyophonus* prevalence in Prince William Sound (18%) was less than in 1996 (21%) or in 1994 and 1995 (29%). However, much of the decrease in prevalence was a result of younger, less infected fish, recruiting into the fishery in 1997. The spring prevalence of *Ichthyophonus* in the 1988 year class has not changed much in four years: spring sample prevalence from 1994 to 1997 was 30, 35, 26, and 30%, respectively. In spring 1997, *Ichthyophonus* prevalence was higher in 9-year-old fish (30%) than in 4- and 5-year-old fish (20%) or 3-year-old fish (4.3%).

In addition to viral hemorrhagic septicemia virus and *Ichthyophonus*, prevalence of two other organisms was significantly greater in spring samples from 1997 than in 1996: *Epitheliocystis* in the gill (17% in 1996, 36% in 1997), and trematode parasites in the intestine (14% in 1996, 30% in 1997). Several differences in Prince William Sound fish from fall 1996 and spring 1997 were

consistent with minimal feeding during the winter. First, prevalence of some gastrointestinal parasites decreased from fall to spring; e.g., intestinal cestodes (fall = 36%, spring = 3.1%). Second, mild inflammation in the liver--consistent with a functional immune system and/or food material and bacteria in the digestive tract--decreased from fall to spring; e.g., moderate eosinophilic granular leukocytes around blood vessels (fall = 43%, spring = 21%). And third, energy stores decreased from fall to spring. For example, 21% of the fall samples had liver cells with mild or no depletion of energy stores (glycogen depletion), whereas 100% of the spring samples had moderate or severe glycogen depletion. Interestingly, 79% of fall 1996 samples had moderate or severe glycogen depletion, but only 45% of fall 1995 samples had moderate or severe glycogen depletion. Decreased energy stores in fall 1996 samples were correlated with increased viral prevalence in spring 1997 samples (VHSV 0% in spring 1996 vs. 15% in spring 1997).

In the spawn-on-kelp fishery, prevalence of viral hemorrhagic septicemia virus in initial samples varied from 12.5% on day 0 (pound #1), to 0% and 25% on day 1 (pounds 2 and 3). Sample virus prevalence peaked 2 or 4 days after capture (15-28%), and then declined to 0 to 7.5% on the last sample date, 6 to 8 days after capture. Virus isolation was significantly more common in fish with increased scores for caudal fin fraying and focal skin reddening. However, viral prevalence was not associated with fish density within the pounds, and water samples from within pounds at slack tide were negative for virus. Virus prevalence in one pound 5 days after capture was no different in 46 recently dead fish (15%) than in 40 randomly sampled fish (12.5%). Two 40-fish samples of Pacific herring naturally spawning near the spawn-on-kelp pounds were negative for virus.

Discussion

The unusually high prevalence of viral hemorrhagic septicemia virus in Prince William Sound during the spring of 1997 is consistent with observations of suboptimal spawning activity. Although the high virus prevalence may impair population recovery, there is no evidence that the population biomass has significantly declined, and biomass estimates for 1998 are about 10% greater than biomass estimates for 1997 (John Wilcock, ADFG Cordova, personal communication). Continued decrease in *Ichthyophonus* prevalence is consistent with long-term population recovery. Note, however, that the high *Ichthyophonus* prevalence in 1994 followed a period of low prevalence in 1993, when most of the population died. The final field study samples (April 1998) will help determine how *Ichthyophonus* prevalence in 1998 responds to the high virus prevalence in 1997.

Expression of viral hemorrhagic septicemia virus in Prince William Sound was associated with spawn-on-kelp pounds, and affected fish had more severe external lesions, but this virus was not the major cause of fish mortality within the pounds. Lack of virus in water and fish samples outside the pounds is evidence that closed pounds do not represent a severe threat to the feral fish population. However, the lack of virus in water samples may have been a result of suboptimal sample handling from collection to analysis (e.g., multiple freeze-thaw cycles from Prince William

Sound to Seattle before analysis). A second year of study (1998) will help determine the significance of pounding and viral expression, and virus isolation from water samples will begin on site to optimize chances of isolating virus from the water.

Based on multiple years of data, several patterns are emerging with respect to parasites, disease, and Pacific herring. Viral hemorrhagic septicemia is more common in young females, and expression of virus is associated with significant lesions. Expression of virus and development of associated lesions occurs in late winter and early spring, but does not occur in the fall. *Ichthyophonus* prevalence and lesion severity are greater in mature fish than in new recruits. On a population scale, virus probably contributes more to mortality when the population is young, whereas *Ichthyophonus* may contribute more to mortality when the population is old.

Conclusions and Recommendations

Medical findings in Pacific herring from Prince William Sound in 1996 were essentially consistent with a healthy population, but findings in 1997 indicate that the population experienced a disease relapse. The relapse, however, was mostly independent of the spawn-on-kelp pound fishery. The disease relapse was not severe enough to change our recommendation to upgrade the status of Pacific herring in Prince William Sound from "not recovering" to "recovering," but it is too early to consider the population "recovered." According to the restoration objectives, a large year class must fully recruit into the fishery before a population can be reclassified as "recovered." Pacific herring do not fully recruit into the fishery until 5 years old. The last large year class (1988) was part of a near-record population during its fourth year in 1992, yet the population crashed when this year class reached its 5^{th} year in 1993. Our research and other Trustee Council sponsored research has documented large 1994 and 1995 year classes (E.D. Brown, personal communication). This report documents problems with recruitment of the 1994 year class, and study in 1998 will be the first to examine spawning fish from the 1995 year class. Unfortunately, this project will be terminated before the restoration objective can be documented. Because our studies provide evidence that disease affects survival and stock recruitment, we recommend continued study of major diseases until the population has recovered. A proposal for this work (project 99462) has been submitted concurrent to this report.

CHAPTER 1 - Causes of Disease in Pacific herring from Prince William Sound, Alaska, during Fall 1996 and Spring 1997

G.D. Marty, P. K. Hershberger, E. F. Freiberg, T. R. Meyers, G. Carpenter, and D. E. Hinton

Introduction

When the *Exxon Valdez* oil spill occurred in March 1989, the biomass of spawning Pacific herring in **Prince William Sound (PWS)**, Alaska, was the highest in 20 years of reliable estimates (about 11×10^7 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, and fisheries biologists predicted a near-record spawning biomass of 11×10^7 kg before the spawning season (Figure 1). However, when the 1993 spawning season commenced, only 17% of the expected biomass appeared, fish were lethargic, and many had external hemorrhages. Hence, PWS Pacific herring fisheries were severely curtailed in 1993, and were never opened in 1994, 1995, or spring 1996. In PWS, Pacific herring normally support 5 commercial fisheries, with an average annual ex-vessel value of $8.3 million. Roe fisheries, the most valuable, are harvested in April just before spawning.

Toxicants such as crude oil cause more severe damage in younger fish, particularly larvae (McKim 1985); therefore long-term effects of the oil spill were thought most likely to occur in the 1988 and 1989 year classes which entered the spawning population in 1992 and 1993. Indeed, preliminary study of 4-year-old PWS Pacific herring in 1992 revealed less reproductive success in fish spawning in previously oiled sites than in unoiled sites, and fish with poor reproductive success had more severe microscopic lesions (Kocan et al. 1996). In 1993, the North American strain of **viral hemorrhagic septicemia virus (VHSV)** was isolated from pooled samples of Pacific herring from PWS, but no other significant pathogens were isolated (Meyers et al. 1994). Because VHSV had not previously been isolated from Pacific herring in Alaska, its role in population decline could not be determined. By 1994, spawning biomass declined to the lowest level (1.8×10^7 kg) recorded in 20 years of reliable estimates.

This study was initiated in 1994 to determine the cause of morbidity in PWS Pacific herring. Study included thorough necropsy, virology, bacteriology, hematology, and histopathology linked to traditional age-weight-length analysis. Our primary hypothesis was that VHSV was the most important cause of mortality, but the study was designed to diagnose other potential pathogens. We confirmed that VHSV was a significant cause of morbidity, and we also found that the fungus, *Ichthyophonus hoferi*, was important (Marty et al. 1998). Ten other parasites each affected more than 10% of the sampled population, but their role in population decline probably was minimal. Also, prevalence of most parasites was independent of age. We concluded that disease was significantly contributing to population decline, but background disease prevalence and the role of reproductive stage were unknown.

Study was expanded in 1995 to include prespawning samples and samples from a reference site (Sitka Sound). The Pacific herring population in Sitka supports commercial and subsistence fishing, and there is no history of a large oil spill. In PWS in 1995, VHSV was a less important pathogen, but *Ichthyophonus* continued to be significant, and *Ichthyophonus* was also a significant pathogen in Sitka. In PWS and Sitka in 1996, VHSV was never isolated, and the *Ichthyophonus* prevalence decreased slightly at both sites. 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 fall 1996 and spring 1997. Results from study of the 1997 spawn-on-kelp fishery in PWS are reported in Chapter 2 (section I). Results from the laboratory components are reported in sections II and III.

Objectives

Field study had three major objectives:

1) determine the relation among viral hemorrhagic septicemia virus, *Ichthyophonus hoferi*, macroscopic and microscopic lesions, plasma chemistries, and immune status;
2) determine the role of reproductive stage on the general health of herring [Are lesions and viral hemorrhagic septicemia virus more severe during a given reproductive stage?]; and
3) determine the impact of disease on population size and structure [Are fish of a particular year class more likely to be diseased than other year classes?]

Methods

Necropsy

Pacific herring were captured in October 1996, and March or April 1997, at 2 different sites. At the reference site (Sitka), 250 fish were captured by purse seine, cast net, or hook and line during March 22 - 26, 1997. Sitka fish were transported to a heated garage and subjected to complete necropsy. In PWS, fish were sampled by purse seine and subjected to complete necropsy on board contracted vessels on site as follows: 1) 160 fish, October 15-17. 1996 (100 fish from Zaikof Bay of Montague Island, and 60 fish from the eastern side of Green Island); 2) 80 prespawning fish, March 29 and 30, 1997 (40 fish from Rocky Bay, and 40 fish from Stockdale Harbor); and 3) 180 spawning fish, April 12-14, 1997 (80 from Stockdale Harbor and 100 from Rocky Bay).

Each fish was assigned a unique necropsy number: 96HER501 through 96HER660 for fall-sampled fish, and 97HER1 through 97HER510 for spring-sampled fish. After capture, fish were held in plastic fish totes filled with about 300 L of seawater for no more than 4 hours before necropsy. In groups of two, 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 the summary external lesion score. For spring samples, gonadal fullness was estimated and scored as 3 (75-100% full), 2 (50-74% full), 1 (25-49% full), or 0 (0-25% full).

2) about 1.5 mL of blood was drawn from the caudal vein into 3-mL syringes that contained 0.1 mL of lithium heparin (1,000 IU/mL); a capillary tube was filled and centrifuged (5500 × *g* for 5 min) for determination of **packed cell volume (PCV)**, a blood smear was made and air-dried, and remaining blood was centrifuged (13,600 × *g* for 5 min) and plasma was immediately frozen for later analysis. A 100-µL plasma aliquot from each fish was frozen separately for IgM analysis; details of assay development were described previously (Marty et al. 1996).

   Except for the IgM assay, the following analytes were determined by Chris Kennedy's laboratory at Simon Fraser University: osmolality, sodium, potassium, chloride, **alkaline phosphatase (ALP)**, **aspartate aminotransferase (AST)**, **creatine phosphokinase (CPK)**, total protein, albumin, calcium, lactate, phosphate, and glucose.

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

3) for virus isolation, head kidney and spleen from each fish were pooled in a plastic bag and shipped on ice to the Alaska Department of Fish and Game's 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).

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

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

6) a touch preparation of kidney was air-dried, stained with Dipp-Kwik ® (Differential Staining Solution Set, American Histology Reagent Company, Lodi, CA), and examined

for pansporoblasts of the myxosporean *Ortholinea orientalis*; extent of infestation was scored as for external lesions;

7) liver and gonads were weighed;

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

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

At both Sitka and PWS, nearly all fish in the spawning sample had gonads in spawning condition (i.e., "ripe"). Because the PWS prespawning and spawning samples were similar except for spawning status (see Table 1), their numbers were combined for most statistical comparisons.

Histopathology

Tissues from 160 (fall) and 510 (spring) herring were sent to the Aquatic Toxicology Laboratory, University of California, Davis, and randomly assigned a histopathology number (96H12-1 through 96H12-160, and 97H2-1 through 97H2-260) for blind study. Note that funding for histopathological analysis of Sitka samples from March 1997 was not authorized; therefore, those samples were archived, but not examined. 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 analysis of tissues from the next organ 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 of each severity score) were calculated.

Statistical Analysis

The major hypotheses was that fish with lesions were different from fish without lesions. 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 CPK values of fish whose liver's had mild, moderate, and severe *Ichthyophonus*. When necessary, categories were combined to ensure that each group had at

1997. Variables considered included spawning stage, gender, season, and age, with special emphasis on organisms and lesions likely to result in population level effects.

Necropsy Findings and External Gross Lesions - Fall 1996 and Spring 1997 Samples

For spring 1997 samples, spawning fish from PWS were significantly older than other sample groups (Table 1). Significant differences in most other morphometric necropsy variables were consistent with age differences among the sample groups. However, some significant seasonal differences from fall to spring were not related to age or body size: 1) liver weight decreased; 2) ovary and testis weight increased; and 3) IgM concentration and PCV decreased (Table 1). Hold time never exceeded 3.6 hr for any sample group, and difference in mean hold time among sample groups were not significantly different.

Prevalence of ulcers (=severe focal skin reddening) among spring samples was greater in 1997 than in 1996, and greater in fish from PWS than in fish from Sitka (Figure 2). The relation between external lesions and plasma chemistry values was often highly significant (Table 2). Increased sodium concentrations were related to increased scores for caudal fin fraying, caudal fin reddening, and fin base reddening. Focal skin reddening was related to increased *Ichthyophonus* scores and decreased PCV, albumin, and IgM (Table 2). Opercular copepods were more common in younger fish in spring 1996, fall 1996, and spring 1997 samples, but changes in plasma chemistries associated with opercular copepods in 1997 (decreased glucose, calcium, phosphate, and IgM) were probably related more to age than to the opercular copepods (see Table 9). In spring 1996 samples, 59% of prespawning fish and 37% of spawning fish from PWS had opercular copepods. Prevalence of opercular copepods in spring 1997 samples was similar: Sitka spawning (48%), PWS prespawning (57%), and PWS spawning (47%).

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

Microscopic Lesions - 1996 fall and 1997 spring samples

Ichthyophonus

Overall prevalence of *Ichthyophonus* was slightly less in spawning samples from PWS in 1997 (19%, 34 of 180) than in spawning samples from 1994 (29%, 62 of 212), 1995 (29%, 52 of 180), and 1996 (25%, 45 of 180). In 1994, *Ichthyophonus* prevalence among PWS age groups was not significantly different, but in all years after 1994, *Ichthyophonus* was significantly more frequent in the 1988 year class than in the 3-year-old fish (Figure 3). At least some of the decrease in population prevalence of *Ichthyophonus* in 1997 was a result of large numbers of 3-year-olds

recruiting into the fishery in 1997. The spring prevalence of *Ichthyophonus* in the 1988 year class has not changed much in 4 years: prevalence in spring samples from 1994 to 1997 was 30, 35, 26, and 30%. In spring 1997, *Ichthyophonus* prevalence was higher in 9-year-old fish (30%) than in 4- and 5-year-old fish (20%) or 3-year-old fish (4.3%). The lower prevalence of *Ichthyophonus* in Pacific herring sampled in the fall is also consistent with younger fish in these samples.

All organs contained *Ichthyophonus* (Table 2; Figure 4), and the multinucleate resting spore stage was the most common form. Morphology of *Ichthyophonus* and the host reaction were similar to those reported in infections in Atlantic herring (*Clupea harengus*) (Daniel 1933, Sindermann 1970). Scoring, histologic features, and differential diagnoses in Pacific herring were essentially the same as reported previously (Marty et al. 1998).

Although the overall *Ichthyophonus* prevalence in fish from spring 1997 samples was 18%, in no single organ was *Ichthyophonus* prevalence > 15% (Figure 4). Distribution of *Ichthyophonus* in various organs was similar from 1994 through 1997 (Figure 4); heart, liver, kidney, spleen, and skeletal muscle were the most commonly affected organs. A sum*Ichthyophonus* score was calculated for each fish by adding the individual *Ichthyophonus* scores from all 10 organs for that particular fish (Marty et al. 1998). In spring 1997, the highest sum*Ichthyophonus* scores was 19.

Association of *Ichthyophonus* scores with plasma chemistries was fairly consistent (Table 2). In most organs sampled in spring 1997, increased *Ichthyophonus* scores were related to increased age, total protein, albumin, AST, IgM, and sometimes were related to increased ALP. In 1994, increased AST and CPK values were significantly associated with increased *Ichthyophonus* scores in every organ (univariate ANOVA). This trend was consistent in fall 1996, but in spring 1997 CPK values were never related to *Ichthyophonus* scores. In spring 1995, the association of *Ichthyophonus* scores with AST and CPK was less distinct: AST and CPK significantly increased with most Sitka *Ichthyophonus* scores, but only AST significantly increased with most PWS *Ichthyophonus* scores. In spring 1996, the association of *Ichthyophonus* scores with AST and IgM was about the same as in spring 1995, but CPK was significant only for intestinal *Ichthyophonus* in the intestine.

In spring 1997 samples, the sum*Ichthyophonus* score was significantly related to increases in several non*Ichthyophonus* scores (Table 2): gill arch inflammation or hematopoiesis, gill lamellar hyperplasia, pigmented macrophage aggregates in the ovary and kidney, epicarditis, renal interstitial hematopoietic cells, hepatic perivascular eosinophilic granular leukocytes, hepatic granulomatous inflammation, skeletal muscle perivascular leukocytes, and skeletal myositis. In fall 1996, increased scores for ovarian and testicular EGLs were significantly related to increased sum*Ichthyophonus* scores, even though none of the sections of 78 ovaries contained *Ichthyophonus* resting spores; this relation was not significant in spring 1997 samples.

VHSV

VHSV prevalence in spring 1997 samples (15%) was higher than in any other year studied (Figure 2). As shown in the summary table below, VHSV prevalence was not significantly different for prespawning or spawning samples. VHSV prevalence was consistently higher in samples from Rocky Bay, but fish from Rocky Bay were significantly younger than fish from Stockdale Harbor. VHSV prevalence was higher in 3-year-old fish than in other year classes (Figure 5). Note that isolation of VHSV was highly variable (range = 0 to 35%) among the 13 20-fish sets (Figure 6). Sample VHSV prevalence values seem to have a bimodal distribution (Figure 6), similar to that described for daily samples from spawn-on-kelp pounds in the next chapter (compare Figure 6 here with Figures 1, 2, and 3 in Chapter 2). However, many of these samples were taken less than 4 hours apart (e.g., the 1st and 2nd samples from Rocky Bay); therefore, variation probably is more a result of heterogenous distribution of VHSV within the Pacific herring population rather than a result of changes in population prevalence over such a short time.

Site	Sample	n	Mean age (yr.)	VHSV (%)
Rocky Bay	prespawning	40	4.1	20
Stockdale Harbor	prespawning	40	4.9	7.5
Both	prespawning	80	4.5	14
Rocky Bay	spawning	100	5.5	19
Stockdale Harbor	spawning	80	6.3	10
Both	spawning	180	5.9	15
Both	all	260	5.4	15

Several lesions were associated with VHSV (Table 4), but VHSV isolation was associated with few changes in blood or plasma chemistry values (Table 5). VHSV was more commonly isolated from younger fish with external lesions. Affected fish were more likely to have hepatic focal and single cell necrosis, and two parasites were more common in VHSV+ fish (the gall bladder myxosporean *Ceratomyxa auerbachi*, and the renal intraductal myxosporean, *Sphaerospora* sp.?). Inflammation associated with VHSV isolation included submucosal gastritis and gonadal granulomatous inflammation. In contrast, several types of inflammation were decreased in VHSV+ fish: renal interstitial hematopoietic cells, hepatic focal parenchymal leukocytes, and cardiac focal parenchymal leukocytes (Table 4).

Three fish that had the lowest PCV among spring 1997 samples were all positive for VHSV. Two of these fish had the lowest PCVs ever recorded during the course of this project, 4% and 10%. Both were prespawning 3-year-old females sampled from the same set in Rocky Bay on March 29, 1997. They had mild and moderate focal skin reddening, no diffuse skin reddening,

and no opercular copepods and no *Ichthyophonus*. The third fish, also a 3-yr-old female, had a PCV of 21% and concurrent *Ichthyophonus* infection.

Gender-associated Lesions

Gonads had several differences in lesion prevalence (Tables 2 and 6). As in all previous years, lesions more frequent in ovaries included hyalinization of vessel walls and pigmented macrophage aggregates. Prevalence of pigmented macrophage aggregates in fall 1996 (35%) and spring 1997 (29%) was less than in 1995 and 1996 (about 40%), which was slightly less than in 1994 (about 60%). Prevalence of hyalinization of vessel walls in ovaries of spawning fish in PWS has been highly variable over the years of the study (1994, 61%; 1995, 41%; 1996, 5.8%; 1997, 26%).

Granulomatous inflammation in the gonads continues to be highly variable. Prevalence in ovaries ranged from 1.8% in spawning PWS females in 1994 to 92% in immature prespawning fish in 1995. Prevalence of granulomatous inflammation in testes ranged from 2.0% in spawning fish from Sitka and PWS in 1996 to 43% in immature prespawning fish from PWS in 1995. As in previous years, *Ichthyophonus* was rare in either gonad in fall 1996 and spring 1997 samples (Table 2, Figure 4).

Gender differences within fall 1996 and spring 1997 were significant for several nongonadal lesions (Table 6). In fall 1996 samples, gender differences in *Ichthyophonus* prevalence were not significant; however, in spring 1997, *Ichthyophonus* prevalence in the stomach and brain was significantly greater in females than males. Gall bladder myxosporeans (*Ceratomyxa auerbachi*) were significantly more prevalent in females in spring 1994, 1995, and 1996 samples, but differences were no longer significant in spring 1997 samples; gender differences for *Ceratomyxa auerbachi* have never been significant in fall samples. In fall 1996, intestinal coccidians and intestinal trematodes were more common in males than females, but gender differences in prevalence of these parasites were no longer significant in spring 1997. In previous spring samples, females were significantly more likely than males to have hepatic lipidosis, and males were significantly more likely to have hepatocellular glycogen depletion, but differences were not significant in spring 1997 samples. In spring 1996 and spring 1997 samples, males were more likely to have vacuolated renal tubular epithelial cells, but differences were not significant in fall samples from 1995 or 1996. Hepatocellular single cell necrosis and focal hepatic necrosis were more common in females than in males in spring 1996 and 1997, but not different in fall samples.

Gender differences within fall 1996 and spring 1997 were also significant for several plasma chemistries and blood values (Table 7). Mean age, length, and body weight of each gender were not significantly different for either the fall or spring samples. However, liver weight was significantly greater for females in both seasons; gonad weight was greater in males in fall samples but was greater in females in spring samples (Table 7). Gender differences were significant for three plasma chemistries in both fall and spring, but the gender with the greatest concentration changed from fall to spring. In fall 1996 samples, total protein and glucose were greater in

females and ALP was greater in males; in spring 1997 samples, total protein and glucose were greater in males (consistent with increased energy needs for egg production in females).

Iris reddening

Some lesions significantly associated with iris reddening were more prevalent in fish with moderate iris reddening than in fish with mild or no iris reddening (Table 8). Examples include intestinal foreign body granuloma in fall 1995 and fall 1996 fish, and hepatic granulomatous inflammation in spring 1997 fish. Splenic congestion was significantly more likely in spring 1996 fish with no iris reddening; the same pattern occurred in spring 1994 and spring 1995, but differences were not significant in fall 1995, fall 1996, or spring 1997 samples. In fall 1995 and fall 1996, increased scores for iris reddening were significantly associated with increased values for lactate and osmolality, consistent with a stress-induced response; however, these relationships were not significant in spring samples.

Intraperitoneal Herring Worms (Anisakidae)

More than 98% of the Pacific herring sampled from Sitka and PWS had larval parasites of the family Anisakidae within their peritoneal cavities: 1) PWS, fall 1996, 160 of 160; 2) Sitka, spring 1997, 247 of 250; and 3) PWS, spring 1997, 259 of 260. 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). Numbers of Anisakidae in the peritoneal cavity were significantly related to increased lesion scores for cholangitis and biliary hyperplasia, hepatic perivascular eosinophilic granular leukocytes, hepatic pericholangial leukocytes, gastric serositis, splenic arterial intimal hyperplasia, and splenic congestion (Table 2). Interestingly, numbers of these parasites in the peritoneal cavity was not significantly related to scores for these same parasites within histologic sections of the liver and skeletal muscle.

Other Lesions and Potential Pathogens

No significant bacterial pathogens were isolated. Only one fish had erythrocyte inclusions characteristic of VEN. That fish, a 3-year-old female sampled on October 16, 1996, had severe fin base reddening and a PCV of 54%. The mean PCV for fall 1996 fish from PWS was 48%.

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

1) intraepithelial intestinal coccidian *Goussia*? sp.
   1994: PWS = 91%;
   1995: PWS = 95%, Sitka = 91%;
   1996: PWS = 94%, Sitka = 92%;
   1997: PWS = 94%;

2) hepatic coccidian *Goussia* [*Eimeria*] *clupearum*
   1994: PWS = 61%;
   1995: PWS = 73%, Sitka = 71%;
   1996: PWS = 80%, Sitka = 79%;
   1997: PWS = 70%;
3) testicular coccidian *Eimeria sardinae*
   1994: PWS = 57%;
   1995: PWS = 85%, Sitka = 66%
   1996: PWS = 74%, Sitka = 81%;
   1997: PWS = 94%;
4) gall bladder myxosporean *Ceratomyxa auerbachi*
   1994: PWS = 19%;
   1995: PWS = 39%, Sitka = 32%
   1996: PWS = 29%, Sitka = 23%;
   1997: PWS = 20%;
5) renal intraductal myxosporean *Ortholinea orientalis*
   1994: PWS = 19%;
   1995: PWS = 29%, Sitka = 20%
   1996: PWS = 20%, Sitka = 21%;
   1997: PWS = 16%;
6) branchial *Epitheliocystis*
   1994: PWS = 10%;
   1995: PWS = 15%, Sitka = 25%
   1996: PWS = 17%, Sitka = 21%;
   1997: PWS = 36%;
7) renal intraductal myxosporean (*Sphaerospora* sp.?)
   1994: PWS = 11%;
   1995: PWS = 11%, Sitka = 3.8%
   1996: PWS = 9%, Sitka = 8%;
   1997: PWS = 12%;
8) gastric trematodes
   1994: PWS = 8.6%;
   1995: PWS = 12%, Sitka = 10%
   1996: PWS = 15%, Sitka = 22%;
   1997: PWS = 13%;
9) branchial monogenetic trematodes
   1994: PWS = 13%;
   1995: PWS and Sitka = 11%
   1996: PWS = 1.5%, Sitka = 2.1%;
   1997: PWS = 7.7%;
10) intestinal trematodes, e.g., *Lecithaster gibbosus*
   1994: PWS = 2.9%;
   1995: PWS = 8.9%, Sitka = 2.1%

1996: PWS = 14%, Sitka = 1.7%;
1997: PWS = 30%; and

11) intestinal cestodes, e.g., *Nybelinia surmenicola*
1994: PWS = 2.4%;
1995: PWS = 3.3%, Sitka = 2.5%
1996: PWS = 1.5%, Sitka = 3.8%
1997: PWS = 3.1%.

Prevalence of two of these organisms was significantly greater in spring samples from 1997 than in 1996: *Epitheliocystis* in the gill, and trematode parasites in the intestine. Several differences in Prince William Sound fish from fall 1996 and spring 1997 (see Table 2) were consistent with minimal feeding during the winter. First, prevalence of some gastrointestinal parasites decreased from fall to spring; e.g., intestinal cestodes (fall = 36%, spring = 3.1%). Second, mild inflammation in the liver--consistent with a functional immune system and/or food material and bacteria in the digestive tract--decreased from fall to spring; e.g., moderate eosinophilic granular leukocytes around blood vessels (fall = 43%, spring = 21%). And third, energy stores decreased from fall to spring. For example, 21% of the fall samples had liver cells with mild or no depletion of energy stores (hepatocellular glycogen depletion), whereas 100% of the spring samples had moderate or severe hepatocellular glycogen depletion. Interestingly, 79% of fall 1996 samples had moderate or severe glycogen depletion, but only 45% of fall 1995 samples had moderate or severe glycogen depletion. Decreased energy stores in the fall were correlated with increase viral prevalence the next spring (0% had VHSV in spring 1996, 15% had VHSV in spring 1997).

Intestinal coccidians were common in small numbers throughout the intestine, including the intestinal cecae. In 1994, only 1% of the PWS spawning fish had moderate infestation (i.e., >15 organisms per 400× field), and infestation was not associated with alterations in plasma chemistry values. In spring 1995, overall prevalence was not different in PWS and Sitka. However, 31% of the PWS prespawning fish and 12% of the PWS spawning fish and had moderate infestations, compared to only 2% of Sitka fish with moderate infestations. In spring 1996, overall prevalence was the same at each site, but fish with moderate infestations were again more prevalent in PWS than in Sitka (10.4% vs. 1.7%). Severity of intestinal coccidians in 1995 was significantly related to greater CPK and AST values in PWS fish but not in Sitka fish. In spring 1997, overall prevalence in PWS was the same as in previous years, and 17% had moderate infestation. In spring 1996 and 1997, intestinal coccidians were not associated with clear trends in plasma chemistries (Table 2).

Morphologic features and distribution of the hepatic coccidian were very similar to descriptions of *Goussia clupearum* in Atlantic and Pacific herring (Morrison and Hawkins 1984, Marty et al. 1995). Despite the relatively large volume of hepatic parenchyma displaced by the parasites in severe cases, inflammation was minimal. For 1996 Sitka fish only, increased lesion scores were associated with decreased plasma cholesterol levels, but this difference was not consistent with 1995 findings, where increased lesion scores were associated with decreased plasma glucose levels. In 1997, changes in severity scores were associated with changes in IgM, ALP, and CPK.

As in previous years, diagnosis of the renal intraductal myxosporean *Ortholinea orientalis* was less sensitive by histopathology. In 1995, as histopathology scores for *Ortholinea orientalis* in spawning PWS fish increased, values for PCV, neutrophils, and basophils significantly increased, but these trends were no longer significant in 1996 or 1997 (Table 2).

In 1995, prevalence of 4 subtle inflammatory lesions was significantly greater in spawning fish from PWS than from Sitka, but differences were no longer significant in 1996, and levels in spawning fish from both PWS and Sitka in 1996 were more like Sitka in 1995. In 1997 spawning samples, prevalence of these inflammatory lesions was most similar to PWS in 1996:

1) perivascular leukocytes in skeletal muscle
   1995: PWS = 77%, Sitka = 65 %,
   1996: PWS = 92%, Sitka = 92%;
   1997: PWS = 63%;
2) focal parenchymal leukocytes in the liver
   1995: PWS = 81%, Sitka = 49%,
   1996: PWS = 37%, Sitka = 35%;
   1997: PWS = 34%;
3) focal parenchymal leukocytes in the heart
   1995: PWS = 32%, Sitka = 24%,
   1996: PWS = 11%, Sitka = 17%;
   1997: PWS = 8.9%;
4) foci of leukocytes in the submucosa and muscularis of the stomach
   1995: PWS = 13%, Sitka = 1.7%,
   1996: PWS = 2.2%, Sitka = 2.1%.
   1997: PWS = 3.9%;

Age-associated Changes

As in previous years, the most consistent age-related microscopic change was increased severity of pigmented macrophage aggregates in older fish. Indeed, age-related changes were significant in all organs in which pigmented macrophage aggregates were scored: exocrine pancreas, liver, ovary, spleen, and trunk kidney (Table 2). Among external lesions in spring 1997 fish, caudal fin fraying decreased with age, and moderate iris reddening was more common in older fish. Other microscopic lesion scores that were significantly related to increased age included: 1) hyalinization of vessel walls in the ovary; 2) epicarditis; 3) foreign body granulomas in the intestine; 4) renal tubular epithelial vacuolation; 5) perivascular eosinophilic granular leukocytes in the liver; 6) pericholangial leukocytes; 7) focal intimal arteriolar hyperplasia in skeletal muscle and spleen; 8) splenic ellipsoid hyalinization or hypertrophy; and 9) *Ichthyophonus* in most organs. Older fish had significantly decreased severity scores for splenic congestion, opercular copepods, and intestinal trematodes in spring 1996 and 1997 (ANOVA).

Several values for plasma chemistries, weights, and length were significantly related to age (Table 9). Values that significantly increased with age during both seasons included albumin, calcium,

IgM, total protein, all weights (body, gonad, and liver), and length. No plasma chemistry values significantly decreased with age in both sample groups, and this finding was the same in 1996 samples. Hematology values did not consistently change with age in fall 1996, but in spring 1997 % lymphocytes was lowest in 5-yr-old fish and higher in other age groups; trends in the % thrombocytes were opposite (i.e., frequency was highest among 5-yr-old fish).

Because of the relatively small sample size in the fall, most seasonal comparisons will not be statistically analyzed until the final report. However, Table 9 reveals several interesting trends within the same year class from fall 1996 to spring 1997: 1) sum*Ichthyophonus* scores stayed the same for 2- and 3-yr-old fish, but decreased for fish 4 years old and older; 2) numbers of peritoneal Anisakidae did not change over the winter; 3) albumin and IgM decreased for all age groups, but total protein did not change; and 4) body weight decreased 5 to 8%, but standard length did not change. The calcium values for fall 1996 are about ½ the calcium values for all other groups of Pacific herring sampled since 1994. These values may not be correct; their determination is being rechecked, and the values may be revised or discarded for the final report.

Leukocyte Differential Counts

Although interpretation of leukocyte differential counts is limited without knowledge of the total white blood cell count, the values provide useful information for generating hypotheses that can be further examined with laboratory study, particularly in Section III of this project (C. Kennedy, Simon Fraser University). Several lesions were significantly related to changes in the frequency of various leukocytes, but significant relationships were often inconsistent between samples (Table 2). For example, in spring 1995 samples, increased frequency of neutrophils was significantly related to *Ichthyophonus* scores in PWS samples from several organs, but differences were not significant in spring 1996 or in spring 1997 samples. In contrast, severity of *Ichthyophonus* scores was significantly related to decreased neutrophil frequency in fall 1995 samples from PWS. Increased frequency of neutrophils was associated with increased scores for hepatic Anisakidae, hepatic and pigmented macrophage aggregates, and gastric foreign body granulomas in spring 1997 samples but not in previous years' samples.

Lymphocyte frequency significantly increased with scores for several lesions in 1997 samples from PWS (Table 2): diffuse skin reddening, focal skin reddening, gill arch inflammation or hematopoiesis, gill lamellar telangiectasis, and submucosal lymphocytic gastritis. Note that none of these lesions were related to changes in lymphocyte frequency in 1996 samples. Trends in thrombocyte frequency were usually opposite that of lymphocyte frequency. One lesion unique to changes in thrombocyte frequency was hepatic focal necrosis, which was related to decreased frequency of thrombocytes (Table 2). The frequency of eosinophils significantly increased with only one lesion: atresia of mature oocytes (Table 2). Monocytes and basophils were rare, and the only significant trend was for decreased frequency of basophils with increased scores for ovarian eosinophilic granular leukocytes.

Plasma chemistries

Holding fish for up to 4 hours as they awaited necropsy consistently resulted in increased plasma potassium and decreased PCV (Table 10). In fall 1996 samples, phosphorus and total protein significantly increased as hold time decreased, whereas in spring 1997, IgM (and not total protein) significantly decreased as hold time increased. Changes in other plasma chemistries were not significant in relation to hold time.

Increases IgM values were significantly related to increased scores for area of renal hematopoietic cells in all samples and seasons from fall 1995 through spring 1997.

Annual Trends in Spawning Biomass and Pathogen Prevalence

Sample prevalence of *Ichthyophonus* decreased from spring to fall 1995, and the decrease was maintained through spring 1997, with fall prevalence slightly lower than spring prevalence (Table 11). Preliminary results from fall 1997 samples indicate that *Ichthyophonus* prevalence has dropped dramatically (to about 4%); however, fall 1997 samples were much younger than spring 1997 samples (1997 mean age in years = 2.7 in fall vs. 5.4 in spring). *Ichthyophonus* prevalence in spring 1997 samples was within the range of samples from 1989 and 1990. Prevalence of *Goussia clupearum* was fairly constant between 41 and 63% for most years before 1995, but prevalence has been closer to 70% since 1994, with a peak of 80% in spring 1996 samples from PWS (Tables 2 and 11). Based only on histopathology, the prevalence of *Ortholinea orientalis* has been quite variable over the years (Table 10); 17% of the 80 Pacific herring sampled from PWS in fall 1997 had *Ortholinea orientalis* on histopathology. Note: since the last annual report, sections from the 1990 and 1991 samples were re-examined, additional *Ortholinea orientalis* were found, and prevalence values have been corrected; also, an error in the *Goussia clupearum* prevalence for October 1991 samples has been corrected.

Discussion

Note on the contents the discussion section in this report: The annual reports for project 94320-S (Marty et al. 1995) and 95320-S (Marty et al. 1996) and 96162 (Marty et al. 1997) contain detailed discussion, including historical perspective, on most of the significant lesions and plasma chemistry changes in Pacific herring. That discussion is not repeated in this annual report, but will be included in the final synthesis report. This report concentrates on significant differences in lesions, necropsy findings, and plasma chemistry values in samples from fall 1996 and spring 1997. Variables, considered included spawning stage, season, site of capture, and year of capture, with special emphasis on organisms and lesions likely to result in population level effects.

