Exxon Valdez Oil Spill
Restoration Project Final Report

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

Restoration Project 98162
Final Report

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July 1999

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

Restoration Project 98162
Final Report

Study History: An unexplained disappearance of approximately 110K tons of spawning herring occurred in Prince William Sound, AK in 1993-'94. An emergency project was authorized by the *Exxon Valdez* Trustee Council and funded through Alaska Department of Fish and Game in 1994 to investigate the cause of the massive herring loss. This study revealed that VHS, a viral disease previously unreported from Pacific herring was present in < 6% of surviving fish. However, the prevalence of *Ichthyophonus hoferi*, protist pathogen of fish increased from 5% to 29% between 1994 and 1995. As a result of these findings, the Alaska Department of Fish and Game initiated a second study which covered: 1) Annual field surveys in PWS; 2) Controlled experimental disease studies and 3) Physiological studies. The three project components 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%), but it was higher than in any previous year since 1993. A high viral prevalence in PWS in 1998 (15%) was associated with a population decline in the spring of 1999. 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 prevalence of *Ichthyophonus* in the '88 year-class remained constant at 26-30% over four years. Studies in PWS demonstrated that closed pounds play a role in transmission of VHSV to susceptible fish, resulting in the rapid spread of virus and frequently causing significant mortality within the pens. Studies on *Ichthyophonus hoferi* demonstrated that the organism is a potential serious pathogen of herring but no evidence for its role in the herring declines in PWS could be found. Likewise, no evidence for oil-related increased susceptibility to VHSV was found in either wild or lab-reared herring. A natural immunity developed in fish at 2-years-old, about the time of sexual maturity, and fish of all ages developed an acquired immunity following infection and recovery from VHS.

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

Project Data: Refer to Sections I, II, and III for detailed information relating to project data for each sub-project.

Citation:

Kocan, R.M., G.D. Marty, and C.J. Kennedy. 1999. Investigations of disease factors affecting declines of Pacific herring populations in Prince William Sound, *Exxon Valdez* Oil Spill Restoration Project Final Report (Restoration Project 98162), Alaska Department of Fish and Game, Habitat and Restoration Division, Anchorage, Alaska.

Executive Summary (98162)

Introduction

In 1993 an estimated 80% of the Prince William Sound (PWS) spawning herring population disappeared without a trace. Viral hemorrhagic septicemia virus (VHSV), hitherto unreported from Pacific herring, was isolated from 5% of the survivors the following year. Subsequently the virus has been isolated from all age classes of herring from Prince William Sound south to Puget Sound. Other species also found to be infected include the Pacific cod (*Gadus morhua*), Pacific sandlance (*Ammodytes hexapterus*), English sole (*Parophrys vetulus*) and shiner perch (*Cymatogaster aggregata*). In 1992 herring held in the spawn-on-kelp (SOK) fishery in Prince William Sound were observed to have hemorrhages on the skin, fin bases and mouth; they swam erratically and did not spawn properly. Although no virus was isolated at that time, it was noted that the lesions closely resembled those of VHS in wild herring. In 1994, the year after the severe losses, 23% of PWS herring were also found to be infected with *Ichthyophonus hoferi* , originally believed to be a systemic pathogenic fungus, but now known to be related to the "rosette agent" of salmonids.

In 1995, a study was initiated to investigate the disease implications of VHS and *I. hoferi* on herring populations. The project was a collaborative effort among four groups: Alaska Department of Fish and Game, U.C. Davis, Univ. of Washington and Simon Fraser University. These groups approached the disease problem in herring from several aspects; including annual on-site surveys, controlled laboratory and field experiments, and physiological studies to understand the effects of oil and disease on Pacific herring. Field studies focused on annual fluctuations of VHSV and *I. hoferi* in PWS, while controlled experimental studies on laboratory-reared and wild herring demonstrated that VHS virus was unequivocally pathogenic for nonimmune herring, causing extensive mortality in juvenile fish, and that net pen confinement resulted in increased disease transmission via water exposure. Physiological studies showed that oil affected the herring's immune system, but not susceptibility to disease in adult fish, but that post-exercise mortality was affected by oil exposure.

Objectives

Studies were designed to monitor changes in infection and disease status of PWS herring from 1994 through 1997, determine if reproductive status affected the general health of herring and relate oil exposure to prevalence and severity of disease in herring. Controlled experimental studies were designed to describe the natural history of VHSV and *I. hoferi* in herring from hatching to sexual maturity and to confirm that these organisms are actually pathogenic to Pacific herring. Physiologic studies evaluated immune system alteration in herring exposed to oil as well as the effects of infection by VHSV and *I. hoferi* on swimming ability, white blood cell changes and blood chemistry.

Methods

Adult herring from PWS and Sitka Sound were sampled at random in the spring and fall from 1994 through 1998 and subjected to complete necropsy. The significance of gender, weight, length, site and season on changes in plasma chemistry values and disease/parasite prevalence was determined by subjecting the data to multiple regression statistical analysis.

Controlled laboratory studies were carried out by rearing specific-pathogen-free (SPF) herring from egg to adult under sterile conditions and experimentally infecting them with known amounts of virus and *I. hoferi.* Survival, virus replication, transmission, pathogenesis and virus survival were all evaluated under controlled conditions. Wild herring were captured and examined each year from 1995 through 1998 and the prevalence of VHSV and *I. hoferi* monitored as the fish aged. VHSV and *I. hoferi* were both evaluated by culturing herring tissues in vitro and determining the prevalence of each as well as any pathologic damage to the host.

Physiologic changes and blood chemistry alterations were observed in captive wild herring exposed naturally or experimentally to both pathogens. Fish were also exposed to oil to determine if any of the pathologic changes seen in wild PWS fish could be attributed to exposure to *Exxon Valdez* oil or if exposure to oil exacerbated infections by either of the two disease agents being studied.

Herring tissues and water from SOK pounds in PWS and Puget Sound were examined for the presence of VHS virus during confinement and at the time of release from the pounds Both tissues and water were evaluated by in vitro plaque assay on EPC cells.

Ichthyophonus was studied in SPF herring by injecting 8-month-old fish IP with spores. Tissues from all fish were also cultured and processed for histology in order to determine the efficiency of both methods in accurately identifying infected fish. Field studies consisted of sampling wild 0-year and 1+, 2+ and 3+ herring from Prince William Sound and Puget Sound. Wild-caught fresh water sculpins were exposed by feeding infected tissue to determine if carnivory was a route of transmission.

Results

In 1995 -'96 no VHS-virus was isolated from Sitka Sound samples, but in 1997 < 1% of Sitka Sound and 15% of PWS fish were positive. No detectable change in biomass were observed with this increase in VHSV in PWS. The same prevalences recurred in 1998 in PWS and this time it was associated with a population decline, but not until the spring of 1999. VHSV was not isolated from fish from either site from fall samples, but was most prevalent in females, younger fish and fish with external lesions when it was detected in spring samples. Prevalence did not appear to be associated with spawning status.

Prevalence of *Ichthyophonus hoferi* was consistent over 4 years (16% - 23%) but not correlated with population declines. Twelve other parasites ranging from 10 - 100% prevalence were also detected - none significant on population level.

Under controlled experimental conditions viral hemorrhagic septicemia virus (VHSV) was conclusively shown to cause disease and extensive mortality in nonimmune juvenile Pacific herring by 4 to 7 days post-exposure. Unlike wild field-exposed herring, signs of disease in experimental herring were limited to 1-2 mm hemorrhagic areas on the lower jaw, isthmus and around the eye. Only 4 of 130 infected fish had detectable cutaneous hemorrhaging. Fish began shedding new virus 48 h post-exposure with maximum shedding occurring on days 4-5 post exposure. Histopathology revealed primarily: 1) multifocal coagulative necrosis of liver hepatocytes, 2) diffuse necrosis of the kidney interstitial hematopoietic tissues, and 3) diffuse necrosis of the spleen, epidermis and subcutis.

Multi-year studies on three age classes of wild herring captured in Puget Sound demonstrated that once in captivity most schools undergo an epizootic which was highly lethal to 0-year fish (>50%) and produced up to 15% mortality in fish $\geq$ 2 years. A single school of disease-resistant 0-year fish offered evidence that some herring can be exposed to the virus by 6 months of age and develop a solid acquired immunity. Herring exposed to lethal concentrations of virus 6-8 weeks following an epizootic exhibited no mortality in any age class and no virus could be isolated from their tissues.

Infected wild herring transmitted VHSV to SPF herring via water. Controlled studies demonstrated that VHSV could survive for 2 hours in raw, filtered or oiled sea water and as long as 100 hr in the presence of ovarian fluid in seawater.

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, irrespective of their previous exposure history.

VHSV studies in Prince William Sound spawn-on-kelp (SOK) pounds showed a rapid initial increase in the percent of virus-positive fish followed by a second peak in prevalence due to the infection of susceptible fish with virus from the initial shedding. A sample of naturally spawning herring taken from the same vicinity as an active pound was not positive for VHSV even though the pound fish were highly infected. There was no support for the hypothesis that overcrowding increases VHSV prevalence. Pounds with significantly fewer fish per volume of water exhibited a significantly greater prevalence of virus. 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 pounds could be a source of virus for other marine species in the vicinity of the pounds.

These data indicate that closed pound SOK fisheries may: 1) Activate latent infections in previously infected herring, and 2) Enhance VHSV transmission to non-immune fish in the pounds 3) spread the virus to wild fish outside the pounds directly via contaminated water or by release of infected fish.

Laboratory-reared SPF herring injected IP with *Ichthyophonus* spores began dying by 11 days post exposure and had visible lesions on the heart, liver and spleen. Skin lesions (small ulcers in the epidermis) were detectable after 36 days, as were spores in the musculature under the skin. By 56 days post exposure 90% of the fish were dead. Injection of rainbow trout with these same spores did not result in any detectable infection when fish were held at 10 ^{o}C.

Three year classes of wild herring from Puget Sound were examined by gross examination and in vitro culture of tissues. External skin lesions ranged from 4% to 6% in all age groups while 6% of 0-year, 23% of 1+ and 52% of 2+ ages cultured positive for *Ichthyophonus*. There was no significant difference in mortality between the infected and uninfected individuals within age classes. Infected tissues from these herring were fed to Coast range sculpins which became infected and died.

Petroleum hydrocarbons were not acutely lethal to adult herring but did produce significant mortalities in juveniles. No significant alterations in adult herring plasma biochemistry was associated with hydrocarbon exposure but juveniles exhibited a transient classical stress response when exposed for 24 h. Significant mortality was associated with swimming exercise in juveniles following exposure to petroleum hydrocarbons. Adult swimming ability was not affected but significant mortality followed strenuous exercise. Both juveniles and adults exhibited changes in white blood cell populations and phagocytic activity following oil exposure - effects which persisted for over 8 weeks post-exposure.

Discussion

Multiple years of data show several patterns of disease within PWS herring. VHSV was more common in young and females and was associated with external lesions in feral fish, while *I.hoferi* prevalence and severity was greater in mature fish. VHSV probably contributed more to mortality in young fish while *I. hoferi* was more lethal in older individuals.

The pathogenicity of VHSV for Pacific herring was demonstrated by fulfilling Koch's Postulates and establishing it as a possible cause of the extensive losses that occurred in Prince William Sound in 1993-'94. The course of the disease is very rapid, with mortality peaking by 6-8 days post-exposure. Transmission was shown to occur by exposure to virus in the water column and the characteristic

epidermal hemorrhaging described for wild herring did not occur in SPF herring, suggesting that the lesions and ulcers are the result of secondary microbial invaders.

All age classes of herring were shown to be infected with VHSV in PWS and Puget Sound. Capture and captivity resulted in severe epizootics with the most sever mortality occurring in the 0-year fish. By 3-4 weeks post-capture virus was no longer detectable and the fish were solidly immune to challenge infection. Studies with nonimmune herring demonstrated the minimum lethal dose of virus to be ~ 1 X 10^2 pfu*ml^{-1}*hr^{-1}.

Wild herring are infected with VHSV during their first year of life and apparently die or recover and become immune to reinfection. What triggers the disease caused by these pathogens in nature is not clearly understood at this time. Any "stress" condition that affects the immune system could be the trigger; such as confinement, exposure to toxic substances, malnutrition or a combination of these. Confinement would also increase the probability of spreading the virus from fish to fish. Young-of-the-year (0-year) herring appear to suffer high mortality when exposed to VHS virus 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 6 hours in natural sea water and for up to 100 hours in water containing ovarian fluid, supports the hypothesis that water-born transmission may be responsible for the high prevalence rates of VHS observed in the spawn-on-kelp fishery.

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

Wild herring are infected with *I. hoferi* by 2-4 months post-hatch with the highest prevalence (52-75%) in adult spawners. There was no evidence that the organism affected the survival of wild fish, but different environmental conditions and levels of infection could produce different results.

Based on the sculpin feeding studies, carnivorous fish are potentially at risk of becoming infected by eating infected herring. The growth of the I. hoferi following the death of the host also suggests that the parasite is capable of infecting other fish even after the death of it's host.

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 due, at least in part, to infection by one or both of these organisms.

Exposure to oil has not been linked to any long-term affects on herring health or susceptibility to VHSV or *I. hoferi*. It has, however, been linked to changes in the cellular immune response in herring and to severe exercise-associated mortality in both adult and juvenile herring.

Exxon Valdez Oil Spill
Restoration Project Final Report

Causes of Disease in Pacific Herring from Prince William Sound, Alaska, Spring 1994 through Fall 1998

Restoration Project 98162
Section I - Field Component
Final Report

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August 1999

Causes of Disease in Pacific Herring from Prince William Sound, Alaska, Spring 1994 through Fall 1998

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, which included spawn-on-kelp studies, under the title Causes of Disease in Pacific Herring from Prince William Sound, Alaska, during Fall 1996 and Spring 1997. The project effort was continued as the field component of Restoration Project 98162 and 99462; results from these studies are included in this section of the report (note that project 99462 included study in fall 1998 and spring 1998, but only fall 1998 results are included in this report). All results from spawn-on-kelp studies are included in section II 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 are In press: Davis, C. R., G. D. Marty, M. A. Adkison, E. F. Freiberg, and R. P. Hedrick. In press. 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*) population biomass in Prince William Sound declined nearly 80% from 1992 to 1994. Viral hemorrhagic septicemia virus probably contributed most to population decline in 1994. Viral prevalence in spring samples (n = 233-260/year) steadily decreased from 1994 to 1996 (4.7% → 1.9% → 0.0%), but prevalence increased to 15% in 1997 and 1998. Viral disease is acute (days to weeks) and affects mostly young fish. Virus was never isolated from fish sampled in the fall (1995-1998, n = 80-160/year), and virus was isolated from only 2 of 750 fish sampled from the reference site, Sitka Sound (1995-1997). Another disease, caused by the fungus *Ichthyophonus hoferi*, is chronic and infected fish have decreased life expectancy. In Prince William Sound spring samples, annual spring *Ichthyophonus* prevalence slowly decreased from a peak in 1994 (23%) to a low in 1997 (16%) and 1998 (18%), and levels were comparable to Sitka Sound in 1995 (20%) and 1996 (21%). The 1998 viral outbreak in

Prince William Sound was associated with significant population decline, and most Pacific herring fisheries were closed in 1999. More than 10 other parasites occurred in 10 to 100% of the fish, but none were significant at the population level.

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

Project Data: Data include date, location, and time of capture; sex, age, standard length, body weight, gonad weight, and liver weight; gross necropsy findings; plasma chemistry values, white blood cell differential counts; virus analysis (viral hemorrhagic septicemia virus, VHSV, and viral erythrocytic necrosis virus, VEN), and semiquantitative scores from histopathological analysis. All project data is stored in an Excel spreadsheet (188 columns and 2536 rows). The spreadsheet is stored and maintained by Gary D. Marty, VM:APC, Univ. of CA, 1 Shields Ave., Davis, CA 95616; 530-754-8062; e-mail: gdmarty@ucdavis.edu. Data are available on a case by case basis.

Citation: Marty, G. D., T. R. Meyers, G. Carpenter, and N.H. Willits. 1999. Causes of disease in Pacific herring from Prince William Sound, Alaska, spring 1994 through fall 1998, *Exxon Valdez* Oil Spill Restoration Project Final Report (Restoration Project 98162), University of California, Davis, California.

TABLE OF CONTENTS (Field Component - Section I)

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. The population biomass declined again in 1998, resulting in the closure of most fisheries in 1999.

In 1994, 233 Pacific herring were sampled from Prince William Sound: 23% 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 sampled from Prince William Sound and Sitka Sound from spring 1994 through fall 1998. Results from laboratory study exploring details of viral hemorrhagic septicemia virus and *Ichthyophonus* infections under controlled conditions are reported in sections II and III. The effects of the Prince William Sound spawn-on-kelp pound fishery on expression of viral hemorrhagic septicemia virus were studied, and significant findings are reported in section II.

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 if a history of previous oil exposure correlates with prevalence and severity of disease.

Methods

Adult Pacific herring from Prince William Sound were sampled at random and subjected to complete necropsy during two seasons: 1) spring - 1994 through 1998, sample size varied from 233 to 260 fish per year; and 2) fall - 1995 through 1998, sample size varied from 80 to 160 fish per year. Fish in Prince William Sound were all sampled from bays on the north end of Montague Island (Stockdale Harbor, Rocky Bay, and Zaikof Bay). Fish were sampled from a reference site, Sitka Sound, in the spring of 1995 and 1996 (250 fish per year) and in the fall of 1995 (30 fish). Analysis on all fish included gross examination, white blood cell differential counts,

histopathology on 10 organs, a panel of plasma chemistries, IgM levels, and culture of head kidney and spleen for virus isolation. At Sitka Sound in March 1997, 250 Pacific herring were sampled 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 determine significance of gender, age, weight, length, site, and season on plasma chemistry values and disease/parasite prevalence, results were subject to multiple regression statistical analysis. All analyses that included plasma chemistries were corrected for any differences in the laboratory that performed the analysis (3 different laboratories analyzed plasma chemistries during the study). Comparisons were considered significant when $P < 0.050$ and highly significant when $P < 0.010$.

Results

Among all the parasites and diseases identified during 5 years of study, viral hemorrhagic septicemia virus was the organism most highly correlated with changes in population biomass. As population biomass began to recover in 1995, viral prevalence in spring samples decreased from 5.0% in 1994 to 1.9% in 1995 and 0.0% in 1996. During 1995 and 1996, virus was never isolated from Sitka Sound Samples. During all years of study, virus was never isolated from Pacific herring in the fall (either site). In spring 1997 samples, virus prevalence was significantly greater in fish from Prince William Sound (15%) than Sitka (C.8%), but the increase in viral prevalence was not associated with detectable changes in population biomass. However, high viral prevalence in spring 1998 samples from Prince William Sound (15%) was associated with population decline, but the decline was not detected until the fish failed to return to spawn in the spring of 1999. Study as part of another project (99462) revealed that viral prevalence in spring 1999 samples was only 1%, and fish in 1999 were nearly as healthy as they were in 1996. Viral hemorrhagic septicemia virus was more common in females (1997 only), younger fish, and fish with external lesions. Viral-infected fish were equally prevalent in different bays in Prince William Sound, and viral prevalence was not affected by spawning status (prespawning, spawning, and postspawning). Virus prevalence 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 the blood and tissues of the heart and liver (evidence of immunosuppression, or a decreased ability to fight off disease).

Prevalence of *Ichthyophonus* was fairly constant and did not seem to contribute to population decline. However, lesions associated with *Ichthyophonus* were significant (granulomatous inflammation in many organs), and it is likely that *Ichthyophonus* decreased the lifespan of infected fish. In Prince William Sound samples, annual spring prevalence of *Ichthyophonus* peaked in 1994 (23%), and then slowly decreased through 1995 (19%) and 1996 (20%) to lows in 1997 (16%) and 1998 (18%). Levels were comparable to Sitka Sound in 1995 (20%) and 1996 (21%). Prevalence of *Ichthyophonus* was low among 2- and 3-year-olds, increased until fish

were about 5 years old, and then remained fairly constant until age groups disappeared from the population. For example, spring prevalence of *Ichthyophonus* in the 1988 year class did not changed much in from 1994 to 1997 (30, 35, 26, and 30%, respectively). Fish sampled in the Fall were significantly younger than fish sampled in the spring, and this was reflected in a lower *Ichthyophonus* prevalence among fall samples for each year: 1995 (17%), 1996 (14%), 1997 (3.8%), and 1998 (16%).

Twelve other parasites occurred at a prevalence that varied from 10 to 100%. Although some of these parasites were associated with severe lesions in affected fish, none were significant on a population level. Several differences in parasite prevalence were associated with season of capture. For example, prevalence of some parasites decreased from fall to spring; e.g., intestinal cestodes (fall = 7.0 to 36%, spring = 1.5 to 6.5%) and gastric trematodes (fall = 17 to 36%, spring = 5.6 to 15.4%). By comparison, prevalence of other parasites increased from fall to spring; e.g. the renal myxosporean *Sphaerospora* sp. (fall = 3.8 to 5.4%, spring = 9.3 to 16%) and the intestinal coccidian *Goussia* sp.? (fall = 15to 28%, spring = 91 to 96%).

Changes in seasonal prevalence of parasites were associate with microscopic changes in tissues. For example, 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 = 35 to 45%, spring = 4.7 to 29%). Also, energy stores decreased from fall to spring. For example, 4 to 55% of the fall samples had liver cells with only mild or no depletion of energy stores (glycogen depletion), whereas 100% of the spring samples had moderate or severe glycogen depletion.

Discussion

Disease involves a complex interaction of host, environment, and the disease-causing agent (pathogen). The unusually high prevalence of viral hemorrhagic septicemia virus in Prince William Sound during the spring of 1997 was associated with relatively low prevalence of external ulcers, indicating that the fish were not very sick. This is consistent with population estimates by ADFG, which provided no evidence that the population biomass significantly declined between 1997 and 1998. By comparison, the prevalence of virus in 1998 was not different from 1997, but the prevalence of external ulcers was significantly greater in 1998, indicating that fish were sick in 1998. This is also consistent with population estimates by ADFG, which failed to demonstrate enough biomass in 1999, resulting in closure of most of the Pacific herring fisheries. The high *Ichthyophonus* prevalence in 1994 followed a period of low prevalence in 1993, when most of the population died. *Ichthyophonus* prevalence did not increase in 1998, but preliminary histopathological analysis provides evidence that the prevalence of *Ichthyophonus* in 1999 is comparable to the prevalence in 1994: a finding consistent with significant mortality in the previous year (1998).

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 the young fish in the spring, and expression of virus is associated with significant lesions. Expression of virus and development of associated lesions 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.

We considered whether the oil spill could have been linked to disease outbreak 4 years later (in 1993). Fish that were hatched or were yearlings in 1989 at the time of the spill (1988 and 1989 year classes) might have incurred permanent damage to their ability to fight disease (i.e., irreversible immunosuppression). Under normal growth conditions, minor deficiencies in their immune system might have been insignificant. However, disease might have become a serious problem when fish experienced additional stress upon first spawning (1992 and 1993). Stress is well-documented as a cause of immunosuppression, but stress-induced changes usually are reversible if the fish survives. To address the hypothesis of age-related immunosuppression, we examined the association of lesions with age in 1994. Several lesions were significantly associated with age (e.g., pigmented macrophage aggregates), but nearly all these lesions were more severe in older fish (i.e., fish hatched before 1988). Also, among VHSV, *Ichthyophonus*, and ten other common parasites, none were more prevalent in the 1988 and 1989 year classes than in the entire sampled population. Annual age-weight-length analysis by ADFG documented that the population decreased in the absence of abnormal changes in age distribution. Therefore, the weight of evidence suggests that the disease outbreak in PWS was not a result of permanent immune suppression caused by hydrocarbon exposure when fish were larvae or yearlings.

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 and 1998 indicate that the population experienced a disease relapse. The relapse, however, was mostly independent of the spawn-on-kelp pound fishery. Results from continuing study in 1999 indicate that virus prevalence is again down to low levels and the population is healthy. Because population biomass is very low, and recruitment of the 1996 year class was poor, it will be many years before the population recovers. 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 they are 5 years old. Even if initial recruitment of the 1997 year class is strong, population recovery based on restoration objectives cannot be confirmed until at lease the year 2002. Our studies provide evidence that disease affects survival and stock recruitment, and we recommend continued study of major diseases until the population has recovered.

CHAPTER 1 - Causes of Disease in Pacific herring from Prince William Sound, Alaska

G.D. Marty, G. Carpenter, and T.R. Meyers

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 before 1993, Pacific herring supported 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). Twelve 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. This chapter reports the findings from field disease studies from spring 1995 through fall 1998. Details of the results from the first year of study, spring 1994, are already published; they are included as Appendix 1 (Marty et al. 1998).

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 several samples from 1994 - 1998:

Year	Site	Season	Sample size
1994	PWS	spring - spawning	233
1994	PWS	fall	0
1995	Sitka	spring - spawning	240
1995	PWS	spring - prespawning	80
1995	PWS	spring - spawning	180
1995	Sitka	fall	30
1995	PWS	fall	130
1996	Sitka	spring - spawning	240
1996	PWS	spring - prespawning	80
1996	PWS	spring - spawning	180

Year	Site	Season	Sample size
1996	PWS	fall	160
1997	Sitka	spring - spawning	250 (gross necropsy only)
1997	PWS	spring - prespawning	80
1997	PWS	spring - spawning	180
1997	PWS	fall	80
1998	PWS	spring - spawning	250
1998	PWS	fall	100
			Total = 2493

At the reference site (Sitka), fish were captured by purse seine, cast net, or hook and line. 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. Each fish was assigned a unique necropsy number. 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. 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 (Davis et al. 1999).

   Except for the IgM assay, the following analytes were determined by three different diagnostic laboratories -
   Lab #1 - all samples from spring 1994 and spring 1995 were analyzed at Med Veterinary Laboratory, Concord, California. Analyses were done using about 200 µL of sample in a Monarch-plus analyzer from Instrumentation Laboratories (IL®) that was calibrated and

run at a stabilized 25° C. Plasma was analyzed for total protein (biuret method), albumin (bromocresol green method), and CO_2 (enzymatic method); IL® substrates were used to analyze calcium, cholesterol, glucose, phosphorus, total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine phosphokinase (CPK); Sigma® substrates were used to analyze gamma glutamyltransferase (GGT); ion selective electrodes were used to analyze sodium, potassium, and chloride. Osmolality was analyzed at Lab #2 on a Micro Osmometer Model 3MO-plus from Advanced Instruments (Norwood, MA) using 20 µL of sample. Med Veterinary Laboratory went out of business, so all plasma analysis was transferred to Lab #2.

Lab #2 - all plasma samples from fall 1995 and spring 1996 were analyzed by the clinical chemistry laboratory at the Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis. All analyses, including osmolality as before, were done using about 200 µL of sample. Electrolytes (sodium, potassium, chloride, total CO_2) were analyzed using ion selective electrodes on a Beckman Instruments EL-ISE electrolyte analyzer. Enzymes ALP, ALT, AST, and CPK were analyzed at 27°C on a Cobas Mira Analyzer (Roche) using Sigma® Chemical substrates. Total Protein (Biuret method), albumin (bromcresol green method) and calcium were analyzed on a Dacos Analyzer (Coulter Electronics) at 37°C using reagents by Trace America®. Lactate, phosphate, cholesterol, total bilirubin, and glucose were analyzed on a Dacos Analyzer at 37°C using Sigma® Chemical substrates. The Executive Director requested that we cut our budget; therefore, sample analysis was transferred to Lab #3.

Lab #3 - all plasma samples collected from fall 1996 through fall 1998 were analyzed by Chris Kennedy's laboratory at Simon Fraser University using standard techniques: osmolality, sodium, potassium, chloride, ALP, AST, CPK, total protein, albumin, calcium, lactate, phosphate, and glucose.

Blood smears from all samples collected in 1995 through 1998 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; and

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

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, their numbers were combined for most statistical comparisons.

Histopathology

Tissues were sent to the Aquatic Toxicology Laboratory, University of California, Davis, and randomly assigned a processing number 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.

Since Marty et al. (1998) was published, all *Ichthyophonus* cases were re-examined if the organism had been found only in one organ. The diagnosis of *Ichthyophonus* was redone based on more conservative criteria. Several of previously diagnosed *Ichthyophonus* cases were reclassified as not likely to be *Ichthyophonus*. Most commonly, changes in diagnoses were a result of classifying foreign body granulomas in the stomach as *Ichthyophonus* when they probably were not *Ichthyophonus*. Rescoring resulted in a significant decrease in *Ichthyophonus* prevalence, particularly in 1994 and 1995, and the more conservative diagnoses are reported here.

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

For VHSV, *Ichthyophonus hoferi*, and 12 other common parasites, gross and microscopic findings, plus plasma chemistry values, were subjected to multiple stepwise regression to identify a subset of variables that were predictive for infection/infestation by each organism. Several variables were forced into the equation: 1) dummy laboratory variables accounted for systematic differences in the three laboratories where samples were analyzed; 2) a dummy "crowded" variable accounted for the small size of holding containers used only for fish sampled in Sitka in 1995; 3) age (in years); 4) gender (male or female); 5) season (spring or fall); 6) site (PWS or Sitka); 7) holding time (time from capture until necropsy, in minutes); 8) standard body length (mm); and 9) body weight (g). After the initial analysis with all variables, gross and microscopic findings with the strongest predictive values were selected for further stepwise analysis. It was necessary to run at least 2 stepwise analyses in order to minimize the number of cases that were deleted due to missing values. In this way, only cases missing values for one or more of the variables strongly predictive of a plasma chemistry were deleted. For a full list of variables, see Marty et al. 1998. For all analyses, comparisons were considered significant when $P < 0.050$, and the model reported includes only predictors with $P < 0.050$. Use of the term "prevalence" refers to the sample prevalence.

Results

Detailed descriptions and micrographs of most of the significant lesions in Pacific herring have already been published (Marty et al. 1998). Prevalence of ulcers (=severe focal skin reddening), combined with prevalence of VHSV, seemed to be the best indicators of population decline. The combination of ulcers and VHSV was highest in 1994 and 1998, when population biomass was decreasing (Figures 1 and 2). By comparison, high prevalence of VHSV in 1997 was associated with low prevalence of ulcers, consistent with lack of population decline during 1997 (Figures 1 and 2).

VHSV

Several variables were significantly associated with expression of VHSV. Fish from PWS were more likely to have VHSV than fish from Sitka; only 2 of 750 spring samples from Sitka were positive for VHSV. At both sites, VHSV was isolated only from spring samples (all fall samples were negative), and VHSV was more common in lighter fish. Fish with VHSV were more likely to have lower plasma IgM, higher total plasma protein, lower plasma glucose, and a lower PCV.

Fish with VHSV were more likely to have myxosporean parasites in the gall bladder (odds ratio = 3.5).

VHSV prevalence in spring 1997 samples (14.6%) and spring 1998 samples (13.6%) were higher than in any other year studied (Figure 2). As shown in the summary on the next page, VHSV prevalence in 1997 was not significantly different for prespawning or spawning samples. VHSV prevalence in 1997 was consistently higher in samples from Rocky Bay, but fish from Rocky Bay were significantly younger than fish from Stockdale Harbor. In 1997 and 1998, VHSV prevalence was higher in 3-year-old fish than in other year classes (Figure 3). Females were more likely to have VHSV in 1997, but not in 1998 (Figure 4). Gender differences in VHSV prevalence for the entire project were not significant.

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. Affected fish were more likely to have hepatic focal and single cell necrosis (Marty et al. 1998). 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.

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 hoferi*. The third fish, also a 3-yr-old female, had a PCV of 21% and concurrent *Ichthyophonus hoferi* infection.

Ichthyophonus hoferi

Trends in prevalence of *Ichthyophonus hoferi* generally correlated with the mean age of fish in the sample (Figures 5 and 6), although age differences were not significant in the multiple regression analysis. For example, at least some of the decrease in population prevalence of *Ichthyophonus* in

1997 might have been 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 in PWS changed little between 1994 and 1997, but then increased in 1998 (Figure 7). In general, prevalence of *Ichthyophonus hoferi* was less than 10% when fish were first sampled (age 2 or 3), but then increased to over 20% by the time fish reached age 4 or 5 (Figure 7). In 1993 and 1994 year classes sampled in 1998., *Ichthyophonus* prevalence increased during the summer (between spring and fall samples). However, seasonal trends for the entire project were significant for higher *Ichthyophonus* scores in the spring than in the fall.

Severity scores for *Ichthyophonus hoferi* were significantly greater in males and fish captured in the spring. Fish with *Ichthyophonus hoferi* were more likely to have higher concentrations of plasma AST, IgM, and total protein, a lower PCV, and lower plasma cholesterol, osmolality, total bilirubin, and glucose.

All organs contained *Ichthyophonus*, 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).

Other Lesions and Potential Pathogens

No significant bacterial pathogens were isolated. Only three fish, all females less than 5 years old, had erythrocyte inclusions characteristic of VEN, and none of these fish had VHSV or *Ichthyophonus hoferi*. The first fish, a 3-year-old sampled in the fall of 1995 from Sitka, had a PCV of 41% and no unusual lesions. The second fish, a 4-year-old sampled in the spring of 1996 from Sitka, had moderate fin base reddening, a low PCV (32%), a high plasma IgM concentration (1990 mg/L), severe interstitial or hematopoietic cell hyperplasia in the trunk kidney, severe atrophy of zymogen granules in the exocrine pancreas, and abundant hepatic macrophage aggregates. The white blood cell count was low enough that in 200 oil-immersion fields examined, only 2 thrombocytes were observed. The third fish, a 3-year-old sampled from PWS in the fall of 1996, had severe fin base reddening, a PCV of 54%, and the highest CPK value of the entire study (27,400 IU/L).

Pacific herring had 15 other common parasites, and prevalence of these parasites was highly variable based on age, gender, season, site of capture, length, weight, other parasites, and plasma chemistry values (multiple logistic regression). Herring worms, members of the family Anisakidae, were in nearly 100% of the fish from all samples, seasons, and sites. By comparison, prevalence of several other parasites varied by season, and changes in prevalence of some of the parasites were associated with the population crash of 1998. Basic descriptions and micrographs of many of these parasites in Pacific herring have already been published (Marty et al. 1998).

The intestinal coccidian parasite *Goussia* sp.(?) occurred in greater than 90% of the fish in every spring sample from PWS and Sitka (Figure 8), but less than 30% of the fish in fall samples had intestinal coccidians, and seasonal differences were highly significant (P <0.0001). Fish with the intestinal coccidian parasite were more likely to have gastric trematodes (odds ratio = 1.7) and hepatic coccidians (odds ratio = 1.6), but they were less likely to have intestinal cestodes (odds ratio = 0.6). Prevalence of intestinal coccidian parasites did not vary by site of capture, gender, age, or size.

Prevalence of the hepatic coccidian parasite *Goussia* [*Eimeria*] *clupearum* was consistently greater than 60%, but seasonal variation depended on the year of study (Figure 9). In 1995 and 1996, the trend was towards greater sample prevalence in spring than in fall samples; by comparison in 1997 and 1998, the trend was towards greater sample prevalence in fall than in spring samples (Figure 9). Based on stepwise logistic regression, prevalence was significantly greater in PWS than in Sitka samples (P = 0.03). Also, fish with the hepatic coccidian parasite had greater prevalence of intestinal coccidians (odds ratio = 1.4) and decreased PCV. Prevalence of hepatic coccidian parasites did not vary by season, gender, age, or size.

Prevalence of the testicular coccidian parasite *Eimeria sardinae* in adult males was significantly greater in the spring than the fall (Figure 10), and prevalence was greater in younger fish regardless of season. Within a given age group, heavier fish were more likely to have testicular coccidians. Fish with testicular coccidians had greater prevalence of *Ichthyophonus hoferi*. Among plasma chemistries, fish with testicular coccidians had significantly greater ALP and calcium, but AST and osmolality were significantly less. Prevalence did not vary by site of capture or by length.

Overall prevalence of the gall bladder myxosporean *Ceratomyxa auerbachi* was significantly greater in spring samples (odds ratio = 1.8). Interestingly, the highest prevalence for fall samples was in 1997, the season before the population crashed in 1998. By comparison, the lowest prevalences for fall samples were in 1996 and 1998, consistent with healthy populations during those years (Figure 11). The gall bladder myxosporean was significantly more likely in females than in males (odds ratio = 1.8), in longer fish, in fish with VHSV titer ≥1000 (odds ratio = 3.3), in fish with intestinal cestodes (odds ratio = 1.6), but less likely in fish with *Ichthyophonus hoferi*. Among plasma chemistries, fish with the gall bladder myxosporean had less plasma total protein, albumin, and potassium, but greater lactate concentrations. Prevalence did not vary by site of capture or by weight.

Prevalence of the myxosporean parasite *Ortholinea orientalis* in the archinephric ducts of the kidney was highly variable between 10 and 30% Figure 12). *Ortholinea orientalis* was significantly more likely in males (odds ratio = 1.3) and in fish with gastric trematode parasites (odds ratio = 1.4), but was less likely in fish with the other intraductal renal myxosporean parasite *Sphaerospora* sp.? (odds ratio = 0.61). Among plasma chemistries, fish with *Ortholinea orientalis* had less total protein but greater CPK and lactate concentrations. Prevalence did not vary by age, season, site of capture, length, or weight.

Prevalence of the other myxosporean parasite *Sphaerospora* sp. (?) in the renal archinephric ducts (Figure 13) was significantly greater in spring samples (odds ratio = 3.2). *Sphaerospora* sp. (?) was less likely in fish with the other intraductal renal myxosporean parasite *Ortholinea orientalis* (odds ratio = 0.61). Prevalence of *Sphaerospora* sp. (?) was not significantly associated with age, gender, site of capture, or size.

Prevalence of the gill parasite *Epitheliocystis* (Figure 14) was significantly greater in spring samples (odds ratio = 4.8) and in younger fish. *Epitheliocystis* was more likely in fish with monogenetic trematode parasites in the gill (odds ratio = 1.9), and fish with *Epitheliocystis* had higher plasma ALP. *Epitheliocystis* was not significantly associated with gender, site of capture, or size.

Prevalence of digenetic trematode parasites in the stomachs was significantly greater in fall samples (Figure 15; odds ratio = 2.9), fish from Sitka (odds ratio = 1.9), longer fish, heavier fish, and in younger fish. Fish with digenetic trematodes in the stomach had less plasma chloride and lactate, and they were more likely to have intestinal coccidians (*Goussia* sp.?; odds ratio = 1.6) and intestinal cestodes (odds ratio = 1.8). Digenetic trematodes in the stomach were not significantly associated with gender.

Prevalence of the intestinal trematode parasites (e.g., *Lecithaster gibbosus*) was significantly greater in the spring (Figure 16; odds ratio = 7.2), in fish from PWS (odds ratio = 10.4), and in younger fish. Seasonal differences were not consistent; as evidence, 1996 prevalence of intestinal trematodes was greater in the fall than in the spring. Fish with intestinal trematodes had higher plasma chloride and total protein, but lower plasma osmolality and calcium. Intestinal trematodes were not significantly associated with size or gender.

Prevalence of the intestinal cestode parasites (e.g., *Nybelinia surmenicola*) was significantly greater in the fall (Figure 17; odds ratio = 8.0) and in shorter fish. Fish with intestinal cestodes were more likely to have monogenetic trematodes in the gill (odds ratio = 3.1). Intestinal cestodes were not significantly associated with age, weight, gender, or site of capture.

The ciliated protozoan parasites (e.g., *Trichodina* sp.) occurred in the gills of Pacific herring sampled in the spring only (Figure 18). Ciliated protozoans never occurred in fish sampled in the fall or in fish sampled from Sitka. Prevalence was also highest during periods of population decline (e.g., 1994 and 1998). Ciliated protozoans were more likely in longer fish, but less likely in heavier fish. Fish with ciliated protozoans had significantly less plasma total protein than fish without ciliated protozoans. Prevalence of ciliated protozoans was not associated with age or gender.

Prevalence of monogenetic trematode parasites on the gills was significantly greater in the spring (Figure 19; odds ratio = 7.2), in fish from PWS (odds ratio = 4.4), and in longer fish. Fish with monogenetic trematodes were more likely to have Epitheliocystis in the gills (odds ratio = 2.0)

and intestinal cestodes (odds ratio = 3.2). Monogenetic trematodes were not significantly associated with age, length, or gender.

Prevalence of cysts of unknown etiology in the gills was similar in spring and fall, although in 1996 the prevalence was higher in fish sampled in the fall (Figure 20). These cysts were not systematically scored until 1995, so prevalence in 1994 was not determined. Additional statistical analysis of these poorly characterized structures were not done.

Prevalence of copepod parasites on the medial operculum ranged from 40 to 65%, and seasonal variation was minimal (Figure 21). These parasites were not systematically scored until 1996, so prevalence in 1994 and 1995 was not determined, and site comparisons were not possible. Additional statistical analysis was not done.

Trends in variables affecting parasite prevalence can be summarized as follows:

Parasites with significantly greater prevalence in the spring:

1) intestinal coccidian *Goussia* sp.(?)
2) testicular coccidian *Eimeria sardinae*
3) gall bladder myxosporean *Ceratomyxa auerbachi*
4) myxosporean *Sphaerospora* sp. (?) in the renal archinephric ducts
5) gill *Epitheliocystis*
6) digenetic trematodes in the intestine
7) ciliated protozoan *Trichodina* sp.
8) monogenetic trematodes in the gills

Parasites with significantly greater prevalence in the fall:

1) digenetic trematodes in the stomach
2) cestodes in the intestine

Parasites more common in older fish: none

Parasites more common in younger fish:

1) testicular coccidian *Eimeria sardinae*
2) gill *Epitheliocystis*
3) digenetic trematodes in the stomach
4) digenetic trematodes in the intestine

Parasites more common in males:

1) testicular coccidian *Eimeria sardinae*
2) myxosporean *Ortholinea orientalis* in the archinephric ducts of the kidney

Parasites more common in females:

1) gall bladder myxosporean *Ceratomyxa auerbachi*

Parasites more common in PWS:
1) hepatic coccidian *Goussia* [*Eimeria*] *clupearum*
2) digenetic trematodes in the intestine
3) ciliated protozoan *Trichodina* sp.
4) monogenetic trematodes in the gills

Parasites more common in Sitka:
1) digenetic trematodes in the stomach

Parasites more common in heavier fish:
1) testicular coccidian *Eimeria sardinae*
2) digenetic trematodes in the stomach

Parasites more common in lighter fish:
1) ciliated protozoan *Trichodina* sp.

Parasites more common in longer fish:
1) gall bladder myxosporean *Ceratomyxa auerbachi*
2) digenetic trematodes in the stomach
3) ciliated protozoan *Trichodina* sp.
4) monogenetic trematodes in the gills

Parasites more common in shorter fish:
1) cestodes in the intestine

Discussion

VHSV

The unusually high prevalence of viral hemorrhagic septicemia virus in Prince William Sound during the spring of 1997 and 1998 is consistent with observations of suboptimal spawning activity and with significant decline of the population biomass between spawning in 1998 and 1999 (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. Evidence from fall 1998, and preliminary evidence from spring 1999 indicates a slight increase in *Ichthyophonus* prevalence that may be partly in response to high virus prevalence in 1998.

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 seemed 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 probably play a role in determining the effect of a VHSV outbreak on population health.

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 (<15%, Marty et al. 1999) 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.

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 25% in 1994. 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 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. 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).

Other parasites

Changes in prevalence of the other parasites provide clues to the health of Pacific herring populations. Although these parasites are probably of minimal population significance as primary pathogens, many seem to act as markers of fish health. For example, increased prevalence of 8 parasites in the spring is consistent with lack of feeding and a depressed immune system during the long Alaskan winter. Increase prevalence of two gastrointestinal parasites in the fall (gastric trematodes and intestinal cestodes) may be a general indication of active feeding and good health in the population. Parasites that were more common during population decline in 1994 and 1998 include gill parasites (ciliated protozoans *Trichodina* sp. and monogenetic trematodes), and *Sphaerospora* sp.? in the trunk kidney. The gall bladder myxosporean *Ceratomyxa auerbachi* was increased in fall 1997, possibly indicating stressed fish entering the winter of 1997-98. Consistent as in indicator of poor population health, spring sample prevalence of gastric trematodes was lowest in PWS samples in 1994 and 1998. Also, the prevalence of gastric trematodes was lower in spring samples from PWS than in spring samples from Sitka.

Acknowledgments

We thank S.D. Moffitt, K. St. Jean, N.J. Speer, C.T. Stack, and J. Wilcock for technical assistance. D. Janka captained the *Auklet*, W. Widmann sectioned tissues, F.C. Teh performed the IgM assay with assistance from C.R. Davis. K.A. Burek, K. Mero, and S.J. Teh performed necropsies.

Literature Cited - All literature cited in this chapter is listed in a single section for the end of the entire report, just before the appendices.

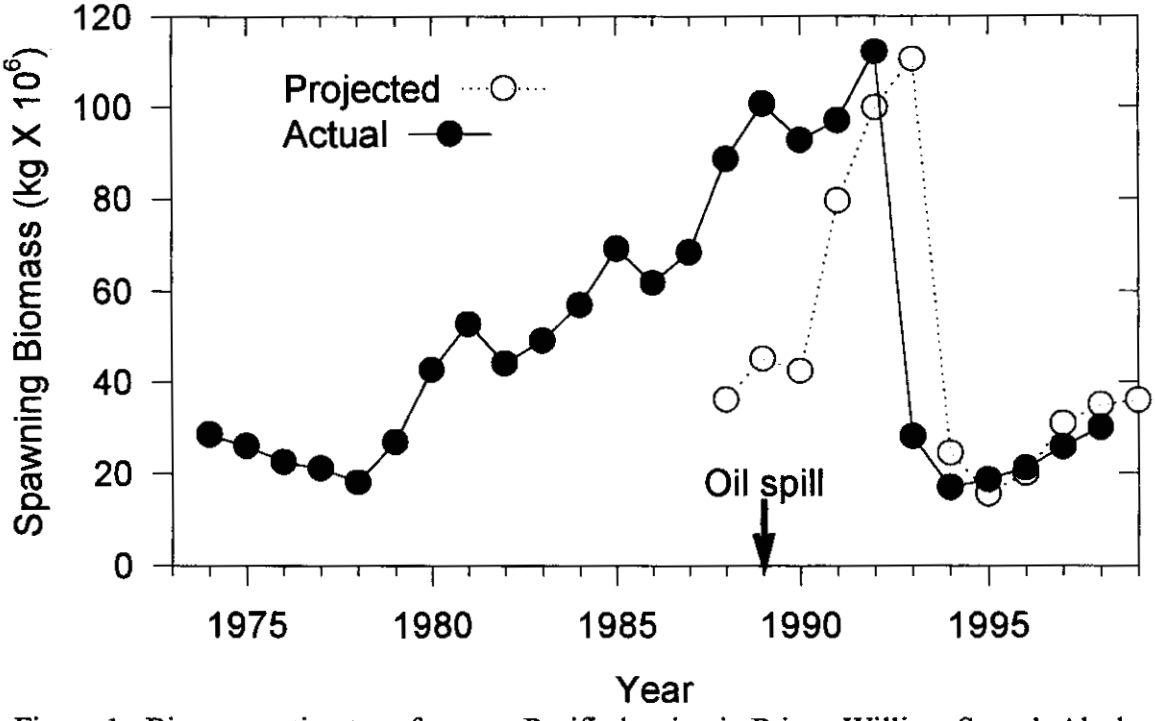

Figure 1. Biomass estimates of mature Pacific herring in Prince William Sound, Alaska. Unexploited spawning biomass projected in the year before spawning (PROJECTED) and calculated after spawning (ACTUAL) using the age-structure assessment model. Estimates were made by John Wilcock, Alaska Department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

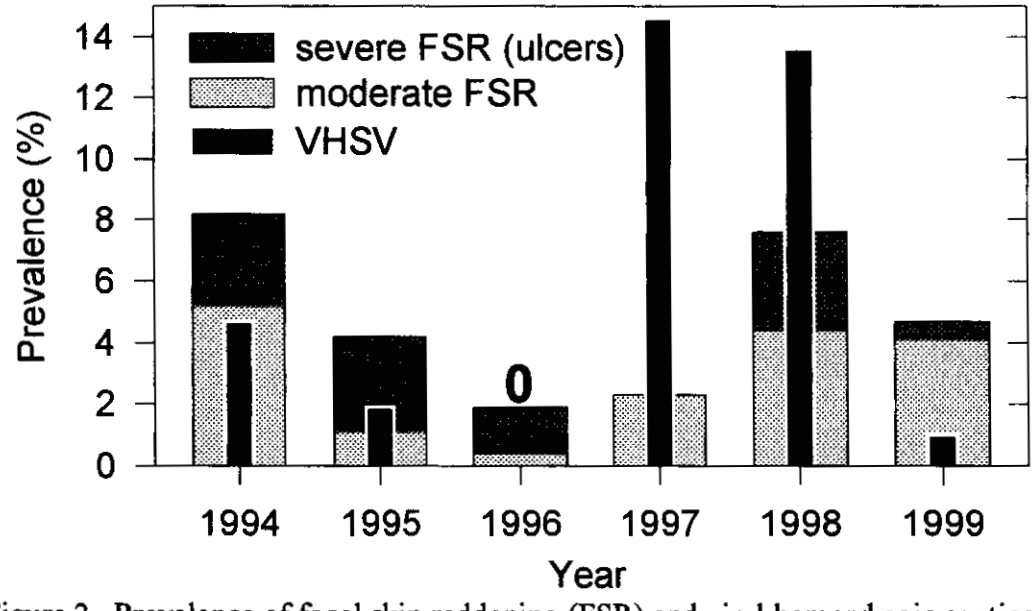

Figure 2. Prevalence of focal skin reddening (FSR) and viral hemorrhagic septicemia virus (VHSV) in adult Pacific herring sampled in the spring from Prince William Sound, Alaska (n = 233-260 per year). Compare with VHSV prevalence in Pacific herring from Sitka Sound (n = 240-250 per year) in spring 1995 (0%), spring 1996 (0%), and spring 1997 (0.8%).

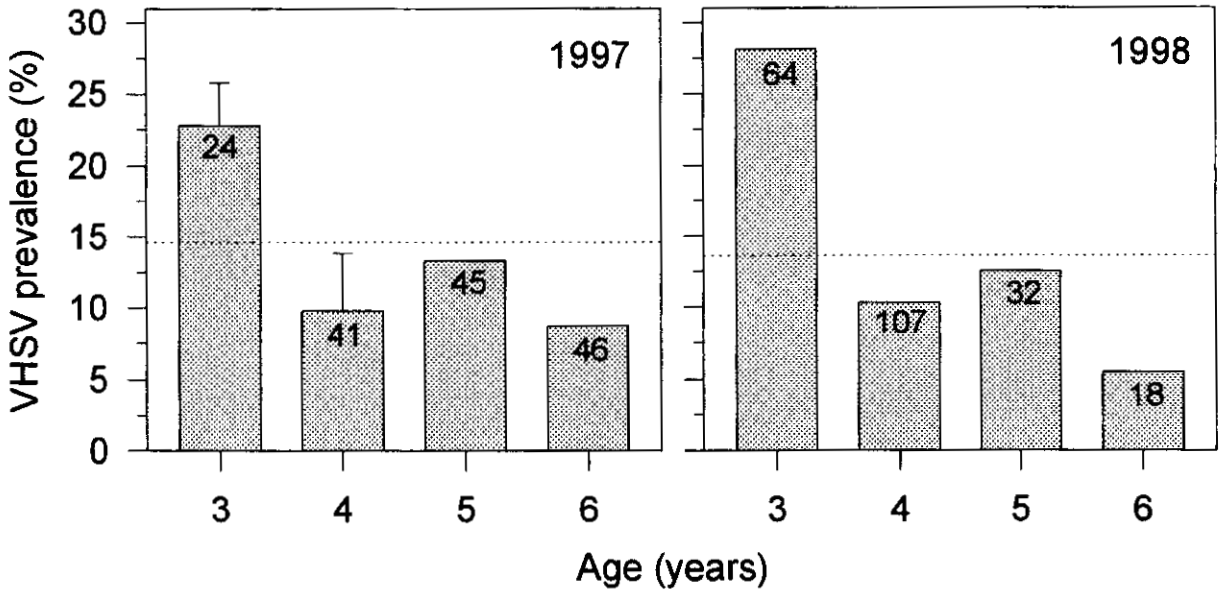

Figure 3. Age prevalence of viral hemorrhagic septicemia virus (VHSV) in adult Pacific herring sampled in the spring from Prince William Sound, Alaska (n = 250-260 per year). The reference line is at the VHSV prevalence for the entire sample (all ages). Numbers within the bars indicates the number of fish examined within each age group.

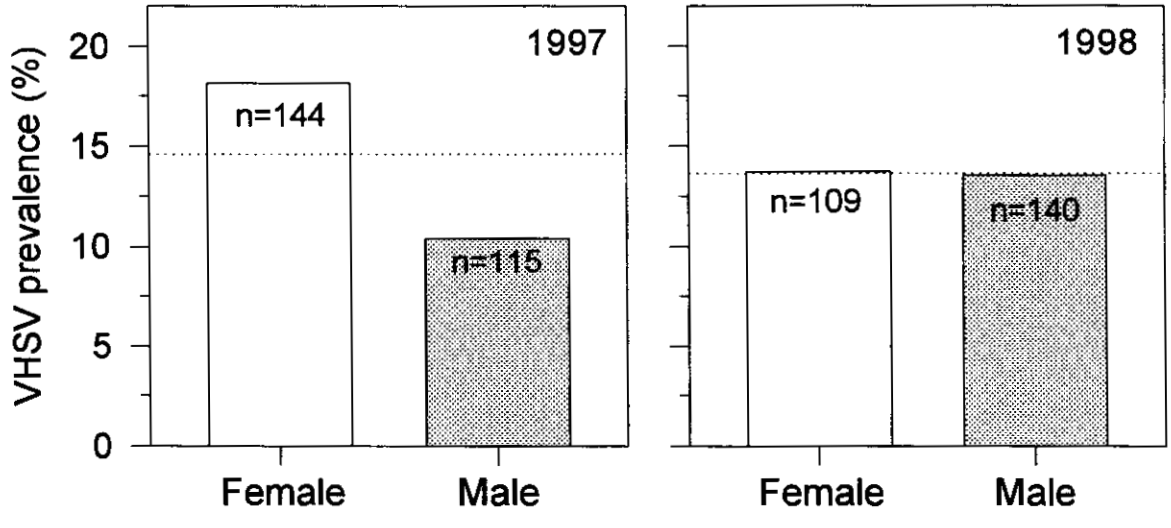

Figure 4. Gender prevalence of viral hemorrhagic septicemia virus (VHSV) in adult Pacific herring sampled in the spring from Prince William Sound, Alaska (n = 250-260 per year). The reference line is at the VHSV prevalence for the entire sample. Numbers within the bars indicates the number of fish examined within each gender.

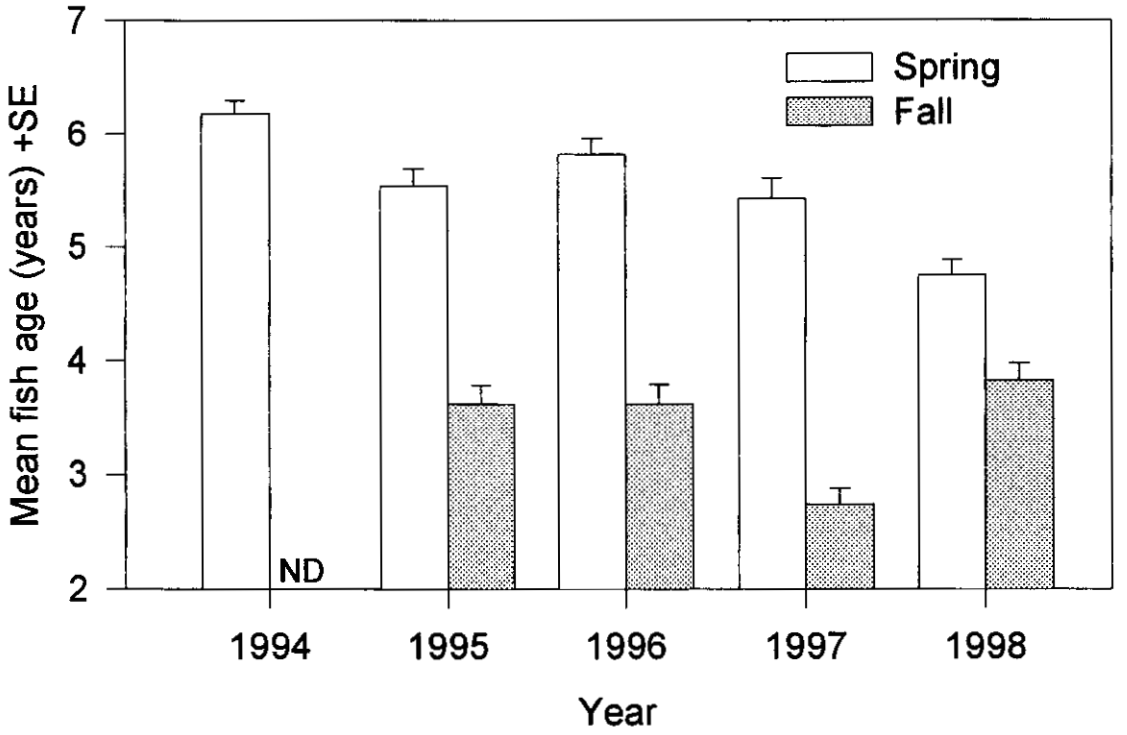

Figure 5. Age of Pacific herring sampled from Prince William Sound, Alaska (n = 233-260 per spring, 80-160 per fall). Compare mean age of Pacific herring from Sitka Sound (n = 240-250 per year) in spring 1995 (5.1 ± 0.13), spring 1996 (4.6 ± 0.12), and spring 1997 (4.9 ± 0.15).

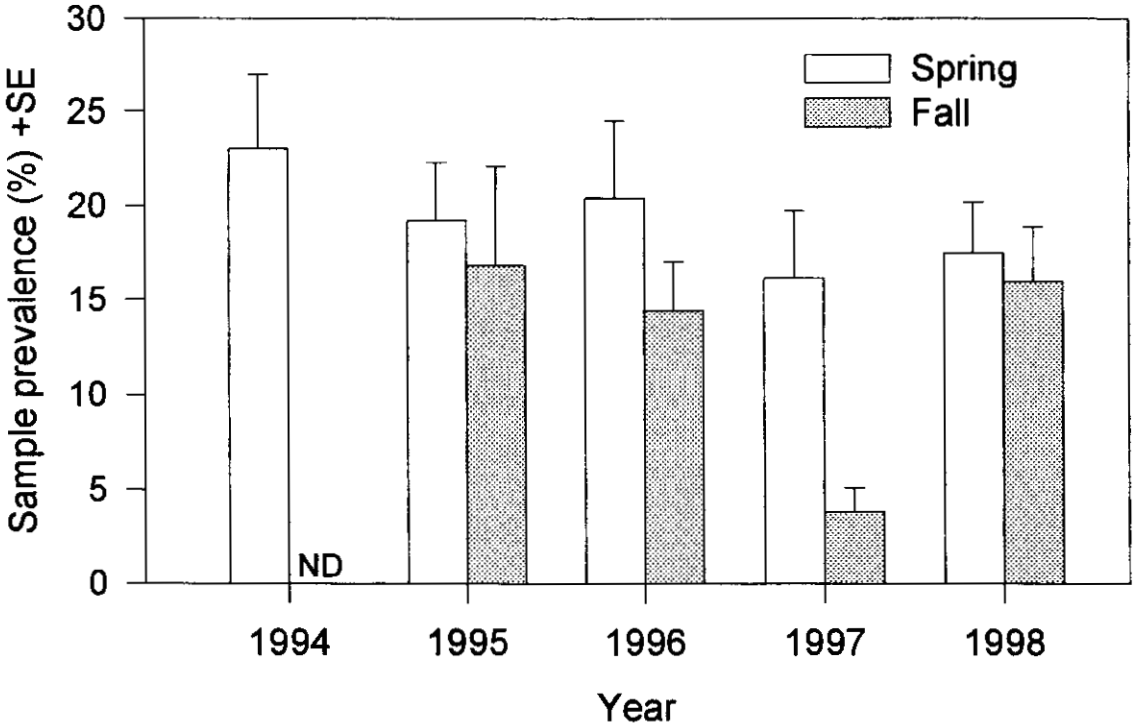

Figure 6. Mean sample prevalence of *Ichthyophonus hoferi* in Pacific herring sampled from Prince William Sound, Alaska (n = 233-260 per year). Compare with *I. hoferi* prevalence in Pacific herring from Sitka Sound (n = 240-250 per year) in spring 1995 (19.6 ± 2.6 %) and spring 1996 (21.3 ± 3.0 %). ND = no data.