Ichthyophonus hoferi

In Pacific herring from PWS, the large increase in prevalence of *Ichthyophonus* in 1994 was not associated with an unusual population decline between 1994 and 1996 (Figure 1). The main

difference in *Ichthyophonus* epidemiology between 1994 and spring 1995 was the age distribution of the fungus. In 1994, all age groups were infected in equal proportions. But in spring 1995, prevalence of *Ichthyophonus* was significantly greater in older fish. This trend towards higher prevalence in older fish continued in all samples through spring 1997, including those from Sitka in 1995 and 1996. No other pathogens were significantly more common in older fish, and *Ichthyophonus* seems the most likely cause of differential mortality in older fish. In the 9-year database of *Ichthyophonus* prevalence among Pacific herring in PWS (1989 - 1997), the relatively low prevalence in 1991, 1992, and 1993 seems to be more of an anomaly than the prevalence since 1994. It may be that *Ichthyophonus* takes several months to years to cause mortality after a Pacific herring is infected, and mortality may require interaction with other variables such as ageing, predation, or other parasites.

In Atlantic herring populations as recently as 1991, major population decline in the North Sea was attributed to *Ichthyophonus* (Lang 1992). Mathematical analysis of population trends following this outbreak revealed that the use of *Ichthyophonus* prevalence significantly improved the fit of the catch and survey observations to the conventional assessment model (Patterson 1996). Because *Ichthyophonus* seems to cause mortality more readily in Atlantic herring than in Pacific herring, the role of *Ichthyophonus* in population decline may be less in Pacific herring than in Atlantic herring. Use of *Ichthyophonus* prevalence in the age-structured assessment model has not yet been attempted with Pacific herring, but a revised proposal for this work was submitted to the United States National Science Foundation, Division of Biological Oceanography, on Feb. 15, 1998 (G.D. Marty, principal investigator).

A potential problem with studies of marine fish epizootics in the North Atlantic (Rahimian and Thulin 1996, Mellergaard and Spanggaard 1997) is that research usually focused on a single organism, possibly allowing for other significant organisms or environmental variables go undetected. In PWS Pacific herring, prevalence of *Ichthyophonus* was nearly 30% in 1994 (Marty et al. 1998). This was similar to reports of *Ichthyophonus* prevalence in Atlantic herring epizootics (Sindermann 1970). However, when most PWS adult mortality occurred in 1993, the *Ichthyophonus* prevalence in Pacific herring was significantly lower (5%; Meyers et al. 1994) than reports of *Ichthyophonus* epizootics in Atlantic herring. This was evidence that the increase in *Ichthyophonus* prevalence in PWS may have been more of a response to the population crash rather than the cause. During intensive study of I. hoferi in the 1991 Atlantic herring epizootic in the North Sea, little effort was expended to determine if other pathogens, particularly viruses, were contributing to the epizootic. The recent isolation of the European strain of VHSV from Atlantic herring (Dixon et al. 1997) introduces a new hypothesis into the interpretation of the *Ichthyophonus* findings. Was *Ichthyophonus* the primary cause of the epizootic in the North Sea? Or, did an increase in *Ichthyophonus* prevalence follow an outbreak of VHSV that went undetected?

Ichthyophonus infections in both Sitka in 1995 and 1996, and in PWS in 1995 through 1997 were significantly related to increased IgM levels–consistent with the chronic nature of the disease. Further definition of the IgM response would require development of an ELISA specific for anti-

Ichthyophonus antibodies, but development is beyond the current scope of the project. Infection with *Ichthyophonus* in 1995, 1996, and 1997 was less commonly related to changes in plasma CPK than in 1994, particularly in PWS samples, although AST was about equally effective all 4 years. The role of CPK in *Ichthyophonus* infections could not be further defined because isozymes could not be consistently separated using commercial tests that had been developed for use in mammalian plasma (C.J. Kennedy, personal communication).

VHSV

The unusually high prevalence of viral hemorrhagic septicemia virus in Prince William Sound during the spring of 1997 is consistent with observations of suboptimal spawning activity, and the high virus prevalence may impair population recovery. However, there is no evidence that the population biomass has significantly declined, and biomass estimates for 1998 are about 10% greater than biomass estimates for 1997 (John Wilcock, ADFG Cordova, personal communication). Continued decrease in the *Ichthyophonus* prevalence is consistent with long-term population recovery. Note, however, that the high *Ichthyophonus* prevalence in 1994 followed a period of low prevalence in 1993, when most of the population died. The final field study samples (April 1998) will help determine how *Ichthyophonus* prevalence in 1998 responds to the high virus prevalence in 1997.

The lack of VHSV in spawning samples from both PWS and Sitka in 1996 clearly demonstrated that Alaskan populations of Pacific herring can spawn without expressing significant quantities of VHSV. Likewise, finding of VHSV in both populations in spring 1997 samples is evidence that VHSV is endemic in both populations. Because the populations at both sites seem to be unaffected by the higher prevalence of VHSV in 1997, it is clear that isolation of VHSV is not sufficient evidence of population decline. Other variables such as environmental conditions, population density, size at age, and tissue energy stores may play a role in determining the effect of a VHSV outbreak on population health.

External Lesions and Iris Reddening

External lesions are too nonspecific to be consistently related to any single cause. As evidence, in 1994 the external lesion fin base reddening was significantly associated with VHSV, and no external lesions were significantly associated with *Ichthyophonus*. In spring 1995, fall 1996, and spring 1997, focal skin reddening was significantly associated with *Ichthyophonus* infection. In spring 1996, focal skin reddening was not associated with *Ichthyophonus* infection. Because many of the spring 1996 fish had already spawned, and 1995 and 1997 were ripe but had not yet spawned, focal skin reddening may be a useful *Ichthyophonus* marker during the spring only for fish before they spawn. Although external lesions are useful indicators of population health, the interannual inconsistency of external lesions associated with various internal lesions (e.g., Table 3) provide evidence that external lesions are not reliable indicators of specific internal lesions.

Other Potential Pathogens

The best candidate for significant pathogens among the other common parasites in 1995 was the unidentified intestinal coccidian (*Goussia*? sp.), particularly in fish from PWS. In all samples since fall 1995, however, infection with the intestinal coccidian was not consistently related to any changes in plasma chemistries. As a secondary candidate for a significant pathogen, infection with the renal intraductal myxosporean *Ortholinea orientalis* was related to decreased total protein levels in fall 1995 samples from PWS, and to decreased IgM levels in fall 1996 fish. Although differences were not highly significant, decreased protein levels could potentially inhibit overwinter survival. *Ortholinea orientalis* infection was not consistently related to any plasma chemistry changes in spring 1996 or spring 1997 samples, but analysis of fall 1997 samples (to be done as part of the final report) may provide further evidence of the effect of *Ortholinea orientalis* on fall fish.

Plasma Chemistries

Plasma chemistries were highly sensitive to changes in several lesions, reproductive status, and other variables (e.g., age and season). The significant site-related differences in lactate and CO_2 from spring 1995 samples provided information that was used to improve fish holding techniques as early as fall 1995 samples. Fish for sampling are now held in about 300 L of seawater (in large totes used by fish processors) to allow for swimming space while fish are held from capture to necropsy. Now, plasma chemistries provide evidence that initial capture and transport are stressful, but that holding conditions allow for at least a partial return to resting values. The negative correlation of hold time to PCV, plasma total protein, and plasma IgM may be explained by gradual recovery from the initial stress of capture. Within a few minutes after acute stress due to capture, catecholamine and cortisol release and muscle lactic acidosis result in elevated intracellular osmolarity, which causes a fluid shift from the circulation to the intracellular compartment(McDonald and Milligan 1992). This fluid shift results in increased PCV and concentrations of plasma proteins, including IgM. Over time, if the stress level is decreased, the fluid shift reverses so that PCV and protein concentrations return to normal.

Conclusions

Disease was probably the primary force driving Pacific herring population decline in 1993 and 1994. No other variables–food availability, predation, water temperature, currents, or recruitment–were needed to explain this significant decline; however, these variables may have contributed to conditions favorable for initiation of the epidemic. The conclusion that disease was significant is based on integration of results from this project, literature review including Meyers et al. (1994), plus information from biologists, fishers, and laboratory study where both VHSV and *Ichthyophonus* killed Pacific herring in the absence of other diseases (e.g., Kocan et al. 1997). Among the 2 significant diseases, VHSV was most important in 1993. By 1994, *Ichthyophonus* prevalence had increased, possibly as a result of VHSV-induced immunosuppression, so that *Ichthyophonus* and VHSV were of about equal importance. By 1995, VHSV prevalence had

decreased to where it was a less important cause of mortality, and by 1996, VHSV was not an important cause of mortality. The effect of the large increase in VHSV prevalence in 1997 is unknown, but does not seem to be linked to population decline. *Ichthyophonus* seems to be fairly normal in older Pacific herring, but it may be one of the major limiting variables in fish longevity. The long-term effects on population change, however, are still not well understood. Continued monitoring of disease in PWS will increase our knowledge of how disease interacts with pelagic schooling fish like Pacific herring, and further study (proposed as project 94962) 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 Prince William Sound in October 1996, March 1997, and April 1997. Group means within each variable were compared using one-way analysis of variance and Tukey's multiple comparison procedure; groups with the same letter are not significantly different ($P \leq 0.050$). Comparisons in which Levene's test for equality of variance was significant ($P \leq 0.05$) are marked (*).

Variable	Month	Spawning status	# examined	Mean	±SE	ANOVA *P* value
age (yrs)	October	none	160	3.6[C]	0.2	<0.001*
	March	prespawning	80	4.5[B]	0.2	
	April	spawning	180	5.9[A]	0.2	
standard length (mm)	October	none	160	207.8[B]	1.7	<0.001
	March	prespawning	80	203.3[B]	2.2	
	April	spawning	180	218.1[A]	1.6	
body weight (g)	October	none	160	125.0[A]	3.5	0.005
	March	prespawning	80	110.0[B]	4.5	
	April	spawning	180	129.0[A]	3.2	
liver weight (g)[c]	October	none	160	1.4[A]	0.1	<0.001
	March	prespawning	80	1.1[B]	0.1	
	April	spawning	180	1.1[B]	0.1	
ovary weight (g)[a,c]	October	none	78	3.3[B]	0.5	<0.001
	March	prespawning	44	20.3[A]	3.4	
	April	spawning	97	25.8[A]	2.7	
testis weight (g)[a,c]	October	none	79	7.8[C]	1.5	<0.001*
	March	prespawning	34	14.8[B]	2.5	
	April	spawning	77	23.1[A]	3.1	
hold time (min)	October	none	160	108.5[A]	3.1	NS[b]
	March	prespawning	80	102.4[A]	4.4	
	April	spawning	180	108.3[A]	2.9	
IgM (µg/mL)[c]	October	none	160	450[A]	44.2	<0.001
	March	prespawning	80	262[C]	39.7	
	April	spawning	180	342[B]	34.2	

Variable	Month	Spawning status	# examined	Mean	±SE	ANOVA *P* value
PCV (%)[d]	October	none	160	47.8[A]	0.8	<0.001*
	March	prespawning	80	40.9[B]	1.9	
	April	spawning	180	42.7[B]	0.6	
peritoneal cavity - number of herring worms (Anisakidae)[c]	October	none	160	9.4[B]	1.1	0.031
	March	prespawning	80	11.5[A,B]	1.7	
	April	spawning	179	11.2[A]	1.1	
gills - number of 0.5-mm-diameter white foci	October	none	157	0.4[A]	0.1	0.002*
	March	prespawning	79	0.3[A,B]	0.1	
	April	spawning	179	0.1[B]	0.0	

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

Table 2. Lesion severity (% of fish classified in each lesion score) and lesion prevalence (% of sample having lesion score >0) in Pacific herring sampled from Prince William Sound, Alaska, during October 1996 (F96) or April 1997 (S97). Lesions were scored as none (0), mild (1), moderate (2), or severe (3). Age, hold time, and blood values were compared for groups based on lesion scores using one-way analysis of variance and Tukey's multiple comparison procedure. Significant trends ($P \leq 0.050$) were based on rank order of mean responses for fish groups classified by lesion scores. Compared to fish with the lowest lesion score, mean response for the fish group with the highest lesion score was significantly higher (↑), lower (↓), or there was no significant trend (NT) in the rank order. For comparisons in which Levene's test for equality of variance was significant (*), only ANOVA comparisons with $P \leq 0.010$ are shown.

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
External gross lesions:								
caudal fin fraying	F96-all	160	9.4	71.9	18.8	0.0	90.6	NT- ALP (0.016)
	S97-all	260	13.5	73.5	11.9	1.2	86.5	↑- sodium (<0.001) ↓- age (<0.001), glucose (<0.001) NT- albumin (0.006), calcium (0.021), lnIgM (<0.001)
	S97-prespawning	80	1.2	67.5	30.0	1.2	98.8	ND
	S97-spawning	180	18.9	76.1	3.9	1.1	81.1	ND
caudal fin reddening	F96-all	160	35.0	56.2	6.9	1.9	65.0	NT- lactate (0.050), osmolality (0.022)
	S97-all	260	36.9	55.0	7.3	0.8	63.1	↑- sodium (0.010*) ↓- lnIgM (0.013) NT- age (0.010*), albumin (0.039), glucose (0.010*)
	S97-prespawning	80	18.8	62.5	17.5	1.2	81.2	ND
	S97-spawning	180	45.0	51.7	2.8	0.6	55.0	ND
fin base reddening	F96-all	160	18.1	48.8	23.1	10.0	81.9	NT- hold time (0.029), ALP (0.019), calcium (0.020)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	260	71.2	25.4	1.5	1.9	28.8	↑- sodium (0.005) ↓- PCV (0.025) NT- albumin (0.003), ALP (0.046)
	S97-prespawning	80	62.5	30.0	3.8	3.8	37.5	ND
	S97-spawning	180	75.0	23.3	0.6	1.1	25.0	ND
iris reddening	F96-all	160	36.2	58.8	5.0	0.0	63.8	↑- age (0.001*), lactate (<0.001*), phosphorus (0.015), calcium (0.003), osmolality (<0.001), lnIgM (0.045) NT- albumin (0.041)
	S97-all	260	16.2	80.4	3.5	0.0	83.8	NT- age (<0.001*), PCV (0.003*), total protein (0.037), lnAST (0.039), lnIgM (0.002)
	S97-prespawning	80	27.5	68.8	3.8	0.0	72.5	ND
	S97-spawning	180	11.1	85.6	3.3	0.0	88.9	ND
opercular copepod	F96-all	157	38.2	61.8	0.0	0.0	61.8	↓- age (<0.001*), total protein (0.006), phosphorus (0.006), lnIgM (0.008)
	S97-all	260	50.0	50.0	0.0	0.0	50.0	↓- age (<0.001*), calcium (0.032), phosphorus (0.021), glucose (<0.001*), lnIgM (0.044)
	S97-prespawning	80	42.5	57.5	0.0	0.0	57.5	ND
	S97-spawning	180	53.3	46.7	0.0	0.0	46.7	ND
skin reddening, diffuse	F96-all	160	63.1	24.4	10.6	1.9	36.9	↑- hold time (0.001) ↓- lactate (0.034)
	S97-all	260	96.2	3.8	0.0	0.0	3.8	↑- % lymphocytes (0.003*) ↓- total protein (0.017), albumin (0.036), % thrombocytes (0.009), lnIgM (0.018)
	S97-prespawning	80	95.0	5.0	0.0	0.0	5.0	ND
	S97-spawning	180	96.7	3.3	0.0	0.0	3.3	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
skin reddening, focal	F96-all	160	61.9	33.8	1.9	2.5	38.1	↑- sum*Ichthyophonus* (0.015*) ↓- chloride (0.038)
	S97-all	260	80.4	17.3	2.3	0.0	19.6	↑- sum*Ichthyophonus* (<0.001*), % lymphocytes (0.001*) ↓- PCV (<0.001*), albumin (0.004*), % thrombocytes (0.001*), lnIgM (0.006)
	S97-prespawning	80	75.0	20.0	5.0	0.0	25.0	ND
	S97-spawning	180	82.8	16.1	1.1	0.0	17.2	ND
Other Gross findings:								
eggs or other food in stomach	F96-all	160	26.9	16.9	27.5	28.8	73.1	↑- lactate (0.003*), calcium (0.001) ↓- total protein (0.011) NT- hold time (0.005), ca (0.007)
	S97-all	260	65.8	13.8	6.5	13.8	34.2	NT- % thrombocytes (<0.001), % lymphocytes (<0.001), lnAST (0.001)
	S97-prespawning	80	37.5	26.2	12.5	23.8	62.5	ND
	S97-spawning	180	78.3	8.3	3.9	9.4	21.7	ND
gills, 0.5-mm-diameter white foci (3 = % with 3 or more foci)	F96-all	157	74.5	17.8	3.2	4.5	25.5	↓ - sodium (0.003); chloride (0.027) NT -lnPotassium (0.047)
	S97-all	258	83.7	14.7	0.8	0.0	15.5	↑ - lnCPK (0.035)
	S97-prespawning	79	79.7	15.2	2.5	0.0	17.7	ND
	S97-spawning	179	85.5	14.5	0.0	0.0	14.5	ND
gonadal development	F96-all							ND (all were developing, but unripe)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	150	5.3	36.7	57.3	0.7	94.7	↑- age (<0.001*), % thrombocytes (0.034) ↓- % lymphocytes (0.008), % eosinophils (0.003*) NT- PCV (0.039), sodium (0.001), glucose (0.001*), lnCPK (0.005), lnAST (<0.001), lnIgM (0.001)
	S97-prespawning	46	4.3	80.4	15.2	0.0	95.7	ND
	S97-spawning	104	5.8	17.3	76.0	1.0	94.2	ND
gonad fullness	F96-all	154	0.0	0.0	0.0	100.0	100.0	ND (too little variation)
	S97-all	251	0.4	0.0	0.0	99.6	99.6	ND (too little variation)
	S97-prespawning	78	0.0	0.0	0.0	100.0	100.0	ND
	S97-spawning	173	0.6	0.0	0.0	99.4	99.4	ND
Ichthyophonus (multifocal white foci, 0.5-1 mm in diameter)	F96-all	157	84.7	15.3	0.0	0.0	15.3	↑- sum*Ichthyophonus* (<0.001*), lnAST (0.001), lnIgM (<0.001*)
	S97-all	258	92.2	7.8	0.0	0.0	7.8	↑- age (0.003), total protein (0.001), ALP (0.045), calcium (0.012), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (0.002) ↓- PCV (0.004), lnPotassium (0.013)
	S97-prespawning	79	94.9	5.1	0.0	0.0	5.1	ND
	S97-spawning	179	91.1	8.9	0.0	0.0	8.9	ND
Brain microscopic lesions:								
Ichthyophonus	F96-all	159	90.6	6.9	2.5	0.0	9.4	↑- age (0.002), total protein (0.037), sum*Ichthyophonus* (<0.001*), lnCPK (0.036), lnAST (<0.001), lnIgM (<0.001) ↓- PCV (0.022)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	260	92.3	7.7	0.0	0.0	7.7	↑- sum*Ichthyophonus* (<0.001*), lnAST (0.005), lnIgM (0.004) ↓- lnPotassium (0.040)
	S97-prespawning	80	92.5	7.5	0.0	0.0	7.5	ND
	S97-spawning	180	92.2	7.8	0.0	0.0	7.8	ND
meningeal eosinophilic granular leukocytes	F96-all	159	3.1	59.1	37.7	0.0	96.9	↑- sum*Ichthyophonus* (0.002*), lnCPK (0.003)
	S97-all	260	6.2	66.2	27.7	0.0	93.8	NT- % thrombocytes (0.017), % lymphocytes (0.013)
	S97-prespawning	80	5.0	62.5	32.5	0.0	95.0	ND
	S97-spawning	180	6.7	67.8	25.6	0.0	93.3	ND
meningoencephalitis	F96-all	159	98.1	1.9	0.0	0.0	1.9	ND (too few responses)
	S97-all	260	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S97-prespawning	80	100.0	0.0	0.0	0.0	0.0	ND
	S97-spawning	180	100.0	0.0	0.0	0.0	0.0	ND
Gall bladder microscopic lesions:								
eosinophils, submucosal	F96-all	158	11.4	75.3	13.3	0.0	88.6	none
	S97-all	251	36.3	59.8	4.0	0.0	63.7	NT- lnAnisakidae (0.024)
	S97-prespawning	78	29.5	65.4	5.1	0.0	70.5	ND
	S97-spawning	173	39.3	57.2	3.5	0.0	60.7	ND
myxosporean (*Ceratomyxa auerbachi*)	F96-all	156	91.7	7.7	0.6	0.0	8.3	none
	S97-all	250	79.6	14.4	6.0	0.0	20.4	NT- % lymphocytes (0.045), % basophils (0.010*)
	S97-prespawning	78	83.3	10.3	6.4	0.0	16.7	ND
	S97-spawning	172	77.9	16.3	5.8	0.0	22.1	ND
Gill microscopic lesions:								

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
ciliated protozoa (e.g., *Trichodina* spp.)	F96-all	160	91.9	8.1	0.0	0.0	8.1	↑- hold time (0.025), % basophils (0.017), lnAnisakidae (0.036) ↓- ALP (0.031)
	S97-all	260	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S97-prespawning	80	100.0	0.0	0.0	0.0	0.0	ND
	S97-spawning	180	100.0	0.0	0.0	0.0	0.0	ND
Epitheliocystis	F96-all	160	92.5	6.9	0.6	0.0	7.5	↓- lnIgM (0.022)
	S97-all	260	64.2	34.2	1.2	0.4	35.8	↑- lactate (0.032)
	S97-prespawning	80	57.5	40.0	2.5	0.0	42.5	ND
	S97-spawning	180	67.2	31.7	0.6	0.6	32.8	ND
foreign body granuloma	F96-all	160	91.9	7.5	0.6	0.0	8.1	↓- sodium (0.047)
	S97-all	260	95.0	5.0	0.0	0.0	5.0	none
	S97-prespawning	80	96.2	3.8	0.0	0.0	3.8	ND
	S97-spawning	180	94.4	5.6	0.0	0.0	5.6	ND
gill arch inflammation or hematopoiesis	F96-all	160	0.0	92.5	7.5	0.0	100.0	↑- sum*Ichthyophonus* (<0.001*), lnCPK (0.044), lnAST (0.001)
	S97-all	260	0.0	88.5	11.5	0.0	100.0	↑- sum*Ichthyophonus* (0.006*), % lymphocytes (0.040), lnCPK (0.027) ↓- hold time (0.041), % thrombocytes (0.037)
	S97-prespawning	80	0.0	90.0	10.0	0.0	100.0	ND
	S97-spawning	180	0.0	87.8	12.2	0.0	100.0	ND
Ichthyophonus	F96-all	160	90.0	3.1	2.5	4.4	10.0	↑- age (0.004), sum*Ichthyophonus* (<0.001*), lnCPK (0.032), lnAST (<0.001*), lnIgM (<0.001*)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	260	91.5	5.4	3.1	0.0	8.5	↑- sum*Ichthyophonus* (<0.001*) ↓- lnPotassium (0.028) NT- age (0.024), total protein (0.006), albumin (0.017), lnAST (0.003), lnIgM (<0.001)
	S97-prespawning	80	92.5	3.8	3.8	0.0	7.5	ND
	S97-spawning	180	91.1	6.1	2.8	0.0	8.9	ND
lamellar hyperplasia	F96-all	160	99.4	0.0	0.6	0.0	0.6	ND (too few responses)
	S97-all	260	95.4	2.3	1.5	0.8	4.6	↑- sum*Ichthyophonus* (0.001*), lnAST (0.030)
	S97-prespawning	80	96.2	1.2	2.5	0.0	3.8	ND
	S97-spawning	180	95.0	2.8	1.1	1.1	5.0	ND
lamellar telangiectasis	F96-all	160	91.2	8.1	0.6	0.0	8.8	↑- lnIgM (0.032)
	S97-all	260	92.7	6.9	0.4	0.0	7.3	↑- phosphorus (0.047), % lymphocytes (0.007) ↓- % thrombocytes (0.009)
	S97-prespawning	80	91.2	8.8	0.0	0.0	8.8	ND
	S97-spawning	180	93.3	6.1	0.6	0.0	6.7	ND
monogenetic trematodes (e.g., *Gyrodactylus* spp.)	F96-all	160	98.1	1.9	0.0	0.0	1.9	ND (too few responses)
	S97-all	260	92.3	7.7	0.0	0.0	7.7	none
	S97-prespawning	80	95.0	5.0	0.0	0.0	5.0	ND
	S97-spawning	180	91.1	8.9	0.0	0.0	8.9	ND
Gonad - female microscopic lesions:								
eosinophilic granular leukocytes	F96-all	78	1.3	85.9	12.8	0.0	98.7	↑- sum*Ichthyophonus* (<0.001*), % neutrophils (<0.001), % basophils (0.004*), lnIgM (0.005) ↓- chloride (0.017)
	S97-all	141	34.8	62.4	2.8	0.0	65.2	↓- glucose (0.010*), % basophils (0.003*)
	S97-prespawning	44	27.3	70.5	2.3	0.0	72.7	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-spawning	97	38.1	58.8	3.1	0.0	61.9	ND
granulomatous inflammation	F96-all	78	98.7	1.3	0.0	0.0	1.3	ND (too few responses)
	S97-all	141	81.6	18.4	0.0	0.0	18.4	none
	S97-prespawning	44	75.0	25.0	0.0	0.0	25.0	ND
	S97-spawning	97	84.5	15.5	0.0	0.0	15.5	ND
hyalinization of vessel walls	F96-all	78	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S97-all	141	73.8	26.2	0.0	0.0	26.2	↑- age (0.014), glucose (0.046), % neutrophils (0.027)
	S97-prespawning	44	97.7	2.3	0.0	0.0	2.3	ND
	S97-spawning	97	62.9	37.1	0.0	0.0	37.1	ND
Ichthyophonus	F96-all	78	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S97-all	141	98.6	1.4	0.0	0.0	1.4	ND (too few responses)
	S97-prespawning	44	100.0	0.0	0.0	0.0	0.0	ND
	S97-spawning	97	97.9	2.1	0.0	0.0	2.1	ND
macrophage aggregates (pigmented)	F96-all	78	65.4	34.6	0.0	0.0	34.6	↑- age (<0.001*), total protein (0.048), albumin (0.010) ↓- lnCPK (0.039)
	S97-all	141	70.9	28.4	0.7	0.0	29.1	↑- age (<0.001*), albumin (0.025), ALP (0.001), calcium (0.020), glucose (0.005), sum*Ichthyophonus* (0.003*), lnIgM (<0.001) ↓- lnPotassium (0.037)
	S97-prespawning	44	70.5	29.5	0.0	0.0	29.5	ND
	S97-spawning	97	71.1	27.8	1.0	0.0	28.9	ND
oocyte atresia, mature follicles	F96-all	78	56.4	32.1	9.0	2.6	43.6	NT- age (0.044), hold time (0.050)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	141	90.8	9.2	0.0	0.0	9.2	↑- % eosinophils (0.003*) ↓- PCV (0.003*), lnAnisakidae (0.029), lnCPK (0.017)
	S97-prespawning	44	84.1	15.9	0.0	0.0	15.9	ND
	S97-spawning	97	93.8	6.2	0.0	0.0	6.2	ND
Gonad - male microscopic lesions:								
Eimeria sardinae	F96-all	78	57.7	39.7	2.6	0.0	42.3	↑- lnAnisakidae (<0.001) ↓- lnAST (0.024)
	S97-all	111	6.3	82.9	10.8	0.0	93.7	none
	S97-prespawning	34	5.9	82.4	11.8	0.0	94.1	ND
	S97-spawning	77	6.5	83.1	10.4	0.0	93.5	ND
eosinophilic granular leukocytes	F96-all	78	28.2	60.3	11.5	0.0	71.8	↑- sum*Ichthyophonus* (<0.001*), lnCPK (0.008) NT- lnIgM (0.011)
	S97-all	111	53.2	46.8	0.0	0.0	46.8	↑- lnIgM (0.013)
	S97-prespawning	34	38.2	61.8	0.0	0.0	61.8	ND
	S97-spawning	77	59.7	40.3	0.0	0.0	40.3	ND
granulomatous inflammation	F96-all	78	94.9	5.1	0.0	0.0	5.1	ND (too few responses)
	S97-all	111	95.5	3.6	0.9	0.0	4.5	ND (too few responses)
	S97-prespawning	34	94.1	2.9	2.9	0.0	5.9	ND
	S97-spawning	77	96.1	3.9	0.0	0.0	3.9	ND
Ichthyophonus	F96-all	78	96.2	3.8	0.0	0.0	3.8	ND (too few responses)
	S97-all	111	99.1	0.9	0.0	0.0	0.9	ND (too few responses)
	S97-prespawning	34	100.0	0.0	0.0	0.0	0.0	ND
	S97-spawning	77	98.7	1.3	0.0	0.0	1.3	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
macrophage aggregates (pigmented)	F96-all	78	98.7	1.3	0.0	0.0	1.3	ND (too few responses)
	S97-all	111	98.2	0.9	0.9	0.0	1.8	ND (too few responses)
	S97-prespawning	34	100.0	0.0	0.0	0.0	0.0	ND
	S97-spawning	77	97.4	1.3	1.3	0.0	2.6	ND
spermatocyte numbers (3 = abundant)	F96-all	78	3.8	70.5	24.4	1.3	96.2	↑- age (<0.001*), PCV (0.007), total protein (0.003), albumin (0.014), lnIgM (<0.001)
	S97-all	111	0.9	0.0	0.0	99.1	99.1	ND (too little variation)
	S97-prespawning	34	0.0	0.0	0.0	100.0	100.0	ND
	S97-spawning	77	1.3	0.0	0.0	98.7	98.7	ND
Heart microscopic lesions:								
epicarditis	F96-all	158	3.8	87.3	8.9	0.0	96.2	↑- sum*Ichthyophonus* (<0.001*), lnAST (0.007), lnIgM (<0.001)
	S97-all	260	12.3	77.3	10.0	0.4	87.7	↑- age (0.012), sum*Ichthyophonus* (<0.001*), lnAST (0.001), lnIgM (<0.001) ↓- PCV (0.034) NT- total protein (<0.001), calcium (0.025)
	S97-prespawning	80	15.0	80.0	5.0	0.0	85.0	ND
	S97-spawning	180	11.1	76.1	12.2	0.6	88.9	ND
Ichthyophonus	F96-all	160	88.1	1.2	3.1	7.5	11.9	↑- age (0.010), sum*Ichthyophonus* (<0.001*), lnCPK (0.003), lnAST (<0.001), lnIgM (<0.001*) ↓- PCV (0.032), sodium (0.032)
	S97-all	260	85.4	6.2	2.7	5.8	14.6	↑- age (<0.001), total protein (<0.001), ALP (0.028), calcium (0.042), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001) NT- albumin (0.025)
	S97-prespawning	80	87.5	3.8	3.8	5.0	12.5	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-spawning	180	84.4	7.2	2.2	6.1	15.6	ND
leukocytes, focal, parenchymal	F96-all	160	76.2	22.5	1.2	0.0	23.8	none
	S97-all	260	91.9	8.1	0.0	0.0	8.1	none
	S97-prespawning	80	93.8	6.2	0.0	0.0	6.2	ND
	S97-spawning	180	91.1	8.9	0.0	0.0	8.9	ND
thrombosis	F96-all	160	84.4	13.8	1.9	0.0	15.6	↑- albumin (0.040), phosphorus (0.007), sum*Ichthyophonus* (<0.001*), lnAnisakidae (0.018), lnIgM (0.031) ↓- ALP (0.026)
	S97-all	260	90.4	8.8	0.4	0.4	9.6	↓- osmolality (0.013)
	S97-prespawning	80	83.8	16.2	0.0	0.0	16.2	ND
	S97-spawning	180	93.3	5.6	0.6	0.6	6.7	ND
Intestine and intestinal cecae, microscopic lesions:								
Anisakidae	F96-all	160	13.1	64.4	18.1	4.4	86.9	↑- lnAnisakidae (<0.001)
	S97-all	260	15.8	65.8	16.5	1.9	84.2	↑- lnAnisakidae (<0.001)
	S97-prespawning	80	17.5	65.0	15.0	2.5	82.5	ND
	S97-spawning	180	15.0	66.1	17.2	1.7	85.0	ND
arteriolar hyperplasia, focal, intimal	F96-all	160	48.8	45.0	6.2	0.0	51.2	↑- age (<0.001*) NT- lnPotassium (0.007)
	S97-all	260	55.4	43.8	0.8	0.0	44.6	↑- chloride (0.041) ↓- lnPotassium (0.022)
	S97-prespawning	80	52.5	46.2	1.2	0.0	47.5	ND
	S97-spawning	180	56.7	42.8	0.6	0.0	43.3	ND
cestodes	F96-all	160	64.4	15.0	20.6	0.0	35.6	↓- age (<0.001*)
	S97-all	260	96.9	1.5	1.5	0.0	3.1	none

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-prespawning	80	96.2	1.2	2.5	0.0	3.8	ND
	S97-spawning	180	97.2	1.7	1.1	0.0	2.8	ND
coccidian, intraepithelial (*Goussia*? sp.)	F96-all	160	85.0	15.0	0.0	0.0	15.0	↓- phosphorus (0.016)
	S97-all	260	6.5	76.2	17.3	0.0	93.5	NT- age (<0.001*), ALP (0.015), lnIgM (0.037)
	S97-prespawning	80	6.2	76.2	17.5	0.0	93.8	ND
	S97-spawning	180	6.7	76.1	17.2	0.0	93.3	ND
eosinophilic granular leukocytes, submucosal	F96-all	160	0.6	97.5	1.9	0.0	99.4	ND (too little variation)
	S97-all	260	0.4	97.7	1.9	0.0	99.6	ND
	S97-prespawning	80	0.0	100.0	0.0	0.0	100.0	ND
	S97-spawning	180	0.6	96.7	2.8	0.0	99.4	ND
foreign body granuloma	F96-all	160	73.8	26.2	0.0	0.0	26.2	none
	S97-all	260	74.2	25.8	0.0	0.0	25.8	↑- age (0.002*)
	S97-prespawning	80	75.0	25.0	0.0	0.0	25.0	ND
	S97-spawning	180	73.9	26.1	0.0	0.0	26.1	ND
Ichthyophonus	F96-all	160	93.8	5.6	0.6	0.0	6.2	↑- age (0.049), total protein (0.024), sum*Ichthyophonus* (<0.001), lnCPK (0.004), lnAST (<0.001), lnIgM (<0.001) ↓- PCV (0.004)
	S97-all	260	92.3	7.7	0.0	0.0	7.7	↑- total protein (0.002), sum*Ichthyophonus* (<0.001*), lnAST (0.002), lnIgM (0.001)
	S97-prespawning	80	95.0	5.0	0.0	0.0	5.0	ND
	S97-spawning	180	91.1	8.9	0.0	0.0	8.9	ND
steatitis	F96-all	160	95.6	4.4	0.0	0.0	4.4	ND (too few responses)
	S97-all	260	3.1	47.3	49.6	0.0	96.9	NT- % thrombocytes (0.012), % lymphocytes (0.016)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-prespawning	80	5.0	43.8	51.2	0.0	95.0	ND
	S97-spawning	180	2.2	48.9	48.9	0.0	97.8	ND
trematodes (e.g.,	F96-all	160	76.2	23.1	0.6	0.0	23.8	↑- % neutrophils (0.029)
Lecithaster gibbosus), cecal	S97-all	260	69.6	30.0	0.4	0.0	30.4	↓- age (<0.001*), calcium (0.025), % lymphocytes (0.040)
	S97-prespawning	80	65.0	35.0	0.0	0.0	35.0	ND
	S97-spawning	180	71.7	27.8	0.6	0.0	28.3	ND
Kidney (trunk) microscopic lesions:								
congestion, interstitial, vascular	F96-all	160	94.4	5.6	0.0	0.0	5.6	↑- total protein (0.028), albumin (0.011) ↓- % thrombocytes (0.007)
	S97-all	260	90.0	8.5	1.5	0.0	10.0	↓- PCV (<0.001*), lnAST (0.023)
	S97-prespawning	80	78.8	16.2	5.0	0.0	21.2	ND
	S97-spawning	180	95.0	5.0	0.0	0.0	5.0	ND
granulomatous inflammation	F96-all	160	79.4	19.4	1.2	0.0	20.6	↑- % monocytes (0.005*) ↓- hold time (0.042), total protein (0.008), lnCPK (0.002)
	S97-all	260	83.5	15.0	1.2	0.4	16.5	none
	S97-prespawning	80	80.0	16.2	3.8	0.0	20.0	ND
	S97-spawning	180	85.0	14.4	0.0	0.6	15.0	ND
hematopoietic cells (relative area)	F96-all	160	3.1	70.0	25.0	1.9	96.9	↑- age (0.028), sum*Ichthyophonus* (<0.001*), lnIgM (0.004)
	S97-all	260	7.7	81.9	10.4	0.0	92.3	↑- PCV (<0.001*), sum*Ichthyophonus* (<0.001*), lnIgM (<0.001) NT- total protein (0.003), % thrombocytes (0.008), % lymphocytes (0.033)
	S97-prespawning	80	11.2	83.8	5.0	0.0	88.8	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-spawning	180	6.1	81.1	12.8	0.0	93.9	ND
Ichthyophonus	F96-all	160	88.1	1.9	3.8	6.2	11.9	↑- sum*Ichthyophonus* (<0.001*), lnCPK (0.006), lnAST (<0.001), lnIgM (<0.001*) NT- age (0.037), phosphorus (0.035)
	S97-all	260	85.8	8.1	3.5	2.7	14.2	↑- age (0.001), total protein (<0.001*), calcium (0.012), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001) NT- ALP (0.040), % thrombocytes (0.046), % lymphocytes (0.024), % basophils (0.005*)
	S97-prespawning	80	87.5	7.5	5.0	0.0	12.5	ND
	S97-spawning	180	85.0	8.3	2.8	3.9	15.0	ND
intraductal myxosporean (*Sphaerospora* sp.?)	F96-all	160	95.0	5.0	0.0	0.0	5.0	↓- PCV (0.044), albumin (0.001*)
	S97-all	259	88.4	10.4	0.8	0.4	11.6	↓- lnPotassium (0.041)
	S97-prespawning	79	88.6	11.4	0.0	0.0	11.4	ND
	S97-spawning	180	88.3	10.0	1.1	0.6	11.7	ND
intratubular mineral, with associated tubular hyperplasia	F96-all	160	98.1	1.9	0.0	0.0	1.9	ND (too few responses)
	S97-all	260	98.1	1.2	0.8	0.0	1.9	ND (too few responses)
	S97-prespawning	80	97.5	1.2	1.2	0.0	2.5	ND
	S97-spawning	180	98.3	1.1	0.6	0.0	1.7	ND
macrophage aggregates, pigmented	F96-all	160	3.1	63.1	28.8	5.0	96.9	↑- age (<0.001*) NT- lnCPK (0.043)
	S97-all	260	4.6	62.3	24.6	8.5	95.4	↑- age (<0.001*), PCV (0.003*), phosphorus (0.007), glucose (<0.001*), lnIgM (<0.001) NT- total protein (0.004), albumin (0.010), calcium (0.005), sum*Ichthyophonus* (0.001*), % neutrophils (0.044), lnCPK (0.023)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-prespawning	80	8.8	72.5	13.8	5.0	91.2	ND
	S97-spawning	180	2.8	57.8	29.4	10.0	97.2	ND
Ortholinea orientalis (intraductal myxosporean), histopathology	F96-all	160	80.6	11.2	5.6	2.5	19.4	↓- lnIgM (0.023)
	S97-all	260	91.2	3.8	3.5	1.5	8.8	none
	S97-prespawning	80	95.0	1.2	3.8	0.0	5.0	ND
	S97-spawning	180	89.4	5.0	3.3	2.2	10.6	ND
Ortholinea orientalis (intraductal myxosporean), kidney touch preparation	F96-all	160	90.0	6.2	0.6	3.1	10.0	↑- % basophils (0.041), % monocytes (<0.001*) ↓- PCV (0.032)
	S97-all	260	85.8	8.1	2.7	3.5	14.2	↓- sodium (0.017) NT- lnIgM (0.012)
	S97-prespawning	80	93.8	3.8	1.2	1.2	6.2	ND
	S97-spawning	180	82.2	10.0	3.3	4.4	17.8	ND
Ortholinea orientalis (intraductal myxosporean); sum of both techniques (3 = % with sum score >2)	F96-all	160	78.1	10.0	3.8	8.1	21.9	↑- % monocytes (0.001*) ↓- lnIgM (0.019)
	S97-all	260	83.8	7.3	2.7	6.2	16.2	NT- lnPotassium (0.048)
	S97-prespawning	80	91.2	3.8	2.5	2.5	8.8	ND
	S97-spawning	180	80.6	8.9	2.8	7.8	19.4	ND
tubular dilation (of lumen)	F96-all	160	99.4	0.6	0.0	0.0	0.6	ND (too few responses)
	S97-all	260	98.8	1.2	0.0	0.0	1.2	ND (too few responses)
	S97-prespawning	80	98.8	1.2	0.0	0.0	1.2	ND
	S97-spawning	180	98.9	1.1	0.0	0.0	1.1	ND
tubular epithelial vacuolation	F96-all	160	58.1	40.6	1.2	0.0	41.9	↑- % basophils (0.020) ↓- sum*Ichthyophonus* (0.007*), % neutrophils (0.026)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	260	47.7	50.0	2.3	0.0	52.3	↑- age (<0.001*), albumin (<0.001), glucose (<0.001*), % neutrophils (0.001*), lnIgM (0.028) ↓- lnPotassium (0.004)
	S97-prespawning	80	48.8	48.8	2.5	0.0	51.2	ND
	S97-spawning	180	47.2	50.6	2.2	0.0	52.8	ND
Liver microscopic lesions:								
Anisakidae	F96-all	160	77.5	21.9	0.6	0.0	22.5	none
	S97-all	260	75.4	23.8	0.8	0.0	24.6	↑- % neutrophils (0.029)
	S97-prespawning	80	78.8	21.2	0.0	0.0	21.2	ND
	S97-spawning	180	73.9	25.0	1.1	0.0	26.1	ND
cholangitis or biliary hyperplasia	F96-all	160	94.4	5.0	0.6	0.0	5.6	↑- % basophils (0.040)
	S97-all	260	90.8	8.5	0.8	0.0	9.2	↑- lnAnisakidae (0.016)
	S97-prespawning	80	90.0	8.8	1.2	0.0	10.0	ND
	S97-spawning	180	91.1	8.3	0.6	0.0	8.9	ND
coccidiosis (*Goussia* [*Eimeria*] *clupearum*)	F96-all	160	30.0	49.4	13.1	7.5	70.0	NT - age (0.030), glucose (0.039)
	S97-all	260	30.8	48.8	11.5	8.8	69.2	NT- age (0.004*), ALP (0.023), lnCPK (0.007*), lnIgM (0.002)
	S97-prespawning	80	33.8	47.5	10.0	8.8	66.2	ND
	S97-spawning	180	29.4	49.4	12.2	8.9	70.6	ND
eosinophilic granular leukocytes, perivascular	F96-all	160	1.9	55.6	42.5	0.0	98.1	↓- sodium (0.009), osmolality (0.011)
	S97-all	260	4.2	75.0	20.8	0.0	95.8	↑- age (<0.001), sum*Ichthyophonus* (<0.001*), lnAnisakidae (0.002) NT- calcium (0.034), % basophils (0.007*), lnIgM (<0.001)
	S97-prespawning	80	2.5	81.2	16.2	0.0	97.5	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-spawning	180	5.0	72.2	22.8	0.0	95.0	ND
glycogen depletion, hepatocellular	F96-all	160	3.1	18.1	63.8	15.0	96.9	↑- hold time (0.001), sum*Ichthyophonus* (<0.001*), lnAST (0.001) NT- age (0.010*), PCV (0.035), lnCPK (0.038), lnIgM (0.049)
	S97-all	260	0.0	0.0	4.6	95.4	100.0	↓- lactate (0.002)
	S97-prespawning	80	0.0	0.0	8.8	91.2	100.0	ND
	S97-spawning	180	0.0	0.0	2.8	97.2	100.0	ND
granulomatous inflammation	F96-all	160	61.9	36.2	1.2	0.6	38.1	↑- phosphorus (0.043)
	S97-all	260	69.2	30.4	0.4	0.0	30.8	↓- sum*Ichthyophonus* (0.002*)
	S97-prespawning	80	70.0	28.8	1.2	0.0	30.0	ND
	S97-spawning	180	68.9	31.1	0.0	0.0	31.1	ND
Ichthyophonus	F96-all	160	88.8	1.9	6.2	3.1	11.2	↑- age (0.005), total protein (0.026), sum*Ichthyophonus* (<0.001*), lnCPK (0.009), lnAST (<0.001), lnIgM (<0.001) ↓- sodium (0.037)
	S97-all	260	87.3	8.1	3.1	1.5	12.7	↑- age (<0.001), total protein (<0.001), albumin (0.009), glucose (0.024), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001) ↓- PCV (0.042)
	S97-prespawning	80	91.2	7.5	0.0	1.2	8.8	ND
	S97-spawning	180	85.6	8.3	4.4	1.7	14.4	ND
leukocytes, focal, parenchymal	F96-all	160	28.8	70.0	1.2	0.0	71.2	↑- PCV (0.005), calcium (0.042), lnAnisakidae (0.017)
	S97-all	260	67.3	32.7	0.0	0.0	32.7	↑- PCV (0.008), glucose (0.032), lnIgM (0.002)
	S97-prespawning	80	70.0	30.0	0.0	0.0	30.0	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-spawning	180	66.1	33.9	0.0	0.0	33.9	ND
leukocytes, pericholangial	F96-all	160	92.5	7.5	0.0	0.0	7.5	none
	S97-all	260	93.5	6.5	0.0	0.0	6.5	↑- age (0.019), lnAnisakidae (0.034) ↓- hold time (0.006)
	S97-prespawning	80	93.8	6.2	0.0	0.0	6.2	ND
	S97-spawning	180	93.3	6.7	0.0	0.0	6.7	ND
lipidosis, hepatocellular	F96-all	160	0.0	33.8	66.2	0.0	100.0	↑- lnAnisakidae (0.008) ↓- sum*Ichthyophonus* (0.004*), lnCPK (0.006), lnAST (0.006)
	S97-all	260	78.1	18.1	2.7	1.2	21.9	↓- lnIgM (0.030) NT- sodium (<0.001*), lnAST (0.037)
	S97-prespawning	80	58.8	33.8	6.2	1.2	41.2	ND
	S97-spawning	180	86.7	11.1	1.1	1.1	13.3	ND
macrophage aggregates, pigmented	F96-all	160	9.4	75.0	12.5	3.1	90.6	↑- age (<0.001*), % basophils (0.003), lnIgM (0.013) ↓- sodium (0.032), % thrombocytes (0.002)
	S97-all	260	2.3	63.8	20.4	13.5	97.7	↑- age (<0.001*), sodium (0.016), total protein (0.001), albumin (<0.001), calcium (<0.001), phosphorus (0.003*), glucose (<0.001*), % neutrophils (<0.001*), lnIgM (<0.001)
	S97-prespawning	80	5.0	70.0	17.5	7.5	95.0	ND
	S97-spawning	180	1.1	61.1	21.7	16.1	98.9	ND
megalocytosis, hepatocellular	F96-all	160	92.5	7.5	0.0	0.0	7.5	none
	S97-all	260	99.2	0.8	0.0	0.0	0.8	ND (too few responses)
	S97-prespawning	80	100.0	0.0	0.0	0.0	0.0	ND
	S97-spawning	180	98.9	1.1	0.0	0.0	1.1	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-spawning	180	65.6	34.4	0.0	0.0	34.4	ND
zymogen granule depletion	F96-all	160	30.6	47.5	21.9	0.0	69.4	↑- age (<0.001*)
	S97-all	260	0.0	7.3	65.4	27.3	100.0	↑- calcium (0.048) ↓- PCV (0.005*), lnCPK (<0.001) NT- % lymphocytes (0.049)
	S97-prespawning	80	0.0	3.8	67.5	28.8	100.0	ND
	S97-spawning	180	0.0	8.9	64.4	26.7	100.0	ND
Skin and skeletal muscle, microscopic lesions:								
Anisakidae	F96-all	160	98.1	1.9	0.0	0.0	1.9	ND (too few responses)
	S97-all	260	97.7	1.9	0.4	0.0	2.3	ND (too few responses)
	S97-prespawning	80	98.8	1.2	0.0	0.0	1.2	ND
	S97-spawning	180	97.2	2.2	0.6	0.0	2.8	ND
arteriolar hyperplasia, focal, intimal	F96-all	160	80.6	19.4	0.0	0.0	19.4	↑- hold time (0.012)
	S97-all	260	77.7	22.3	0.0	0.0	22.3	↑- age (0.002*)
	S97-prespawning	80	81.2	18.8	0.0	0.0	18.8	ND
	S97-spawning	180	76.1	23.9	0.0	0.0	23.9	ND
Ichthyophonus	F96-all	160	88.1	5.6	5.6	0.6	11.9	↑- sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001*) ↓- PCV (0.047) NT- age (0.017), lnCPK (0.030)
	S97-all	260	87.3	6.9	5.4	0.4	12.7	↑- age (0.001), total protein (<0.001*), albumin (0.038), ALP (0.046), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001) NT- calcium (0.034)
	S97-prespawning	80	88.8	7.5	3.8	0.0	11.2	ND
	S97-spawning	180	86.7	6.7	6.1	0.6	13.3	ND

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
leukocytes, perivascular	F96-all	160	16.9	83.1	0.0	0.0	83.1	none
	S97-all	260	37.3	62.7	0.0	0.0	62.7	↑- sum*Ichthyophonus* (<0.001*), lnIgM (0.010)
	S97-prespawning	80	38.8	61.2	0.0	0.0	61.2	ND
	S97-spawning	180	36.7	63.3	0.0	0.0	63.3	ND
myodegeneration or myonecrosis	F96-all	160	100.0	0.0	0.0	0.0	0.0	ND (too few responses)
	S97-all	260	99.6	0.4	0.0	0.0	0.4	ND (too few responses)
	S97-prespawning	80	98.8	1.2	0.0	0.0	1.2	ND
	S97-spawning	180	100.0	0.0	0.0	0.0	0.0	ND
myositis	F96-all	160	95.0	5.0	0.0	0.0	5.0	↑- calcium (0.046), osmolality (0.026), lnAST (0.003)
	S97-all	260	95.8	4.2	0.0	0.0	4.2	↑- chloride (0.013), sum*Ichthyophonus* (0.007*), lnPotassium (0.034), lnAST (0.016), lnIgM (0.043)
	S97-prespawning	80	96.2	3.8	0.0	0.0	3.8	ND
	S97-spawning	180	95.6	4.4	0.0	0.0	4.4	ND
Spleen microscopic lesions:								
arteriolar hyperplasia, focal, intimal	F96-all	160	81.2	18.8	0.0	0.0	18.8	none
	S97-all	260	72.7	27.3	0.0	0.0	27.3	↑- age (0.041), ALP (0.031), lnAnisakidae (0.006)
	S97-prespawning	80	77.5	22.5	0.0	0.0	22.5	ND
	S97-spawning	180	70.6	29.4	0.0	0.0	29.4	ND
congestion, vascular	F96-all	160	18.1	34.4	20.0	27.5	81.9	↑- hold time (<0.001), lnPotassium (0.003) ↓- age (<0.001*), PCV (0.004), total protein (<0.001), albumin (0.001), lactate (0.001) NT- calcium (0.049)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	260	40.0	28.1	16.2	15.8	60.0	↑- hold time (<0.001*) ↓- age (<0.001*), total protein (0.001), ALP (0.004), lactate (0.002), phosphorus (0.007), glucose (0.006*), lnAnisakidae (<0.001) NT- PCV (0.031)
	S97-prespawning	80	36.2	28.8	13.8	21.2	63.8	ND
	S97-spawning	180	41.7	27.8	17.2	13.3	58.3	ND
ellipsoid hyalinization or hypertrophy	F96-all	160	7.5	68.1	24.4	0.0	92.5	↑- age (<0.001*) ↓- % eosinophils (0.005*) NT- total protein (0.005)
	S97-all	260	9.6	71.2	19.2	0.0	90.4	↑- age (<0.001*), calcium (0.012), phosphorus (0.016), lnIgM (0.027) NT- albumin (0.023), ALP (0.030), glucose (0.019)
	S97-prespawning	80	12.5	68.8	18.8	0.0	87.5	ND
	S97-spawning	180	8.3	72.2	19.4	0.0	91.7	ND
granulomatous inflammation	F96-all	160	92.5	7.5	0.0	0.0	7.5	↑- phosphorus (0.016) ↓- lactate (0.028), % neutrophils (0.022)
	S97-all	260	96.9	2.7	0.4	0.0	3.1	none
	S97-prespawning	80	96.2	3.8	0.0	0.0	3.8	ND
	S97-spawning	180	97.2	2.2	0.6	0.0	2.8	ND
Ichthyophonus	F96-all	160	87.5	5.6	1.2	5.6	12.5	↑- age (0.004), sumICH (<0.001*), lnAST (<0.001), lnIgM (<0.001*) NT- chloride (0.038), lnK (0.044)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	260	85.8	6.2	3.1	5.0	14.2	↑- age (0.001), total protein (<0.001), albumin (0.012), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001) ↓- ALP (0.013)
	S97-prespawning	80	88.8	2.5	5.0	3.8	11.2	ND
	S97-spawning	180	84.4	7.8	2.2	5.6	15.6	ND
macrophage aggregates, pigmented	F96-all	160	13.1	33.8	40.6	12.5	86.9	↑- age (<0.001*), % basophils (<0.001), lnIgM (0.002) ↓- % thrombocytes (<0.001) NT- hold time (0.015), PCV (0.010), total protein (0.012), albumin (0.035), % neutrophils (0.020), lnAnisakidae (0.036)
	S97-all	260	15.0	33.8	30.4	20.8	85.0	↑- age (<0.001*), total protein (0.001), albumin (0.002), calcium (0.004*), phosphorus (0.003*), glucose (<0.001*), lnIgM (<0.001) ↓- lnPotassium (0.045) NT- chloride (0.040), ALP (0.006), % neutrophils (0.009)
	S97-prespawning	80	23.8	35.0	26.2	15.0	76.2	ND
	S97-spawning	180	11.1	33.3	32.2	23.3	88.9	ND
Stomach microscopic lesions:								
foreign body granuloma	F96-all	160	92.5	7.5	0.0	0.0	7.5	↑- age (0.028), lactate (0.049), lnCPK (0.014) ↓- % thrombocytes (0.045)
	S97-all	260	86.2	13.8	0.0	0.0	13.8	↑- % neutrophils (0.045)
	S97-prespawning	80	86.2	13.8	0.0	0.0	13.8	ND
	S97-spawning	180	86.1	13.9	0.0	0.0	13.9	ND
gastritis, submucosal	F96-all	160	0.0	68.8	30.6	0.6	100.0	↑- age (0.038), albumin (0.034)

Organ - lesion	Date-Group	n	Lesion Score Prevalence %=0	%=1	%=2	%=3	%>0	Significant trends (*P*-value)
	S97-all	260	1.2	88.5	10.4	0.0	98.8	↑- % lymphocytes (<0.001) ↓- % thrombocytes (0.007)
	S97-prespawning	80	0.0	90.0	10.0	0.0	100.0	ND
	S97-spawning	180	1.7	87.8	10.6	0.0	98.3	ND
Ichthyophonus (includes only cases with organisms)	F96-all	160	87.5	5.6	5.0	1.9	12.5	↑- sum*Ichthyophonus* (<0.001*), lnCPK (0.030), lnAST (0.001), lnIgM (<0.001) ↓- PCV (0.015) NT- age (0.041)
	S97-all	260	90.4	8.1	1.5	0.0	9.6	↑- age (0.009), total protein (0.001), albumin (0.045), ALP (0.044), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001) ↓- PCV (0.039)
	S97-prespawning	80	93.8	3.8	2.5	0.0	6.2	ND
	S97-spawning	180	88.9	10.0	1.1	0.0	11.1	ND
Ichthyophonus+ (includes cases with characteristic inflam-mation, but no organisms)	F96-all	160	85.0	8.1	5.0	1.9	15.0	↑- sum*Ichthyophonus* (<0.001*), lnAST (<0.001*), lnIgM (<0.001) ↓- PCV (0.022)
	S97-all	260	88.5	10.0	1.5	0.0	11.5	↑- age (0.006), total protein (0.001), ALP (0.026), sum*Ichthyophonus* (<0.001*), lnAST (<0.001), lnIgM (<0.001) ↓- PCV (0.019)
	S97-prespawning	80	91.2	6.2	2.5	0.0	8.8	ND
	S97-spawning	180	87.2	11.7	1.1	0.0	12.8	ND
leukocytes, focal, parenchymal	F96-all	160	88.1	11.9	0.0	0.0	11.9	↑- phosphorus (0.023)
	S97-all	260	95.8	4.2	0.0	0.0	4.2	↑- chloride (0.001*), osmolality (<0.001*)
	S97-prespawning	80	95.0	5.0	0.0	0.0	5.0	ND

Table 3. Other lesions associated with external lesions in Pacific herring sampled from Prince William Sound, Alaska, in October 1996 (fall, n = 160) and in March and April 1997 (spring, n = 260). Chi-square test for association. For lesions with minimum expected cell frequency <1 (*), only chi-square tests with $P \leq 0.010$ are included. Trends in the associated lesion scores were classified in comparison to an increase in the given external lesion score. As the external lesion score increased, the associated lesion score either increased (↑), decreased (↓), or changes in the associated lesion score were not simple (NS; e.g., as scores for the external lesion increased, associated lesion scores initially increased and then later decreased). Lesions not listed were not significant.

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion or change	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
caudal fin fraying	fall			↑	<0.001						
	spring			↑	<0.001						
caudal fin reddening	fall	↑	<0.001					↑	0.025		
	spring	↑	<0.001					↑	<0.001*		
diffuse skin reddening	fall							↑	<0.001		
	spring							↑	<0.001*	↑	<0.001
fin base reddening	fall			↑	0.025	↑	<0.001				
	spring			↑	<0.001*	↑	<0.001*			↑	<0.001
focal skin reddening	spring					↑	<0.001	↑	<0.001		
iris reddening	spring					↑	0.005*	NS	<0.001*		
gross score	spring	↑	<0.001	↑	<0.001	↑	0.005	↑	<0.001	↑	<0.001
gross *Ichthyophonus*	fall							NS	0.019	↑	0.028
	spring									↑	0.003

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion or change	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
age	fall							↑	0.031		
	spring	↓	0.002								
food in stomach	fall							↓	0.008		
brain *Ichthyophonus*	fall									↑	0.018
	spring									↑	0.017
heart epicarditis	spring									↑	0.002
heart *Ichthyophonus*	fall									↑	0.004
	spring									↑	0.004
heart thrombosis	fall			↑	0.023						
	spring	↑	0.009								
stomach submucosal	fall					NS	0.022				
gastritis	spring					↑	0.002				
stomach *Ichthyophonus*	spring									↑	<0.001
gill foreign body granuloma	fall					↑	0.006				
gill *Ichthyophonus*	fall									↑	0.008
	spring									↑	0.005
gill lamellar telangiectasis	spring	↑	0.035								
gonad development	spring	↓	0.003*	↓	0.008*	↓	0.004*	↓	0.010*	↓	0.005

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion or change	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
gonadal pigmented macrophage aggregates	fall							NS	0.050		
liver Anisakidae	fall	↑	0.034	NS	0.008						
liver focal necrosis	spring			↑	0.002*			↑	<0.001*	↑	0.007
liver focal parenchymal leukocytes	fall									↓	0.050
	spring	↓	0.045	↓	0.002						
liver granulomatous inflammation	spring									↓	0.001
liver *Ichthyophonus*	fall									↑	0.008
	spring									↑	0.001
liver hepatocellular lipidosis	spring	↑	0.002								
liver pigmented macrophage aggregates	spring	↓	0.018								
liver hepatocellular single cell necrosis	spring			↑	0.031					↑	0.001
liver hepatocellular staining pattern (eosinophilic → basophilic)	spring	↑	0.011	↑	0.004			NS	0.032		
intestinal foreign body granulomas	spring	NS	0.025	NS	0.012						

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion or change	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
Intestinal *Ichthyophonus*	spring									↑	<0.001
intestinal mesenteric steatitis	spring					↓	0.005*			↑	0.012
intestinal trematodes	fall									↑	0.035
	spring	↓	0.028								
pancreatic zymogen granule depletion	spring									↑	0.039
opercular copepod	spring									↑	0.042
kidney interstitial hematopoietic cells	fall									↓	0.048
	spring							NS	0.015		
kidney *Ichthyophonus*	fall									↑	0.011
	spring									↑	0.005
kidney pigmented macrophage aggregates	spring	NS	0.032								
kidney tubular epithelial vacuolation	fall	↑	0.013								
splenic congestion	spring	NS	0.045								
splenic granulomatous inflammation	fall			↑	0.006						

		↑ caudal fin fraying		↑ caudal fin reddening		↑ diffuse skin reddening		↑ fin base reddening		↑ focal skin reddening	
Associated lesion or change	Season	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value	Trend	*P*-value
splenic *Ichthyophonus*	fall									↑	0.043
	spring									↑	0.012
skin/skeletal muscle *Ichthyophonus*	fall									↑	0.015
	spring									↑	0.006
skin/skeletal muscle arteriolar focal intimal hyperplasia	fall					NS	0.005				
	spring									↓	0.044
skin/skeletal muscle perivascular leukocytes	fall	↑	0.042								

Table 4. Age and lesion frequency (%) compared with status of viral hemorrhagic septicemia virus (VHSV) in Pacific herring sampled from Prince William Sound, Alaska, in March and April 1997. 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	score	VHSV+ (n≈38)	VHSV− (n≈222)	χ^2 *P*-value[a]	Odds ratio[b]	95% Confidence interval for odds ratio
age	3	55	32	0.0414	NC[c]	NC
	4	11	17			
	5	16	18			
	6+	18	34			
gross score	0	24	27	0.0055	NC	NC
	1	50	64			
	2+3	26	9			
kidney interstitial hematopoietic cells	0	18	6	0.0261	NC	NC
	1	74	83			
	2	8	11			
	1+2[d]	82	94	0.0072	0.28	0.10, 0.74
kidney interstitial congestion	0	79	92	0.0140	3.0	1.2, 7.6
	1+2	21	8			

Variable	score	VHSV+ (n≈38)	VHSV− (n≈222)	χ^2 *P*-value[a]	Odds ratio[b]	95% Confidence interval for odds ratio
kidney intratubular myxosporean (*Sphaerospora*?)	0	79	90	0.0483	2.4d	0.99, 5.9
	1+2+3	21	10			
liver focal parenchymal leukocytes	0	84	64	0.0162	0.34	0.14, 0.85
	1	16	36			
liver focal necrosis	0	74	99	0.0000	39	8.2, 188
	1+2+3	26	1			
liver hepatocellular single cell necrosis	0	63	94	<0.001	NC	NC
	1	8	5			
	2+3	29	1			
	1+2+3	37	6	<0.001	9.4	4.0, 22
gall bladder myxosporean (*Ceratomyxa auerbachi*)	0	61	83	0.0119	NC	NC
	1	28	12			
	2	11	5			
	1+2	39	17	0.0029	3.0	1.4, 6.5

Variable	score	VHSV+ (n≈38)	VHSV- (n≈222)	χ^2 *P*-value[a]	Odds ratio[b]	95% Confidence interval for odds ratio
liver hepatocellular staining pattern (1 = eosinophilic, 2 = intermediate, and 3 = basophilic)	1	13	19	0.0050	NC	NC
	2	50	66			
	3	37	15			
heart focal parenchymal leukocytes	0	100	91	0.0480	Approximate odds ratio=0.25[c]	
	1	0	9			
stomach submucosal gastritis	0+1	76	92	0.0036	3.517	1.4, 8.6
	2	24	8			
gonadal granulomatous inflammation	0	76	90	0.0155	2.821	1.2, 6.7
	1+2	24	10			

[a]*P*-value. For lesions with minimum expected cell frequency <1 (*), only comparisons with $P \leq 0.010$ were considered significant. Note that for comparisons with a low expected cell frequency, the odds ratio has a wide confidence interval.

[b]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 VHSV-positive versus VHSV-negative. For example, VHSV-positive fish were 39 times more likely to have hepatic focal necrosis than were VHSV-negative fish.

[c]NC = odds ratios were not calculated for lesions with more than 2 groups.

[d]In selected cases, scores for all positive cases (1+2+3) were combined and compared against all negative cases (score = 0) so that odds ratios could be calculated.

[e]The maximum odds ratio can be approximated by replacing the 0% frequency with a 1% frequency (e.g., calculate as if there is one VHSV-positive fish that has mild focal parenchymal leukocytes in the heart).

Table 5. Plasma chemistry values and white blood cell frequencies that were significantly different ($P < 0.05$) based on status of viral hemorrhagic septicemia virus (VHSV). Pacific herring were sampled in March or April 1997, from Prince William Sound, Alaska. One-way analysis of variance; for comparisons in which Levene's test for equality of variance was significant (*), only comparisons with $P \leq 0.010$ are shown. Plasma chemistries not shown were not significant.

	VHSV+ (n≈38)		VHSV- (n≈222)		
Variable	Mean	SE	Mean	SE	*P*-value
PCV (%)[a]	39.3	3.6	42.6	0.6	0.002*
Chloride (mmol/L)	221.4	4.5	234.8	2.3	0.024
Thrombocytes (%)	71.7	6.3	77.2	2.0	0.050
Lymphocytes (%)	21.9	5.7	15.7	1.6	0.009

[a]Values for PCV and other % data (e.g., thrombocytes) were arcsine square root transformed. Mean values were retransformed. First-order Taylor series approximations of standard errors of the means.

Table 6. Lesion frequency (%) and age within gender in Pacific herring sampled from Prince William Sound, Alaska, in October 1996 and March/April 1997. 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[a]	Odds ratio[b]	95% Confidence interval for odds ratio
October 1996 Samples		Female (n≈78)	Male (n≈79)			
splenic granulomatous inflammation	0	87	97	0.0153	5.7	1.2, 26.7
	1	13	3			
kidney interstitial hematopoietic cells	0+1	65	80	0.0436	2.1	1.0, 4.3
	2+3	35	20			
liver hepatocellular glycogen depletion	0	17	3	0.0060	NC[c]	NC
	1	72	77			
	2+3	12	20			
liver perivascular eosinophilic granular leukocytes	0+1	73	44	0.0003	0.29	0.15, 0.57
	2	27	56			
liver hepatocellular staining pattern (0 = eosinophilic; 1 = intermediate)	0	41	87	<0.001	9.9	4.4, 22.1
	1	59	13			
Intestinal coccidian (unclassified)	0	92	78	0.0143	0.30	0.11, 0.82
	1	8	22			
pancreatic zymogen granule depletion	0	41	20	0.0027	NC	NC
	1	46	48			
	2	13	32			

Variable and lesion	Lesion score	Frequency		χ^2 *P*-value[a]	Odds ratio[b]	95% Confidence interval for odds ratio
intestinal trematodes	0	85	68	0.0164	0.39	0.18, 0.85
	1+2	15	32			
skin/skeletal muscle perivascular leukocytes	0	10	23	0.0347	2.6	1.0, 6.4
	1	90	77			
gonadal eosinophilic granular leukocytes	0	1	28	<0.001	NC	NC
	1	86	60			
	2	13	12			
gonadal macrophage aggregates	0	65	99	<0.001	40.8	5.4, 309
	1	35	1			
March/April 1997 samples		Female (n≈141)	Male (n≈111)			
gross score	0	21	35	0.0189	NC	NC
	1	64	58			
	2+3	15	7			
caudal fin reddening	0	30	47	0.0085	NC	NC
	1	59	49			
	2+3	11	5			
splenic pigmented macrophage aggregates	0	21	8	0.0442	NC	NC
	1	32	36			
	2	29	31			
	3	18	25			
kidney tubular epithelial vacuolation	0	57	34	0.0002	0.39	0.23, 0.65
	1+2	43	66			

Variable and lesion	Lesion score	Frequency		χ^2 *P*-value[a]	Odds ratio[b]	95% Confidence interval for odds ratio
March/April 1997 samples		Female (n≈141)	Male (n≈111)			
liver pigmented macrophage aggregates	0+1	72	57	0.0306	NC	NC
	2	18	25			
	3	10	18			
liver focal parenchymal leukocytes	0	75	60	0.0118	0.50	0.29, 0.86
	1	25	40			
liver focal necrosis	0	93	98	0.050	4.2	0.89, 19
	1+2+3	7	2			
liver hepatocellular single cell necrosis	0	82	98	<0.001	NC	NC
	1	9	2			
	2+3	9	0			
liver hepatocellular staining pattern (1 = eosinophilic, 2 = intermediate, and 3 = basophilic)	1	2	32	<0.001	NC	NC
	2	67	65			
	3	31	3			
stomach *Ichthyophonus*	0	87	95	0.030	2.7	1.1, 7.1
	1+2	13	5			
intestinal mesenteric Anisakidae	0	18	14	0.034	NC	NC
	1	69	61			
	2+3	13	25			
pancreatic zymogen granule depletion	1	1	14	<0.001	NC	NC
	2	60	73			
	3	40	14			

Variable and lesion	Lesion score	Frequency		χ^2 P-value[a]	Odds ratio[b]	95% Confidence interval for odds ratio
March/April 1997 samples		Female (n≈141)	Male (n≈111)			
intestinal mesenteric steatitis	0	1	0	<0.001	NC	NC
	1	66	25			
	2	33	75			
skin/skeletal muscle perivascular leukocytes	0	42	30	0.0474	0.59	0.35, 1.0
	1	58	70			
brain *Ichthyophonus*	0	88	97	0.0064	4.9	1.4, 17
	1	12	3			
gonadal granulomatous inflammation	0	82	95	0.0008	4.8	1.8, 12
	1+2	18	5			
gonadal eosinophilic granular leukocytes	0	35	53	0.0034	2.1	1.3, 3.5
	1+2	65	47			
gonadal pigmented macrophage aggregates	0	71	98	<0.001	22	5.3, 95
	1+2	29	2			

[b]P-value. For lesions with minimum expected cell frequency <1 (*), only comparisons with $P \leq 0.010$ were considered significant. Note that for comparisons with a low expected cell frequency, the odds ratio has a wide confidence interval.

[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, in October 1996 samples, females were 5.7 times more likely to have splenic granulomatous inflammation than were males.

[c]NC = odds ratios were not calculated for lesions with more than 2 groups.

Table 7. Mean plasma chemistry and hematology values in adult Pacific herring sampled from Prince William Sound in fall 1996 (F96) and spring 1997 (S97). Analysis of variance (NS = not significant). Sample size varied slightly for some variables, but usually was as follows: F96 males (n = 79), F96 females (n = 78); S97 males (n = 111), S97 females (n = 141).

		Males		Females		
Variable	Date	mean	SE	mean	SE	*P*-value
Age (years)	F96	3.5	0.3	3.8	0.2	NS[a]
	S97	5.7	0.3	5.4	0.2	NS
Length (mm)	F96	205.6	2.6	211.0	2.1	NS
	S97	213.7	2.2	214.8	1.8	NS
Body weight (g)	F96	123.7	5.5	128.3	4.5	NS
	S97	123.2	4.2	126.0	3.5	NS
$\log_e$ Gonad weight (g)[a]	F96	7.8	1.5	3.3	0.5	<0.001*
	S97	20.2	2.3	23.9	2.2	0.020
$\log_e$ Liver weight (g)[a]	F96	1.1	0.1	1.9	0.2	<0.001
	S97	0.9	0.1	1.3	0.1	<0.001
Hold time (min)	F96	114.2	4.7	103.1	4.1	NS
	S97	104.2	3.5	107.7	3.3	NS
Sum*Ichthyophonus*	F96	1.6	0.6	2.1	0.6	NS
	S97	1.0	0.3	2.1	0.4	0.028*
PCV (%)[b]	F96	48.0	1.2	47.5	1.4	NS
	S97	43.5	0.8	41.0	1.2	0.002
Albumin (g/dL)	F96	2.3	0.1	2.3	0.1	NS
	S97	1.9	0.1	1.8	0.1	NS
$\log_e$ IgM (µg/mL)[a]	F96	449.4	56.5	459.4	71.4	NS
	S97	358.9	45.9	289.2	34.1	0.016
Total protein (g/dL)	F96	3.95	0.09	4.21	0.10	0.051
	S97	4.2	0.1	3.9	0.1	0.040
ALP (U/L)	F96	45.75	1.10	42.05	1.31	0.032
	S97	80.7	3.6	95.0	4.3	0.014*
$\log_e$ AST (U/L)[a]	F96	364.7	40.1	366.1	35.9	NS
	S97	63.9	8.0	84.2	9.9	0.002
$\log_e$ CPK (U/L)[a]	F96	1742.4	368.1	1600.4	357.4	NS
	S97	1478.8	218.2	1227.8	181.1	NS

Variable	Date	Males mean	Males SE	Females mean	Females SE	*P*-value
Calcium (mg/dL)	F96	4.2	0.1	4.8	0.1	0.001*
	S97	10.8	0.5	10.8	0.4	NS
Chloride (mmol/L)	F96	273.5	5.1	268.8	4.2	NS
	S97	232.4	3.2	231.7	2.6	NS
Glucose (mg/dL)	F96	148.0	5.6	164.9	5.6	0.033
	S97	134.4	6.7	90.3	3.6	<0.001*
Lactate (mmol/dL)	F96	6.9	0.5	7.2	0.5	NS
	S97	7.0	0.3	5.8	0.2	0.002
Osmolality (mOsm/kg)	F96	379.9	2.5	382.2	2.2	NS
	S97	415.0	4.4	406.0	3.3	NS
Phosphorus (mg/dL)	F96	7.4	0.2	7.7	0.2	NS
	S97	5.8	0.2	5.8	0.2	NS
Potassium (mmol/L)	F96	1.4	0.0	1.3	0.0	NS
	S97	2.2	0.06	2.4	0.08	NS
Sodium (mmol/L)	F96	192.3	1.4	195.4	2.0	NS
	S97	196.0	3.4	194.8	3.2	NS
Basophils (%)[b]	F96	0.1	0.1	0.1	0.1	NS
	S97	0.06	0.05	0.02	0.02	NS
Eosinophils (%)[b]	F96	1×10^{-5}	4×10^{-4}	2×10^{-3}	6×10^{-3}	NS
	S97	0.1	0.1	0.01	0.01	NS
Lymphocytes (%)[b]	F96	46.5	2.9	41.9	3.1	0.042
	S97	19.3	2.3	14.0	1.9	0.001
Monocytes (%)[b]	F96	4×10^{-4}	2×10^{-3}	9×10^{-4}	2×10^{-3}	NS
	S97	1×10^{-3}	1×10^{-2}	1×10^{-3}	1×10^{-2}	NS
Neutrophils (%)[b]	F96	8.3	1.6	6.9	1.7	NS
	S97	6.5	1.3	3.8	0.8	<0.001
Thrombocytes (%)[b]	F96	42.8	2.5	48.3	3.1	0.010
	S97	71.9	3.0	80.5	2.3	<0.001

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

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

Table 8. Lesion frequency (%) within iris reddening (IR) in Pacific herring sampled from Prince William Sound, Alaska. Lesions were scored as none (0), mild (1), moderate (2), or severe (3). Chi-square test for homogeneity. For lesions with minimum expected cell frequency <1 (*), only chi-square tests with $P \leq 0.010$ are included. Lesions not listed were not significant. For some lesions, sum of individual frequencies within a category is different from 100% due to rounding differences.