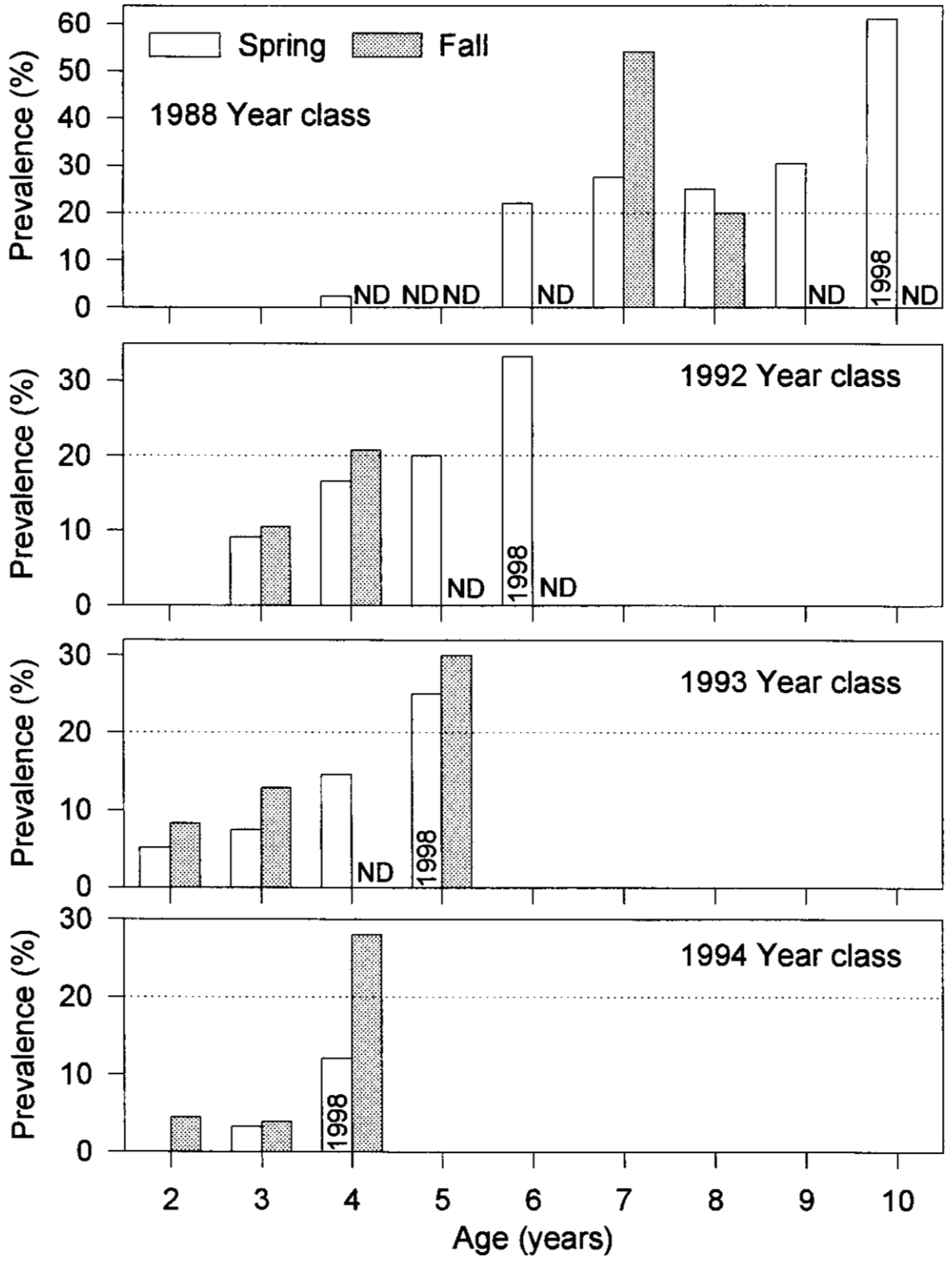

Figure 7. Mean sample prevalence of *Ichthyophonus hoferi* in selected Pacific herring year classes sampled from Prince William Sound, Alaska. Samples that contained less than 10 fish of a given year class are designated as "ND" (no data). The reference line is at 20% for each year class.

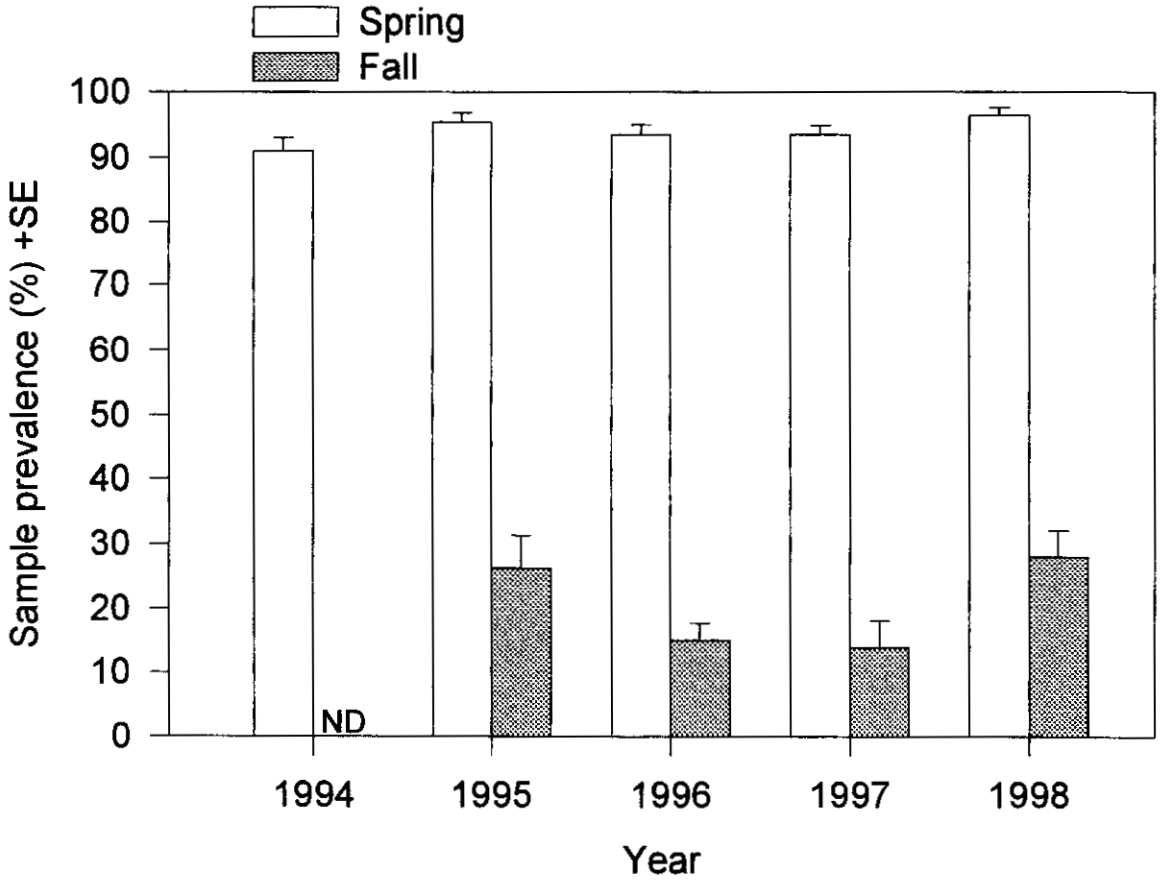

Figure 8. Prevalence of the intestinal coccidian parasite *Goussia* sp.? in adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (91.3 ± 1.7) and spring 1996 (92.1 ± 1.3). ND = no data.

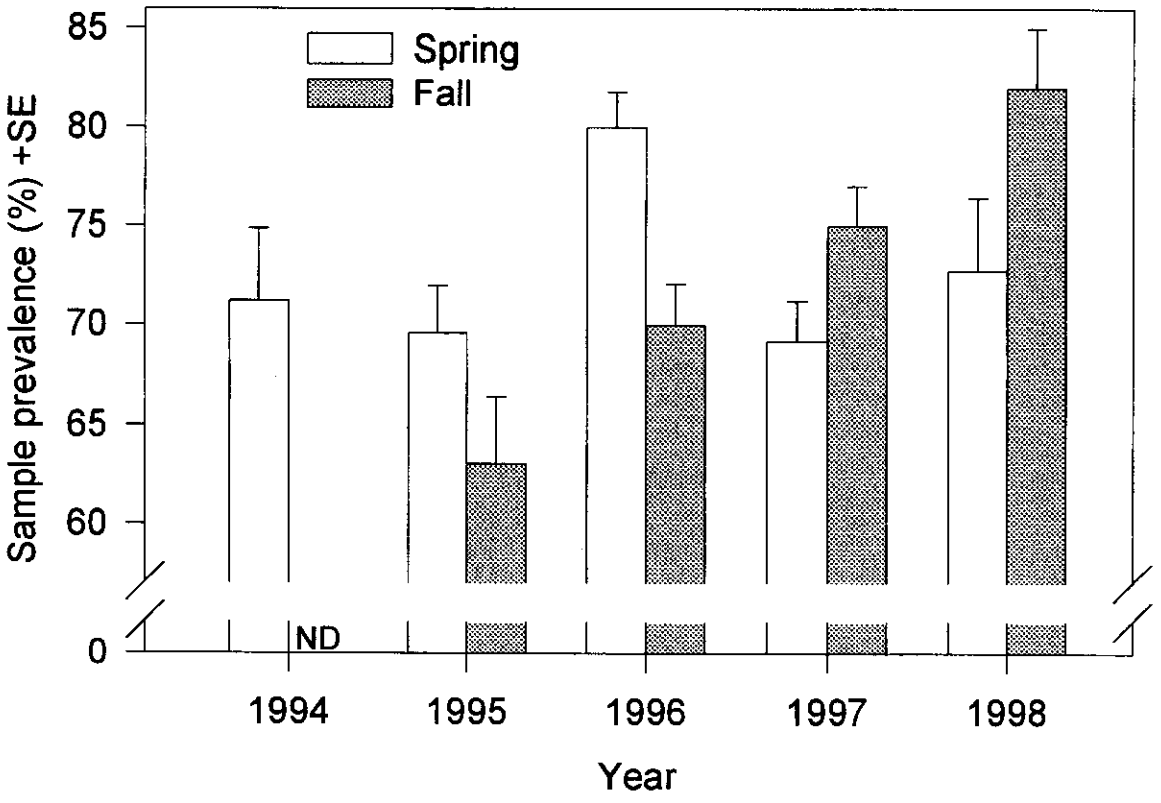

Figure 9. Prevalence of the hepatic coccidian parasite *Goussia* [*Eimeria*] *clupearum* in adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (72.9 ± 3.3) and spring 1996 (79.2 ± 2.8). ND = no data.

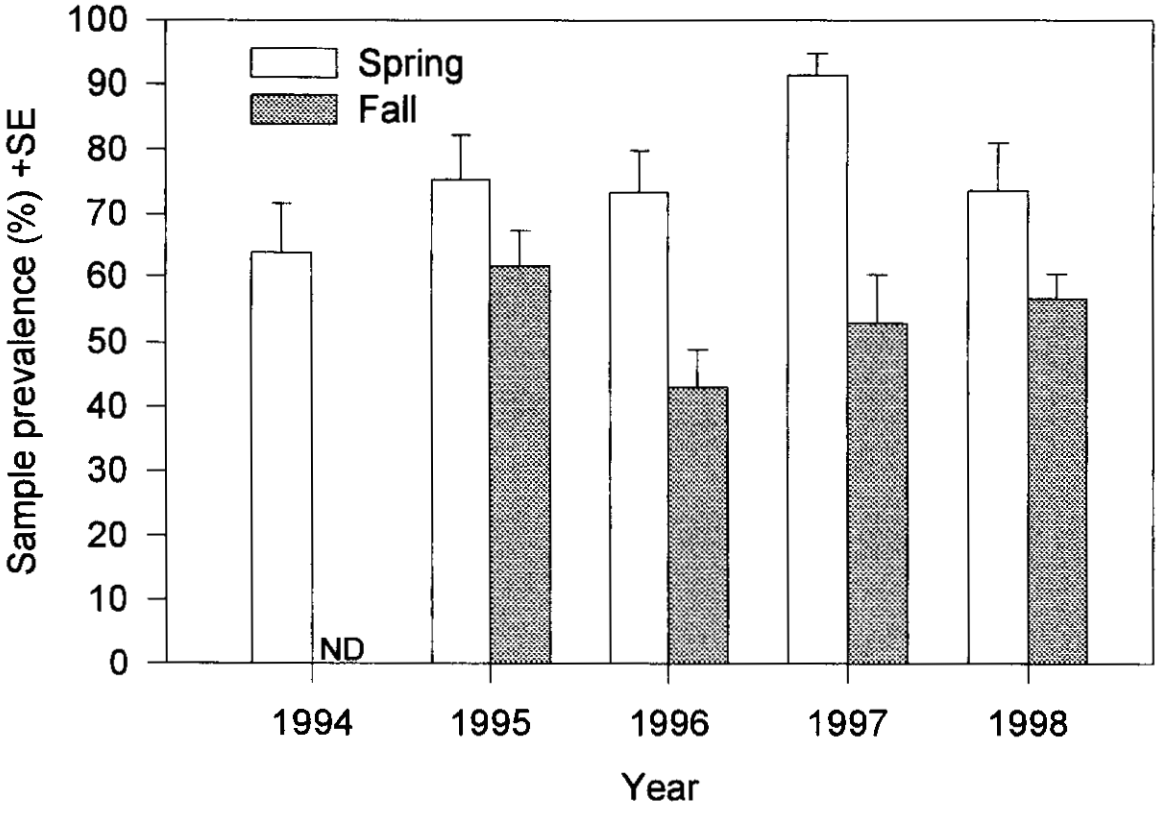

Figure 10. Prevalence of the testicular coccidian parasite *Eimeria sardinae* in adult male Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (65.5 ± 4.8) and spring 1996 (81.1 ± 3.7). ND = no data.

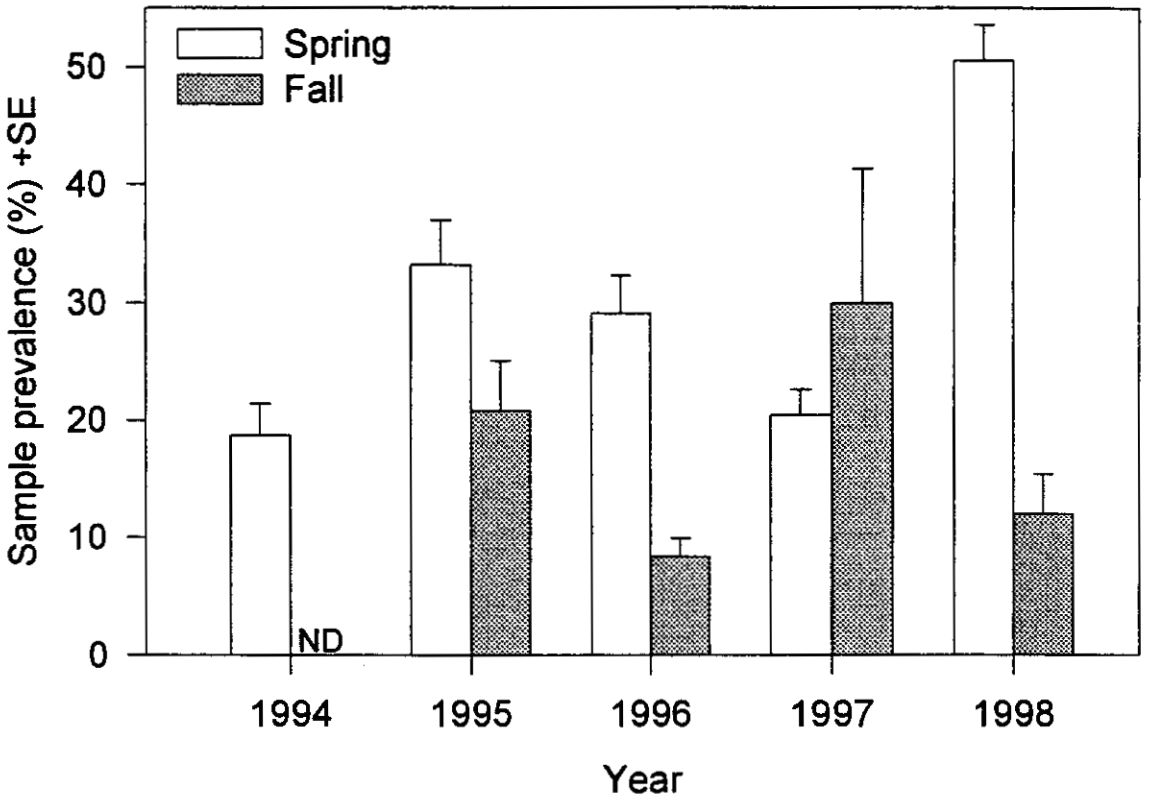

Figure 11. Prevalence of the gall bladder myxosporean *Ceratomyxa auerbachi* in adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (70 ± 32.4) and spring 1996 (54 ± 23.4). ND = no data.

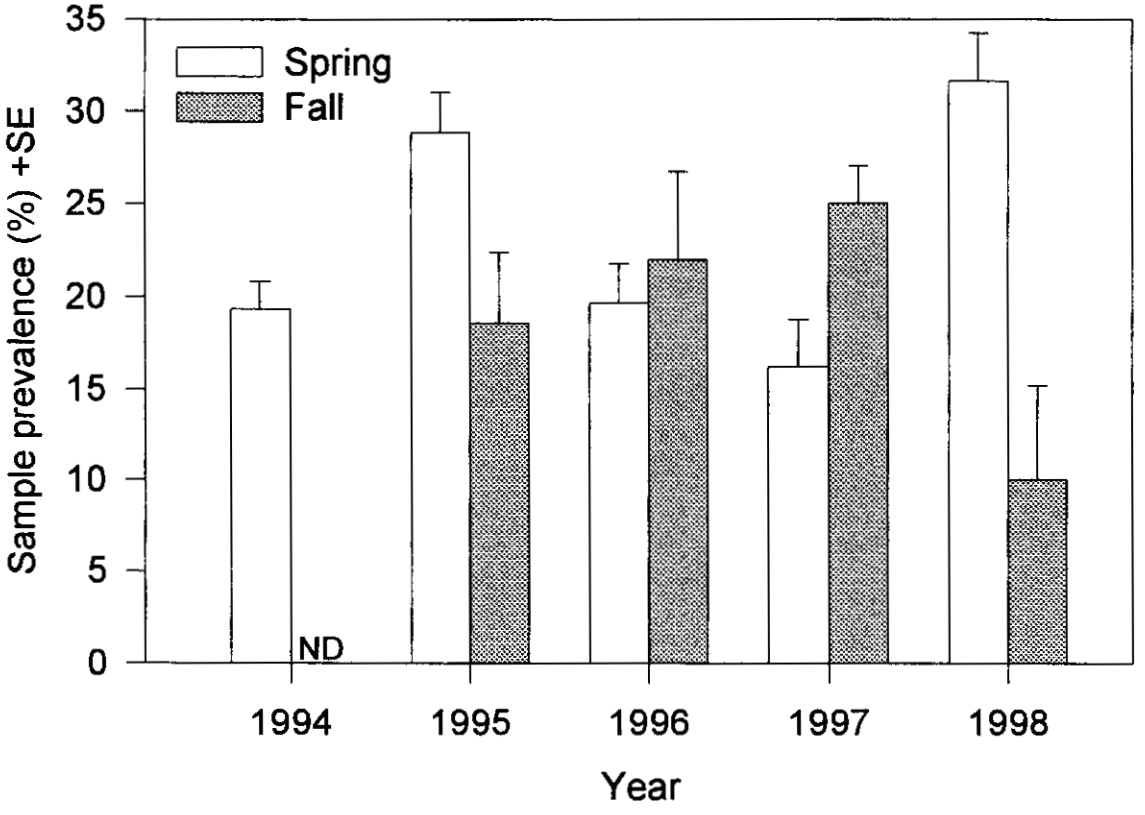

Figure 12. Prevalence of the myxosporean parasite *Ortholinea orientalis* in the archinephric ducts (kidney) of adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (20.4 ± 3.8) and spring 1996 (27.5 ± 2.3). ND = no data.

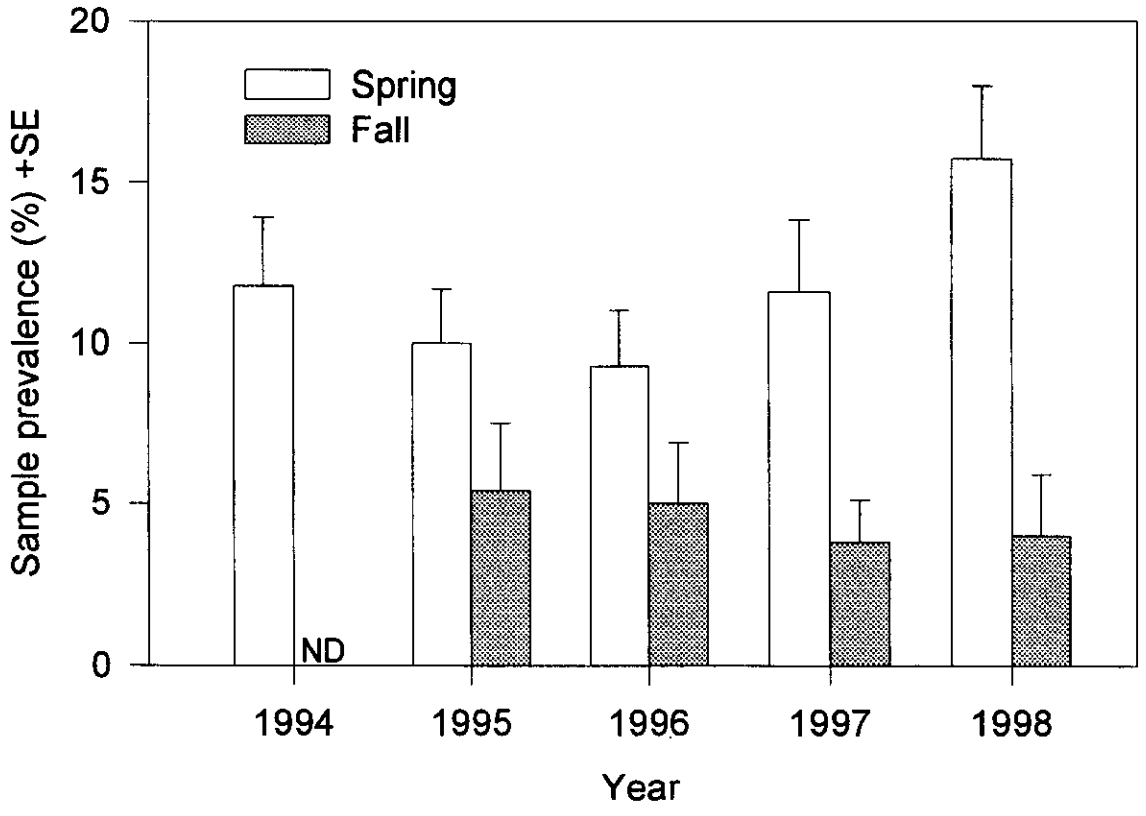

Figure 13. Prevalence of the myxosporean parasite *Sphaerospora* sp.? in the archinephric ducts (kidney) of adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (3.8 ± 1.1) and spring 1996 (7.9 ± 1.8). ND = no data.

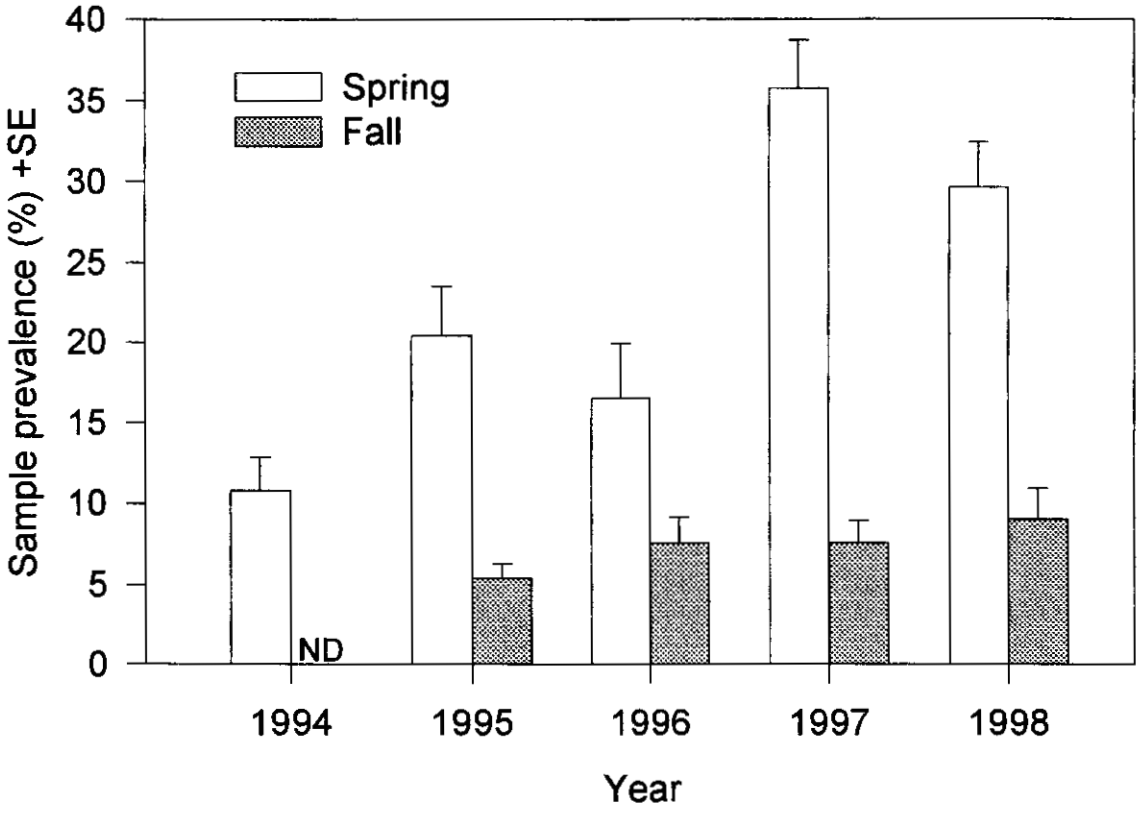

Figure 14. Prevalence of the rickettsia-like organism *Epitheliocystis* sp. in the gill (and rarely in the skin) of adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (25.0 ± 3.2) and spring 1996 (21.3 ± 2.4). ND = no data.

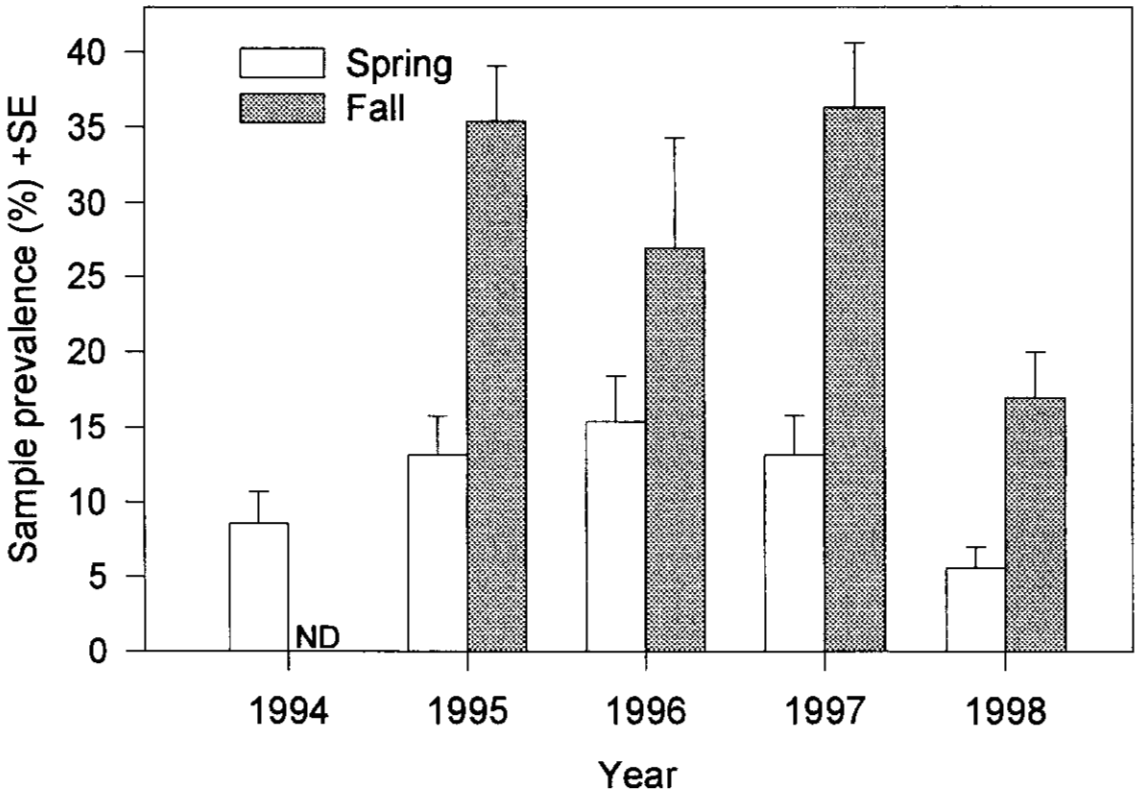

Figure 15. Prevalence of digenetic trematode parasites in the stomachs of adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (10.4 ± 2.8) and spring 1996 (22.1 ± 2.2). ND = no data.

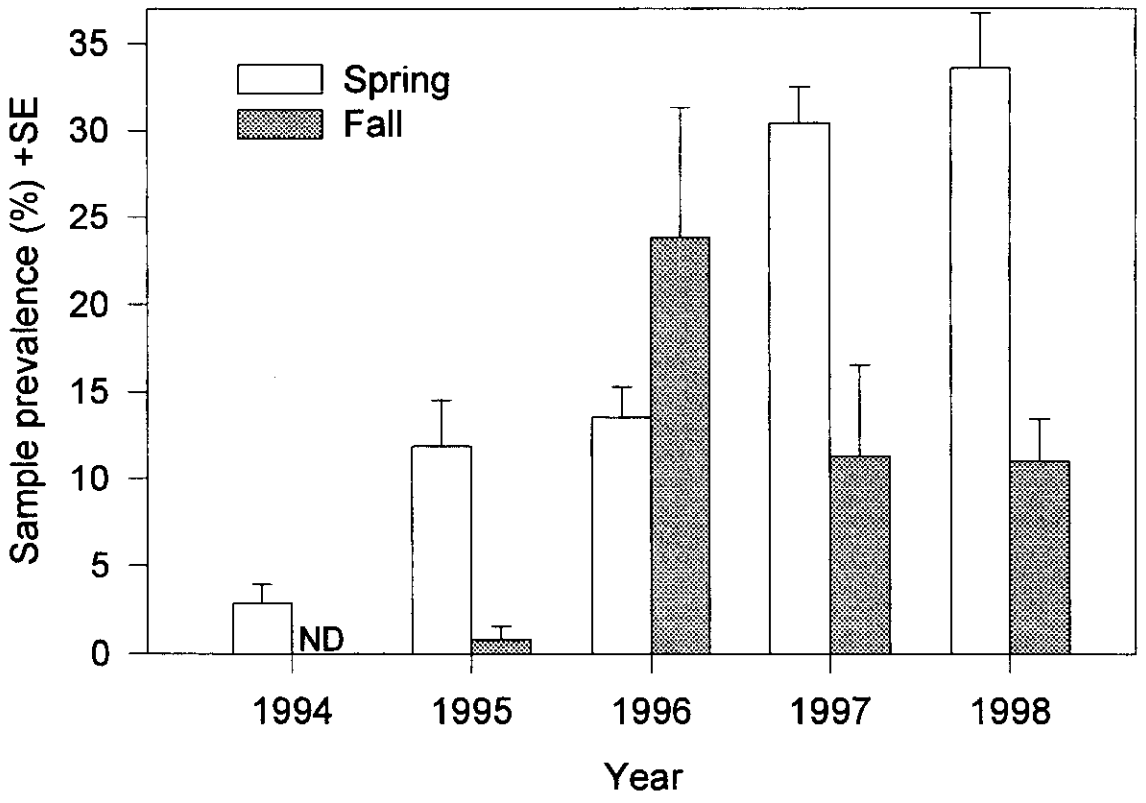

Figure 16. Prevalence of the intestinal trematode parasites (e.g., *Lecithaster gibbosus*) in adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (2.1 ± 1.4) and spring 1996 (1.7 ± 0.94). ND = no data.

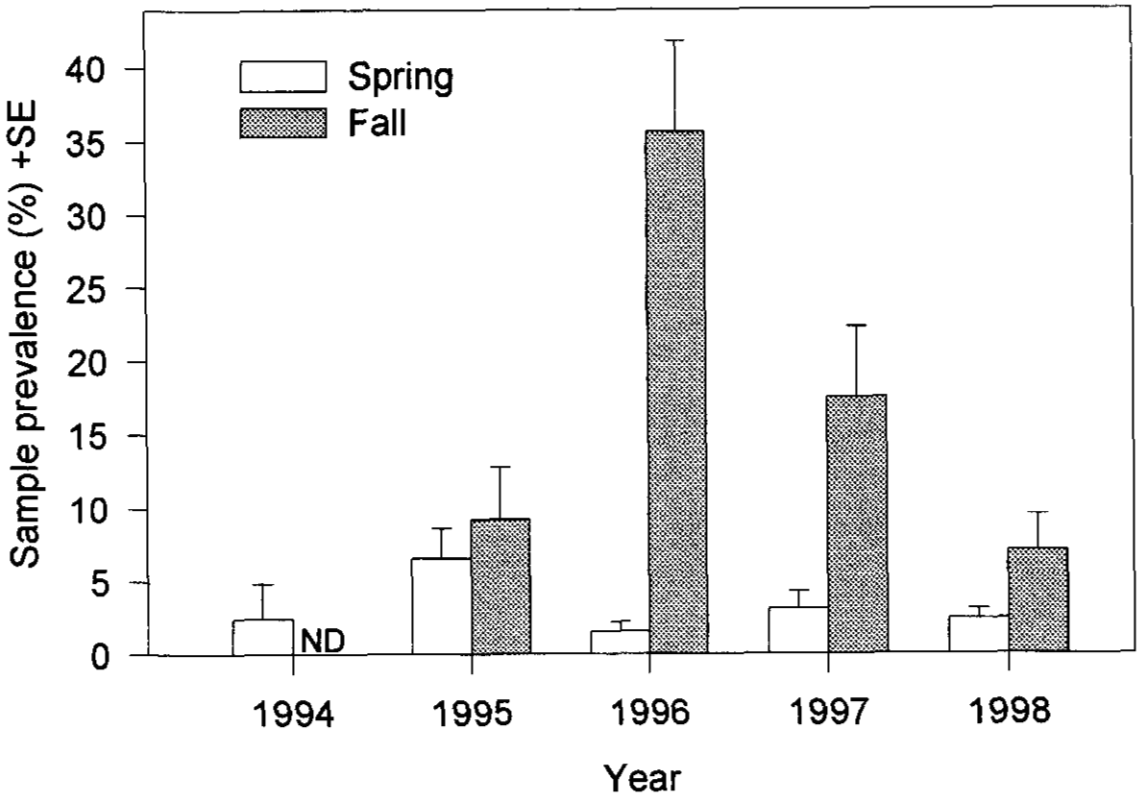

Figure 17. Prevalence of the intestinal cestode parasites (e.g., *Nybelinia surmenicola*) in adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (2.5 ± 1.2) and spring 1996 (3.8 ± 1.3). ND = no data.

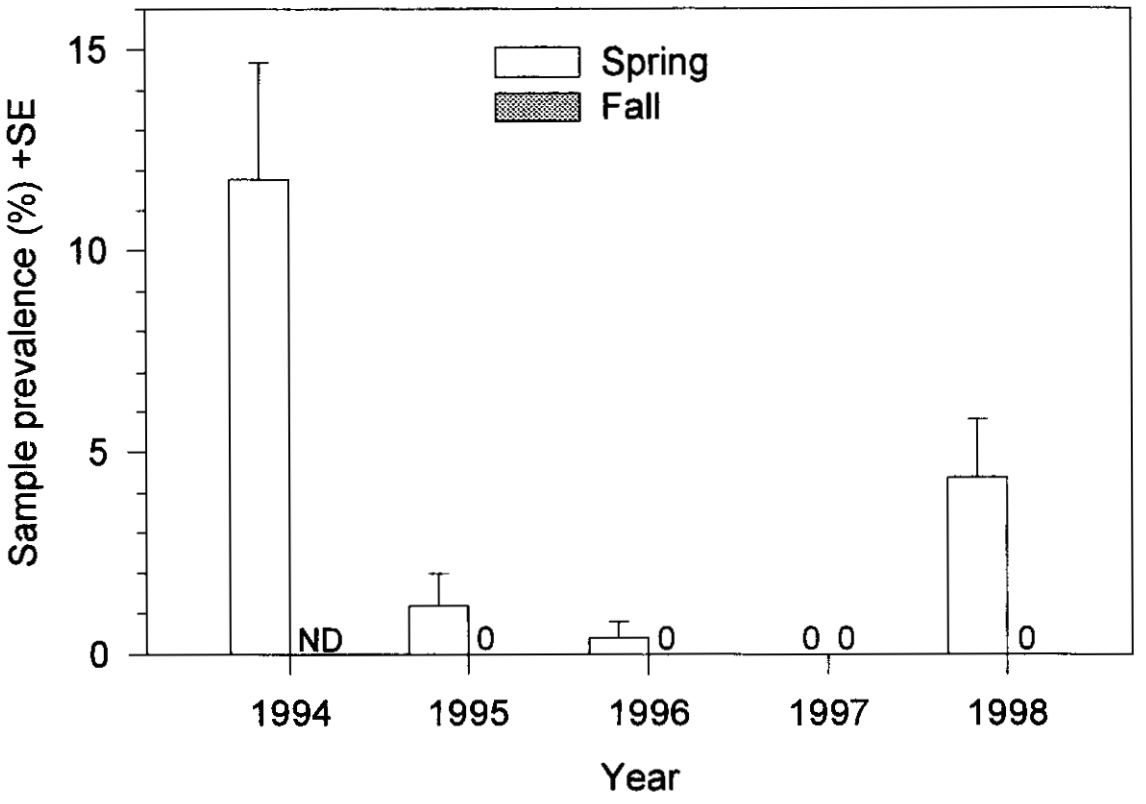

Figure 18. Prevalence of ciliated protozoan parasites (e.g., *Trichodina* sp.) in the gills of adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (1.3 ± 0.73) and spring 1996 (0.0). ND = no data.

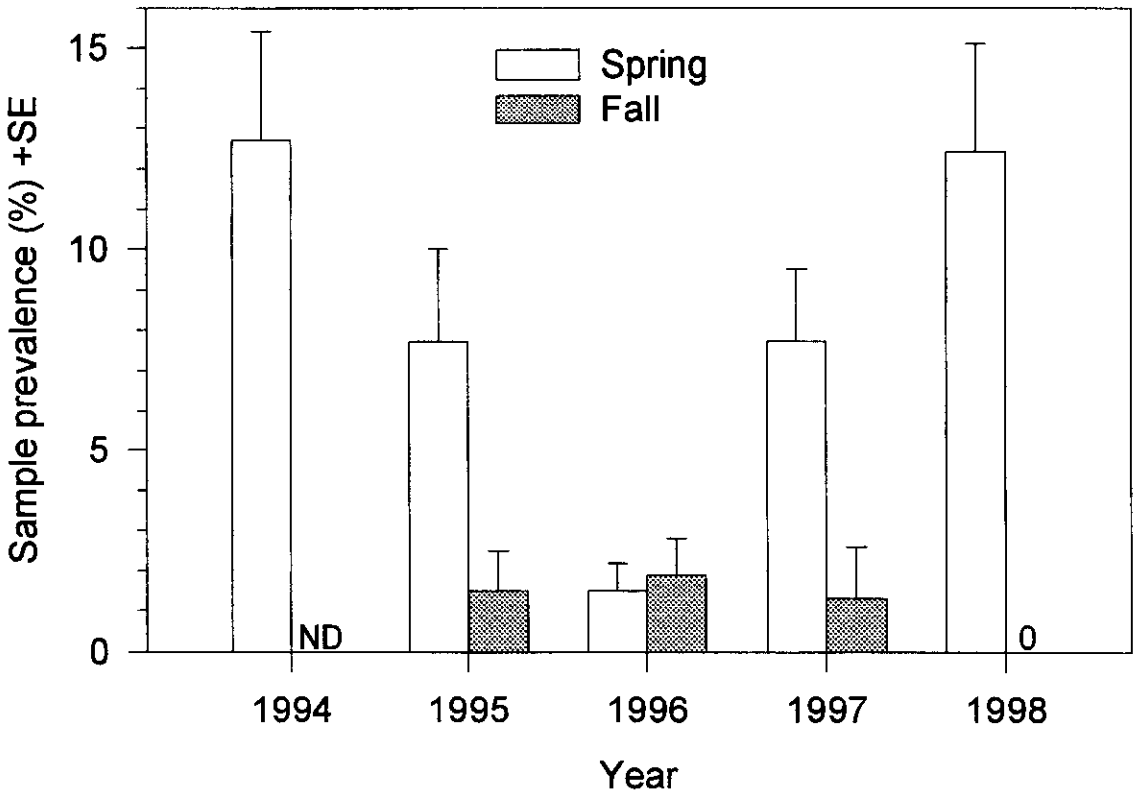

Figure 19. Prevalence of monogenetic trematode parasites on the gills of adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (10.8 ± 1.8) and spring 1996 (2.1 ± 0.96). ND = no data.

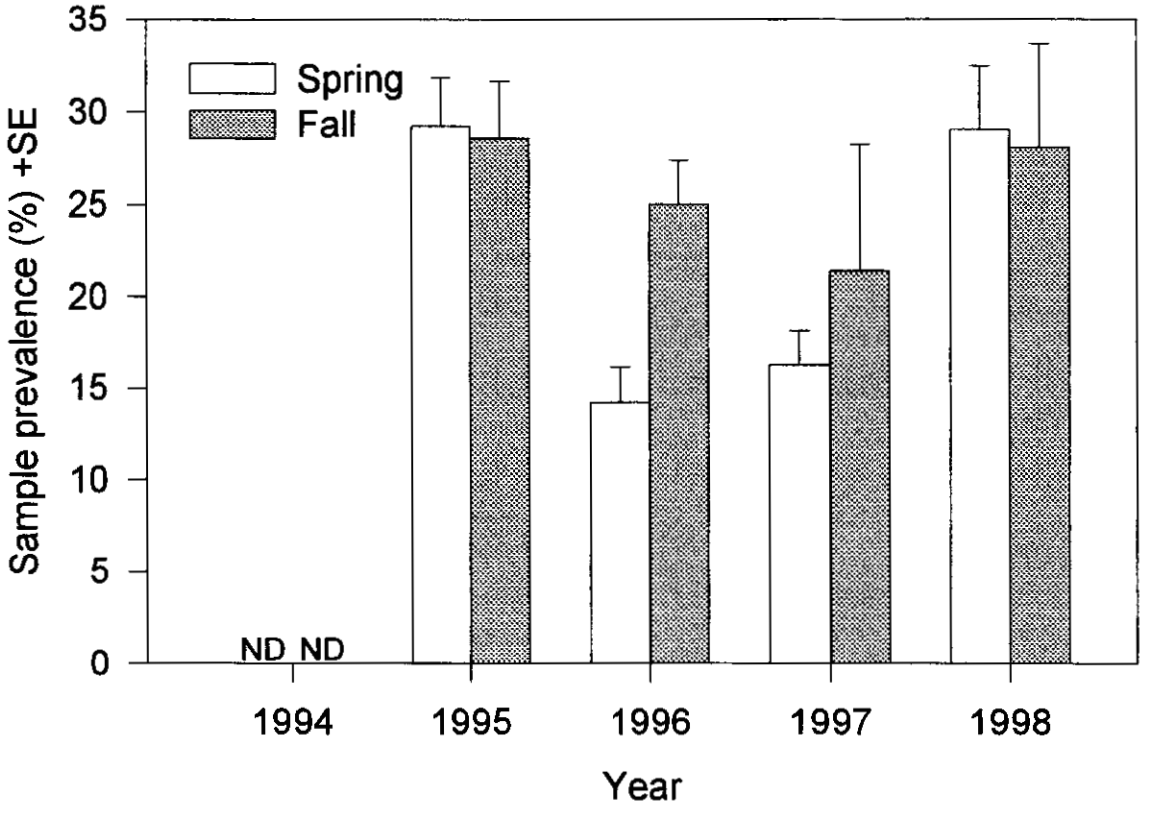

Figure 20. Prevalence of cysts of unknown etiology in the gills of adult Pacific herring from Prince William Sound, Alaska. Compare with prevalence in Pacific herring from Sitka Sound in spring 1995 (30.4 ± 2.5) and spring 1996 (34.6 ± 2.2). ND = no data.

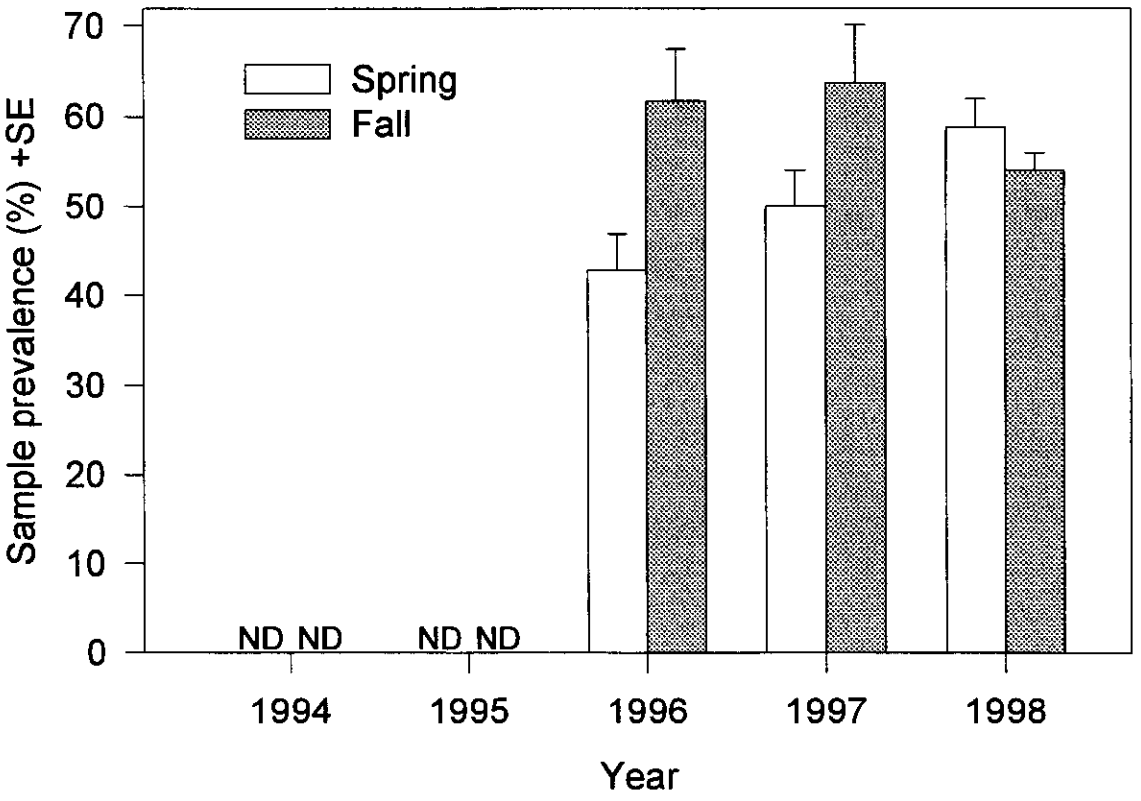

Figure 21. Prevalence of copepod parasites on the medial operculum of adult Pacific herring from Prince William Sound, Alaska. Prevalence of the opercular copepod was not determined in Pacific herring from Sitka Sound. ND = no data.

CHAPTER 2 -Effect of age, gender, size, season, and lesions on plasma chemistries in free-ranging Pacific herring

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Introduction

Plasma chemistry analysis has long been used in medicine to aid in the interpretation of clinical findings and to pinpoint sources of disease. The use of plasma chemistries in individual fish has been limited by the lack of knowledge of normal values and the high variability resulting from differences in collection technique, season, reproductive status, and age of fish being sampled. To increase our knowledge of variability in plasma chemistry values in free-ranging Pacific herring *Clupea pallasi*, we studied the relation of plasma chemistries to age, gender, size, season, and lesions. The Pacific herring population of Prince William Sound (PWS) was the primary population of interest, studied from 1994 through 1998, but we also studied the population of Pacific herring in Sitka Sound in 1995 and 1996. The purpose of this chapter is to identify variables associated with changes in plasma chemistry values.

Methods

Methods for determination of plasma chemistry concentrations are described in Chapter 1. Plasma chemistry values were compared using Spearman partial correlation coefficients. Effects of several variables were controlled for before the correlations were done: 1) dummy laboratory variables accounted for systematic differences in the laboratory where samples were analyzed (lab #1 = spring 1994 and spring 1995 samples; lab #2 = fall 1995 and spring 1996 samples; and lab #3 = fall 1996 through fall 1998 samples); 2) a dummy "crowded" variable accounted for the small size of holding containers used only for fish sampled in Sitka in 1995; 3) age (in years); 4) gender (male or female); 5) season (spring or fall); 6) site (PWS or Sitka); holding time (time from capture until necropsy, in minutes); 7) standard body length (mm); and 8) body weight (g).

Gross and microscopic findings were subjected to multiple stepwise regression to identify a subset of variables that were predictive for each plasma chemistry. Variables were forced into the equation were the same 8 variables used in the partial correlations. After the initial analysis with all variables, gross and microscopic findings with the strongest predictive values were selected for further stepwise analysis. It was necessary to run at least 2 stepwise analyses in order to minimize

the number of cases that were deleted due to missing values. In this way, only cases missing values for one or more of the variables strongly predictive of a plasma chemistry were deleted. For a full list of variables, see Marty et al. 1998. For all analyses, comparisons were considered significant when $P < 0.050$, and the model reported includes only predictors with $P < 0.050$. Use of the term "prevalence" refers to the sample prevalence.

Results

Several plasma chemistries were highly correlated with each other (Tables 1a - 1c). For example, total protein was highly correlated with albumin, ALP, and cholesterol; sodium was more highly correlated with phosphorus than it was with potassium; and, both AST and CPK were highly correlated with ALT. Multiple stepwise regression identified significant predictors for each plasma chemistry (Tables 2 - 19). Significant trends in variables affecting plasma chemistry values can be summarized as follows:

Plasma chemistries related to differences in the laboratory that analyzed the samples: total protein, albumin, IgM, ALT, ALP, AST, CPK, total bilirubin, sodium, chloride, potassium, calcium, phosphate, lactate, and osmolality. Plasma chemistries NOT related to the laboratory that analyzed the samples: glucose.

Plasma chemistries with greater values associated with crowding: AST, CPK, total bilirubin, sodium, calcium, and lactate. Plasma chemistries with lower values associated with crowding: IgM, glucose, and chloride.

Plasma chemistries greater in older fish: IgM, glucose, and calcium. Plasma chemistries greater in younger fish: cholesterol, AST, CPK, total bilirubin, chloride, phosphate, and lactate.

Plasma chemistries greater in males: total protein, albumin, IgM, cholesterol, PCV, glucose, sodium, chloride, and lactate. Plasma chemistries greater in females: ALP, AST, and phosphate

Plasma chemistries with greater values in the spring: sodium, chloride, potassium, calcium, and lactate. Plasma chemistries with greater values in the fall: total protein, albumin, IgM, cholesterol, PCV, ALT, AST, CPK, total bilirubin, glucose, and phosphate.

Plasma chemistries greater in PWS: sodium, calcium, and osmolality. Plasma chemistries greater in Sitka: albumin, IgM, PCV, and glucose.

Plasma chemistries greater in fish that were held longer: ALP, sodium, potassium, calcium, lactate, and osmolality. Plasma chemistries less in fish that were held longer: total protein, IgM, cholesterol, PCV, ALT, AST, and glucose.

Plasma chemistries greater in heavier fish: total protein, albumin, cholesterol, PCV, and glucose. Plasma chemistries greater in lighter fish: potassium, phosphate, and osmolality.

Plasma chemistries greater in longer fish: phosphate, and osmolality. Plasma chemistries greater in shorter fish: cholesterol, ALT, and glucose.

Discussion

Multiple stepwise regression was useful for identifying relationships among plasma chemistries and age, gender, length, weight, season, and lesions. Variability among the three laboratories analyzing the plasma was significant for all but one chemistry, indicating that use of published values for determination of normal and abnormal has limited value with Pacific herring. Instead, control values need to be determined for each laboratory and each technique—a standard recommendation for interpretation of values from veterinary laboratories (Duncan and Prasse 1986). Because the breadth of the data in this study is unprecedented, there are no published studies with which to compare these results. Even Marty et al. (1998) provided only univariate analysis for interpretation of individual lesions with plasma chemistry values. Therefore, this study provides the baseline for beginning to understand variables that effect plasma chemistries in schooling marine fish.

Literature Cited

All literature cited in this chapter is listed in a single section for the end of the entire report, just before the appendices.

Table 1a. Pearson partial correlation coefficients (top number) and *P*-value (bottom number) of plasma chemistries analyzed in Pacific herring (n = 2135) from Prince William Sound and Sitka Sound, Alaska. Before correlations were done, all plasma chemistries were corrected for systematic effects of age, weight, length, site of capture, season, gender, the laboratory where samples were analyzed, and crowding before necropsy.

Variable	Osmolality	Total protein	Albumin	Glucose	ALP	AST	CPK
Osmolality	1.00000	0.25101 <.0001	0.11181 <.0001	0.04817 0.0264	0.27206 <.0001	-0.16643 <.0001	-0.06687 0.0020
Total protein	0.25101 <.0001	1.00000	0.47011 <.0001	0.22653 <.0001	0.42350 <.0001	-0.13438 <.0001	-0.02290 0.2912
Albumin	0.11181 <.0001	0.47011 <.0001	1.00000	0.22403 <.0001	0.22677 <.0001	-0.03565 0.1003	-0.01929 0.3739
Glucose	0.04817 0.0264	0.22653 <.0001	0.22403 <.0001	1.00000	0.15448 <.0001	-0.13765 <.0001	-0.05287 0.0148
ALP	0.27206 <.0001	0.42350 <.0001	0.22677 <.0001	0.15448 <.0001	1.00000	-0.15510 <.0001	-0.07034 0.0012
AST	-0.16643 <.0001	-0.13438 <.0001	-0.03565 0.1003	-0.13765 <.0001	-0.15510 <.0001	1.00000	0.32696 <.0001

Variable	Osmolality	Total protein	Albumin	Glucose	ALP	AST	CPK
CPK	-0.06687 0.0020	-0.02290 0.2912	-0.01929 0.3739	-0.05287 0.0148	-0.07034 0.0012	0.32696 <.0001	1.00000
Sodium	0.25557 <.0001	0.04423 0.0414	0.04584 0.0346	-0.07934 0.0003	0.11207 <.0001	0.00683 0.7529	-0.06854 0.0016
Potassium	0.28084 <.0001	0.12217 <.0001	0.02154 0.3209	-0.12742 <.0001	0.16651 <.0001	-0.07323 0.0007	-0.02016 0.3528
Chloride	0.11486 <.0001	-0.05852 0.0070	-0.00896 0.6795	-0.02554 0.2391	-0.07078 0.0011	-0.00700 0.7470	-0.08039 0.0002
Phosphorus	0.19397 <.0001	0.07455 0.0006	-0.03022 0.1636	-0.08093 0.0002	0.14169 <.0001	0.10884 <.0001	0.02281 0.2931
Calcium	0.28037 <.0001	0.23325 <.0001	0.10482 <.0001	0.01606 0.4592	0.24281 <.0001	-0.04675 0.0311	-0.07629 0.0004
PCV	-0.01743 0.4217	0.16713 <.0001	0.04068 0.0608	0.12430 <.0001	0.05984 0.0058	-0.06476 0.0028	0.00110 0.9597

Table 1b. Pearson partial correlation coefficients (top number) and *P*-value (bottom number) of plasma chemistries analyzed in Pacific herring (n = 2135) from Prince William Sound and Sitka Sound, Alaska. Before correlations were done, all plasma chemistries were corrected for systematic effects of age, weight, length, site of capture, season, gender, the laboratory where samples were analyzed, and crowding before necropsy.

Variable	Sodium	Potassium	Chloride	Phosphorus	Calcium	PCV
Osmolality	0.25557 <.0001	0.28084 <.0001	0.11486 <.0001	0.19397 <.0001	0.28037 <.0001	-0.01743 0.4217
Total protein	0.04423 0.0414	0.12217 <.0001	-0.05852 0.0070	0.07455 0.0006	0.23325 <.0001	0.16713 <.0001
Albumin	0.04584 0.0346	0.02154 0.3209	-0.00896 0.6795	-0.03022 0.1636	0.10482 <.0001	0.04068 0.0608
Glucose	-0.07934 0.0003	-0.12742 <.0001	-0.02554 0.2391	-0.08093 0.0002	0.01606 0.4592	0.12430 <.0001
ALP	0.11207 <.0001	0.16651 <.0001	-0.07078 0.0011	0.14169 <.0001	0.24281 <.0001	0.05984 0.0058
AST	0.00683 0.7529	-0.07323 0.0007	-0.00700 0.7470	0.10884 <.0001	-0.04675 0.0311	-0.06476 0.0028
CPK	-0.06854 0.0016	-0.02016 0.3528	-0.08039 0.0002	0.02281 0.2931	-0.07629 0.0004	0.00110 0.9597
Sodium	1.00000	0.18737 <.0001	0.15643 <.0001	0.39758 <.0001	0.20353 <.0001	-0.14113 <.0001

Variable	Sodium	Potassium	Chloride	Phosphorus	Calcium	PCV
Potassium	0.18737 <.0001	1.00000	-0.03182 0.1424	0.21106 <.0001	0.14478 <.0001	-0.21065 <.0001
Chloride	0.15643 <.0001	-0.03182 0.1424	1.00000	0.10395 <.0001	-0.10230 <.0001	0.03535 0.1032
Phosphorus	0.39758 <.0001	0.21106 <.0001	0.10395 <.0001	1.00000	0.17054 <.0001	0.02901 0.1812
Calcium	0.20353 <.0001	0.14478 <.0001	-0.10230 <.0001	0.17054 <.0001	1.00000	-0.01491 0.4919
PCV	-0.14113 <.0001	-0.21065 <.0001	0.03535 0.1032	0.02901 0.1812	-0.01491 0.4919	1.00000

Table 1c. Pearson partial correlation coefficients (top number) and *P*-value (bottom number) of plasma chemistries analyzed in Pacific herring from Prince William Sound and Sitka Sound, Alaska. Before correlations were done, all plasma chemistries were corrected for systematic effects of age, weight, length, site of capture, season, gender, the laboratory where samples were analyzed, and crowding before necropsy. Unlike chemistries included in Table ??a, these chemistries were not analyzed for all samples; therefore, sample size for comparisons is less.