Sample date and lesion	Lesion score	Lesion score frequency (%)			χ^2 P-value
Fall 1996		IR moderate (n = 8)	IR mild (n = 94)	IR none (n = 58)	
food in stomach (3 = abundant)	0	13	22	36	0.017
	1	25	16	17	
	2	0	27	33	
	3	63	35	14	
intestinal foreign body granuloma	0	38	81	67	0.010
	1	63	19	33	
splenic macrophage aggregates	0	0	14	14	0.024
	1	13	31	41	
	2	38	46	33	
	3	50	10	12	
pancreatic zymogen granule depletion	0	0	30	36	0.035
	1	38	48	48	
	2	63	22	16	
testis spermatocyte numbers (3 = abundant)	0+1	33	76	82	0.044
	2+3	67	24	18	

Sample date and lesion	Lesion score	Lesion score frequency (%)			χ^2 *P*-value
Spring 1997		IR moderate (n≈9)	IR mild (n ≈ 209)	IR none (n ≈ 42)	
age	3	11	30	67	<0.001
	4	11	15	19	
	5	33	20	2	
	6+	44	35	12	
diffuse skin reddening	0	78	98	93	0.005*
	1	22	2	7	
fin base reddening	0	56	74	62	<0.001*
	1	11	23	38	
	2+3	33	3	0	
gill arch inflammation or hematopoiesis	1	89	91	76	0.024
	2	11	9	24	
liver granulomatous inflammation	0	33	69	79	0.028
	1+2	67	31	21	

Table 9. Significantly different values (ANOVA, $P \leq 0.05$) based on year class (age). Pacific herring were sampled from Prince William Sound, Alaska, in October 1996 (F96) and in March/April 1997 (S97). For comparisons in which Levene's test for equality of variance was significant (*), only comparisons with $P \leq 0.010$ are shown. variables not shown were not significant.

		Year class = 1994 F96, n = 66 S97, n = 92		Year class = 1993 F96, n = 31 S97, n = 41		Year class = 1992 F96, n = 29 S97, n = 45		Yr. classes = 1983-1991 F96, n = 33 S97, n = 82		
Variable	Date	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P*-value
Hematology										
lymphocytes[b] (%)	S97	18.76	2.91	16.55	3.93	12.41	2.71	16.78	2.63	0.042
neutrophils[b] (%)	S97	4.39	1.04	3.95	1.30	3.83	1.58	6.76	1.38	0.006
thrombocytes[b] (%)	S97	74.81	3.40	77.70	4.40	81.80	3.78	74.46	3.24	0.038
Parasites										
Anisakidae[a] (# in peritoneal cavity)	F96	6.27	1.02	13.33	3.08	14.08	3.57	10.65	2.57	<0.001
	S97	9.24	1.22	11.51	2.48	14.41	2.38	12.22	1.71	0.001
Ichthyophonus hoferi (sum of scores)	F96	0.26	0.20	1.77	0.97	3.03	1.21	4.30	1.36	0.002*
	S97	0.39	0.25	1.66	0.69	1.53	0.54	2.88	0.60	0.001*
Plasma chemistries										
albumin (g/dL)	F96	1.98	0.07	2.27	0.12	2.68	0.18	2.66	0.15	<0.001*
	S97	1.46	0.09	1.66	0.12	1.89	0.14	2.30	0.12	<0.001*
ALP (U/L)	S97	72.34	4.50	78.37	6.02	92.27	6.47	106.28	5.51	<0.001
calcium (mg/dL)	F96	4.20	0.13	4.53	0.21	4.77	0.20	4.79	0.22	0.033
	S97	9.29	0.38	8.95	0.51	10.56	0.78	13.30	0.62	<0.001*
chloride (mmol/L)	F96	260.05	4.06	265.10	7.07	284.53	9.13	283.63	7.64	0.008
glucose (mg/dL)	S97	77.94	3.25	95.95	7.16	98.20	6.51	156.79	7.65	<0.001*

Variable	Date	Year class = 1994 F96, n = 66 S97, n = 92 Mean	SE	Year class = 1993 F96, n = 31 S97, n = 41 Mean	SE	Year class = 1992 F96, n = 29 S97, n = 45 Mean	SE	Yr. classes = 1983-1991 F96, n = 33 S97, n = 82 Mean	SE	*P*-value
IgM[a] (µg/mL)	F96	333.62	43.94	439.22	81.38	540.23	113.05	724.15	157.34	<0.001
	S97	216.37	27.64	349.67	66.88	342.41	66.17	434.85	61.57	<0.001
phosphate (mg/dL)	S97	5.42	0.19	5.25	0.31	5.88	0.31	6.33	0.26	0.009
potassium (mmol/L)	F96	1.46	0.04	1.33	0.06	1.30	0.04	1.25	0.08	0.019
sodium (mmol/L)	S97	185.90	3.37	198.56	6.77	194.17	5.98	202.12	3.90	0.029
total protein (g/dL)	F96	3.72	0.09	3.92	0.13	4.35	0.11	4.69	0.14	<0.001
	S97	3.56	0.11	3.88	0.15	4.02	0.18	4.54	0.13	<0.001
Weight and Length										
body weight (g)	F96	88.88	1.46	116.83	3.37	142.93	4.35	191.00	6.08	<0.001*
	S97	81.26	1.10	108.32	2.22	127.56	2.47	175.11	3.03	<0.001*
gonad weight[a] (g)	F96	2.75	0.49	5.03	1.22	7.11	1.89	11.60	3.46	<0.001
	S97	11.69	1.93	20.53	1.61	27.80	1.80	35.45	4.18	<0.001*
liver weight[a] (g)	F96	1.07	0.07	1.39	0.17	1.72	0.19	2.40	0.26	<0.001
	S97	0.78	0.06	0.98	0.09	1.25	0.13	1.52	0.10	<0.001
standard length (mm)	F96	189.03	0.94	206.61	1.79	218.21	1.81	238.52	2.08	<0.001
	S97	190.67	0.74	208.44	1.35	218.80	1.26	238.85	1.23	<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 10. Linear correlations (r) of age (yr), body weight and gonad weight (g), standard length (mm), hold time (min), sum-*Ichthyophonus* (sumICH) scores, albumin (g/dL), lnIgM (µg/mL), and blood values in Pacific herring sampled from Prince William Sound, Alaska, in fall 1996 (F96; n ≈ 160) and spring 1997 (S97; n ≈ 260). Significant correlations are denoted for $P < 0.050$ (*) and $P < 0.010$ (**). Values for PCV and white blood cells were arcsine square root transformed for analysis.

Variable	Date	Age	Body weight	Standard Length	Gonad weight	Hold time	sumICH	Albumin	lnIgM
Body weight	F96	0.892**							
	S97	0.890**							
Length	F96	0.865**	0.967**						
	S97	0.886**	0.970**						
Gonad weight	F96	0.679**	0.765**	0.694**					
	S97	0.765**	0.927**	0.889**					
Hold time	F96	-0.072	-0.044	-0.044	0.079				
	S97	-0.054	-0.044	-0.033	-0.016				
Ichthyophonus	F96	0.225**	0.122	0.159*	0.005	0.024			
(sum of scores)	S97	0.216**	0.211**	0.259**	0.158*	-0.073			
Albumin	F96	0.335**	0.402**	0.422**	0.289**	-0.091	0.047		
	S97	0.344**	0.407**	0.383**	0.318**	-0.005	0.170**		
lnIgM	F96	0.430**	0.373**	0.409**	0.249**	-0.047	0.575**	0.160*	
	S97	0.325**	0.293**	0.325**	0.221**	-0.127*	0.310**	0.204**	
Liver weight	F96	0.697**	0.707**	0.738**	0.174*	-0.147	0.267**	0.295**	0.385**
	S97	0.580**	0.634**	0.626**	0.540**	-0.107	0.371**	0.232**	0.190**
PCV	F96	0.044	0.102	0.092	0.121	-0.207**	-0.192*	0.045	0.045
	S97	0.012	0.028	0.036	0.047	-0.155*	-0.132*	-0.009	0.110
Total protein	F96	0.447**	0.520**	0.502**	0.315**	-0.224**	0.159*	0.419**	0.235**
	S97	0.291**	0.326**	0.326**	0.245**	-0.103	0.248**	0.365**	0.242**

Variable	Date	Age	Body weight	Standard Length	Gonad weight	Hold time	sumICH	Albumin	lnIgM
lnAST	F96	0.026	-0.025	-0.017	-0.020	0.047	0.247**	0.067	0.097
	S97	0.014	0.049	0.091	0.081	0.094	0.294**	0.055	0.254**
ALP	F96	0.046	0.104	0.096	0.132	-0.101	-0.125	0.221**	0.035
	S97	0.263**	0.384**	0.334**	0.371**	-0.030	0.160**	0.179**	0.051
lnCPK	F96	-0.117	-0.087	-0.096	-0.079	-0.014	0.156*	0.122	0.037
	S97	-0.115	-0.130*	-0.097	-0.093	-0.120	0.009	-0.076	0.100
Calcium	F96	0.221**	0.235**	0.273**	0.048	0.096	0.036	0.169*	0.178*
	S97	0.369**	0.404**	0.364**	0.335**	0.039	0.147*	0.278**	0.104
Chloride	F96	0.262**	0.307**	0.274**	0.282**	0.081	0.064	0.040	0.062
	S97	-0.024	-0.009	-0.030	-0.068	-0.094	0.043	0.160**	0.047
Glucose	F96	0.036	0.077	0.128	-0.094	0.075	0.038	0.148	0.111
	S97	0.564**	0.573**	0.564**	0.487**	-0.058	0.082	0.294**	0.171**
Lactate	F96	0.147	0.175*	0.148	0.134	-0.068	-0.100	0.296**	0.063
	S97	0.035	0.009	0.030	0.002	-0.069	-0.053	-0.048	0.040
Osmolality	F96	0.136	0.188*	0.165*	0.157*	0.138	-0.050	0.128	0.050
	S97	-0.001	-0.004	-0.011	-0.037	-0.060	-0.085	0.002	-0.011
Phosphorus	F96	0.043	0.059	0.028	-0.026	-0.204**	-0.076	0.080	-0.032
	S97	0.213**	0.252**	0.241**	0.217**	-0.040	0.040	0.261**	-0.007
Potassium	F96	-0.203**	-0.187*	-0.212**	-0.128	0.382**	-0.108	-0.188*	-0.079
	S97	-0.087	-0.085	-0.081	-0.092	0.214**	-0.111	-0.077	-0.023
Sodium	F96	-0.003	0.079	0.052	0.016	0.041	-0.153	-0.016	0.004
	S97	0.185**	0.219**	0.148*	0.145*	0.107	-0.000	0.210**	-0.049
%	F96	-0.073	-0.050	-0.031	-0.064	0.066	0.132	-0.060	0.108
Basophils	S97	0.013	0.007	0.019	0.029	0.019	0.125*	-0.033	0.081

Variable	Date	Age	Body weight	Standard Length	Gonad weight	Hold time	sumICH	Albumin	lnIgM
%	F96	-0.078	-0.052	-0.037	-0.071	-0.131	-0.036	0.070	0.020
Eosinophils	S97	-0.120	-0.113	-0.139*	-0.161**	-0.155*	-0.084	-0.045	-0.064
%	F96	0.103	0.148	0.122	0.230**	-0.017	-0.018	0.058	-0.019
Lymphocytes	S97	-0.046	-0.105	-0.072	-0.132*	-0.038	0.074	-0.080	0.021
%	F96	-0.123	-0.125	-0.129	-0.095	-0.042	-0.056	-0.060	-0.119
Monocytes	S97	0.027	0.038	0.055	-0.010	-0.134*	0.078	0.103	0.243**
%	F96	-0.044	-0.093	-0.077	-0.082	0.138	0.086	-0.105	0.074
Neutrophils	S97	0.201**	0.148*	0.122*	0.081	-0.025	-0.057	0.078	0.075
%	F96	-0.098	-0.130	-0.110	-0.208**	-0.046	-0.045	-0.039	-0.058
Thrombocytes	S97	-0.033	0.036	0.018	0.088	0.052	-0.055	0.037	-0.043
# Anisakidae	F96	0.159*	0.228**	0.276**	0.139	0.086	0.035	0.133	0.076
	S97	0.100	0.164**	0.173**	0.151*	-0.027	0.151*	0.069	0.120

Table 11. Sample prevalence (%) of parasites and virus in adult Pacific herring in Prince William Sound, Alaska, 1989-1997.

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	12	TNE
1991 April[c]	59	54	3.4	20	TNE
1991 October[c]	48	40	2.1	31	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[g]	212	61	24 (29)	5.7 (19)	4.7
1995 April (spawning)	180	73	23 (29)	7.2 (29)	0.0
1995 November	130	63	13 (19)	12 (19)	0.0
1996 April	260	80	19 (21)	11 (20)	0.0
1996 October	160	70	13 (16)	19 (22)	0.0
1997 April	260	70	16 (18)	8.8 (16)	15

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

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

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

[d]TNE = Tissue not examined

[e](Kocan et al. 1996)

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

[g](Marty et al. 1998)

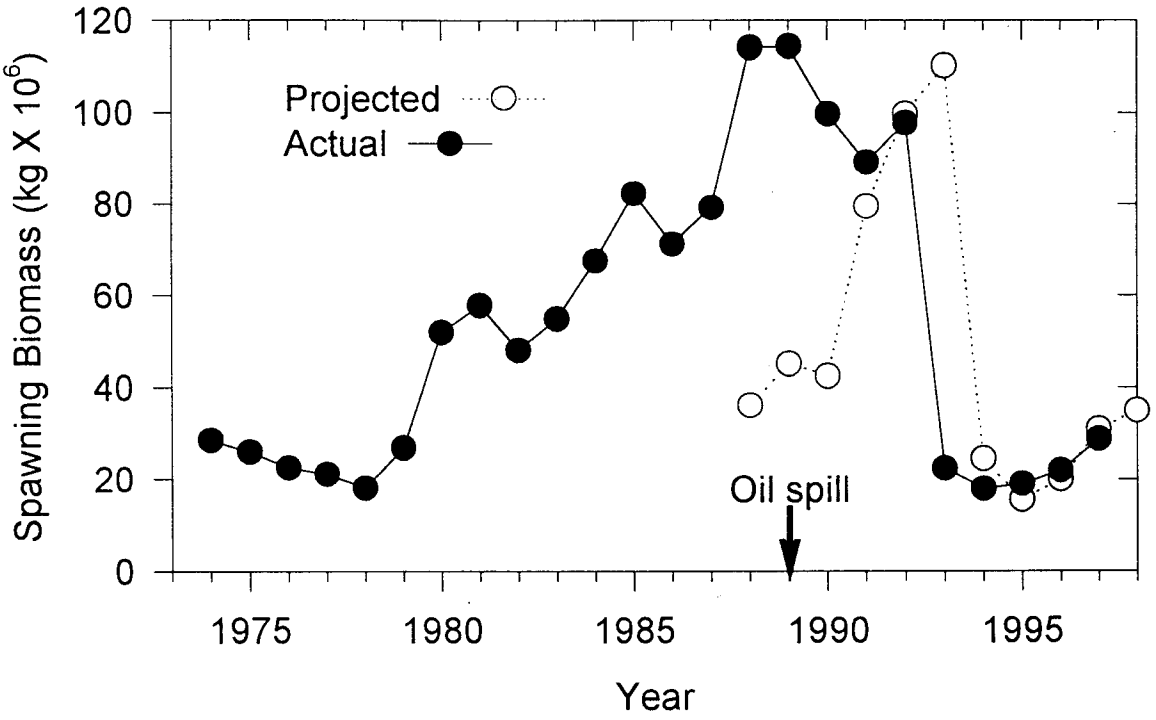

Figure 1. Biomass estimates of mature Pacific herring in Prince William Sound, Alaska. Unexploited spawning biomass projected in the year before spawning (PROJECTED) and calculated after spawning (ACTUAL) using the age-structure assessment model. Estimates were made by John Wilcock, Alaska Department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

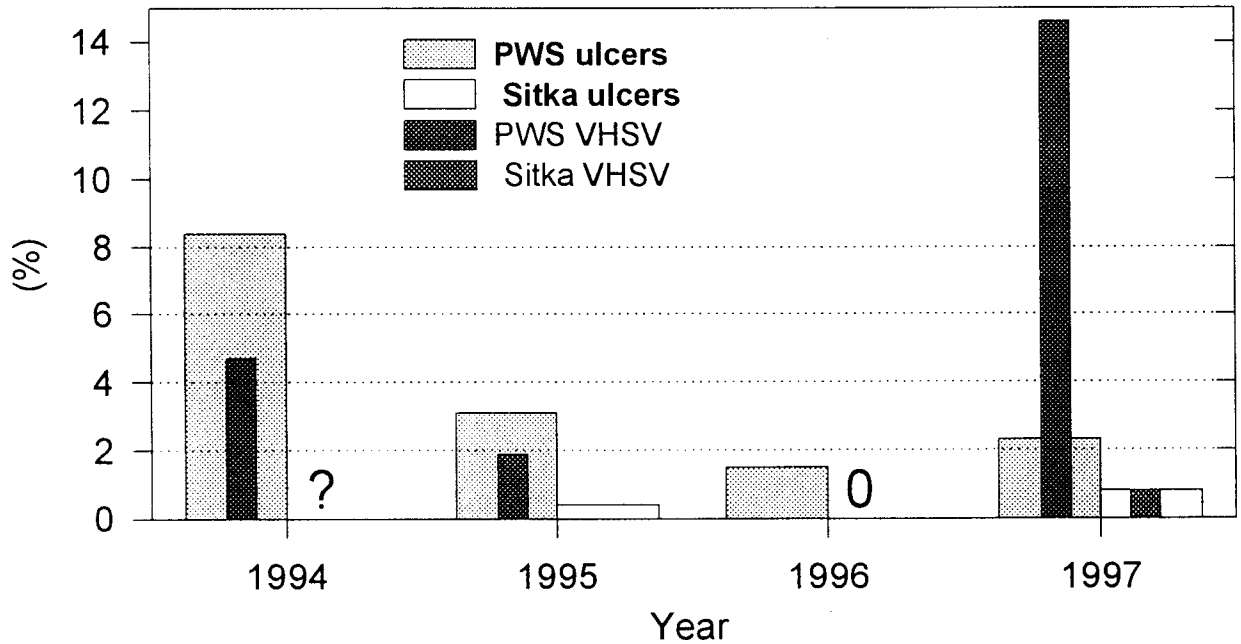

Figure 2. Prevalence of ulcers and viral hemorrhagic septicemia virus (VHSV) in adult Pacific herring sampled from Prince William Sound (PWS) and Sitka, Alaska.

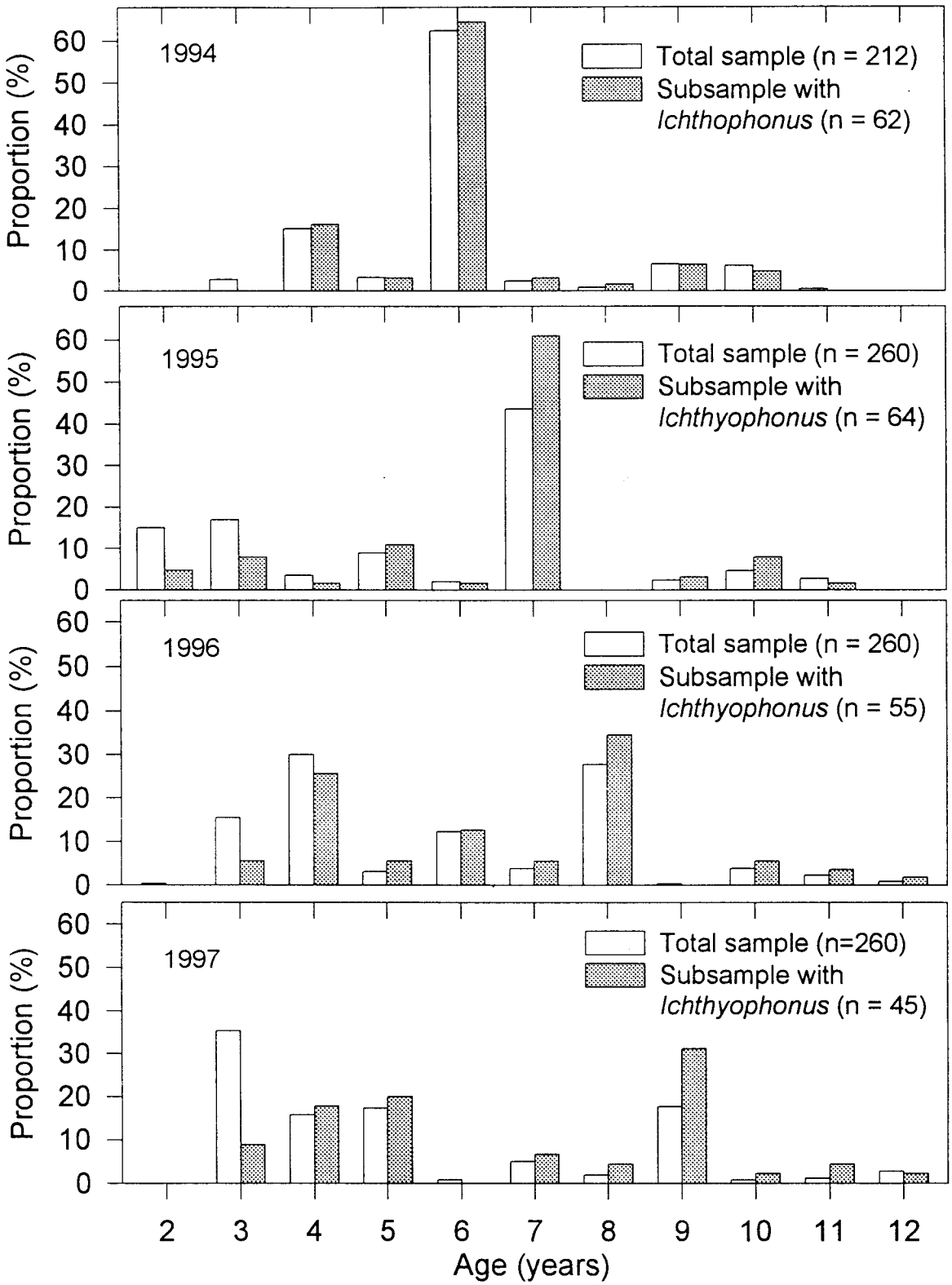

Figure 3. Annual age distribution of Pacific herring sampled in the spring from Prince William Sound, Alaska, compared with the age distribution of the subset of sampled fish with *Ichthyophonus* infection.

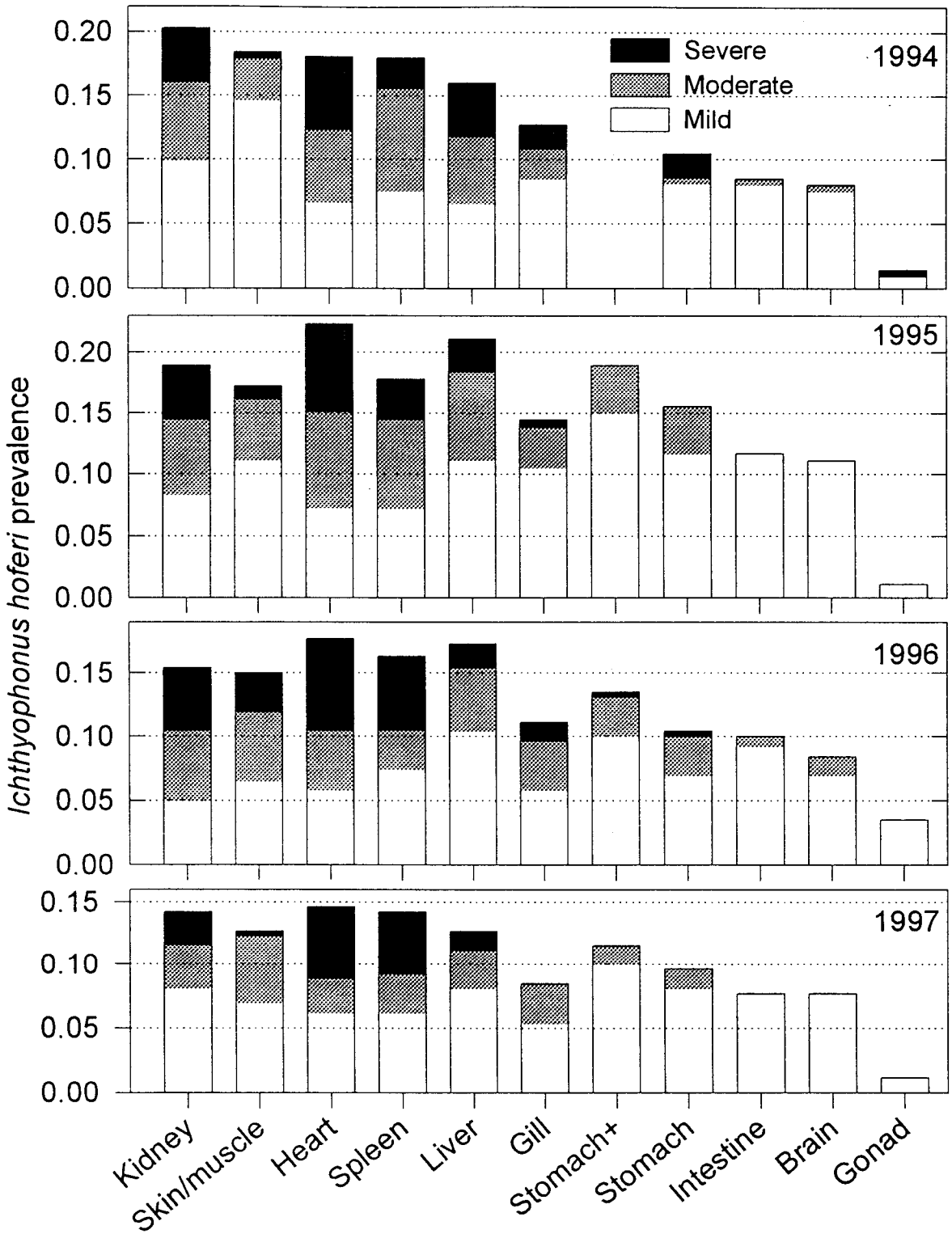

Figure 4. Sample prevalence of *Ichthyophonus* lesion scores in various organs of mature Pacific herring sampled in the spring from Prince William Sound, Alaska. Lesions were scored as none (0), mild (1), moderate (2), or severe (3). The Stomach+ score includes stomachs with inflammation characteristic of *Ichthyophonus*, but organisms were not in the section examined.

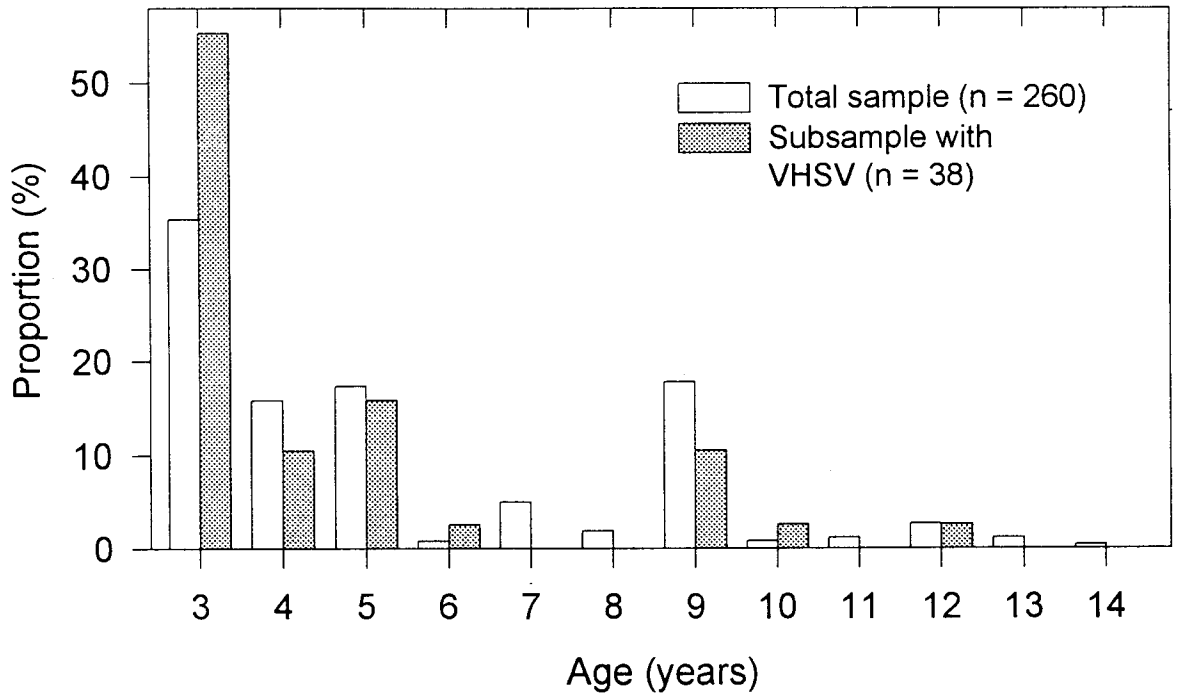

Figure 5. Age distribution of Pacific herring randomly sampled in spring 1997 from Prince William Sound, Alaska, compared with the age distribution of the subset of sampled fish that were positive for viral hemorrhagic septicemia virus (VHSV).

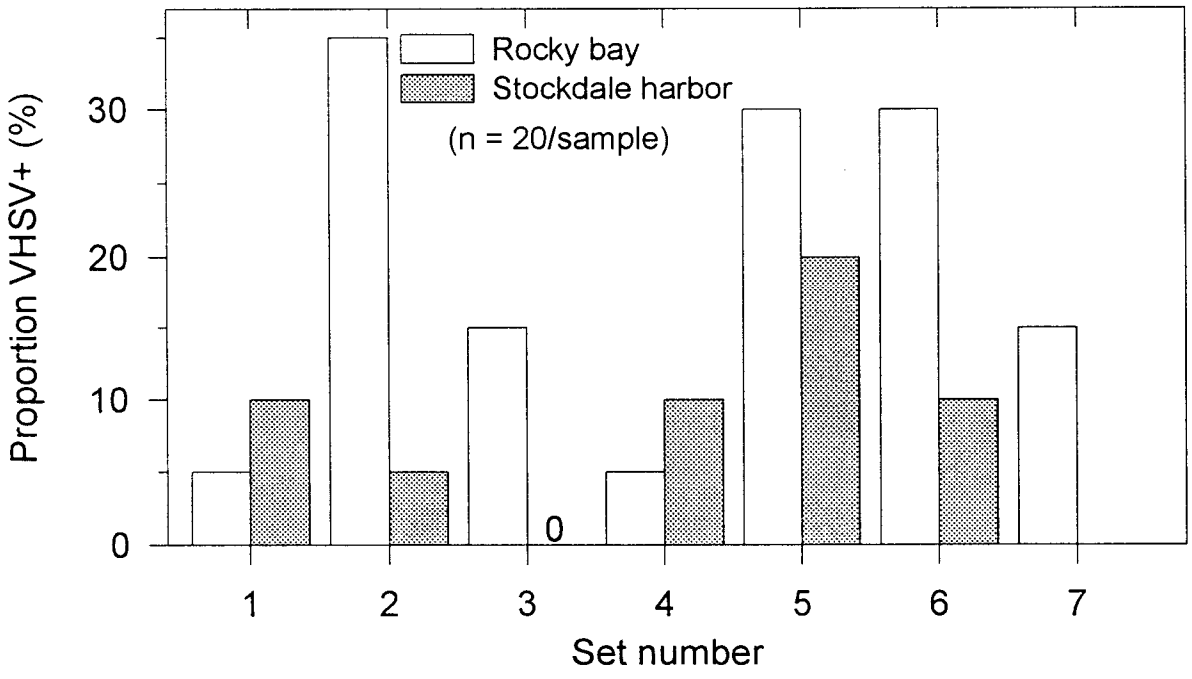

Figure 6. Proportion of VHSV+ Pacific herring within each 20-fish sample collected from two sites in Prince William Sound, Alaska, in March or April of 1997.

CHAPTER 2 - Expression of Viral Hemorrhagic Septicemia Virus in Pacific herring from the Spawn-on-Kelp Pound Fishery

P. K. Hershberger, R.M. Kocan, and G.D. Marty

Introduction

Recent discovery of viral hemorrhagic septicemia virus (VHSV) in several fish species from the west coast of North America (Eaton et al. 1991, Meyers et al. 1992, Meyers et al. 1994, Meyers and Winton 1995) has raised new concerns regarding host range, viral virulence and transmission, and management of affected populations. Differences that may affect virulence include strain differences within the same species of fish (Kaastrup et al. 1991), as well as genetic (Bernard et al. 1992, Winton et al. 1991, and Bernard et al. 1990) and serological (Jorgensen 1972) differences. Questions still remain regarding natural transmission and management of certain fisheries that may contribute to virus proliferation.

This project was designed to determine whether activities associated with the closed pound spawn-on-kelp fishery (= pound fishery) are responsible for initiating active VHSV infections within the captured and confined herring. In the pound fishery, sexually mature pre-spawn herring are purse seined, transported to a floating net pen (or pound) containing suspended kelp fronds, and held in the pound for several days to spawn upon the kelp. The kelp fronds with adherent herring eggs attached are then harvested and sold to Japanese markets as kazunoko.

Pounds may foster VHSV infections for several reasons. First, stressors such as temperature and crowding have been reported to exacerbate VHSV infections in European rainbow trout farms (Enzmann and Konrad 1984). Conditions in the pounds are often kept very crowded in an attempt to acquire a high quality product with multiple layers of eggs on the kelp surface. Second, other investigators have noted the presence of "*Vibrio*-like disease" or unusually high mortality in crowded net pens containing herring (Brett and Solmie 1982, Krieberg et al. 1982, Krieberg et al. 1984, and Hay et al. 1988). Affected fish had subcutaneous hemorrhage, but bacterial isolation from these fish was unsuccessful. Interestingly, the clinical signs of the reported disease were very similar to those reported by Meyers et al. (1994) for VHS in wild Pacific herring. Also, recent experiments with Pacific herring in Puget Sound suggest that captured herring shed VHSV into the surrounding water within 2 hours of capture (Hershberger and Kocan, unpublished data). Thus, pounds may create a situation highly conducive to rapid shedding of large quantities of VHSV into the water, followed by viral exposure to large numbers of confined fish.

Based on this knowledge, a pilot study was conducted on the 1996 spawn-on-kelp fishery in Craig, Alaska (SE Alaska) to determine whether pounds may foster VHSV infections. Of 38 fish sampled nonrandomly from the fishery, 21% were positive for VHSV. When the PWS pound fishery was reopened in 1997, the Trustee Council funded a more comprehensive study to examine the epizootiology of VHSV in pounds in PWS.

Objectives

The major objective was to determine the role of spawn-on-kelp pound fisheries on expression of viral hemorrhagic septicemia virus. This involved several specific objectives:

1. Determine VHSV prevalence in fish entering into pound fisheries and in unpounded spawning fish in the population;
2. Determine time course of VHSV infection while herring are impounded;
3. Determine VHSV prevalence in dead fish in pounds.
4. Determine VHSV prevalence at the time when fish would have been released from pounds [in previous years, fish were released from the pounds, but new ADFG regulations for 1997 required that no fish be released].
5. Determine VHSV titer in water samples within and around closed pounds.
6. Determine if VHSV prevalence varies with capture, handling, and pounding conditions (based on visual observations by management biologists).
7. Determine the effect of age and gender on VHSV activation/expression.
8. Determine the effect of environmental conditions in pounds (dissolved oxygen, temperature, and salinity).

Methods

The 1997 closed pound fishery in PWS consisted of 8 fishers who consolidated into three pounds. All pounds were located in Fidalgo Bay, in the Northeast region of PWS, and designated pound #1, #2, and #3 (Table 1). Experimental design for the pound study consisted of dip netting 40 Pacific herring from each of the three pounds on consecutive days after capture and introduction of the herring into the pounds. Data from each fish included age (from scales), weight, length, gender, external lesion scores, and VHSV tissue titer in spleen and kidney pools. External lesions were scored the same as for field study of Pacific herring disease (Marty et al. 1998; chapter 1, this report). VHSV tissue titrations were performed by Dr. Ted Meyers using a plaque assay, as described in Meyers et al. (1994). Water samples were also taken from each pound every other day at slack tide (± 1h), frozen, shipped to the University of Washington, and assayed by Paul Hershberger for VHSV titer. Dissolved oxygen and water temperature were measured inside and outside the pounds at slack tide (± 1h). In addition to the daily 40-fish samples, two 40-fish samples of naturally spawning Pacific herring from outside the pounds were assayed. A 40-fish sample of recently deceased herring floating inside one of the pounds was also analyzed for virus.

Table 1. Sampling schedule for spawn-on-kelp pounds in Prince William Sound, Alaska.

	Date (1997)								
	4/11	4/12	4/13	4/14	4/15	4/16	4/17	4/18	4/19
Pound 1	day 0	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
Pound 2			NS	day 1	day 2	day 3	day 4	day 5	day 6
Pound 3	NS	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8

*NS= no sample

For statistical analysis, the association between 2 selected categorical variables (e.g., VHSV status versus age, gender, or external lesion scores) 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.

Results

The owners of pound # 1 were the first to catch fish and load their pound (4/11/97). All fish in this pound were caught at the head of Irish Cove in one set and transported only a few hundred meters to the pound. The fish in pound # 2 were added in two different loading events and all herring in this pound were caught in Two Moon Bay and transported 2.2 km to the closed pound. Herring were first introduced to pound #2 at 04:00 hrs on 4/13/97, but too few fish were in the pound to sample with a dip net. Hence, no day 0 sample was taken from this pound. The next group of herring (approximately 16 metric tons) was added to this pound just before midnight on 4/13/97, and a day 1 sample was taken on 4/14/97 at 06:00 hrs. All the fish in Pound #3 (approximately 100 Kg) were taken in one set at the head of Landlocked Bay on 4/11/97 and transported only a few hundred meters to the pound. Too few fish were in this pound to sample with a dip net on day 0 without disturbing the kelp. The pound owners were subsequently unable to catch any more herring so the pound was abandoned and the kelp removed. This enabled sampling of the fish in the pound because the sides of the pound could be lifted during sampling events and the fish captured using either a dip net or cast net.

All 3 pounds were different dimensions and loaded at various crowding densities (Table 2). Pound #2 was by far the most crowded; it contained the most fish (18 metric tons) and lacked corner weights to hold the sides of the pound down, enabling the sides to float up and crowd herring to the surface. Predators including kittiwakes, eagles, and sea lions captured fish from this pound throughout the study. Pound #3 was the deepest pound, containing the fewest and youngest fish.

Table 2. Physical and biological characteristics of the spawn-on-kelp pounds in Prince William Sound, Alaska.