Variable	ALT n = 1286	Cholesterol n = 1286	Bilirubin n = 1286	IgM n = 1873	Lactate n = 1873
Osmolality	0.01091 0.6969	0.04941 0.0774	-0.02623 0.3488	0.01891 0.4146	-0.04467 0.0538
Total protein	0.06749 0.0158	0.63020 <.0001	0.07070 0.0115	0.17271 <.0001	-0.19813 <.0001
Albumin	0.11642 <.0001	0.54646 <.0001	0.04409 0.1151	0.08154 0.0004	0.01140 0.6228
IgM	0.10256 0.0010 n = 1039	0.08231 0.0082 n = 1039	0.01880 0.5465 n = 1039	1.00000	-0.02305 0.3199
Cholesterol	-0.05579 0.0461	1.00000	0.14265 <.0001	0.08231 0.0082 n = 1039	0.10605 0.0006 n = 1039
Glucose	-0.05128 0.0669	0.34359 <.0001	0.03096 0.2687	0.08017 0.0005	-0.11325 <.0001
Total bilirubin	0.04346 0.1204	0.14265 <.0001	1.00000	0.01880 0.5465 n = 1039	-0.00971 0.7555 n = 1039
ALP	-0.03031 0.2789	0.30596 <.0001	0.01443 0.6063	0.05441 0.0188	-0.24353 <.0001

Variable	ALT n = 1286	Cholesterol n = 1286	Bilirubin n = 1286	IgM n = 1873	Lactate n = 1873
ALT	1.00000	-0.05579 0.0461	0.04346 0.1204	0.10256 0.0010 n = 1039	0.07774 0.0125 n = 1039
AST	0.37170 <.0001	-0.09991 0.0003	-0.07621 0.0064	0.09302 <.0001	0.31624 <.0001
CPK	0.39683 <.0001	-0.03385 0.2266	0.00689 0.8055	0.05460 0.0184	0.10561 <.0001
Sodium	-0.03629 0.1948	-0.10443 0.0002	-0.09281 0.0009	-0.06028 0.0092	0.16661 <.0001
Potassium	0.05506 0.0491	-0.15796 <.0001	0.06195 0.0268	-0.03700 0.1103	-0.22885 <.0001
Chloride	-0.14669 <.0001	-0.06495 0.0202	-0.13828 <.0001	-0.00358 0.8772	0.06006 0.0095
Phosphorus	0.04591 0.1009	0.06477 0.0206	0.01816 0.5166	0.00001 0.9995	0.23783 <.0001
Lactate	0.07774 0.0125 n = 1039	0.10605 0.0006 n = 1039	-0.00971 0.7555 n = 1039	-0.00922 0.6908	1.00000
Calcium	-0.01037 0.7112	0.15494 <.0001	0.01829 0.5135	-0.03450 0.1365	0.03249 0.1608
PCV	0.00687 0.8063	0.31589 <.0001	0.11684 <.0001	0.08013 0.0005	-0.07336 0.0015

Table 2. Significant predictors of **total** plasma **protein** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.53; n = 1898.

Variable	Total protein estimate (g/dL)[a]	*P*-value
Model intercept	+3.53	<0.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	+0.093	0.34
Laboratory; #1 = no change; laboratory #2 =	-0.26	<0.0001
laboratory #3 =	+0.63	<0.0001
Age (multiply estimate by age, in years)	+0.013	0.41
Gender; female = no change; male =	+0.24	<0.0001
Season; fall = no change; spring =	-0.82	<0.0001
Site; Sitka = no change; Prince William Sound =	-0.10	0.12
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	+0.0001	.77
Length (multiply estimate by standard length, in mm)	-0.0046	0.040
Weight (multiply estimate by weight, in g)	+0.0055	<0.0001
Other variables (in order of selection by model; multiply estimate by lesion score)		
Gonad fullness	+0.14	<0.0001
Intestinal mesenteric inflammation (steatitis)	+0.35	<0.0001
Focal skin reddening	-0.19	<0.0001
Liver weight (g)	+0.24	<0.0001
Ichthyophonus hoferi, spleen	+0.19	0.0002
Caudal fin fraying	-0.13	<0.0001
Perivascular leukocytes in skeletal muscle	-0.15	0.0006

Variable	Total protein estimate (g/dL)[a]	*P*-value
Zymogen granule atrophy, exocrine pancreas	-0.14	<0.0001
Splenic congestion	-0.07	0.0001
Gall bladder myxosporean (*Ceratomyxa auerbachi*)	-0.11	0.0002
Ichthyophonus hoferi, gill	-0.17	0.0057
Pigmented macrophage aggregates, kidney	-0.09	0.0016
Renal intraductal myxosporean (*Ortholinea orientalis*)	-0.04	0.0017
Intestinal autolysis (or autodigestion)	-0.09	0.0076
Iris reddening	+0.09	0.0055
Hepatocellular glycogen depletion	-0.10	0.0179
Hepatocellular single cell necrosis (apoptosis)	+0.14	0.0042
Hepatic perivascular eosinophilic granular leukocytes	-0.08	0.0293
Ichthyophonus hoferi, kidney	+0.12	0.0134
Ichthyophonus hoferi, stomach	-0.14	0.0198
Hepatic focal parenchymal leukocytes	+0.07	0.0425

[a]The model predicts the contribution of each variable to total protein concentration assuming all other variables are constant. For example, the model predicts that total protein will be 0.24 g/dL greater in a male than in a female, and 0.82 g/dL less in the spring than in the fall. As another example, intestinal mesenteric inflammation (steatitis) increases total protein values 0.35 g/dL for each increase in severity score.

Table 3. Significant predictors of plasma **albumin** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.; n = 1959.

Variable	albumin estimate (g/dL)	*P*-value
Model intercept	0.866	0.0007
Variables forced into the model		
Crowded holding conditions; if crowded, add	0.079	0.2858
Laboratory; #1 = no change; laboratory #2 =	0.260	<.0001
laboratory #3 =	1.139	<.0001
Age (multiply estimate by age, in years)	0.012	0.3311
Gender; female = no change; male =	0.082	0.0021
Season; fall = no change; spring =	-0.453	<.0001
Site; Sitka = no change; Prince William Sound =	-0.116	0.0270
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.000357	0.1565
Length (multiply estimate by standard length, in mm)	-0.000063	0.9685
Weight (multiply estimate by weight, in g)	0.00398	<.0001
Other variables (in order of selection by model; multiply estimate by lesion score)		
Cytoplasmic vacuolation, renal tubular epithelial cells	.101	.0004
Focal skin reddening	-.085	<.0001
Ichthyophonus hoferi, spleen	.063	.0010
Gall bladder myxosporean (*Ceratomyxa auerbachi*)	-.067	.0025
Gastritis, submucosal	-.104	.0006
Caudal fin reddening	-.052	.0074
Granulomatous inflammation, liver	.056	.0210

Variable	albumin estimate (g/dL)	*P*-value
Zymogen granule atrophy, exocrine pancreas	-.050	.0248
Mongenetic trematodes, gill	-.120	.0241
Meningeal eosinophilic granular leukocytes, brain	-.048	.0311
Serositis, stomach	.058	.0365
Pigmented macrophage aggregates, kidney	-.045	.0389

Table 4. Significant predictors of plasma **IgM** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1995-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.36; n = 1861.

Variable	IgM estimate (μg/mL)	*P*-value
Variables forced into the model		
Model intercept	14.9	0.949
Crowded holding conditions	-140.	0.012
Laboratory; #1 = no change; laboratory #2 =	+2.2	0.95
laboratory #3 =	-394.	<0.0001
Age (multiply estimate by age, in years)	+29.5	0.0017
Gender; female = no change; male =	+63.9	0.0015
Season; fall = no change; spring =	-193.	<0.0001
Site; Sitka = no change; Prince William Sound =	-111.	0.0037
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.53	0.0042
Length (multiply estimate by standard length, in mm)	+1.6	0.22
Weight (multiply estimate by weight, in g)	-0.48	0.51
Other variables (in order of selection by model; multiply estimate by lesion score)		
Ichthyophonus hoferi, kidney	+79.8	0.0043
Focal skin reddening	-106.74	<0.0001
Gonad fullness	+46.7	0.0001
Renal interstitial (hematopoietic) cell hyperplasia	+87.2	<0.0001
Pigmented macrophage aggregates, liver	+60.9	0.0002
Focal coagulative necrosis, liver	+135.12	0.0013
Hepatocellular megalocytosis	+112.0	0.0098

Variable	IgM estimate (μg/mL)	*P*-value
Gill arch inflammatory cells or hematopoiesis	+100.8	0.011
Brain artifact	+157.2	0.0071
Dilation of renal tubules	-111.2	0.0047
Meningitis, brain	+134.6	0.013
Skeletal muscle degeneration or necrosis	+202.4	0.0001
Splenic congestion	+82.1	0.001
Hepatic focal parenchymal leukocytes	+50.1	0.016
Renal intraductal myxosporean (*Ortholinea orientalis*)	-19.1	0.018

Table 5. Significant predictors of plasma **cholesterol** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1996) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.57; n = 1194.

Variable	cholesterol estimate (mg/dL)	*P*-value
Model intercept	386.	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	-15.5	0.0576
Laboratory; #1 = no change; laboratory #2 =	-5.8	0.2738
laboratory #3 = (did not analyze for cholesterol)		
Age (multiply estimate by age, in years)	-3.5	0.0259
Gender; female = no change; male =	66.1	<.0001
Season; fall = no change; spring =	-59.3	<.0001
Site; Sitka = no change; Prince William Sound =	-9.3	0.1080
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.12	<.0001
Length (multiply estimate by standard length, in mm)	-0.64	0.0058
Weight (multiply estimate by weight, in g)	1.3	<.0001
Other variables (in order of selection by model; multiply estimate by lesion score)		
Focal skin reddening	-20.6	<.0001
Zymogen granule atrophy, exocrine pancreas	-11.7	0.0002
Intestinal mesenteric inflammation (steatitis)	-18.3	0.0012
Hepatocellular glycogen depletion	-25.9	<.0001
Pigmented macrophage aggregates, kidney	-8.6	0.0021
Caudal fin reddening	-9.9	0.0001

Variable	cholesterol estimate (mg/dL)	*P*-value
Myositis, skeletal muscle	-21.6	<.0001
Viral hemorrhagic septicemia virus (culture positive)	-46.9	0.0037
Gonad fullness	10.2	<.0001
Gonad weight	-0.85	0.0064
Liver weight (g)	-14.6	0.0034
Skeletal muscle degeneration or necrosis	-21.2	0.0025

Table 6. Significant predictors of packed cell volume (**PCV**) generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.24; n = 1954.

Variable	PCV estimate (%)	*P*-value
Model intercept	51.8	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	0.29	0.6686
Laboratory; #1 = no change; laboratory #2 =	0.90	0.0360
laboratory #3 =	-0.52	0.1667
Age (multiply estimate by age, in years)	-0.18	0.0824
Gender; female = no change; male =	0.93	0.0007
Season; fall = no change; spring =	-0.97	0.0480
Site; Sitka = no change; Prince William Sound =	-1.5	0.0010
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.019	<.0001
Length (multiply estimate by standard length, in mm)	-0.017	0.2943
Weight (multiply estimate by weight, in g)	0.027	0.0019
Other variables (in order of selection by model; multiply estimate by lesion score)		
Focal skin reddening	-1.0	<.0001
Hepatocellular single cell necrosis (apoptosis)	-0.89	0.0240
Hepatic focal parenchymal leukocytes	0.91	0.0002
Splenic congestion	-0.64	<.0001
Fin base reddening	1.05	<.0001
Gonad fullness	0.48	0.0005
Zymogen granule atrophy, exocrine pancreas	-0.83	<.0001

Variable	PCV estimate (%)	*P*-value
Dilation of renal tubules	-1.7	0.0003
Hepatocellular megalocytosis	-1.7	0.0022
Liver weight (g)	-1.02	0.0023
Intestinal autolysis (or autodigestion)	0.74	0.0013
Anisakidae (multiple estimate by # of herring worms in visceral cavity)	-0.030	0.0106
Focal coagulative necrosis, liver	-1.6	0.0019
Coccidian in liver (*Goussia clupearum*)	-0.28	0.0279
Ichthyophonus hoferi, intestine	-1.68	0.0076
Viral hemorrhagic septicemia virus (titer ≥1000)	-2.2	0.0134
Cestodes, intestinal lumen	0.60	0.0487
Heart artifact	-1.8	0.0272
Ichthyophonus hoferi, gill	0.82	0.0400
Ichthyophonus hoferi, liver	-0.99	0.0021

Table 7. Significant predictors of plasma alanine aminotransferase (**ALT**) concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1996) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.13; n = 1125.

Variable	ALT estimate (U/L)	*P*-value
Model intercept	9.0	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	0.095	0.8260
Laboratory; #1 = no change; laboratory #2 =	1.7	<.0001
laboratory #3 = (did not analyze for ALT)		
Age (multiply estimate by age, in years)	-0.10	0.2049
Gender; female = no change; male =	-0.059	0.7909
Season; fall = no change; spring =	-1.4	0.0009
Site; Sitka = no change; Prince William Sound =	-0.20	0.5224
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.0038	0.0326
Length (multiply estimate by standard length, in mm)	-0.031	0.0078
Weight (multiply estimate by weight, in g)	0.0051	0.4021
Other variables (in order of selection by model; multiply estimate by lesion score)		
Liver weight (g)	1.2	<.0001
Skeletal muscle degeneration or necrosis	1.5	0.0008
Myocardial foci of leukocytes	-0.67	0.0020
Ichthyophonus hoferi, skeletal muscle	0.49	0.0030
Myocardial mineralization	9.3	0.0033
Foreign body granuloma, focal, intestinal wall	0.50	0.0158
Hepatic perivascular eosinophilic granular leukocytes	0.43	0.0407

Table 8. Significant predictors of plasma alkaline phosphatase (**ALP**) concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.31; n = 1960.

Variable	ALP estimate (U/L)	*P*-value
Model intercept	66.6	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	-4.9	0.1128
Laboratory; #1 = no change; laboratory #2 =	15.9	<.0001
laboratory #3 =	16.7	<.0001
Age (multiply estimate by age, in years)	0.74	0.1457
Gender; female = no change; male =	-6.6	<.0001
Season; fall = no change; spring =	-5.5	0.0571
Site; Sitka = no change; Prince William Sound =	-3.2	0.1414
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.03	0.0030
Length (multiply estimate by standard length, in mm)	-0.091	0.2104
Weight (multiply estimate by weight, in g)	0.083	0.0855
Other variables (in order of selection by model; multiply estimate by lesion score)		
Intestinal mesenteric inflammation (steatitis)	16.8	<.0001
Perivascular leukocytes in skeletal muscle	-8.9	<.0001
Focal skin reddening	-4.3	<.0001
Gall bladder myxosporean (*Ceratomyxa auerbachi*)	-3.1	0.0007
Pigmented macrophage aggregates, kidney	-2.9	0.0015
Food (including eggs) in stomach	2.4	<.0001
Gonad weight	0.30	<.0001

Variable	ALP estimate (U/L)	*P*-value
Zymogen granule atrophy, exocrine pancreas	-3.5	0.0001
Stomach artifact	-7.3	0.0021
Caudal fin fraying	-3.2	0.0014
Ichthyophonus hoferi, spleen	8.7	<.0001
Ichthyophonus hoferi, sum of scores from all organs	-0.96	0.0032
Intestinal submucosal eosinophilic granular leukocytes	-6.1	0.0038
Iris reddening	2.9	0.0054
Hepatocellular single cell necrosis (apoptosis)	3.7	0.0169
Cytoplasmic vacuolation, renal tubular epithelial cells	2.8	0.0175
Hepatocellular lipid	2.2	0.0119
Focal intimal hyperplasia, large vessels, intestinal mesenteries	-2.0	0.0293
Granulomatous inflammation, kidney	2.4	0.0404
Epitheliocystis, gill (and rarely, in skin)	2.4	0.0440

Table 9. Significant predictors of plasma aspartate aminotransferase (**AST**) concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.26; n = 2098.

Variable	AST estimate (U/L)	*P*-value
Model intercept	270.	0.0492
Variables forced into the model		
Crowded holding conditions; if crowded, add	297.	<.0001
Laboratory; #1 = no change; laboratory #2 =	-43.7	0.0369
laboratory #3 =	-40.2	0.0280
Age (multiply estimate by age, in years)	-13.9	0.0051
Gender; female = no change; male =	-27.6	0.0203
Season; fall = no change; spring =	-245.	<.0001
Site; Sitka = no change; Prince William Sound =	-5.7	0.8036
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.26	0.0154
Length (multiply estimate by standard length, in mm)	0.72	0.3408
Weight (multiply estimate by weight, in g)	-0.46	0.2754
Other variables (in order of selection by model; multiply estimate by lesion score)		
Ichthyophonus hoferi, skeletal muscle	129.8	<.0001
Zymogen granule atrophy, exocrine pancreas	59.0	<.0001
Perivascular leukocytes in skeletal muscle	67.3	<.0001
Myositis, skeletal muscle	82.6	<.0001
Skeletal muscle degeneration or necrosis	85.4	0.0015
Cytoplasmic vacuolation, renal tubular epithelial cells	-47.6	0.0002

Variable	AST estimate (U/L)	*P*-value
Ichthyophonus hoferi, intestine	-64.7	0.0095
Intestinal mesenteric inflammation (steatitis)	-49.6	0.0034
Hepatocellular glycogen depletion	48.8	0.0009
Liver artifact	90.9	0.0001
Gonad fullness	-16.1	0.0130
Intestinal autolysis (or autodigestion)	23.8	0.0312
Renal interstitial congestion	46.7	0.0033
Foreign body granuloma, focal, stomach wall	28.8	0.0255
Hepatic perivascular eosinophilic granular leukocytes	28.8	0.0194
Spleen artifact	-57.0	0.0338
Hepatic focal parenchymal leukocytes	-25.0	0.0324
Intestinal coccidian (*Goussia* sp.?)	31.0	0.0289

Table 10. Significant predictors of plasma creatine phosphokinase (**CPK**) concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as *none* (0), *mild* (1), moderate (2), or severe (3). The model R-square = 0.16; n = 2128.

Variable	CPK estimate (U/L)	*P*-value
Model intercept	1600.	0.0983
Variables forced into the model		
Crowded holding conditions; if crowded, add	546.	0.0380
Laboratory; #1 = no change; laboratory #2 =	-221.	0.1754
laboratory #3 =	1146.	<.0001
Age (multiply estimate by age, in years)	-91.9	0.0239
Gender; female = no change; male =	-49.0	0.6043
Season; fall = no change; spring =	-1043.	<.0001
Site; Sitka = no change; Prince William Sound =	290.	0.1308
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-1.1	0.2042
Length (multiply estimate by standard length, in mm)	-7.6	0.1821
Weight (multiply estimate by weight, in g)	6.1	0.0554
Other variables (in order of selection by model; multiply estimate by lesion score)		
Hepatocellular glycogen depletion	408.	0.0007
Iris reddening	276.	0.0022
Ichthyophonus hoferi, kidney	310.	<.0001
Perivascular leukocytes in skeletal muscle	301.	0.0114
Food (including eggs) in stomach	-87.	0.0446
Caudal fin fraying	203.	0.0214

Table 11. Significant predictors of **total** plasma **bilirubin** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1996) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.34; n = 1303.

Variable	total bilirubin estimate (mg/dL)	*P*-value
Model intercept	0.14	0.0029
Variables forced into the model		
Crowded holding conditions; if crowded, add	0.034	0.0009
Laboratory; #1 = no change; laboratory #2 =	-0.039	<.0001
laboratory #3 = (did not analyze for bilirubin)		
Age (multiply estimate by age, in years)	-0.0046	0.0172
Gender; female = no change; male =	0.0048	0.3014
Season; fall = no change; spring =	-0.18	<.0001
Site; Sitka = no change; Prince William Sound =	0.0066	0.3958
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.000071	0.0752
Length (multiply estimate by standard length, in mm)	0.00043	0.1179
Weight (multiply estimate by weight, in g)	-0.00015	0.3074
Other variables (in order of selection by model; multiply estimate by lesion score)		
Hepatic focal parenchymal leukocytes	0.012	0.0074
Intestinal submucosal eosinophilic granular leukocytes	0.027	0.0049
Ichthyophonus hoferi, liver	-0.0096	0.0054
Gastritis, submucosal	-0.013	0.0093
Myositis, skeletal muscle	-0.016	0.0376

Table 12. Significant predictors of plasma **glucose** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.40; n = 2000.

Variable	glucose estimate (mg/dL)	*P*-value
Model intercept	149.	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	-17.4	0.0003
Laboratory; #1 = no change; laboratory #2 =	2.0	0.5154
laboratory #3 =	3.5	0.1723
Age (multiply estimate by age, in years)	2.0	0.0064
Gender; female = no change; male =	19.2	<.0001
Season; fall = no change; spring =	-15.0	0.0003
Site; Sitka = no change; Prince William Sound =	-23.0	<.0001
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.060	<.0001
Length (multiply estimate by standard length, in mm)	-0.36	0.0010
Weight (multiply estimate by weight, in g)	0.38	<.0001
Other variables (in order of selection by model; multiply estimate by lesion score)		
Zymogen granule atrophy, exocrine pancreas	-12.8	<.0001
Gonad fullness	7.5	<.0001
Renal interstitial congestion	-8.4	0.0003
Caudal fin reddening	-2.9	0.0389
Dilation of renal tubules	-9.8	0.0022
Intestinal mesenteric inflammation (steatitis)	-6.4	0.0099

Variable	glucose estimate (mg/dL)	*P*-value
Gastritis, submucosal	-4.1	0.0319
Liver weight (g)	8.2	0.0004
Focal skin reddening	-3.8	0.0066
Viral hemorrhagic septicemia virus (titer ≥ 1000)	-15.3	0.0116
Cysts of unknown etiology, gill	-6.2	0.0305
Caudal fin fraying	-3.2	0.0668
Cytoplasmic vacuolation, renal tubular epithelial cells	3.8	0.0323
Gill arch inflammatory cells or hematopoiesis	6.2	0.0444
Ichthyophonus hoferi, heart	-5.2	0.0007
Ichthyophonus hoferi, brain	11.7	0.0023
Coccidian in liver (*Goussia clupearum*)	-1.7	0.0449

Table 13. Significant predictors of plasma **sodium** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.26; n = 2081.

Variable	sodium estimate (mmol/L)	*P*-value
Model intercept	164.3	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	5.0	0.0185
Laboratory; #1 = no change; laboratory #2 =	13.4	<.0001
laboratory #3 =	3.0	0.0101
Age (multiply estimate by age, in years)	0.31	0.3454
Gender; female = no change; male =	1.6	0.0341
Season; fall = no change; spring =	11.7	<.0001
Site; Sitka = no change; Prince William Sound =	4.4	0.0042
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.050	<.0001
Length (multiply estimate by standard length, in mm)	0.015	0.7672
Weight (multiply estimate by weight, in g)	0.052	0.1204
Other variables (in order of selection by model; multiply estimate by lesion score)		
Gonad weight	-0.33	<.0001
Hepatocellular lipid	4.26	<.0001
Gastritis, submucosal	4.1	<.0001
Food (including eggs) in stomach	1.3	0.0003
Splenic congestion	-2.0	<.0001
Caudal fin reddening	1.5	0.0071

Variable	sodium estimate (mmol/L)	*P*-value
Stomach artifact	-6.8	<.0001
Iris reddening	2.5	0.0007
Viral hemorrhagic septicemia virus (positive titer <1000)	-7.8	0.0032
Hepatic focal parenchymal leukocytes	-2.2	0.0037
Skeletal muscle degeneration or necrosis	4.2	0.0150
Renal interstitial congestion	3.0	0.0042
Hepatocellular single cell necrosis (apoptosis)	-2.3	0.0357

Table 14. Significant predictors of plasma **chloride** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.77; n = 2077.

Variable	chloride estimate (mmol/L)	*P*-value
Model intercept	162.	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	-6.8	0.0347
Laboratory; #1 = no change; laboratory #2 =	-18.3	<.0001
laboratory #3 =	79.8	<.0001
Age (multiply estimate by age, in years)	-1.1	0.0442
Gender; female = no change; male =	2.5	0.0287
Season; fall = no change; spring =	9.6	0.0024
Site; Sitka = no change; Prince William Sound =	0.96	0.6791
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.018	0.0879
Length (multiply estimate by standard length, in mm)	-0.051	0.5092
Weight (multiply estimate by weight, in g)	0.17	0.0009
Other variables (in order of selection by model; multiply estimate by lesion score)		
Gonad weight	-0.42	<.0001
Splenic congestion	-2.3	0.0003
Pigmented macrophage aggregates, spleen	1.9	0.0094
Food (including eggs) in stomach	1.5	0.0082
Gastritis, submucosal	5.1	0.0001
Intestinal mesenteric inflammation (steatitis)	-5.2	0.0028

Variable	chloride estimate (mmol/L)	*P*-value
Viral hemorrhagic septicemia virus (titer ≥ 1000)	-9.8	0.0214
Intestinal coccidian (*Goussia* sp.?)	-4.3	0.0029
Cestodes, intestinal lumen	3.1	0.0352
Heart artifact	-7.9	0.0402
Ichthyophonus hoferi, intestine	4.2	0.0307
Hepatocellular lipid	2.7	0.0033

Table 15. Significant predictors of plasma **potassium** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.36; n = 1908.

Variable	potassium estimate (mmol/L)	*P*-value
Model intercept	0.79	0.0363
Variables forced into the model		
Crowded holding conditions; if crowded, add	0.044	0.6421
Laboratory; #1 = no change; laboratory #2 =	-0.24	0.0001
laboratory #3 =	-0.66	<.0001
Age (multiply estimate by age, in years)	-0.0075	0.5904
Gender; female = no change; male =	-0.046	0.1617
Season; fall = no change; spring =	0.37	<.0001
Site; Sitka = no change; Prince William Sound =	0.0093	0.8861
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.0045	<.0001
Length (multiply estimate by standard length, in mm)	0.0041	0.0513
Weight (multiply estimate by weight, in g)	-0.0041	0.0006
Other variables (in order of selection by model; multiply estimate by lesion score)		
Intestinal mesenteric inflammation (steatitis)	0.39	<.0001
Hepatocellular lipid	0.18	<.0001
Iris reddening	0.14	<.0001
Food (including eggs) in stomach	0.070	<.0001
Spleen autolysis	-0.85	0.0028
Liver artifact	-0.16	0.0203

Variable	potassium estimate (mmol/L)	*P*-value
Focal intimal hyperplasia, large vessels, intestinal mesenteries	-0.060	0.0341
Granulomatous inflammation, liver	0.073	0.0164
Dilation of renal tubules	0.15	0.0156
Myositis, skeletal muscle	0.15	0.0083
Perivascular leukocytes in skeletal muscle	-0.13	0.0016
Gall bladder myxosporean (*Ceratomyxa auerbachi*)	-0.062	0.0259
Intestinal autolysis (or autodigestion)	-0.064	0.0359
Gonad fullness	0.046	0.0197

Table 16. Significant predictors of plasma **calcium** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.51; n = 1983.

Variable	calcium estimate (mg/dL)	*P*-value
Model intercept	8.9	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	1.2	0.0008
Laboratory; #1 = no change; laboratory #2 =	-1.2	<.0001
laboratory #3 =	-3.4	<.0001
Age (multiply estimate by age, in years)	0.16	0.0045
Gender; female = no change; male =	0.0034	0.9808
Season; fall = no change; spring =	2.2	<.0001
Site; Sitka = no change; Prince William Sound =	0.74	0.0026
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.016	<.0001
Length (multiply estimate by standard length, in mm)	0.0080	0.3384
Weight (multiply estimate by weight, in g)	-0.0059	0.1961
Other variables (in order of selection by model; multiply estimate by lesion score)		
Hepatocellular lipid	-0.86	<.0001
Hepatocellular glycogen depletion	-1.1	<.0001
Intestinal mesenteric inflammation (steatitis)	0.69	0.0002
Fin base reddening	-0.36	<.0001
Caudal fin reddening	0.33	0.0004
Splenic congestion	-0.32	<.0001

Variable	calcium estimate (mg/dL)	*P*-value
Pigmented macrophage aggregates, spleen	-0.20	0.0125
Hepatocellular single cell necrosis (apoptosis)	0.50	0.0063
Splenic ellipsoid hypertrophy	-0.48	0.0001
Perivascular leukocytes in skeletal muscle	-0.52	0.0008
Hepatocellular megalocytosis	-0.71	0.0111
Focal intimal hyperplasia, large vessels, skeletal muscle	0.41	0.0018
Gonad fullness	0.27	0.0001
Gastric muscularis, foci of leukocytes	0.56	0.0120
Spleen autolysis	4.8	<.0001
Liver autolysis	0.6	0.0257
Liver weight (g)	0.41	0.0151

Table 17. Significant predictors of plasma **phosphate** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.38; n = 1897.

Variable	phosphate estimate (mg/dL)	*P*-value
Model intercept	5.3	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	-0.15	0.6144
Laboratory; #1 = no change; laboratory #2 =	2.9	<.0001
laboratory #3 =	1.1	<.0001
Age (multiply estimate by age, in years)	-0.095	0.0435
Gender; female = no change; male =	-0.35	0.0030
Season; fall = no change; spring =	-1.9	<.0001
Site; Sitka = no change; Prince William Sound =	-0.33	0.1139
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	-0.0016	0.1310
Length (multiply estimate by standard length, in mm)	0.024	0.0005
Weight (multiply estimate by weight, in g)	-0.013	0.0008
Other variables (in order of selection by model; multiply estimate by lesion score)		
Gastritis, submucosal	1.1	<.0001
Splenic congestion	-0.45	<.0001
Hepatocellular lipid	0.50	<.0001
Food (including eggs) in stomach	0.22	<.0001
Iris reddening	0.46	<.0001
Stomach artifact	-1.0	<.0001

Variable	phosphate estimate (mg/dL)	*P*-value
Serositis, stomach	-0.42	0.0001
Branchial ciliates (e.g., *Trichodina*)	2.0	<.0001
Intestinal autolysis (or autodigestion)	0.42	<.0001
Renal interstitial congestion	0.47	0.0010
Caudal fin fraying	-0.25	0.0077
Focal intimal hyperplasia, large vessels, skeletal muscle	0.29	0.0068
Liver artifact	-0.61	0.0055
Skeletal muscle degeneration or necrosis	0.65	0.0134
Pigmented macrophage aggregates, spleen	0.16	0.0141
Anisakidae (herring worms) between intestinal ceca	-0.14	0.0342
Fin base reddening	-0.16	0.0294
Gonad fullness	-0.16	0.0133
Intestinal mesenteric inflammation (steatitis)	0.31	0.0431
Liver weight (g)	-0.29	0.0322

Table 18. Significant predictors of plasma **lactate** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1995-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.62; n = 1910.

Variable	lactate estimate (mmol/dL)	*P*-value
Model intercept	-50.8	0.0008
Variables forced into the model		
Crowded holding conditions; if crowded, add	26.7	<.0001
Laboratory; #1 = no change; laboratory #2 =	63.3	<.0001
laboratory #3 =	16.4	<.0001
Age (multiply estimate by age, in years)	-2.7	<.0001
Gender; female = no change; male =	7.2	<.0001
Season; fall = no change; spring =	12.8	<.0001
Site; Sitka = no change; Prince William Sound =	4.5	0.0538
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.027	0.0170
Length (multiply estimate by standard length, in mm)	0.14	0.0852
Weight (multiply estimate by weight, in g)	0.081	0.0654
Other variables (in order of selection by model; multiply estimate by lesion score)		
Intestinal mesenteric inflammation (steatitis)	-10.3	<.0001
Splenic congestion	-3.1	<.0001
Food (including eggs) in stomach	-2.6	<.0001
Hepatocellular megalocytosis	9.4	0.0003
Cytoplasmic vacuolation, renal tubular epithelial cells	-3.0	0.0164

Variable	lactate estimate (mmol/dL)	*P*-value
Anisakidae (multiple estimate by # of herring worms in visceral cavity)	0.17	0.0042
Iris reddening	4.0	0.0005
Zymogen granule atrophy, exocrine pancreas	2.4	0.0169
Perivascular leukocytes in skeletal muscle	3.1	0.0362
Granulomatous inflammation, kidney	-3.8	0.0059
Focal intimal hyperplasia, large vessels, skeletal muscle	3.3	0.0082
Cestodes, intestinal lumen	-4.3	0.0041
Hepatocellular lipid	3.1	0.0016
Gill artifact	10.4	0.0307
Intestinal submucosal eosinophilic granular leukocytes	6.2	0.0051
Skeletal muscle degeneration or necrosis	7.7	0.0121
Skeletal muscle artifact	-6.6	0.0122
Splenic ellipsoid hypertrophy	-3.2	0.0083
Gonad fullness	-1.6	0.0399
Kidney autolysis	-25.1	0.0491
Pigmented macrophage aggregates, kidney	2.1	0.0402
Foreign body granuloma, focal, stomach wall	3.3	0.0243

Table 19. Significant predictors of plasma **osmolality** concentration generated using multiple stepwise regression. Pacific herring were sampled from Prince William Sound, Alaska (1994-1998) and Sitka Sound, Alaska (1995 and 1996). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3). The model R-square = 0.34; n = 2029.

Variable	osmolality estimate (mOsm/kg)	*P*-value
Model intercept	338.	<.0001
Variables forced into the model		
Crowded holding conditions; if crowded, add	5.7	0.1584
Laboratory; #1 = no change; laboratory #2 =	4.6	0.0715
laboratory #3 =	-37.3	<.0001
Age (multiply estimate by age, in years)	-0.10	0.8688
Gender; female = no change; male =	0.75	0.6119
Season; fall = no change; spring =	1.3	0.7011
Site; Sitka = no change; Prince William Sound =	12.4	<.0001
Hold time (multiply estimate by time, in minutes, from capture to necropsy)	0.065	<.0001
Length (multiply estimate by standard length, in mm)	0.24	0.0066
Weight (multiply estimate by weight, in g)	-0.12	0.0166
Other variables (in order of selection by model; multiply estimate by lesion score)		
Intestinal mesenteric inflammation (steatitis)	16.7	<.0001
Iris reddening	9.9	<.0001
Food (including eggs) in stomach	3.5	<.0001
Perivascular leukocytes in skeletal muscle	-8.9	<.0001
Splenic congestion	-2.8	0.0003
Cytoplasmic vacuolation, renal tubular epithelial cells	4.2	0.0081

Variable	osmolality estimate (mOsm/kg)	*P*-value
Ichthyophonus hoferi, heart	-2.0	0.0247
Dilation of renal tubules	8.0	0.0044
Zymogen granule atrophy, exocrine pancreas	-3.9	0.0016
Cestodes, intestinal lumen	4.3	0.0201
Hepatic focal parenchymal leukocytes	3.8	0.0100
Heart artifact	13.8	0.0033

Conclusions

Viral hemorrhagic septicemia virus was the most significant pathogen in Pacific herring from Prince William Sound. When combined with poor fish health, as identified by ulcers and changes in plasma chemistries, high VHSV prevalence was associated with population decline. *Ichthyophonus hoferi*, by comparison, is a significant pathogen that seems to cause low-grade mortality of older fish on a regular basis. *Ichthyophonus hoferi* has not been associated with unexpected population decline in Pacific herring. Other clues to population health include the prevalence of other parasites, macroscopic and microscopic lesions, and changes in plasma chemistries and immune status.

Spawning status in the spring was not associated with changes in susceptibility to VHSV. Fish that survive the long Alaska winter are more susceptible to disease, and gonadal maturation and spawning were associated with changes in plasma chemistry values. However, VHSV prevalence in prespawning and postspawning fish was not significantly different in our samples.

Disease had a significant impact on population size and structure. A disease outbreak caused severe decline of the population of Pacific herring in PWS in 1993 and 1994, and another disease outbreak in 1998 killed nearly half the fish in the population. Disease was more common among younger fish, and none of the 15 common parasites were more common in older fish. Finally, we found no evidence that potential exposure to *Exxon Valdez* oil was related to prevalence or severity of disease in 1994 through 1998.

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Appendix 1. Viral hemorrhagic septicemia virus, *Ichthyophonus hoferi*, and other causes of morbidity in Pacific herring *Clupea pallasi* spawning in Prince William Sound, Alaska, USA.

The first publication resulting from this work is included here in reprint form:

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.

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Vol. 32: 15–40, 1998 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published February 26

Viral hemorrhagic septicemia virus, *Ichthyophonus hoferi*, and other causes of morbidity in Pacific herring *Clupea pallasi* spawning in Prince William Sound, Alaska, USA

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ABSTRACT: Pacific herring *Clupea pallasi* populations in Prince William Sound, Alaska, USA, declined from an estimated 9.8×10^7 kg in 1992 to 1.5×10^7 kg in 1994. To determine the role of disease in population decline, 233 Pacific herring from Prince William Sound were subjected to complete necropsy during April 1994. The North American strain of viral hemorrhagic septicemia virus (VHSV) was isolated from 11 of 233 fish (4.7%). VHSV was significantly related to myocardial mineralization, hepatocellular necrosis, submucosal gastritis, and meningoencephalitis. *Ichthyophonus hoferi* infected 62 of 212 (29%) fish. *I. hoferi* infections were associated with severe, disseminated, granulomatous inflammation and with increased levels of plasma creatine phosphokinase (CPK) and aspartate aminotransferase (AST). *I. hoferi* prevalence in 1994 was more than double that of most previous years (1989 to 1993). Plasma chemistry values significantly greater ($p < 0.01$) in males than females included albumin, total protein, cholesterol, chloride, glucose, and potassium; only alkaline phosphatase was significantly greater in females. Hypoalbuminemia was relatively common in postspawning females; other risk factors included VHSV and moderate or severe focal skin reddening. Pacific herring had more than 10 species of parasites, but they were not associated with significant lesions. Two of the parasites have not previously been described: a renal intraductal myxosporean (11% prevalence) and an intestinal coccidian (91% prevalence). Transmission electron microscopy of a solitary mesenteric lesion revealed viral particles consistent with lymphocystis virus. No fish had viral erythrocytic necrosis (VEN). Prevalence of external gross lesions and major parasites was not related to fish age, and fish that were yearlings at the time of the 1989 'Exxon Valdez' oil spill (1988 year class) had no evidence of increased disease prevalence.

KEY WORDS: *Clupea pallasi* · 'Exxon Valdez' · Histopathology · Hypoalbuminemia · *Ichthyophonus hoferi* · Pacific herring · Plasma chemistries · Viral hemorrhagic septicemia virus (VHSV)

INTRODUCTION

Pacific herring *Clupea pallasi* are among the most abundant fish species in coastal regions of the North Pacific, where they are important for commercial and subsistence fishing and as prey for many marine fish, birds, and mammals. In Prince William Sound (PWS), Alaska, 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. Pacific herring in PWS first spawn when 3 or 4 yr old; they rarely live

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more than 12 yr, and abundant year classes recruit into the fishery about once every 4 yr. When the 'Exxon Valdez' oil spill occurred in March 1989, the biomass of spawning Pacific herring in PWS was the highest in 20 yr of reliable estimates (about 102×10^6 kg; Fig. 1). The population declined about 10% each of the first 2 yr after the spill, but then increased to 98×10^6 kg in 1992 (Fig. 1).

Because toxicants such as crude oil cause relatively more severe damage in younger fish, particularly larvae (McKim 1985), 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-yr-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). Pacific herring biomass was stable in 1992, and recruitment from the 1988 year class was expected to be excellent; therefore, fisheries biologists predicted a record spawning biomass of 110×10^6 kg before the 1993 spawning season (Fig. 1). However, when the 1993 spawning season commenced, barely 20% of the expected biomass appeared. Fish were lethargic, and many had external hemorrhages. Unlike reported disease outbreaks in Atlantic herring *Clupea harengus* (Fish 1934, Sindermann 1958, Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997) and Pacific herring (Tester 1942), there were no reports of dead fish to explain differences in predicted and actual biomass in PWS. The North American strain of viral hemorrhagic septicemia virus (VHSV) was isolated from pooled samples of Pacific herring, but no other significant pathogens were isolated (Meyers et al. 1994). Because VHSV isolation had not previously been reported from Pacific herring, the role of VHSV in population decline could not be determined. By 1994, spawning biomass declined to the lowest level in 20 yr (15×10^6 kg). Because of the reduced biomass and the presence of external lesions, commercial fisheries were severely curtailed in 1993 and all Pacific herring fisheries were closed in PWS in 1994. Interpretation of the 1993 VHSV isolates in PWS was confounded by the subsequent isolation of VHSV in several Pacific herring populations throughout the northeastern Pacific (Meyers & Winton 1995). Was VHSV the primary cause of mortality? Or, was VHSV expressed only in otherwise sick fish?

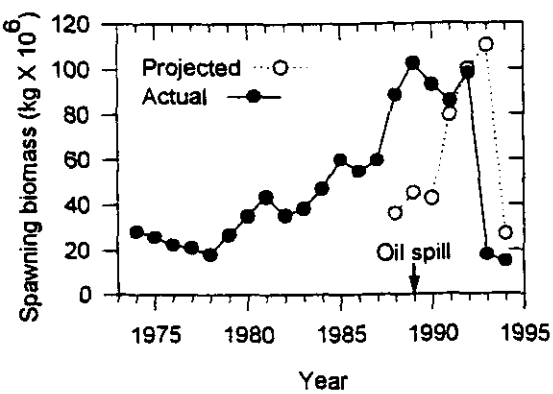


Fig. 1. *Clupea pallasi*. Biomass estimates of mature Pacific herring in Prince William Sound, Alaska. Unexploited biomass projected in the year before spawning (Projected) and estimated during spawning (Actual). Estimates were made by Fritz Funk, Alaska Department of Fish and Game, Juneau, Alaska; unpubl. data. Note that the model for projecting population biomass was not used before 1988

A comprehensive study was initiated to determine the causes of morbidity in PWS Pacific herring. This paper describes the first results from this multiyear study. Our primary hypothesis was that VHSV was the most important cause of mortality, but the study was designed to identify other pathogens. We had 4 specific objectives: (1) assess the general health of Pacific herring in PWS; (2) assess the primary or secondary invader role of VHSV in producing disease in PWS Pacific herring; (3) assess the influence of gender and spawning on Pacific herring health; and (4) determine whether fish of a particular year class were more likely to be diseased than other year classes. Petroleum hydrocarbons decreased steadily after the oil spill, and were detected in mussel tissues only in the most contaminated bays in 1991 (Short & Harris 1996). Continued exposure of Pacific herring to 'Exxon Valdez' oil was considered unlikely in our 1994 samples, and testing for hydrocarbon contamination was not done. Because of the potential for litigation, another research team conducted a separate study of Pacific herring health in Prince William Sound in 1994 (Elston et al. 1997).

This paper describes the pathogens and parasites of Pacific herring in PWS, emphasizing their potential role in population decline. Significant gender differences in plasma chemistries and lesion prevalence are identified. Finally, we discuss research needs to better understand population health and prevention of epizootics.

MATERIALS AND METHODS

Necropsy. Pacific herring were captured in Rocky Bay of Montague Island, PWS, Alaska, from April 21 through 26, 1994. To obtain a sample representative of the spawning population in PWS, fish were collected by gill net, beach seine, or purse seine in 17 different sets (8 to 18 fish per set). After capture, fish were held

in plastic containers filled with about 100 l of seawater for no more than 4 h before processing. In groups of 2, herring were anesthetized in tricaine methane sulfonate (Finquel®), weighed and measured (standard length), and a scale was removed for age determination. Each fish was assigned a unique identifying number. Several diagnostic procedures were done as part of complete necropsy and subsequent analysis on each of 233 fish:

(1) External lesions—scored as none (0), mild (1), moderate (2), or severe (3). Also, each fish was assigned a summary 'external lesion score' equal to the most severe score for fin base reddening, caudal fin reddening, or focal skin reddening.

(2) Blood—about 1.5 ml of blood was drawn from the caudal vein into 3 ml syringes containing 0.1 ml of sodium heparin (10 000 IU ml^{-1}). 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. Remaining blood was centrifuged (13 600 × *g* for 5 min) and resultant plasma was frozen for storage until analysis.

Osmolality was analyzed on a Micro Osmometer Model 3MO-plus (Advanced Instruments, Norwood, MA, USA) using 20 µl of plasma. All other analyses were done using about 200 µl of sample in a Monarch-plus analyzer (Instrumentation Laboratories, Lexington, MA, USA) calibrated and run at a stabilized 25°C. Plasma was analyzed for total protein (biuret method), albumin (bromocresol green method), and CO_2 (enzymatic method). Instrumentation Laboratories substrates were used to analyze calcium, cholesterol, glucose, phosphorus, total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine phosphokinase (CPK). Sigma® (St. Louis, MO, USA) substrates were used to analyze gamma glutamyltransferase (GGT). Ion-selective electrodes were used to analyze sodium, potassium, and chloride. Blood smears were stained with Diff-Quik (Dade Diagnostics, Inc., Aquada, Puerto Rico) and 30 fields (1000×) were examined for cytoplasmic inclusions of viral erythrocytic necrosis (VEN).

(3) Virus isolation—head kidney and spleen from each fish were pooled in a plastic bag and shipped on ice to the Alaska Department of Fish and Game Fish Pathology Laboratory in Juneau, Alaska. Skin lesions, if present, were sampled and bagged separately for individual virus assay. Propagation of EPC cell lines, media formulation, and tissue preparation for cell line inoculation were as described by Meyers et al. (1994).

(4) Tissue preservation—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—for fish with moderate or severe external lesions; kidney tissues were aseptically inoculated onto trypticase soy agar (TSA) and plates were incubated at 25°C for at least 5 d.

(6) Kidney parasite identification—a touch preparation of kidney (junction of head and trunk kidney) was air-dried, stained with Diff-Quik, and examined for pansporoblasts of the myxosporean *Ortholinea orientalis*; extent of infection was scored as for external lesions.

(7) Organ weights—liver and gonad were weighed.

(8) Herring worms (Anisakidae)—larvae in the peritoneal cavity were counted.

Histopathologic analysis. Tissues from 233 Pacific herring were sent to the University of California, Davis, and randomly assigned an individual histopathology number for blind study. Tissues from 21 herring had been inadvertently put in water rather than fixative. Therefore, data on histopathology reflect the 212 herring that were adequately fixed. After routine paraffin processing, tissue blocks were sectioned at 5 µm and stained with hematoxylin and eosin. Lesions were scored using a 4-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 4-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. Differentiation of severity scores for each lesion was based on written criteria and 'type specimen' examples. Not all scores were used for each lesion, because many lesions had no examples that were 'severe'. After all organs were examined and lesions scored, data were rearranged by necropsy number and subjected to statistical analysis.

Transmission electron microscopy. After determining that 2 fish had gross and microscopic lesions consistent with lymphocystis virus, more detailed analysis was needed to confirm the presence of viral particles in the lesions. A stained histological section of one suspect cell was removed from the glass slide and processed for transmission electron microscopy as previously described (Meyers et al. 1990).

Statistical analysis. The primary hypothesis was that fish with lesions were different from fish without lesions. The association of categorical variables (e.g. none, mild, moderate, and severe) with continuous variables (e.g. CPK values) was determined using 1-way analysis of variance (1-way ANOVA). For example, the CPK values for fish with a liver *Ichthyophonus* score of zero were compared to CPK values in fish with mild, moderate, and severe hepatic *Ichthyophonus*. When necessary, categories were combined to ensure that each group had at

least 6 fish. Category-specific means and standard errors were calculated for each continuous variable and compared using Tukey's Studentized range method. Levene's test was used to evaluate the homogeneity of variance assumption for the ANOVA.

The association between 2 selected categorical variables (e.g. *Ichthyophonus* scores versus scores for hepatic focal necrosis) was evaluated using chi-square methods for categorical data analysis; comparisons were considered valid only if individual expected cell frequencies were >1. Odds ratios were calculated for standard (2 × 2) 2-way contingency tables only. To measure the strength of the linear relationship between 2 continuous variables, the correlation coefficient r was calculated.

In the initial univariate analysis, some plasma chemistries were significantly associated with several lesions or other variables. In selected cases, multiple regression analysis was used to model a multifactor ANOVA, examining the relationships between the dependent variable (e.g. plasma albumin) and associated variables (e.g. focal skin reddening, splenic congestion, and VHSV). Lesion scores were forced into a multiple regression equation using stepwise regression to determine their joint impact in the prediction of the dependent variable (e.g. albumin level), while controlling for gender, gonad weight, hold time, and length. Criteria used for inclusion of variables in the evaluation included significance in the univariate analysis and postulated association of the equation variable with the dependent variable. Length was used rather than age or weight for 2 reasons: (1) length was more normally distributed than was age; and (2) length was more consistent in spawning fish than was weight.

To determine if certain age classes of fish were more likely to be infected by certain parasites, the association of fish age with common parasites was evaluated using the chi-square test for homogeneity. Fish were grouped into 3 categories for analysis: <5 yr old, 5 or 6 yr old, or >6 yr old. Regardless of severity of infestation, fish with a given parasite were classified as positive, and fish without the parasite were classified as negative.

For all analyses, comparisons were considered significant when $p < 0.05$ and highly significant when $p < 0.01$. For this report, use of the term 'prevalence' refers to the sample prevalence.

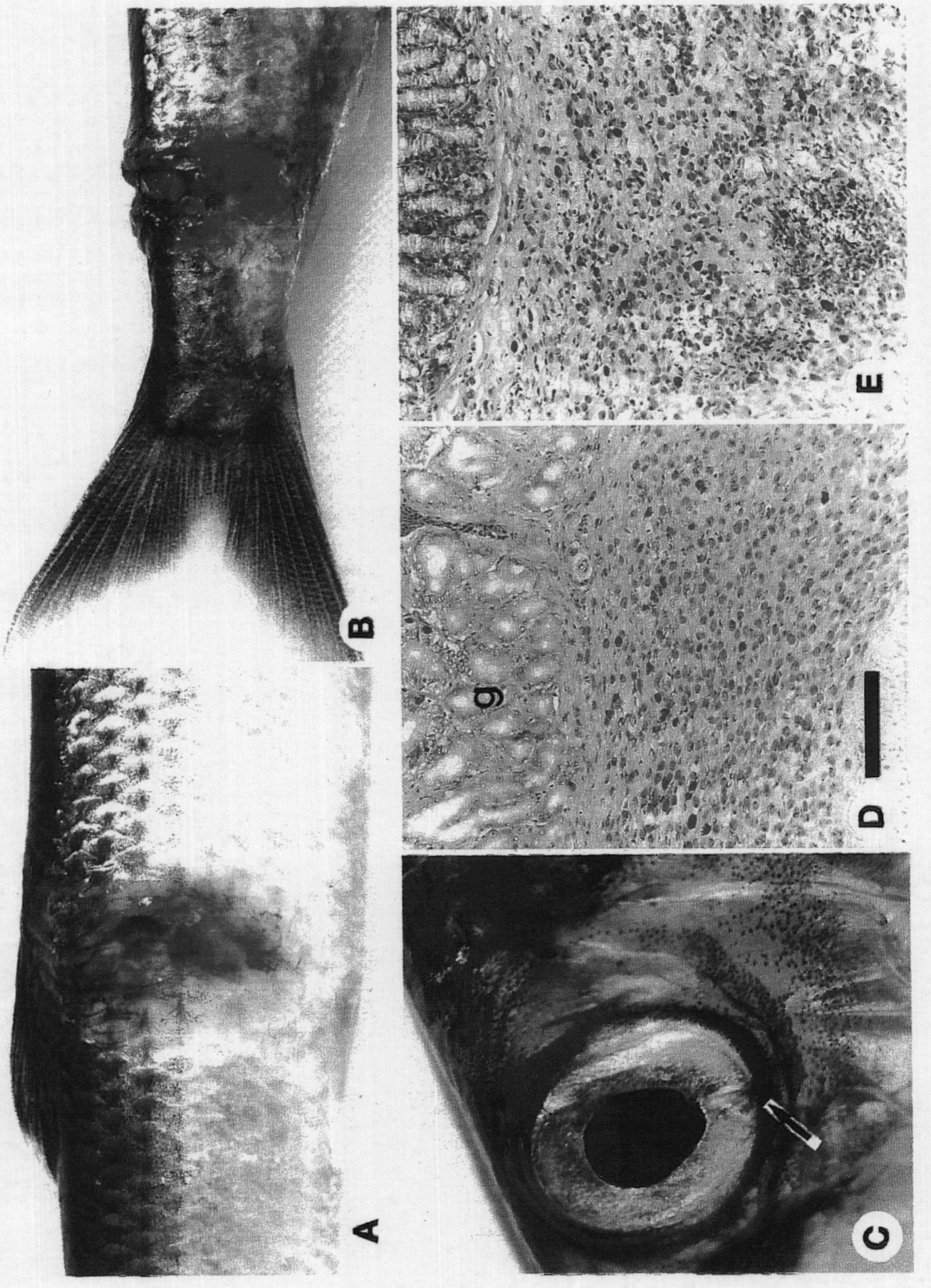
RESULTS

External gross lesions

The summary external lesion score was moderate or severe in 47 of 233 fish (20%), and several of these fish concurrently had more than one lesion graded as moderate or severe. Seven of 233 (3.0%) had ulcers (scored as severe focal skin reddening; Table 1, Fig. 2A, B). Some ulcers penetrated to underlying bone and one ulcer perforated into the peritoneal cavity, resulting in adhesions of viscera to the body wall. External lesions were significantly associated with several microscopic lesions. For example, increased scores for focal skin reddening were associated with increased scores for gill arch inflammation or hematopoiesis, submucosal gastritis, intestinal mesenteric steatitis, and renal hematopoietic cells. By comparison, scores for hepatic parenchymal leukocytes decreased as scores for focal skin reddening increased. The relationship among other gross lesions and histologic lesions were not consistent.

Because of the lack of published information on normal Pacific herring gross and microscopic anatomy, some findings were scored without knowledge of whether they were in fact lesions. Iris reddening is a good example. The inferior margin of the iris has a blood vessel about 3 mm long and 0.5 mm in diameter. Iris reddening occurred when the vessel contained enough blood to be detected by gross observation (Fig. 2C). Scores for iris reddening were assigned as follows: no reddening (0); reddening was limited to the primary vessel (1); reddening extended beyond the margins of the primary vessel, probably due to congestion of connecting venules (2); and reddening involved the entire iris (3). No fish had severe iris reddening, and mild iris reddening probably was normal. Several lesions were more prevalent in fish with no iris reddening than in fish with mild or moderate iris reddening (Table 2). For example, branchial ciliated protozoa and meningoencephalitis were more likely in fish with no iris reddening. Also, mean albumin and total protein were significantly lower in fish with no iris reddening than in fish with mild iris reddening (albumin, 0.46 vs. 0.54 g dl^{-1}; total protein, 2.0 vs 2.3 g dl^{-1}).

Fig. 2. *Clupea pallasi*. Gross and histologic lesions in Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994. (A) The ulcer with neovascularization on the right lateral side of this 198 mm long female was positive for viral hemorrhagic septicemia virus (VHSV). (B) A similar ulcer on the dorsal caudal peduncle of a 245 mm long female was negative for VHSV. (C) Mild reddening of the ventral region of the iris (arrow) was considered normal; this fish was released and not cultured for VHSV. (D) Normal gastric submucosa with large numbers of eosinophilic granular leukocytes. (E) Gastric submucosa with increased numbers of lymphocytes and macrophages (i.e. submucosal gastritis). (D) and (E): hematoxylin and eosin stain; g: gastric glands; same magnification, bar length = 100 µm


A
B
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E

Table 1. *Clupea pallasi*. Lesion severity (number of fish classified in each lesion score) and prevalence (% of sample having lesion score >0) in mature Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994. Lesions were scored as none (0), mild (1), moderate (2), or severe (3). Age, hold time, and blood values were compared for groups of fish based on lesion scores using 1-way analysis of variance and Tukey's multiple comparison procedure. Significant trends 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 comparisons with $p \leq 0.010$ are shown

Organ; lesion or tissue type	Lesion score 0	1	2	3	Sample prevalence	Significant trends (p-value)
External gross lesions						
Caudal fin fraying (n = 233)	39	177	15	2	83	↑[a] calcium (0.005*), osmolality (0.008)
Caudal fin reddening (n = 233)	127	95	9	2	45	↓[b] ALP (0.022)
Fin base reddening (n = 233)	112	89	29	3	51	↑ hold time (< 0.001), osmolality (0.005) ↓ chloride (0.050)
Iris reddening (n = 205)	100	100	5	0	51	↑ albumin (0.003), ALP (< 0.001), Calcium (< 0.001), chloride (< 0.001), cholesterol (0.017), osmolality (< 0.001), phosphorus (< 0.001*), potassium (< 0.001*), total protein (< 0.001) ↓ CO_2 (0.006)
Skin reddening, focal (includes ulcers; n = 227)	148	60	12	7	35	↑ chloride (0.002) ↓ albumin (< 0.001), ALP (< 0.001), calcium (0.034), cholesterol (< 0.001*), total protein (< 0.001*) NT: PCV (0.043)
Brain microscopic lesions (n = 212)						
Ichthyophonus	195	16	1	0	8.0	↑ AST (0.002*), $\log_e$ AST (< 0.001), CPK (< 0.001), $\log_e$ CPK (< 0.001), potassium (0.021), total protein (0.023) ↑ PCV (0.049)
Meningeal eosinophilic granular leukocytes	28	142	39	3	87	NT:[c] GGT (0.007)
Meningoencephalitis	205	6	1	0	3.3	↑ age (0.003*)
Gall bladder microscopic lesions (n = 171)						
Myxosporean (*Ceratomyxa auerbachi*) *Ichthyophonus* (score combined with liver *Ichthyophonus*)	139	31	1	0	19	↑ age (0.005*)
Gill microscopic lesions (n = 212)						
Ciliated protozoa (e.g. *Trichodina* spp.)	187	25	0	0	12	↓ chloride (0.035)
Epitheliocystis	190	20	2	0	10	none
Foreign body granuloma	193	19	0	0	9.0	none
Gill arch inflammation or hematopoiesis	1	161	39	0	100	↓ albumin (0.003), ALP (0.004), calcium (0.011), cholesterol (0.009), osmolality (0.048)
Ichthyophonus	185	18	5	4	13	NT: AST (0.003*), $\log_e$ AST (0.003), CPK (< 0.001*), $\log_e$ CPK (< 0.001), total protein (0.001)
Lamellar hyperplasia	204	7	1	0	3.8	↑ glucose (0.048)
Monogenetic trematodes (e.g. *Gyrodactylus* spp.)	185	27	0	0	13	none
Gonad - female (n = 110) microscopic lesions						
Eosinophilic granular leukocytes	38	53	19	0	65	NT: phosphorus (0.006)
Granulomatous inflammation	108	1	1	0	1.8	none
Hyalinization of vessel walls	43	57	10	0	61	none
Ichthyophonus	108	2	0	0	1.8	none
Macrophage aggregates (pigmented)	40	68	2	0	64	↑ age (< 0.001)
Gonad - male (n = 102) microscopic lesions						
Eimeria sardinae	44	52	6	0	57	↑ ALT (0.006*),
Eosinophilic granular leukocytes	45	34	22	1	56	↓ calcium (0.051)
Granulomatous inflammation	93	8	0	1	8.8	none
Hyalinization of vessel walls	102	0	0	0	0.0	ND[d]
Ichthyophonus	101	0	0	1	1.0	ND
Macrophage aggregates (pigmented)	99	3	0	0	2.9	none
Spermatocyte numbers (3 = abundant)	9	23	30	40	NA[e]	↑ glucose (< 0.001), osmolality (< 0.001), total protein (< 0.001*) NT: albumin (0.001), ALP (0.001), chloride (0.021)
Heart microscopic lesions (n = 210)						
Epicarditis	105	105	0	0	50	↓ age (0.017)
Ichthyophonus	172	14	12	12	18	↑ CPK (< 0.001*), $\log_e$ CPK (< 0.001) NT: AST (< 0.001*), $\log_e$ AST (< 0.001*), total protein (0.001)
Leukocytes, focal, parenchymal	107	103	0	0	49	↓ glucose (0.020), total protein (0.009)
Mineralization, myocardial	208	2	0	0	0.9	↑ ALT (0.003*)
Thrombosis	193	16	0	0	8.1	↑ AST (< 0.001*), $\log_e$ AST (0.008*), CPK (< 0.001*), $\log_e$ CPK (0.004) ↓ PCV (0.026)
Intestine and intestinal cecae, microscopic lesions (n = 211)						
Anisakidae	51	137	23	0	76	none
Arteriolar hyperplasia, focal, intimal	133	76	2	0	37	none
Cestodes	206	1	4	0	2.4	ND
Coccidian, intraepithelial (*Goussia* sp.?)	19	190	2	0	91	↓ osmolality (0.028)
Eosinophilic granular leukocytes, submucosal	0	202	9	0	100	↑ ALP (0.033)
Foreign body granuloma	133	78	0	0	37	none
Ichthyophonus	193	17	1	0	8.5	↑ $\log_e$ AST (0.031), CPK (< 0.001*), $\log_e$ CPK (0.008)
Steatitis	0	184	27	0	100	↑ AST (< 0.001*), $\log_e$ AST (0.002*)
Trematodes (e.g. *Lecithaster gibbosus*), cecal	205	6	0	0	2.9	none

Table 1 (continued)

Organ; lesion or tissue type	Lesion score 0	1	2	3	Sample prevalence	Significant trends (p-value)
Kidney (trunk) microscopic lesions (n = 212)						
Congestion, interstitial, vascular	156	55	1	0	26	↑ AST (0.008*), $\log_e$ AST (0.002)
Granulomatous inflammation	139	43	15	15	34	NT: age (0.004)
Hematopoietic cells (relative area)	16	156	40	0	92	NT: ALP (0.016), cholesterol (0.034)
Ichthyophonus	169	21	13	9	20	↑ $\log_e$ AST (0.028), CPK (< 0.001*), $\log_e$ CPK (0.002) NT: total protein (< 0.001*)
Interstitial cell necrosis	194	18	0	0	8.5	none
Intratubular mineral, with associated tubular hyperplasia	206	4	2	0	2.8	none
Intraductal unclassified myxosporean	188	23	1	0	11	↓ age (0.031)
Macrophage aggregates, pigmented	0	81	110	21	100	↑ age (< 0.001) NT: glucose (0.023)
Ortholinea orientalis (intraductal myxosporean)	200	6	4	2	5.7	↑ calcium (< 0.001*)
Tubular dilation (of lumen)	204	8	0	0	3.8	none
Tubular epithelial vacuolation	202	9	1	0	4.7	↑ age (0.044), albumin (0.004), calcium (0.002), chloride (0.011), cholesterol (0.026), osmolality (0.004), phosphorus (0.035)
Liver microscopic lesions (n = 212)						
Cholangitis or biliary hyperplasia	191	20	1	0	9.9	↓ chloride (0.004*)
Coccidiosis (*Goussia clupearum*)	83	58	43	28	61	none
Eosinophilic granular leukocytes	15	187	10	0	93	↓ CO_2 (0.009) NT: AST (0.001*), $\log_e$ AST (0.003*)
Glycogen depletion	0	0	2	210	100	none
Granulomatous inflammation	131	57	10	14	38	↑ $\log_e$ AST (0.018), potassium (0.006) ↓ age (0.028)
Ichthyophonus	178	14	11	9	16	↑ AST (< 0.001*), $\log_e$ AST (< 0.001*) NT: CPK (< 0.001*), $\log_e$ CPK (< 0.001)
Leukocytes, focal, parenchymal	119	93	0	0	44	↑ albumin (0.015), cholesterol (< 0.001), glucose (0.021), phosphorus (0.003*)
Lipidosis, hepatocellular	145	49	15	3	32	↑ AST (0.003*), $\log_e$ AST (0.010*), CPK (0.011), osmolality (< 0.001), phosphorus (< 0.001*), potassium (< 0.001*) ↓ glucose (0.012), PCV (< 0.001*) NT: ALP (0.039), cholesterol (0.019)
Macrophage aggregates, pigmented	0	85	95	32	100	↑ age (< 0.001*)
Necrosis, focal	206	3	2	1	2.8	none
Necrosis, hepatocellular, single cell	196	11	3	2	7.5	none
Pancreas, exocrine, microscopic lesions						
Macrophage aggregates, pigmented	78	131	2	0	63	↑ age (0.018) ↓ ALT (0.006), $\log_e$ ALT (0.007)
Zymogen granule depletion	0	4	70	137	100	↑ age (0.045)
Skin and skeletal muscle, microscopic lesions (n = 212)						
Anisakidae	205	7	0	0	3.3	↓ potassium (0.038)
Arteriolar hyperplasia, focal, intimal	82	127	1	0	61	none
Ichthyophonus	173	31	7	1	18	↑ AST (< 0.001*), $\log_e$ AST (0.001), CPK (< 0.001*), $\log_e$ CPK (< 0.001), total protein (0.003)
Leukocytes, perivascular	27	183	2	0	87	↑ osmolality (0.002), total bilirubin (0.016) ↓ phosphorus (0.010*)
Myodegeneration or myonecrosis	202	9	1	0	4.7	↑ AST (< 0.001*), GGT (0.029)
Myositis	193	19	0	0	9.0	↑ AST (0.008*)
Spleen microscopic lesions (n = 211)						
Arteriolar hyperplasia, focal, intimal	150	57	4	0	29	↓ CO_2 (0.023)
Congestion, vascular	81	90	37	3	62	↑ hold time (< 0.001), CO_2 (0.045) ↓ albumin (< 0.001), ALP (0.001), calcium (0.032), cholesterol (< 0.001), GGT (0.010), total protein (0.006)
Ellipsoid hyalinization or hypertrophy	30	147	33	1	86	↑ age (< 0.001*)
Ichthyophonus	173	16	17	5	18	↑ AST (< 0.001*), CPK (< 0.001*), total protein (0.014) NT: PCV (0.020)
Macrophage aggregates, pigmented	0	33	122	56	100	↑ age (< 0.001*)
Serosal cell thickening	44	139	27	1	79	↓ ALP (0.033)
Stomach microscopic lesions (n = 210)						
Eosinophilic granular leukocytes, (submucosal gastritis)	0	157	53	0	100	↓ albumin (0.025), ALP (0.005), cholesterol (0.020), phosphorus (0.002*)
Foreign body granuloma	150	60	0	0	29	↓ osmolality (0.034)
Ichthyophonus	188	17	1	4	10	↑ AST and $\log_e$ AST (< 0.001*), CPK (< 0.001*), $\log_e$ CPK (< 0.001)
Leukocytes, focal, parenchymal	171	38	1	0	19	none
Serositis	163	46	1	0	22	↑ chloride (0.015)
Trematodes, intraluminal (e.g. Hemiuridae)	192	17	1	0	8.6	↑ total protein (0.014) ↓ PCV (0.025) NT: CPK (< 0.001*)

[a] ↑ e.g. when plasma calcium values (mg dl^{-1}) were separated into 3 groups based on scoring of caudal fin fraying, mean (±SE) scores increased as follows: none (11.7^{A} ± 0.4), mild (11.5^{A} ± 0.1), and moderate/severe (13.1^{B} ± 0.7). (Means with a superscript in common were not significantly different; Tukey's analysis, $p > 0.05$)

[b] ↓ e.g. when ALP values (U l^{-1}) were separated into 3 groups based on scoring of caudal fin reddening, mean (±SE) scores decreased as follows: none (57.8^{A} ± 1.8), mild ($53.3^{A,B}$ ± 2.0), and moderate/severe (41.6^{B} ± 8.9). (Means with a superscript in common were not significantly different; Tukey's analysis, $p > 0.05$)

[c] NT: e.g. when GGT values (U l^{-1}) were separated into 3 groups based on scoring of meningeal eosinophilic granular leukocytes, mean (±SE) scores for the least affected group (none) were not significantly different from mean scores for the most affected group (moderate/severe) as follows: none ($6.5^{A,B}$ ± 0.7), mild (6.5^{A} ± 0.3), and moderate/severe (8.6^{B} ± 0.6). (Means with a superscript in common were not significantly different; Tukey's analysis, $p > 0.05$)

[d] ND: not done

*NA: not applicable

Table 2. *Clupea pallasi*. Lesion frequency (%) within variables of gender, iris reddening, and viral hemorrhagic septicemia virus (VHSV) in Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994. 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[b]	Odds ratio[a]	95% Confidence interval for odds ratio
Gender		Female (n = 110)	Male (n = 102)			
Gall bladder myxosporeans (*Ceratomyxa auerbachi*)	0	73	90	0.003		
	1+2	27	10		3.5	1.5, 8.4
Gonadal granulomas (or focal granulomatous inflammation)	0	98	91	0.022		
	1+2+3	2	9		0.2	0.0, 0.9
Gonadal hyalinized vessel walls	0	39	100	<0.001		
	1	52	0		NC[c]	NC
	2	9	0			
Gonadal pigmented macrophage aggregates	0	36	97	<0.001		
	1+2	64	3		58	17, 190
Intestinal mesenteric steatitis	1	92	82	0.036		
	2	8	18		0.4	0.2, 1.0
Renal proximal tubular epithelial vacuolation	0	99	91	0.007		
	1+2	1	9		0.1	0.0, 0.8
Renal tubular dilation (of lumen)	0	99	93	0.023		
	1	1	7		0.1	0.0, 1.0
Splenic *Ichthyophonus*	0	83	82	0.031		
	1	4	12		NC	NC
	2+3	14	7			
Iris reddening		Mild/moderate (n = 93)	None (n = 94)			
Branchial ciliated protozoa	0	95	82	0.007		
	1	5	18		0.3	0.1, 0.7
Caudal fin fraying	0	10	22	0.049		
	1	85	73		NC	NC
	2+3	6	5			
Fin base reddening	0	59	36	0.002		
	1	32	44		NC	NC
	2+3	9	20			
Meningoencephalitis	0	100	96	0.044		
	1+2	0	4		0.0	NC
Pancreatic zymogen granule depletion	1+2	27	44	0.019		
	3	73	56		2.1	1.1, 3.8
Renal congestion	0	67	80	0.043		
	1+2	33	20		2.0	1.0, 3.8
Splenic congestion	0	45	28	0.037		
	1	35	52		NC	NC
	1+2	20	20			
Splenic ellipsoid hyalinization	0	9	23	0.013		
	1	78	60		NC	NC
	1+2	13	17			
VHSV		Positive (n = 11)	Negative (n = 200)			
Fin base reddening	0	18	50	0.005		
	1	36	38		NC	
	2+3	45	12			
Gastritis, submucosal	2	27	77	<0.001		
	3	73	23		9.1	2.3, 36
Gill arch inflammation or hematopoiesis	0+1	45	83	0.002		
	2	55	17		5.7	1.6, 20
Meningoencephalitis	0	82	98	0.005*		
	1+2	18	2		8.7	1.5, 51
Hepatic focal necrosis	0	82	98	0.002*		
	1+2+3	18	2		11	1.8, 68
Intestinal arteriolar focal intimal hyperplasia	0	27	75	0.012		
	1+2	73	35		5.0	1.3, 19
Myocardial mineralization	0	90	99	0.003*		
	1	10	1		22	1.3, 380

[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 or VHSV-positive) to the corresponding odds for the other category of the variable (e.g. male or VHSV-negative). For example, females were 58 times more likely to have pigmented gonadal macrophage aggregates than were males; fish with mild/moderate iris reddening were 2 times more likely to have renal congestion than were fish with no iris reddening, and VHSV-positive fish were 11 times more likely to have hepatic focal necrosis than were VHSV-negative fish

[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

[c]NC: odds ratios were not calculated for lesions with more than 2 groups (e.g. splenic *Ichthyophonus*)

Ichthyophonus hoferi

All organs contained *Ichthyophonus hoferi* (hereafter referred to as *Ichthyophonus*) (Table 1), 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 (Daniel 1933b, Sindermann 1970). Most resting spores were surrounded by a rim of fibroblasts and maturing collagenous connective tissue, but some were surrounded by activated macrophages. Severe granulomatous inflammation, common in the heart, was usually associated with developing spores (Fig. 3C). Occasionally, resting spores had burst and released multinucleate endospores (Fig. 4A). A consistent scoring system was used for *Ichthyophonus* in each organ: score = 0 (no *Ichthyophonus*); score = 1 (<1 resting spore per 100× field); score = 2 (≥1 but <3 resting spores per 100× field, but inflammation was limited to a thin rim of fibrous connective tissue); score = 3 (≥1 resting spore per 100× field, with prominent granulomatous inflammation, or ≥3 resting spores per 100× field, regardless of the amount of inflammation).

Granulomatous inflammation associated with *Ichthyophonus* had to be differentiated from other forms of macrophage aggregates. Pigmented macrophage aggregates at least 60 µm in diameter were common in liver, spleen, and kidney. Pigment varied from yellow-brown (Fig. 5A, B) to green-brown, but aggregates did not contain melanin. Pigmented macrophage aggregates were more common in older fish, and some aggregates were as large as 300 µm in diameter (Fig. 5B). Aggregates of nonpigmented activated macrophages were classified as nonspecific granulomatous inflammation (Fig. 5C). Granulomatous inflammation was composed of activated macrophages with pale eosinophilic cytoplasm. Activated macrophages sometimes infiltrated and expanded foci of pigmented macrophage aggregates. Small numbers of lymphocytes and eosinophilic granular leukocytes were scattered throughout foci of granulomatous inflammation.

Lesions associated with *Ichthyophonus* occurred in 62 of 212 (29%) fish, but no single organ had greater than 21% prevalence (Fig. 6). Prevalence of *Ichthyophonus* in skin and skeletal muscle was the second highest after kidney, but most cases in skin and skeletal muscle were mild (31 of 39, 79%). By comparison, prevalence of *Ichthyophonus* in the heart was similar to that in skin and skeletal muscle, but relatively few cases in the heart were mild (14 of 38, 37%).

A sum-*Ichthyophonus* (sumICH) score was calculated for each fish by adding the individual *Ichthyophonus* scores from all 10 organs for that particular fish. For example, *Ichthyophonus* scores in organs of fish #106 included spleen (score = 2), kidney (score = 1), and a combined score for skin and skeletal muscle (score = 1), but the other 7 organs had no *Ichthyophonus* (score = 0); therefore, the sumICH score for fish #106 was 4. Because the maximum *Ichthyophonus* score for each organ was 3 (severe), the maximum possible sumICH score for a fish was 30. The highest actual score was 24. SumICH scores significantly increased with increased severity of several internal lesions, but sumICH scores were not associated with any external lesions. Several lesions were significantly associated with greater sumICH scores: cardiac thrombosis, gastric foreign body, gastric focal parenchymal leukocytes, hepatic eosinophilic granular leukocytes, intestinal foreign body granuloma, intestinal mesenteric steatitis, and skeletal myositis. Note that Levene's test for equality of variances was significant for all comparisons except skeletal myositis.

Association of *Ichthyophonus* scores with plasma chemistries was variable (Table 1), but AST and CPK, enzymes commonly used in mammalian medicine as part of the evaluation of general health, were significantly associated with *Ichthyophonus* scores in every organ (univariate ANOVA). Increases in CPK in mammals result from disruption in muscle cell membranes (Willard et al. 1989). By comparison, AST is present in significant quantities in mitochondria of hepatocytes, muscle, erythrocytes, and other blood-rich organs. The most common causes of increased AST in small domestic mammals are hepatic disease, muscular disease (inflammation or necrosis), and hemolysis (Willard et al. 1989).

The significant increase in CPK and AST in every organ was inconsistent with distribution of these enzymes in mammals. Therefore, multiple regression analysis was used to model a multifactor ANOVA, examining the linear relationships between the dependent variable CPK (or AST) and *Ichthyophonus* lesion scores in 9 organs (brain, gill, heart, intestine, kidney, liver, skin/skeletal muscle, spleen, and stomach). Gonad scores were not analyzed because only 3 gonads contained *Ichthyophonus*. For CPK, brain *Ichthyophonus* status, gender, and gonad weight were the only significant predictors when all organs were included in the multiple regression equation. For AST, renal *Ichthyophonus* status and gonad weight were the significant predictors; however, in the final model, predicted values for AST decreased when a fish had renal *Ichthyophonus*.

As a relative measure of the severity of *Ichthyophonus* in individual organs, a mean sumICH score was computed as follows for each organ: all fish with *Ichthyophonus* in an organ were selected, their sumICH scores were totaled, and this sum of sumICH scores was divided by the number of fish in which the organ was infected. For example, of 212 kidneys examined, 43 had *Ichthyophonus*; the mean sumICH

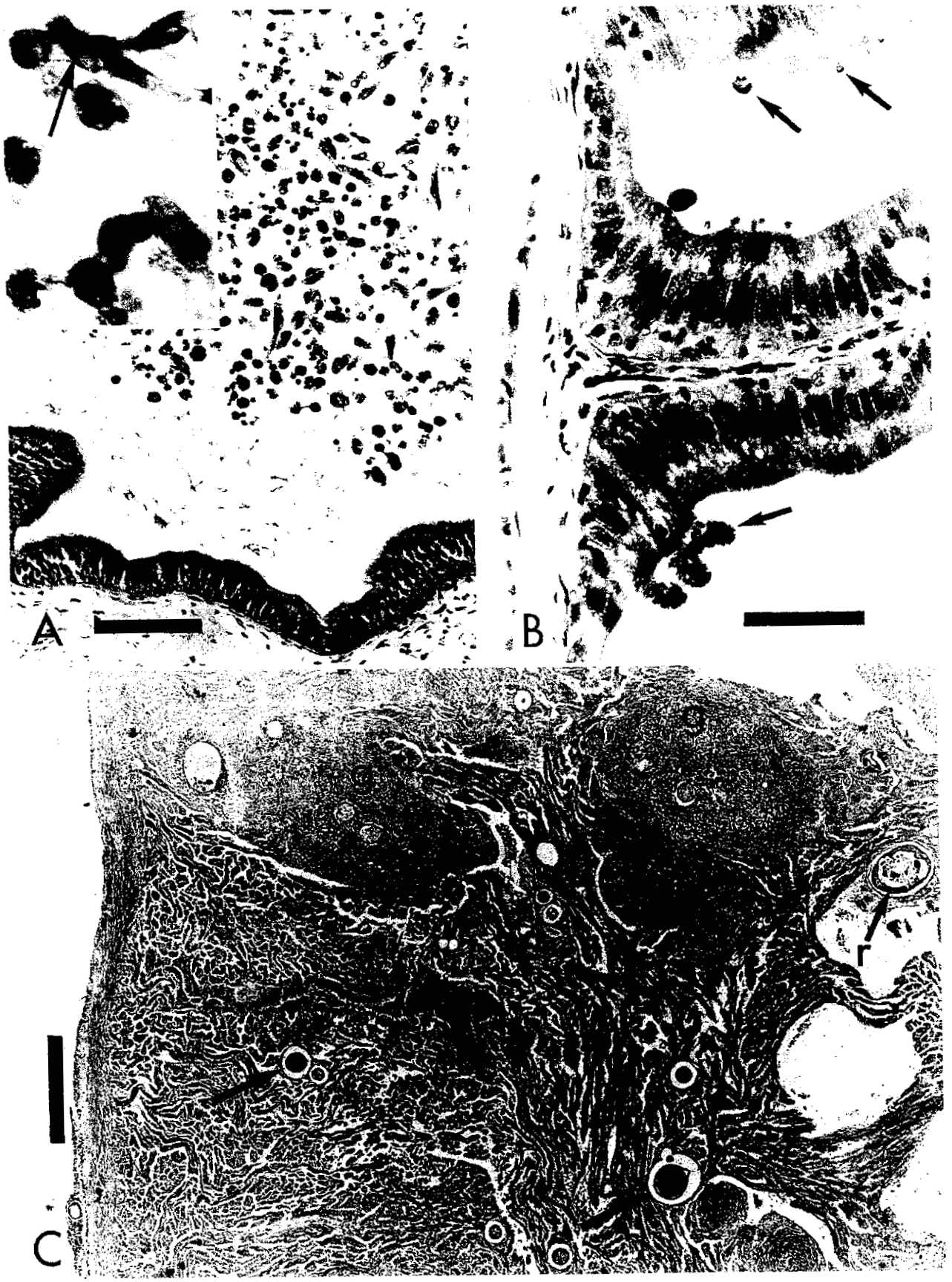
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Fig. 3. *Clupea pallasi*. Internal parasites of Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994; hematoxylin and eosin stain. (A) The myxosporean *Ceratomyxa auerbachi* in the gall bladder lumen; despite large numbers of organisms, inflammation in the gall bladder wall is minimal; bar length = 80 µm. Inset: trophozoites and maturing spores (arrow points to polar capsules); bar in larger print is 30 µm long at inset magnification. (B) Several stages of an unclassified coccidian (*Goussia* sp.?) in the apical margin of epithelial cells of intestinal cecae. Note different stages of development (arrows) and lack of inflammation; bar length = 30 µm. (C) Forms of *Ichthyophonus* in the heart include multinucleate resting spores with minimal inflammation (arrows), remnants of ruptured resting spores (r) with small endospores, and developing spores surrounded by severe granulomatous inflammation (g); bar length = 400 µm

score for those 43 fish was 9.4; by comparison, the mean sumICH score for the 17 fish with brain *Ichthyophonus* was 14.2. Generally, organs with the lowest *Ichthyophonus* prevalence (e.g. brain) had the highest mean sumICH scores (Fig. 6).

VHSV

Eleven of 233 Pacific herring (4.7%) were positive for VHSV. Virus was isolated from 7 of 233 spleen-kidney pools and from 5 of 15 skin lesions. One fish had VHSV isolated from both the spleen-kidney pool and a skin lesion. Several lesions and alterations in blood chemistries were associated with VHSV infection (Tables 2 & 3). Among external lesions, fin base reddening was significantly associated with VHSV infection. Also, VHSV was significantly associated with focal skin reddening ($p = 0.03$, chi-square test for homogeneity), but the minimum expected cell frequency was <1. The low minimum expected cell frequency resulted from having only 11 positive fish out of 233 fish sampled. Among chemistries, decreased plasma levels of albumin, ALP, and cholesterol were associated with VHSV infection (Table 3). Loss of albumin might have resulted from leakage from external lesions. Albumin was highly correlated with cholesterol ($r = 0.895$) and ALP ($r = 0.587$) regardless of VHSV status.

The normal gastric submucosa contained diffuse infiltrates of large numbers of eosinophilic granular leukocytes, but these cells did not extend into the adjacent muscularis or mucosa (Fig. 2D). Similar infiltrates have been described in intestine of Atlantic herring (Morrison et al. 1986). In 53 Pacific herring, the gastric submucosa also contained small to moderate numbers of lymphocytes and macrophages (Fig. 2E), and these infiltrates were significantly associated with VHSV infection (Table 2).

Sheets of mononuclear cells within gill arches were significantly associated with VHSV infection (Table 2). Gill arches normally contained scattered mononuclear cells that had densely basophilic nuclei and relatively scant basophilic cytoplasm (Fig. 7A). Not all cells could be identified, but they included mature inflammatory cells and hematopoietic cells in various stages of development. In 39 fish, these mononuclear cells were more abundant, but the cells did not alter tissue architecture (Fig. 7B).

Meningoencephalitis was significantly associated with VHSV infection (Table 2), and eosinophilic meningitis was marginally associated with VHSV infection ($p = 0.06$). In the brain, meninges usually contained 2 to 25 eosinophilic granular leukocytes in at least one 400× field, but normal meninges did not contain macrophages or lymphocytes. Forty-two fish had more than 25 eosinophilic granular leukocytes in at least one 400× field. In 7 fish, the meninges and perivascular space within the neuropile contained foci of inflammation (lymphocytes and macrophages) that were not associated with *Ichthyophonus* infection, but these foci of meningoencephalitis were <400 µm in diameter in all but one fish.

Table 3. *Clupea pallasi*. Plasma chemistry values that were significantly different ($p < 0.05$) based on status of viral hemorrhagic septicemia virus (VHSV) or gender. Pacific herring were sampled during spawning in Prince William Sound, Alaska, 1994. 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

Plasma chemistry	Mean	SE	Mean	SE	p-value
	VHSV status				
	Negative (n = 222)		Positive (n = 11)		
Albumin (g dl^{-1})	0.52	0.01	0.36	0.05	0.007
ALP (U l^{-1})	56.1	1.4	36.6	4.5	0.002
Cholesterol (mg dl^{-1})	221.4	4.7	156.9	21.0	0.003
	Gender				
	Female (n = 117)		Male (n = 116)		
Albumin (g dl^{-1})	0.47	0.02	0.56	0.02	<0.001
ALP (U l^{-1})	59.3	2.1	51.1	1.6	0.002
Chloride (mmol l^{-1})	160.4	0.9	165.6	1.2	0.001*
Cholesterol (mg dl^{-1})	202.1	6.3	234.8	6.6	<0.001
CO_2 (mmol l^{-1})	5.6	0.2	6.5	0.2	0.004
Glucose (mg dl^{-1})	75.9	2.6	90.0	4.3	0.001
Potassium (mmol l^{-1})	2.13	0.10	2.45	0.11	0.029
Total protein (g dl^{-1})	2.14	0.06	2.30	0.05	0.042

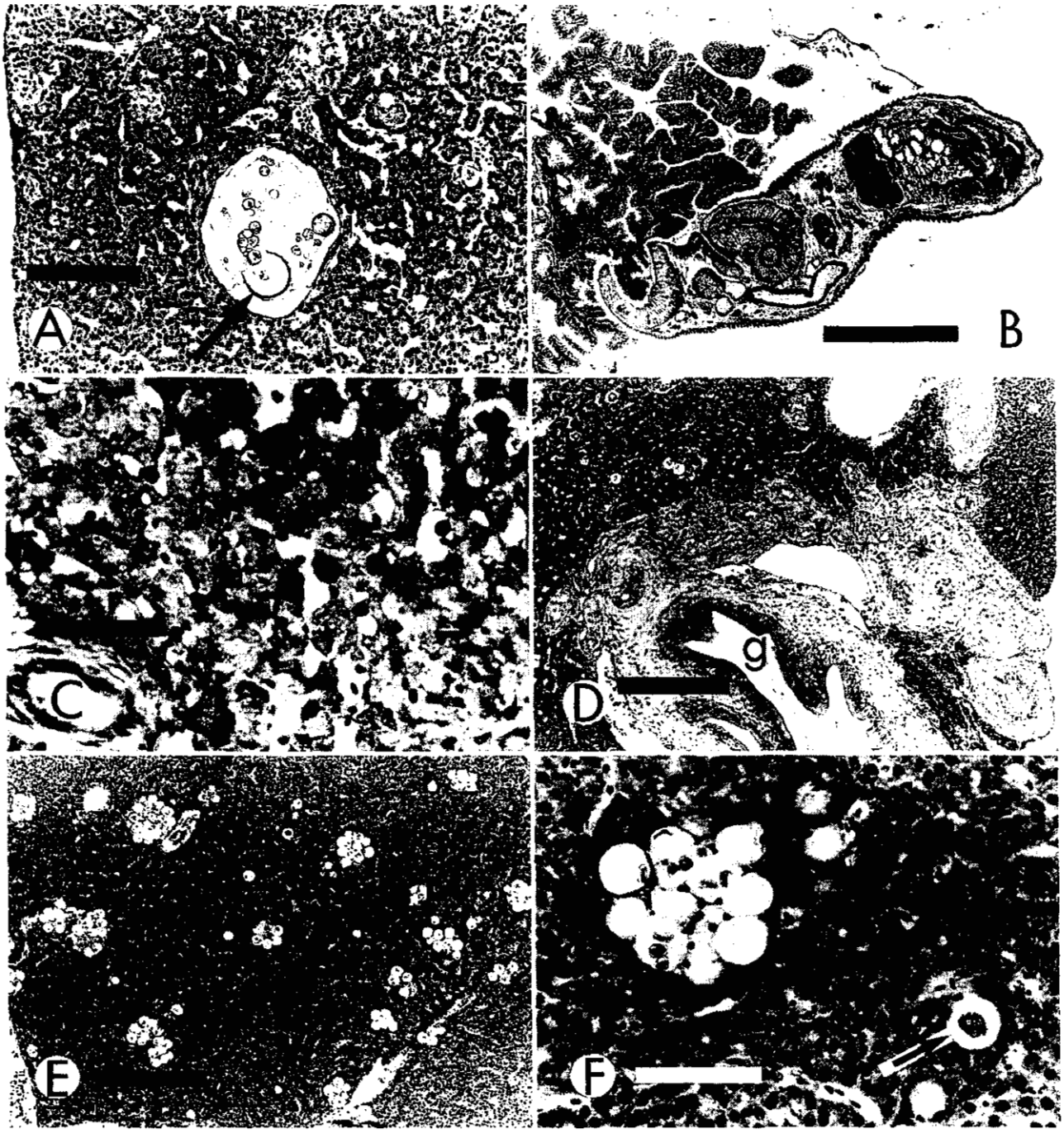
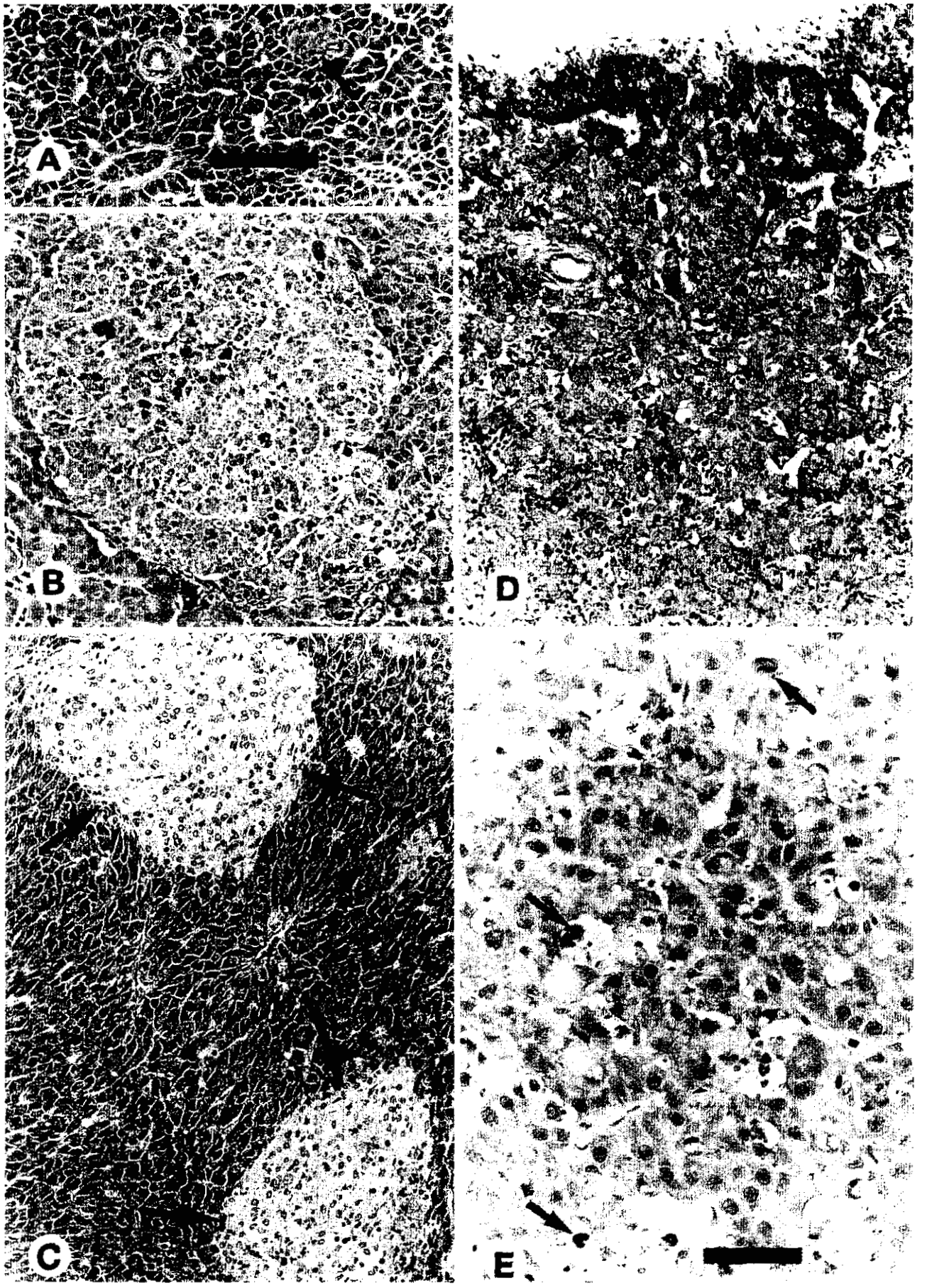


Fig. 4. *Clupea pallasi*. Microscopic lesions in the liver and stomach of Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994; hematoxylin and eosin stain. (A) Liver with a ruptured *Ichthyophonus* resting spore (arrow) that has released several multinucleate endospores; bar length = 100 µm. (B) Trematode (probably Hemiuridae) attached to the gastric mucosa with an oral sucker. Note the prominent acetabulum (a); bar length = 300 µm. (C) Hepatic coagulative necrosis; note pyknosis and karyolysis within a broad band of hepatocytes; bar length = 40 µm. (D) Biliary hyperplasia at the base of the gall bladder (g); bar length = 200 µm. (E) Multiple foci of *Goussia clupearum* scattered throughout the hepatic parenchyma; bar length = 200 µm. (F) Sporulated oocysts and an unsporulated oocyst (arrow) of *Goussia clupearum* in the liver. Note minimal inflammation; bar length = 50 µm

Fig. 5. *Clupea pallasi*. Normal liver histology and hepatic lesions in Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994; hematoxylin and eosin stain. (A) A small pigmented macrophage aggregate (arrow); bar length = 150 µm. (B) A large pigmented macrophage aggregate; magnification same as (A). (C) Two foci of granulomatous inflammation (arrows) that were unrelated to *Ichthyophonus*. Note that pale foci of activated macrophages contain scattered lymphocytes but pigment is minimal; magnification same as (A). (D) Severe, acute, zonal, coagulative necrosis with small irregular foci of viable hepatocytes (e.g. v and arrows); magnification same as (A). (E) Severe single cell hepatocellular necrosis (apoptosis). Several hepatocytes have condensed nuclei with contracted hypereosinophilic cytoplasm (arrows); bar length = 30 µm ►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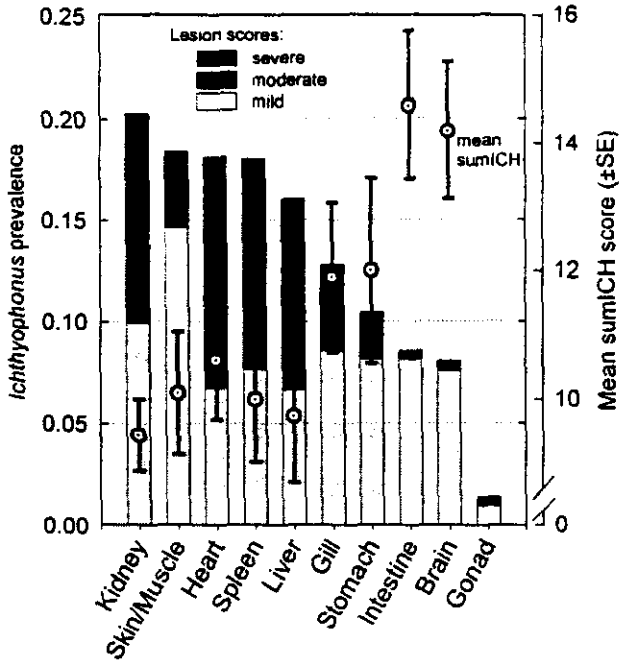


Fig. 6. *Clupea pallasi*. Sample prevalence of *Ichthyophonus* lesion scores in various organs compared with mean sum-*Ichthyophonus* (mean sumICH) score for each organ. Lesions were scored as none (0), mild (1), moderate (2), or severe (3), and the sumICH score was calculated for each fish by adding the *Ichthyophonus* score for all organs in that fish. The mean sumICH score was calculated for each organ. For example, the mean sumICH score for the brain was the average of sumICH scores for all 17 fish that had brain *Ichthyophonus*; fish without brain *Ichthyophonus* were not used for calculations of the mean sumICH score for the brain. Sample size varies from 210 to 212

Focal hepatic necrosis was not common (6 fish affected) but was significantly associated with VHSV infection (Table 2). Broad bands of affected hepatocytes had hypereosinophilic cytoplasm and pyknotic, karyorrhectic, or karyolytic nuclei characteristic of coagulative necrosis (Figs. 4C & 5D). By comparison, single cell hepatocellular necrosis was more common (16 fish affected) but was not significantly associated with VHSV infection. Individual necrotic (or apoptotic) cells had condensed hypereosinophilic cytoplasm and pyknotic nuclei. Necrotic (or apoptotic) cells were often surrounded by a pericellular clear space (Fig. 5E).

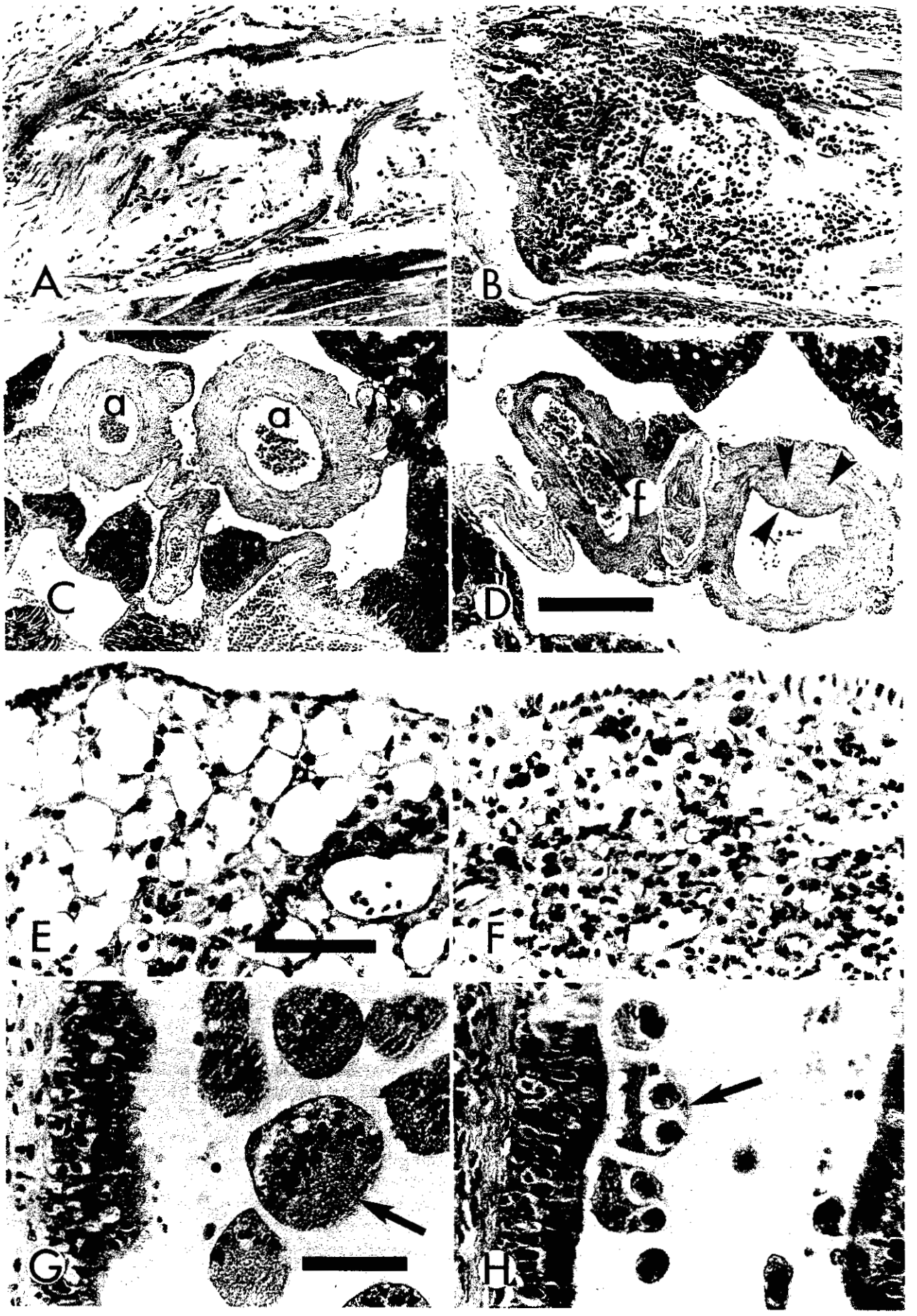
Focal intimal hyperplasia of arteriolar walls was relatively common and was scored in sections of intestine, skin and skeletal muscle, and spleen. In the intestine only, this lesion was significantly associated with VHSV infection (Table 2). Normal arteries and arterioles had a smooth intimal surface without valves (Fig. 7C). In some cases, however, the intima contained one or more foci of connective tissue that projected into the lumen from a narrow base in mild cases, and from a broad base in moderate cases (Fig. 7D). The origin of these foci is unknown, but they may have been sequella to endothelial damage.

Gender-associated lesions

Lesions significantly more frequent in ovaries included hyalinization of vessel walls and pigmented macrophage aggregates. By comparison, granulomatous inflammation was significantly more common in testes than in ovaries (Table 2). Except for one female with severe ovarian *Ichthyophonus*, germ cells were mature in all fish and lesions were not severe enough to have impaired spawning.

Gender differences were significant for several nongonadal lesions (Table 2). Myxosporeans in the gall bladder (*Ceratomyxa auerbachi*) were significantly more frequent in females. Males had a significantly greater frequency of severe intestinal mesenteric steatitis, renal proximal tubular epithelial vacuolation, and renal tubular dilation. Splenic *Ichthyophonus* prevalence was similar in males and females, but associated lesions were more likely to be severe in females. Isolation of VHSV was more frequent from males (7 of 116) than from females (4 of 117), but differences were not significant (chi-square test, 2 × 2 contingency table).

Fig. 7. *Clupea pallasi*. Microscopic lesions in various organs of Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994; hematoxylin and eosin stain. (A) and (B) Gill arches normally contained scattered inflammatory or hematopoietic cells (A), but some fish had more abundant inflammatory or hematopoietic cells (B); same magnification, bar length = 100 m. (C) and (D) Small arteries and nerves were common near exocrine pancreatic tissue between intestinal cecae. Normal arteries (a) had a smooth intimal surface (C), but arteries in some fish had focal intimal hyperplasia (D) that varied from mild (f and arrow) to moderate (arrowheads); same magnification, bar length = 150 m. (E) and (F) Intestinal mesenteries normally had mild infiltrates of inflammatory cells and moderately sized adipocytes (E), but some fish had moderate infiltrates of inflammatory cells (steatitis) and atrophied adipocytes (F); same magnification, bar length = 40 m. (G) and (H) Renal archinephric ducts contained intraluminal parasites, but associated inflammation was minimal. Pansporoblasts of the myxosporean *Ortholinea orientalis* (G, arrow) were free within the lumen, whereas unidentified myxosporeans (H, arrow) were smaller and adhered to the luminal epithelium; same magnification, bar length = 40 m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Intestinal mesenteric steatitis involved peritoneal fat throughout the mesenteries of the viscera. Lipid volume of adipocytes varied from moderately abundant to minimal. In moderate cases of steatitis, lipid volume was often less than the volume of adipocyte nuclei (Fig. 7F). Inflammatory infiltrates included macrophages, lymphocytes, and eosinophilic granular leukocytes. All fish had at least some inflammatory cells within the peritoneal fat (Fig. 7E), but 19 males and 8 females had more than 30% of the volume of peritoneal fat infiltrated by inflammatory cells. The cause of these inflammatory infiltrates was not determined.

Proximal renal tubular epithelium was considered vacuolated if intracytoplasmic clear spaces were larger than adjacent nuclei. Kidneys from 9 males and 1 female contained vacuolated tubular epithelial cells, but in only one case (a male) were more than 20% of the proximal tubular epithelial cells affected. Renal tubules were considered dilated when luminal diameter was more than twice the thickness of tubular epithelial cells. Kidneys from 7 males and 1 female contained dilated tubules, but in no cases were more than 50% of the tubules dilated. Causes for these tubular changes are unknown. Although pansporoblasts of the renal myxosporean *Ortholinea orientalis* sometimes nearly filled archinephric ducts (Fig. 7G), only one of 44 cases was associated with dilated tubules, i.e. *Ortholinea orientalis* was not associated with dilated tubules.

In addition to these lesions, gender differences were significant for several plasma chemistries (Table 3). Compared to males, females had significantly lower values for albumin, chloride, cholesterol, CO_2, glucose, potassium, and total protein, and significantly higher values for ALP. Gender differences were not significant for other plasma chemistries.

Intraperitoneal herring worms (Anisakidae)

All 233 Pacific herring contained larval parasites of the family Anisakidae within their peritoneal cavities. No attempt was made to differentiate species (e.g. *Anisakis* vs *Contracecum*), and parasite morphology and inflammatory response were consistent with previous descriptions (Hauck & May 1977). Herring worm numbers were significantly greater in females than in males, and numbers significantly increased with increasing severity of several lesions. For example, fish with more severe hepatic cholangitis or biliary hyperplasia (Fig. 4D) had increased numbers of herring worms. Also, increased numbers of intraperitoneal Anisakidae were associated with increased scores for Anisakidae in the liver, intestine, and skeletal muscle. Fish with renal interstitial cell necrosis had fewer herring worms than did fish without renal interstitial cell necrosis.

Lymphocystis virus

Two Pacific herring had internal lesions consistent with lymphocystis virus, but the skin of these fish was normal. Affected fish had 1 or 2 spherical, white foci, each about 2 mm in diameter. One focus was in the cranial part of the peritoneal cavity, and the other focus expanded the intestinal mesenteries. Histologically, each white focus was composed of a single hypertrophic fibroblast. The affected fibroblast had a multilayered, 12 µm thick, hyaline capsule, with abundant granular basophilic cytoplasm, and a large nucleus (500 µm in diameter) with vacuolated and marginated chromatin (Fig. 8A, B). The infected fibroblast was not associated with any inflammatory cells. Ultrastructurally, the cytoplasm contained abundant icosahedral viral particles, each about 200 nm in diameter, with an electron-dense viroplasm (Fig. 8C). The ultrastructural features of the virus are characteristic of lymphocystis virus.

Other potential pathogens

No significant bacterial pathogens were isolated, and none of the blood smears had evidence of VEN. Ulcers often contained variable amounts of granulation tissue with a surface layer of filamentous bacteria; however, culture results indicated that the bacteria had not spread to the kidney.

Pacific herring had 12 other parasites, most of which were associated with few lesions. These parasites in descending order of prevalence included: (1) an intestinal coccidian (*Goussia* sp.?) that has not previously been described, 91%; (2) a coccidian in the liver, *Goussia* (*Eimeria*) *clupearum*, 61%; (3) a testicular coccidian, 57% of males; (4) a myxosporean in renal tubules, *Ortholinea orientalis*, 19%; (5) a myxosporean in the gall bladder, *Ceratomyxa auerbachi*, 19%; (6) branchial monogenetic trematodes *Gyrodactylus* spp., 13%; (7) branchial ciliated protozoans, probably *Trichodina* and *Cryptokaryon* spp., 12%; (8) unclassified renal intraductal myxosporean (?), 11%; (9) branchial *Epitheliocystis*, 10%; (10) gastric intraluminal trematodes, e.g. Hemiuridae, 8.6%; (11) intestinal trematodes, e.g. *Lecithaster gibbosus*, 2.9%; and (12) intestinal cestodes, 2.4%. Infestation with these branchial and gastrointestinal parasites did not significantly alter plasma chemistry values or inflammatory changes.

Morphologic features and distribution of the intestinal coccidian were very similar to descriptions of *Goussia zarnowskii* in the 3-spined stickleback *Gasterosteus*

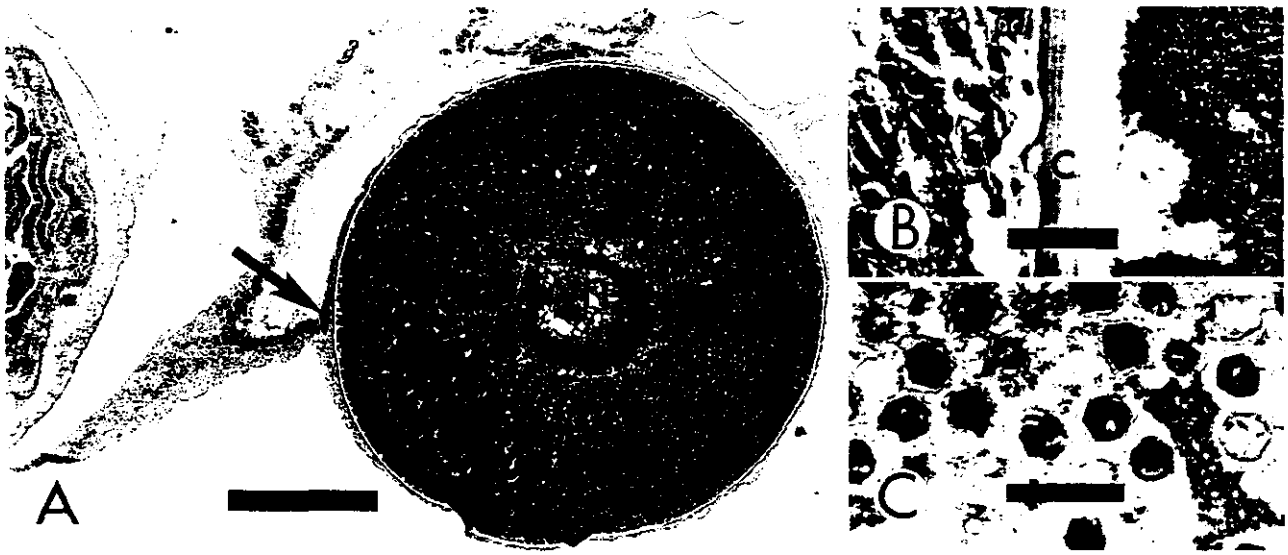

Fig. 8. *Clupea pallasi*. Lymphocystis virus in a spawning Pacific herring from Prince William Sound, Alaska, 1994. (A) intestine (left) and hypertrophied fibroblast expanding intestinal mesenteries (arrow); note hypertrophic fibroblast nucleus (n); arrow = margin of hypertrophic fibroblast and area shown in detail in (B); bar length = 600 µm. (B) Multilayered hyaline cell membrane (c) of hypertrophied fibroblast; atrophic exocrine pancreas is on the left and the expanded granular basophilic cytoplasm is on the right; bar length = 25 µm. (C) Transmission electron micrograph of the cytoplasm of the hypertrophic fibroblast; note icosahedral viral particles; bar length = 0.5 µm

aculeatus (Jastrzebski & Komorowski 1990). In Pacific herring, the coccidians were common in small numbers throughout the intestine, including the intestinal cecae. Only 2 fish had more than 15 organisms per 400× field in several fields examined. In affected intestines, the surface of epithelial cells contained spherical to ovoid, basophilic organisms (Fig. 3B). Small forms of the parasite, about 8 µm in diameter and densely basophilic, were probably meronts or trophozoites. By comparison, larger forms of the organism, up to 15 µm in diameter and 20 µm long, were less intensely stained. Some contained densely basophilic 1 to 2 µm diameter spherical structures, whereas others contained eosinophilic granules that were 2 to 4 µm in diameter. The larger forms were probably microgamonts or microgametes. Oocysts were not present, and there was no inflammatory response to the forms that were present. Also, infections did not significantly alter plasma chemistry values.

Morphologic features and distribution of the hepatic coccidian were very similar to descriptions of *Goussia clupearum* in Atlantic herring (Morrison & Hawkins 1984). In Pacific herring, sporulated oocysts (about 18 × 12 µm) were the most abundant stage and were often in small clusters of 2 to 10 organisms (Fig. 4E), whereas unsporulated oocysts (about 35 µm in diameter) were rare and usually solitary (Fig. 4F). Severity scores were based almost entirely on numbers of foci of sporulated oocysts per 100× field: score = 0 (no parasites); score = 1 (≤2 foci); score = 2 (>2 but ≤6 foci); and score = 3 (>6 foci). Despite the relatively large volume of hepatic parenchyma displaced by the parasites in severe cases, inflammation was minimal and severity of infestation was not significantly associated with changes in plasma chemistry values.

Diagnosis of the renal tubular myxosporean *Ortholinea orientalis* was less sensitive by histopathology (12 of 212, 5.7%) than by examination of kidney touch preparations (41 of 229, 18%). However, 3 cases diagnosed on histopathology were not diagnosed on touch preparations, resulting in a combined total prevalence of 19%. Pansporoblasts, the most common form, were roughly spherical, 60 to 80 µm in diameter, and were free in the lumen of the archinephric duct (Fig. 7G). Multiple nuclei within the pansporoblast were eccentric or polar, depending on the plane of section. Another parasite, an unidentified myxosporean (?), was in the archinephric duct of 24 fish. The parasites were multicellular and usually attached to the surface of ductular epithelial cells (Fig. 7H). They were 25 to 40 µm wide and 15 to 30 µm high.

For the renal myxosporean *Ortholinea orientalis*, the 5 most severely affected fish had plasma calcium levels significantly higher than other groups ($p < 0.001$, with significant Levene's test). The mean ±SE calcium value for the 5 most severely affected fish was 15.3 ± 2.0 mg dl^{-1}, whereas mean calcium values for groups of fish that were less severely affected ranged from 10.8 ± 0.4 to 11.7 ± 0.14 mg dl^{-1}. The proportion of fish with *Ortholinea orientalis* infection was significantly higher in fish with renal *Ichthyophonus* (chi-square test for

homogeneity). Infection with the renal intraductal parasite (probably a myxosporean) was not significantly associated with any changes in plasma chemistries or any other renal lesions.

The gall bladder sometimes contained large numbers of the myxosporean *Ceratomyxa auerbachi* (Fig. 3A). Most common were forms that were roughly spherical, multicellular, and 15 to 30 µm in diameter with 1 to 6 nuclei. Less common were spindle-shaped forms that were 50 to 80 µm long, 15 to 20 µm in diameter, and had pale eosinophilic to vacuolated cytoplasm. Sections of the elongate structures often contained 1 or 2 spherical structures (spores?), about 7 µm in diameter, that stained intensely eosinophilic. Severe infestations sometimes had mild mononuclear inflammation in the lamina propria of the gall bladder, but infestations were not significantly associated with liver lesions or with changes in plasma chemistries.

Age-associated changes

The most consistent age-related 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 1). Lesion scores that significantly increased with age included meningoencephalitis, epicarditis, renal tubular epithelial vacuolation, pancreatic zymogen granule depletion, and splenic ellipsoid hyalinization (Table 1). Interestingly, in the liver, scores for increased granulomatous inflammation were significantly associated with decreased age.

Among common parasites, *Ichthyophonus*, *Goussia clupearum*, and *Ortholinea orientalis* were not significantly associated with age (chi-square test for homogeneity). By comparison, *Ceratomyxa auerbachi* was significantly more frequent in older fish, and the renal intraductal parasite was more common in younger fish (Fig. 9). The number of positive VHSV cases was too small for statistical analysis of age distribution, but the 11 positive cases were distributed among two 3-yr-olds, three 4-yr-olds, three 6-yr-olds, one 9-yr-old, and two 10-yr-olds. In general, VHSV-positive cases were over-represented in younger and older fish in the sample; for example, the 1988 year class (6-yr-old fish) comprised 60% of the sample but only 27% of the VHSV-positive cases.

Plasma chemistries

As hold time increased, plasma potassium and CO_2 significantly increased, but plasma glucose significantly decreased (Table 4). Changes in several other plasma chemistries were not as significant in relation to hold time ($|r| < 0.25$). A complicating factor was that hold time was significantly longer on the last day of sampling when most fish had completed spawning. Therefore, many of the marginally significant changes might have been related to spawning condition rather than hold time. For example, using multifactor regression, hold time was not a significant predictor of albumin levels even though their values were significantly correlated in univariate analysis.

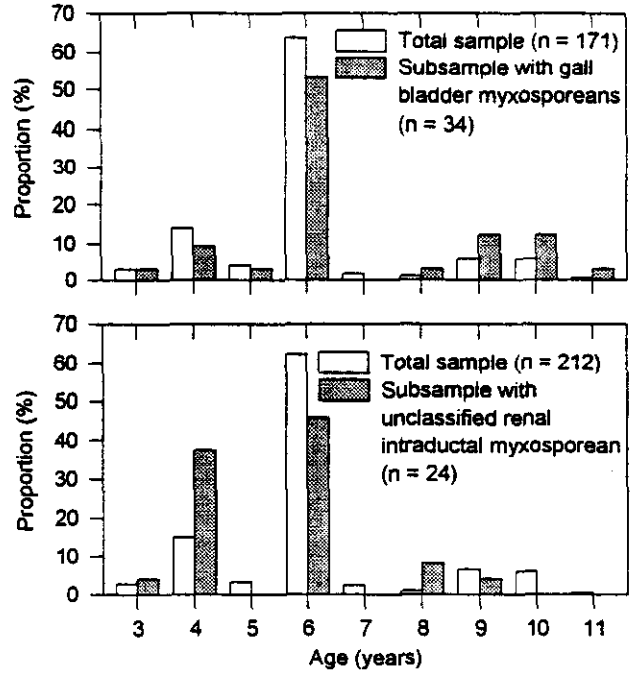

Fig. 9. *Clupea pallasi*. Age composition of Pacific herring with common parasites compared with the age composition of the sample. Pacific herring were sampled from Prince William Sound, Alaska, during spawning, 1994. Top: intraluminal gall bladder myxosporean (*Ceratomyxa auerbachi*). Bottom: unclassified renal intraductal myxosporean

Among enzymes, AST and CPK values were most variable, and differences in lesion scores (particularly *Ichthyophonus*) could be discerned on the basis of AST and CPK (Table 1). Variability of ALP was intermediate, and only rarely could lesion scores be differentiated on the basis of ALP values. Variability of ALT and GGT was minimal and measured values were never greater than 17 U l^{-1} (Table 5). However, correlations of $\log_e$ ALT with total bilirubin ($r = 0.493$) and gonad weight ($r = 0.335$) were highly significant.

Albumin and total protein were unusually low (Table 5) when compared to published values for other fish species (McDonald & Milligan 1992), and albumin was particularly low after fish had finished spawning. For example, 16 fish had albumin ≤0.3 mg dl^{-1}, but only one of these fish had a gonad weight greater than

Table 4. *Clupea pallasi*. Linear correlations (r) of age (yr), body weight and gonad weight (g), standard length (mm), hold time (min), albumin (g dl^{-1}), sum-*Ichthyophonus* (sumICH) scores, and blood values in Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994. Highly significant correlations ($p < 0.01$) are denoted (*); sample size varies from 208 to 233

Variable	Age	Body weight	Length	Gonad weight	Hold time	sumICH	Albumin
Body weight	0.67*						
Length	0.71*	0.90*					
Gonad weight	0.36*	0.75*	0.50*				
Hold time	-0.15	-0.24*	-0.20*	-0.16			
SumICH	-0.06	-0.04	-0.07	0.08	0.03		
Albumin	0.13	0.30*	0.18*	0.34*	-0.13	0.04	
PCV (%)	0.07	0.27*	0.17*	0.29*	-0.14	-0.16	0.38*
Total protein (g dl^{-1})	0.11	0.36*	0.18*	0.52*	-0.08	0.21*	0.75*
log_e AST (U l^{-1})	-0.04	0.09	-0.01	0.23*	-0.03	0.26*	0.17
ALP (U l^{-1})	0.03	0.30*	0.11	0.46*	-0.19*	0.10	0.58*
log_e ALT (U l^{-1})	-0.03	0.02	-0.04	0.33*	0.19*	0.02	0.05
log_e CPK (U l^{-1})	0.06	0.20*	0.14	0.24*	-0.10	0.30*	0.34*
GGT (U l^{-1})	<0.01	0.09	0.06	0.02	-0.23*	0.09	0.16
Calcium (mg dl^{-1})	<0.01	0.14	0.04	0.28*	-0.13	-0.03	0.44*
Chloride (mmol l^{-1})	0.16	0.17*	0.24*	0.05	-0.14	-0.03	0.09
Cholesterol (mg dl^{-1})	0.10	0.27*	0.14	0.34*	-0.17	0.09	0.90*
CO_2 (mmol l^{-1})	-0.02	-0.12	-0.09	-0.21*	0.44*	<0.01	-0.03
Glucose (mg dl^{-1})	0.16	0.22*	0.16	0.26*	-0.33*	-0.05	0.37*
Osmolality (mOsm kg^{-1})	0.08	0.27*	0.20*	0.28*	-0.11	-0.07	0.30*
Phosphorus (mg dl^{-1})	0.01	0.15	0.03	0.31*	-0.10	0.02	0.24*
Potassium (mmol l^{-1})	-0.05	-0.03	-0.06	0.03	0.48*	0.08	0.03
Total bilirubin (mg dl^{-1})	-0.05	-0.03	-0.10	0.11	0.13	-0.14	0.06

5 g. Total protein values derived from refractometer readings were consistently greater than values derived from the biuret method (mean difference = 3.1 g dl^{-1}, range = 1.6 to 4.4 g dl^{-1}); therefore, only values derived from the biuret method were used.