	Number of permits*	mean age (yrs)	Estimated biomass (metric tons)	Pound size (m) (L × W × D)	Density (kg/m^3)
Pound #1	1	7	4-5	5.5 × 11.6 × 4.6	17.0
Pound #2	3	7	>18	17.7 × 8.5 × 6.1	19.6
Pound #3	4	4	0.9	6.1 × 7.3 × 9.1	2.2

* each permit holder was allowed 5.67 metric tons of herring

Water temperature and dissolved oxygen (DO) remained near optimal in each of the pounds (Tables 3-5). Measured DO never dropped below 12mg/L inside the pounds and was not significantly different from DO outside the pounds.

Table 3. Water quality in spawn-on-kelp pound # 1.

Date	Sampling Time (hours)	Dissolved oxygen (mg/L) inside the pound	Dissolved oxygen (mg/L) outside the pound	Temp. (C)
4/10 (pre-fish)	08:00	14.0-14.7	-	4.6-4.9
4/12 (Day 1)	11:40	14.0	15.1	-
4/14 (Day 3	14:15	15.0	15.3	5.7
4/16 (Day 5)	10:10	15.0	14.8	5.7
4/18 (Day 7)	11:45	12.2	13.7	5.2

Table 4. Water quality in spawn-on-kelp pound # 2.

Date	Sampling Time (hours)	Dissolved oxygen (mg/L) inside the pound	Dissolved oxygen (mg/L) outside the pound	Temp. (C)
4/10 (pre-fish)	08:00	14.1-14.6	-	4.9-5.1
4/13 (Day 0)	13:45	15.2	15.0	6.1
4/15 (Day 2)	13:50	14.7	14.9	6.0
4/17 (Day 4)	10:50	13.9	14.6	5.1
4/19 (Day 6)	06:35	12.9	13.2	5.8

Table 5. Water quality in spawn-on-kelp pound # 3.

Date	Sampling Time (hours)	Dissolved oxygen (mg/L) inside the pound	Dissolved oxygen (mg/L) outside the pound	Temp. (C)
4/10 (pre-fish)	07:30	13.4-13.7	-	4.7
4/12 (Day 1)	18:00	15.7	15.5	-
4/14 (Day 3)	10:15	15.1	15.3	6.1
4/16 (Day 5)	06:30	14.5	14.9	5.8
4/18 (Day 7)	16:25	13.8	13.8	6.5

VHSV prevalence in herring from pound # 1 had bimodal peaks on days 1 and 4 (Figure 1). Initially (day 0), 12.5% of the sampled herring tested positive for VHSV, and by day 8 virus was undetectable in any fish from the pound. VHSV prevalence in herring from pound #2 increased from 0% on day 1 to a high of 15% by day 4 and decreased thereafter (Figure 2). Again, a bimodal viral prevalence occurred in the herring of pound #3, on days 2 (27.5%) and 5 (12.5%) then returned to low levels (Figure 3). No VHSV was detected in tissues samples of unpounded, naturally spawning, Pacific herring. Also, prevalence of VHSV in pound # 2 on day 5 was not significantly different in recently dead fish (15%) or randomly collected live fish (12.5%).

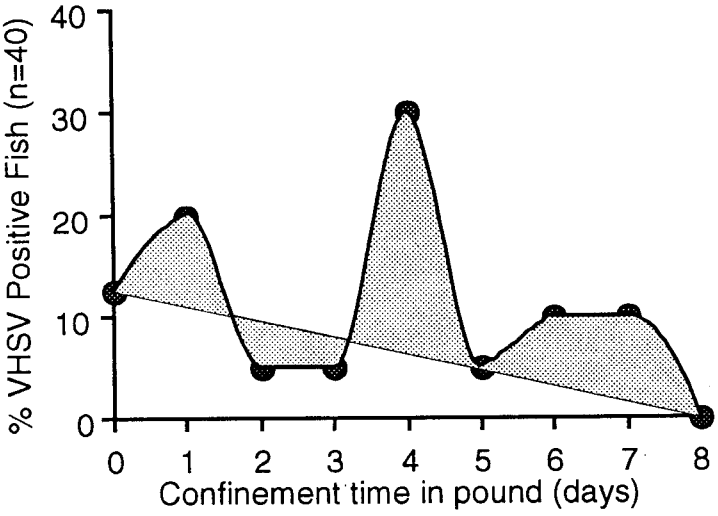

Figure 1. VHSV prevalence in Pacific herring from spawn-on-kelp pound # 1.

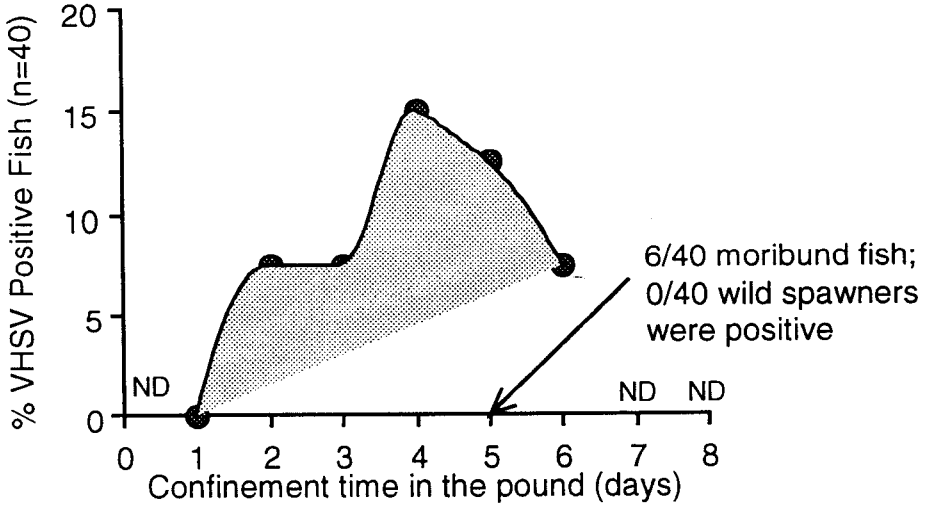

Figure 2. VHSV prevalence in Pacific herring from spawn-on-kelp pound # 2.

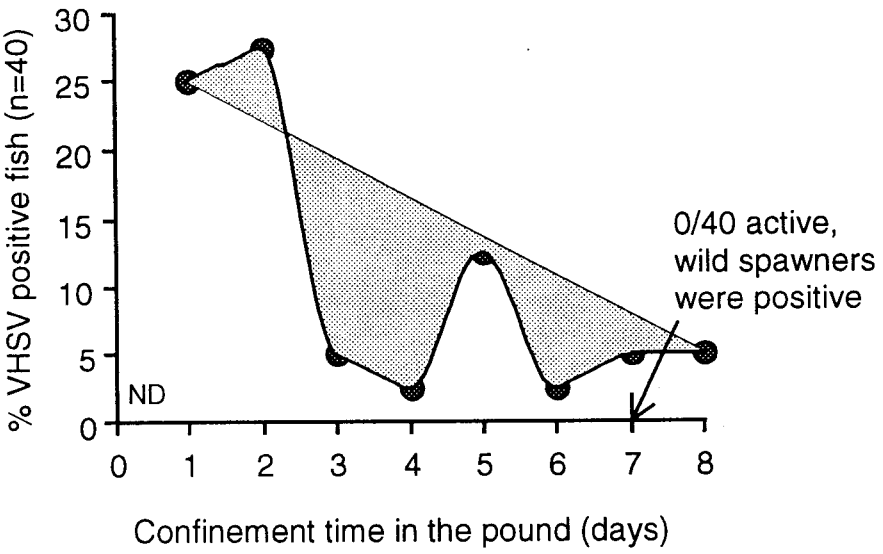

Figure 3. VHSV prevalence in Pacific herring from spawn-on-kelp pound # 3.

The year classes with the largest percentage of virus-positive fish were the 4-6 year olds and possibly the 11-year-olds (Figure 4).

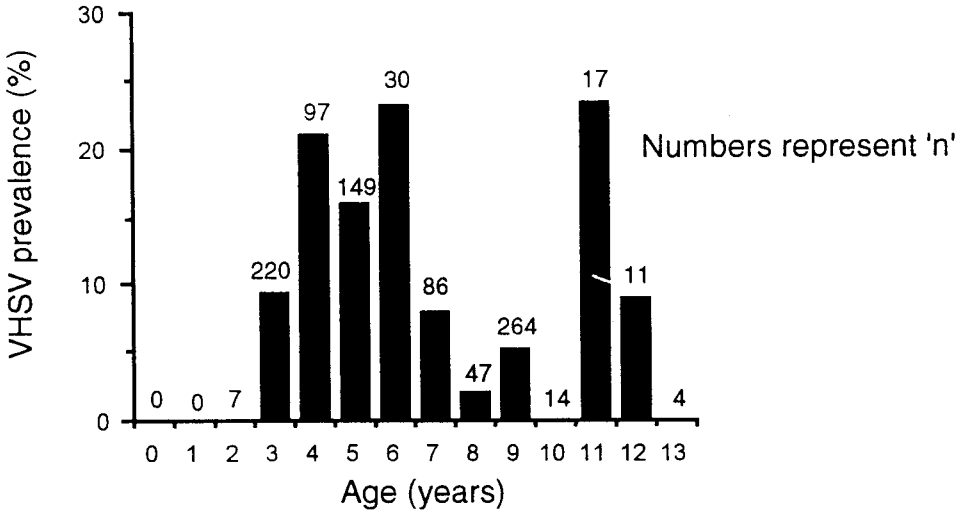

Figure 4. Year-class VHSV prevalence among Pacific herring sampled from spawn-on-kelp pounds in Prince William Sound, Alaska.

VHSV prevalence was significantly greater in females (11.8%, 51 of 431) than in males (7.8%, 48 of 614; $P = 0.019$, odds ratio for females = 1.7). Prevalence of external lesions was greater in VHSV+ fish (Table 6) for caudal fin fraying ($P = 0.016$) and focal skin reddening ($P = 0.028$; odds ratio for VHSV = 2.4).

Table 6. Prevalence of external lesions in Adult Pacific herring Sampled from pounds in Prince William Sound, April 1997.

	CFF*	CFR	FBR	FSR	DSR
VHSV+ (n = 99)					
%>0	51.5	61.6	70.7	10.1	36.4
%>1	13.1	9.1	22.2	2.0	9.1
VHSV- (n = 941)					
%>0	40.6	56.5	62.3	4.0	27.5
%>1	6.3	13.3	18.3	0.64	6.7

*CFF = caudal fin fraying; CFR = caudal fin reddening, FBR = fin base reddening, FSR = Focal skin reddening, DSR = diffuse skin reddening

Discussion

VHSV prevalence data from this study indicate that Pacific herring within the pounds undergo confined epizootics. VHSV prevalence increased after introduction of fish to the pounds, peaked, and then decreased to near background levels by day 6 to 8. Alternatively, VHSV prevalence from herring tissues in pounds #1 and #3 could be interpreted to be high initially, decreasing to low levels by the end of the study. This alternative interpretation suggests that conditions within the pounds may have contributed to reduced viral prevalence in the captured herring.

Stating that the pounds contributed to recovery of the captured herring is improbable for several reasons. First, the observation that 25% of the herring from pound #3 tested positive for VHSV after 1 day in the pound is not surprising considering the young age of the herring in this pound. Data from pound studies in Puget Sound as well as Figure 4 confirm that younger fish are more susceptible to VHSV infections than older fish. The mean age of the herring in Pound #3 was only 4 years and the median age was 3 years, whereas the mean age of the fish in the other pounds was 7 years. A 1996 pound study in Puget Sound revealed 0% VHSV-positive fish on Day 0, followed by 12.2% VHSV-positive herring on Day 1. Thus, the rapid onset of virus expression in pound #3 is not unprecedented. Second, a 40-fish sample of naturally wild-spawning herring from near where the fish in pound #3 were captured was negative for VHSV. Because these fish were caught in the same vicinity, of the same age structure, and in the same reproductive condition as those in the pound, it is likely that the wild-spawning fish were from the same school as those in the pound. Also, it is uncertain whether 12.5% of the herring in pound #1 were actually positive at the time of capture (Figure 1). Since this study, experiments in Puget Sound have revealed rapid shedding of VHSV after capture of wild herring. VHSV has been found in transport water containing wild herring after only 2 hours, while no virus could be detected in 100 fish sampled at the time of capture. This may be significant in understanding the high virus prevalence in the

initial sample from pound #1 because this sample took almost 8 hours to process. A sample of 40 herring was taken for virus analysis at 16:30 hours and not finished processing until 22:00 hours. These herring were kept alive in totes until processed, thus potentially swimming in virus-infected water for several hours. Similar rapid VHSV infection was reported by Baroni et al. (1982) who found VHSV antigen in cells infected with VHSV only 8h postinfection. Thus, a more feasible explanation of the data would be to conclude that VHSV prevalence of confined herring initially increased in the pounds and then decreased to near-background levels by day 6 to 8.

Evidence of a bimodal peak in VHSV prevalence was observed in pounds 1 and 3, with a second peak occurring on day 4 or 5. Such a pattern could result from initial shedding of VHSV by some of the herring introduced to the pound, exposing other fish to the virus. The second peak could then result from previously uninfected herring expressing the virus after being exposed to water-born virions in the pound. Semblance of bimodal viral prevalence in confined herring has also been seen in laboratory studies of Puget Sound Pacific herring held in tanks (Hershberger and Kocan unpublished data).

Data from these experiments demonstrate that VHSV is not responsible for all herring mortality in the pounds. Thus, either: 1) most of the herring died from something other than VHS, or 2) virulence of VHSV is rapidly reduced in the host post mortem. Puget Sound studies performed by Hershberger and Kocan (unpublished data) suggest the former explanation because VHSV virulence was retained for at least 4 hours in raw seawater, suggesting a resilient nature of the virion. Also, data from this study do not indicate that overcrowding in the pounds affects VHSV prevalence. Pound #2 had almost an order of magnitude more fish biomass per volume of water than pound # 3, yet the herring in pound #3 had a greater prevalence of virus. Comparison of these 2 pounds is limited due to the different age structure of fish, but studies in Puget Sound also showed that crowding had little effect in activating infection. By comparison, Krieberg et al. (1982) found that crowding was associated with increased disease prevalence in pounded Pacific herring.

There was no indication of poor water conditions within the pounds. Dissolved oxygen concentrations within the pounds remained above 12mg/L around slack tides, a time when water exchange should have been at a minimum. These levels of dissolved oxygen are more than adequate for survival.

Although no VHSV was detected in water samples from within the pounds, this can not be interpreted to mean that no virus was present. Recent studies (unpublished data) have shown approximate 10-fold decreases in VHSV titers following a freeze-thaw episode of water samples. Due to the remote nature of the study site, the water samples went through 3 partial freeze-thaw episodes prior to being analyzed for virus titer. Such temperature fluctuations might have resulted in a 1,000-fold decrease in the number of virulent virus particles in the samples and may explain why no VHSV was found in the water. To avoid problems from freezing in 1998 study of spawn-on-kelp pounds, water samples will be inoculated directly onto cell lines.

Female herring may be more susceptible to active infections with VHSV. Greater prevalence of VHSV in females from pounds was consistent with a similar trend in gender prevalence from wild

herring in the Montague area (G.D. Marty, chapter 1, this report). Also, younger herring (4-6 year olds) were more susceptible to VHSV than older herring (Figure 4), a finding also consistent with VHSV in wild herring from the Montague area. Superficially, there appears to be a high susceptibility of the 11 year olds to VHSV, but the sample size of this age class was too small (17 fish) to make a conclusive statement of viral susceptibility. Interestingly, the 11-year-old fish would have returned to spawn for the first time in 1989, the year of the *Exxon Valdez* oil spill.

Conclusions

Data from this study indicate that closed pound fisheries may: 1) activate latent or subclinical infections in previously infected herring, and 2) spread VHSV to non-immune fish in the pounds. Although we were not able to document the presence of VHSV in the water around the pounds, the potential is high for spreading VHSV to fish outside the pounds because wild herring are attracted to the spawn emitted by herring within the pounds (Stacey and Hourston 1982). This study was not designed to determine what particular component of the fishery is responsible for initiating the VHSV epizootic within captured herring. The pound fishery involves several potentially stressful events for the captured herring such as purse seining, transport to the pound, crowding, and confinement. It remains uncertain which processes are responsible for triggering VHSV epizootics within the captured herring. If, for example, initial purse seining of the fish is sufficient to cause VHSV shedding within captured herring, other fisheries such as herring sac roe may also contribute to VHSV proliferation. We plan to address these research questions in study during 1998.

Acknowledgements

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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:

Project

II

Controlled Field and Laboratory Studies on VHS & *Ichthyophonus* in Pacific herring

Restoration Project (97162)

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

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

Section II.

Controlled Field and Laboratory Studies on VHS & *Ichthyophonus* in Pacific herring

Restoration Project (97162)

Study History: Between 1993-94 there was an unexplained disappearance of approximately 110K tons of spawning herring in Prince William Sound (PWS). An emergency project (94320 S) was authorized by the Alaska Department of Fish and Game and funded in April 1994 to investigate the cause of the massive herring loss. As a result it was found that VHS, a viral disease previously unreported from Pacific herring was present in some surviving herring. In 1994 VHSV was present in < 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 initiated a second study. A detailed work plan was written which covered: 1) Field surveys in PWS; 2) Controlled experimental studies and 3) Physiological studies. The three components of the study were designed to interact and supply information to each other in order to answer questions regarding infection, pathogenicity and recovery prospects of Prince William Sound herring.

Abstract: In 1997 the controlled field and laboratory studies covered a wide range of topics including virus survival in seawater, effects of oil exposure on immunity, net pen effects on disease, antibody production and the development of age-related natural resistance and acquired immunity to VHSV.

No evidence for increased susceptibility, mortality or loss of immunity was observed in wild or laboratory-reared herring exposed to oil either prior to or following exposure to VHSV. However, a natural age-related resistance to VHS virus was observed in fish that reached two-years-old while in captivity. These fish survived an initial infection and developed an acquired immunity evident by in vitro plaque neutralizing plasma antibodies of 1/80. These same fish were also resistant to challenge infection with 10 - 100 times the known minimum lethal dose of virus and produced antibody titers as high as 1/640 following challenge infection.

Three years of consecutive monthly sampling of wild 0-year herring in Puget Sound, demonstrated that the VHSV carrier rate is probably less than 1%, but this level of infection appears to be adequate to initiate an epizootic in confined as well as free-ranging fish. Virus shed by VHSV carriers was recoverable from seawater after 2 hours, and was still detectable for as long as six hours.

Studies in PWS demonstrated that closed pounds play a role in activating latent infections in carrier fish, resulting in the spread of virus to uninfected individuals. Recoverable virus was identified as early as 24 hours after fish were introduced into pens, but was not detected after 8 days.

A comparison of in vitro culture and histopathology demonstrated that the former was significantly more sensitive in detecting *Ichthyophonus*. Studies in PWS and Puget Sound resulted in similar results.

Key Words: *Clupea pallasi*, herring, Exxon *Valdez*, *Ichthyophonus*, Prince William Sound, Viral Hemorrhagic Septicemia Virus (VHSV), cell culture.

Citation:

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Executive Summary (97162)

Introduction

In 1993 the Prince William Sound herring population declined over 80% and viral hemorrhagic septicemia virus (VHSV) was isolated from 5% of the survivors. This was the first report of this viral pathogen from wild Pacific herring, and it has subsequently been isolated from bait fish in Puget Sound and herring collected in the vicinity of a diesel fuel spill in Prince Rupert, B.C. In 1992 herring held in the roe-on-kelp fishery in Prince William Sound were observe to have hemorrhages on the skin, fin bases and mouth - the fish swam erratically and did not spawn properly. Although no virus isolations were attempted, it was noted that these lesions closely resembled the lesions observed in confirmed cases of VHS in wild herring the following year. Since VHS had not previously been reported in Pacific herring, this suggested the possibility that VHS was responsible for the heavy losses observed in 1993-'94. In 1995, a study was initiated to study the implications of disease factors on herring populations. This project was a collaborative effort among four separate groups: Alaska Department of Fish and Game, U.C. Davis, U. of Washington and Simon Fraser University. These groups approached the disease problem in herring from several aspects: 1) By conducting annual on-site surveys of Prince William and Sitka Sound herring it was possible to follow changes in annual prevalence of VHS and *Ichthyophonus* in each age class, and to compare PWS herring, with Sitka Sound herring where no previous oil contamination occurred and no losses of herring were apparent. 2) By conducting controlled experimental disease studies on laboratory-reared and wild herring it was demonstrated that VHS virus was unequivocally pathogenic for nonimmune herring, causing sever mortality in juvenile fish, that net pen confinement resulted in increased disease transmission, that VHS virus could survive for up to 6 hours in sea water, and that herring recovered from VHS were solidly immune to reinfection. 3) By measuring biochemical and physiological changes in diseased and healthy herring it was demonstrated that hematological changes occurred in oiled fish that could potentially compromise their resistance to infection and that swimming stamina was affected.

Objectives

1. Establish SPF model systems for studying VHSV and *Ichthyophonus* (continuing)
2. Determine age-related prevalence of VHSV in wild herring from 0-year to spawning (complete for Puget Sound)
3. Determine age-related prevalence of *Ichthyophonus* in wild herring from 0-year to spawning (complete for Puget Sound - continuing for Prince William Sound)
4. Determine the effects of capture and confinement (pound fishery simulation) on the course of VHSV infection in wild herring (on schedule and continuing in PWS)
5. Determine the immune status of lab-reared herring before and after VHS exposure (complete)
6. Determine the immune status of wild herring before and after an epizootic of VHS (complete)

Methods

Field studies consisted of sampling wild 0-year and 1+ herring from Puget Sound for both VHS and *Ichthyophonus*. Tissues from fish held in SOK pounds were examined for the presence of VHS virus during confinement and at the time of release from the pounds, and water from the pounds was sampled for the presence of virus.

Experimental studies on immune response relied on both specific-pathogen-free (SPF), laboratory-reared herring as well as captive and wild free-ranging herring. Laboratory-reared fish were exposed to virus every 6 months until they reached 2-years-old to establish a pattern of natural immunity development and were exposed to oil to determine if immunosuppression occurred. Wild herring captured from Puget Sound were assayed for the presence of VHSV at the time of capture and at regular intervals for the first 30 days post capture. Immunity was determined by exposing surviving herring to 10 - 100 times the known lethal dose of VHS virus for 1 hr and by examining the virus

neutralizing capability of plasma from pre and post-infected fish. Serologic data was compared with that obtained form SPF fish which had no immunity to the virus.

Ichthyophonus was studied in wild herring by culturing tissues from different age classes and from the same age-class over a period of a year. Changes in prevalence were noted in terms of increasing age and difference in geographic location of the fish. A comparison of visual examination, histologic examination and in vitro culture was made to determine the most sensitive and economic technique for monitoring *Ichthyophonus* in the field.

Results

Field sampling: The spawn-on-kelp study demonstrated that with one exception, newly captured fish did not have detectable levels of VHS virus, but by 2-4 days post-capture 15-30% had significant levels of virus in their tissues, and by 6-8 days the prevalence dropped to 0 - 7.5%. Of 40 wild spawning fish captured just outside the pounds, none were positive for VHSV.

Controlled experimental studies: No evidence could be found to support the hypothesis that exposure to oil resulted in increased susceptibility to VHS in wild or laboratory-reared herring. It was demonstrated however, that herring have little natural immunity to VHS until they are 2-years-old, at which time they develop a clear natural resistance to the virus. Conversely, fish of all ages that survived an initial exposure to VHS virus developed an acquired protective immunity that could be demonstrated by plaque neutralization. Titers in fish challenged with VHSV following initial recovery from infection ranged from 1/80 dilutions to as high as 1/640 dilutions. Control fish dying with VHS did not develop a detectable neutralizing antibody. Herring plasma contained a natural toxicity to EPC cells at dilutions below 1/40, thus preventing the use of lower plasma dilutions in the study. This problem is being addressed for the FY98 study.

Between 1995 and 1997 no evidence of VHSV could be found in wild free-ranging Puget Sound herring of any age at the time of year. However, once in captivity, an epizootic occurred which was highly lethal to 0-year fish and produced up to 15% mortality in older fish.

In only one case, a single infected 0-year wild herring was detected in a school captured in late September of 1997. The remainder of the school did not experience an epizootic once in captivity, but rather was solidly immune to experimental reinfection by VHS virus. This indicated that the school had already been exposed to the virus, but gave no indication of how many fish were lost as a result of this exposure.

Studies on the survival of VHS virus in sea water demonstrated that virus could survive for up to 2 hours in raw, filtered or oiled sea water. In one study a reduction of 20% of PFUs was observed after 1 hour and 40% after 2 hours in sea water at 8°C. A second study resulted in about a 60% reduction in PFUs during the first hour and a loss of 90% by 6 hours.

Pound studies: VHSV prevalence in each of 3 pounds followed a similar pattern of initial increase in the percent of virus-positive fish, peaking at 1-4 days, followed by a subsequent decrease to near-0% levels by day 6-8. A bimodal VHSV prevalence with time is observed in pounds 1 and 3, with a second peak occurring on day 4 or 5. Such a pattern may result from initial shedding of VHSV by herring carrying active infections, with the second peak resulting from uptake and release of virus by herring that were previously uninfected.

A day 0 sample from pound #3 was unobtainable due to the low herring loading density. A 40-fish sample of naturally spawning herring was taken from the same vicinity where the fish in pound #3 were caught and none tested VHSV+. The fact that these fish were caught in the same vicinity, were of the same age structure, and in the same reproductive condition as those in the pound indicates that the wild-spawning fish were from the same school as those in the pound and hence the fish in the pound were not expressing virus when they were caught.

The high percentage of VHSV+ fish in pound #3 (25%) after 1 day is not surprising considering the young age of the herring in this pound. Data from pound studies in Puget Sound confirm that younger fish are more susceptible to active VHSV infections than older fish and often show signs of disease within 24-48 hours of capture. The mean age of the herring in Pound #3 was only 4 years and the median age was 3 years, while the mean age of the fish in the other pounds was 7 years. A 1996 pound study in Puget Sound revealed 0% VHSV+ fish on Day 0 in the pound, followed by 12.2% VHSV+ herring in the pound 24 hours later. Hence, the rapid onset of virus expression in this pound is not unusual.

Only 15% of the herring mortalities were VHSV positive, indicating that most of the herring died from something other than VHS. Data from Puget Sound studies showed that VHSV survived for extended periods in tissues of fish that died naturally, indicating that the virus in dead fish from the pound studies was not lost because of inactivation in the tissues post mortum.

Data from this study does not support the hypothesis that overcrowding increases VHSV prevalence. One pound had an order of magnitude more fish per volume of water than another, yet the herring in the later pound exhibited a greater prevalence of virus.

Data from this study indicate that closed pound SOK fisheries may: 1) activate latent infections in previously infected herring, and 2) VHSV is transmitted to non-immune fish in the pounds. The potential for spreading the virus to wild fish outside the pounds is potentially high due to field observations that wild herring are attracted to the spawn emitted by contained herring within the pounds (Stacey and Hourston 1982)

Conclusions and recommendations:

Experimental studies show clearly that herring develop both a natural age-related resistance and an acquired immunity to VHS. How long this resistance lasts or how it might be compromised is unknown.

Juvenile herring in the 0-year age class appear to be very susceptible to high mortality when exposed to VHS virus. This results in heavy losses of these young fish under captive conditions, and may represent a situation which goes unnoticed in wild fish because of the difficulty in tracking these populations. If heavy losses do occur and go unnoticed in 0-year herring, this may explain the dramatic differences observed in egg biomass and predicted spawner biomass of an age class.

The observation that VHS virus can survive for up to 6 hours in natural sea water supports the hypothesis that water-born transmission may be responsible for the high prevalence rates of VHS infection observed in the spawn-on-kelp fishery. A few infected individuals shed virus into the water under crowed conditions and susceptible fish become infected.

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

Study 97-1 Age resistance to VHS virus in laboratory-reared herring

Objectives:
To determine whether a natural age-related resistance to VHSV infection develops in

herring without prior exposure to the virus.

Methods:
At varying ages from 6 months old to 24 months-old, specific-pathogen-free (SPF) herring were exposed to waterborne virus at nominal concentrations of 2 X 10^2 PFU*ml^{-1} and 2 X 10^3 PFU*ml^{-1} for 1 hour; actual concentrations were slightly less. Survival, antibody production (see study 97-11) and tissue virus were evaluated by plaque assay to determine whether changes in resistance to VHS occurred as the fish aged.

Results:
No obvious differences in survival or susceptibility were noted in SPF herring up to 18 months-old. However, at 24 months a significant increase in natural resistance was evident at two doses of virus (Table I) [see Kocan et al, 1997 for earlier age resistance studies]. Fish survived at both doses and 20% of the mortalities in the lower dose had no virus evident in their tissues. Antibodies were present at 1/80 dilutions after 6 months and proved protective to fish when they were re-exposed at 2 X 10^3 PFU*ml^{-1} for 1 hour.

Table I. Demonstration of age resistance to VHS in 2-year-old SPF herring 21 days post-exposure to water-born virus.

Treatment (1) (PFU*ml^{-1})	mm (2) (SD)	gm (SD)	% morts	% VHS + morts
Controls	131.4 (13.38)	23.9 (5.06)	0	0
0.7 X 10^2	130.4 (7.25)	21.9 (3.49)	33	80
1.3 X 10^3	133.0 (9.36)	23.9 (8.50)	50	100

(1) N = 30 per treatment group
(2) Data taken only on morts; survivors used for challenge exposures in Study 97-11

Conclusions: At approximately the time Puget Sound herring become sexually mature, an obvious age-resistance to VHS occurs that reduces mortality and results in the production of protective serum antibodies that protect the fish from subsequent infection by VHSV. This is more probable than not the case with PWS herring as well. However, due to the difference in maturation age of herring from Puget Sound and PWS, the exact time that this natural resistance occurs in PWS herring remains to be determined. Once this is determined, it should be possible to monitor herring populations for the presence of neutralizing antibody to VHSV and evaluate their vulnerability to epizootics should they become exposed to the virus.

Study 97- 3: Immunosuppression of herring exposed to Prudhoe Bay crude oil

Wild juvenile herring (0-1 year-old)

Objective:
To determine if exposure to crude oil results in increased susceptibility to initial infection or relapse of VHS in herring.

Methods:
0-year herring were captured by dip net and 1-year-olds were captured by purse seine. Both groups were transferred in oxygenated seawater to the Marrowstone Island lab where they were housed in flowing sea water tanks. Within 3-7 days of capture, the fish experienced an epizootic of VHS which subsided by 3 weeks post capture. After 30 days, one tank was fitted with an oil generator similar to that described by Carls (1996) and exposed to 10-20 ppb oil for 21 days, during which time mortalities were collected and assayed for VHSV. Thirty days following the epizootic, the surviving fish were challenged with 5×10^3 PFU$*$ml^{-1} for 1 hr and observed for mortality and assayed for virus. Controls consisted of unexposed survivors of VHS epizootic and challenge infection.

SPF herring were exposed to a similar oil generator for 14 days then exposed to $2X10^2$ PFU$*$ml^{-1} for 1 hour. Fish were monitored for signs of disease and mortalities were assayed for VHSV by plaque assay. Surviving fish were held for 6 months then challenged with $5X10^3$ PFU$*$ml^{-1} for 1 hour as a challenge exposure to determine their state of immunity (see Study 97-11).

Results:
No difference in morbidity or mortality between groups (eg. no oil effect) and minimal evidence of VHSV in morts was observed in all ages of fish exposed to oil both before and after virus exposure. Wild herring did not relapse at a greater rate when exposed to oil than when held in clean sea water. No significant difference in survival was noted in SPF fish exposed to oil prior to VHS exposure.

Conclusions:
We were unable to verify that herring exposed to oil are immunocompromised as measured by exposure to VHS virus prior to or following recovery from infection. If oil exposure does play a role in increasing disease susceptibility in herring, then some factor or factors in addition to oil must be involved, and these are presently unknown.

Study 97-6: Net pen spawners: SOK and laboratory simulation

Objectives
To determine whether activities associated with the closed pound spawn-on-kelp (SOK) fishery are responsible for initiating active viral hemorrhagic septicemia virus (VHSV) infections within the captured and confined herring.

Methods
The 1997 closed pound SOK fishery in Prince William Sound (PWS) consisted of 8 permit holders who consolidated into 3 pound structures. Herring were sampled from each pound with a dip net on consecutive days after the pounds were loaded and fish were analyzed for age (from scales), weight, length, and VHSV tissue titer in spleen and kidney pools (plaque assay (Meyers et al 1994)). Water samples were also taken from each pound every other day, frozen, and later assayed for VHSV. Measurements of dissolved oxygen and water temperature were taken from inside and outside the pounds at slack tide ($\pm$ 1h). In addition to the daily 40 fish samples, two 40-fish samples of naturally beach-spawning herring from outside the pounds were taken. A 40 fish sample of dead herring floating inside one of the pounds was also assayed for VHSV.

Results

The 3 SOK pounds were located in Fidalgo Bay, on the northern shore of PWS, and designated pound #1, #2, and #3 (Table II). The operators of pound # 1 were the first to catch fish and load their pound. All fish in this pound were caught at the head of Irish Cove in 1 set and transported only a few hundred yards to the pound. The fish in pound # 2 were loaded in 2 different events and all herring in this pound were caught in Two Moon Bay before being transported 1.2 nautical miles to the pound. Herring were first introduced to pound #2 at 04:00 hrs on 4/13 but too few fish were added to sample with a dip net and no day 0 sample was taken from this pound. The next group of herring (approximately 18 tons) was added to this pound just before midnight on 4/13 and a day 1 sample was taken on 4/14 at 06:00 hrs. All the fish in Pound #3 (approximately 1 ton) were taken in 1 set at the head of Landlocked Bay on 4/11 and transported only a few hundred yards to the pound. Too few fish were in this pound to sample with a dip net on day 0 without disturbing the kelp. The pound operators were subsequently unable to catch more herring so the pound was abandoned and the kelp removed. Sampling of the fish in the pound commenced since the sides of the pound could be lifted and the herring captured with either a dip net or cast net.

Table II. Pound Sampling Dates

	Date (1997)								
	4/11	4/12	4/13	4/14	4/15	4/16	4/17	4/18	4/19
Pound 1	day 0	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
Pound 2			NS	day 1	day 2	day 3	day 4	day 5	day 6
Pound 3	NS	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8

NS= no sample

All 3 pounds were different dimensions and loaded at various crowding densities (Table III). Pound #2 was by far the most crowded since it contained the most fish (20 t) and lacked corner weights to hold the sides of the pound down, thus permitting the sides to float up and crowd the herring to the surface. Predators including kittiwakes, eagles, and sea lions captured herring from this pound throughout its operation. Pound #3 was the deepest pound, containing the fewest (1 t) and youngest herring (mean age 4 years). Measured DO never dropped below 12mg/L inside the pounds and, along with water temperature, was not significantly different from water outside the pounds.

Table III. Physical and Biological Characteristics of Pounds

	# permit holders*	mean herring age	estimated herring biomass (tons)	pound dimensions (LxWxD)	herring density (lbs/ft^3)
Pound #1	1	7	4-5	18x38x15'	1.0
Pound #2	3	7	20+	58x28x20'	1.2
Pound #3	4	4	1	20x24x30'	0.1

VHSV prevalence in herring from pound #1 showed a bimodal peak with the percent positive fish peaking on days 1 and 4 (Figure 1). Initially 12.5% of the sampled herring tested VHSV+ and by day 8 virus was undetectable in any sampled fish. VHSV prevalence in herring from pound #2 increased from 0% on day 1 to a high of 15% by day 4 and decreased thereafter (Figure 2). A bimodal viral prevalence was also seen in the herring of pound #3 where VHSV prevalence peaked on days 2 and 5 at 27.5% and 12.5% respectively, then returned to low levels (Figure 3). No VHSV was detected in

tissue samples of naturally beach-spawning herring and only 15% of the dead fish sampled from pound #2 tested VHSV+.

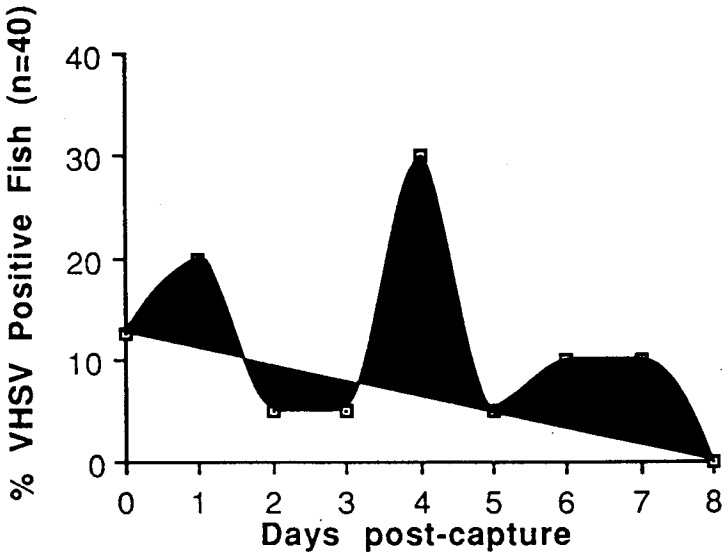

Figure 1. VHSV prevalence in herring of Pound #1

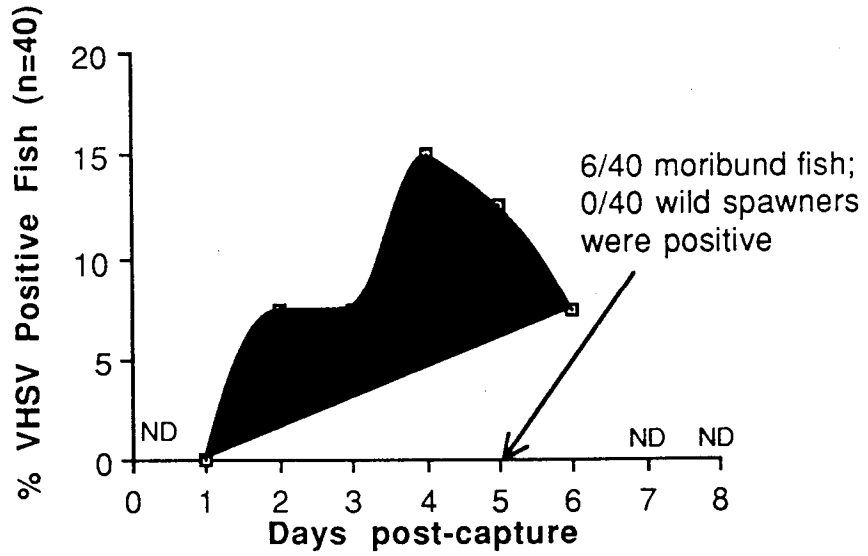

Figure 2. VHSV prevalence in herring of pound #2

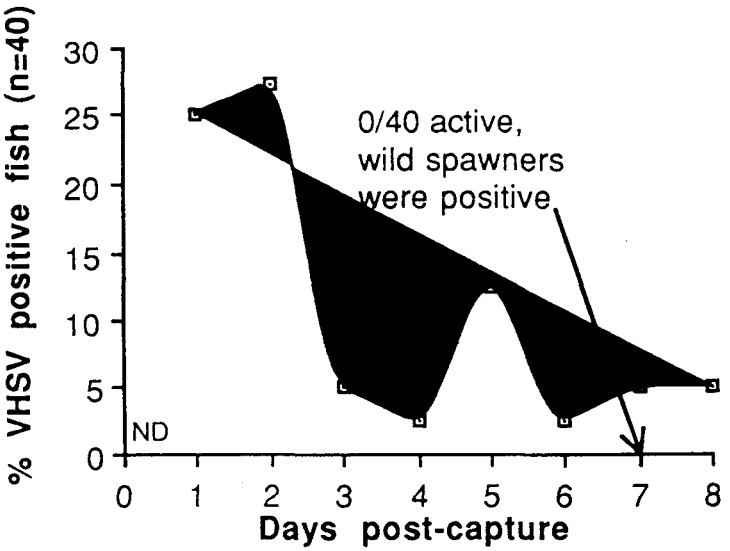

Figure 3. VHSV prevalence in herring of Pound #3

The year classes with the largest percentage of virus-positive fish were the 4-6 year olds and possibly the 11 year olds (Figure 4). Data also indicated that females were more susceptible to VHSV infection than males. Of the 99 herring that tested VHSV+ in this study, 48% were males and 52% were females, while only 39% of the 904 total fish sampled were females and 60.6% were males.

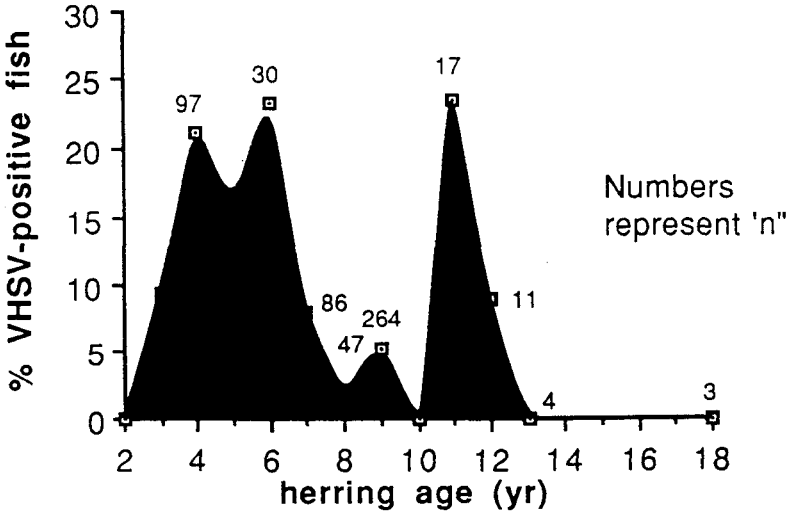

Figure 4. % VHSV+ herring in the pounds by age

Discussion

VHSV prevalence in each of the 3 pounds followed a similar pattern of initial increase in the percent of virus-positive fish, peaking around day 1-4, followed by a subsequent decrease to near-0% levels by day 6-8. Semblance of a bimodal VHSV prevalence with time is observed in pounds 1 and 3, with a second peak occurring on day 4 or 5. Such a pattern may conceivably result from initial shedding of VHSV by herring carrying active infections, with the second peak resulting from uptake and expression of the water-born virions by herring that previously failed to test positive.

A day 0 sample from pound #3 was unobtainable due to the low herring loading density. A 40-fish sample of naturally spawning herring was taken from the same vicinity where the fish in pound #3 were caught and none tested VHSV+. The fact that these fish were caught in the same vicinity, were of the same age structure, and in the same reproductive condition as those in the pound indicates that the wild-spawning fish were from the same school as those in the pound and hence the fish in the pound were not expressing virus when they were caught.

The high percentage of VHSV+ fish in pound #3 (25%) after 1 day is not surprising considering the young age of the herring in this pound. Data from pound studies in Puget Sound as well as Figure 4 confirm that younger fish are more susceptible to active VHSV infections than older fish. The mean age of the herring in Pound #3 was only 4 years and the median age was 3 years, while the mean age of the fish in the other pounds was 7 years. A 1996 pound study in Puget Sound revealed 0% VHSV+ fish on Day 0 in the pound, followed by 12.2% VHSV+ herring in the pound 24 hours later. Hence, the rapid onset of virus expression in this pound is not unprecedented.

It is uncertain whether 12.5% of the herring in pound #1 were actually positive at the time of capture (Figure 1). Since this study, experiments in Puget Sound have revealed rapid shedding of VHSV after capture of wild herring. VHSV has been found in transport water containing wild herring after only 2 hours, while no virus could be detected in 100 fish sampled at the time of capture. This may be significant in understanding the high virus prevalence in the initial sample from pound #1 since this sample took almost 8 hours to process. Similar rapid VHSV infection was reported by Baroni et al (1982) who found VHSV antigen in cells infected with VHSV only 8h post infection.

Only 15% of the morts tested VHSV+, indicating that either :1) most of the herring died from something other than VHS, or 2) virulency of VHSV is rapidly reduced in the host post mortum. Data from Puget Sound studies suggest the former explanation since VHSV virulency was retained for at least 4 hours in raw seawater (unpublished data), suggesting a resilient nature of the virion.

Data from this study do not indicate that overcrowding increases VHSV prevalence. Pound #2 had an order of magnitude more fish per volume of water than pound # 3, yet the herring in the later pound exhibited a greater prevalence of virus. Although caution should be taken in comparing these 2 pounds due to the different age structure of fish, other studies in Puget Sound (unpublished data) also showed that crowding had little effect in activating infection as previously suggested (Krieberg et al 1982).

Dissolved oxygen concentrations within the pounds remained above 12mg/L around slack tides, a time when water exchange into the pounds should have been at a minimum. Such levels of DO are more than adequate for adult herring survival. Although no VHSV was detected in water samples from within the pounds, one should not conclude that no virus was shed. Subsequent studies have shown approximate 10-fold decreases in VHSV titers following a freeze-thaw episode of water samples. Due to the remote nature of the study site, the water samples went through 3 partial freeze-thaw episodes prior to being analyzed for virus titer. Such temperature fluctuations should have resulted in a 1,000-fold decrease in the number of virulent virus particles in the samples and may explain why no VHSV was found in the water. Additionally, the bimodal nature of VHSV prevalence in pounds 1 and 3 indicate possible horizontal transmission within these pounds.

Data from this study indicate that closed pound SOK fisheries may: 1) activate latent infections in previously infected herring, and 2) Spread VHSV to non-immune fish in the pounds. The potential for spreading the virus to wild fish outside the pounds is high due to field observations that wild herring are attracted to the spawn emitted by contained herring within the pounds (Stacey and Hourston 1982).

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Study 97-7: *Ichthyophonus* survey in Prince William Sound and Puget Sound herring: A comparison of methodology.

Objectives:
To determine the most sensitive and economical method for surveying wild herring populations for the presence of *Ichthyophonus*.

Methods:
Adult spawning herring were captured by purse seine from Rocky Bay (Montague Island), PWS, and returned to the laboratory for age, weight and length determination (AWL). A subsample of 60 fish was then necropsied and evaluated for the presence of *Ichthyophonus* by external and internal visual examination. Tissues from these fish were cut into equal parts with half evaluated histologically and the other half cultured in L-15 medium for in vitro evaluation.

A similar group of 2+ herring were captured in Puget Sound and examined for *Ichthyophonus* . Juvenile herring (0-year & 1+) were also captured in Puget Sound and similarly examined for the presence of *Ichthyophonus*. The tissues from 2+ Puget Sound fish were processed for histology by cutting at 3 levels with 5 sections per level. All 15 sections were examined for the presence of *Ichthyophonus* and the results compared with those from the in vitro culture of the same tissues.

Results:
Ichthyophonus was first detected in Puget Sound herring at approximately 4 months-post hatch at a prevalence rate of 5-6%. This increased to 24% by the time they were 1-year-old and as high as 50-70% by 2-3 years-old (Tables IV & V).

The overall prevalence of *Ichthyophonus* from Rocky Bay (PWS) spawners was 28% (17/60) with a male: female ratio of 26:34 of which 27% and 29% respectively were infected with *Ichthyophonus*. The largest age class represented in the PWS sample was the 9-year-olds, with 4-year-olds representing the second strongest year-class (Fig.1). On a population basis, the 9-year-old year class contributed the most infected fish to the population (16%) while the remaining 7 year-classes each contributed less than 5% to the overall prevalence. When viewed by age-class, the 9 and 11 year-olds had the highest prevalence (> 90%), 50% of the 6-year-olds were infected, while the 3,4, 5, and 8 year-olds each had less than 20% prevalence (Fig. 1).

When in vitro culture and histopathology were evaluated for sensitivity in detecting *Ichthyophonus*, the results were 28% positive by in vitro culture and 17% by histology, a 40% difference in detection rate. Because of this difference, a second experiment was conducted to verify the results, using Puget Sound herring.

The sex ratio in adult Puget Sound herring was 49:51 (males:females) with 33% and 31% infected respectively. The hearts and livers of 30 2-year-old wild herring were split into equal parts and evaluated by histology and in vitro culture. The results showed 21/30 (70%) positive by culture and 2/30 (7%) positive by histology, an even greater discrepancy than that seen in PWS.

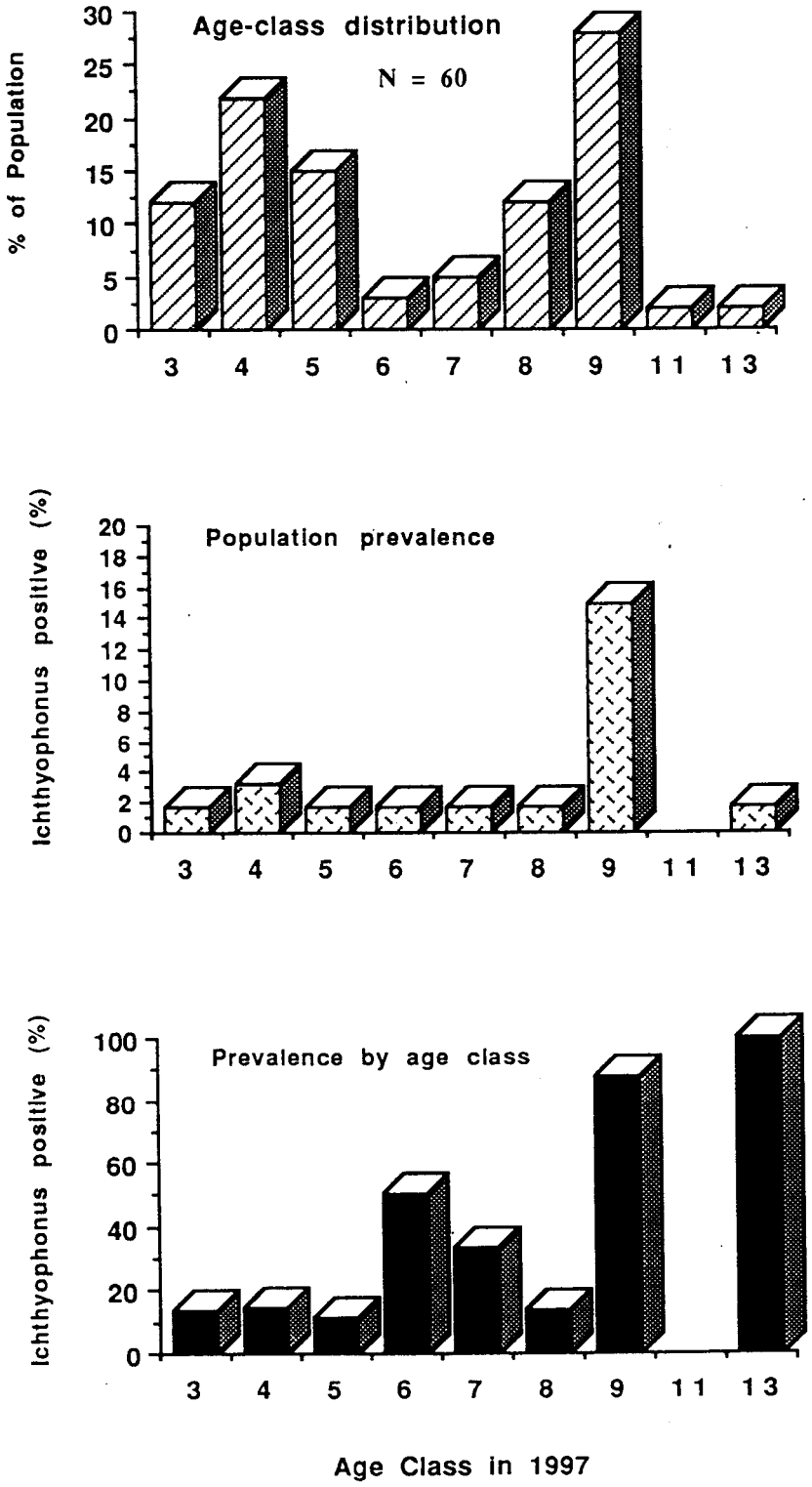

Figure 5. Age class distribution, population prevalence and age-class prevalence of *Ichthyophonus* in adult herring captured at Rocky Bay (Montague Island), Prince William Sound in April 1997.

Conclusions:
A significant number of adult herring in both Prince William Sound and Puget Sound carry *Ichthyophonus*, primarily as subclinical infections. In vitro culture was found to be the most sensitive technique for detecting these infections at both locations. The exceptional difference in detection rate observed in Puget Sound fish is likely the result of less sever infections in Puget Sound fish, as indicated by the relatively low number of grossly visible lesions relative to PWS fish (Table IV). The hypothesis here is that mild or subclinical infections are more likely to be detected by in vitro culture of tissues. Although histologic examination is not as sensitive in detecting total prevalence of *Ichthyophonus*, it is the only way to evaluate the extent and severity of pathogenesis resulting from infection.

Table IV. *Ichthyophonus* Surveys in wild juvenile Puget Sound herring

1996 - 1997

	length (mm)	weight (gm)	gross skin lesions (%)	gross visceral lesions (%)	in vitro culture (%)
0-year (N=100) Sept. '96	79 (± 4.7)	3.6 (± 0.7)	6	0	6
0-year (N= 60) June - July '97	63 (± 6.6)	2.0 (± 0.6)	2	0	5
1+ juveniles (N=100) Oct. '96	152 (± 8.3)	36.0 (± 6.5)	5	0	24

Table V. Prevalence of *Ichthyophonus* in adult Puget Sound and Prince William Sound herring.

	gross lesions (%)	% positive cultures	% positive histologically	+ males (+ / total)	+ females (+ / total)
Prince Wm Sound[1] (Montague Island)	18	28	17	7/34	10/26
Puget Sound-1 [2] (Port Townsend)	8	52	ND[4]	22/49	19/51
Puget Sound-2 [3]	7	70	7	ND	ND

1) N = 60
2) N = 100
3) N = 30
4) ND - not done

Study 97-11: Antibody production in VHS-challenged SPF herring

Objective:
To determine if serum antibodies to VHSV can be induced and detected in herring exposed to VHSV.

Methods:
Forty lab-reared SPF herring were exposed to $2X10^2$ PFU$*$ml^{-1} of VHSV for 1 hour on 23 Dec '96 and observed for signs of VHS for 6 months. After six months, 8 uninfected controls and 2 survivors of the initial exposure were bled to obtain background plasma samples for virus neutralization titers, then the fish were challenged with $5X10^3$ PFU$*$ml^{-1} for 1 hour. Controls for the challenge exposure consisted of similar age fish which had not been previously exposed to VHSV.

Mortalities were collected daily and assayed for VHSV. At the termination of the study 5 controls and 6 experimental herring were bled and their plasma assayed for the presence of antibodies to VHS virus.

Antibodies were assayed by plaque neutralization without the addition of exogenous complement. The plasma was serially diluted, mixed with a constant amount of VHS virus for 1 hour, then the virus was placed on EPC cells and incubated. The minimum plasma dilution used was 1/80 because many of the herring plasmas were toxic to EPC cells at 1/40 and lower. Virus plaques was then quantified for each plasma dilution and the antibody dilution titer expressed as LC_{50} for virus neutralization.

Results:
All of the fish surviving the initial exposure of $2X10^2$ PFU$*$ml^{-1} had antibody titers to VHSV of 1/80 dilutions after 6 months. Following challenge infection, no mortality was observed in the previously exposed fish and only one of 6 fish had detectable virus in its tissues after 7 days ($1.2 X10^6$ PFU$*$gm^{-1}). This fish also had the highest antibody titer - 1/640. In contrast, 4 of 10 control fish died with virus titers of > $5X10^6$ PFU$*$gm^{-1} of tissue and none had demonstrable virus antibody. Of the 6 surviving control fish, 4 had virus titers of $6.4 X 10^3$ PFU$*$gm^{-1} to $3 X 10^5$ PFU$*$gm^{-1} of tissue and none had detectable antibody to the virus (Table VI).

Conclusions: Antibody to VHSV increases to detectable levels in herring that recover from an initial infection, and increase further (anamnestic response) soon after challenge infection with high titers of virus. No antibody could be detected in the plasma of newly infected fish 7 days post exposure even though virus was present in the tissues at very high levels. This suggests that the absence of antibody in wild herring can not be interpreted to mean that they have not been exposed to VHS virus, but that the presence of antibody indicates that the individual has been exposed, recovered and is immune to VHS. Because of the presence of cytotoxic factors in herring plasma, it is possible that protective antibodies are present at dilutions lower than 1/80, but go undetected because these concentrations of herring plasma are toxic to the indicator cells. Current studies are underway to remove the toxic factor(s) or to find alternate cell lines that are not sensitive to the toxic factor(s). If successful, this may increase the sensitivity of the plaque-neutralizing antibody assay and make it more amenable to field use.

Table VI. Neutralizing serum antibodies produced in SPF herring after initial and challenge infection with VHS virus.

Pre-challenge	mm (SD)	gms (SD)	# VHS positive	VHS titer (PFU*gm^{-1})	antibody titer (dilutions)
Controls (1)					
N = 8	149.3 (5.25)	37.5 (3.32)	0	ND	ND (2)
Previously exposed (3)					
N = 2	132 (12.0)	23.9 (4.5)	0	ND	2 @ 1/80
Post challenge					
Controls N = 8	151.7 (10.61)	39.7 (10.36)	6	6 X 10^3 - 6 X 10^7	ND
Previously exposed (3) N = 6	147.5 (18.11)	30.1 (13.49)	1	1 X 10^6	2 @ 1/80 3 @ 1/160 1 @ 1/640

1) SPF herring not previously exposed to VHS virus
2) ND - antibody detection limit = 1/80 dilutions; plasma toxic to cells at 1/40 dils
3) SPF herring recovered from previous exposure to VHS virus

Study 97-12: Background prevalence of VHSV in wild herring

Objective:
- o To determine at what age wild herring become infected
- o To determine what percent of each age-class carries the virus
- o To determine what percent of each age-class is immune or recovered from a prior infection

Methods: From 1995 through 1997, wild juvenile Puget Sound herring were captured by purse seine or netted from bait-balls when possible from June through October. They were immediately assayed for VHSV, then held in 200 g flowing seawater tanks and observed for 3 - 6 months. Periodic subsamples of live fish, as well as all recently dead fish were assayed for VHS virus by plaque assay. To determine if fish were immune to VHS, they were challenged with 10 times the known minimum lethal dose of VHSV between 30 and 60 days post-capture and observed for signs of disease and assayed for tissue virus.

Results: Of 9 bait balls sampled between 1995 and 1997, all but one were highly susceptible to VHS and suffered heavy mortality when placed in captivity (Table VII). Within 3-5 days post-capture fish began dying and showing signs of skin, jaw and fin-base hemorrhaging. Mortality frequently exceeded 50% and virtually all moralities were positive for VHS, as were the majority of live-sampled fish. Mortality subsided by 3 weeks post-capture, although many fish continued to exhibit hemorrhaging. Little or no virus could be found in the tissues of these fish by 21-30 days post-capture. The total number of 0-year fish sampled over the three year study was in excess of 1,000 individuals.

Table VII. VHSV Prevalence in Juvenile Wild Puget Sound Herring

Date	N	age	initial sample	positive live fish day1-21	positive morts day 1-21	positive survivors day >21	total mortality (%)
8/95	300	0-yr	0/30 (day-0) [1]	NS	100%	0	> 60%
9/96	375	0-yr	0/30 (day-0)	NS	11%	0	~ 23%
10/97	500	0-yr	0/30 (day-0)	40%	6 %	0	~ 80%
8/96	300	1 +	17/84 (day-5)	7%	36%	0	~ 10%
9/96	150	1 +	1/30 (day-2)	22%	74%	0	~ 10%

1) days fish held in captivity prior to sampling
NS - no sample

These survivors became solidly immune to challenge infection, with no virus-related mortality and no virus in their tissues after being challenged with > 5 X 10^5 PFU*ml^{-1}. One-year-old and older herring exhibited the same disease course, but with significantly less mortality. About 15% tested positive for the virus within one week following capture, then returned to "0" prevalence with < 1% mortality. After one month, these fish were also solidly immune to challenge infection with VHSV.

In June and July of 1997, 0-year herring responded exactly as described above. However, the first isolate of VHSV from a wild free-ranging Puget Sound herring came in September of 1997, when a single fish (1/100) tested positive for VHSV with a titer of $3X10^5$ PFU*gm^{-1} tissue. Significantly, there was no subsequent epizootic in the remaining fish from this school, no dead fish tested positive for VHSV, and when the group was challenged with VHS virus, they were all solidly immune to reinfection.

Conclusions: VHSV can be isolated from wild herring very soon after metamorphosis when they are extremely susceptible to the pathologic effects of the virus. Based on virus assays performed on groups of 100 newly captured wild fish, it appears that under normal circumstances < 3% of the 0-year fish are carrying active infections. Because 0-year herring undergo a massive epizootic when confined, and subsequently become solidly immune as survivors, it is highly probable that a very small percentage of wild fish carry active infections, and that under conditions of confinement or close proximity, they transmit the disease to the remaining nonimmune fish, thus initiating epizootics.

The single infected wild 0-year individual in a school of immune herring is strong evidence that supports the hypothesis that epizootics occur in 0-year fish and that the survivors represent fish that did not succumb to the infection. These surviving fish were immune to reinfection and exhibited no signs of disease. Three possible scenarios exist for development of disease resistance in wild herring: 1) The virus is transmitted at a low rate to relatively few fish and along with age resistance the population becomes immune over a long period of time; 2) Epizootics occur in wild 0-year herring with varying degrees of mortality and the survivors become immune to reinfection: 3) Little or no virus transmission occurs during early life stages and a large portion of the adult population has no acquired immunity and must rely on age-related immunity to ward off infection by VHSV. The ultimate effect of disease on herring populations of any age depends on which of these situations prevailed in prior years.

This study offers evidence that natural unnoticed epizootics can cause mortality in 0-year fish and this may have a profound influence on the subsequent recruitment of that particular year-class into the spawning population.

Study 97-18: Survival of VHSV in seawater

Objectives: To determine how long VHS virus survives in seawater and remains infective

Methods: VHS virus was placed into replicates of 10°C raw sea water, filtered sea water, and sea water + oil from an oil generator, at an initial concentration of $1X10^5$ PFU*ml^{-1}. Water samples were taken at time-0, every 10 minutes for one hour, then every 30 minutes for the next hour. Water samples were titrated for virus on EPC cells immediately after collection. The assayed concentration of oil in the water from the generator was approximately 8 ppb (range 6-10 ng/ml).

Results: In the first study, virus titers decreased from $1X10^5$ PFU*ml^{-1} to $6X10^5$ PFU*ml^{-1} after 2 hours in seawater, regardless of whether it was raw, filtered or containing oil (Table VIII). A second study, initiated with only 400 PFU*ml^{-1} demonstrated a virus decrease of about 62% after 1 hour, then continued a slow decrease to 5% by 6 hours and was undetectable after 24 hours (Table IX).

Table VIII. Survival of VHS virus in raw, filtered and oil-exposed sea water for 2 hours

	Virus titer over time in sea water (X 10^5 PFU*ml^{-1})								
Seawater (1) minutes ->	0	10	20	30	40	50	60	90	120
raw	1.01	0.91	0.71	0.73	0.88	0.75	0.84	0.73	0.58
filtered	1.00	0.78	0.67	0.64	0.80	0.67	0.58	0.61	0.58
oiled	1.08	0.90	0.85	0.78	0.84	0.75	0.79	0.67	0.71

1) background virus titer = 0

Table IX. Survival of VHS virus in raw seawater for 24 hours

	Virus titer over time in raw sea water (PFU*ml^{-1})							
hours ->	0	1	2	3	4	5	6	24
	400	153	113	60	67	33	50	0

Conclusions: The survival time of VHS virus in seawater is adequate to sustain transmission of the virus from fish to fish under crowded conditions. Previous EVOS studies (96162) demonstrated that a single infected juvenile herring could shed over 1X10^6 PFU*hr^{-1} and that only 1 - 2 X10^2 PFU*ml^{-1} are required to initiate a water-born infection. Conditions where herring are held in nets, such as prior to bait processing or during the SOK fishery, are adequate for transmission of VHS virus to uninfected fish if infected fish are present and shedding virus. The distance that infectious virus can travel in seawater is not known, but probably depends on currents and the proximity of uninfected fish.

Exxon Valdez Oil Spill
Restoration Project Annual Report

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

Section III: The Effects of Oil and Disease on Various Aspects of Herring Fitness

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

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April 1998

Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound
Section III: The effects of oil and disease on various aspects of herring fitness

Restoration Project 97162
Annual Report

Study History: This project was initiated under Restoration Project 95320-S in response to a request for proposals to investigate disease factors affecting Pacific herring decline in Prince William Sound and continues research from projects 95320, 96162 and 97162. The proposal is a joint effort of Simon Fraser University, the University of Washington, University of California, Davis and the Alaska Department of Fish & Game.

Abstract: Cause and effect relationships between hydrocarbon and disease exposure on various aspects of herring fitness, continue to be examined in order to determine their role in population declines in Prince William Sound. An oil-water dispersion (OWD) of North Slope crude oil was not acutely lethal to adult herring although it initiated a measurable, classical and transient biochemical 'stress' response which returned to preexposure values by 72 hours and remained at baseline levels through 22 days of exposure. To begin an assessment of the effects of fish density on the responses of herring to pollution and disease, baseline levels of biochemical parameters were measured at various stocking densities. At both very high and low densities, fish were more 'stressed' than when stocked at medium densities. Adult herring exposed to OWD showed alterations in several components of their immune systems. Significant alterations in white blood cell populations, phagocytic and respiratory burst activity of macrophages, and lysozyme activity were seen. Adults exposed to OWD were subsequently challenged with VHSV. These fish did not display any signs of this disease indicating that this population had already been exposed to VHSV and was solidly immune, conditions which may more realistically emulate field conditions. However, these studies may indicate that OWD exposure does not always alter the expression of VHSV in immune fish. Juvenile herring showed varied responses when challenged by VHSV and oil separately or together. No increased mortality due to VHSV occurred in oil-exposed fish when compared to control fish, and mortalities through the expression of VHSV-induced disease was variable. These results indicate that the age of fish, previous immunity to disease and other unknown factors are important in the relationship between contaminants and disease. The swimming ability of adult herring was not affected by OWD, although there was a significant increase in herring mortalities following strenuous exercise in exposed fish. As well, OWD affected the recovery of herring biochemistry which is typically altered during exercise.

Key Words: *Exxon Valdez*, oil, Viral Hemorrhagic Septicemia Virus, *Ichthyophonus hoferi*, *Clupea harengus pallasi*, herring, fitness

Project Data: *Description of data*- Several sets of data were gathered by laboratory experiments and include: effects of oil exposure and Viral Hemorrhagic Septicemia Virus on juvenile and adult Pacific herring biochemistry, immunology and disease resistance, and

swimming performance and exercise recovery. *Format*- Data regarding experimental data are stored in Microsoft Excel and text files in WordPerfect 6.1. *Custodian*- Contact Dr. Chris Kennedy at the Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada, V5A 1S6.)Phone: (604) 291-5640, fax: (604) 291-3496 or email at: ckennedy@sfu.ca). *Availability*- Copies of text in annual reports are available for the cost of duplication. Reprints of any manuscripts will also be available when published.

Citation:

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Executive Summary

Although near-record spawning biomass returns of Pacific herring were predicted for 1993, the population crashed when less than half of the >100,000 tons of spawning herring returned to Prince William Sound. Spawning herring sampled from Prince William Sound (PWS), showed a high prevalence of two pathogens, namely Viral Hemorrhagic Septicemia Virus (VHSV) and *Ichthyophonus hoferi* (ITP). The presence of these pathogens has led to the suggestion that disease was the likely cause of morbidity of herring in PWS. It is also unclear whether the Exxon Valdez oil spill contributed to these population declines.

Stressors such as disease and pollution can affect the longterm survival of fish without being acutely lethal, through reductions in overall 'health' or 'fitness'. The longterm objective of this study, therefore, is to document cause-effect relationships for oil, VHSV and ITP on herring fitness or health to determine probable causes of population declines. The categories of fitness chosen for this study include herring survival, blood biochemistry, performance in terms of the immune system and swimming, and reproduction. In these studies, both juvenile and adult herring were used because previous results indicated significant differences in responses to the examined stressors. The disease state of wild fish was determined: juveniles were negative in all tests for both VHSV and ITP, however, adult herring were only negative for presence of VHSV. Approximately 24% of adult fish were positive for the presence of ITP.

An oil-water dispersion (OWD) of North Slope crude oil was not acutely lethal to adult herring although it initiated a measurable and classical biochemical stress response which included a hypersecretion of corticosteroids, hyperlacticemia and hyperglycemia. By 72 h of exposure to OWD, all of these parameters measured had returned to preexposure values and remained at baseline levels through 21 days of exposure, indicating that the stress response was transient. To begin an assessment of abiotic factors on stressor effects on fish health, the effects of fish density on baseline levels of measured biochemical parameters indicated that increases in the stress response occurred at high fish densities and in the lowest densities.

Immunological parameters were measured in adult fish exposed to OWD for 28 days and following transfer to uncontaminated water. Significant alterations in white blood cell populations, phagocytic and respiratory burst activity of macrophages, and lysozyme activity. Fish challenged with VHSV did not break with disease although the response to the challenge was monitored by changes in tissue viral loads and antibody titres. Juvenile herring exposed to OWD chronically for 28 days showed variable mortality when further challenged by VHSV.

Adult herring were exposed to varying concentrations of OWD for 4 and 22 days to determine effects on swimming performance. Swimming ability was not affected at any OWD concentration, although there was a significant increase in herring mortalities following strenuous exercise in OWD-exposed fish. Due to high mortalities in fish which were swum in these experiments, the effects of OWD exposure on exercise recovery were determined. It was shown that OWD affected the recovery of herring biochemistry which is typically altered during exercise.

These results further explain the possible roles of oil, VHSV and ITP in herring population declines. Exposures of herring to these stressors can affect several aspects of fish fitness at several levels of biological organization. These effects also manifest themselves in juvenile and adult herring to different extents. Understanding how herring respond to these stressors and their recovery from exposure has important implications to herring management strategies and herring fisheries practices and will aid in the recovery of the resource as well as in successful monitoring of fish health in the future.

Introduction

The Pacific herring (*Clupea harengus pallasi*) spawning population in 1989 was the largest in many years when the *Exxon Valdez* oil spill occurred in Prince William Sound (PWS). Although near-record spawning biomass returns were predicted for 1993, the population crashed when less than half of the >100,000 tons of spawning herring returned to PWS. Several hypotheses have been put forward to explain the population decline which include the direct or indirect effects of oil or its components on herring habitat, food resources or their survival and fitness. Pearson et al. (1995) concluded that the levels of hydrocarbons measured in various matrices in PWS were too low to pose a serious risk to either adult or juvenile herring. This conclusion appears to be premature and relies mainly on results of acute toxicity tests. A more comprehensive examination of both lethal and sublethal toxic effects of hydrocarbons on several life stages of Pacific herring is needed to realistically assess the impact of such events on fish populations.

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. Therefore, it has been suggested that VHSV may have played a role in the population decline of the herring populations in Prince William Sound. 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 infection from another pathogen, *Ichthyophonus hoferi* (ITP), were present in about 29% of herring sampled. It has been suggested that ITP may also have been a major cause of herring morbidity between the 1992 and 1993 spawning seasons (Marty et al. 1994).

Stress due to anthropogenic contamination, i.e. the *Exxon Valdez* oil spill, may have 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) can impair immunological responses, possibly 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).

From the information that had existed prior to 1995, there had been no definitive evidence on whether VHSV, ITP or oil exposure through the *Exxon Valdez* oil spill, or some combination of these stressors had caused a decline in herring populations. In this project, Section I has as its objectives to determine the prevalence and severity of VHSV, ITP and other lesions in surviving spawning Pacific herring in PWS through several years. Sections II and III of this proposal have as their combined objectives to determine definitive links and relationships between VHSV, ITP and hydrocarbon exposure and morbidity, mortality, pathogenicity, and overall fitness and 'health' of Pacific herring. In 1996 and 1997, results from these studies showed that fish exposed to low levels of hydrocarbons experienced acute lethality as well as very significant sublethal effects on other aspects of herring fitness which included effects on various aspects of the herring immune system. These results begin to link oil exposure to a reduced immunocompetence in herring which may have contributed to increased disease prevalence observed in the field section of this proposal.

The results of this project in the examination of the effects of combinations of anthropogenic and disease stressors on herring health are aimed at answering many questions regarding herring population dynamics such as: 'Are herring that survive exposure to VHSV, ITP or hydrocarbons 'healthy' or are they surviving at a reduced fitness level? If full recovery occurs, what is the time frame? What are the effects of multiple stressors and recovery from such cumulative stresses?' What are the important abiotic modifiers of herring responses, especially with respect to density and temperature conditions? This information has particular relevance to herring management practices such as the Pound Fishery (Roe-on-Kelp). In the absence of such information, sound management of the herring stock in PWS will be a difficult task.

Objectives

From all of the information that has been made available through laboratory and field studies investigating the decline of herring stocks in Prince William Sound (PWS), there is no definitive evidence on whether viral hemorrhagic septicemia virus (VHSV), *Ichthyophonus hoferi* (ITP) or oil exposure *via* the *Exxon Valdez* oil spill, or some combination of these stressors has caused a decline in herring survival, performance or reproductive fitness. It is also unclear if the fish that survived exposure to one or more of these stressors are 'healthy' or are surviving at a reduced fitness level. In the absence of such information, sound management of the herring stock in PWS will be a difficult task.

The laboratory component of this proposal addresses these important information needs. The objectives of the proposed study will contribute directly towards discovering why herring populations are recovering at their present rate in PWS. The longterm objectives of Section III of this project, therefore, are to document cause-effect and interactive relationships for oil, VHSV and ITP on herring survival, performance and reproduction and to establish the effects of important abiotic modifiers such as density and temperature on herring responses to these stressors.

The overall hypothesis being tested in this project is:

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

Objectives:

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

2) To determine the effects of oil exposure on the biochemistry on adult Pacific herring.

3). To determine the effects of oil exposure on various components of the immune system in herring.

4) To determine the effects of oil and VHSV exposure on adult Pacific herring

5) To determine the effects of oil exposure on the swimming performance and exercise recovery of adult Pacific herring.

6) To further develop immunological assays for potential use in experiments and in future biomonitoring programs for Pacific herring.

7) To begin the assessment of abiotic factors on the responses of herring to stressors.

Methods

General

1) Fish

Adult Pacific herring were caught in Barkley Sound, Vancouver Island, by purse seine by the Department of Fisheries and Oceans, Canada in the Spring of 1997 and donated for our experiments. Juvenile young of the year were caught in Barkley Sound by beach seine in the summer of 1997. Both adults and juveniles were transported to the laboratory without using nets and held at least two weeks until any experiment was performed. Disease status was determined for both VHSV and ITP in wild adult and juvenile fish. Virology was performed on the whole body of herring at the Marrowstone Facility by Dr. Kocan's group. Virology was also performed on juveniles using the whole body and pooled liver, spleen, pyloric cecae and gill of adult herring by the Department of Fisheries and Oceans, Pacific Biological Station, Naniamo, BC. ITP prevalence was performed by histopathology of liver and heart of herring and by procedures recommended by Dr. Kocan of Section II which included grinding heart and liver (pooled for each fish) in a petri dish or test-tube in cell culture medium (L-15) supplemented with 2-10% FBS (fetal bovine serum) and either gentamicin or penicillin/streptomycin combination. Petri dishes were incubated at 14.5°C and observed after 1 to 2 weeks for spores or hyphae characteristic of ITP.

2) Chemical and stressor exposure

Varying times were used to dose herring to oil in order to begin to separate acute v. chronic exposure effects. Exposure times ranged from 24 h acute exposures to 28 day chronic exposures to more fully determine possible effects on herring fitness. In recovery studies, fish were typically sampled up to 6 weeks post-oil or disease exposure.