Postspawning fish commonly had clear, highly proteinaceous fluid in the peritoneal cavity (ascites). In most cases, the fluid clotted as it was aspirated into 1 ml syringes for volume measurements. Forty-three fish had 0.1 to 2.5 ml of ascites, and no fish with gonad weight greater than 5 g had ascites. Ascites was more frequent in males (26 of 116, 22%) than in females (17 of 116, 17%), but differences were not significant (chi-square test, 2 × 2 contingency table). Fish with ascites had albumin levels that varied from 0.0 to 0.6 g dl^{-1}, and albumin levels in fish without ascites ranged from 0.0 to 1.1 g dl^{-1}.

Multiple regression analysis was used to model a multifactor ANOVA, examining the relationships between the dependent variable albumin and 3 variables (focal skin reddening, splenic congestion, and VHSV). Iris reddening, which was significant in the univariate analysis, was left out of the regression because fewer cases were scored on this variable, contributing to a loss of 19 cases in the analysis. Based on the responses from 205 fish, 7 factors were entered in the final model (gender, gonad weight, hold time, length, focal skin reddening, splenic congestion, and VHSV); the adjusted r^2 was 0.38. A stepwise regression equation derived from significant

Table 5. *Clupea pallasi*. Plasma chemistry values in 233 Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994

Plasma chemistry	Mean	Minimum	Maximum	SD	Normal[a] Low	Normal[a] High
Total protein (g dl^{-1})	2.2	0.2	3.8	0.6	1.0	3.1
Albumin (g dl^{-1})	0.5	0	1.1	0.2	0.1	0.8
ALP (U l^{-1})	55	2	116	21	13	95
ALT (U l^{-1})	3.7	0	14	2	0	8
AST (U l^{-1})	346	11	2590	318	0	860
CPK (U l^{-1})	450	10	8080	705	0	1240
GGT (U l^{-1})	7	0	17	4	0	15
Potassium (mmol l^{-1})	2.3	0.6	7.7	1.1	0	4.4
Chloride (mmol l^{-1})	163	141	197	12	139	184
CO_2 (mmol l^{-1})	6	0	17	2	1.7	10.3
Phosphorus (mg dl^{-1})	12.7	5.5	38	4.3	3.7	21.5
Calcium (mg dl^{-1})	11.6	6.7	21	1.9	7.9	14.8
Cholesterol (mg dl^{-1})	218	4	420	71	74	353
Glucose (mg dl^{-1})	83	17	411	39	3	164
Total bilirubin (mg dl^{-1})	0.04	0	0.4	0.08	0	0.2
Osmolality (mOsm kg^{-1})	428	374	512	24.6	378	475

[a]Normal values are the range (mean ± 2SD; n = 140) after removal from the data set of all fish with *Ichthyophonus*, VHSV, or severe *Ortholinea orientalis*

factors only was used to quantify the contribution of each variable to albumin levels (g dl^{-1}). The constant (0.21 g dl^{-1}) is altered as follows:

gender male =	+0.114
gender female =	+0.000
gonad weight (g) =	+0.0045 × (gonad wt)
VHSV-negative =	+0.047
VHSV-positive =	−0.047
focal skin reddening, none =	+0.098
focal skin reddening, mild =	−0.006
focal skin reddening, moderate/severe =	−0.104
splenic congestion, none =	+0.046
splenic congestion, mild =	−0.008
splenic congestion, moderate/severe =	−0.038

For example, a male (+0.114) with a gonad weight of 10 g (+0.045) that was VHSV negative (+0.047) and had no focal skin reddening (+0.098) and mild splenic congestion (−0.008) would be expected to have a plasma albumin level of 0.51 g dl^{-1}. The predicted plasma albumin level in a similar male with moderate focal skin reddening would decrease to 0.30 g dl^{-1}.

Like albumin, scores for several lesions and other variables could be differentiated on the basis of PCV, and PCV was significantly associated with several plasma chemistries (Tables 1 & 4). Multiple regression analysis was used to model a multifactor ANOVA, examining the relationships between PCV and 7 variables. Based on the results from 186 fish, 12 factors were entered in the final model (gender, gonad weight, hold time, length, osmolality, focal skin reddening, splenic *Ichthyophonus*, renal hematopoietic cells, hepatic lipidosis, cardiac thrombosis, gastric trematodes, and VHSV); the adjusted r^2 was 0.29. Because of the potential that dehydration could effect PCV, osmolality was added as a controlling variable. A stepwise regression equation derived from significant factors only was used to quantify the contribution of each variable to PCV (%). The constant (51.14 %) is altered as follows:

gender male =	+2.30
gender female =	+0.00
gonad weight (g)=	+0.1513 × (gonad wt)
osmolality (mOsm/kg) =	−0.0433 × (osmolality)
hepatic lipidosis, none =	+1.49
hepatic lipidosis, mild =	−0.71
hepatic lipidosis, moderate/severe =	−0.77
splenic *Ichthyophonus*, none =	+1.50
splenic *Ichthyophonus*, mild =	−2.43
splenic *Ichthyophonus*, moderate/severe =	+0.92
renal hematopoietic cells, none =	−2.29
renal hematopoietic cells, mild =	+1.63
renal hematopoietic cells, moderate =	+0.67
gastric trematodes, none =	+1.87
gastric trematodes, mild/moderate =	−1.87

For example, a male (+2.30) with a gonad weight of 10 g (+1.51), osmolality of 425 mOsm kg^{-1} (−18.40), no hepatic lipidosis (+1.49), no splenic *Ichthyophonus* (+1.50), mild renal hematopoietic cells (+1.63), and mild gastric trematodes (−1.87) would be expected to have a PCV of 39.3%. By comparison, a similar male with no renal hematopoietic cells and mild splenic *Ichthyophonus* would have a predicted PCV of 31.5%.

Table 6. *Clupea pallasi*. Sample prevalence (%) of parasites and virus in adult Pacific herring in Prince William Sound, Alaska, 1989 to 1994

Sample date	n	*Goussia clupearum*	*Ichthyophonus hoferi*	*Ortholinea orientalis*	Viral hemorrhagic septicemia virus
1989 April[a]	40	63	13	TNE[b]	TNE
1990 October[a]	99	60	15	12	TNE
1991 April[a]	59	54	5.1	17	TNE
1991 October[a]	48	54	2.1	15	TNE
1992 April[c]	105	53	5.7	3.1	TNE
1993 April[d]	79	41	5.1	4.3	2 of 3 5-fish pools
1994 April	212	61	29	5.7[e]	4.7

[a]Unpubl. data from G. D. Marty, M. S. Okihiro, and D. E. Hinton
[b]TNE: Tissue not examined
[c]Kocan et al. (1996)
[d]Meyers et al. (1994), Meyers & Winton (1995)
[e]Prevalence based on histopathology only; total prevalence using touch preparations and histopathology was 19%

Annual trends in spawning biomass and pathogen prevalence

Sample prevalence of *Ichthyophonus* in this study was almost twice that of previous years (Table 6). During the damage assessment phase of study from 1989 through 1992, and disease studies in 1993 (Meyers et al. 1994), prevalence of *Ichthyophonus* in Pacific herring sampled from PWS was never more than 15%. By comparison, prevalence of *Goussia clupearum* has remained fairly constant between 41 and 63%, and *Ortholinea orientalis* prevalence has not exceeded 17%. The slight increase in *Ortholinea orientalis* prevalence in this study (19%) was probably at least partly due to increased efficiency of diagnosis when touch preparations were examined; previous prevalence data were derived from histopathology only. Prevalence of VHSV and other parasites was not determined in previous studies because appropriate tissues were not examined.

DISCUSSION

VHSV

The North American strain of VHSV was a major cause of morbidity in Pacific herring in PWS during spawning in 1994. Fish from which VHSV was isolated had significant gross lesions as well as microscopic lesions in the gills, liver, stomach, arteries, and heart. Most lesions were consistent with a disseminated endotheliotrophic virus, and lesions such as coagulative necrosis in the liver have been attributed to VHSV in natural and laboratory infections in rainbow trout (Amlacher et al. 1980, Wolf 1988b). Because the VHSV outbreak was nearly over in 1994, opportunities to confirm association of lesions with VHSV by further field study have been limited. However, recent study with Pacific herring fulfilled Koch's postulates, demonstrating that VHSV kills laboratory-reared Pacific herring in absence of other pathogens (Kocan et al. 1997).

Although the North American strain of VHSV has been isolated from several populations of Pacific herring (Meyers & Winton 1995), the only other published report of VHSV linked to population decline was from fish sampled in PWS in 1993 (Meyers et al. 1994). Meyers et al. (1994) postulated that several lesions were associated with VHSV: subdermal and renal hemorrhages, kidney tubule degeneration, and active reticuloendothelial cell foci in the kidneys. Also, active reticuloendothelial cell foci in the liver were associated with hepatocellular necrosis. In the present study, we confirmed an association of VHSV with fin base reddening and focal coagulative hepatic necrosis, and we had some evidence for association of VHSV with ulcers (i.e. severe focal skin reddening). Association of VHSV with renal hemorrhage or kidney tubule degeneration could not be confirmed. In the present study, 'active reticuloendothelial cells' were classified as either pigmented macrophage aggregates or granulomatous inflammation, and neither was significantly related to VHSV in the liver or kidney. However, infiltrates of lymphocytes or macrophages in the gastric submucosa, gill arches, and brain were significantly associated with VHSV infection. In a study of PWS Pacific herring from 1992, granulomatous inflammation was associated with decreased reproductive success (Kocan et al. 1996), but based on our results we cannot attribute these lesions to VHSV.

Population fluctuations in Pacific herring are considered normal by management biologists, but in only one other case was population decline attributed to disease. During February and March of 1942, 'several thousands of tons' of Pacific herring were found dead along the southeast coast of Vancouver Island, British Columbia, Canada (Tester 1942). 'The dying fish came to the surface and could, while still alive, be picked up by gulls or by hand.' Mortality involved pre- and post-spawners, and fish continued to be lethargic and school in shallow water near shore until mid-May (Tester 1942). Diagnostic examination included gross necropsy, bacteriology, blood smears, and parasite screen, but no significant pathogens were found. Based on this level of diagnostic detail, *Ichthyophonus* can be ruled out as the cause of mortality in 1942, but many features were similar to the 1993 epizootic in PWS. Both outbreaks had lethargic fish, some of which had reddening of the fins, and both outbreaks followed a year in which commercial harvest was above average. The epizootic near Vancouver Island involved a dominant 1938 year class (4-yr-olds), whereas the PWS epizootic involved a dominant 1988 year class (5-yr-olds). As a difference, the Vancouver Island outbreak had large numbers of dead fish, whereas dead fish were not reported in the PWS epizootic. One other disease, VEN, has been reported to cause significant mortality in juvenile Pacific herring when such year classes are strong. However, VEN has not been associated with significant decline in population biomass (Meyers et al. 1986), and PWS fish in 1994 had no evidence of VEN.

Several questions about the pathogenesis of VHSV in Pacific herring are beginning to be answered with continued field study and focused laboratory study. VHSV is highly infectious, spreads through the water, and readily kills disease-free Pacific herring independent of exposure to other pathogens (Kocan et al. 1997). Preliminary field and laboratory studies indicate that 10 to 15% of Pacific herring have sub-

clinical infections but express VHSV and the associated disease only when subjected to stress (R. M. Kocan pers. comm., G. D. Marty unpubl. obs.). A related virus, infectious hematopoietic necrosis virus (IHNV, also in the family Rhabdoviridae), is commonly carried by salmonids. Disease from IHNV is generally a serious problem only in juvenile fish, and virus is expressed in surviving adults during and after spawning (Wolf 1988a). In a study of Pacific herring at the National Marine Fisheries Service Laboratory in Auke Bay, Alaska, VHSV was expressed in a dose-dependent manner after 17 d of exposure to weathered crude oil (Mark Carls pers. comm., Meyers & Winton 1995). Although the VHSV status of these fish before the study began was unknown, the study provided evidence that oil can act as a stressor that activates VHSV. Several other questions are under investigation. Once VHSV is expressed, can fish mount a successful immune response and overcome the disease? Does virus expression cycle seasonally? What environmental factors contribute to disease and immunity?

Ichthyophonus hoferi

Ichthyophonus has not previously been described as a major cause of mortality in Pacific herring, but in Atlantic herring several epizootics of *Ichthyophonus* have been linked to population decline (Fish 1934, Sindermann 1958, Patterson 1996). Indeed, *Ichthyophonus hoferi* is the most commonly reported and most severe marine fungal pathogen, and 'this disease may be the most important single limiting factor to population growth of herring in the western North Atlantic' (Sindermann 1970). Although recent evidence indicates that *Ichthyophonus* is not a fungus (Spanggaard et al. 1996), its biological significance remains unchanged. Outbreaks in Atlantic herring tend to begin during biomass peaks, usually lasting 2 to 3 yr, and recovery often takes more than 3 yr (Sindermann 1970, Mellergaard & Spanggaard 1997). In Pacific herring in PWS, peak biomass in 1989 did not result in a major *Ichthyophonus*-related population decline, but severe population decline in 1993 was followed by a sharp increase in *Ichthyophonus* prevalence in 1994. Previous declines in Pacific herring biomass have been recorded in PWS, but these were attributed to poor year-class recruitment and over-fishing (Rounsefell & Dahlgren 1932). In Atlantic herring in the Gulf of Maine, *Ichthyophonus* was considered the cause of population declines in 1931 and 1947, and anecdotal evidence was strong for *Ichthyophonus* as the major cause of population declines in 1898 and 1916 (Fish 1934, Sindermann 1965). From 1898 to 1947, outbreaks occurred about every 16 yr and this trend held for 4 cycles; however, no *Ichthyophonus* outbreaks have been documented in the Gulf of Maine since 1947. Sporadic but significant *Ichthyophonus* outbreaks have also been described in the Gulf of St. Lawrence (Sindermann 1970). In Europe, *Ichthyophonus* was not associated with Atlantic herring population decline until a 1991 epizootic in the North Sea (Patterson 1996, Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997).

Some features of *Ichthyophonus* infection in PWS Pacific herring were different from those described in wild Atlantic herring. For example, Atlantic herring with severe infections often had gross lesions in the muscle described as 'rough or granulomatous skin' or 'sandpaper effect' (Post 1987); associated ulcers have been termed 'pepper effect', partly as a result of pigment deposition in the lesions (Fish 1934). By comparison, Pacific herring had no gross external lesions directly associated with *Ichthyophonus*, and microscopic lesions in the skin and skeletal muscles were usually mild. Further, Pacific herring had no pigment associated with *Ichthyophonus* resting spores. Another difference was that epizootics in North American Atlantic herring were always characterized by large numbers of moribund and dead fish in shallow areas (Fish 1934, Sindermann 1958), whereas there were no confirmed reports of dead fish in PWS. Not all features of *Ichthyophonus* were different in Atlantic and Pacific herring; multifocal to coalescing granulomas in internal organs of PWS Pacific herring were similar to the descriptions of gross and histologic lesions reported in Atlantic herring.

The epizootiology of *Ichthyophonus* infection in Pacific herring in PWS is still unclear with only 7 samples in 6 years from 1989 through 1994. Many questions remain unanswered: (1) What is the latency period between *Ichthyophonus* exposure and overt signs of disease? (2) When *Ichthyophonus* is diagnosed histologically, how long will the affected fish live? (3) Can a fish, once infected, initiate a successful immune response and overcome the disease, or are all infected fish destined to die? and (4) Because *Ichthyophonus* prevalence was only 5% in 1993 despite significant population decline (Meyers et al. 1994), how important is *Ichthyophonus* as a cause of Pacific herring mortality? The large spike in *Ichthyophonus* prevalence in this study (29%) was unexpected, but was consistent with infection levels of about 25% described in epizootics affecting Atlantic herring (Sindermann 1970). Sindermann (1970) stated that enzootic levels were about 1%, lower than any samples from Pacific herring in PWS, but Sindermann's observations were based only on gross examination to determine prevalence. Method of

diagnosis can make a significant difference in the number of positive cases identified (Holst 1994, Rahimian & Thulin 1996), and in our study, results from gross examination underestimated the number of infected fish.

In Pacific herring from our study, CPK and AST values could be used to differentiate *Ichthyophonus* lesion scores in nearly every organ. Creatine phosphokinase is a dimeric enzyme with isoenzyme types CK_1 (BB, brain), CK_2 (MB, heart), and CK_3 (MM, skeletal muscle). In mammals, the brain form of CPK is not found in plasma, even during neurologic disease (Duncan & Prasse 1986); therefore, the finding that brain *Ichthyophonus* status was the best predictor for increased CPK in our study was unexpected. Brain *Ichthyophonus* was uncommon, but the high mean sumICH score for fish with brain *Ichthyophonus* (Fig. 6) provided evidence that *Ichthyophonus* was disseminated when it appeared in the brain. That is, if *Ichthyophonus* was disseminated sufficiently that it affected the brain, then the fish probably also had muscle *Ichthyophonus* severe enough to increase CPK. Further, 79% of muscle *Ichthyophonus* cases were mild, and the damage caused by these muscle lesions was probably not sufficient to increase CPK. Alternatively, the brain form of CPK might be released during neurologic disease in Pacific herring. Isoenzyme analysis on plasma from *Ichthyophonus*-positive Pacific herring has not been successful (C. Kennedy, Simon Fraser University, Burnaby, British Columbia, Canada, pers. comm.).

For AST in mammals, lesions in liver, muscle, and blood-rich organs are most highly associated with increased enzyme levels (Duncan & Prasse 1986). For Pacific herring, renal *Ichthyophonus* status was significant in all regressions, but spleen and heart were not. Most likely, the disseminated nature of *Ichthyophonus* infections prevented localization of the source of AST in infected fish.

The effects of *Ichthyophonus* infection on plasma chemistries have not previously been described in natural epizootics. In laboratory-exposed rainbow trout *Oncorhynchus mykiss*, *Ichthyophonus* infection was associated with anemia and leukopenia, but did not change plasma chloride, creatinine, glucose, osmolarity, potassium, total protein, sodium, or T4 (Rand & Cone 1990); enzymes CPK and AST were not measured. In addition to increased CPK and AST in this study, *Ichthyophonus* infection was significantly associated with anemia and variable plasma protein levels; white blood cells were not counted. Based on the equation derived from multifactor analysis, a fish with mild splenic *Ichthyophonus* would be predicted to have a PCV that was 4% less than a similar fish with no splenic *Ichthyophonus*.

Other potential pathogens

A few comprehensive reports are available on the prevalence of parasites in Pacific herring, and their potential role in stock identification (Arthur & Arai 1980, Moser & Hsieh 1992). The purpose of our study was not to repeat these studies, but to determine which of the common parasites of Pacific herring in PWS could potentially contribute to population decline. More than 30 species of parasites have been described from Pacific herring (Arthur & Arai 1980). In our study, 10 parasites occurred in prevalences sufficient to study their role in disease and population decline. Two criteria were used to determine if a parasite caused significant damage to the host: (1) Was the parasite associated with histopathologic damage, particularly inflammation? and (2) Was infection with the parasite associated with alterations in plasma chemistries? Using these criteria, linkage of damage to infections by parasites other than *Ichthyophonus* was not clear.

The intraductal renal myxosporean *Ortholinea orientalis* was not associated with morphologic lesions, nor was there metastatic calcification, but fish with large numbers of organisms had elevated plasma calcium. Because the kidney is one organ that excretes calcium (Dacke 1979), large numbers of organisms might have impaired calcium excretion. The relation of intraductal parasites and calcium levels has not previously been described, and this effect would need to be confirmed by controlled laboratory study.

Lymphocystis virus has been identified in fibroblasts of over 150 species of fish, including Atlantic herring (listed by Lawler et al. 1977), but this is the first description of lymphocystis virus in Pacific herring. Also, in most reported cases, lymphocystis lesions are limited to the skin (Post 1987). Our finding of lymphocystis lesions limited to solitary nodules within the peritoneal cavity is unusual.

Gender- and age-associated lesions

Lower plasma albumin levels in females than in males could partly be explained by vitellogenin synthesis in females. In the hepatocyte, estradiol activates the vitellogenin gene, but production of albumin is depressed (Mommsen & Walsh 1988). Low plasma albumin is commonly associated with ascites in mammals, and Pacific herring with ascites tended to have lower albumin levels than fish without ascites; however, females were not more likely to develop ascites than were males. Several other plasma chemistries and lesion scores had significant gender differences, but little information is available to explain these differences in Pacific herring.

We could critically evaluate only one age-related hypothesis regarding the link between the oil spill and disease in 1994. Fish that were hatched or were yearlings in 1989 at the time of the spill (1988 and 1989 year classes) might have incurred irreversible immunosuppression. Under normal growth conditions, minor deficiencies in their immune system might have been insignificant. However, disease might have become a serious problem when fish experienced additional stress upon first spawning (1992 and 1993). Stress is well-documented as a cause of immunosuppression, but stress-induced changes usually are reversible if the fish survives (Anderson 1990). We found that several changes were significantly associated with age, but scores for nearly all these changes were greater in older fish (i.e. fish hatched before 1988). Also, among VHSV, *Ichthyophonus*, and 10 other common parasites, none were more prevalent in the 1988 and 1989 year classes than in the entire sampled population. Annual age-weight-length analysis by the Alaska Department of Fish and Game has documented that the population decreased in the absence of abnormal changes in age distribution (Fritz Funk unpubl. data). Therefore, the weight of evidence suggests that the disease outbreak in PWS was not a result of permanent immune suppression caused by hydrocarbon exposure when fish were larvae or yearlings. A companion study in Prince William Sound reached similar conclusions (Elston et al. 1997).

Alterations in plasma chemistries

Analysis of plasma chemistry values was inexpensive and provided useful information for evaluating health of Pacific herring in PWS. However, interpretation of results was limited by lack of reference values. In the only published study of normal plasma enzyme values in Pacific herring (Márquez 1976), analysis of electrolytes and other nonenzyme chemistries was not included. Márquez (1976) captured 5 to 12 Pacific herring by angling, held the fish for 12 h, and then drew blood to analyze for plasma enzymes at 30°C. His mean values for CPK (2948 U l^{-1}) and AST (1778 U l^{-1}) were more than twice the maximum values of normal ranges established in our study (Table 5). Differences between the 2 studies probably resulted from Márquez performing analyses at 30°C instead of the 25°C of our study. Also, the 12 h hold time might have been long enough so that increased enzyme levels reflected damage that occurred during capture. In our study, hold time of less than <4 h was not significantly correlated with plasma CPK or AST. Reference values from Pacific herring populations in peak condition (e.g. late summer) are needed to better interpret changes associated with spawning.

Interesting findings in plasma chemistry values included unusually low albumin levels and unusually high osmolality. Altered plasma chemistry values have been associated with spawning in other fish species, but abnormalities were transient (McDonald & Milligan 1992). Because albumin levels in Pacific herring were significantly decreased only at the end of spawning, and ascites occurred most often in spawned out fish, development of ascites was probably related to physiologic changes at the end of spawning.

Total plasma protein values determined using a refractometer were higher than values determined by colorimetry. Similar differences have been documented in other fish species (Hunn & Greer 1990, Hunn et al. 1992), but the molecular cause for this difference has not been determined. Subsequent analysis of total plasma protein in Pacific herring has used only the colorimetry technique.

Plasma glucose, CO_2, and potassium were useful markers of the effects of hold time between capture and necropsy. The increase in plasma CO_2 was indicative of respiratory acidosis, and potassium levels are expected to increase during acidemia (McDonald & Milligan 1992). Decreased glucose levels may have been associated with increased anaerobic glycolysis, but lactate levels were not determined. Normally, capture stress results in hyperglycemia (Hopkins & Cech 1992), but Pacific herring hepatocytes had histologic evidence of minimal glycogen, thereby limiting the ability of the liver to increase plasma glucose levels in response to stress. To determine the relation of hold time to metabolic acidosis and other plasma chemistry values, plasma lactate levels have been analyzed in continuing Pacific herring disease studies (G. D. Marty unpubl. data).

Implications for studies of disease epizootics in fish populations

Disease epizootics have been associated with declining populations of several marine fish species during the past century. Most notably, early work identified *Ichthyophonus* as the major cause of population decline in Atlantic herring in the Northwest Atlantic (Daniel 1933a, Fish 1934, Sindermann 1970). When *Ichthyophonus* prevalence was high in North Sea Atlantic herring in 1991, *Ichthyophonus* was assumed to be the primary cause of population decline. An extensive, multiyear study focused on *Ichthyophonus* to determine the effects of season, fish age, and gear type on sample prevalence (Holst 1996, Patterson 1996, Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997). Comparatively 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 described in Atlantic herring? Or, did an increase in *Ichthyophonus* prevalence follow an outbreak of VHSV that went undetected?

In other epizootics, a broader range of diagnostic techniques was used to identify the source of the epizootic. For dying striped bass *Morone saxatilis* sampled from an estuary, histopathology was combined with plasma chemistry and toxicant analysis, but virus isolation was not attempted and the cause of death was not determined (Young et al. 1994). For dying pilchard *Sardinops sagax*, a herpesvirus was consistently identified on histological and ultrastructural analysis of the gills of sick fish (Hyatt et al. 1997, Whittington et al. 1997), but the virus could not be cultured *in vitro* (study was limited by lack of pilchard cell lines for virus isolation). Underlying causes of these epizootics and the Pacific herring epizootic are not fully understood (Meyers & Winton 1995). However, through comprehensive pathological examination, combined with focused laboratory study, we have shown that significant pathogens and risk factors can be identified and many variables can be ruled out as significant causes of population decline. Study of disease in PWS Pacific herring has been expanded to include a reference site and semiannual study through spring of 1998. Decreasing prevalence of VHSV and *Ichthyophonus* has been accompanied by an increasing fish population. Future papers will detail the results of these studies.

Acknowledgements. We thank J. Vansant, D. Branshaw, D. C. Phipps, and C. T. Stack for finding and capturing the fish, and W. Widmann for sectioning tissues. Corrine R. Davis performed half of the necropsies and all gonad histopathology. Adam Moles helped identify the parasites, and Joseph R. Sullivan and Barbara S. Washburn reviewed the project design. The research described in this paper was supported by the 'Exxon Valdez' Oil Spill Trustee Council through contracts with the Alaska Department of Fish and Game. However, the findings and conclusions presented by the authors are their own and do not necessarily reflect the views or position of the Trustee Council.

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Editorial responsibility: Jo-Ann Leong, Corvallis, Oregon, USA

Submitted: August 14, 1997; Accepted: October 31, 1997
Proofs received from author(s): February 12, 1998

Appendix 2. Association of plasma IgM with body size, histopathologic changes, and plasma chemistries in adult Pacific herring *Clupea pallasi*.

The second publication resulting from this work is included here in reprint form:

Davis, C.R., G.D. Marty, M.A. Atkinson, E.F. Freiberg, and R.P. Hedrick. 1999. Association of plasma IgM with body size, histopathologic changes, and plasma chemistries in adult Pacific herring *Clupea pallasi*. Dis. Aquat. Org. 38:125-133.

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Vol. 38: 125–133, 1999 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published November 8

Association of plasma IgM with body size, histopathologic changes, and plasma chemistries in adult Pacific herring *Clupea pallasi*

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ABSTRACT: Pacific herring *Clupea pallasi* immunoglobulin is an IgM-like molecule comprised of heavy and light chains with molecular weights of 79 and 25 to 27 kD, respectively. Purified immunoglobulin was used to generate highly specific polyclonal antibodies for development of a sandwich ELISA. The ELISA was used to quantify total plasma IgM in 602 Pacific herring captured in Prince William Sound and Sitka Sound, Alaska, USA. Plasma IgM concentrations ranged from 0.13 to 5.32 mg ml^{-1}. Using multiple stepwise regression analysis, plasma IgM was highly correlated ($p \leq 0.01$) with body length, *Ichthyophonus hoferi* infection, plasma albumin, plasma cholesterol, liver macrophage aggregates, and focal skin reddening. *I. hoferi* was the only organism significantly associated with plasma IgM. Gender, site, and season (spring vs fall) did not contribute to significant differences in plasma IgM. This study contributes to the understanding of the interaction of body size, plasma chemistries, and pathological changes upon circulating immunoglobulins in fish.

KEY WORDS: IgM · Immunoglobulin M · Pacific herring · *Clupea pallasi* · *Ichthyophonus hoferi* · ELISA · Euglobulin · Plasma chemistry · Body length · Fish · Histopathology

INTRODUCTION

Total plasma immunoglobulin concentration may be a valuable indicator of general fish health. Circulating immunoglobulin levels in various species of fish vary with body size (Fuda et al. 1991, Sánchez et al. 1993, Estévez et al. 1995), temperature (Wilson & Warr 1992), season (Yamaguchi et al. 1981, Zapata et al. 1992), and pathogenic organisms (Olesen & Jørgensen 1986, Magnadottir & Gudmundsdottir 1992); however, evaluation of the multiple interactive effects of body size, plasma chemistries and pathological changes upon circulating immunoglobulins in fish is lacking. Also, little data are available on plasma IgM in free-ranging fish and the effects of disease on plasma IgM levels in wild populations.

The population of Pacific herring *Clupea pallasi* in Prince William Sound, Alaska, USA, declined nearly 80% from a 20 yr peak biomass in fall 1992 to a 20 yr low in spring 1994 (Marty et al. 1998). Viral hemorrhagic septicemia virus (VHSV) was isolated from pooled samples of these fish in 1993 (Meyers et al. 1994). Further study in 1994 confirmed the association of VHSV with disease, and *Ichthyophonus hoferi* was identified as a significant pathogen (Marty et al. 1998). The degree to which these 2 pathogens contributed to the population decline and the role of any precipitating factors have not been fully elucidated. Intensive study has not identified any direct link between the 1989 'Exxon Valdez' oil spill in Prince William Sound and the Pacific herring population decline.

Public and scientific interest in this wild Pacific herring population presented an unique opportunity to investigate the interaction of total plasma IgM with a variety of physical characteristics, plasma chemistries, histologic changes and pathogens recorded for these

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fish. We initiated this study with 3 main objectives: (1) purification and partial characterization of plasma IgM in Pacific herring; (2) development of a sandwich ELISA to quantify plasma IgM; and (3) identification of physical characteristics, plasma chemistry values, hematologic changes, and gross and microscopic pathologic changes that might be correlated with plasma IgM in free-ranging Pacific herring. In addition to sampling fish from Prince William Sound, sampling was done in Sitka Sound, Alaska, which was chosen as a reference site because it had an increasing Pacific herring population with an age composition similar to the population in Prince William Sound.

MATERIALS AND METHODS

Euglobulin purification, SDS-PAGE, western blotting, and sequence analysis. Pacific herring from Prince William Sound were caught in purse seines and held in 400 l salt water. Blood was collected from the caudal vein into heparinized syringes. Samples from 100 fish were pooled and centrifuged at 3000 × *g* for 10 min. Plasma was harvested and held at −20°C for transport to the laboratory, then stored at −70°C until processed. The plasma was thawed and centrifuged at 4°C at 10000 × *g* for 30 min to remove precipitated fibrin. The euglobulin was purified from plasma by extensive dialysis at 4°C against 5 mM Tris-HCL, pH 7.4 (Dighiero et al. 1985, Partula & Charlemagne 1993, Adkison et al. 1996). Protein content was determined via the Lowry (Folin-Ciocalteau) method (Lowry et al. 1951). The euglobulin fraction was analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and nonreduced conditions with molecular weight standards including human IgM (Sigma). Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) and stained with Coomassie blue. Membranes were also incubated with specific polyclonal rabbit anti-herring euglobulin antibodies (described below). Due to amino terminal blocking, internal sequence analysis was carried out on the heavy chain portion after SDS-PAGE and electrophoretic transfer to PVDF membranes. Peptides were digested off the membrane with lysyl endopeptidase (Wako Bioproducts) as previously described (Fernandez et al. 1994). Fragments were purified on microbore HPLC (ABI Model 172) and isolated. Peptides were sequenced on an HPG1000 or ABI 470 sequence analyzer. Sequences were analyzed for homology with known peptides using the Kabatpro database and the BLASTP alignment tool (Altschul et al. 1990). Densitometry analysis (ImageQuant software; Molecular Dynamics, Inc.) was performed on Coomassie blue-stained gels to determine the percentage that the proposed heavy and light chain bands comprised of the total purified euglobulin fraction.

Polyclonal rabbit antibody. The herring euglobulin fraction was deglycosylated using a modification of a previously described protocol (Mattes & Steiner 1978). Euglobulin (2.5 mg) was resuspended in 0.04 M sodium acetate buffer, pH 5.4, 0.1 M NaCl. An equal volume of 20 mM sodium periodate in the same acetate buffer was added to the eluent and incubated at room temperature for 1 h. Glycerol was added to 50% total volume and the mixture was dialyzed, using Spectrapor 2 tubing, against 4 l PBS, pH 7.4, overnight at 4°C. The dialyzed sample was centrifuged at 14000 × *g* for 2 min. The supernatant was collected, divided into aliquots, and stored at −20 or −70°C. Protein concentration was determined via Lowry (Folin-Ciocalteau) method (Lowry et al. 1951).

A single male New Zealand White rabbit received an initial subcutaneous inoculation of 400 µg deglycosylated euglobulin in complete Freund's adjuvant. A second subcutaneous inoculation of 400 µg deglycosylated euglobulin in incomplete Freund's adjuvant was administered 4 wk later. Rabbit antibodies to herring euglobulin were detected by ELISA at a dilution of 1:51200 in serum collected 10 d after the second inoculation. The rabbit antiserum bound specifically with the euglobulin fraction when reacted with whole Pacific herring plasma on a western blot (data not shown). Polyclonal rabbit immunoglobulin (IgG) was purified by methods previously described (McKinney & Parkinson 1987). A portion of the purified antibody was biotinylated with NHS-LC-Biotin according to the manufacturer's instructions (Pierce).

Immunoprecipitation of Pacific herring and rainbow trout plasma was performed with the purified rabbit immunoglobulin. Pacific herring or rainbow trout plasma was incubated overnight at 4°C with rabbit-anti-herring IgG diluted 1:100 (29 µg ml^{-1}). The IgG-bound proteins were precipitated by addition of Protein G/Protein A agarose (Oncogene Research Products, Cambridge, MA). The precipitate was washed 2 times in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA [ethyleneglycol-bis-tetraacetic acid], 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 µg ml^{-1} leupeptin) and 1 time in PBS, with centrifugation at 10000 × *g* for 10 min after each wash. After reconstitution with PBS, precipitates were run on a 12% polyacrylamide gel and electrophoretically transferred to a PVDF membrane. The membrane was stained with Ponceau S to highlight all proteins in the reaction and then immunoblotted with biotinylated rabbit-anti-herring IgG. The membrane was blocked for 1 h in 5% nonfat dry milk in Tween TBS (TTBS:

50 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl and 1 ml Tween-20 per liter), then incubated with biotinylated rabbit-anti-herring IgG diluted 1:1000 (3 µg ml^{-1}) for 1 h at room temperature. The membrane was washed in TTBS and incubated with streptavidin-alkaline phosphatase conjugate at a 1:2000 dilution at room temperature for 30 min. The membrane was washed in TTBS and incubated with a chemiluminescent substrate (CDP-*Star*™, Tropix, Inc., Bedford, MA) for 5 min, drained, and exposed to X-ray film.

Development of a sandwich ELISA for detection of euglobulin. The ELISA was optimized as previously described (Case et al. 1983). To determine IgM concentrations in herring plasma samples, 96-well immunoassay plates (Falcon®, Pro-bind™) were coated with rabbit anti-herring antibody (50 µl $well^{-1}$) diluted 1:2500 (1 µg ml^{-1}) in 50 mM bicarbonate/carbonate buffer, pH 9.6, and incubated overnight at 4°C or 2 h at 37°C. Following incubation, plates were washed 5 times with TTBS and shaken vigorously to remove excess fluid. Wells were blocked with 5% nonfat dry milk in TTBS and incubated at 37°C for 60 min. Plates were then washed 5 times. Fifty µl of test sample were added to each well and incubated at 37°C for 60 min. Plates were again washed 5 times, followed by addition of 50 µl $well^{-1}$ of biotinylated rabbit anti-herring antibody diluted 1:800 (3.75 µg ml^{-1}) in blocking buffer. After a 60 min incubation at 37°C, the plates were again washed, followed by addition of 50 µl $well^{-1}$ peroxidase-streptavidin diluted 1:1000 in blocking buffer. After a 30 min incubation at 37°C, plates were washed 9 times. The TMB substrate solution was added (100 µl $well^{-1}$) and the plates incubated for 20 to 40 min at 37°C. The reaction was stopped with the addition of 50 µl $well^{-1}$ 1 M H_2SO_4. Each plate contained reference blanks and standard curves in triplicate. The standard curve was constructed using serial dilutions of herring euglobulin of known protein concentration. Plates were read at 450 nm on a Ceres 900 Hdi plate reader (Bio-Tek) and interpreted with Kineti Calc™ version 2.12 software using end point curvilinear regression.

Field study collections. Pacific herring in spawning condition were sampled in the spring of 1995 in 2 groups: (1) 240 fish from Sitka Sound (used as a reference site) and (2) 180 fish from Prince William Sound (PWS). The PWS sample was smaller due to fewer spawning fish captured. In the fall of 1995, 130 fish were sampled from PWS. All fish were captured by purse seine and held no longer than 4 h in ambient temperature salt water before necropsy. The water temperature was 4 to 5°C at the time of capture in the spring and 6 to 10°C in the fall; water temperature was not rechecked during the holding period. All fish were deeply anesthetized with tricaine methane sulfonate (Finquel™) and subjected to complete necropsy, which included determination of weight, standard length, and age (from scale annuli). Blood samples were obtained from the caudal vein at the beginning of the necropsy, effectively exsanguinating the fish. Plasma was harvested and aliquots frozen for (1) later analysis on a Monarch plus analyzer (Instrumentation Laboratories, Lexington, MA, USA) calibrated and run at 25°C to quantify total plasma protein, albumin, CO_2, calcium, cholesterol, glucose, phosphorus, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate animotransferase, creatine phosphokinase, gamma glutamyltransferase, sodium, potassium, and chloride, and (2) IgM quantification. Samples of gill, liver, gonad, spleen, trunk kidney, gastrointestinal tract, heart, skin, skeletal muscle, and brain were fixed in 10% buffered formalin for histopathology. Herring worms (Anisakidae) present in the peritoneal cavity were also counted. Head kidney and spleen from each fish were pooled and shipped on ice to the Alaska Department of Fish and Game Fish Pathology Laboratory (Juneau, AK, USA) for viral isolation; skin lesions, if present, were submitted separately. Propagation of 1 cell line (EPC, epithelioma papillosum cyprini cells), media formulation, and tissue preparation for inoculation was performed as previously described (Meyers et al. 1994). Propagation of a second cell line (PHE, Pacific herring embryo) was similar except media lacked tryptose phosphate broth. Swabs of kidney from fish with severe external lesions were inoculated onto trypticase soy agar and marine agar. Plates were incubated at 23°C for at least 3 d. No viral or bacterial pathogens were isolated from any of the fish.

Formalin-fixed tissues were processed with standard methods for paraffin embedding, sectioned at 5 µm, and stained with hematoxylin and eosin. Tissues were read in ascending numerical order using random histopathology numbers assigned to each fish. Lesions were scored, using a 4 point scale, as none (0), mild (1), moderate (2), or severe (3). Ranking of lesions was the same as previously described (Marty et al. 1998). *Ichthyophonus hoferi* infection was diagnosed by identification of the organisms in tissue sections. After all organs were examined and lesions scored, data were rearranged by necropsy number and subjected to statistical analysis.

Statistical analysis. The association of categorical variables (e.g. none, mild, moderate, and severe) with continuous variables (e.g. ln IgM concentrations) was determined using 1-way analysis of variance (1-way ANOVA). Category-specific means and standard errors were calculated for each continuous variable and compared using Tukey's Studentized range method. Levene's test was used to evaluate the homogeneity of variance assumption for the ANOVA. Most

analyses were run separately for spawning fish from Sitka Sound and PWS, and fish collected in the fall from PWS. To measure the strength of the linear relationship between 2 continuous variables, the correlation coefficient r was calculated.

Multiple stepwise regression analysis was used to identify a subset of variables that were predictive of IgM (log transformed). Those variables with the strongest predictive values were selected for further stepwise analysis. It was necessary to run 2 stepwise analyses in order to minimize the number of cases that were deleted due to missing values. In this way, only cases missing values for 1 or more of the variables strongly predictive of IgM were deleted. For a full report of variables, see Marty et al. 1998. For all analyses, comparisons were considered significant when $p \leq 0.05$. Use of the term 'prevalence' refers to the sample prevalence.

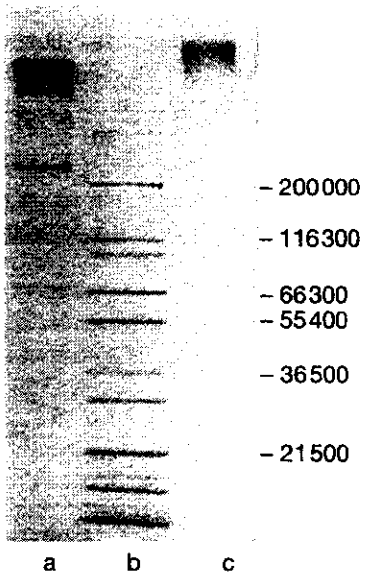

Fig. 1. *Clupea pallasi.* SDS-PAGE analysis of nonreduced Pacific herring putative IgM (lane a), molecular weight markers (lane b) and human IgM for comparison (lane c). 8 to 25% gradient gel

RESULTS

Euglobulin characterization and sequence data

The purified euglobulin fraction consisted primarily of protein with a molecular weight comparable to purified human IgM (Fig. 1). Under reducing conditions, the herring immunoglobulin was comprised of a 79 kD heavy chain and 3 light chains ranging from 25 to 27.5 kD (Fig. 2). With densitometry analysis, 98% of the reduced euglobulin fraction consisted of heavy and light chain bands. Two independent internal heavy chain fragments were sequenced. In the first fragment, 15 residues were sequenced, with poor yield at cycles 6 and 15. The residue at position 6 was either a cysteine or a tryptophan, and the residue at position 15 was most likely a lysine. For the second fragment, 11 residues were sequenced. The 15 amino acid sequence showed significant homology with several IgM heavy chain constant regions (Table 1). No significant matches were made with the 11 amino acid peptide sequence.

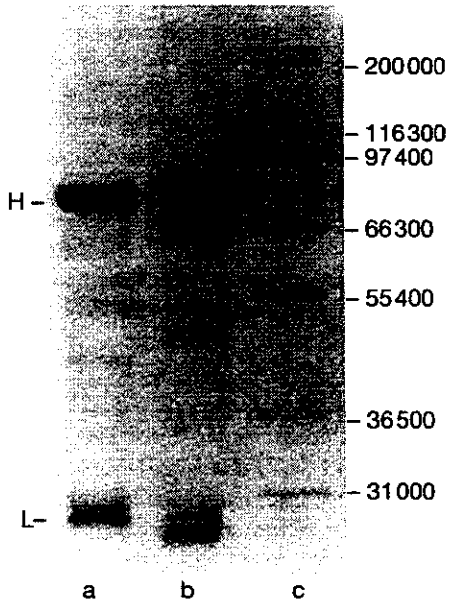

Fig. 2. *Clupea pallasi.* SDS-PAGE analysis of reduced white sturgeon IgM (for comparison, lane a), Pacific herring putative IgM (lane b), and molecular weight markers (lane c). Heavy (H) and light (L) chains are indicated. 10% gel

Table 1. *Clupea pallasi.* Comparison of amino acid sequences of an internal fragment of Pacific herring IgM with heavy chain sequences from different species using the Kabatpro database and BLASTP alignment tool. The underlined tryptophan and lysine were not completely specified. X: no amino acid match. Database accession numbers are given. Amino acid positions of the matching sequences within the heavy chains are included

Species	Accession no.	Amino acid sequence														
Pacific herring		E	V	F	V	S	W	L	A	D	D	E	P	V	A	K
Atlantic salmon, amino acids 350 to 362	KADBID 013655	E	V	L	V	A	W	L	I	D	D	E	P	V	X	X
Rainbow trout, amino acids 349 to 361	KADBID 013652	D	V	L	V	A	W	L	V	D	D	E	P	V	X	X
Elops, amino acids 342 to 354	KADBID 013659	Q	V	L	I	S	W	L	V	D	D	Q	P	V	X	X
Sheep, amino acids 362 to 375	KADBID 013636	D	V	F	V	Q	W	L	Q	K	G	E	P	V	A	X

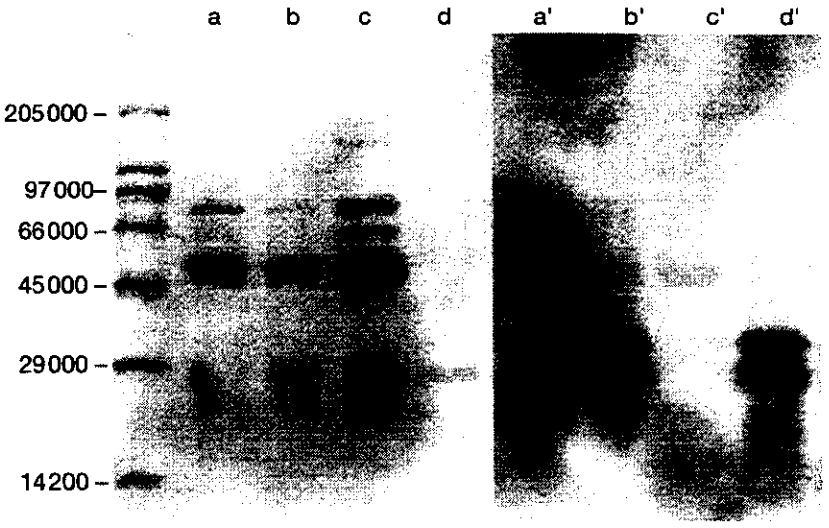

Fig. 3. *Clupea pallasi*. Western blot of Pacific herring and rainbow trout proteins precipitated with rabbit-anti-Pacific herring euglobulin antibody. Lanes a–d: precipitated proteins stained with Ponseau S; lanes a'–d': the same blot probed with biotinylated rabbit-anti-herring IgM antibody. Lanes: a and a', Pacific herring plasma; b and b', rainbow trout plasma; c and c', rabbit-anti-Pacific herring IgM antibody alone; d and d', Protein G/Protein A agarose alone. Molecular weight markers are located in the far left lane. The immunoblot has been overexposed to demonstrate all reactive protein bands

Specificity of the rabbit antibody

Immunoprecipitation of Pacific herring and rainbow trout plasma with the rabbit-anti-herring immunoglobulin demonstrated specificity of the rabbit IgG for a single protein in Pacific herring plasma with an apparent molecular weight consistent with putative IgM heavy chain (Fig. 3, lane a'). Because protein reagents of the immunoprecipitation reactions appeared in the blots, 2 lanes (Fig. 3, lanes c/c' and d/d') were run as controls to demonstrate where the rabbit IgG and the Protein G/Protein A agarose bands are located, allowing differentiation from precipitated plasma components. The Ponceaus S stain (left half, Fig. 3) demonstrates a very strong band in the rabbit IgG (Fig. 3, lane c) near 50 kD representing rabbit Ig heavy chains. Bands of the same molecular weight in lanes a and b are interpreted as rabbit IgG heavy chains from the immunoprecipitation reaction. None of the rabbit protein reacted with biotinylated rabbit-anti-herring IgG (Fig. 3, lane c'). The biotinylated antibody bound strongly to components of the Protein G/Protein A agarose (Fig. 3, lane d'). Corresponding bands in lanes a' and b' are also interpreted as Protein G/Protein A agarose components from the immunoprecipitation reaction. The strong reaction of the rabbit IgG with Protein G/Protein A agarose components in lanes a' and b' may obscure bands in the lower molecular weight regions, where light chains are expected.

ELISA parameters

The intraplate coefficient of variation (same plate, same day, same sample: 5 standard dilutions run in triplicate on a total of 60 plates) for the ELISA ranged from 0.3 to 36.3%, with a mean of 10.9%. The interplate coefficient of variation (different plate, different day, same sample: 9 samples run in triplicate on 3 different days) ranged from 3.7 to 32.6%, with a mean of 16.1%. The ELISA detected IgM concentrations as low as 0.039 μg ml^{-1} and the test can be performed in 4 to 6 h.

IgM levels in Pacific herring

Plasma IgM levels ranged from 0.13 to 5.32 mg ml^{-1} for all samples tested (n = 602). IgM as a percentage of total protein ranged from 0.5 to 20.4%, with an average of 2.9% in *Ichthyophonus hoferi*-negative fish and 4.9% in *I. hoferi*-positive fish. Using univariate analysis, plasma IgM increased significantly as lesion scores increased for *I. hoferi* infection. For example, plasma IgM significantly increased with increasing severity of *I. hoferi* lesions in the heart (Fig. 4). In addition to *I.*

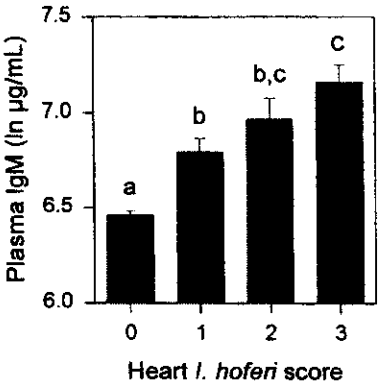

Fig. 4. *Clupea pallasi*. Relationship of heart *Ichthyophonus hoferi* lesion score (0, none; 1, mild; 2, moderate; 3, severe) with ln plasma IgM. Bars with different letter designations are significantly different (Tukey's multiple comparison procedure, $p \leq 0.05$)

Table 2. *Clupea pallasi*. Histologic lesions associated with increasing plasma IgM. Significant trends 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 (ANOVA, $p \leq 0.05$). ↓: downward trend in IgM. PWS: Prince William Sound. SS: Sitka Sound

Histologic finding	Population	Significant trend in IgM (p-value)
Ichthyophonus hoferi		
Heart, kidney, liver, spleen	PWS spawning	≤0.001
	SS spawning	≤0.001
	PWS fall	≤0.001
Skin/skeletal muscle	PWS spawning	≤0.001
	SS spawning	≤0.001
	PWS fall	0.005
Gill arch inflammation	PWS spawning	0.005
Meningoencephalitis	SS spawning	0.036
Epicarditis	PWS spawning	0.021
Steatitis, mesenteric	SS spawning	0.001
	PWS fall	0.015
Trunk kidney hematopoietic cells	SS spawning	≤0.001
	PWS fall	≤0.001
Pigmented macrophage aggregates		
Liver	SS spawning	≤0.001
	PWS fall	0.031
Spleen	SS spawning	≤0.001
	PWS spawning	0.043
	PWS fall	0.017
Splenic vascular congestion	PWS spawning	0.026
Submucosal gastritis	SS spawning	0.016
Granulomatous inflammation, liver	SS spawning	0.013
Hepatic lipidosis	SS spawning	0.013↓

hoferi infection, a variety of nonspecific histologic lesions were also significantly associated with increasing plasma IgM (Table 2). Hepatic lipidosis was the only variable associated with a decreasing trend in plasma IgM. Finally, still using univariate analysis, several variables were significantly correlated with increases in plasma IgM (Table 3): length, weight and age, as well as the plasma chemistries alkaline phosphatase, cholesterol, plasma protein, and albumin.

Using multiple stepwise regression, body length, heart *Ichthyophonus hoferi* score, and plasma albumin were the factors most strongly correlated with plasma IgM (Table 4). In the stepwise regression model, these 3 variables accounted for 29% of the variability in plasma IgM.

Eight parasites occurred at a prevalence of 10 to 90% (Marty et al. 1998), but none were significantly associated with IgM levels. Also, gender, site, and season (spring vs fall) did not contribute to significant differences in plasma IgM.

DISCUSSION

We have shown that Pacific herring immunoglobulin is an IgM-like molecule with a native molecular weight similar to that of human IgM. This finding is in agreement with previous reports of IgM in fish (Kobayashi et al. 1982, Estévez et al. 1993), and with the tetrameric nature of teleost IgM (Wilson & Warr 1992, Rombout et al. 1993). Previously reported values for heavy and light chains in a variety of fish species are all in the range of 70 to 79 kD for heavy chains and 23 to 30 kD for light chains: *Cyprinus carpio* (Marchalonis 1971, Koumans-van Diepen et al. 1995), *Oncorhynchus keta* (Kobayashi et al. 1982), *Carassius auratus* (Marchalonis 1971), *Scophthalmus maximus* (Esétvez et al. 1993), *Acipenser transmontanus* (Adkison et al. 1996), and *Acipenser baeri* (Partula & Charlemagne 1993).

This is the first report of plasma IgM levels in Pacific herring. Serum IgM concentrations have been reported for several other species: *Oncorhynchus mykiss*, 0.6 to 9.3 mg ml^{-1} (Sánchez et al. 1993) and 3.3 mg ml^{-1} (Olesen & Jørgensen 1986); *Salmo trutta*, 7.3 mg ml^{-1} (Ingram & Alexander 1979); *Oncorhynchus keta*, 0.63 to 0.72 mg ml^{-1} (Kobayashi et al. 1982); and *Scophthalmus maximus*, 3.35 to 11.14 mg ml^{-1} (Estévez et al. 1995). The IgM component of total serum protein in fish has been reported as 6.4% (Mochida et al. 1994), 10% (Ingram & Alexander 1979), and 7.5 to 18% (Estévez et al. 1995). Due to variation among different fish species, variable methods of total protein determination, and possible interaction of age as well as infectious disease status on IgM, this parameter may vary widely between studies.

Table 3. *Clupea pallasi*. Correlation coefficients (r) for continuous variables significantly correlated with ln IgM, univariate analysis ($p \leq 0.05$). PWS: Prince William Sound. SS: Sitka Sound

Variable	PWS spring n = 177	SS spring n = 234	PWS fall n = 128
Length (mm)	0.262	0.206	0.377
Weight (g)	0.210	0.174	0.414
Age (yr)	0.264	0.227	0.357
Alkaline phosphatase (U l^{-1})	0.196	0.136	0.204
Cholesterol (mg dl^{-1})	0.182	0.179	0.226
Plasma protein (g dl^{-1})	0.361	0.221	0.478
Albumin (g dl^{-1})	0.362	0.247	0.416

Table 4. *Clupea pallasi*. Significant predictors of IgM concentration ($p \leq 0.05$) generated using multiple stepwise regression. All parameter estimates are positive except for focal skin reddening and hold time (↓: negative correlation coefficient). Scored variables were classified as none (0), mild (1), moderate (2), or severe (3)

Variable	Partial r^2	Model r^2	p
Length (mm)	0.156	0.156	0.0001
Heart *Ichthyophonus hoferi* (score)	0.076	0.232	0.0001
Albumin (g dl^{-1})	0.058	0.290	0.0001
Cholesterol (mg dl^{-1})	0.015	0.305	0.0004
Liver macrophage aggregates (score)	0.012	0.317	0.0017
Focal skin reddening (score)	0.009↓	0.326	0.0053
Kidney hematopoietic cells (score)	0.007	0.333	0.0125
Hold time (min)	0.006↓	0.338	0.0286

The range of IgM concentrations in Pacific herring could be due to a combination of factors. IgM levels were most strongly correlated with body length. Body length, in turn, was highly correlated to body weight and age (Marty et al. 1998). In our regression analysis, body length was a better predictor of IgM than age or body weight. This finding may be due to 2 factors: (1) body length was a better continuous variable than age, and (2) among actively spawning fish, gonad weight (comprising up to 30% of body weight in prespawning fish) was highly variable because some fish had spawned and thus had empty gonads while others were yet to spawn and still had full gonads.

Independent of fish size, total plasma IgM was strongly and positively related to *Ichthyophonus hoferi* infection. Previous studies have shown that total immunoglobulin levels in fish increase with natural exposure to a pathogen (Magnadottir & Gudmundsdottir 1992), and *I. hoferi* has been previously identified as a significant pathogen in Atlantic herring *Clupea harengus* (Daniel 1933) and Pacific herring (Kocan et al. 1999). Also, specific immune responses have been demonstrated in fish in response to *I. hoferi* (McVicar & McLay 1985). Due to the chronic granulomatous inflammatory response to *I. hoferi* in Pacific herring (Elston et al. 1997, Marty et al. 1998), elevated total IgM concentrations in infected fish could reflect chronic exposure to *I. hoferi* antigens. The increase in IgM concentrations with increasing severity of *I. hoferi* infection in this study is consistent with previous reports that precipitating antibodies to *I. hoferi* are not protective against infection in other fish species (McVicar & McLay 1985). In our study, the proportion of total plasma IgM elevation due to a specific antibody response was not determined.

After the effects of body size and *Ichthyophonus hoferi* infection were accounted for, it is likely that nutritional status reflected by plasma albumin, total protein, and cholesterol had an impact on plasma IgM. Serum or plasma chemistry studies in Pacific herring are scarce (Márquez 1976, Marty et al. 1998); thus, interpretation of chemistry values is difficult. The multiple regression analysis identified the collinear relationship of albumin and total protein. The positive association of albumin and total protein with IgM may be related to fasting of herring in the weeks before spawning. Decreases in plasma proteins have been reported in fasting fish (Navarro & Gutierrez 1995), with albumin being the first plasma protein to be depleted. Gamma globulins are reportedly not utilized during fasting, but it is possible that gamma globulin production may be diminished in fasting fish. Decreases in plasma cholesterol have been reported in a variety of teleost species when fasting occurred in conjunction with spawning (McDonald & Milligan 1992). Hepatic lipidosis, which may reflect mobilization of body fat stores during fasting, was also significantly associated with decreasing IgM in Sitka Sound fish (Table 2). Plasma IgM may drop in females coincident with spawning due to incorporation of IgM into the eggs (Castillo et al. 1993, Takemura & Takano 1997); however, this incorporation is likely to occur at the level of nanograms of IgM per gram of egg (Takemura & Takano 1997), thus not significantly contributing to changes in circulating IgM. The positive correlation of plasma alkaline phosphatase with IgM is unexplained.

Unidentified inflammatory processes or tissue damage may have contributed significantly to increased plasma IgM, as reflected by the positive correlation of liver macrophage aggregates with IgM independent of age and *Ichthyophonus hoferi* infection. The pigmented macrophage aggregates of fish are repositories of pigment that likely reflect previous tissue injury with accumulation of membrane breakdown products. These aggregates may also represent primitive germinal centers (Agius 1985). The strong association of kidney hematopoiesis with increased IgM likely reflects a general increase in immune responsiveness because the kidney is the primary hematopoietic tissue in most teleost fishes. Studies using molecular markers to identify specific cell lineages in fish may help clarify processes in hematopoietic tissue in response to pathogens.

Focal skin reddening, negatively correlated with IgM, may be a nonspecific clinical sign associated with systemic disease. Possibly, some types of focal skin reddening reflected processes in which humoral immunity was suppressed.

The negative correlation of holding time to plasma IgM concentrations 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 lactacidosis result in elevated intracellular osmolarity, which causes a fluid shift from the circulation to the intracellular compartment (McDonald & Milligan 1992). This fluid shift results in increased concentrations of plasma proteins, including IgM. Over time, if the stress level is decreased, the fluid shift will reverse and protein concentrations return to normal.

The association of IgM with inflammatory lesions such as gill arch inflammation and meningoencephalitis seen in the univariate, but not the multivariate, analysis may be due to several factors. First, the association may be related to age, because older fish have higher IgM values and these older fish may be exposed to more inflammatory agents as a function of time. Secondly, since IgM was correlated with *Ichthyophonus hoferi* infection, some fish with *I. hoferi* may have suffered from other opportunistic infections that were reflected in the nonspecific inflammation.