Dosing of herring with oil in 1997 was performed using the dosing apparatus or 'oil generators' which had been selected for this research project and were developed by Carls et al. (unpublished method and analysis data). Essentially, this apparatus consists of a 15 cm diameter X 80 cm tall polyvinyl chloride plastic cylinder containing ceramic beads which have been soaked in North Slope Crude oil. Water upwells through the cylinder and over the oil soaked beads and flows into the bottom of an individual treatment tank containing herring. A trap inside the generator prevents slick overflow. Appropriate levels of hydrocarbons are generated through the apparatus by varying the amount of beads in each column. Hydrocarbon analysis using this method has been documented by Carls et al. (unpublished) using Alaska North Slope Crude oil with polycyclic aromatic hydrocarbon (PAH) concentrations in the range of 10 to 100 ppb at the start of water flow to 0.3 to 30 ppb 16 days following the initiation of water flow. Our preliminary analysis includes both total fluorescence analysis coupled with gas chromatography and FID detection. Samples are in the process of being analyzed by GC-FID at Simon Fraser University at no charge to the project. Total hydrocarbon concentrations are given in the results as control (no detectable hydrocarbons), low (3-16 ppb), medium (56-85 ppb) and high (178-328 ppb) concentrations at the beginning of the experiments.

Challenges with VHSV were done using a virus stock obtained from Dr. R. Kocan of the University of Washington, WA, USA. The original stock contained 4.0×10^8 pfu/ml upon thawing. The disease challenge was performed following a protocol outlined in Kocan (1997) and Section II of this report. Water in experimental tanks was lowered (including controls) to facilitate a static bath challenge for one hour. The final challenge dose per VHSV exposed tank was 1×10^4 pfu/ml. All tanks were observed twice daily for mortalities for up to 8 weeks.

3) Biochemical assays

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

4) Hematological and Immunological assays

Immunological assays include both hematological and immunological parameters. Hematological assays included hematocrit (% packed red blood cells), leucocrit (% packed

white blood cells) and differential white blood cell counts. Hematocrit and leucocrit were measured according to Kennedy et al. (1995). Differential white blood cells were performed as follows: Smears were stained with Diff-Quik (Dade Diagnostics, Inc., Aquada, Puerto Rico), using the recommended protocol on the product package. Smears were examined microscopically at 1000X oil immersion magnification. Approximately 100 white blood cells were counted from the randomly selected fields. The number of fields examined varied with the smear, however, on average, 48 fields per slide were counted. The number of red blood cells in each field were not counted but were similar: with approximately 150-175 red blood cells per field. White blood cells were differentiated into six cell types; thrombocytes, lymphocytes, neutrophils, basophils, eosinophils and monocytes. Identification of each type was based on morphology and staining characteristics (Ainsworth 1992: Sherburne 1973). Red blood cells from each smear were examined for viral erythrocytic necrosis (VEN) .

To determine effects on the nonspecific immune system, macrophages were isolated and assayed for their phagocytic and repiratory burst activities. These assays were previously modified for use with herring in this project based on Secombes (1990). The methods are briefly as follows: macrophage isolation begins with the aseptic removal of the head kidney and placed in tissue cell culture medium. Herring macrophages have viability only in L-15 medium. Tissue is then macerated by grinding through a fine mesh screen and the tissue homogenate are resuspended in cell culture medium. Cell suspensions are then centrifuged on discontinuous Percoll density gradients. The density that has been successful for obtaining a macrophage band for herring is 1.075. This corresponds to 53% Percoll (53 ml Percoll:10ml: 1.5M NaCl: 37 ml ddH20). Cell suspensions were then spun for 25 min at 4C at 400 g and bands were collected with Pasteur pipettes. Cells are washed with PBS and resuspended in cell culture media. Cell viability was determined by using 0.4% trypan blue and a hemocytometer to count the number of viable macrophages (95% viability ideal for assay).

The phagocytosis assay was modified and was carried out as follows: yeast was used for assays with adult fish. Yeast suspensions are autoclaved with formalin to produce a formalin-killed solution. The suspension is washed with PBS and then resuspended in PBS/L-15/Hanks. The suspension was then opsonized by incubation for 30 min at 28C in the presence of pooled herring serum. An aliquot of macrophage suspension is placed on a glass slide and allowed to incubate for 90 min in a moist chamber. The slide is then gently rinsed with PBS and yeast suspension is added to the slide and the slide again incubates for 90 min. The slide is washed again with PBS and stained using Diff Quik. 100 macrophages in random fields were counted and scored as to number of yeast ingested.

The respiratory burst assay measures the ability of macrophages to reduce the dye nitroblue tetrazolium (NBT) via generation of reactive oxygen intermediates (ROI's). In this initial assay NBT is reported as a percentage of macrophage cells displaying respiratory burst activity per 100 cells. The NBT assay involved incubation of macrophages with a 0.2% solution of NBT for 90 minutes following by examination microscopically to score reactive versus non-reactive macrophages. Photographs were taken for scoring at a later date due to deterioration of the slides over time. The optimization of a quantitative spectrophotometric analysis method was conducted to increase sensitivity of this assay and will be employed in 1998 experiments.

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

Serum was collected from all fish following VHSV exposures, and bodies were frozen at -70°C. To perform assays for viral load, bodies were partially thawed and viscera, including the stomach, gut, spleen, heart, liver and kidney were dissected, pooled and weighed. Minimum Essential Medium (MEM) with gentamicin, fungizone, penicillin and streptomycin was added to give a ratio of 1:5 tissue to medium, and homogenized. The homogenate was further diluted to give an initial concentration of 1:40 and ten-fold serial dilutions of this homogenate were used. Epithelioma papulosum cyprini (EPC) cells were plated in 24-well Costartissue culture plates in MEM supplemented with 10% fetal bovine serum (FBS) and incubated at 28°C overnight to allow formation of a cell monolayer. Monolayers were pretreated for 15 minutes with polyethyleneglycol (PEG), and inoculated with serial dilutions of homogenates. After 30 minutes at room temperature, 0.05 % methylcellulose in MEM was added to each well, and plates were incubated at 15°C for 6 days. The number of plaques formed in each well was counted, and wells containing the dilution that gave a statistically significant plaque count (30-300 plaques) were used to calculate viral titre, which is expressed as plaque forming units/gram tissue (PFU/g).

For the antibody neutralization assay, serum collected from fish was serially diluted and used to inoculate EPC monolayers pretreated with PEG. VHS was added to each well of the plate. For each 7 samples, one row of wells was inoculated with virus only. If antibodies were present in the serum, they will neutralize the virus and fewer or no plaques will form. After overlay with methylcellulose, and incubation for 6 days at 15°C, plaques were counted and compared to wells with virus only.

5) Swimming performance

The swim chamber apparatus used to swim adult herring was modified according to Nikl and Farrell (1993) and Brett (1964). 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 chamber.

Following exposure to one of the stressors, fish were transferred to a swim chamber without using nets and allowed to acclimate to the chamber for at least 2 hours before a test began. Critical swimming speed (U_{crit}) was measured according to Brett (1964). The

initial velocity was selected for each group of fish, and the speed was increased in increments at 15-min intervals until all fish had fatigued. Fatigued fish were individually removed from the test chamber and time to exhaustion, fish fork length and weight were recorded. U_{crit} was calculated using the method of Brett (1964). A fish was considered exhausted when it rested against the rear grid and did not respond to mechanical stimulation.

In a separate experiment, the effects of OWD on the recovery of herring from 'burst swimming' was examined. Fish were forced to swim in a 'burst' fashion for 6 minutes (Graham and Wood 1982), and recovery from exercise was examined through measurements of hematocrit, plasma lactate and the plasma ions [Cl-] and [Na+].
6) Statistical analysis

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

Specific Objectives Methods

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

Blood smears from 660 Pacific herring sampled in March/April 1997 in Prince William Sound were received from Dr. G. Marty of the University of California at Davis. Smears were stained and analyzed for differential white blood cell counts as described in the section on immunological techniques previously. White blood cells were differentiated into six cell types; thrombocytes, lymphocytes, neutrophils, basophils, eosinophils and monocytes. Red blood cells from each smear were examined for viral erythrocytic necrosis.

In 1997, analytical support for Section I of this project was provided for plasma chemistry analysis. Plasma from 660 Pacific herring sampled in March 1997 in PWS were received from Dr. G. Marty of the University of California, Davis

Objective 2: To determine the effects of oil exposure on the biochemistry of adult Pacific herring.

Previous studies in this project have shown that exposure of juvenile Pacific herring exposed to OWD showed a classical stress response. In order to determine if adult herring show a similar response, fish were exposed a high concentration of OWD as described above using oil 'generators'. In these studies, blood from adult herring were routinely sampled and assayed for various biochemical parameters including plasma cortisol, lactate, glucose, and hematocrit as described previously.

Objective 3: To determine the effects of oil exposure on the immunology on adult Pacific herring.

Adult herring were exposed to OWD as described previously. At various time points herring were sampled for immunological status. Blood was sampled by caudal puncture and then fish were dissected and the head kidney removed for macrophage isolation. The immunological measures taken included hematocrit, leucocrit, differential white blood cell counts, lysozyme activity, macrophage phagocytosis and macrophage respiratory burst activity according to the methods described previously.

Objective 4: To determine the effects of oil and VHSV exposure on adult Pacific herring.

Adult Pacific herring were exposed to control, low, medium and high concentrations of an OWD as described above for 28 days. Herring were then exposed to VHSV titres as outlined by Kocan (1997) and blood and tissues sampled routinely for up to 8 weeks post exposure. Fish were monitored for signs of VHSV and mortality. When sampled, measurements were made of hematocrit, leucocrit, differential white blood cell counts, lysozyme activity, macrophage phagocytosis, macrophage respiratory burst activity, tissue viral loads and antibody titres. The procedures for the measurement of these immunological parameters are as described above.

Objective 5. To determine the effects of oil exposure on the swimming performance of adult Pacific herring.

Adult herring were acutely exposed to three concentrations of an OWD for either 4 days or 22 days as described previously. Upon completion of an exposure period, fish were examined for 1) acute mortality, 2) swimming performance and 3) their recovery from exercise. To determine the acute toxicity of OWD to herring, exposure and control tanks were monitored for mortalities for up to 96 hours. Following an exposure of herring to 96h of OWD, fish were transferred to modified swim chambers and the Ucrit determined. Ucrit is defined as the maximal prolonged swimming speed of a fish. A second group of fish were exposed to OWD and then subject to a bout of 'burst' swimming. These fish were subsequently sampled for biochemical parameters (as described previously) which are indicators of exercise recovery.

Objective 6: To further develop immunological assays for potential use in experiments and in future biomonitoring programs for Pacific herring.

One of the major objectives of this project is to develop a battery of biochemical, hematological and immunological assays which may be used in future monitoring programs to assess fish health. There have been relatively few studies which have utilized standard mammalian and fish immunological assays on marine fish such as the Pacific herring. This has necessitated considerable effort in the optimization of several assays for use in this species for this and future studies.

Objective 7: To assess the effects of density as an abiotic factor on the responses of herring to stressors.

There are several important environmental factors which can modify an organisms response to stress. One of the most important factors to fish is stocking density. In these

preliminary experiments, the effects of fish stocking density on baseline biochemical measures were made. Measurements included plasma cortisol, lactate, glucose, hematocrit, and leucocrit, and were performed as described previously. In further experiments, juveniles were exposed to OWD at varying densities (50, 100 and 300 fish per 1000L tank) and biochemical measures taken to determine effects on the biochemical stress response which has been shown to occur in these fish upon exposure.

Results

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

Statistical analysis and reporting of differential white blood cell counts and presence of viral erythrocytic necrosis are given in Section I (Field studies-Dr. Marty) of this annual report for spring samples from Sitka Sound and Prince William Sound. Plasma chemistry results are also given in Section I of this report.

Objective 2: To determine the effects of oil exposure on the biochemistry of adult Pacific herring.

Adult Pacific herring were exposed to a high OWD concentration to determine effects on adult herring biochemistry through time from 24h to 28 days. Significant differences were seen in the biochemical parameters between oiled and control fish only at the 24h sampling time.

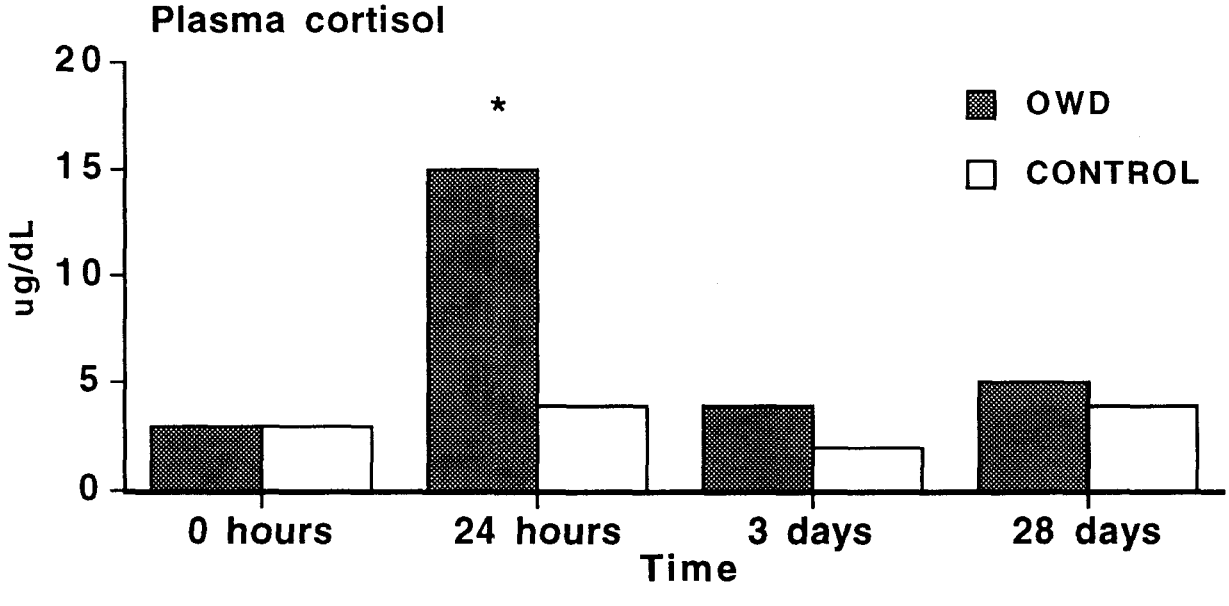

Figure 1. Time course of plasma cortisol in control and OWD exposed fish. Values are means for 10 fish. Significant differences at $p<0.05$ are denoted by *.

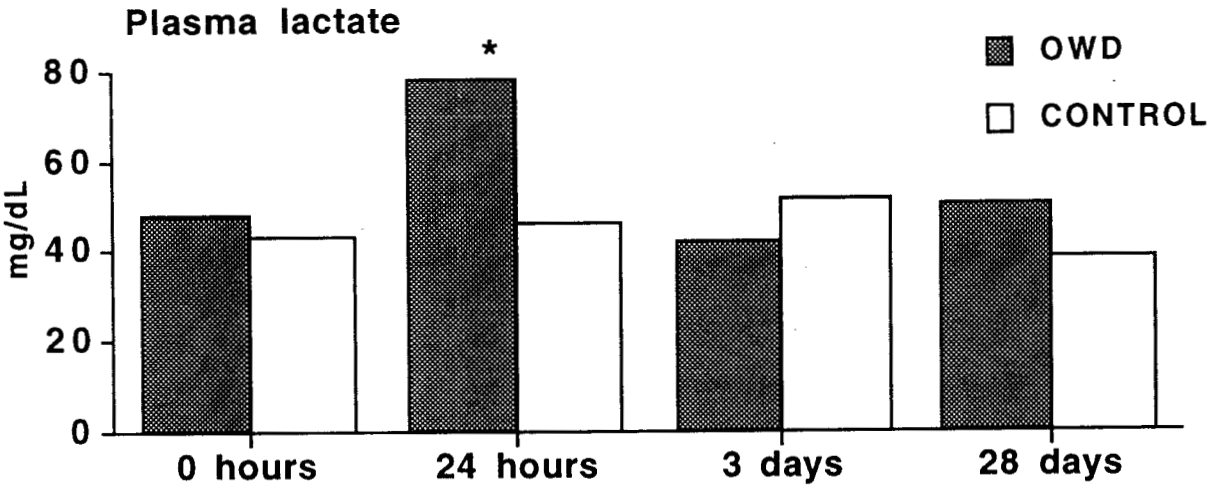

Figure 2. Time course of plasma lactate in control and OWD exposed fish. Values are means for 10 fish. Significant differences at $p<0.05$ are denoted by *.

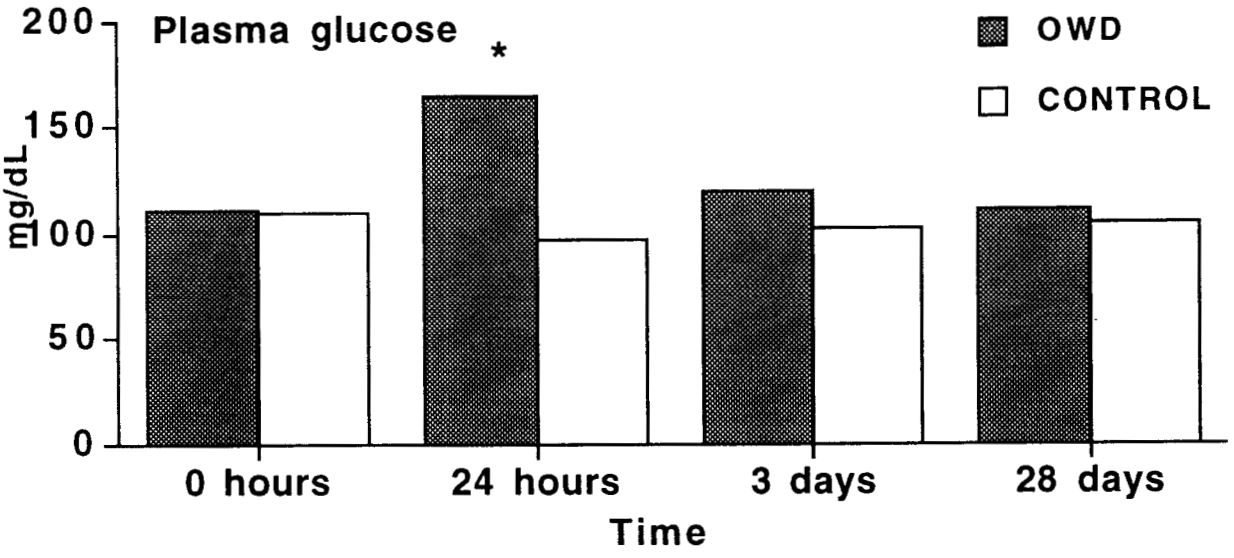

Figure 3. Time course of plasma glucose in control and OWD exposed fish. Values are means for 10 fish. Significant differences at $p<0.05$ are denoted by *.

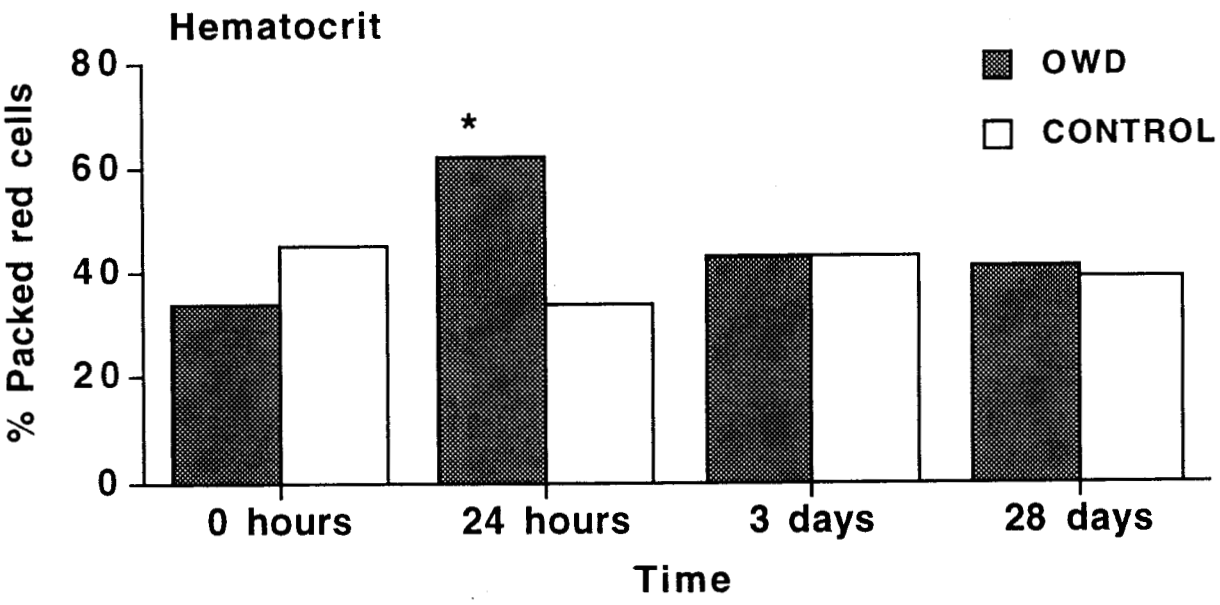

Figure 4. Time course of hematocrit in control and OWD exposed fish. Values are means for 10 fish. Significant differences at $p<0.05$ are denoted by *.

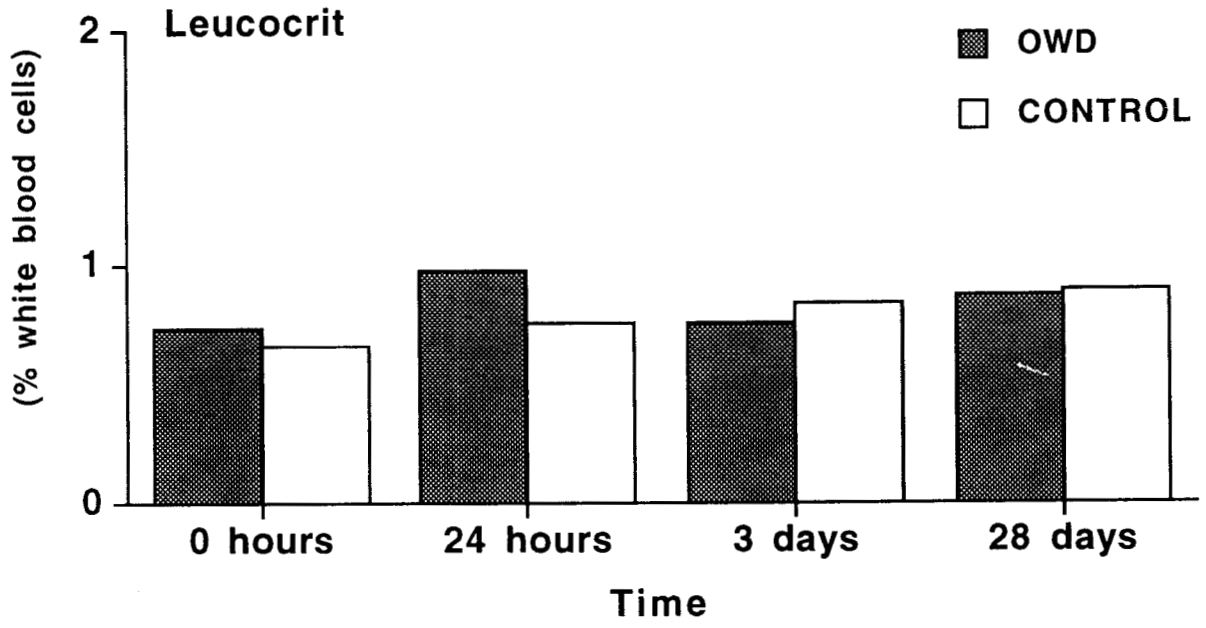

Figure 5. Time course of leucocrit in control and OWD exposed fish as above. Values are means for 10 fish. Significant differences at $p<0.05$ are denoted by *.

Objective 3: To determine the effects of oil exposure on the immunology on adult Pacific herring.

Adult herring were exposed to OWD for 21 days followed by 6 weeks in uncontaminated water. At various time points herring were sampled for immunological status. Blood was sampled by caudal puncture and then fish were dissected and the head kidney removed for macrophage isolation. The immunological measures taken included hematocrit, leucocrit, differential white blood cell counts, lysozyme activity, macrophage phagocytosis and macrophage respiratory burst activity according to the methods described previously. No significant effects were seen in hematocrit and leucocrit. Significant effects were seen following a 21 day exposure in herring lysozyme, macrophage phagocytic activity and macrophage respiratory burst activity. Both lysozyme and macrophage phagocytic ability returned to normal by 6 weeks following transfer to uncontaminated water. Interestingly, respiratory burst activity 6 weeks following the transfer was elevated in previously OWD exposed fish.

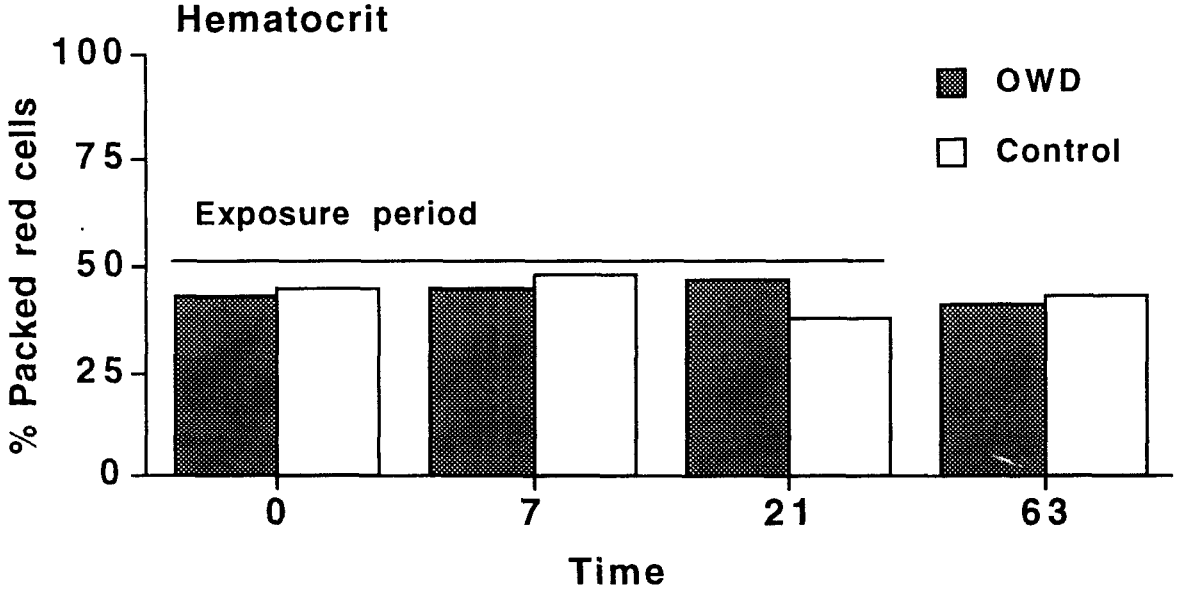

Figure 6. Time course of hematocrit in control and OWD exposed fish. Values are means for 12 fish. Significant differences at $p<0.05$ are denoted by *.

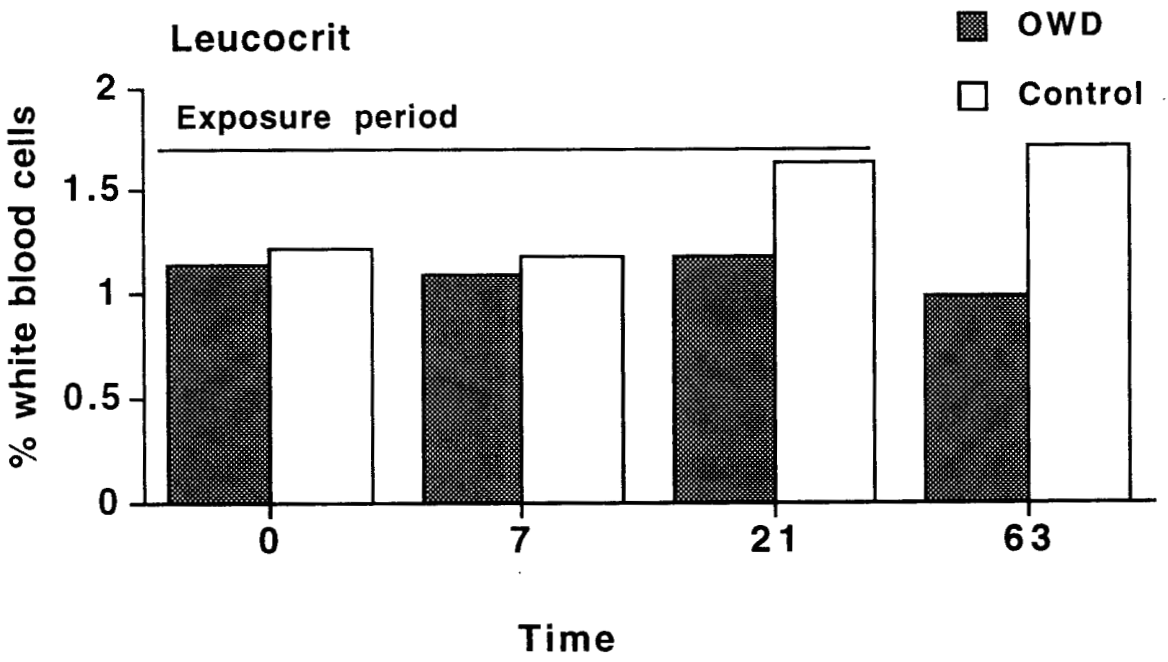

Figure 7. Time course of leucocrit in control and OWD exposed fish. Values are means for 12 fish. Significant differences at $p<0.05$ are denoted by *.

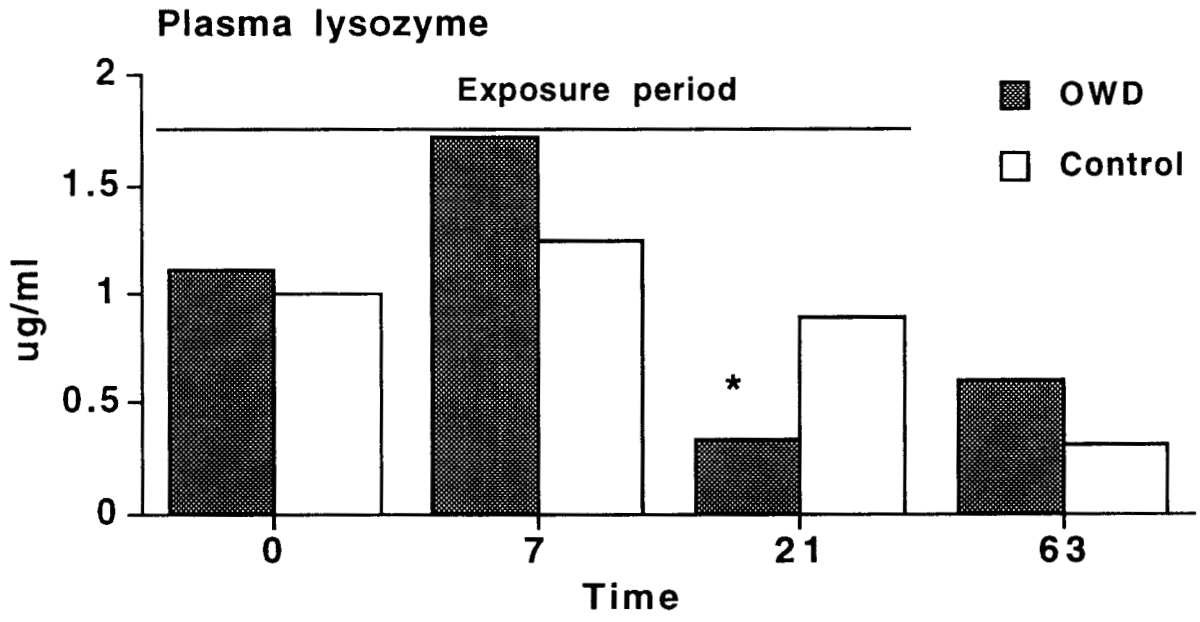

Figure 8. Time course of plasma lysozyme in control and OWD exposed fish. Values are means for 12 fish. Significant differences at $p<0.05$ are denoted by

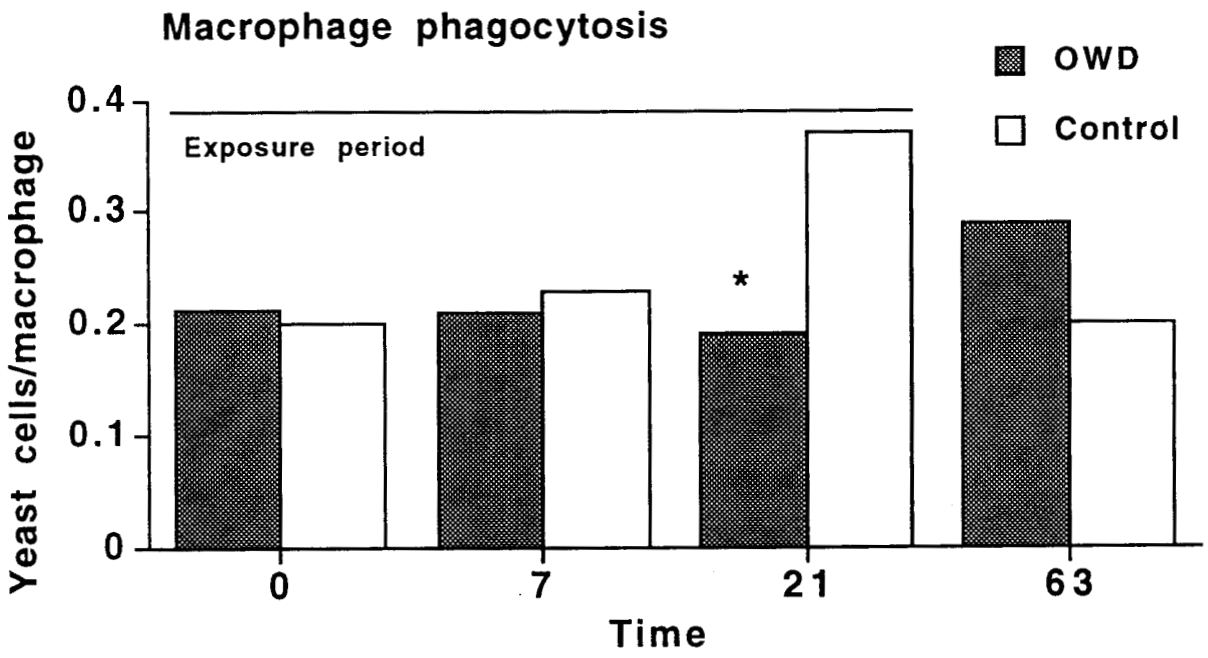

Figure 9. Time course of macrophage phagocytosis in control and OWD exposed fish. Values are means for 12 fish. Significant differences at $p<0.05$ are denoted by *.

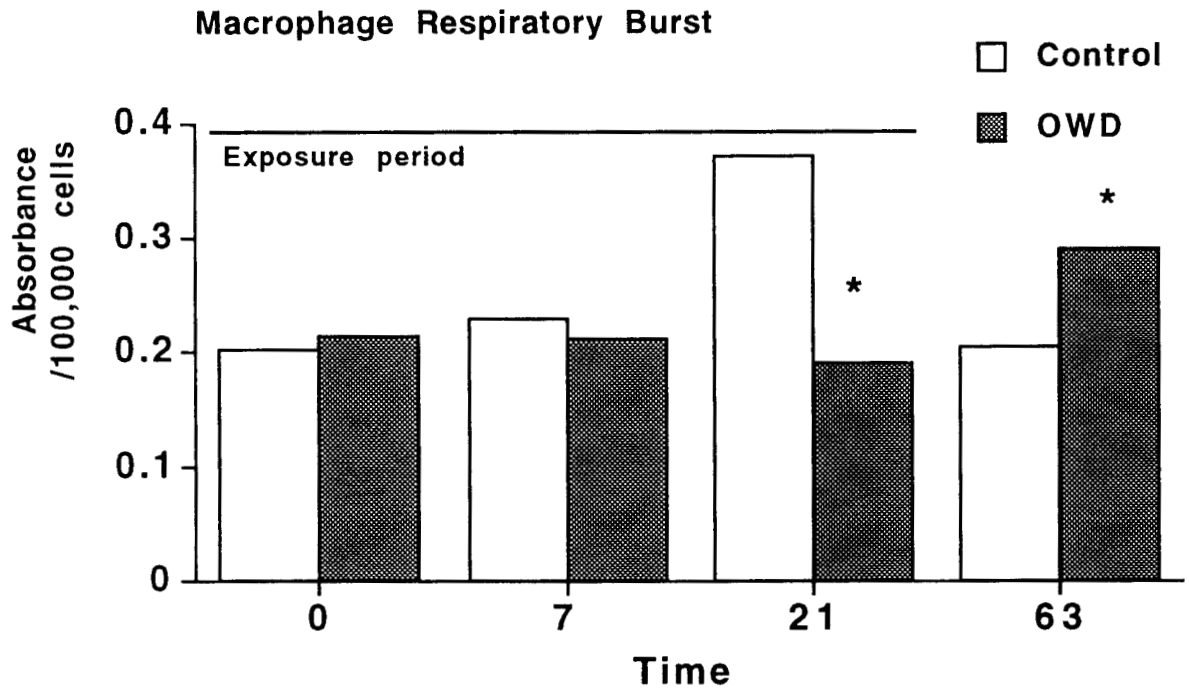

Figure 10. Time course of macrophage respiratory burst activity in control and OWD exposed fish. Values are means for 12 fish. Significant differences at $p<0.05$ are denoted by *.

The results of the differential white blood cell counts shows that a 21 day exposure of adult herring to a high dose of OWD results in a neutrophilia and leucocytopenia which are not evident 7 weeks following transfer to uncontaminated water (Table 1).