Although effects of season on IgM have been reported (Zapata et al. 1992), no seasonal effect on IgM was noted in this study. It is possible that seasonal variations would have been detected if sampling had been done in mid-summer or mid-winter. Antibody responses in fish are generally affected by temperature: fish tend to produce better responses at warmer than colder temperatures (Wilson & Warr 1992). Lower IgM serum concentrations were reported in rainbow trout held at 7°C compared to 19°C (Sánchez et al. 1993). Surface water temperature in this study ranged from 4 to 5°C during spring sampling and from 6 to 10°C in the fall. Although the surface water temperature varies with season, the herring stay in deeper water in the fall; thus, fish collected in the fall may actually be at a temperature similar to that of shallow waters inhabited in the spring.

In this study, we have partially characterized Pacific herring IgM, developed a sensitive ELISA to quantify plasma IgM, and determined a range for total plasma IgM values in a wild fish population. *Ichthyophonus hoferi* is a pathogenic organism whose presence is significantly correlated with Pacific herring plasma IgM. Also, physical characteristics such as body length, as well as plasma chemistry values and histologic changes, are significantly associated with plasma IgM. Finally, we found no evidence that the population decline in Prince William Sound was associated with changes in plasma IgM levels. Further study is needed to determine concentrations of specific *I. hoferi* antibodies in healthy and infected fish and to evaluate cell-mediated immunity.

Acknowledgements. The authors thank Young Moo Lee and Jack Presley for amino acid sequencing, Neil Willits for multiple regression analysis, the Alaska Department of Fish and Game for assistance with herring sampling, and Nancy J. Rooijakkers for technical assistance. The research described in this paper was supported by U.S. National Science Foundation award no. 9871982 and the 'Exxon Valdez' Oil Spill Trustee Council through a contract with the Alaska Department of Fish and Game; however, the findings and conclusions presented by the authors are their own and do not necessarily reflect the views or position of the Trustee Council.

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Editorial responsibility: Donald Evans, Athens, Georgia, USA

Submitted: February 12, 1998; Accepted: June 22, 1999
Proofs received from author(s): October 12, 1999

Exxon Valdez Oil Spill
Restoration Project Final 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 (98162)
Final Report

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

Restoration Project (98162)
Final Report

Study History: Approximately 110K tons of spawning herring in Prince William Sound disappeared between 1993 and 1994. An emergency project (94320 S) was authorized by the ADF&G to investigate this loss. It was discovered that VHS, a viral disease previously unreported from Pacific herring was present in < 6% of surviving herring. In 1994 however, the prevalence of *Ichthyophonus hoferi*, a pathogen of fish increased from 5% to 29%. Consequently, the ADF&G initiated a second study which covered: 1) Annual field surveys in PWS; 2) Controlled experimental disease studies and 3) Physiological studies on diseased and oil-exposed herring. 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: From 1995 through 1998 the controlled disease studies covered a wide range of topics including virus survival in seawater, development of natural immunity, effects of oil exposure on immunity, effect of synthetic immunosuppressants, epizootiology of VHSV in net pens, antibody production and the natural history of VHS in Pacific herring. Both VHSV and *I. hoferi* were shown to be serious pathogens of juvenile herring. A natural age-related resistance to VHS virus was observed in fish that reached two-years-old while in captivity. Prior to 2 years old, fish that survived an initial infection by VHSV developed an acquired immunity detectable by in vitro plaque assay and resistance to reinfection with the known minimum lethal dose of virus.

Three years of consecutive monthly sampling of wild 0-year herring in Puget Sound revealed a VHSV carrier rate below 1%, but that this level of virus shedding was adequate to initiate an epizootic under confined conditions, and probably resulted in infection of free-ranging fish also. Over 50% of the virus was recoverable from seawater after 2 hours, was still detectable after six hours, and survived up to 100 h when ovarian fluid was present in the water.

Studies in PWS demonstrated that closed pounds play a role in transmission of VHSV to susceptible fish, resulting in the rapid spread of virus within the pens. Prevalence of VHSV from impounded fish steadily increased with confinement time and viral tissue titers were at very high levels in fish being released after 8 days in captivity. Viable virus was also recovered from the water inside and outside the net pens at levels adequate to initiate an epizootic in susceptible fish.

Studies on *Ichthyophonus hoferi* demonstrated that the organism is a potential serious pathogen of herring and that vitro culture was the most sensitive method for detecting it.

No evidence for increased susceptibility, mortality or loss of disease resistance was observed in wild or laboratory-reared herring exposed to oil or synthetic corticosteroids, either prior to or following exposure to VHSV.

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

Citation:

Kocan, R.M., P.K. Hershberger, N. Elder and J.R. Winton. 1999. Controlled Field and Laboratory Studies on viral hemorrhagic septicemia (VHS) & *Ichthyophonus* in Pacific herring, Section II in: Investigations of disease factors affecting declines of Pacific herring populations in Prince William Sound. *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 98162), Alaska Department of Fish and Game, Anchorage, Alaska. __ pages.

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Executive Summary (98162)

Introduction

In 1993, when the Prince William Sound herring population declined over 80%, viral hemorrhagic septicemia virus (VHSV) was isolated from 5% of the survivors. This was the first report of this pathogen from wild Pacific herring (*Clupea pallasi*), however it has subsequently been isolated from all age classes of herring in Puget Sound, as well as from herring collected in the vicinity of a diesel fuel spill in Prince Rupert, B.C. Other species also found to be infected include the Pacific cod (*Gadus morhua*), Pacific sandlance (*Ammodytes hexapterus*), English sole (*Parophrys vetulus*) and shiner perch (*Cymatogaster aggregata*). In 1992 herring held in the spawn-on-kelp (SOK) 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 those 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 investigate 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, Univ. of Washington and Simon Fraser University. These groups approached the disease problem in herring from several aspects; including annual on-site surveys, controlled laboratory and field experiments, and physiological studies to understand the effects of oil and disease on Pacific herring. 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 extensive mortality in juvenile fish; that 0-age herring were infected by the time they metamorphosed; that net pen confinement resulted in increased disease transmission; that VHS virus could survive for over 6 hours in sea water; and that herring which recovered from VHS were solidly immune to reinfection.

Objectives

1. Rear specific-pathogen-free herring in the laboratory for studying VHSV and *Ichthyophonus*
2. Describe the natural history of VHSV in wild herring from 0-year to spawning
3. Determine age-related prevalence of *Ichthyophonus* in wild herring from 0-year to spawning
4. Determine the effects of capture and confinement on VHSV infection in wild herring
5. Describe natural and acquired immunity to VHSV in lab-reared herring
6. Determine the immune status of wild herring before and after an epizootic of VHS
7. Determine the stability of VHS-virus in seawater

Methods

Specific-pathogen-free (SPF) herring were reared in the laboratory from egg to 2 years old. When fish reached approximately 5 months of age, studies on their susceptibility to VHSV began. Fish were exposed to various levels of water-borne virus in flowing filtered seawater for 1 hr, then observed for 14 days. Exposure virus was obtained from wild Puget Sound herring and grown in vitro in epithelioma papulosum cytrini (EPC) cells. Natural transmission studies consisted of exposing SPF herring to wild infected herring and observing the SPF fish for signs of disease and / or virus in their tissues.

Wild herring captured from Puget Sound were housed in 70 gal tanks of natural flowing seawater. Age classes of 0-year, 1+, 2+ and 3+ were assayed for the presence of VHSV at the time of capture and at regular intervals for the first 30-90 days post capture. Virus titers were

recorded as plaque forming units (PFU) per gram of tissue ($PFU*g^{-1}$), while water titers were expressed as $PFU*ml^{-1}$. Immunity was determined by exposing surviving herring to known lethal doses of VHS-virus, then quantitating tissue virus load by plaque assay.

Tissues from fish held in SOK pounds in PWS during 1997 and 1998 were examined for the presence of VHS virus during confinement and at the time of release from the pounds, and water from inside and outside the pounds was sampled for the presence of virus. Both tissues and water were evaluated by plaque assay on EPC cells.

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 were assayed for the presence of VHSV at the time of capture and at regular intervals for 30 days post capture. Immunity was determined by exposing surviving herring to 10-100 times the known lethal dose of virus and by examining the virus neutralizing capability of plasma from pre and post-infected fish.

Ichthyophonus was studied in SPF herring by injecting 8-month-old fish IP with spores. Fish were maintained in flowing sterile seawater for 60 days and observed for mortality, lesions, growth and behavior. Tissues from all fish were also cultured and processed for histology in order to determine the efficiency of each method in accurately identifying infected fish. Field studies consisted of sampling wild 0-year and 1+, 2+ and 3+ herring from Puget Sound for *Ichthyophonus*.

Results

Laboratory-reared herring: Viral hemorrhagic septicemia virus (VHSV) was conclusively shown to be capable of causing disease and extensive mortality in nonimmune juvenile Pacific herring. Fish began dying 7 days post-exposure with peak mortality occurring on days 10-11. Mortality began 5 days post-exposure and peaked on day 7, with no mortality observed in SPF herring exposed to VHSV concentrations of $< 1 \times 10^2$ $pfu*ml^{-1}$. External signs of disease was limited to 1-2 mm hemorrhagic areas on the lower jaw, isthmus and around the eye. Only 4 of 130 infected fish had detectable cutaneous hemorrhaging. Virus was first detected in tissues of experimentally infected SPF herring 48 h post-exposure and peaked at 96 h at > 6 million pfu's per gram of tissue. Fish began shedding new virus 48 h post-exposure with maximum shedding occurring on days 4-5 post exposure. Histopathologic examination of tissues from moribund fish revealed primarily: 1) multifocal coagulative necrosis of liver hepatocytes, 2) diffuse necrosis of the kidney interstitial hematopoietic tissues, and 3) diffuse necrosis of the spleen, epidermis and subcutis.

VHSV in wild herring: Three age classes of wild herring were captured in Puget Sound from 1995 through 1997 and held in flowing seawater tanks for observation. Immediately upon capture, a subsample of fish was frozen at -70°C until assayed for virus. The remaining fish were held at various densities ranging from 5 to 300 fish per tank for up to 5 months. During this time dead fish and subsamples of live fish were collected and frozen for later virus assay.

Between 1995 and 1997 a single infected 0-year wild herring was detected in one school captured in October 1997, otherwise no evidence of VHSV was found in wild free-ranging 0-year Puget Sound herring. However, following captivity each school experienced an epizootic which was highly lethal to 0-year fish (>50%) and produced up to 15% mortality in older fish.

In the single instance where a VHSV-positive 0-year herring was found, the remainder of the school did not experience the usual epizootic in captivity, but rather all fish were solidly immune to experimental reinfection with VHS virus. The single VHSV-positive fish and the total immunity of the remainder of the school is evidence that these fish had already been exposed to the virus. How many fish were lost as a result of this exposure is not known.

In 1995 and '96 juveniles (1+ and 2+) as well as adults (3+) were also negative for VHSV when initially captured, but began showing virus by 24-48 hours post-capture. In 1997 and '98 about 1% of older fish were positive at the time of capture, but also converted to higher prevalence levels after capture. Mortality was significantly less (< 10%) in older fish. Virus was detected between days 2 and 11, but undetectable by day 21 post capture. Surviving herring exposed to lethal concentrations of virus 6-8 weeks post-capture exhibited no mortality in any age class and no virus could be isolated from tissues of these fish 10 days post-exposure.

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

No virus was isolated from any wild fish at the time of capture. However, 2-3 weeks post-capture approximately 60% of the 0-year herring died with massive hemorrhages of the skin, fins and mouth. Plaque assays on EPC cells revealed that > 90% of the dead fish had > 1 X 10^6 $PFU*gm^{-1}$ tissue at the time of death. Ninety percent of the live fish sampled from the same tanks carried slightly lower titers of virus from 5 to 14 days post-capture, then virus titers declined until they were undetectable by 4 weeks post-capture.

Immunity to VHSV: 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.

Virus survival in seawater: Studies on the survival of VHS virus in seawater 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. The presence of 0.05 - 0.1 % ovarian fluid in the water resulted in 50% survival of the virus after 60 - 100 hours.

Spawn-on-kelp (pound) studies: Confinement of apparently healthy wild herring into SOK pounds led to increased infections of VHSV which peaked relatively soon after capture. Magnitude, severity, and duration of infection depended upon numerous factors including herring age, immune status, VHSV shedding intensity and crowding density. Although crowding influenced the rate of transmission within the pounds, VHS epizootics were primarily dependent upon the presence of: 1) waterborne virus in the pounds and 2) proportion of susceptible herring. A source of waterborne virus originated from a small percentage of fish carrying the virus when introduced into the pounds. Prolonged crowding and handling stress resulted in waterborne

VHSV shedding and exposure to susceptible fish. Young herring were more susceptible to the virus than older fish.

These data indicate that closed pound SOK fisheries may: 1) Activate latent infections in previously infected herring, and 2) Enhance VHSV transmission to non-immune fish in the pounds 3) spread the virus to wild fish outside the pounds directly via contaminated water or by release of infected fish.

Ichthyophonus in Pacific herring: Laboratory-reared SPF herring injected IP with ca 1,000 *Ichthyophonus* spores began dying by 11 days post exposure and had visible lesions on the heart, liver and spleen. Skin lesions (small ulcers in the epidermis) were detectable after 36 days, as were spores in the musculature under the skin. By 56 days post exposure 90% of the fish were dead. *Ichthyophonus* was cultured in MEM-10 from all but one of the fish which died or presented with lesions.

Three year classes of wild herring (0-year, 1+ and 3+) were captured from Puget Sound between June 1995 and February 1997 and examined for the presence of *Ichthyophonus* by gross examination and in vitro culture of heart, liver and spleen. External skin lesions were observed in 6%, 5% and 4% of the three groups respectively while 6% 23% and 52% of each group cultured positive for *Ichthyophonus*. There was no significant difference in weight or length between infected and uninfected fish within each age class, and when the fish were held in captivity for up to 90 days post-capture, there was no significant difference in mortality between the infected and uninfected individuals within age classes. Infected tissues from these herring were cultured then injected IP into coast-range sculpins (*Cottus aleuticus*), all of which became infected and/or died by 14 days post exposure. Infected tissues from these sculpins were fed to other sculpins which also became infected and cultured positive for *Ichthyophonus*. No control sculpins were found to be infected with *Ichthyophonus*.

Discussion

Laboratory-reared pathogen-free herring: Koch's Postulates have been fulfilled for VHSV, confirming the organism's pathogenicity for nonimmune herring and establishing it as a possible cause of the extensive losses that occurred in Prince William Sound in 1993-'94. The course of the disease is very rapid, with mortality peaking by 6-8 days post-exposure. Transmission occurs by exposure to virus in the water column. The characteristic epidermal hemorrhaging described for wild herring does not occur in SPF herring, suggesting that the lesions and ulcers are the result of secondary invaders.

VHSV in wild herring: All age classes had detectable virus within 24-48 hours post-capture, with the most sever mortality occurring in the 0-year fish. By 3-4 weeks post-capture virus was no longer detectable and the fish were solidly immune to challenge infection. The minimum lethal dose of virus for nonimmune herring was shown to be ~ 1×10^2 pfu$*$ml$^{-1}*$hr^{-1}.

Wild herring are infected with VHSV during their first year of life and apparently die or recover and become immune to reinfection. What triggers the disease caused by this pathogen in nature is not clearly understood at this time. Any "stress" condition that affects the immune system could be the trigger; such as confinement, exposure to toxic substances, malnutrition or a combination of these. Confinement would also increase the probability of spreading the virus from fish to fish.

Young-of-the-year (0-year) herring appear to suffer 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 is stable for up to 6 hours in natural sea water and for over 60 hours in water containing ovarian fluid, supports the hypothesis that water-born transmission may be responsible for the high prevalence rates of VHS observed in the spawn-on-kelp fishery. Virus is shed from a few infected individuals, remains virulent in the seawater and infects susceptible fish.

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

Wild herring were found to be infected with *I. hoferi* by 2-4 months post-hatch, with a prevalence of 6%, while the highest prevalence (52-75%) was found in adult spawners. There was no evidence that the organism affected the health or survival of wild fish, but different environmental conditions and levels of infection could result in a significant level of morbidity and mortality.

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

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 due, at least in part, to infection by one or both of these organisms.

A. Rearing Specific-Pathogen-Free Herring in the Laboratory

Methods

Obtain and hatch herring eggs

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

Peak hatching of fish occurred 12 d post-fertilization with water temperatures ranging from 11.2°C to 13.0°C during incubation. The mean length of newly hatched larvae was 8.54 + 0.29 mm and increased linearly to 90 d when the fry were 28.98 + 1.65 mm (Figure A1). By 40 weeks, the mean length for the fish was >100 mm.

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

Rear herring larvae for experimental use

Pacific herring larvae were continuously maintained on filtered-u.v. sterilized natural seawater. Larvae were initially fed trochophores (oyster), rotifers (*Brachionus* sp) and *Artemia* larvae. The rotifers and brine shrimp were treated with Super Selco@ for 8 hours prior to feeding in order to maintain adequate levels of omega-3 fatty acids in the larval diet. Trochophores were discontinued after two weeks and rotifers after 12 weeks. At three months the fry were introduced to frozen brine shrimp and commercial trout chow (1-2 mm) and have been continuously maintained on these food items. During the first 90 days post-hatch, larval growth data was collected every 5-7 days, then at 30, 35 and 75 weeks (Figure A1). The entire collection was catalogued and deposited in the University of Washington larval fish collection for future study and teaching purposes.

Production of specific pathogen-free (SPF) herring.

Seawater for hatching and rearing SPF herring was pumped from 15 m below mean low water off the north end of Marrowstone Island in northern Puget Sound, Washington where ambient water temperatures during the year typically range from 8-13°C. The water was initially filtered through a 20-40 m non-hydrous silicon dioxide filter medium (Jacuzzi, Little Rock, AR), followed by a 30 m spun polyester cartridge filter, and finally through a 16 m canister filter (Aquatic Eco-systems, Apopka, FL). After filtration, the water was passed through two, in-line, 8 watt, ultraviolet (UV) light sterilization units at 20 L/min (Aquatic Eco-Systems). The filters were back-flushed for 1 h every 48 h and the UV bulbs changed every 6 months when they reached 50% of their effective life. To evaluate the relative

effectiveness of the UV sterilization, six tanks of larvae were reared in UV-sterilized seawater and two tanks of larvae in filtered seawater without UV sterilization. Larval survival and growth were used as endpoints for the effects of UV sterilization on larval rearing water.

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

In the laboratory, the egg racks were suspended in 250 L tanks of ambient (8-10 oC), sand-filtered, UV-sterilized, flowing seawater for incubation and grow-out of the larvae. Egg racks were removed 3 d following the peak hatch and the flow rate in the tanks was adjusted to $2L*min^{-1}$ for the first 30 d post-hatching, then increased to 4 L/min. Larvae were given a mixture of rotifers (*Brachionus* sp), larval brine shrimp (*Artemia*), dry larval fish food, and algae paste for the first 90 d post-hatch. Rotifers and brine shrimp were soaked in an omega-3 fatty acid supplement (Super Selco, Aquaculture Supply, Dade City, FL) prior to feeding. Frozen brine shrimp and dry trout chow were introduced at 4 months and fed continuously for the remainder of the study.

Verification of disease-free laboratory herring

Histopathology

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

Ichthyophonus

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

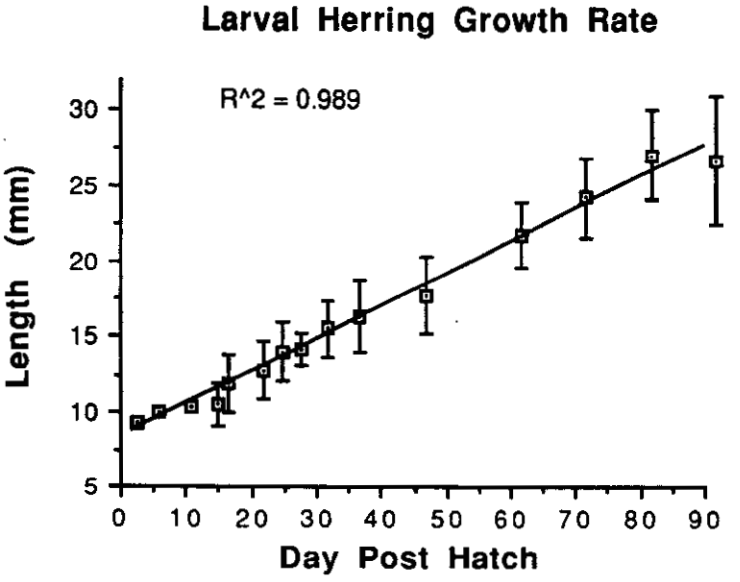

Figure A-1. Three month growth data for Pacific herring larvae reared in captivity in filtered - U.V. sterilized natural seawater

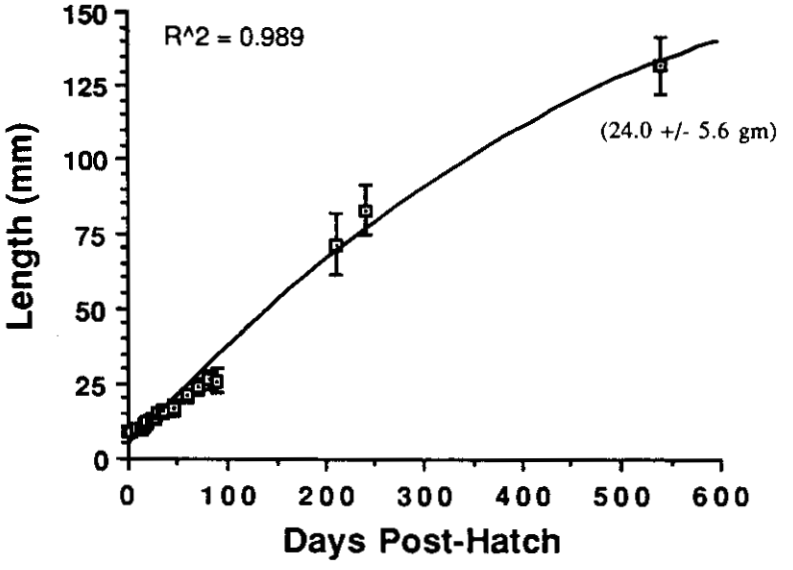

Figure A-2. Growth rate of specific-pathogen-free (SPF) Pacific herring during the first 1.5 years post-hatching. Approximately 100 fish have survived to over 2-years-old (April, 1997).

VHSV

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

B. Pathogenicity of Viral Hemorrhagic Septicemia Virus in Specific Pathogen-Free Pacific Herring (*Clupea pallasi*)

Introduction

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

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

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

Objectives

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

Methods

Production of specific pathogen-free herring.
(See Section A)

Virus propagation and assay

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

The titers of infectious virus in cell culture fluid, aquarium water, or homogenates of herring tissue were determined by plaque assay using monolayer cultures of CHSE-214 or EPC cells pretreated with polyethylene glycol as described by Batts and Winton (1989). Virus titers were expressed as plaque-forming units (pfu) per milliliter of fluid or per gram of tissue. For titration of virus in herring, the head and tail were removed and the entire mid-portion of the fish homogenized in minimum essential medium. Serial dilutions of the homogenates were plated on monolayers of EPC cells.

Exposure of herring to virus

Replicate groups of SPF herring were transferred from grow-out tanks to 40 L aquaria (8-10 fish/aquarium depending upon size), provided with ambient-temperature, sand-filtered, UV-sterilized, seawater at a flow rate of 0.5 L/min, and allowed to acclimate for 24 h. For virus challenge, the water flow was turned off and the volume in the aquaria reduced to 3 L. Dilutions of stock virus in MEM-10 or equivalent volumes of MEM-10 as controls were added to the water (2-3 aquaria/dose) to provide replicate low, medium, and high challenge levels (approximately 10^2, 10^4, and 10^6 pfu$*$ml^{-1}, respectively) of freshly grown VHSV. At the end of the 1 h waterborne challenge, the water flow was resumed for the remainder of each study and fish were then observed twice daily for 21 d. Experimental fish were fed twice daily with frozen brine shrimp and krill and dead fish were removed and frozen at -70°C for later virus assay.

Susceptibility of SPF herring to VHSV

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

Virus shedding by VHSV-infected herring

Virus shedding rates were determined by placing 10 SPF herring into each of two 40 L aquaria, exposing them to $10^{6.7}$ pfu$*$ml^{-1} VHSV for 1 h, then sampling the water for virus during the next 5 d. Immediately following the exposure period, the water was turned on at 0.5 L$*$min^{-1} and 1.0 mL water samples for virus isolation were taken hourly for the next 3 h. At 24 h post-exposure, and at the same time each day

for the next 5 d, the water was turned off and a 1.0 mL water sample was taken immediately and then hourly for 3 h. At the end of the 3 h period, the water was turned on again until the next day. Water samples were placed directly into 1 mL of 2X MEM-10 and frozen at -70°C until assayed. This made it possible to monitor both the level and the rate of virus shedding.

Histopathology and replication of virus

Pathological changes in tissues and replication of VHSV over time were examined using 6 month old, juvenile SPF herring. Following exposure to VHSV at low ($10^{1.5}$ pfu/mL), medium ($10^{3.5}$ pfu$*$ml^{-1}) or high ($10^{5.5}$ pfu$*$ml^{-1}) doses of virus for 1 h, live or moribund fish were collected from replicate aquaria at 2, 4, 6 and 8 days post-exposure and processed for histology and for virus titration.

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

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

Results

Production of SPF herring
(See Section A)

Susceptibility of SPF herring to VHSV

Typically, mortality began 4-6 days post-exposure and peaked about day 7 in groups of herring exposed to high doses (105.5-6.5 pfu*ml^{-1}) of VHSV regardless of the age of the fish (Table B1). Fish exposed to low levels of VHSV began dying 6-12 days post-exposure with peak mortality occurring on days 10-11 (Table B1). Tissues from individual fish that died during the study often had a mean virus concentration in excess of 106 pfu/g while fish surviving to the end of the 21 d experiment had low or undetectable levels of VHSV in their tissues. Mortality and pathology were not observed in control fish or in some groups of SPF herring exposed to the lowest levels of VHSV, and no virus could be isolated from their tissues. Both the onset of mortality and mean-day-to-death showed a dose-response; however, once infected, the total mortality in the groups and the virus titers in infected fish were generally similar regardless of the initial challenge dose (Table B1). Little, if any, effect of increased size upon resistance was noted and the fish remained highly susceptible to infection at more than 1 year of age. Re-isolation of VHSV from the fish completed Koch's postulates.

Virus shedding by VHSV-infected herring

Following waterborne exposure to $10^{6.7}$ pfu*ml^{-1} VHSV for 1 h, the water flow was resumed and the virus titer in the aquaria declined to below detectable levels (5 PFU*ml^{-1}). No virus was found in any sample collected at 24 h post-infection (Figure B1), but infected herring began shedding detectable levels of new virus by 48 h post-exposure, coinciding with the first appearance of virus in the tissues (Table B2). By 72 h post-exposure the fish were shedding enouth virus to produce titers of 10^2 pfu*ml^{-1} in the aquarium water and, when the water flow was turned off, the titer rose to more than 10^3 pfu*ml^{-1} during the 3 h sampling period. Virus shedding peaked on days 4 and 5 post-exposure at levels of $10^{2.5}$ pfu*ml^{-1} of flowing water and the first mortalities were observed on day 5. On both day 4 and day 5, virus titers in the 40 L aquaria increased from $10^{2.5}$ to $10^{3.5}$ pfu*ml^{-1} during the 3 h period, indicating that, on average, the 10 fish were shedding more than $10^{6.7}$ pfu/fish*h^{-1}.

Histopathology and replication of virus

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

Microscopic examination of SPF fish exposed to virus revealed lesions in tissues of both groups of herring exposed to the high dose of VHSV, but in only one of the two groups exposed to the medium dose. No lesions were observed in the low dose or control groups of fish (Table B3). Histopathologic changes consisted of: (1) moderate to severe multifocal coagulative necrosis of liver hepatocytes; (2) moderate diffuse necrosis of the spleen ; (3) infrequent multifocal necrosis of acinar cells in the pancreas; mild to moderate diffuse necrosis of kidney interstitial hematopoietic tissues including occasional glomeruli or tubules and mild to moderate diffuse necrosis of the epidermis and subcutis. Lesions in one fish also included diffuse pyknosis and karyorrhexis of granular cells in the stratum granulosum and lamina propria of the intestine with similar changes in the mucosal epithelium accompanied by some sloughing in the intestine, pyloric caeca and rectum.

Virus was first detected in the tissues of one high dose fish on day 2 post-exposure while tissues from all fish in medium and high dose tanks were positive for virus on days 4 and 6 post-exposure. By day 8 post-exposure, one fish exposed to a medium dose still had detectable virus while the other fish was below the detection limit. The sole surviving high dose fish had detectable virus on day 8. No fish mortality occurred in controls or low dose tanks and no virus was detected in tissues from these fish at the end of the study (Table B2).

Discussion

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

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

Virus could be isolated from tissues of fish exposed to a high dose ($10^{6.5}$ PFU$*$ml^{-1}) of VHSV by 2 d post-exposure and in fish exposed to a medium ($10^{4.5}$) dose of virus by 4 d post-exposure. By the second week post-exposure, tissue concentrations of virus began to drop dramatically in surviving fish, indicating that the fish were beginning to clear the virus. Elimination of the virus was also observed among survivors of outbreaks of VHS in wild-caught, naturally-infected herring held for several months in the laboratory and fish surviving either natural or artificial infection were strongly protected against reinfection (data not shown).

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

Table B1. Mortality in specific-pathogen-free Pacific herring challenged with a North American strain of viral hemorrhagic septicemia virus.

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

(Table B1 cont'd)

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

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

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

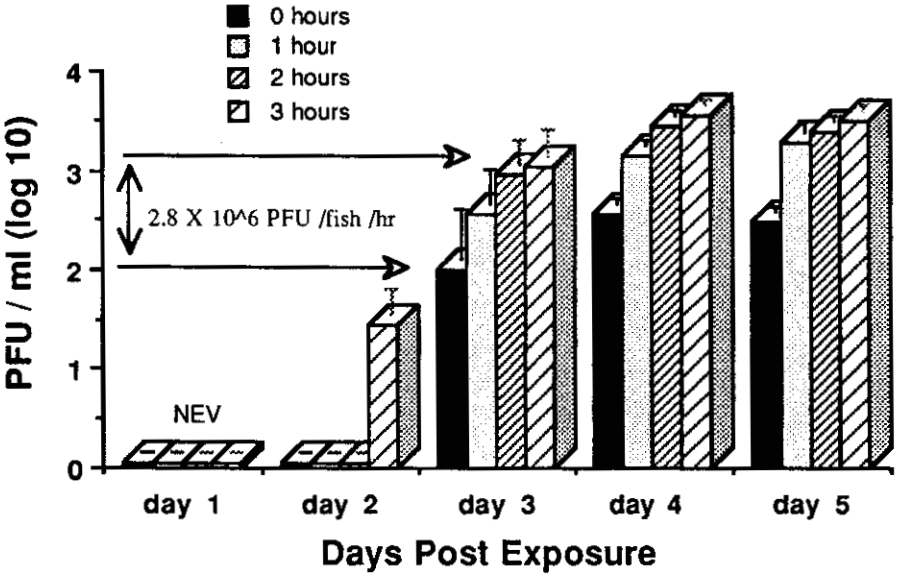

Figure B1. Mean concentrations of the North American strain of viral hemorrhagic septicemia virus shed by specific-pathogen-free Pacific herring. Fish were infected via a 1 h water-borne exposure to $10^{6.7}$ pfu*ml^{-1} of virus and water samples were collected from replicate aquaria at each of three hourly intervals during 5 d post-infection. NEV = no evidence of virus

Table B2. Viral titers in visceral tissues of Pacific herring following challenge with a N. A. strain of viral hemorrhagic septicemia virus. Values expressed as the geometric mean of titers of positive fish (log_{10} $pfu*gm^{-1}$)

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

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

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

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

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

The lesions in these VHSV-infected SPF juvenile herring were somewhat typical of lesions seen in salmonids infected with the European strain of VHSV where major degenerative changes and necrosis occur in the liver, kidney and spleen (Liversidge and Munro 1978, Wolf 1988). However, significant hemorrhaging caused by the endotheliotropic nature of the virus was not apparent in these juvenile fish, unlike the extensive hemorrhaging typical of European VHSV in rainbow trout and for North American VHSV in wild juvenile and adult Pacific herring (Meyers et al. 1994). Although the tissues targeted by VHSV in juvenile SPF herring were also affected in wild, adult herring, the lesions were different.
Table 3. Presence of histologic lesions in selected tissues of SPF Pacific herring following waterborne exposure to a North American strain of VHS virus.

Skin hemorrhages with occasional ulceration were prominent in the adult herring from Prince William Sound and, although some focal hepatic necrosis was evident (Marty et al. 1995), other associated microscopic lesions of the liver, kidney and spleen were of a chronic and proliferative nature (Meyers et al. 1994).

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

Table B3. Presence of histologic VHS-associated lesions in SPF herring tissues

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

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

C. Natural history of viral hemorrhagic septicemia virus (VHSV) in the spawn-on-kelp fishery and other net pen operations.

Introduction

Viral hemorrhagic septicemia virus (VHSV), historically a problem of farmed fish in Europe (Meier et al 1994, Ross et al 1994, Schlotfeldt et al 1991, Meier & Wahli 1988, Jorgensen 1980, Castric & de Kinkelin 1980, Wolf 1988), was first detected in North America among returning adult coho (*Oncorhynchus kisutch*) and chinook salmon (*O. tschawytscha*) of Washington state (Winton et al 1991, Eaton et al 1991, Meyers & Winton 1995). Subsequent studies have shown that VHSV is endemic among a wide range of north Pacific species including Pacific cod (*Gadus macrocephalus*) (Meyers et al 1992), Pacific herring (*Clupea pallasi*) (Meyers et al 1994), Pacific sandlance (*Ammodytes hexapterus*), English sole (*Parophrys vetulus*) and shiner perch (*Cymatogaster aggregata*) (unpublished data). Detection of VHSV in wild herring from Prince William Sound, AK following the 1989 *Exxon Valdez* oil spill (Meyers et al 1994) raised the possibility that VHSV could be highly pathogenic to some North Pacific fishes which was subsequently confirmed by Kocan et al (1997). Further questions were raised concerning environmental conditions and human activities that might increase the severity of VHSV epizootics among wild herring.

The closed pound spawn-on-kelp (SOK) herring fishery in Prince William Sound, AK is operated to produce a high quality specialty product, primarily for Japanese markets. Sexually mature, pre-spawn Pacific herring for this fishery are purse seined, transported to a net pen (closed pound) containing suspended *Macrocystis* blades (Whyte 1979), and confined for 7-10 d until eggs are deposited onto the kelp. Post-spawn fish are then released from the pound and presumably rejoin wild stocks. The crude product consisting of kelp blades overlaid with layers of adherent herring eggs is removed from the pound, trimmed of rough edges, and brined. Activities involved in the SOK fishery, such as rapid transport of herring to the closed pounds are reported to cause bruising and scale loss (Shields et al 1985). High loading densities in the pounds can also result in herring mortalities as great as 33% (Brett & Solmie 1982), "*Vibrio*-like disease" (Hay et al 1988), or physical damage such bruising of the opercula and snout, hemorrhage at the base of fins, fin deterioration, and open ulcers on the body and snout (Shields et al 1985). These signs are similar to the clinical signs of VHSV infection in herring (Meyers et al 1994). Capture of seemingly healthy, wild *C. pallasi* and confinement in laboratory tanks often leads to active VHSV infections after 3-7 d (Kocan et al 1996). Meyers et al (1994) suggest that stressors such as spawning, capture, nutritional deficiency, or other diseases may contribute to periodic epizootics of viral hemorrhagic septicemia (VHS) in wild populations. This project was designed to determine whether impoundment of herring for the closed pound SOK fishery is correlated with increased prevalence of VHSV and to describe the course of virus infection within the pounds.

Methods

The locations of SOK pounds and study sites were determined from data on herring biomass and spawning status collected at the beginning of the 1997 and 1998 herring seasons (Figs. C1,C2). Herring age (from scales), weight, and length were recorded from all sampled fish. Water quality measurements were recorded from the middle and 3 m outside of all pounds and included temperature, salinity and dissolved oxygen (DO).

Titers of VHSV in water samples and homogenized herring tissues were determined by plaque assay. Kidney and spleen tissues from each fish were pooled and homogenized in tris-buffered minimum essential medium (1x MEM) containing 100 IU mL^{-1} penicillin, 100 $\mu g\ mL^{-1}$ streptomycin, 100 $\mu g\ mL^{-1}$ gentamicin, and 2.5 $\mu g\ mL^{-1}$ amphotericin B. Serial 10-fold dilutions of the homogenates were plated on monolayer cultures of *epithelioma papulosum cyprini* (EPC) cells pretreated with polyethylene glycol

(Batts & Winton 1989), overlaid with methyl cellulose, and incubated at 15 °C for 7 d. Virus titers were expressed as plaque-forming units (pfu) g^{-1} of tissue. Virus from infected fish was identified as VHSV using the polymerase chain reaction and VHSV-specific primers (Einer-Jensen et al 1995).

Water samples (2 ml) were diluted 1:1 in MEM containing 10% fetal bovine serum (MEM-10) in 1997 and 2X MEM containing 40% fetal bovine serum (2X MEM-20) in 1998 and returned to the laboratory for virus assay. Virus titers were expressed as pfu ml^{-1} water. All viral prevalences were statistically compared using the Z test for proportions, (Zar 1984) and comparisons with $p \leq 0.05$ were considered significant.

1997 Studies

All 1997 study pounds were located in the Port Fidalgo region (Fig. C1) and designated Pounds #1997-1 in Irish Cove, #1997-2 at the mouth of Landlocked Bay, and #1997-3 at the head of Landlocked Bay. Random samples of 40 active herring $pound^{-1}$ day^{-1} were collected on consecutive days, beginning when the pounds were loaded until the fish were released 6-8 d later. All herring in Pound #1997-1 (4-5 metric tons) were caught at the head of Irish Cove in one purse seine set and transported only a few hundred meters to the pound on 11 April. Herring in Pound #1997-2 were captured from Two Moon Bay and transported 2.2 km across Port Fidalgo to the closed pound in two loads on 13 and 14 April. The limited number of fish added to this pound from the first load (≈2 metric tons) prevented 0 d sampling, so the first fish sample was taken the following day (1 d) after an additional 16 metric tons was added (Table C1). A 40 fish sample of moribund herring swimming listlessly on the surface of Pound #1997-2 was removed on 18 April, corresponding to 5 d of impoundment. All herring in Pound #1997-3 (≈ 0.9 metric tons) were loaded from one purse seine set at the head of Landlocked Bay on 11 April and transported only a few hundred meters to the pound. The limited number of fish added to the pound prevented 0 d sampling without disturbing the kelp, and subsequently the pound operators were unable to catch more herring. The kelp was later removed, and the pound was abandoned. However, sampling of the fish continued and was facilitated by lifting the sides of the pound to concentrate the herring during sampling events. Spawning herring were also sampled from 2 free-ranging schools of wild fish (40 herring per school) in Landlocked Bay on 18 April.

Herring collected from each pound were placed in static water live-tanks and necropsied within 2 to 8 h. Spleen and kidney tissues from each fish were placed in sterile plastic bags, packed on ice, and shipped to the Alaska Department of Fish and Game fish pathology laboratory in Juneau, AK, where samples were frozen at -80 °C until assayed for VHSV. Herring tissues identified as VHSV-positive on primary isolation were distinguished from those that became positive following blind passage.

Duplicate water samples for VHSV analysis were collected from both the center and 3 m outside each pound every other day at 1 m below the surface and within 1 h of slack tide. Samples were passed through a 0.45 µm filter, diluted 1:1 in MEM-10, shipped to Cordova, AK where they were frozen at -80 °C, then shipped to the University of Washington and finally to the Marrowstone Marine Station where they were assayed for virus. The 1997 water samples underwent 3 partial freeze-thaw events prior to assay.

1998 Studies

Modifications to the 1997 study design were implemented in 1998 to address possible problems with pre-necropsy handling and virus stabilization techniques which were encountered the previous year. Herring were placed on ice immediately after capture rather than held in static live tanks as was done in 1997. Necropsies were performed on herring within 2 h of capture and tissues were immediately placed in MEM containing 5% fetal bovine serum (MEM-5). Water samples were passed through a 0.45 µm filter and 2 ml of the filtrate diluted 1:1 in 2 ml of 2X MEM-20 to stabilize the virus. All water samples were kept on ice (not frozen) until plaque assays were performed less than 2 wk later at the Marrowstone Marine Station.

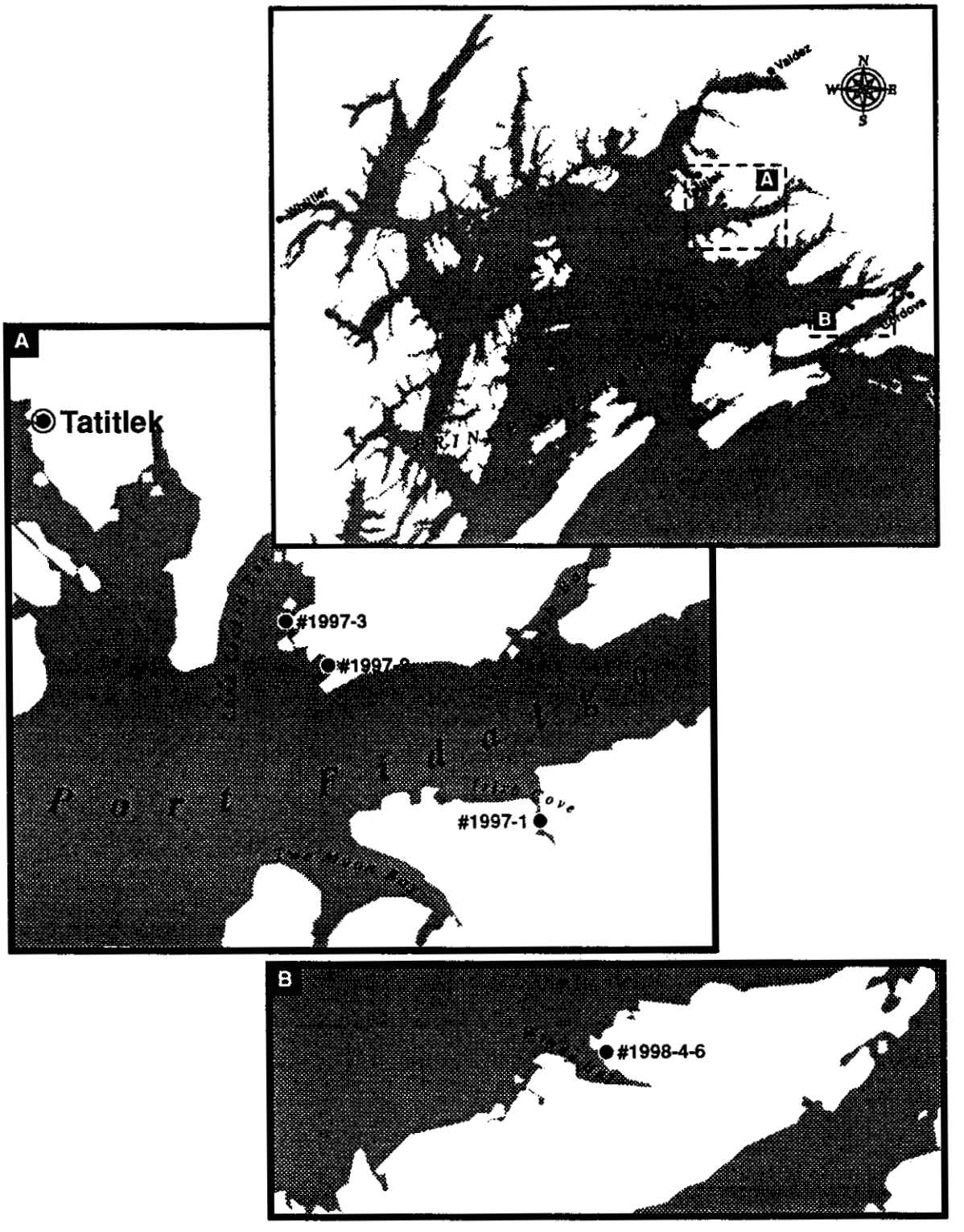

Figure C1. Map of Prince William Sound, AK, including 1997 (A) and 1998 (B) study areas.

Table C1. Physical and biological characteristics of the 1997 PWS pounds.

pound ID	# permit holders*	estimated biomass (metric tons)	pound size (m) (LxWxD)	fish density (kg/m^3)
#1997-1	1	4-5	5.5 x 11.6 x 4.6	17.0
#1997-2	3	>18.0	17.7 x 8.5 x 6.1	19.6
#1997-3	4	0.9	6.1 x 7.3 x 9.1	2.2
#1998-4	3	1.8	5.5 x 11.6 x 4.6	61.4
#1998-5	4	24.0	9.7 x 8.5 x 9.1	32.0
#1998-6	4	16.0	6.1 x 17.1 x 6.1	25.1

* each permit holder is allowed 5.67 metric tons of herring

All 1998 pounds, designated #1998 (4, 5, and 6), were located in Windy Bay (Fig. C2) and loaded at different crowding densities (Table C1) with herring captured on 16 April less than 1 km away near the head of the bay. Herring in Pounds #1998-5 and #1998-6 were loaded from single purse seine sets while Pound #4 was filled with herring captured in 2 sets, made approximately 3 h apart. Random samples of 40 herring pound^{-1} day^{-1} were removed on consecutive days beginning at d 0 when the pounds were loaded and continuing until the fish were released 8 d later. Moribund herring from Pound #4 were sampled daily after their initial appearance on d 2.

A large natural herring spawn in Port Gravina (Fig. C1) on 11 April, 1998 was followed by migration of the post-spawn herring to Two Moon Bay the next day. Wild, post-spawn herring (40 fish day^{-1}) were sampled with either a cast net or purse seine on consecutive days following the major spawning event. Actively spawning, free-ranging herring, presumably members of the same school from which impounded fish were captured, were sampled with a cast net near the head of Windy Bay on 21 April, 1998.

All sampled herring were immediately placed on ice and transported to a fishing vessel where necropsies were performed. Spleen and kidney samples were removed within 1.5 h; tissues from each fish were pooled in sterile plastic bags containing MEM-5 and shipped on ice to the Marrowstone Marine Station where they were frozen at -80 °C until assayed for VHSV.

Duplicate daily water samples for VHSV analysis were collected from both the center and 3 m outside each pound at 1 m below the surface and within 1 h of slack tide. Water samples were also taken from the center of two actively spawning, free-ranging herring schools, one in Fish Bay (Fig. C1) on 11 April and another from the head of Windy Bay on 21 April. Water was also collected from the middle of two closed purse seines of post-spawn herring in Two Moon Bay on 12 April and assayed for VHSV.

Dissolved oxygen remained at or above 8.2 mg$*$L^{-1} inside the pounds throughout the collection period, but was generally 1 mg$*$L^{-1} higher outside the pounds.

RESULTS

1997 Studies

The prevalence of VHSV in herring tissues from all pounds increased and peaked following 1-4 d of confinement, then returned to low levels after 5-6 d. The prevalence of VHSV among herring from Pound #1997-1 was 5% on d 0 and peaked significantly higher ($p < 0.025$) at 20% after 4 d (Fig. C2). Virus prevalence among herring from Pound #1997-2 increased from 0% on d 1 to a high of 12.5% on d 4 (Fig. C3) with significantly greater viral prevalences ($p < 0.05$) on d 2-5. The prevalence of VHSV in both moribund and apparently healthy fish from Pound #1997-2 was similar on d 5 (12.5% and 10% respectively). No 0 d samples were taken from Pound #1997-3, but VHSV prevalence peaked at 25% after 2 d of confinement, then dropped to less than 10%, with significantly fewer ($p<0.05$) VHSV-positive fish on d 3-4 and 6-8 of confinement (Fig. C4). No VHSV was detected in 80 tissue samples of wild, naturally spawning herring collected from Landlocked Bay on 18 April. A slight increase in prevalence (0-15%) was noted when tissues were passed blind, reflecting fish with very low titers (< 50 pfu g^{-1}) that were probably infected while in holding tanks prior to necropsy; however, prevalence patterns following primary isolation and secondary passage were similar.

Herring age distributions in Pounds #1997-1 and 2 were nearly identical, consisting primarily of 9 yr-olds (40%), while Pound #1997-3 contained primarily 3 yr-olds (60%) with few (5%) 9 yr-olds (Fig. C5). Virus prevalence was associated primarily with the 4 to 6 yr-olds (≈20% VHSV-positive in each year class) and decreased with age (Fig. C6). Prevalence of VHSV was significantly greater in females (11.8%, 51/431) than in males (7.8%, 48/614; $P<0.05$) from these groups.

No virus was isolated from any of the water samples taken inside or outside the pounds during the 1997 season. Dissolved oxygen remained at or above 12 mg L^{-1} throughout the 1997 sampling period, but was about 1 mg L^{-1} lower inside each pound than outside.

1998 Studies

Modifications to the 1997 study design were made in 1998 resulting in fewer variables and easier data interpretation. The prevalences of VHSV among herring from each of the 3 study pounds followed similar kinetic patterns and were initially low, followed by peaks between 57-87% after 6-8 d of confinement (Figs. C7, C8, C9). Only 10-22.5% of the herring from Pound #1998-4 tested positive for VHSV from d 0 through d 5, which increased significantly ($p < 0.001$) on d 6-8 to 55-87.5% (Fig. C7). Virus prevalence among herring tissues from Pound #1998-5 was significantly greater ($p < 0.02$) after d 2 and 4-8 of confinement. A bimodal pattern of VHSV prevalence was indicated by the significant increases ($p < 0.02$) in virus prevalence on d 2 and 6 (Fig. C8). Prevalence of VHSV among herring from Pound #1998-6 steadily increased from 7.5% on d 0 to 57.5% on d 8, with significant differences ($p < 0.02$) from d 0 occurring on d 5-8 (Fig. C9).

Nearly all moribund herring (90-100%) sampled from the surface of Pound #1998-4 had detectable levels of VHSV (Fig. C10). No moribund herring were sampled from the pound on d 0 and 1 because so few were present, and none were sampled on d 8 when the pounds were emptied.

The prevalence of VHSV among free-ranging, post-spawn herring from Two Moon Bay decreased from 17.5% 1 d after the major spawning event to 7.5% 4 d later, but the decrease was not significant ($p > 0.10$). No pre-spawn wild herring were sampled from this school on d 0 because it was not anticipated that the post-spawn herring would remain in the area. The sample of free-ranging, spawning herring from Windy Bay, collected 5 d after capture of fish for the pounds, indicated that only 5% (2 / 40) of the unimpounded cohorts tested positive for VHSV.

Significantly more herring from the 1998 pounds ($p < 0.02$) had high virus titers (10^4-10^8 pfu g^{-1}) than low titers (400-9,999 pfu$*g^{-1}$) only on d 7 and 8, while significantly more moribund herring in Pound #1998-4 had high virus titers than low on each sampling day ($p < 0.001$). There were no differences between the percentages of fish with high and low virus titers in the free-ranging, post-spawn herring from Two Moon Bay. The two virus-positive herring of the 40 fish sampled from the actively spawning school in Windy Bay had low titers.

No VHSV was detected in water from inside the pounds prior to introduction of herring, but virus was found inside each pound as early as 1 d and outside the pounds as early as 2 d after herring were introduced. Daily concentrations of waterborne VHSV inside each pound followed a bimodal pattern, with the initial, smaller peak occurring after 1-4 d, and the second, higher peak occurring just prior to release of the fish from the pounds on d 8 (Figs. C11, C12, C13). Concentrations of VHSV in the water increase through the final sampling date inside each pound, reaching levels of 700 pfu$*mL^{-1}$ in Pound #1998-4 after 8 d of confinement. Waterborne virus concentrations 3 m outside Pound #1998-4 increased to over 200 pfu$*mL^{-1}$ after 8 d of confinement, but remained below 20 pfu$*mL^{-1}$ outside Pounds #1998-5 and 6 through the final sampling day (Figs. C11, C12, C13).

Low titers of VHSV were found in 2 water samples near free-ranging herring, but virus concentrations were significantly below those detected near the pounds. Water from a closed purse seine in Two Moon Bay containing 1 d post-spawn herring was positive for VHSV, with 15 pfu mL^{-1}. No VHSV was isolated from a second purse seine in the same area 30 min later . Water from the middle of a wild herring spawn in Windy Bay was positive for VHSV, but no VHSV was recovered from water sampled in the middle of another wild herring spawn in Fish Bay.

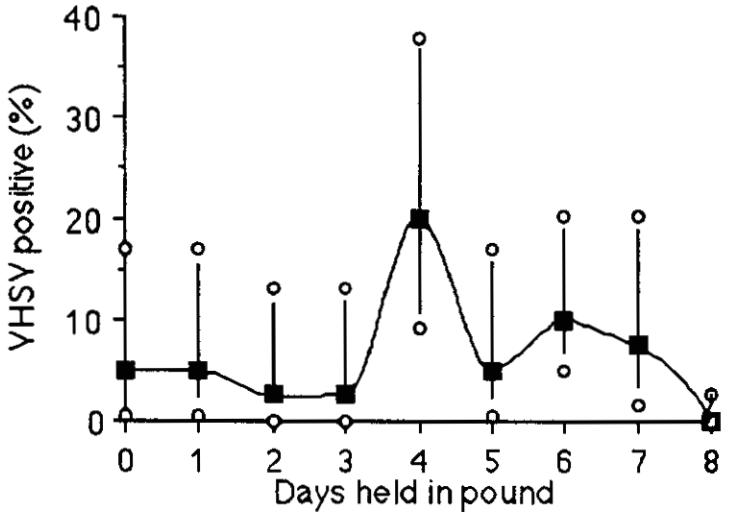

Figure C2. Daily VHSV prevalences in herring from Pound #1997-1.

Daily "n" = 40. Bars indicate 95% confidence limits.

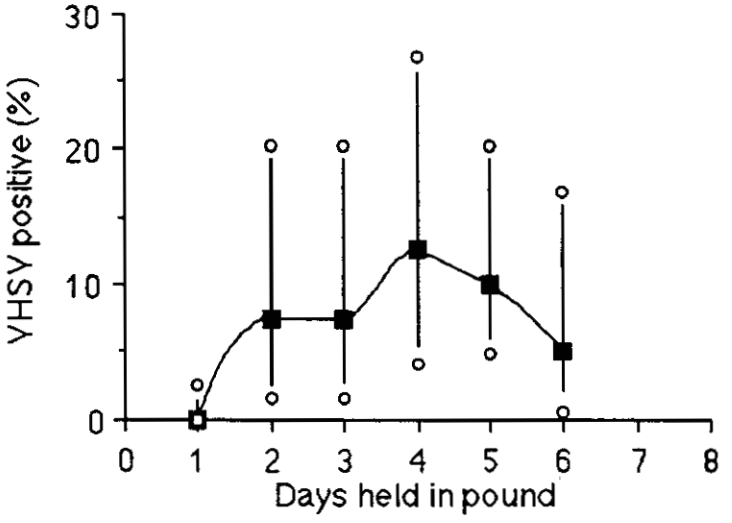

Figure C3. Daily VHSV prevalences in herring from Pound #1997-2.

Daily "n" = 40. Bars indicate 95% confidence limits.

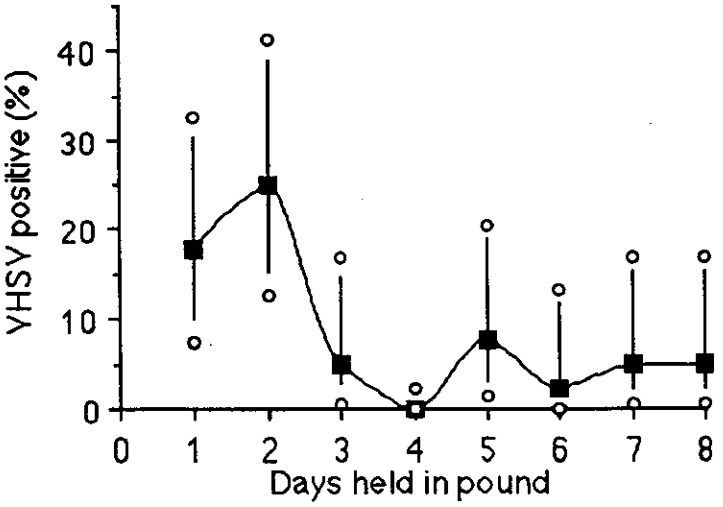

Figure C4. Daily VHSV prevalences in herring from Pound #1997-3.

Daily "n" = 40. Bars indicate 95% confidence limits.

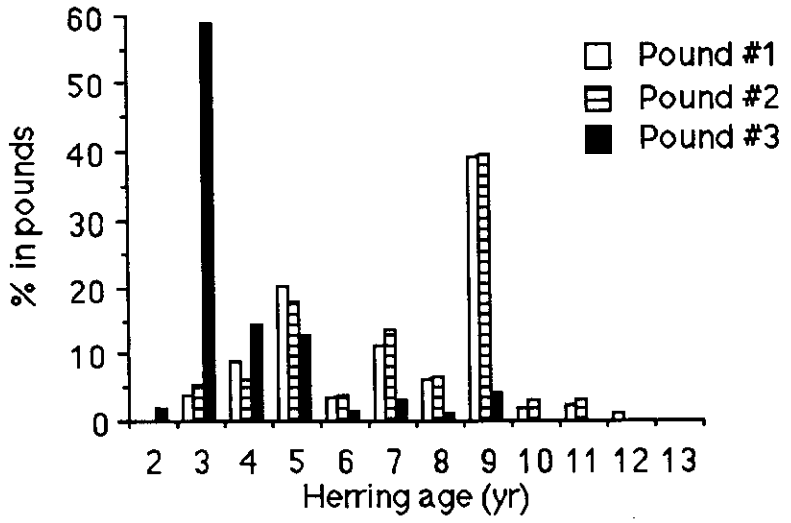

Figure C5. Age composition of herring in each of the 1997 pounds (from scales).

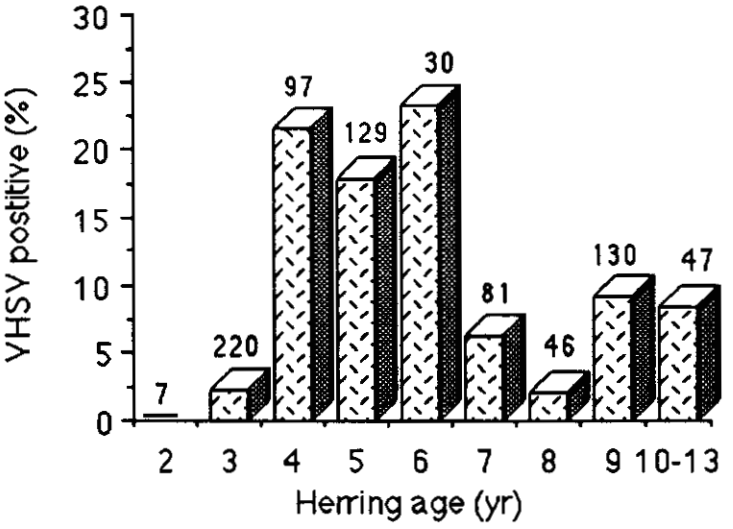

Figure C6. Age-related VHSV prevalence among 1997 impounded herring.

Numbers represent "n."

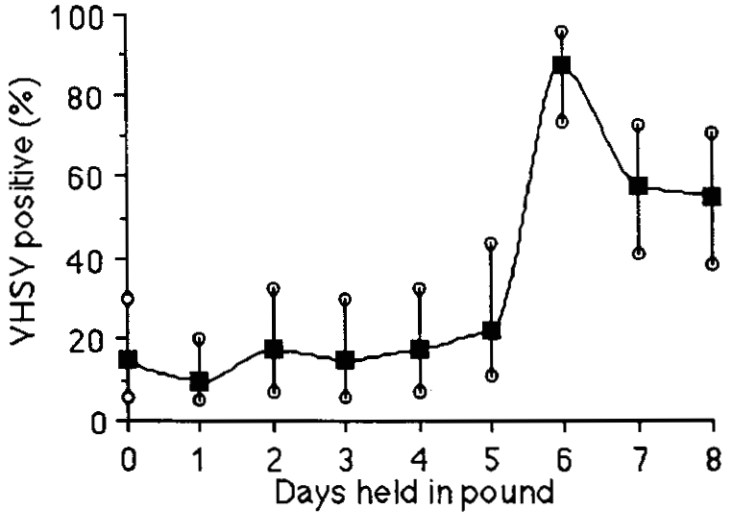

Figure C7. Prevalence of VHSV among herring tissues from Pound #1998-4.

Daily "n" = 40. Bars indicate 95% confidence limits.

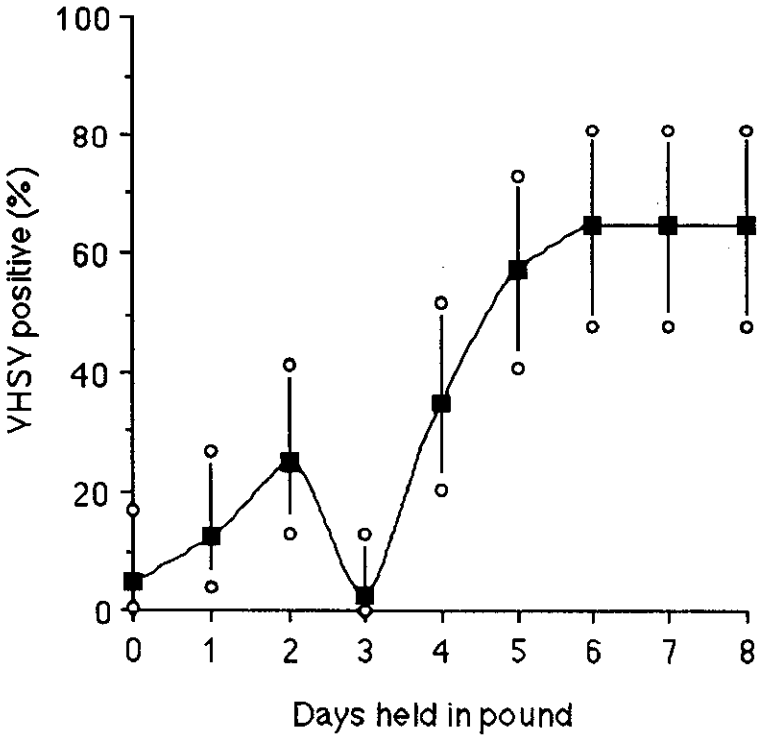

Figure C8. Prevalence of VHSV among herring tissues from Pound #1998-5. Daily "n" = 40. Bars indicate 95% confidence limits.

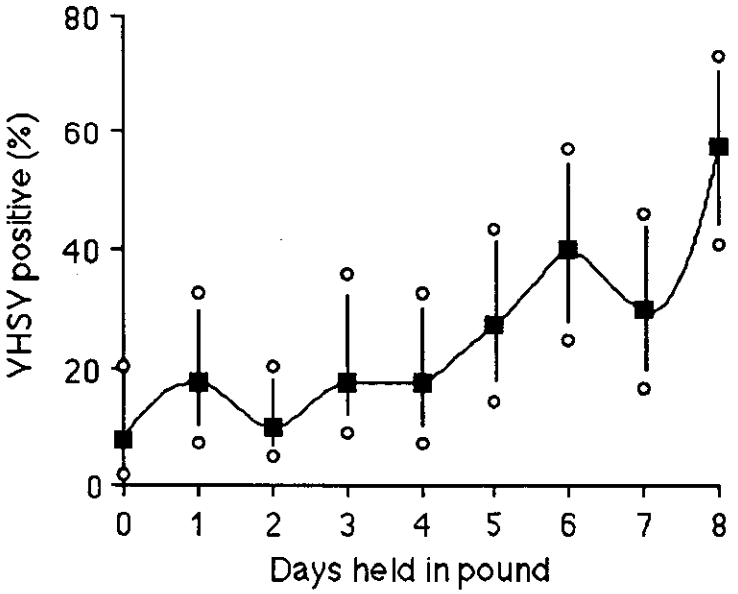

Figure C9. Prevalence of VHSV among herring tissues from Pound #1998-6. Daily "n" = 40. Bars indicate 95% confidence limits.

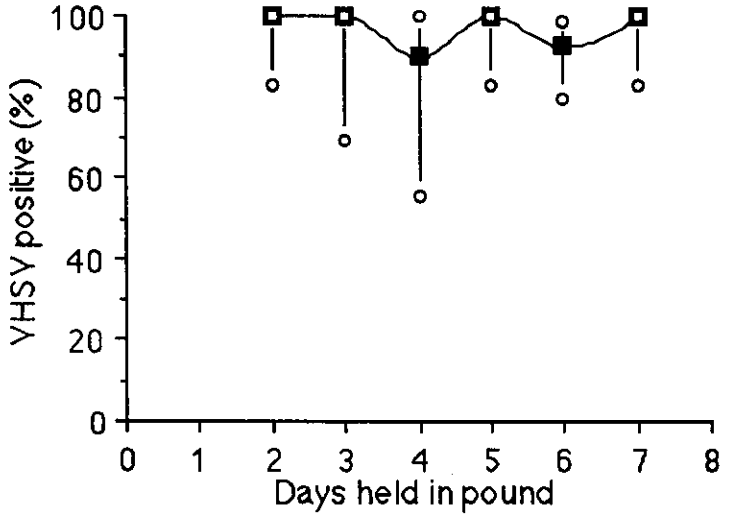

Figure C10. Prevalence of VHSV among tissues from Pound #1998-4 moribund herring.

Bars indicate 95% confidence limits.

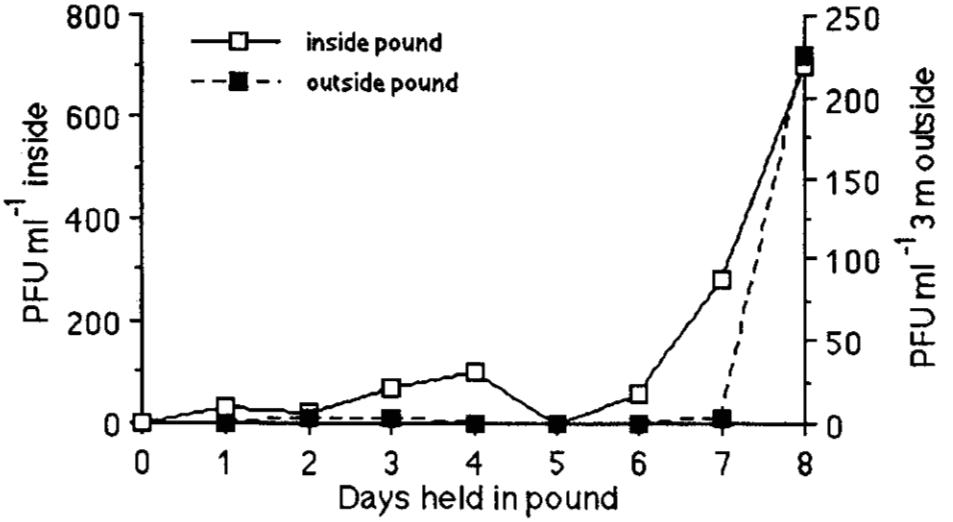

Figure C11. Daily VHSV concentrations in the water inside and 3 m outside Pound #1998-4. Daily concentrations are reported as duplicate means.

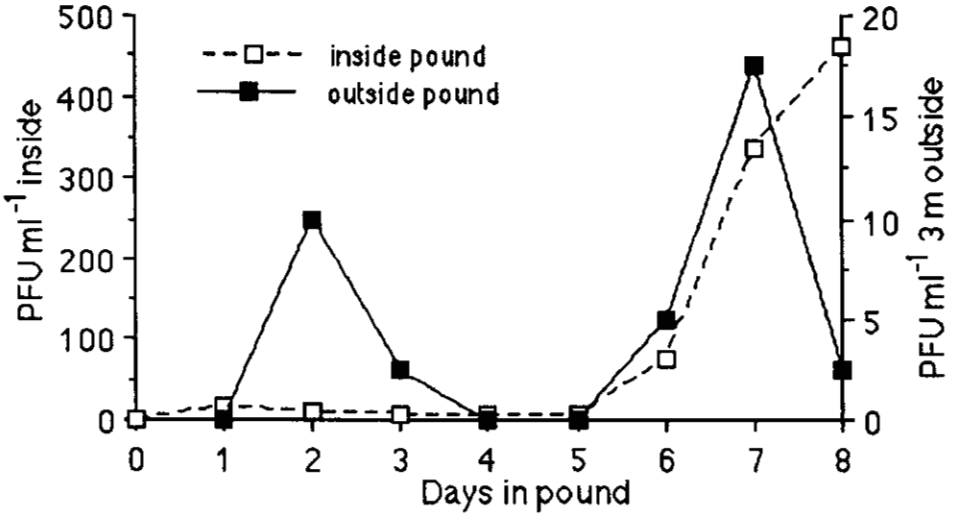

Figure C12. Daily VHSV concentrations in the water inside and 3 m outside Pound #1998-5. Daily concentrations are reported as duplicate means.

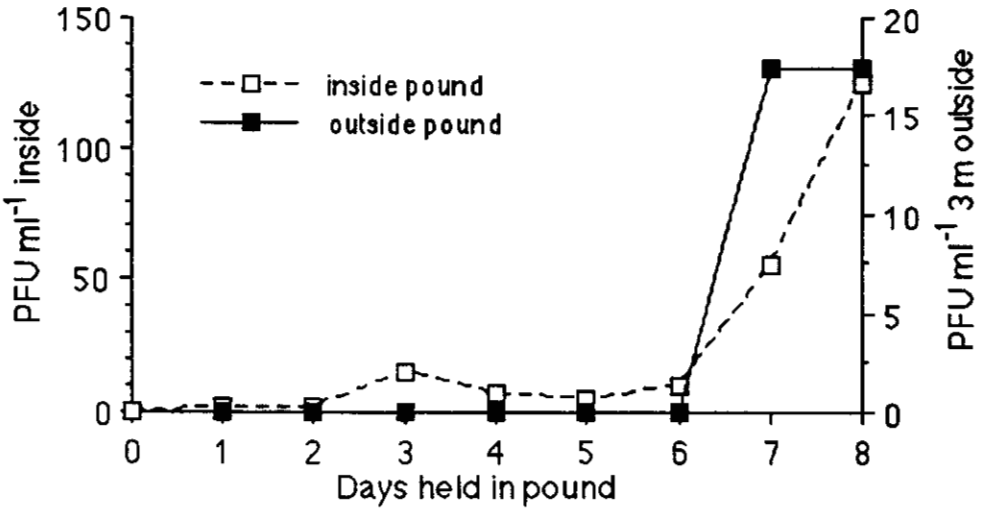

Figure C13. Daily VHSV concentrations in the water inside and 3 m outside Pound #1998-6. Daily concentrations are reported as duplicate means.

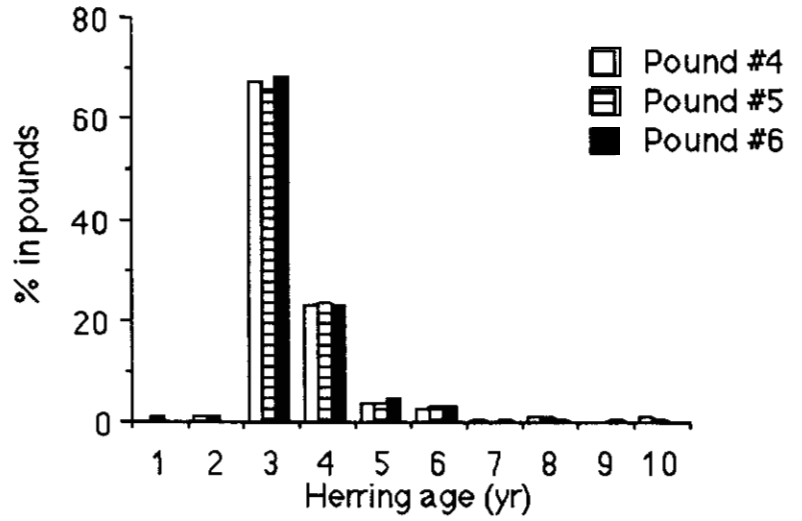

Figure C14. Age structure of herring in each of the 1998 pounds (from scales).

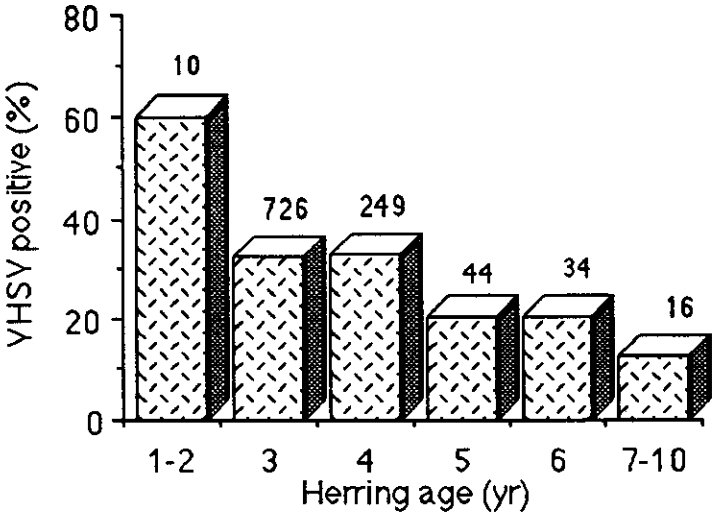

Figure C15. Age-related VHSV prevalence among 1998 impounded herring.

Numbers represent "n."

Nearly identical herring age distributions were loaded into each of the 3 pounds, dominated by 3 yr-olds (65-68%) and a few 7+ yr-olds (Fig. C14). Prevalence of VHSV was highest (60%) in the 1-2 yr-olds and steadily decreased with age to 12.2% in the 7-10 yr-olds (Fig. C15). Prevalence of VHSV among males (36.4%, 246/675) and females (33.7%, 247/733) was not significantly different ($p > 0.20$).

Discussion

The prevalence of VHSV among Pacific herring in SOK pounds consistently increased with confinement time in the pounds, a phenomenon not seen in free-ranging herring from the same stocks. However, the magnitude, temporal occurrence, and duration of the peaks in prevalence varied from year-to-year. The higher peaks of VHSV prevalence in 1998 (60 - 85.5% after 6-8 d)) than in 1997 (12.5 - 25% after 1-4 d) is believed to be due to a greater susceptibility to VHSV among the younger age classes present in 1998. Interannual procedural differences in handling of the tissue samples are not believed to account entirely for the differences in peak viral prevalences because preliminary data from disease monitoring studies in PWS also indicate increased VHSV prevalence among free-ranging herring in 1998. Additionally, unpublished data from our laboratories suggest that increased prevalence of VHSV among impounded herring is not correlated with loading density. Viral prevalence in 1997 was greatest (25%) among herring taken from Pound #3, which contained the lowest biomass (2.2 kg m^{-3}), but which had the highest proportion of newly recruited 3 year-old fish (Figure C5).

The highest prevalences of VHSV typically occurred in the youngest herring and steadily decreased with fish age (Figs. 4 & 8). Only 2/16 of the impounded herring in the 7-10 yr age classes tested positive for VHSV in 1998 and both were found near the end of the study (d 6 and 7), when the virus prevalence peaked among the impounded fish. Similarly, only 4 herring older than 10 yr tested positive for VHSV in 1997, 3 of which had low level infections that were detected only after secondary passage, and the fourth came from Pound #1997-1 on d 4, when the viral prevalence peaked at 30% (Fig. C2). Such a pattern of decreasing VHSV prevalence with increasing age is indicative of an increased immunity among older herring, permitting the older fish to clear the virus before significant viral replication occurs. Total mortality rates among the impounded herring were not recorded, but nearly all moribund fish sampled from the surface of Pound #1998-4 tested positive for VHSV with high viral tissue titers, indicating that mortality was associated with VHSV.

Differences in peak viral prevalences among impounded herring from the same SOK season were detected but were not as pronounced as differences between years. Prevalence of VHSV among fish from Pound #1997-3 peaked at 25% (Fig. C4), higher than any other 1997 pound, presumably due to the predominance of 3 yr old herring (Fig. C5). The high prevalence of VHSV found in these herring after only 1 d was also observed among impounded herring in Puget Sound, WA where none tested positive for VHSV on d 0, but the prevalence increased to 12.2% after only 1 d (Hershberger 1999).

Increased prevalences of VHSV among impounded herring (Figs. C2, C3, C4 and C7, C8, C9) appeared to be correlated with activities associated with operation of the closed pound SOK fishery, because virus prevalence among wild, free-ranging herring failed to increase, and possibly decreased, after the 1998 spawn. Wild, actively spawning herring captured from the head of Windy Bay in 1998 were taken from the same vicinity and were of the same age structure as those loaded into all pounds on 16 April, suggesting that all impounded and free-ranging fish came from the same school. Differences in viral prevalence between confined (Figs. C7, C8, C9) and free-ranging herring from the same school indicate that increased viral prevalence was correlated with impoundment .

A greater prevalence of VHSV in females from 1997 pounds was consistent with a similar trend reported among wild herring from the Montague area of PWS in 1997 (Marty et al 1998). In contrast, no difference in prevalence was correlated with gender among 1998 herring.

In 1998, waterborne VHSV both inside and outside pounds rapidly increased during confinement (Figs. C11, C12, C13) to concentrations capable of producing lethal infections among juvenile laboratory-reared, specific pathogen-free herring (Kocan et al 1997). The lower concentrations in water samples collected from outside Pounds #1998-5 and 6 may have resulted from samples being taken on the upstream side of slight currents circulating through the pounds during tide changes. Nevertheless, VHSV concentrations in water samples collected within 1998 pounds near the end of the confinement period were substantially higher than natural background levels associated with wild herring, where concentrations never exceeding 15 $pfu*ml^{-1}$ were found in only 1 of 4 water samples from wild spawning schools and in 1 of 2 samples from purse seines. The peaks in waterborne VHSV concentrations did not coincide with herring spawning actively within the pounds; rather, they corresponded with increased titers of VHSV in tissues sampled from impounded herring. High concentrations of waterborne virus in and around the pounds provided the most probable exposure route for uninfected impounded herring and for free-ranging fish which were attracted to the spawn emanating from the pounds (personal observation). Post-spawn, herring released from the pounds back into the wild while still shedding virus would constitute a further source of infection for wild stocks. Transfer of virus by direct contact between fish in the pounds is improbable because only small percentages of the herring in each pound had visible subdermal hemorrhages and ulcers similar to those previously associated with VHSV infections (Meyers et al 1994).

Whether significant levels of virus were actually present in the water within or outside the pounds in 1997 is not known because the samples collected that year underwent several freeze-thaw cycles prior to being assayed, and with the methods employed, each freeze-thaw event resulted in a reduction in recoverable VHSV of as much as 90%. The method of transporting virus in 1998 proved to be superior because there was little loss of titer during transport.

It is unlikely that all VHSV-positive herring entered the pounds as latent carriers of infection. Hershberger (1999) showed that VHSV prevalence (17.5%) among *C. pallasi* confined into individual laboratory aquaria was significantly less ($p<0.001$) than the prevalence among the same lot of herring grouped in a community tank (>77%), demonstrating that transmission was responsible for increased viral prevalences after confinement. Additionally, detectable levels of waterborne virus were found in all 1998 pounds as early as 1 d after introduction of the herring, representing the most probable source of infection for susceptible fish.

Based on the available evidence, we propose the following explanation for the epizootics of viral hemorrhagic septicemia (VHS) which occur among impounded herring. A small percentage of pre-spawn, wild herring carry and shed VHSV at the time they are introduced to the SOK pounds. These early virus-positive fish constitute the first prevalence peak and either die from VHS after 2 d of impoundment, as observed in 1998, or recover from infection, as occurred in 1997 (Figs. C2, C3, C4). Relatively few additional fish are infected immediately after impoundment because waterborne VHSV titers are initially low. Impoundment of herring results in prolonged crowding of fish, thereby increasing both the stress and the probability of exposure to waterborne virus. VHSV shed during the first prevalence peak provided a waterborne source of infection for the remaining susceptible fish. These fish then undergo infections and shed higher levels of VHSV, which explains the increasing viral titers in the surrounding water. The age structure of impounded fish also influences infection rates, because older fish are more likely to have developed non-specific resistance and/or specific humoral immunity from prior exposures to natural background levels of VHSV. Such immunity would render older fish refractory to infection by VHSV.

The prevalences and tissue titers of VHSV, shedding intensity, and duration of infection all vary depending on the susceptibility of fish in the population. The latter may be a function of age and immunity status. Younger fish will have had less opportunity to encounter VHSV than older fish, and as the population ages, generally to a maximum of 10 yr in PWS, a greater proportion of surviving

individuals in each age group will have been exposed to VHSV. This may explain, in part, both the decrease in the numbers of older individuals in the population and their increased resistance to VHSV.

Worldwide, Clupeoid stocks have been prone to large, cyclic population fluctuations with declines attributed to overfishing, predation, food availability, El Niño, and species interactions (Blaxter and Hunter 1982). In addition, pathogenic organisms including the systemic fungus *Ichthyophonus hoferi* (Rahimian and Thulin 1996) and VHSV (Meyers and Winton 1995) have been proposed as factors that influence the population size. Hudson et al (1998) report cyclic fluctuations in a population of wild birds, the red grouse (*Lagopus lagopus scoticus*), which were prevented by removal of the parasitic nematode, *Trichostrongylus tenuis*. Thus, it is possible that the cyclic fluctuations observed in *C. pallasi* populations may be due, at least in part, to the effects of a natural disease process, and that operation of inherently stressful SOK pounds during spawning years predominated by susceptible fish may amplify the role of VHS in this process.

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D. Survival of VHS-virus (VHSV) in seawater, ovarian fluid and culture medium

Introduction

The successful spread of most viral diseases requires the presence of: 1) a susceptible host, 2) a virulent virus strain and 3) environmental conditions suitable for the spread of the virus. Viral hemorrhagic septicemia (VHS) has been found in wild populations of Pacific and Atlantic herring as well as numerous other salt water species (Brunson, et al. 1989; Meyers et al. 1992; Traxler & Kieser, 1994).

One route of transmission for viral hemorrhagic septicemia virus (VHSV) has been shown to be via water exposure (Kocan et al, 1997). The exposure of nonimmune fish to as little as 100 PFU / ml of water for one hour was sufficient to produce lethal infections in juvenile herring as was placing a single infected fish into a tank with 10 uninfected SPF herring. Because herring travel in large schools and spawn in high densities, any infected individual can potentially transmit VHSV to other nonimmune individuals if conditions are right and the virus can survive and remain infectious sufficiently long in seawater. During studies on wild 0-year herring in Puget Sound, it was observed that VHSV could regularly be isolated from transport water when no virus nor disease was detected in the fish at the time of transport. When these same fish later experienced an epizootic of VHS, it was hypothesized that virus was being shed by a very small number of newly captured fish, and that conditions were adequate in the transport tanks to cause large numbers of fish to become infected after less than two hours exposure. We also demonstrated that live infectious VHS-virus could be isolated from water both inside and outside SOK net pens, as well as from water in the vicinity of naturally spawning herring.

Another related question was that of virus survival in culture during transport. As previously noted, freezing and thawing of virus stocks resulted in a loss of one half to a whole log of virus with each freeze-thaw cycle. Consequently a study was designed to determine the stability of VHSV in seawater and culture medium under various physical and chemical conditions.

Methods

The VHS virus used in this study was originally isolated in 1993 from bait herring held in net pens in south-central Puget Sound and was propagated on the epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (MEM-10) and buffered with Tris (Batts et al. 1993). The master stock of virus was passed less than five times in cell culture before being frozen in aliquots at -70 ^{o}C, which were subsequently shown to be highly pathogenic to nonimmune juvenile herring (Kocan et al. 1997). Working stocks of virus were prepared from frozen pre-titered (1 X 10^{8} pfu $*ml^{-1}$) aliquots as needed and incubated at 15 ^{o}C in MEM-10.

Three seawater treatments were used to assess the stability of VHSV; 1) raw seawater, 2) filtered seawater and 3) Prudho Bay crude oil-exposed seawater. Raw seawater was collected in March, 1998 from 10 m below the surface of Puget Sound, WA at the north end of Marrowstone Island, and filtered to 5 µm. Oiled seawater was produced by exposing filtered seawater to an oil generator based on a design described by (Carls et al 1997). Oil hydrocarbons in the seawater were quantified by total hydrocarbon fluorescence (Mason 1988). Briefly, 50 ml volumes of oiled seawater were extracted with 5 ml of hexane. The hexane extracts were excited at 280 nm and the emission read at 374 nm with no barrier filter in a Perkin-Elmer Model MPF-2A fluorescence spectrophotometer. The slit width was 10 nm for both the excitation and emission wave length. The concentrations of extractable hydrocarbons were determined from a standard curve using North Slope crude oil dissolved in hexane. The concentration of hydrocarbons in undiluted crude oil was determined gravimetrically. Fluorescence was directly proportional to hydrocarbon concentration and ranged from 0.0023 to 0.075 $\mu g*ml^{-1}$. The oil

concentration for this study was 3-5 ng * ml^{-1} (ppb), derived from the mean of samples collected on five successive days.

Ten ml of each seawater treatment was inoculated with pre-titered stock virus and incubated at 15 °C. At each time period following inoculation, beginning at time-0 (t_0), 0.1 ml of water was inoculated onto polyethylene glycol-treated EPC cell cultures and incubated for 7 days, then evaluated by plaque assay. Virus titers were expressed as plaque-forming units (pfu) per milliliter of fluid.

Filtered natural seawater: Seawater was pumped from 10 m below the surface of Admiralty Inlet near Marrowstone Island (Nordland, WA), filtered to 5 µm and sterilized with uv light. Two virus concentrations (6 X 10^2 and 4 X 10^4 pfu*ml^{-1}) were used and incubated at 15 °C. Samples were collected and assayed for virus regularly, until virus could no longer be detected.

Seawater and oil: A water-accommodated-fraction of North Slope crude oil was prepared and virus added to give two concentrations of 6 X 10^2 and 4 X 10^4 pfu*ml^{-1}. As with seawater alone, samples were taken over time to determine virus survival in the presence of oil.

Ovarian fluid: Pooled ovarian fluid from six chinook salmon was added to seawater at varying concentrations ranging from 1% to 0.05%. Solutions were filter sterilized to remove cellular debris and bacteria then virus added to give a final concentration of 5 X 10^3 pfu*ml^{-1}. The ovarian fluid was also titrated on EPC cells to verify that it was not contaminated with virus from the donor fish.

Enriched culture medium: Standard minimal essential medium (MEM) normally used to culture EPC cells for virus propagation, was enriched with 20% fetal bovine serum and VHSV added at ca 5 pfu*ml^{-2}. The mixture was maintained at 15 °C and sampled for 35 days.

Results:

The LC_{50} for both high and low concentrations of VHSV in seawater and oiled seawater was approximately 10h (Figures D1, D2). After 36 - 40 h incubation there was still 10 percent virus survival, but no virus could be isolated by 45-50 h. Virus stability was not significantly affected by the presence of North Slope crude oil in any of the three tests.

The presence of ovarian fluid in seawater resulted in a stabilization of the virus over the entire range of concentrations tested, with seawater controls dying out by 40 h, with significant virus survival occurring in ovarian fluid cultures at all concentrations tested Fig. D3.

Survival of VHSV in serum-enriched Eagle's Minimal Essential Medium (MEM-20) resulted in prolonged survival and stability of the virus without the need for freezing.

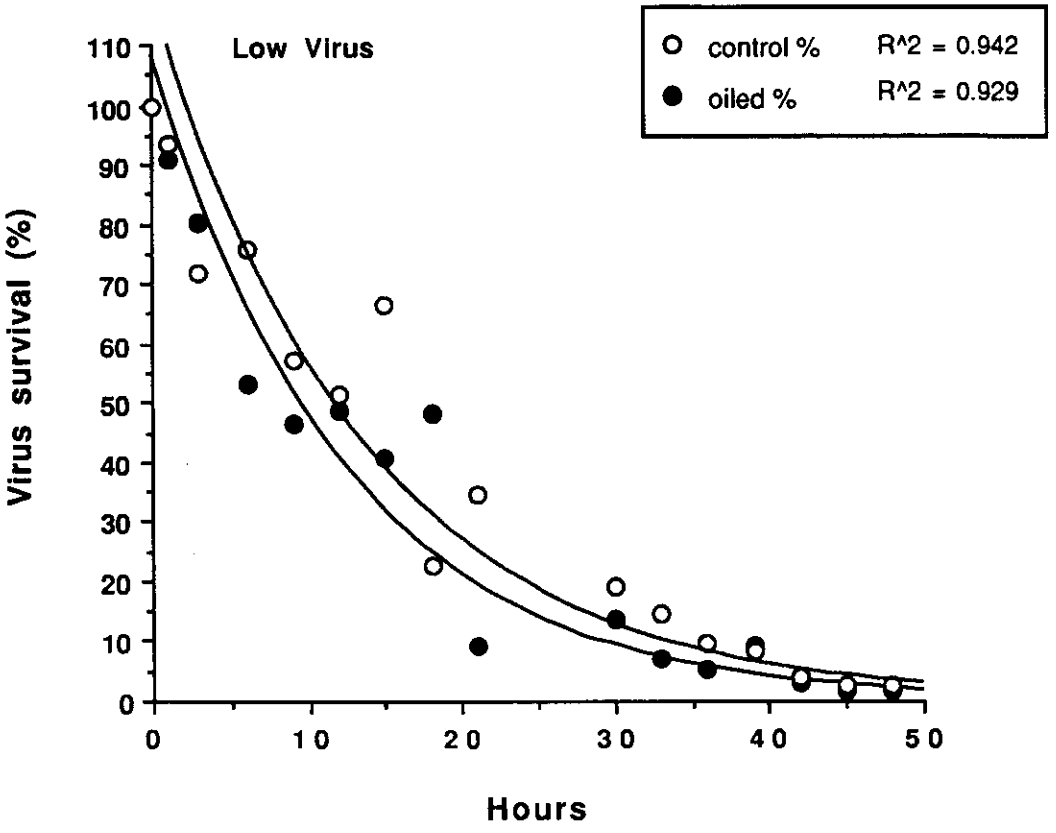

Figure D1. Survival of VHSV over time while incubated in filtered seawater (27ppt & 15°C) and seawater + North Slope crude oil (ppb). Initial virus concentration = 6 X 10^2 pfu*ml^{-1}.

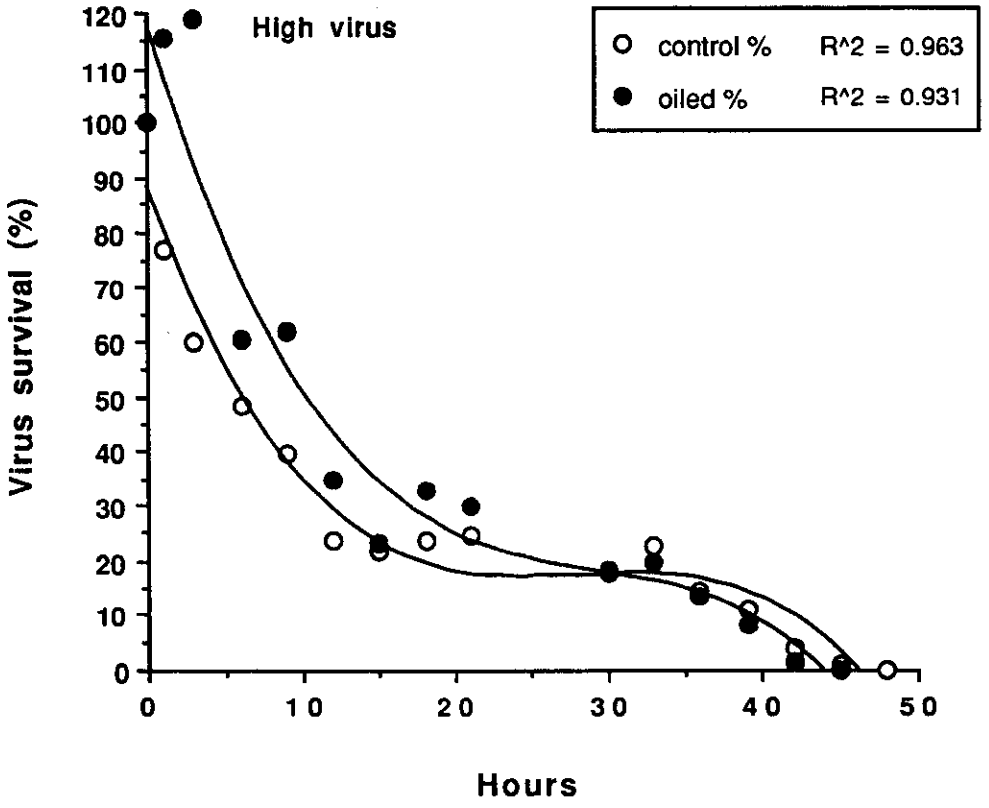

Figure D2. Survival of VHSV over time while incubated in filtered seawater (27ppt & 15°C) and seawater + North Slope crude oil (ppb). Initial virus concentration = 4 X 10^4 pfu*ml^{-1}.

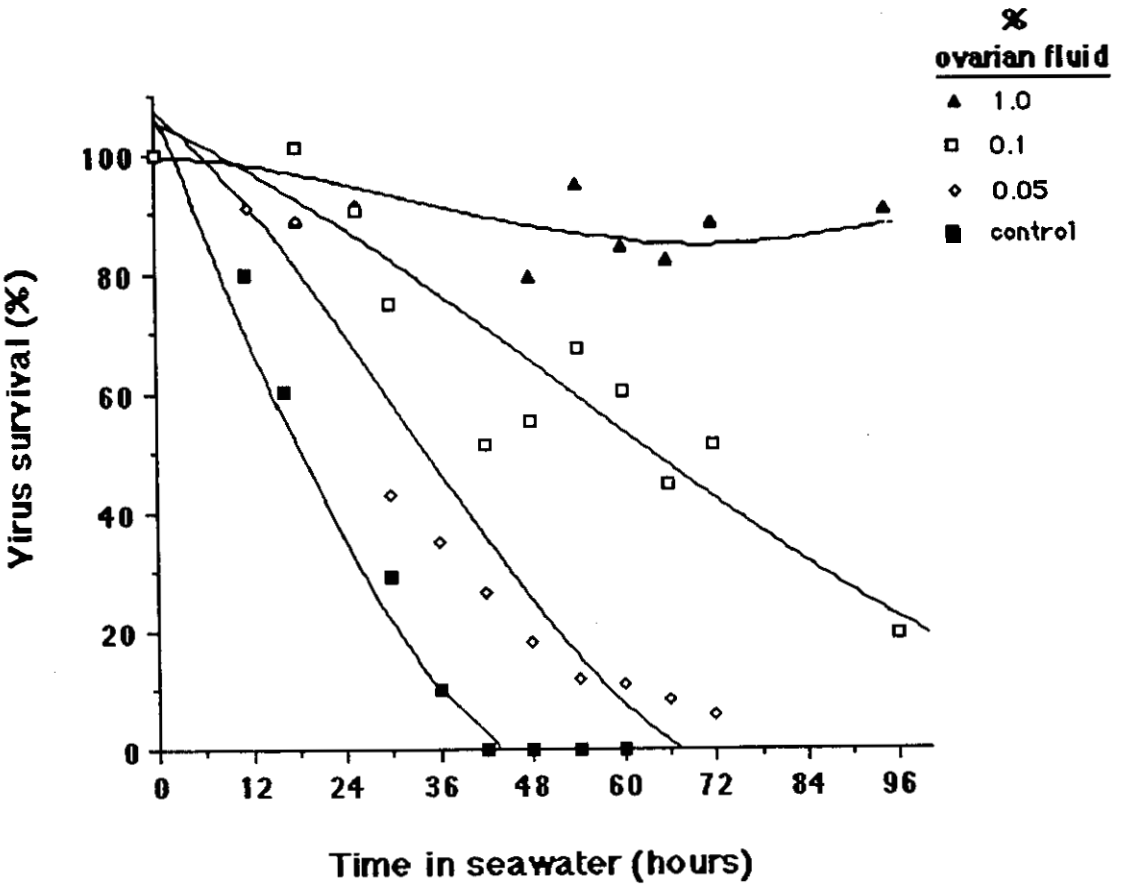

Figure D-3. Survival of VHSV in four concentrations of ovarian fluid. Initial virus concentration was 5 X 10^3 pfu*ml^{-1}.

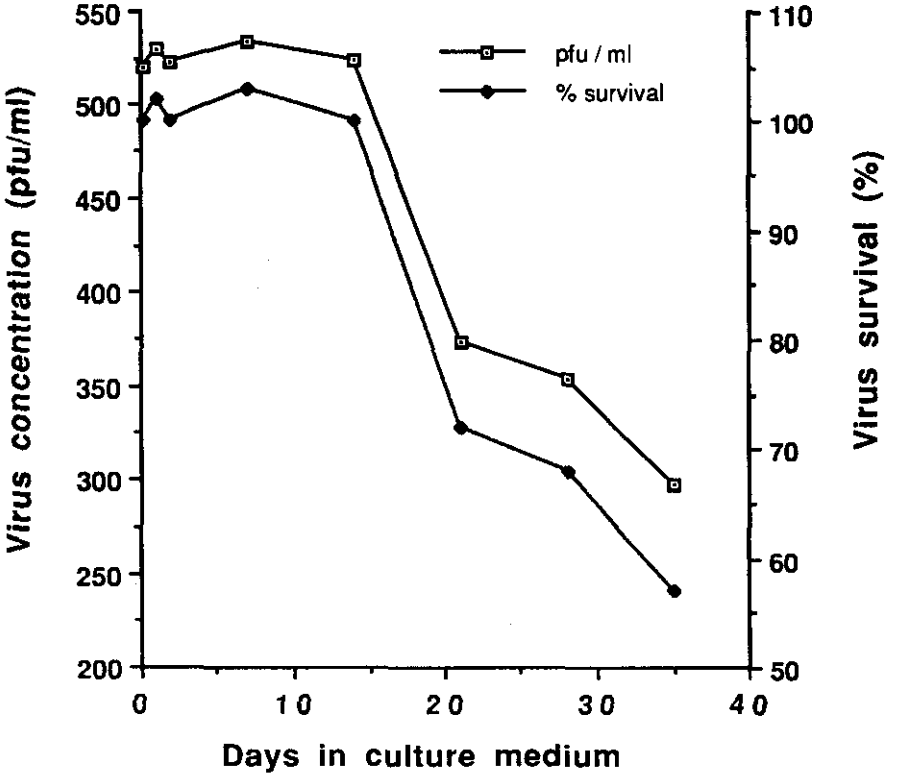

Figure D4. Survival of VHSV in serum-enriched culture medium (MEM-20) Initial virus concentration was 5 X 10^2 *pfu*ml*$^{-1}$. Plaque assays were performed on EPC cells as described above.

Discussion

VHSV was stabilized and maintained it's infectivity for EPC cells for up to 40 h at 15 ^{o}C, with 50 percent of the virus being lost after 10h. The presence of proteinaceous materials such as ovarian fluid and fetal bovine serum significantly increased the survival of virus in the unfrozen state. At 0.05 percent ovarian fluid virus maintained infectivity twice as long as in seawater alone, while at 0.5 percent it remained virtually unchanged for up to 100 h.

Because freezing and thawing VHSV results in titer losses of up to 90 percent, this method of transporting field-collected samples, especially when low titers exist, can give false negatives when the virus is assayed. By stabilizing the virus in the presence of high-serum culture medium and maintaining the samples in a chilled (not frozen) environment, they can be successfully transported from the field to the laboratory with little or no loss of titer for up to 30 d. This is especially important when field samples have initially low titers, such as the water samples collected from SOK pounds in 1997 (this report). Freezing these samples initially resulted in no detectable virus when assayed, while virus could readily be identified in 1998 samples transported from PWS to the laboratory in Seattle, WA when transported in MEM-20 and kept cool with ice.

E. Oil generator studies

Objective

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

Methods

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

Results

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

Discussion

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

Figure E1. Crude oil profiles in natural seawater that had been passed over oiled substrate for 25 days. Background < 0.03 ppm; 24 hr = 0.07 ppm; 10 days < 0.03 ppm; 25 days = 0.52 ppm. Compared with the crude oil reference, the primary missing component after 25 days was the LMW (gasoline) range hydrocarbons which were lost during the weathering process. The large component of hydrocarbons with MW > C24 were presumably the result of microbial metabolism and solubilization.

F. 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 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 with mortality essentially 100 percent for each exposure. However, at 24 months a significant increase in natural resistance was evident at two doses of virus (Table FI) [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 F-I. Demonstration of age resistance to VHS in 2-year-old SPF herring 21 days post exposure to water-born virus. These fish had not been previously exposed to VHSV.

Treatment (1) (pfu*ml^{-1})	mm (2) (SD)	gm (SD)	% mortality	% VHS positive mortalities
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.

G. 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.

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. It was also observed that VHSV prevalence in herring injected with near-lethal levels of synthetic corticosteroid immunosuppressants was not significantlydifferent from the prevalence in fully immunocompetant fish.

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.

H. VHSV infection and development of acquired immunity in juvenile Pacific herring (*Clupea pallasi*) and Pacific Sandlance (*Ammodytes hexapterus*)

Introduction
Viral hemorrhagic septicemia virus (VHSV) was isolated from Pacific herring soon after it was first recognized in North America (Meyers et al. 1994). The first herring isolates were from penned bait fish being held prior to processing and wild fish did not seem to carry the virus or be affected by it. In 1992 a massive disappearance of spawning herring in Prince William Sound occurred and subsequent surveys identified VHSV in both herring and Pacific cod in Prince William Sound as well as other non-salmonids (Meier et al 1994). Disease surveys were undertaken on herring from 1993 through 1997 and VHSV was consistently identified in spawning herring, but with no identifiable pattern of occurrence.

Since the prevalence of VHSV in herring did not seem to correlate with any recognizable events, it was felt that a thorough understanding of the life history of this virus in herring populations would help clarify some of the uncertainties. A study commenced in 1995 to track the virus from it's earliest appearance in juvenile herring Puget Sound herring through spawning age fish and to document it's effect on each life-stage. Earlier studies by Kocan et al (1997) demonstrated that 0-year herring suffered extensive morbidity and mortality when exposed to VHSV in the absence of other pathogens. Since 0-year herring are the least understood of all the herring life stages, it seemed that disease-related mortality might explain the extreme fluctuations in recruitment for different year-classes of herring.

Methods

Herring capture and transport

From 1995 through 1998 personnel from the Washington Department of Fish and Game (WDFW) captured wild 0-year herring (*Clupea pallasi*) and sandlance (*Ammodytes hexapterus*) estimated to be from 4 to 8 months old. These fish were collected by dip net from northern Puget Sound near Port Townsend, WA, while juvenile (1+) and adult (2+) herring from the same area were obtained from a local bait dealer on the day of capture. All herring were transported to the laboratory in tanks of filtered seawater aerated with pure O_2 and subsequently maintained in flowing seawater at ambient Puget Sound temperature (8 - 12 °C) and salinity (27 $^{o}/_{oo}$). Captive herring were fed frozen brine shrimp and krill twice daily during the study. These same culture conditions and diet were used for laboratory-reared herring for two years without evidence of disease transmission (Kocan et al 1997).

Fish arrived at the Marrowstone Island Field Station in 100 L transport tanks between 1 and 2 h from the time of capture, then were wet-brailed into 200 g tanks at a density of approximately 300 -800 fish per tank. Upon arrival 60 - 100 fish were randomly selected and frozen at -70 °C until assayed. On all but two occasions the transport water was sampled for virus when the fish arrived at the laboratory. This was done by passing 2 ml of water through a 0.45 µm filter, mixing with an equal volume of 2 X MEM-10, then refrigerating at 4 °C until assayed for virus.

Herring age was estimated using the mean length (mm) of 20-30 fish from each collection. These measurements were then compared with data on wild herring (Blaxter & Hunter 1982; Hourston & Haegele 1980) and laboratory reared herring (Talbot & Johnson 1972; Kocan et al 1997). A second factor used to estimate age was the known time of spawning and hatch of local herring.

A mixed population of approximately equal numbers of 0-year herring and sandlance were captured in July of 1997 from a single bait ball. The two species were maintained in the same tanks and observed for mortality and signs of disease. A second group of approximately 400 sandlance was captured in the same vicinity one month later and were maintained in the laboratory under the same conditions as the mixed population. From June through October of 1998 no 0-year herring could be located in the same areas sampled during 1995 to 1997. However, numerous bait balls of sandlance were present and a single sample of these was collected and maintained as described above.

Virus assay

Virus from herring tissues and transport water was propagated on the *epithelioma papulosum cyprini* (EPC) cell line (Fijan et al. 1983) cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (MEM-10), buffered with Tris and treated with penicillin-gentamicin (Batts et al. 1993). Herring tissues were frozen at -70 °C until assayed for virus. Transport water was collected when fish arrived at the laboratory and mixed 1:1 with MEM-10 than refrigerated until assayed. The titers of infectious virus was determined in cell culture fluid, transport water and homogenates of herring tissue via plaque assay using monolayer cultures of EPC cells pretreated with polyethylene glycol (Batts and

Winton 1989). Virus titers were expressed as plaque-forming units (pfu) per milliliter of fluid or per gram of tissue.

Tissue samples
For titration of virus in herring tissue, the head and tail were removed and the entire midportion of the fish was homogenized in MEM. Serial 10-fold dilutions of the homogenates were plated on monolayers of EPC cells and the recovered virus identified as VHSV by using PCR and VHSV-specific primers .

Challenge infection
To determine if survivors of confinement-induced VHS epizootics developed an active immunity or merely were not exposed to the virus, survivors were exposed to a known lethal concentration of VHSV (Kocan et al., 1997). Sixty days after the last virus could be isolated from their tissues, 90 fish were exposed to 5×10^3 pfu$*$ml^{-1} of VHSV for 60 min, then observed for mortality for 30 days. Subsamples of 60 live fish were taken 10 d after exposure to determine tissue virus levels. Dead fish were frozen at -70 ^{o}C and the tissues later assayed to determine whether VHSV was responsible for the mortality.

Results

Transport to the laboratory
When loading densities were at or below 10 fish$*$L^{-1} and pure O_2 was supplied, 0-year herring and sandlance experienced less than 1 percent mortality during a 1 - 2 hr transport in 100 L tanks. Older fish were likewise successfully transported to the laboratory with little or no mortality at loading densities of 2 - 3 fish$*$L^{-1} provided O_2 was continuously supplied.

Laboratory epizootics
In September of 1995 several thousand 0-year herring housed in flowing seawater tanks began dying with massive hemorrhages on the skin, fin bases and around the mouth and eyes after two days in captivity. Dead fish were collected weekly until the epizootic ended, then survivors were collected until gross lesions were no longer evident. Virus assays on the dead fish revealed extremely high tissue titers of virus (Table H-I). Total mortality exceeded 60% with 93% of the dead fish testing positive for VHSV.

Virus titers exceeding 1×10^8 pfu $*$ gm^{-1} of tissue by 1 wk post-confinement gradually declined to slightly over 1×10^6 pfu $*$ gm^{-1} by the third week and were undetectable by 30 days post-confinement. (Figure H-1).

In September of 1996 a second group of 0-year herring was captured and treated the same as in 1995. The pattern of VHS-related morality in this group of fish was similar to that seen in 1995, with no virus detected at the time of capture, peaking by 7 d in captivity and becoming undetectable by the end of the third week (Table HII). Mortality in the 1996 fish was 18 percent (20/110) with 15 percent (3/20) positive for VHSV.

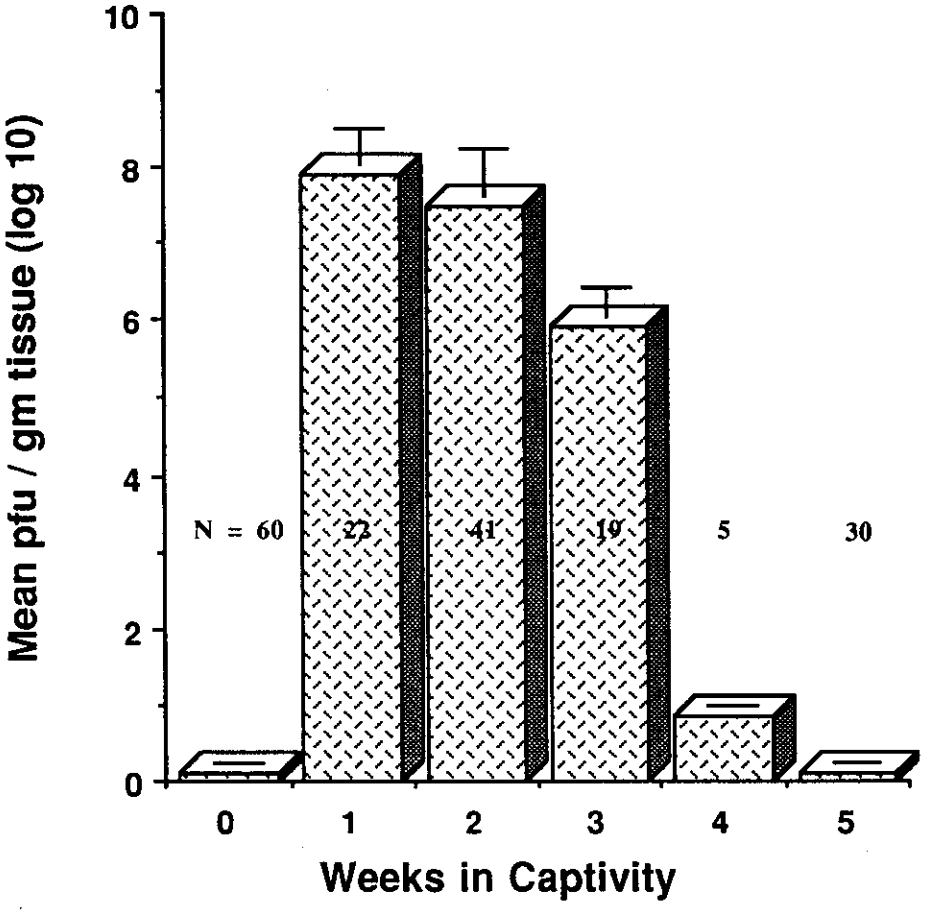

Figure H-1. VHSV concentration in tissues of dead 0-year herring for 5 weeks following captivity in 1995. Mortality began 3-4 days post-capture and ended by week four. (Weeks 0 and 5 are live fish)

Table H-1. VHS in 5-month-old wild Puget Sound herring (Aug. 1995)

days in captivity	N (1)	% VHSV positive
0	30 (2)	0
14	30	100
30	30	0

1) live subsamples
2) subsampled fish not included in total mortality calculations
Total mortality = 60% (316/527)
VHSV positive morts = 93% (294/316)

Table H-2. VHSV isolated from 6-month-old wild herring (Sept. 1996)

days in captivity	subsample N (1)	% VHSV positive	# VHS-pos / # of morts (2)
0	100	0	0/0
7	60	40	3/21
14	60	55	12/18
> 21	60	0	0/7

1) live subsamples
2) subsampled fish not included in total mortality calculations
Total mortality = 18% (46/255)

Table H-3. VHSV isolated from 18-month-old wild herring held in captivity for 30 d (1996)

Days in Captivity	Subsample N (1)	% VHSV+ subsample	# VHSV pos # mortalities (2)
0	60	0	-
2	30	3	0/0
8	30	33	19/19
14	30	10	9/17
30	30	0	0/2

1) live subsamples
2) subsampled fish not included in total mortality calculations
Total mortality = 25% (38/152)

Table H-4. VHSV expression in wild 0-year Puget Sound herring captured over a 4 month period (1997)

date collected	date sampled	number assayed live	number assayed morts (4)	% positive	tissue titer (1)
July 8	July 8	100 (2)	-	0	0
(4 mo old)	July 15	60 (3)	-	76.7	$1.5X10^7$
	July 9-15	-	11	100	$4.7X10^7$
Aug. 7	Aug 7	100	-	0	0
(5 mo old)	Aug 14	40	-	100	$7.7X10^6$
	Aug 8-15	-	16	100	$4.0X10^7$
Sept. 30	Sept 30	100	-	1	$3.3X10^5$
(7 mo old)	Oct 7	15	-	0	0
	Oct 1-7	-	30	0	0

1) pfu * gm^{-1} tissue
2) Initial samples taken immediately after capture (< 1h)
3) Subsample of live herring after one week in captivity
4) Mortalities are cumulative for 7 days following capture

Simultaneously, a group of 18-month-old herring were obtained from a local bait dealer who captured the fish in the same general area as the 0-year fish. The 18-month-old fish exhibited a similar pattern of VHSV progression within the captive population, beginning with no detectable virus at the time of capture, peaking at the end of one week and returning to undetectable by week four (Table H III). Total mortality was 25 percent (38/152) with 74 percent of these (28/38) positive for VHSV.

Three groups of 0-year herring were collected on July 8, August 7 and September 30 1997 in order to follow the progression of VHSV as the fish matured. Each group was transported and housed as described above. The July and August samples followed the same VHS epizootic pattern seen in the previous two years, however, the late September sample deviated significantly from that seen previously. For the first time in three years VHSV was isolated from a single newly captured free-ranging 0-year herring. More significantly, the remaining 500 fish showed no signs of VHS or any other disease during the next 30 days in captivity (Table H IV).

Although extensive efforts were made to locate and capture 0-year herring during the Summer of 1998, none could be found in the same areas that were productive from 1995 through 1997, however numerous "bait balls" of sandlance were present in the area from July through October.

Sandlance

Sandlance (*Ammodytes hexapterus*) captured in July 1997 were housed with the 0-year herring captured from the same bait-ball. Both species began exhibiting hemorrhages on the mouth and fin bases about seven days post-capture. Virus assays conducted on dead and moribund sandlance reveled that they died of VHS with a course of disease indistinguishable from that seen in the herring (Table H IV). Virus titers in the sandlance tissue reached or exceeded 4 X 106 $pfu*ml^{-1}$.

Sandlance captured in August, 1997 measured 77.2 mm (+ 5.8) and weighed 1.8 gm (+ 0.37). A sub-sample of 30 randomly selected fish were all negative for VHSV at the time of capture. The remaining fish were housed as a single species for 60 d during which no mortality occurred in approximately 300 fish.

An additional 300 - 400 sandlance were obtained from a single bait ball in July, 1998. None of the fish assayed for virus at the time of capture tested positive for VHSV and no epizootic occurred during the first 30 d the fish were held in captivity.

Transport water

Transport water was sampled from six shipments of 0-year, 1+ and 2+ herring in 1996 and 1997 and all tested positive for VHSV at or greater than 2 X 10^2 $pfu*ml^{-1}$ of water. A sub-sample of 60 - 100 fish was also assayed from each shipment, and only one 0-year fish from one shipment (Sept. 30, 1997) tested positive for VHSV (Table H IV).

Challenge infection

Herring

All age classes of herring which survived an initial epizootic or for more than 30 days in captivity were solidly immune to challenge infection by $\geq$ 5 X 10^3 $pfu*ml^{-1}$ of VHS virus. No morbidity or mortality was observed in challenged fish by 30 d post-challenge. Tissue samples taken from 60 live fish 7 - 10

day post challenge resulted is relatively few fish being VHSV-positive and tissue titers were below that normally found in fish infected for the first time (Table H-V).

Sandlance

Challenge infection of sandlance which survived an epizootic resulted in no morbidity or mortality, and no virus could be isolated from their tissues 7d post-challenge. These fish had been housed with 0-year herring which also experienced an epizootic of VHS. Likewise, sandlance captured late in 1997 and in 1998, but which had not experienced an epizootic following capture, were solidly immune to challenge infection with 5×10^3 pfu$*$ml^{-1} of VHS virus.

Table H-5. Challenge infection of VHS survivors three months post-capture

estimated age[1]	days in captivity	exposure conc.[2]	exposure time	N	% VHSV+ subsample[3]	cumulative mortalities[4]
5 mo	90	$5X10^3$	1 hr	5	-	0
	90	$5X10^6$	1 hr	5	-	0
6 mo	90	$5X10^3$	1 hr	90	3	0
18 mo	90	$5X10^3$	1 hr	90	4	0
24 mo	90	$5X10^3$	1 hr	90	0	0

1) age at time of capture
2) pfu $*$ ml^{-1} water
3) 30 fish at 10 days post-challenge
4) 30 days post-challenge

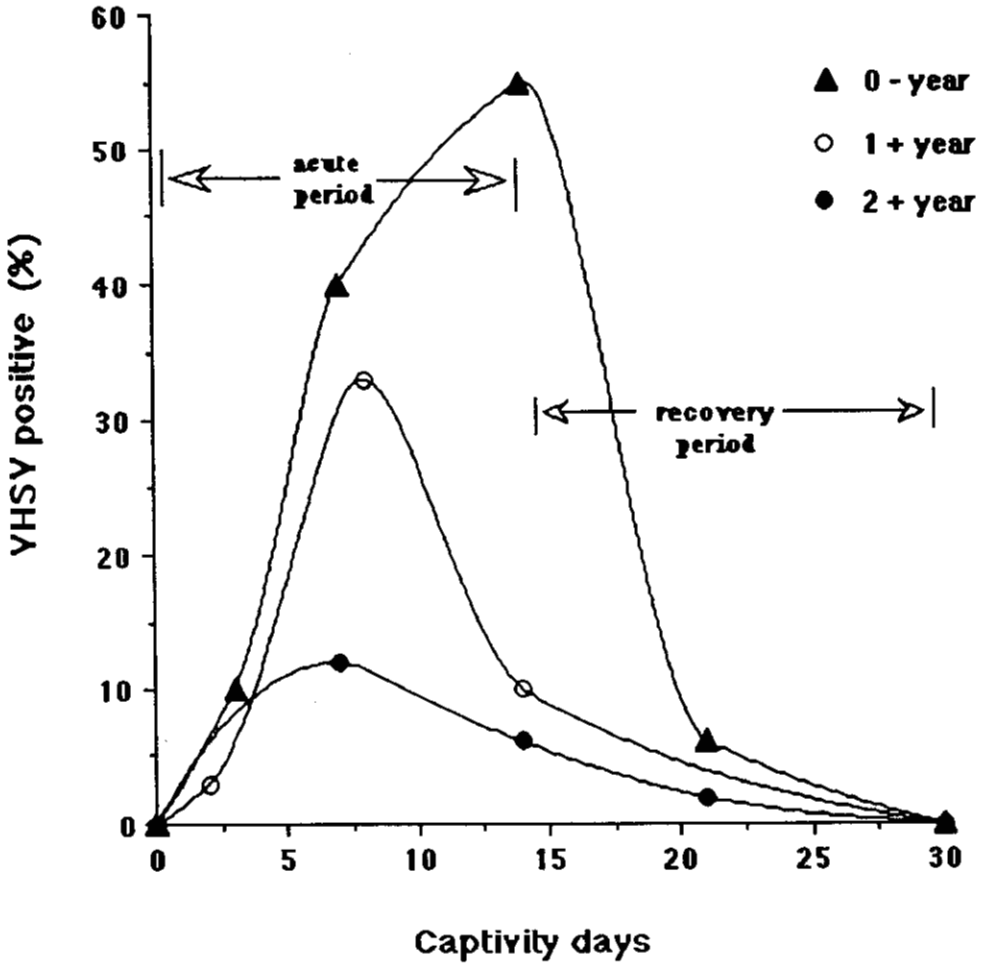

Figure H-2. Changes in prevalence of infection as a function of herring age. Fewer fish become VHSV-positive and few die as the population ages.

Discussion

Based on data obtained from wild Pacific herring in Puget Sound and data derived from experimentally infected herring (Kocan et al 1997) it appears that 0-year fish are exposed and infected by VHSV very shortly after they metamorphose at 3 - 4 mo post-hatch, and that there is a potential for extremely high VHS-associated mortality rates during their fist year of life. As the herring aged they appeared to become more resistant to the effects of VHSV. The course of laboratory-induced epizootics in 0-year fish frequently resulted in morbidity and mortality rates in excess of 50 percent, while following challenge infection these same fish demonstrated an acquired immunity expressed as resistance to reinfection, no clinical signs of VHS and little or no associated mortality (Figure H-2).