Table 1. Differential white blood cell counts in control and OWD exposed fish. Values are means for 12 fish. Significant differences at $p<0.05$ are denoted by *

Exp. Conditions	Lymphocytes (%)	Basophils (%)	Neutrophils (%)	Thrombocytes (%)	Monocytes (%)	Eosinophils (%)
0 days						
Control	29.0±3.3	0.12±0.06	21.5±1.6	49.1±6.3	0.3±0.2	0
OWD	29.2±3.2	0.13±0.05	19.6±1.5	50.7.±4.3	0.4±0.2	0
7 days						
Control	31.7±2.8	0.14±0.04	22.3±2.0	45.6±3.3	0.3±0.1	0
OWD	29.4±2.1	0.14±0.06	22.4±1.7	47.8±4.7	0.3±0.2	0
21 days						
Control	30.3±3.7	0.09±0.09	19.5±2.1	49.9±2.7	0.2±0.3	0
OWD	21.8±3.2*	0.14±0.03	24.4±0.7*	53.3±3.9	0.4±0.3	0
6 weeks post exposure						
Control	27.3±5.1	0.10±0.04	20.0±1.2	52.3±5.7	0.3±0.2	0
OWD	36.94±4.7	0.06±0.06	17.5±1.9	45.1±5.0	0.4±0.2	0

Objective 4: To determine the effects of oil and VHSV exposure on adult Pacific herring.

Adult Pacific herring were exposed to control, low, medium and high concentrations of an OWD as described above for 28 days. Herring were then exposed to VHSV titres as outlined by Kocan (1997) and blood and tissues sampled routinely for up to 8 weeks post exposure. Fish were monitored for signs of VHSV and mortality. When sampled, measurements were made of hematocrit, leucocrit, differential white blood cell counts, lysozyme activity, macrophage phagocytosis, macrophage respiratory burst activity, tissue viral loads and antibody titres. The procedures for the measurement of these immunological parameters are as described above. No effect of either an OWD exposure, VHSV exposure or combination was seen in hematocrit or leucocrit, results which, along with the lack or clinical signs of VHSV and virus in tissues, indicated that these fish were solidly immune to the virus at this stage under these conditions.

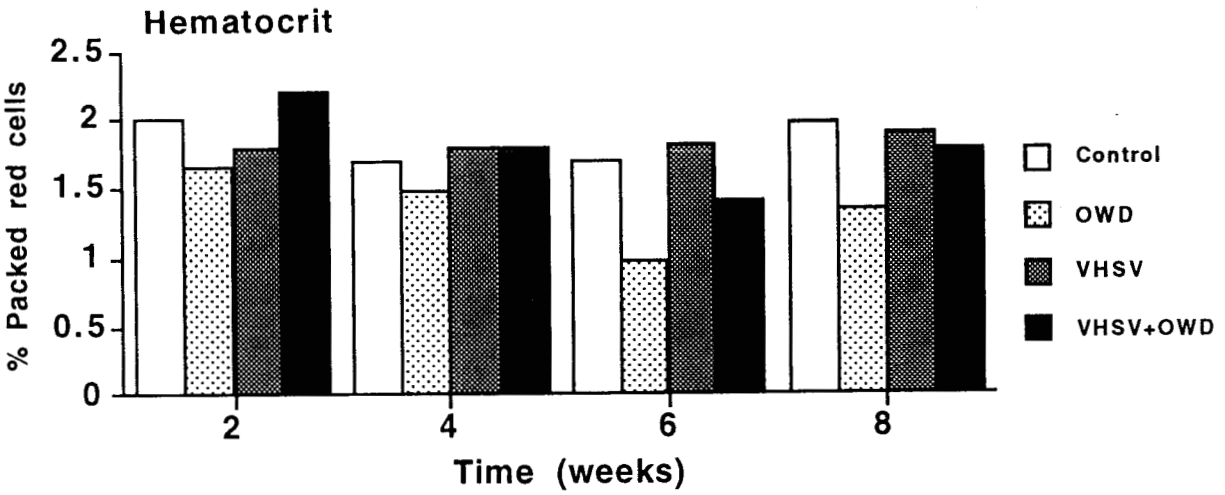

Figure 11. Time course of hematocrit in control and OWD exposed fish. Fish were exposed to OWD for 28 days and subsequently challenged with VHSV. Values are means for 6 fish.

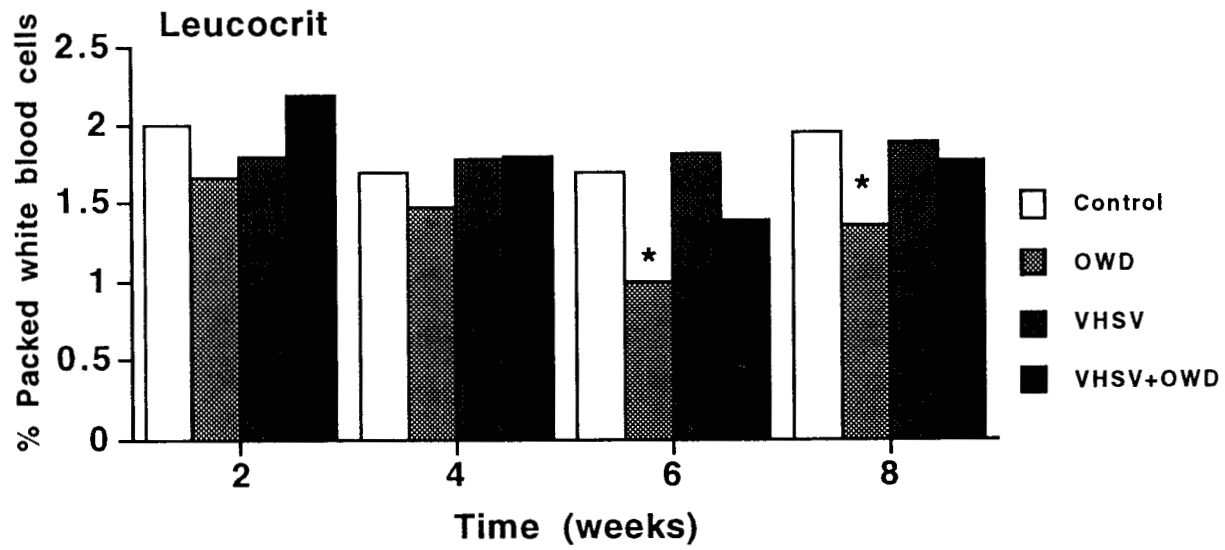

Figure 12. Time course of leucocrit in control and OWD exposed fish. Fish were exposed to OWD for 28 days and subsequently challenged with VHSV. Values are means for 6 fish. Significant differences at $p<0.05$ are denoted by *.

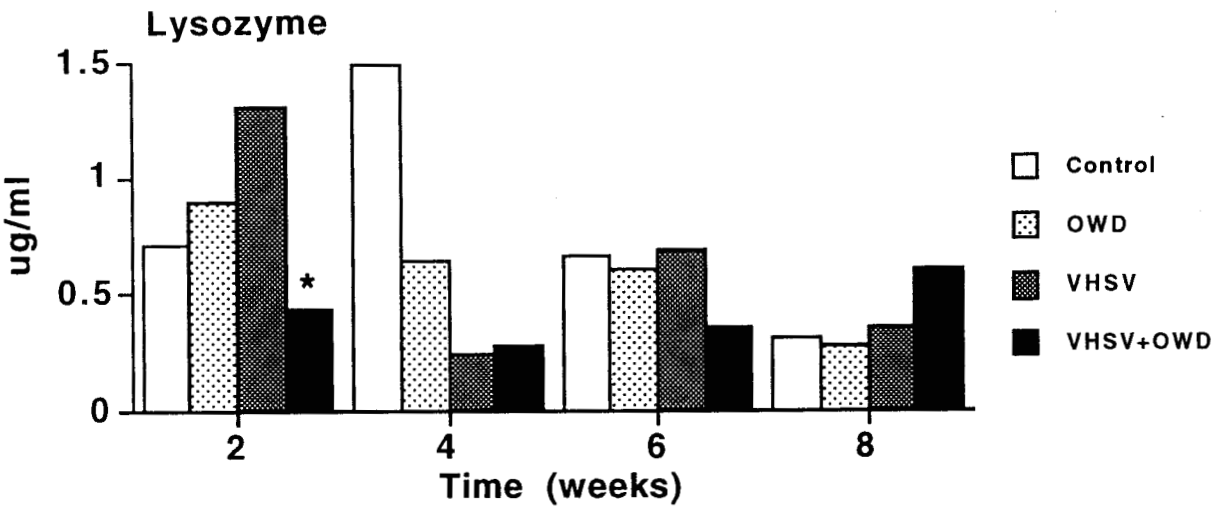

Figure 13. Time course of plasma lysozyme in control and OWD exposed fish. Fish were exposed to OWD for 28 days and subsequently challenged with VHSV. Values are means for 6 fish. Significant differences at $p<0.05$ are denoted by *.

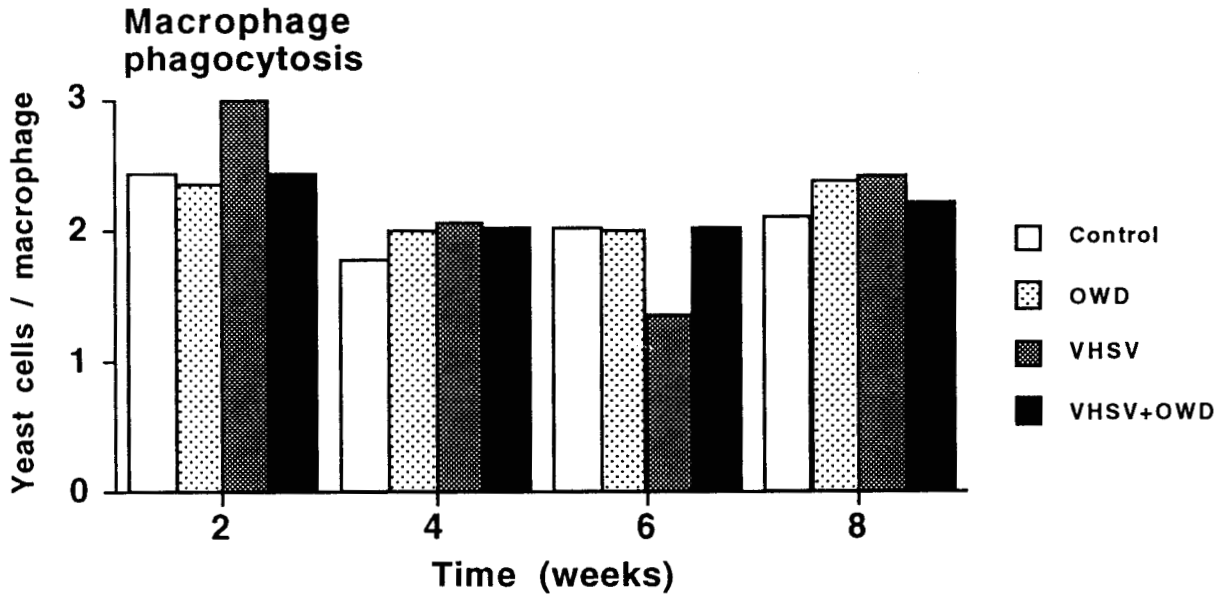

Figure 14. Time course of macrophage phagocytosis in control and OWD exposed fish. Fish were exposed to OWD for 28 days and subsequently challenged with VHSV. Values are means for 6 fish.

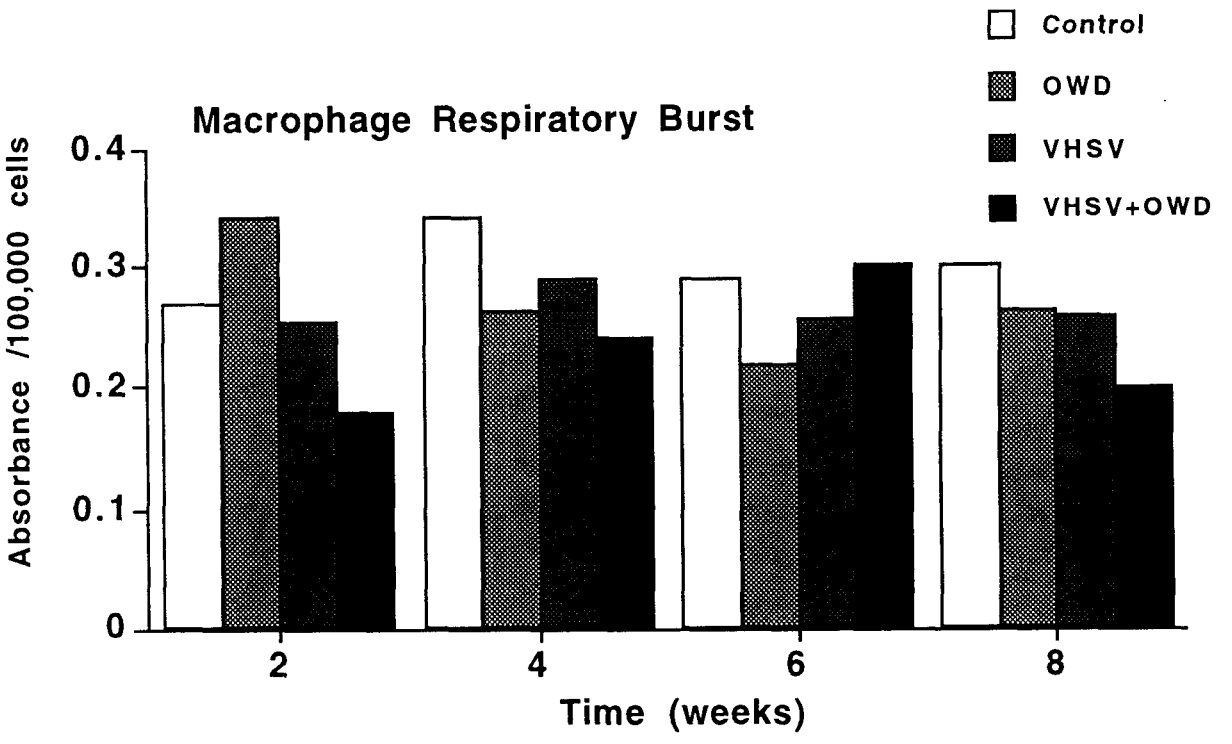

Figure 15. Time course of macrophage respiratory burst activity in control and OWD exposed fish. Fish were exposed to OWD for 28 days and subsequently challenged with VHSV. Values are means for 6 fish.

Juvenile herring were challenged with VHSV as described previously for adult fish following exposure to varying doses of OWD for 21 days.The results of these experiments were highly variable, and no effect of OWD exposure on herring mortlity due to VHSV could be determined. In one experiment, all juvenile fish died within one week of VHSV exposure, although virus titres for VHSV were low and signs of the disease were questionable according to Marty et al. (1994). In a second, experiment, no mortalities occurred under the same conditions. These experiment will be repeated in 1998.

Objective 5. To determine the effects of oil exposure on the swimming performance of adult Pacific herring.

Adult herring were exposed to OWD to determine effects on swimming performance and recovery of fish from exercise. OWD in this experiment resulted in significant mortalities which are shown in Figure 16. The highest percentage of herring died in the highest OWD concentration. No effects of sublethal OWD exposure on herring swimming performance as measured by critical swimming speed (Ucrit) were seen (Figure 17). Due to the high mortalities in fish which had been forced to swim, a separate experiment was set up to determine OWD effects on the recovery of herring from 'burst's swimming'. Figures 18, 19, 20 and 21 shows the effects of exercise on hematocrit, plasma lactate and [Cl-] and [Na+]. Exposure of fish to high doses of OWD caused more disturbance in most

parameters measured and appeared to inhibit a return of these values to 'normal' following exercise.

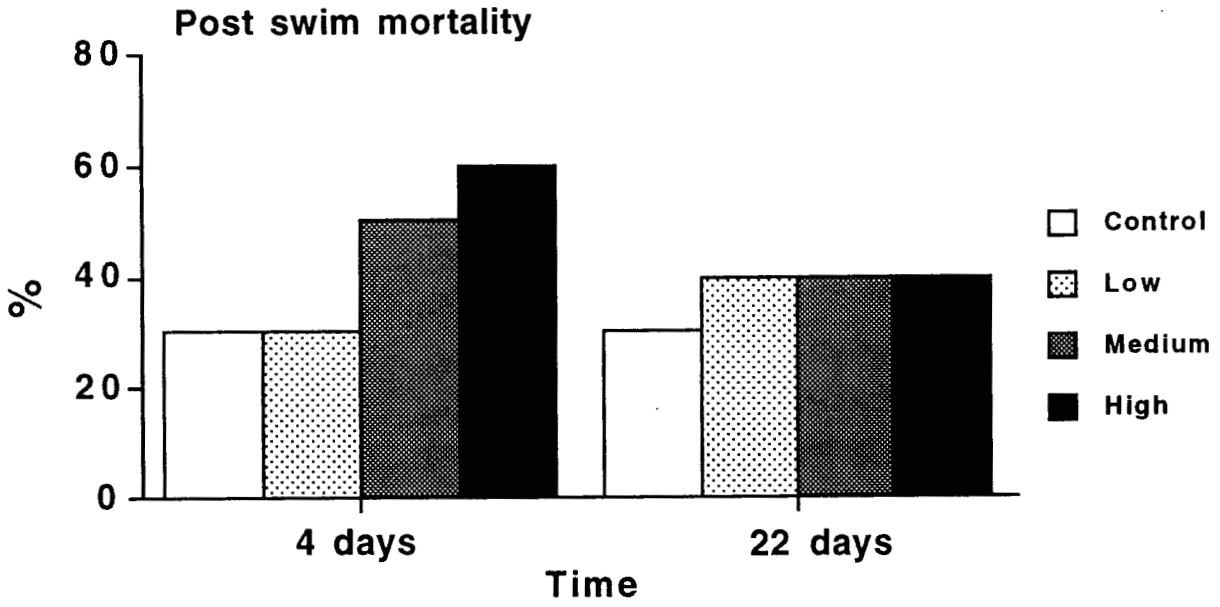

Figure 16. Acute mortality in adult Pacific herring exposed to varying concentrations of OWD and forced to swim.

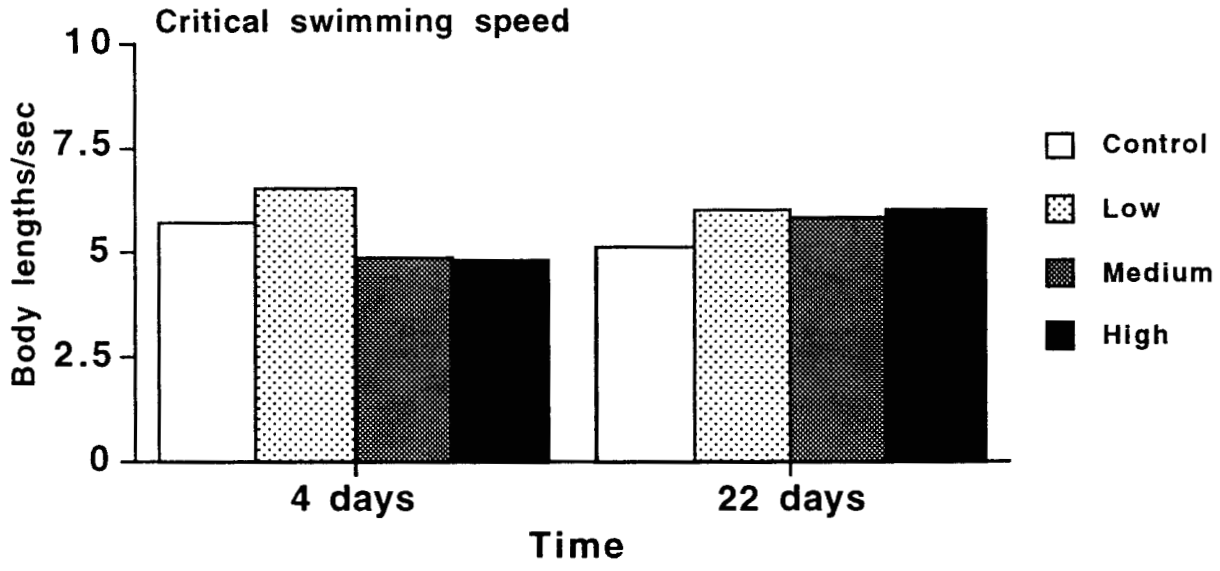

Figure 17. Effects of an OWD exposure on the critical swimming speed of adult herring following a 24 or 96 h exposure. Values are means±SE of three fish.

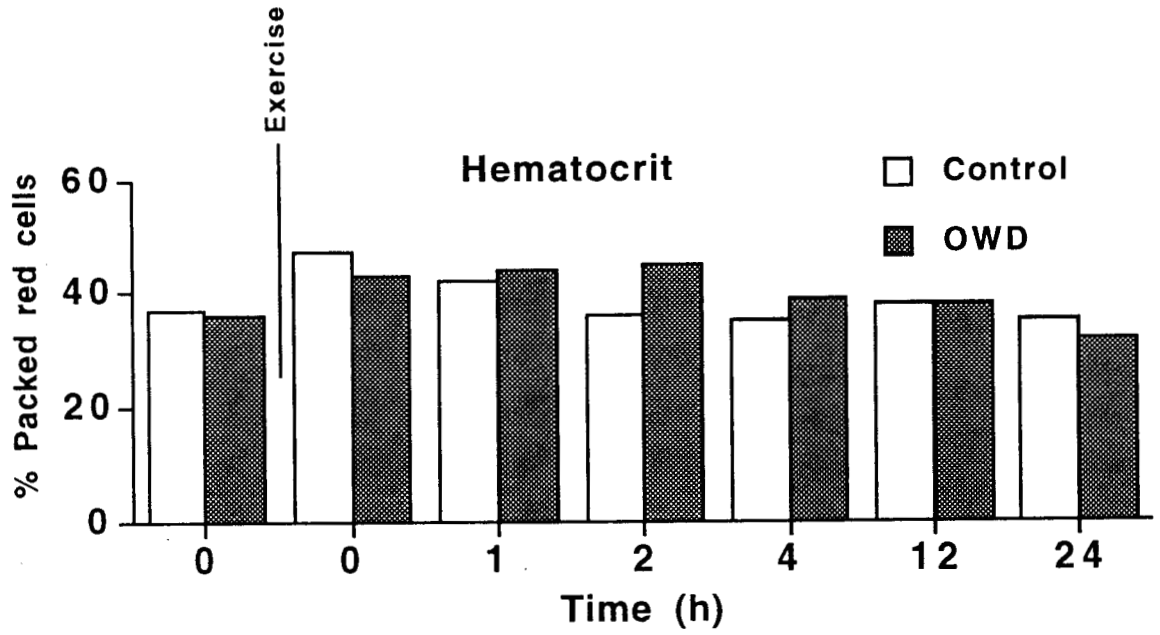

Figure 18. Effects of an OWD exposure on adult herring hematocrit following 6 minutes of "burst' swimming. Values are means±SE of three fish.

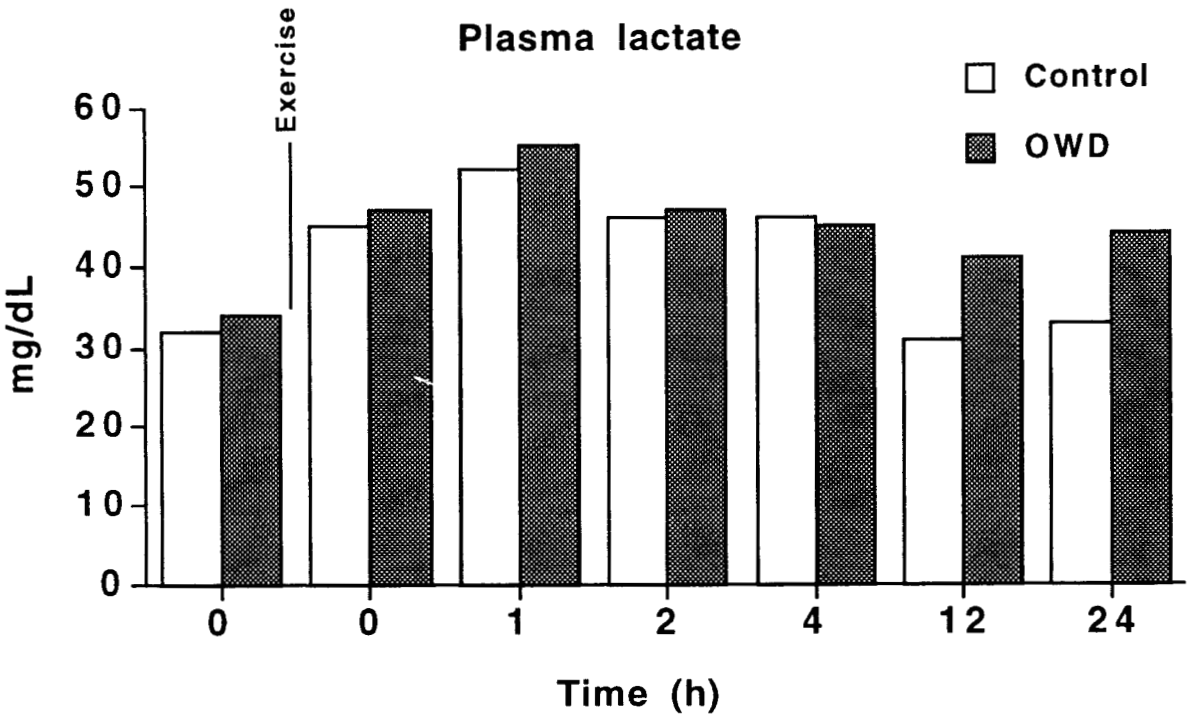

Figure 19. Effects of an OWD exposure on adult herring plasma lactate concentrations following 6 minutes of "burst' swimming. Values are means±SE of three fish.

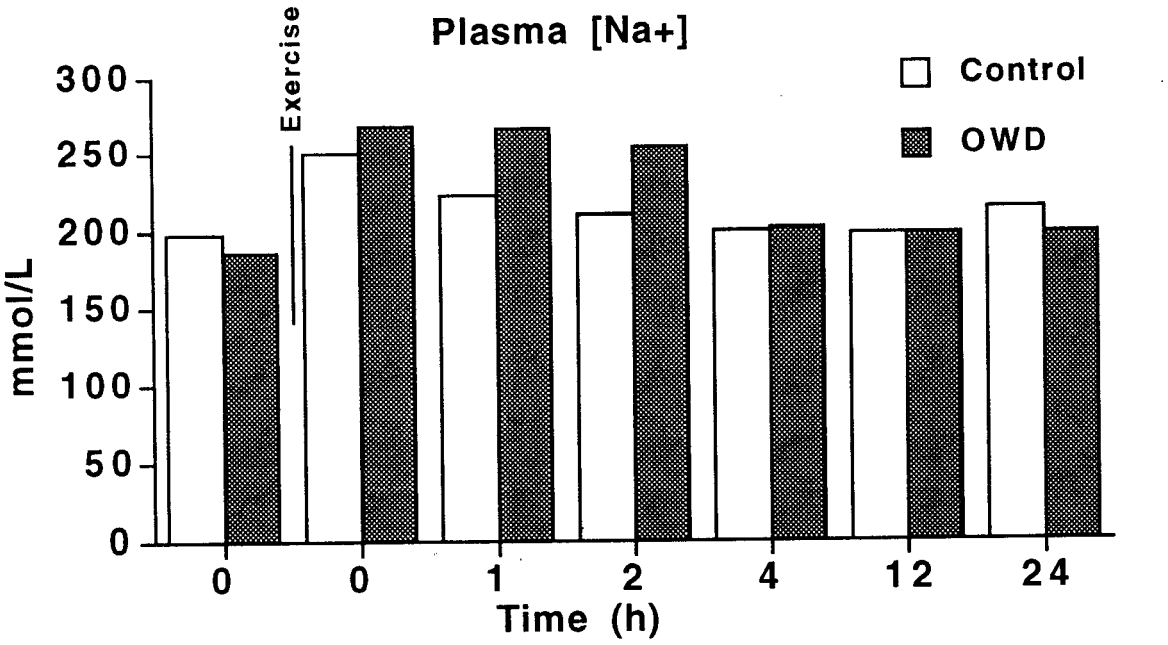

Figure 20. Effects of an OWD exposure on adult herring plasma Na+ concentrations following 6 minutes of 'burst' swimming. Values are means±SE of three fish.

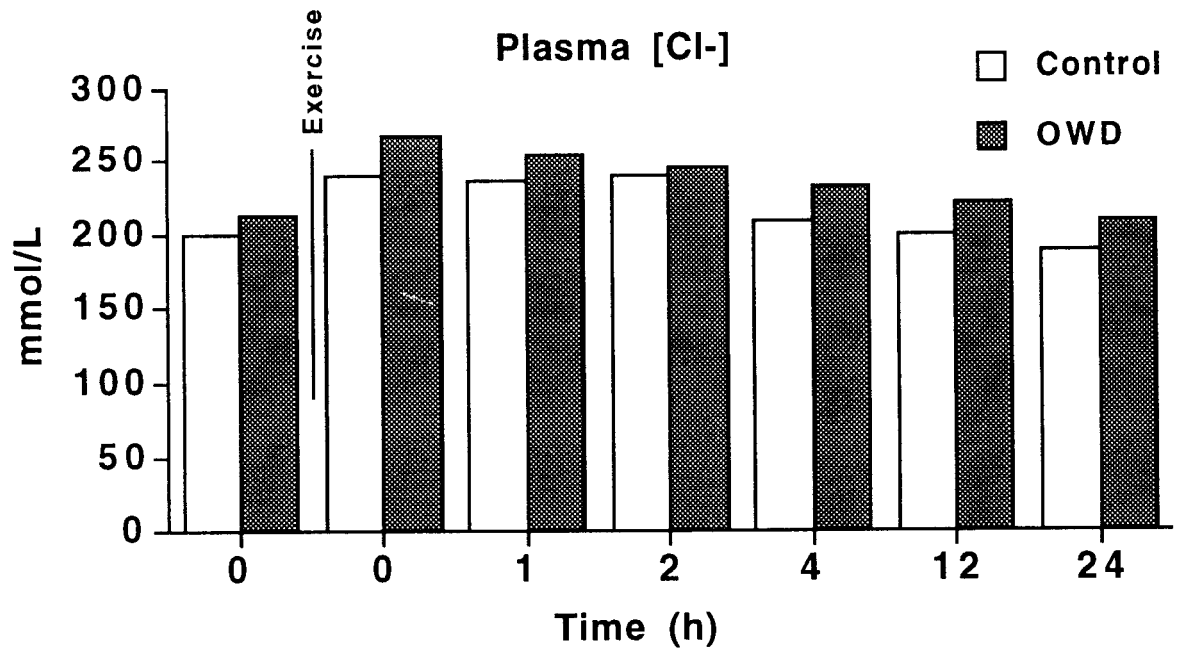

Figure 21. Effects of an OWD exposure on adult herring plasma Cl- concentrations following 6 minutes of 'burst' swimming. Values are means±SE of three fish.

Objective 6: To further develop immunological assays for potential use in experiments and in future biomonitoring programs for Pacific herring.

One of the major objectives of this project is to develop a battery of biochemical, hematological and immunological assays which may be used in future monitoring programs to assess fish health. In the final report of this project, a complete and comprehensive methods section for all assays for use in Pacific herring will be appended.

Objective 7: To assess the effects of density as an abiotic factor on the responses of herring to stressors.

Fish stocking density had a significant effect on several biochemical parameters in juvenile herring. The highest levels of 'stress' parameters were seen in fish in low and high stocking densities (Table 2).

Table 2. The effects of stocking density on various baseline levels of hematological and biochemical parameters in juvenile herring. Values are means±SE for 6 fish. Significant differences at $p<0.05$ were noted by an *.

Parameter	Low Density	Medium Density	High Density
Cortisol (ng/ml)	6.7±1.5	1.3±0.2	3.6±0.9
Glucose (mg/dL)	143.4±10.6	96.7±5.6	129.1±8.4
Lactate (mg/dL)	61.3±6.3	40.2±9.1	66.8±7.8
Hematocrit	34.2±3.7	23.5±3.4	31.3±3.2
Leucocrit	0.51±0.14	0.51±0.11	0.34±0.21

Discussion

This section of the project has as one of its main aims to perform analytical support for Section I of the project (field studies). Analytical services were performed and were successful. Statistical analysis and conclusions regarding the results of the plasma chemistry analysis, differential white blood cell counts and presence/absence of viral erythrocytic necrosis from Prince William Sound are discussed in Section I of this annual report. Considerable effort was placed into ensuring that the plasma chemistry analysis methods used were appropriate for herring plasma. Any developed methods will be reported as an appendix to the final report.

Preliminary studies in this project revealed that significant differences exist between adult and juvenile herring in their responses to both oil and disease. Therefore, in order to more adequately address the general objective of this project, significant focus was given to understanding the responses of adult herring to the stressors, and to highlight results which may be different from those obtained with juvenile fish.

Adult and juvenile wild herring caught at Barkley Sound on Vancouver Island have shown to be negative with respect to the VHSV virus, although the adult population showed an ITP prevalence exceeding 24%. This value is as high as that of herring sampled in PWS (Marty 1994). In all adult fish used in any study, no identifiable signs of progressive ITP infection were noted.

The sensitivity of fish to environmental contaminants is known to be altered by age and stage of development. In the present studies, adult Pacific herring were more tolerant of OWD exposure than juvenile fish. No acute mortality occurred in adult fish, even at the highest concentrations of hydrocarbons used. These results suggest that, at least on the short term, the acute effect of oil exposure may be significantly more dramatic on younger fish. However, it should be noted that longer exposures to low levels of hydrocarbons may illicit sublethal effects that may be equally as devastating.

A wide variety of adverse environmental conditions including pollution and disease can induce a characteristic series of endocrine and other biochemical and physiological changes in fishes (Mazeaud et al. 1977). Corticosteroid hormone release after exposure to stressors often triggers a variety of biochemical and physiological responses called secondary stress responses. Typical secondary stress responses elicited by increases in plasma cortisol levels include hyperglycemia, depletion of tissue glycogen reserves, catabolism of muscle protein and altered blood levels of protein and cholesterol. As was the case with juvenile herring exposed to OWD in previous experiments, adult herring exhibited the 'classical' stress response when exposed, however, the response was transient and shorter lived than that in younger fish. Sublethal exposure to oil evoked increases in plasma cortisol which has been linked to immunosuppression and increased susceptibility to disease (Thomas 1990). However, the transient nature of the response indicates that any effects of hydrocarbons on the immune system in herring are probably due to direct effects and not by indirect effects of increased corticosteroids. Moreover, other studies with fish have shown that increased corticosteriod production may be due to a particular fraction of oil, and the changing composition of the OWD in this study may have yielded different results than under conditions of a more constant hydrocarbon profile. Increased corticosteroid levels was followed by a hyperlacticemia and hyperglycemia, indicating an increase in energy expenditure in herring as they mobilize energy reserves to compensate for the stress. Again, the transient nature of these responses indicates that this may not be significant to the fish in the longterm unless the compostition of hydrocarbons results in a chronic biochemical response. It should be noted that even if these parameters are returned to normal, other tertiary effects on herring fitness could become evident with these short exposures, and especially with longer sublethal exposures. Clearly, the sublethal exposure duration and hydrocarbon profile has a direct implication in the selection of biochemical parameters to be used as indicators of aquatic contamination and should be the focus of further research. Recommendations for biochemical parameters (and their relevant time frames) that have potential as biomonitoring tools of population recovery will be made in the final report once all data has been synthesized.

Components of oil such as polycyclic aromatic hydrocarbons can affect the immune systems of fish and may result in increases in disease susceptibility. In this study, sublethal exposures of adult Pacific herring to oil resulted in effects on specific components of their

immune system. As in previous studies with juvenile fish, exposures were longterm in nature in these experiments to better mimic conditions which may have occurred during the EVOS. Several important aspects of the herring immune system were affected by oil exposure and included alterations in the population of circulating white blood cells, plasma lysozyme levels, the ability of macrophages in phagocytosis foreign particles, and the respiratory burst activity of macrophages. These alterations may not return to preexposure values up to seven weeks following transfer to uncontaminated water. These results seem significant biologically, however, when adult fish were exposed to oil and subsequently challenged with VHSV, no fish developed the disease or showed significant tissue viral loads. These results indicate that many adult fish may be immune to VHSV at this stage of their lives (Kocan, 1997). The high prevalence of VHSV in spawning herring in PWS noted by Marty et al. (1994), and the outbreaks of VHSV in herring exposed to oil (Meyers et al. unpublished) may indicate that severe stress is needed to overcome the natural defenses of these fish. In both of those situations, the added burden of spawning may be an important factor in disease susceptibility in adult fish. It has been suggested that the costs of reproduction in some species may compromise the immune system (T. Williams pers. comm.), therefore, VHSV or similar disease outbreaks may only occur during spawning or other very stressful events.

Although the concentrations of hydrocarbons in these studies did not result in the mortality of adult herring, significant effects on herring survival were seen when the fish performed the swimming trials and thus were challenged with conditions which may be more realistic in terms of their natural environment. Appropriate swimming performance is paramount to herring as it is imperative in foraging for food, escaping predators, migration etc. Exposure to oil did not affect the ability of herring to swim although, significantly higher mortalities were observed in fish exposed to oil and forced to swim. Due to these increased mortalities in swum fish, it was decided to investigate the effects of oil on the ability of fish to recover from exercise. Significant alterations in biochemistry are known to occur during 'burst' swimming in fish, changes which return to preexercise values shortly after. An inhibition of exercise recovery was seen in this study and would severely inhibit a herring's ability to exercise repeatedly, adding to the reduction in fitness seen in the reduced survival following swimming.

Conclusions

These studies show that exposure of both juvenile and adult herring to oil, VHSV or ITP can alter various aspects of herring fitness. Significant alterations in biochemistry and immunology were noted, although the susceptibility of adult herring to VHSV was far less than juveniles and may only be significant during extremely stressful events such as spawning. The biochemical alterations brought about by stressors seems to be transient, while longer term alterations may occur in the immune system. The acutely lethal effects of oil on herring may be age dependent with older fish being more tolerant, however, factors such as activity levels and fish density may modify the susceptibility of herring to hydrocarbons and disease. The results of these studies continue to explain the roles and mechanisms of oil, VHSV and ITP in herring population dynamics, and will increase the understanding how environmental stress can be monitored and predicted and subsequently used in fisheries management practices.

Acknowledgments

The authors wish to acknowledge Dr. Richard Kocan (author of Section II of this report) for VHSV virus and techniques in viral tissue loads and antibody titres. We would also like to acknowledge Dr. Sue Sanders and Dr. Rosemarie Ganassin for their work on the project. Several technicians including Joanne Scherba and Keith Tierney are also thanked.

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