With one exception, tissue samples taken from newly captured 0-year herring and sandlance over a four year period were negative for VHSV by plaque assay, but virus was consistantly isolated from transport water at levels capable of producing lethal infections in nonimmune fish (eg 1-2 X 10^2 pfu*ml^{-1}). Since 100 fish were sampled and assayed for virus within 1 - 2 h of capture the prevalence rate for carriers had to be below 1 percent. High levels of virus in the transport water, which often contained $\geq$10 fish*L^{-1}, indicated that a very few infected fish were shedding large amounts of virus, which is supported by previous laboratory studies which demonstrated that a single infected 0-year herring shed more than $10^{6.5}$ pfu*h^{-1} (Kocan et al 1997). With over 1,000 fish transported in a 100 L tank for 1 h it can be demonstrated that if 0.5 percent of these fish were VHSV-positive they could produce enough virus to reach titers of > 200 pfu per ml.

The potential for transmission of the virus under confined conditions is considerably greater than that which occurs in the open sea. Even with flow-through conditions, a portion of the holding water is recirculated, resulting in the build-up of virus within the tanks. Consequently, exposure to virus during transport is believed to be the initiator for the epizootics observed immediately following confinement and transmission within the holding tanks perpetuates the infection following the initial exposure during transport.

The very low prevalence levels of VHSV in wild fish suggests that within a school, under natural conditions, the virus is transmitted to very few fish at any one time. In the open sea the dilution factor is enormous and fish rarely encounter the same water twice, resulting in very low virus titers and few fish being exposed at any one time.

There are two probable scenarios for VHSV transmission in wild juvenile herring. The first possibility is that very few individuals are carrying active infections and shedding virus at any one time. This results in extremely low virus titers in the water surrounding the school and very low transmission rates to uninfected individuals. Consequently, a few fish are constantly being infected and shedding virus, resulting in a slow increase in the number of infected and ultimately recovered individuals within each school. By the time the fish reach sexual maturity a high proportion of the population has been exposed and either died or recovered with an acquired immunity to VHS. The second possibility is that once the virus gains entrance to the population (school), it spreads rapidly causing high rates of mortality but ultimately resulting in a portion of the population surviving with a high level of immunity. In reality, both of these scenarios are possible and which one predominates in any one year may influence juvenile survival and ultimately the strength of the year-class.

At this time there is only circumstantial evidence to support the first hypothesis of slow transmission with the ultimate development of immunity. This can be seen in the fish collected in 1995, 1996 and the first two samples of 1998, where most of the fish were susceptible to the virus at the time of capture. However the one sample of 0-year herring captured on September 30, 1997 in which a single VHSV-positive fish (3.3 X 10^5 pfu*ml^{-1}) was detected on initial sample is evidence to support the second hypothesis of mass exposure and recovery (Table H-IV). The remaining fish in this group exhibited none

of the clinical signs of VHS or mortality during the first 30 d in captivity, and none succumbed to challenge exposure to high levels of virus. This indicates that the entire population had been previously exposed to VHSV, and the survivors developed a lasting immunity. There is no way to determine what proportion of the original school was lost, but based on laboratory studies it is not outside the realm of possibility that at least 50 percent died.

Two different groups of sandlance captured in 1997 exhibited similar disease resistance, but without any detectable VHSV-positive individuals at the time of capture. Since sandlance are as susceptible to VHS as are herring, this indicates that they had also been previously exposed to VHSV and recovered.

Both the 0-year and 18-months-old herring examined in 1996 exhibited similar increases in VHSV prevalence following capture and confinement. Their mortality rate however was 18 and 25 percent respectively, of which 3 percent and 18 percent were attributable to VHSV. This is suggestive of the possibility that these fish were already resistant to VHSV.

There is no clear evidence as to the source of the infection in 0-year herring or sandlance. Both species are found in large pelagic schools (eg bait balls) and are not directly associated with adults. Sandlance are frequently associated with herring schools, but both species can be captured as a pure stock. In the one case where a mixed population of sandlance and herring were captured, the sandlance exhibited clinical signs identical to the herring, suffered high mortality and developed a similar resistance to reinfection following recovery. Because of their similar behavior and juvenile habitat, it is probable that they are exposed to virus from the same source.

These data demonstrate that estimates of VHSV prevalence or even it's presence in a population can not be obtained by examination of newly captured herring. Although the occasional positive fish may be detected by examining newly captured individuals, it is more probable than not to miss diagnose a population as being uninfected because of the very low prevalence rate normally encountered in wild fish - epizootics being the exception.
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I. *Ichthyophonus hoferi* in Pacific herring

Introduction

Approximately 80,000 tons (60%) of spawning Pacific herring *Clupea pallasi* failed to return to Prince William Sound, Alaska in 1993, following which, the prevalence of *Ichthyophonus* in survivors reached 27%, more than double that seen in previous years (Marty et al. 1998). Because both mortality and an increase in prevalence occurred closely together, it was proposed that *I. hoferi* might be responsible for the heavy loss of herring. Prior to this time *I. hoferi* was not recognized as a serious pathogen of Pacific herring, although it was know to occur in many North Pacific fishes. Conversely, epizootics of *I. hoferi* often accompanied by mass mortality, have occurred sporadically in Atlantic herring *Clupea harengus* populations of the North Atlantic since 1898 (Sinderman & Chenoweth 1993) and more recently in the North Sea and Baltic Sea (Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997). Although there has been a correlation between the prevalence of *I. hoferi* and mortalities reported in these epizootics, the pathogenicity of *I. hoferi* for herring had not been unequivocally demonstrated.

Conclusive proof that an organism is responsible for a disease requires that experimental infections of known specific-pathogen-free host organisms be carried out to confirm Koch's Postulates. A study was designed to confirm the pathogenicity of *I. hoferi* for Pacific herring by rearing specific-pathogen-free (SPF) herring and exposing them to pure cultures of *I. hoferi*. Parallel field studies were designed to track the development of the organism from its earliest appearance in 0-year through spawning age fish. Identification of the organism in wild fish was attempted by several different diagnostic methods, and transmission to carnivorous fish was attempted using the coast range sculpin *Cottus aleuticus*.

Methods

Culture conditions for primary isolation: *Ichthyophonus hoferi* was cultured from heart, liver and spleen tissue of herring with visible gross lesions, collected from Prince William Sound or Puget Sound, Washington. Tissues were aseptically excised from infected fish and cultured in Leibovitz L-15 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 $\mu g*ml^{-1}$ Gentamicin, 100 units penicillin, 25 $\mu g*ml^{-1}$ streptomicin and buffered to pH 7.2 with 10 mM HEPES buffer. A reference culture originally isolated from North Sea herring and maintained in culture for approximately two years was supplied by Danish investigators (Spanggaard et al. 1994). Cultures were maintained at 12 °C for 10 - 14 d, during which time they were examined under a dissecting microscope at 100X magnification for the presence of *I. hoferi* spores and hyphae. Each culture was evaluated on different days by two independent observers to insure that low-level infections were not overlooked.

Wild herring: Wild 0-year herring ranging from 4 to 8 months old, were captured by dip net in northern Puget Sound near Port Townsend, WA between June and September, while juvenile (1+) and adult (2+ & 3+) herring were obtained from a local bait dealer on the day of capture. All herring were transported to the laboratory in tanks of filtered seawater aerated with pure O_2 and subsequently maintained in flowing natural seawater at ambient Puget Sound temperature (8 - 11 °C) and salinity (27 $^{o}/_{oo}$). Captive herring were fed frozen brine shrimp (*Artemia* sp) and krill twice daily during the study. This same diet was fed to laboratory-reared herring for up to two years without evidence of transmission of *I. hoferi* nor any other pathogen.

Experimental infections in herring: Laboratory-reared herring were infected at 6 months of age by intraperitoneal (IP) injection of 1,000 *Ichthyophonus* cells of various stages and sizes at a final injection volume of 0.1 ml. Following exposure, the herring were maintained in flowing filtered seawater tanks at ambient Puget Sound temperature and salinity. Tanks were observed daily and moribund or dead fish were removed for necropsy and in vitro culture. The study was terminated when mortality reached 90%.

The remaining fish were euthanized with an overdose of MS-222, necropsied and examined for the presence of *Ichthyophonus*.

Ichthyophonus prevalence in wild herring: *Ichthyophonus* prevalence in wild herring between 0 and 3+ years was determined by visually examining fish for skin lesions (eg. sandpaper skin or ulcers) and gross lesions of internal organs, histologic examination of heart, liver and spleen and in vitro culture of tissues. Tissues for histology were fixed in 10% formalin. Heart, liver and spleen were cut and three sections of each organ from each fish were stained with hematoxylin-eosin. Tissues for in vitro culture were aseptically removed, minced and placed into 15 ml tubes containing 5 ml of L-15 medium. Heart, liver and spleen from individual fish were pooled and cultured for 10-14 d at 12 °C, then examined at 100 X magnification for the presence of spores and hyphae. Pilot studies demonstrated that tissues that were not culture-positive by 10 d would not convert if maintained for longer periods.

To evaluate the relative sensitivity of different diagnostic techniques, one group of 30 adult Puget Sound spawners showing no evidence of gross visceral lesions was necropsied and the heart, liver and spleen cut into two equal parts. Half of each tissue was fixed in 10% formalin while the other half was cultured in L-15 medium. Three sections from two different levels of each organ (6 sections total) were stained with hematoxylin-eosin and examined microscopically for the presence of *Ichthyophonus* . The cultured half of the tissue was examined for spores and hyphae at 100X magnification after 14 d in culture. A similar study was conducted with Prince William Sound herring, which had a high prevalence of grossly visible visceral lesions. A separate laboratory (G Marty; Univ. California, Davis, CA) examined the histological sections of these fish, while the cultures were maintained and evaluated at the University of Washington (Seattle, WA).

Experimental infections in coast range sculpins: Wild coast range sculpins *Cottus aleuticus* were captured from Pipers Creek (Seattle, WA; October, 1995) and Issaquah creek, (Issaquah, WA; May, 1996). These were maintained at the University of Washington's School of Fisheries in flowing dechlorinated city water and fed frozen brine shrimp.

A pilot study was designed to verify the susceptibility of sculpins to infection by *Ichthyophonus* by injecting three fish IP with 130 - 550 spores recently cultured from infected herring tissues, while two control fish were injected IP with phosphate-buffered saline (PBS). Prior to injection, spores were washed with three changes of PBS to remove all traces of culture medium. The culture medium was decanted and replaced with PBS, after which the spores were allowed to settle (1 X g) for 1 h between washes. All fish were monitored for up to 4 months and mortalities were necropsied and their heart, liver and spleen cultured in L-15 medium.

A second study was designed to determine whether sculpins could be infected orally with *Ichthyophonus*. Four sculpins from Issaquah Creek were injected IP with 2,500 *Ichthyophonus* spores per fish. The internal organs of two of these sculpins that subsequently died were cultured for one month, after which the spores were removed, rinsed in PBS and injected into four additional sculpins at a concentration of 360 spores per fish. The remaining two infected sculpins were sacrificed, their bodies minced and the infected tissues fed to six sculpins. The orally exposed sculpins were given a single feeding of fresh heavily infected tissues containing visible *Ichthyophonus* lesions, and monitored for 30 d. As the organism was still embedded in the tissues no attempt was made to quantify the dose. Simultaneously, four control sculpins, housed in a separate tank, were injected with PBS and fed pieces of uninfected tissue. All three groups were monitored for 30 d, at which time the first *Ichthyophonus*-exposed fish died. The remaining fish were then sacrificed and their tissues cultured in L-15.

RESULTS

Laboratory-reared experimentally infected herring: The Prince William Sound isolate of *Ichthyophonus* was pathogenic for SPF herring, infecting 90% of the fish and killing 80% within two months of

exposure. Fish exposed by IP injection with approximately 1,000 spores began dying 7 d post-exposure with a mean-day-to-death of 36 d. By 56 d post exposure 8 of 10 fish were dead and the study was terminated (Table 1). Gross and histologically visible lesions on the heart, liver and spleen first appeared on day 11 post exposure, while large spores (150 - 190 μm) were observed just under the skin 36 d post-exposure. These spores appeared to migrate through the body musculature to the skin where by 46 days post-exposure they eroded through the skin, ruptured and presumably released their spores into the surrounding water. After the spores ruptured, dark pigmented ulcers remained in the skin where spores had originally occurred.

In vitro culture of heart, liver and spleen resulted in 90% (9/10) of the SPF fish culturing positive for *Ichthyophonus*. One fish surviving until 56 d post-exposure showed no signs of infection by any of the diagnostic methods employed.

No mortality was observed in a second group of 10 SPF herring injected IP with a North Sea herring isolate of *Ichthyophonus*, which had been in culture for approximately two years. However, spores were observed under the skin of two fish, none had gross visceral lesions, one was histologically positive (1 spore seen in liver) and all were positive for *Ichthyophonus* by in vitro culture. None of the control (sham-injected) fish died and no signs of disease were detected in the controls when the study was terminated (Table I-1).

All stages of parasite development seen in naturally infected fish were also observed in cultured tissues from infected fish. The large spores occurring under the skin and in sections of infected tissue were indistinguishable from those seen in mature cultures. Hyphal-like structures were evident growing from the spores by four days in culture. Microspores measuring 10 μm in diameter developed at the hyphal tips and were released into the medium by 11 d in culture. The microspores then grew to 20 - 47 μm by 15 days, and by three months were approximately 200 μm (197.8 $\pm$ 22.2 μm). These large spores appeared identical to those that began the cycle. The organism underwent a similar growth sequence in tissues obtained from freshly dead herring.

The mean weight of the SPF fish infected with the Prince William Sound strain was significantly less than either of the other two groups at the end of the study, even though they were indistinguishable at the beginning of the study ($P = 0.01$; t-Test). The mean length of the Prince William Sound group was also less than the other two groups, however the difference was not significant ($P > 0.05$) (Table I-1), indicating significant weight loss of infected fish during the course of the study.

Wild Puget Sound herring: *Ichthyophonus* was isolated from Puget Sound herring by 4-6 months post-hatch (June-July) and its prevalence increased over the next 3 years, ultimately reaching 70% (Table I-2). Skin lesions were observed at approximately the same rate (4-6%) in all age groups from 0-year to 3+ years, suggesting a constant rate of infection (exposure), while gross visceral lesions were relatively rare, occurring primarily in fish 2-year-old and older at $\leq 5\%$. Culture of tissues revealed a steady increase in prevalence from 6% in 0-year fish to > 70% in 3+ year spawning adults. Equal numbers of spawners were examined (49M, 51F) and their length (185 mm $\pm$ 16.5 and 185 mm $\pm$ 10.9) and disease prevalence were not significantly different. Unlike the SPF herring however, no difference in weight was evident between infected and uninfected wild fish, possibly reflecting a difference between clinical and subclinical infections.

In vitro culture as a diagnostic tool: In vitro culture of herring tissue proved to be more sensitive than either gross or histologic examination for detecting subclinically infected fish. Of 30 adult herring simultaneously evaluated by in vitro culture and histology, 70% (21/30) were positive by culture, but only 7% (2/30) of the same fish were positive by histologic examination. Both of the histologically positive fish were also positive by in vitro culture. A similar study involving 60 spawning herring from

Rocky Bay (Montague Island) in Prince William Sound, AK showed a significant but less dramatic discrepancy between histologic examination and in vitro culture (17% vs 28%) (R. Kocan, Univ. of Wash., Seattle, WA and G. Marty, U.C. Davis, CA. unpublished data). However, of the known positive Prince William Sound fish, 53% (9/17) exhibited obvious gross lesions on their viscera, while only 6% (5/78) of known positive fish from Puget Sound exhibited gross lesions. This difference in severity of disease between Puget Sound fish and Prince William Sound fish may explain the difference in sensitivity of these techniques between the two sites.

Transmission to sculpins: The coast range sculpin proved to be susceptible to infection and disease following exposure to *I. hoferi* by both IP injection of spores as well as ingestion of infected tissues. Of three sculpins injected IP with spores, one died four weeks post exposure, a second was sacrificed and the third died 3.5 months post-exposure. All three presented with similar *Ichthyophonus* lesions on their heart, liver and posterior kidney and cultured positive for the organism in L-15 medium.

All six of the sculpins given a single feeding of infected tissue developed visible visceral lesions and died or cultured positive for *Ichthyophonus* within 30 d of exposure. None of the controls fed uninfected tissues died or cultured positive for the organism. *Ichthyophonus* was not cultured from tissues of any control fish injected with PBS or fed uninfected tissues.

Since the course of the disease appeared to be the same regardless of the route of infection, injection is probably a reasonable experimental technique for studying this organism and the disease it causes in the vertebrate host.

Discussion

Laboratory-reared experimentally infected herring: *Ichthyophonus* hoferi has been shown to be highly pathogenic for juvenile SPF Pacific herring, capable of causing extensive mortality. This confirms the cause-and-effect predictions of earlier investigators that this organism is a serious pathogen of herring, capable of causing massive losses under natural conditions (Fish 1934, Sinderman & Scattergood 1954, Sinderman & Rosenfield 1954, McVicar 1982, Sinderman & Chenoweth 1993). Laboratory studies serve to demonstrate the potential of an organism to cause morbidity and mortality in a host species under appropriate conditions. The actual pathogenicity in nature however, is dependent on many variables, including multiple infections with other pathogens, nutritional status as well as exposure to environmental contaminants.

The appearance of subcutaneous spores following infection of internal organs in IP infected fish suggests that under natural conditions the parasite first targets the internal organs, then migrates to the skin where it exits as small spores that may infect or be ingested by an invertebrate or larval fish. Alternatively, since herring are schooling fish and occur in high densities, the spores may directly infect individuals in close proximity to infected fish. The exact fate of the parasite after it exits the skin remains to be experimentally determined. The "sandpaper skin" described in Atlantic herring appears to be the result of *I. hoferi* spores located under the skin. However, when these spores rupture and release their contents, their location can still be seen as eroded holes (ulcers) in the fish's skin, but lacking the "sandpaper" texture. This process appears to be the same in naturally infected Atlantic and Pacific herring, as well as in experimentally infected herring.

Since the turn of the century *I. hoferi* has been associated with massive herring mortalities in the North Atlantic (Fish 1934, Sinderman & Scattergood 1954, Sinderman & Rosenfield 1954, McVicar 1982), Prince William Sound, Alaska (Meyers et al. 1994) and the North Sea and Baltic Sea (Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997). Although the constant association of this organism with heavy mortality is highly suggestive of a cause-and-effect relationship, hard evidence for this relationship was not available, and rarely did investigators look for other potential pathogens. Marty et al (1998) describe over 10 potential pathogens associated with a massive herring disappearance in Prince William Sound, Alaska, which initially appeared to be associated with an increase in prevalence in *I. hoferi*.

Subsequently, viral hemorrhagic septicemia virus (VHSV), also a serious pathogen of herring (Kocan et al. 1997), was also found in these same fish, suggesting a possible alternative explanation for the massive mortality. This same virus has recently been identified from herring in the English Channel (Dixon et al. 1997). Although some field evidence for cause-and-effect may seem overwhelming, the variability associated with infection by multiple pathogens makes it essential to conduct controlled studies with pure pathogen cultures as well as specific pathogen-free host animals in order to confidently assign pathogen status to a specific organism.

Wild Puget Sound herring: Wild Puget Sound herring were found to be subclinically infected with *I. hoferi* as early as 4 months post-hatch, and exhibited a steady yearly increase in prevalence of infection with age, with rates reaching 50% to 70% by the time fish were 3 years old. This is in contrast to Atlantic herring, where all age groups were reported to be equally infected (70%) through winter and spring, but declined to 18% in July when uninfected "fingerlings" migrated inshore and diluted the infected population (Fish 1934). Sinderman & Chenoweth (1993) suggest that following epizootics, the prevalence drops due to mortality of infected individuals, then gradually increases until the next epizootic. A relatively low disease prevalence (average 27%) at the epizootic peak was reported as well as site prevalence rates as low as 1.3%. Since there are no confirmed reports of epizootics caused by *Ichthyophonus* in North Pacific herring, it is not possible to determine whether the high adult prevalence observed in adult Puget Sound herring would decrease following an epizootic. The 29% prevalence report from Prince William Sound immediately following the massive herring losses in 1993 (Marty et al. 1998) similar to that reported at the peak of one epizootic in Atlantic herring (Sinderman 1958) but is much high than that observed in Prince William Sound the previous year. This is the opposite of what Sinderman & Chenoweth (1993) predicted following an epizootic. Multi-year surveys in Prince

Table I-1. Experimental *Ichthyophonus* infection in lab-reared Pacific herring using two strains of the parasite

	mean day to death	mortality (%)	length (mm)	weight (g)	gross lesions (%) skin	gross lesions (%) viscera	histologically positive (%)	positi cultu (%
Controls[a]	--[b]	0	82.2 (±8.6)	5.76 (±1.3)	0	0	0	0
Danish strain	--[b]	0	85.0 (±7.5)	6.10 (±1.1)	20	0	10	10
Prince William Sound strain	36	90	78.8 (±7.0)	4.52 [c] (±1.2)	50	80	80	9

a) N = 10 for all treatment groups
b) no mortalities
c) Significantly different from other treatments (P = 0.01, t-test)

Table I-2. Age class differences in *Ichthyophonus* prevalence in Wild Puget Sound Herring: 1996 - 1997

	length (mm)	weight (g)	skin lesions (%)	visceral lesions (%)	positive cultures (%)
0-year (N=100) Sept. '96	79 (± 4.7)	3.6 (± 0.7)	6	0	6
0-year (N=30) June '97	63 (± 6.6)	2.0 (± 0.6)	0	0	5
Yr-1 juveniles (N=100) Oct. '96	152 (± 8.3)	36 (± 6.5)	5	0	24
Yr ≥ 2 adults (N=100) Feb. '97	185 (± 13.7)	--- (a)	4	5	78

(a) Weights not taken

Table I-3. *Ichthyophonus* survey of wild herring from Puget and Prince William Sound (1996)

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

(a) Eaglet, Zaikof, Hidden and Wells Bay were negative by culture
(b) Minimum - maximum prevalence estimate
ND No Data

William Sound have shown annual changes in prevalence as well as differences in year-class prevalence (Marty et al. 1998). From past and present observations the prevalence rate of *I. hoferi* infection in herring populations is highly variable with no hard evidence available to account for the variability, and although *I. hoferi* infection is often associated with high mortality, no cause-and-effect relationship between *I. hoferi* prevalence and mortality has been established in wild herring. Perhaps the best way to resolve this question would be to track the prevalence rate of individual year-classes from the same geographic area over a period of years, thus focusing on temporal changes in prevalence within a single population.

The health of subclinically infected free-ranging Puget Sound herring did not appear to be different from uninfected fish, but relatively few wild fish exhibited heavy infections characterized by gross (visible) visceral lesions. Conversely, laboratory-reared herring experimentally infected with *Ichthyophonus* lost significantly more weight than their uninfected cohorts after 60 d, but they all exhibited gross visceral lesions which appear to be indicative of terminal infections. It is possible that heavily infected free-ranging herring die without being detected unless a significant epizootic occurs that kills large numbers of fish. Unlike Atlantic herring (Sinderman & Chenoweth 1993), 0-year Puget Sound herring are infected as early as four months post-hatch, prior to their inshore migration. Pelagic "herring balls" consisting of 0-year herring can be found from June through October in Puget Sound, and all groups examined between 1995 and 1997 were infected with *Ichthyophonus*, albeit at lower levels than adult fish.

Development in culture: A predictable sequence of development similar to that observed by Okamoto et al. (1985) occurred when fish tissue infected with *Ichthyophonus* was placed into L-15 culture medium. Initially, large resting spores began to germinate and produce hyphae. Once hyphae developed, cytoplasm flowed into the tips where "microspores" were produced and released into the culture medium, and ultimately developed into more large "resting" spores. This cycle of development occurred very rapidly following culture of infected tissue, but this cycle is rarely repeated under the culture conditions used here. This suggests that this is a normal process following the death of infected fish (McVicar & McLay 1985) and that manipulation of the culture medium is necessary to initiate a new cycle of development (Spanggaard et al. 1994). The small endospores released from hyphal tips may play a role in transmission of the organism following the death of infected host, although Okamoto et al (1987) believe that the large multinucleated spores are the infectious stage.

In vitro culture as a diagnostic tool: The most sensitive method for identifying subclinically infected individuals is in vitro culture. Based on the differences in prevalence detected by gross visual examination and light microscopy, light microscopy appears to be more sensitive than visual examination. The use of in vitro cultivation for determining prevalence rate is supported by McVicar (1990), who noted that for infectious diseases, prevalence levels are relatively easy to determine from subsamples, ideally by direct isolation of the causative agent. Furthermore, the incidence (eg. rate of occurrence of new cases) could be determined from frequent samples of prevalence levels in juvenile herring over a period of several months to several years, as noted in the current study. The limitation of in vitro culture is that it only gives prevalence rate but not pathogenic status nor long-term prognosis.

Subclinically infected fish may serve as reservoirs of infection, developing patent infections only under stress conditions that compromise their immune system (Rice et al. 1996, Tort et al. 1996). Using in vitro culture techniques, large-scale monitoring could include different age classes, geographic areas and temporal changes in prevalence of the organism. This kind of temporal and geographic data would be beneficial to managers in their decisions regarding the effects of this organism on health of fish populations.

Transmission to sculpins: The coast range sculpin proved to be a useful laboratory animal for studying *I. hoferi* transmission in the laboratory, being susceptible to both IP injection and oral exposure to infected fish tissues. Although sculpins were successfully infected with a single feeding of infected tissues,

previous studies concluded that transmission only occurred in herring (Sinderman and Scattergood 1954, Sinderman & Chenoweth 1993) and trout (McVicar & McLay 1985) when massive numbers of spores are fed on successive days.

The natural route of infection for herring has not been conclusively demonstrated, but there is circumstantial evidence for both direct transmission and transmission via an intermediate host (Sinderman & Scattergood 1954, McVicar & McLay 1985). It seems probable that herring become infected directly by free fungal spores, or eating prey items carrying an infective stage of the parasite. If an intermediate host is necessary for *Ichthyophonus* transmission to herring, epizootics might result from changes in prey selection or abundance, where more heavily infected prey become available to the herring. The wide host range and ease of transmission via contaminated food suggests that carnivory and/or scavenging of infected dead fish may play a significant role in the life cycle of *I. hoferi* under natural conditions.

Table I-3 summarizes the *Ichthyophonus* data from all age classes of wild herring from both Puget Sound and Prince William Sound. The prevalence of *Ichthyophonus* appeared to increase with age in Puget Sound fish, with a low of 6% in 0-year fish to a high of 52% in 3+ spawners. PWS 1+ herring showed a minimum prevalence of 1.7% and a high of 23.1%. Because the PWS fish were examined in pools of 5 fish per culture, these are minimum values.

Wild herring were also collected by Evelyn Brown and Malcolm McEwen as a part of the SEA juvenile herring study. Tissues from the PWS fish were removed on ship-board and placed into culture tubes containing MEM-10 with antibiotics. Tissues from 5 fish were placed into each tube, then they were shipped back to the University of Washington for incubation and evaluation. No data on external or internal lesions were taken from the PWS fish, but this project is proposed for April 1997.

Non herring studies:

Rainbow trout were resistant to infection by IP injection of up to 10,000 spores originally isolated from PWS herring. None of the 12 fish died and no *Ichthyophonus* was cultured from their hearts, livers or spleens. The one English sole died within 30 days of exposure and cultured positive for *Ichthyophonus*. Sixty chinook salmon (captured at the mouth of the Yukon River were positive by gross examination of tissues (4%) as well as culture of tissues (18%). Additional studies on upriver changes in disease status and infection rate are currently underway. It is presently assumed that these chinook become infected while at sea.

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

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

Final 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 includes research from projects 95320, 96162, 97162 and 98162. This project was 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 were 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 acutely lethal to juvenile herring, but not to adult herring, although in both it initiated a measurable, classical and transient biochemical 'stress' response. Differences between adults and juveniles was also evident in the effects of OWD on herring immune systems which included alterations in the proportions of different white blood cells, lysozyme activity, but was most consistently evident in alterations in macrophage function. OWD affected disease resistance in herring, however, it appears that the effects of OWD are pathogen-specific; increasing resistance to some pathogens while increasing susceptibility to others. Juvenile herring were susceptible to VHSV, which caused a biochemical stress response, immune system effects and mortality, however, adult herring were immune to the virus under all experimental conditions. *Ichthyophonus hoferi* was found in both wild juvenile and adult herring, and did not affect herring in any of the measured fitness categories. Exposure of adult herring to OWD did not affect swimming ability as it did in juvenile fish, but did result in increases in mortality of fish which were exercised. Exposure of herring to OWD significantly reduced the ability of herring to recover biochemically after swimming which may contribute to post-exercise mortality. 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 which may have ramifications for fish health under certain circumstances such as spawning. The results from this study indicate that the recovery of the effects from stressors are slowest in the immune system, followed by swimming performance and then biochemistry. This study also indicates that the age of fish, disease history and abiotic factors are important in the relationship between contaminants and disease.

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, viral hemorrhagic septicemia virus and *Ichthyophonus hoferi* on juvenile and adult Pacific herring biochemistry, immunology, and swimming performance, and reproduction. *Format-* Data regarding experimental data are stored in Microsoft Excel and text files in MicrosoftWord 6.0. *Custodian-* Contact Dr. Chris Kennedy at the Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada, V5A 1S6.)Phone: (604) 291-5640, fax: (604) 291-3496 or email at: ckennedy@sfu.ca). *Availability-* Copies of text in annual reports are available for the cost of duplication. Reprints of any manuscripts will also be available when published.

Citation:

Kennedy, C.J., A.P. Farrell and S. Sanders. 1999. Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound. Section III: Survival, performance and reproduction in the Pacific herring, *Clupea harengus pallasi*: effects of environmental contamination, viral hemorrhagic septicemia virus and *Ichthyophonus hoferi*. Final Report (Restoration Projects 95320, 96162, 97162, 98162), Simon Fraser University, Burnaby, British Columbia, Canada.

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Executive Summary

It has been hypothesized that the pathogens VHSV and ITP, or oil exposure through the *Exxon Valdez* oil spill, or some combination of these stressors may be responsible for the declines in herring populations in PWS. 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 was to document cause-effect relationships for oil, VHSV and ITP on various aspects of herring fitness. The categories of fitness chosen for this study include herring survival, blood biochemistry, performance in terms of the immune system and swimming, and reproduction.

These studies used both laboratory-raised specific-pathogen-free herring raised from eggs collected from PWS and wild caught juvenile and adult herring. Small but significant differences existed between SPF and wild caught fish in some aspects of hematology. The disease state of wild fish was also determined. Wild juveniles and adults were negative in all tests for VHSV. Both wild adult and juvenile herring were found to be positive for the presence of ITP.

In order to determine the effects of oil on herring fitness, an oil-water dispersion (OWD) of North Slope crude oil was used in both shorterm and longterm exposures. Oil was not acutely lethal to adult herring at the concentrations used (50 to 300 ppb), although it resulted in significant mortalities in juvenile fish in a dose-dependent fashion at concentrations as low as 100 ppb. Thus, the significant differences in the response of herring to oil are age-dependent.

Experiments to determine the effects of an oil-water dispersion (OWD) of North Slope crude oil on herring biochemistry were performed with both juvenile and adult herring. A classical 'stress' response was seen in both juvenile and adult fish exposed to OWD for 24 h which included a hypersecretion of corticosteroids, hyperlacticemia and a hyperglycemia. By 96 h of exposure to OWD, all of these parameters measured had returned to preexposure values and remained at baseline levels through 22 days of exposure indicating that the stress response was transient. The magnitude of the stress response was much less in adult fish, highlighting age again as an important factor in stressor effects in herring. To begin an assessment of abiotic factors which may modify the effects of oil on herring health, experiments with varying densities of fish indicated that baseline biochemical stress parameters were elevated at both high fish densities and in the lowest densities.

Juvenile and adult herring were exposed to varying concentrations of OWD to determine effects on swimming performance. In these experiments, significant mortalities occurred when juvenile herring were swum following oil exposure. Following a 96 h exposure to OWD, swimming ability was reduced in the two highest OWD concentrations. Due to high mortalities in fish which were swum in these experiments, the effects of OWD exposure on exercise recovery were determined. It was shown that OWD affected the recovery of aspects of herring biochemistry which are typically altered during exercise including: lactate clearance and ion disturbances. The swimming ability of adults was not affected at any OWD concentration, however, there was a significant increase in herring mortalities following strenuous exercise in OWD-exposed fish which may have been due to a reduced biochemical recovery similar to juvenile fish.

The effects of oil exposure on the immune systems of both adult and juvenile herring was examined by assessing specific components of the immune system and disease resistance. Effects of oil on the immune system occurred in herring only under subchronic

(i.e. > 7 day) exposures. Significant alterations in white blood cell populations, phagocytic and respiratory burst activity of macrophages, and lysozyme activity occurred in adults. OWD appeared to affect juvenile immune systems to a lesser extent: significant alterations were only seen in macrophage phagocytosis. In both cases, immunological effects persisted for longer than 8 weeks post exposure. Juvenile fish were subjected to a disease challenge with the marine bacteria *Vibrio anguillarum* following a chronic exposure to OWD. Fish exposed to the highest concentration of OWD, were the least susceptible to *Vibrio* and showed the lowest mortalities. Experiments in which juvenile herring were exposed to either VHSV or ITP indicated that infection with either of these pathogens can alter herring hematology and the immune system including hematocrit, leucocrit and differential white blood cell counts, however, results were not consistent.

Adults exposed to oil were subsequently challenged with VHSV did not break with the disease. It appears that adult herring may be immune to VHSV under normal conditions. Juvenile herring exposed to OWD showed significant oil-related mortality effects when further challenged by VHSV: both oil and VHSV alone were acutely lethal to juveniles, with significant increases in mortality when both stressors were applied simultaneously. Infection of adult and juvenile herring to ITP was not consistent or affected by exposure of fish to OWD.

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 herring biochemistry, immunology and swimming performance were noted, although the time frames of disturbance of these systems is both time and age-dependent. Biochemical alterations were the most transient, followed by swimming effects and then immunological alterations. 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. Also, 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 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, predicted and subsequently used in fisheries management. This project was also successful in developing and identifying several assays and techniques which can be used in the monitoring of fish health in other situations.

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 had 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 had 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 addition to lethality, we used ecologically relevant stress responses as endpoints to determine cause-effect relationships between the three stressors (see Figure 1) and herring fitness. Conventional methods of evaluating stress to aquatic organisms often only examine one stress variable or a single level of organization and have been criticized as 'lacking ecological realism' (Cairns, 1981; Schreck, 1981; Adams, 1990). The extrapolation of laboratory bioassays to the natural environment is difficult. It is therefore imperative to use ecologically relevant endpoints in laboratory-based bioassays. The review by Adams (1990) suggests a bioindicator approach, involving measurements of a suite of selected stress responses at several levels of biological organization ranging from the subcellular and biochemical levels to those at the ecosystem level. We used such an approach in our studies. In the long-term we examined four major ecologically relevant classes; 1) immunological fitness, 2) biochemical fitness (blood chemistry), 3) physiological fitness (swimming performance) and, 4) reproductive fitness (see Figure 2).

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 conditions as may occur during spawning? 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 would 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 was 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 was also unclear if the fish that survived exposure to one or more of these stressors are 'healthy' or were surviving at a reduced fitness level. In the absence of such information, sound management of the herring stock in PWS would be a difficult task. The laboratory component of this project addressed these important information needs. The objectives of this study will contribute directly towards discovering why herring populations are recovering at their present rate in PWS.

The general objectives of this study were:

1) To determine cause-effect and interactive relationships for oil exposure, VHSV and ITP on herring survival, performance and reproduction. Even though exposure to one or more of these three stressors may not cause direct mortality in herring, overall fitness of the fish can be reduced, resulting in delayed mortality or lowered reproductive success.

2) To determine the influence of important abiotic factors, such as fish density, on the above cause-effect and interactive relationships for oil, VHSV and ITP on herring. Information regarding the modulatory effects of physical factors on stressor effects is especially important for herring management practices.

3) To determine the baseline levels of fitness indicators for Pacific herring from PWS. Information regarding 'normal' levels of fitness measures will aid in biomonitoring programs of the herring population when recovered.

The overall hypothesis tested in this study was:

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, or 4) reproduction.

The specific objectives of this study were:

1) To supply analytical support for Section I (the field component: Dr. G. Marty) of this research project (1995-1998).

2) To develop and build an exposure system for the delivery of an oil-water dispersion or water soluble fraction to holding aquaria, and to design and build a swim tunnel apparatus for examining the swimming performance of herring.

3) To develop and modify analytical techniques for measuring biochemical and immunological indicators in Pacific herring.

4) To determine the effects of oil exposure, VHSV or ITP on the survival of juvenile and adult Pacific herring.

5) To determine the effects of oil exposure, VHSV or ITP on the biochemistry of juvenile and adult Pacific herring.

6) To determine the effects of oil exposure, VHSV or ITP on various components of the immune system in juvenile and adult herring.

7) To determine the effects of oil exposure on disease resistance in juvenile and adult herring.

8) To determine the effects of oil exposure on the swimming performance of juvenile and adult Pacific herring.

9) Describe the effects of density on Pacific herring biochemistry and immunology.

10) To determine the effects of oil, VHSV and ITP on herring egg survival.

Methods

1) Analytical support for field studies

Blood smears from Pacific herring sampled in 1995-98 in Prince William Sound and Sitka Sound were received from Dr. G. Marty of the University of California at Davis. Smears were stained and analyzed for differential white blood cell counts. 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 and 1998, analytical support for Section I of this project was provided through plasma chemistry analysis. Plasma from Pacific herring sampled in PWS and Sitka Sound were received from Dr. G. Marty of the University of California, Davis.

The data from Freiberg and Farver (1995) indicated that measurements of creatine phosphokinase (CPK) in various tissues is highly correlated with fish lesions. In fish, CPK

levels are elevated in ITP-infected herring indicating cellular damage in infected tissue. In rainbow trout and mammals it is possible to measure CPK isoforms to identify the specific tissues damaged (CPK1, CPK2, CPK3: brain, cardiac and skeletal). Since we predict that cardiac tissue damage may have a proximate linkage to herring survival, we attempted to measured these isoforms electrophoretically in plasma samples in herring sampled from PWS collected as above. It was determined that herring plasma is not amenable to the use of the same procedure developed for trout.

2) Fish

Adult Pacific herring were caught in Barkley Sound, Vancouver Island, by purse seine by the Department of Fisheries and Oceans, Canada and donated for our experiments. Juvenile young of the year were caught in Barkley Sound by beach seine. 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.

3) Chemical and stressor exposure

In this study, the effects of either oil, VHSV or ITP or combinations on herring were examined, thus the experimental matrix (Figure 1) has seven exposure cells and a control cell. Each exposure cell utilized approximately 100 fish. The 3X3 design takes into account the three stressors (oil, VHSV and ITP) alone or in combination. The exposures are: 1) VHSV only, 2) VHSV and ITP, 3) VHSV and oil, 4) ITP only, 5) ITP and oil, 6) oil only, 7) oil, VHSV, and ITP and 8) control fish. This exposure scenario will allow the determination of the relevant parameter or combination which affects herring fitness. These experiments were performed in conjunction with the experiments performed by Dr. Kocan. Dr. Kocan's group examined disease parameters in these fish and our group quantified herring fitness quality. The studies began with cells 1, 4 and 6 of Figure 1, which examine the effects of oil only, VHSV only and ITP only. Cell 3 was also examined, however, cells with ITP (2,4, and 5) were only performed with juvenile fish, as adult fish were infected heavily (24%). However, juveniles did not infect easily or with a short time period between exposure and disease.

Figure 1. Various exposure scenarios and parameters. Superimposed upon this matrix are various doses of the stressors and different stocking densities.

	VHSV	ITP	OIL
VHSV	1. VHSV only	2. VHSV+ITP	3. VHSV+OIL
ITP		4. ITP only	5. ITP+OIL
OIL			6. OIL only

VHSV+ITP+OIL

Controls

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 was performed using the dosing apparatus or oil generators developed by Carls et al. (unpublished method and analysis data) and modified for use with herring. 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 as an oil-water dispersion (OWD). 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. Analysis includes both total fluorescence analysis coupled with gas chromatography and FID detection. 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 exposure.

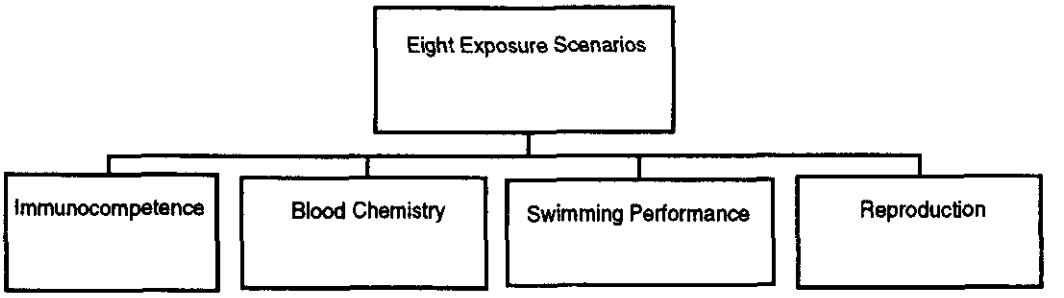
Challenges with VHSV were done using a virus stock obtained from Dr. R. Kocan of the University of Washington, WA, USA. The original stock contained 4.0×10^8 pfu/ml upon thawing. Disease challenges were performed following the protocol outlined in Kocan (1997) and Section II of this report. Water in 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.

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.

4) Fitness measurements

Figure 2 illustrates the generic set of fitness tests and measurements that were applied following exposure of Pacific herring to a given stress parameter. For each exposure cell, endpoints may include: 1) immunological fitness, 2) biochemical fitness, 3) physiological fitness, and 4) reproductive fitness. All exposures were performed in triplicate.

Figure 2. Generic fitness tests to be examined in each of the exposure scenarios shown in Figure 1.



Details of tests and their rationales:

Biochemistry

A wide variety of molecular and biochemical responses to adverse environmental stimuli have been described for teleosts (Thomas 1990). Biochemical alterations can be used as sensitive indicators of stress and show a more rapid response to environmental stressors than most other biological measurements. As well, measurements of molecular and biochemical indicators can often provide specific information on the nature of the stressor and its mechanism of action. This information may be used in subsequent field biomonitoring programs to determine the status of herring once the population has recovered.

Biochemical parameters assayed in these experiments integrated with field studies (Section I of this annual report) included: plasma glucose, lactate, electrolytes including chloride and sodium and were analyzed according to standard techniques outlined 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, and 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).

Immunology

Fish combat pathogenic microorganisms using an immune system that is comparable to other vertebrates. There is little direct evidence to link the contamination event of the oil spill with the increased occurrence of VHSV or ITP in herring in PWS, and yet it has been shown that exposure to contaminants such as hydrocarbons can affect the immune system of fish and compromise their ability to resist disease (Adams, 1990). Moreover, results from preliminary work (unpublished) indicate that exposure of rainbow trout and

herring to the hydrocarbon pristane alters several components of fish immune systems such as hematocrit leucocrit and white blood cell counts.

In view of this preliminary work, immunocompetence in fish was assessed following stressor exposure by measuring several immunological indicators such as; hematocrit, leucocrit, differential white blood cell counts, macrophage phagocyte activity and lysozyme concentration. Since it has been suggested by Meyers et al. (1993) that the progressive ulcerating skin lesions which occur in herring during an VHSV epizootic may act as portals of entry for secondary microbial infections, immunocompetence will also be measured by a disease challenge with the marine bacterium *Vibrio anguillarum* and VHSV to determine the potential for a secondary infection. Methods for these measurements are described in Johansen et al. (1994) and Stolen et al. (1992).

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 respiratory burst activities. These assays were modified for use with herring in this project and were 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 was ideal for this assay).

The phagocytosis assay was modified and was carried out as follows: 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 28°C 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. 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.

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. VHSV 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.

Physiology: swimming performance

Many stress-induced physiological events alter the capacity of fish to perform various physiological functions. Performance tests can be viewed as a form of bioassay that measures the capacity of fish to carry out essential life processes such as the ability to swim. These tests are particularly powerful tools for assessing stress as they incorporate several levels of biological organization and are therefore integrative in nature (Schreck 1990). In this section, we examined the effects of the stressors on the swimming

performance of herring. Ultimately swimming performance affects the ability of herring to forage and avoid predation.

One of the signs of VHSV infection in fish is lethargy and listlessness and frenzied swimming in circles at the terminal stages of disease. It is obvious that a reduced swimming performance may directly affect survival by increasing the possibility of predation and reducing the ability to secure food. Estimates of maximum aerobic swimming ability have provided a sensitive index to general health and stress in fish and an index of the ability to avoid predation (Adams 1990), since many physiological systems must work maximally in a coordinated fashion.

The assessment of swimming performance seems particularly relevant for the present study. ITP infection is high in both skeletal muscle and cardiac muscle of herring sampled from PWS (Freiberg and Farver, 1995), both of which are critical to swimming. It is likely that the ITP infection causes significant muscle tissue damage since high serum CPK levels correlate with ITP infection (Freiberg and Farver, 1995). We predicted that cardiac ITP infection and damage will be particularly damaging to swimming performance and survival.

Maximum aerobic swimming performance was examined by determining the critical swimming speed of fish following exposure. Methods of determining swimming performance are described in Nikl and Farrell et al. (1993). The apparatus for determining the swimming ability of herring was developed for this project.

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

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 separate experiments, the effects of oil 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+] as described previously.

Reproduction

Any stressor, including disease and contamination, that interferes with the process of reproduction at the individual or population level is likely to affect the survival of that species in a habitat. Reproductive development is a continuous process and may be subject to the effects of environmental perturbations at several stages of an organisms life cycle. Through this development there are several parameters which may be useful indicators of reproductive 'fitness' in fish. In this study eggs of mature herring were exposed to oil, VHSV and ITP and examined for the percent of eggs surviving to hatch. Rearing of larvae to fry stage was not possible at the Bamfield Marine Station, as specific water quality

criteria were needed to raise Pacific herring as detailed in Section II of this report (Dr. Kocan).

Statistical analysis

All values are reported as means ± SE. 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.

Results

Objective 1: To supply analytical support for Section I (the field component: Dr. G. Marty) of this research project (1995-1998).

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 final report for blood samples from Sitka Sound and Prince William Sound. Plasma chemistry results are also given in Section I of this report.

Objective 2: To develop and build an exposure system for the delivery of an oil-water dispersion or water soluble fraction to holding aquaria, and to design and build a swim tunnel apparatus for examining the swimming performance of herring.

The dosing apparatus (Figure 3) developed were termed 'oil generators' and were modified from those developed by Carls et al. (unpublished method and analysis data). As described earlier in Methods, 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.

The swim chamber apparatus (Figure 4) developed 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.

Objective 3: To develop and modify analytical techniques for measuring biochemical and immunological parameters in Pacific herring.

Pacific herring, as well as other marine fish, pose special difficulties in performing several routine teleost hematological and immunological assays. The modification of several published assays was successfully applied to Pacific herring and are outlined in the

methods section in detail. A 'suite' of hematological and immunological methods was developed and may be used in future determinations of herring 'health' in biomonitoring programs.

Objective 4: To determine the effects of oil exposure, VHSV or ITP on the survival of juvenile and adult Pacific herring.

Experiment 1: Effects of oil on juvenile herring survival

Juvenile herring were exposed for 96 h to control, low, medium and high concentrations (for concentrations, see methods) of an OWD as described above for 96h. Mortality of herring was monitored for up to 96h. OWD was found to be acutely toxic to juvenile herring in a dose-dependent manner (Figure 5).

Experiment 2: Effects of oil on adult herring survival

Adult herring were exposed to control, low, medium and high concentrations of an OWD as described above for up to 6 days. Mortality of herring was monitored for the same time period. OWD was not found to be acutely toxic to adult herring at any concentration.

Experiment 3: Effects of VHSV and ITP on herring survival

For these results see Objective 7

Objective 5: To determine the effects of oil exposure, VHSV or ITP on the biochemistry of juvenile and adult Pacific herring.

Experiment 1: Effects of oil on adult herring biochemistry

Adult Pacific herring were exposed to control, low, medium and high concentrations of an OWD as described above for 6 days. Fish were sacrificed and sampled via the caudal vasculature. Fish were analyzed for selected biochemical parameters as described above. Biochemical measures are given in Table 1. No significant differences were seen in the biochemical parameters between oiled and control fish.

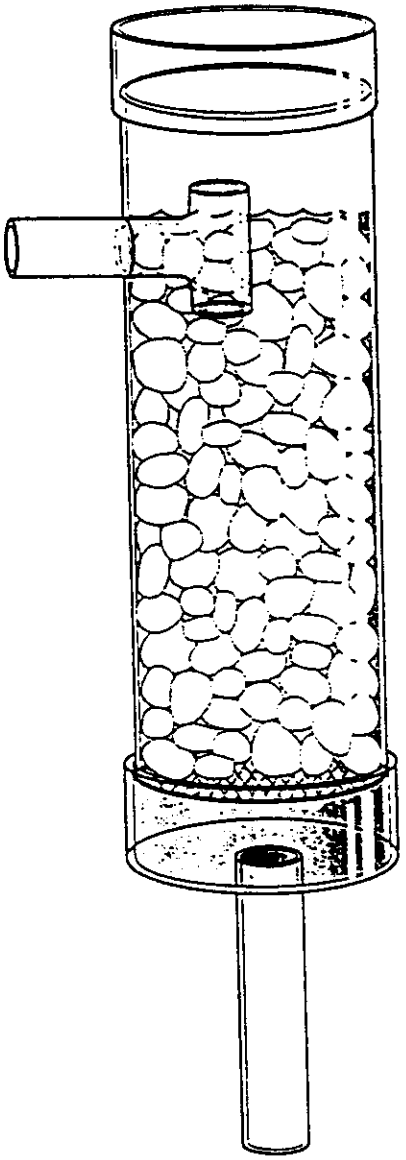


Figure 3. Schematic diagram of an oil generator designed after Carls et al. (unpublished) for dosing herring with an oil water dispersion (OWD) of North Slope crude oil in single and multiple stressor experiments.

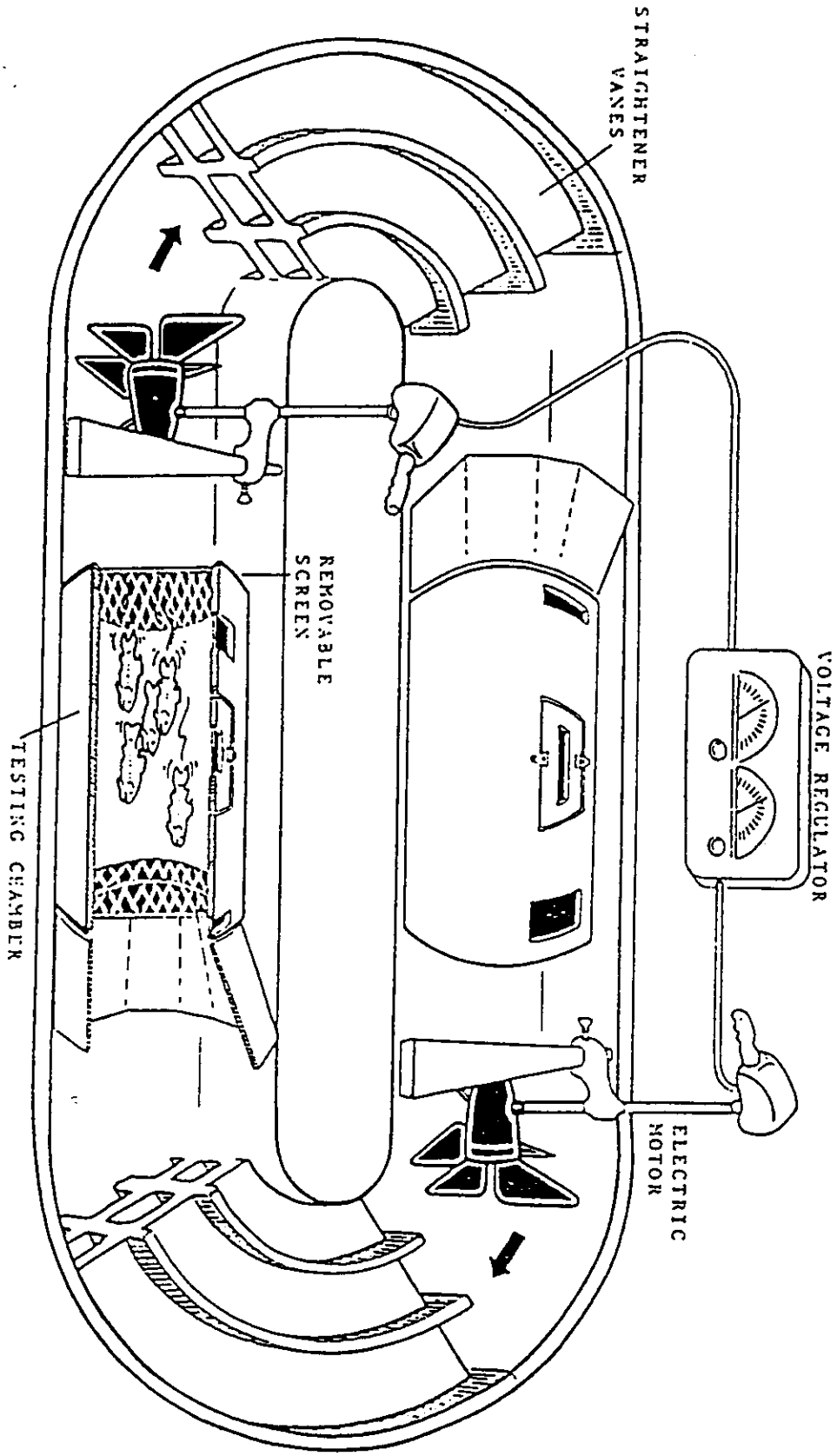


Figure 4. Schematic diagram of the swimming raceway modified from Nikl and Farrell (1993) used in physiological fitness testing of herring following exposure to single or multiple stressors.

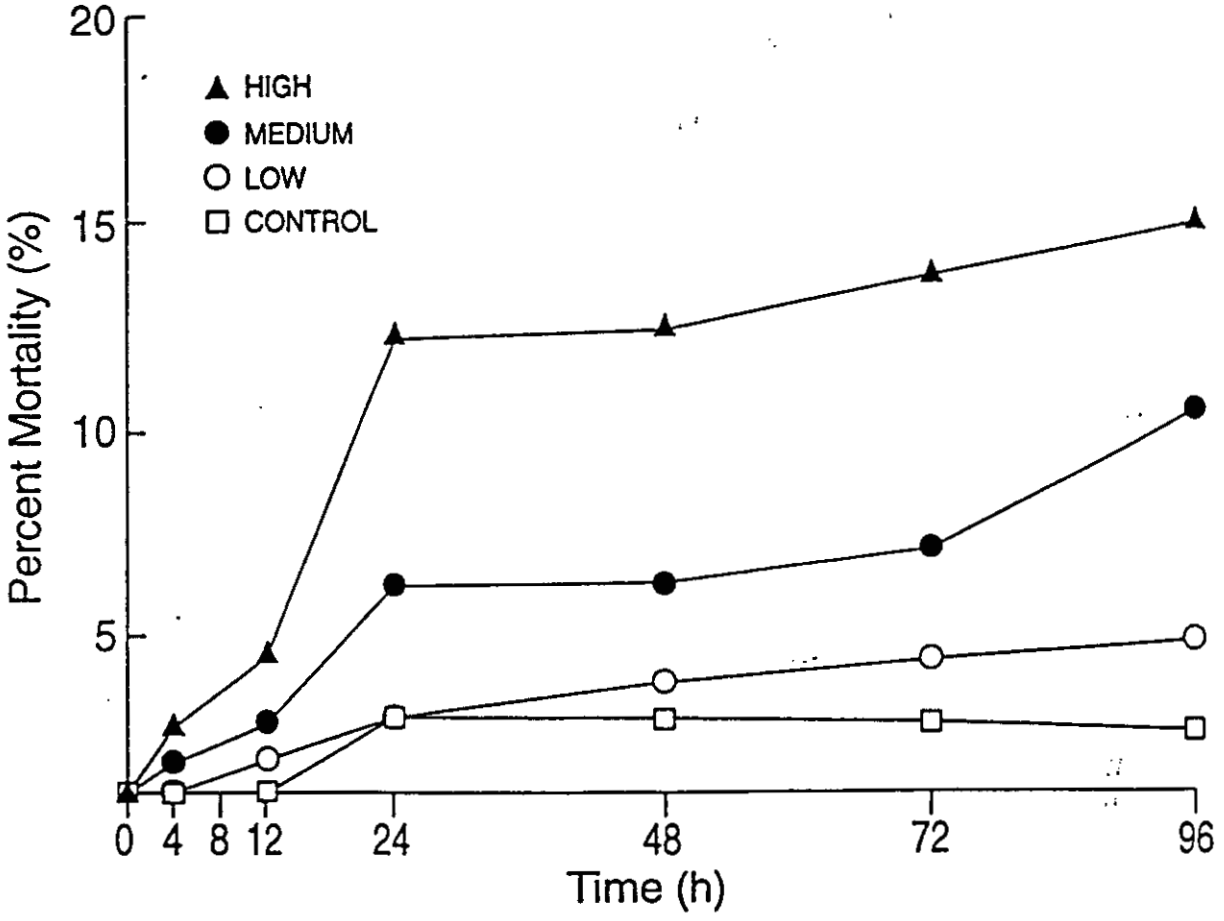


Figure 5. Effects of varying concentrations of an OWD on the survival of juvenile herring for up to 96 h of exposure. Values are means of triplicate experiments.

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

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

Due to the lack of any stress response at 6 days, this experiment was repeated from 24 h to 28 days. Methods are as above. The biochemical measures are shown in Table 2. Significant differences were seen at 24 hours in plasma cortisol, lactate, glucose and hematocrit at the highest dose of oil.

Experiment 2: Effects of oil on juvenile herring biochemistry

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

Juvenile herring exposed to OWD for 96 h showed different responses than adults in that a more pronounced stress response occurred. Figure 6 shows the time course of changes in plasma cortisol, lactate, glucose and hematocrit. Significant initial increases occur in fish exposed to OWD, however, values appear to return to pre exposure level by 96 h, even with continued exposure.

Table 2. The effects of the highest dose of OWD on biochemical parameters in adult herring. Significant differences ($p<0.05$) are shown by an *.

Parameter	0 hours		24 hours		72 hours		28 days	
	Control	OWD	Control	OWD	Control	OWD	Control	OWD
Cortisol (ug/dl)	3.9±0.7	3.7±1.0	4.1±1.3	15.2±2.9*	2.6±0.7	4.1±0.9	4.0±0.6	4.9±1.0
Lactate (mg/dl)	45.3±6.9	48.1±9.4	46.2±7.7	75.6±8.5*	48.7±6.0	41.3±4.8	39.6±6.1	48.3±4.9
Glucose (mg/dl)	110.4±13.5	112.3±8.4	99.2±9.9	169.8±11.5*	100.9±8.6	114.3±10.0	100.3±15.3	102.6±12.1
Hematocrit	44.7±6.1	36.3±7.2	32.2±6.6	61.3±18.5*	37.6±6.5	37.7±8.1	33.1±9.2	35.2±5.3
Leucocrit	0.64±0.17	0.76±0.23	0.66±0.18	1.12±0.42	0.72±0.21	0.69±0.21	0.83±0.17	082±0.31

Table 3. The effects of VHSV exposure on the biochemical parameters in juvenile herring. Significant differences ($p<0.05$) are shown by an *.

Parameter	0 hours		24 hours		21 days		12 weeks	
	Control	VHSV	Control	VHSV	Control	VHSV	Control	VHSV
Cortisol (ug/dl)	3.3±0.6	4.2±1.4	12.7±3.3	17.4±53	4.3±1.4	8.9±2.1*	3.5±0.9	3.0±1.6
Lactate (mg/dl)	45.6±10.8	50.2±9.5	78.5±12.3	81.2±10.1	47.0±8.7	67.2±10.3*	42.8±7.0	50.1±12.3
Glucose (mg/dl)	112.2±12.8	108.8±16.6	156.7±14.2	145.3±23.2	99.0±10.7	114.6±12.4	121.5±9.8	111.8±13.2
Hematocrit	31.0±4.6	35.6±5.4	34.2±6.1	29.4±3.9	26.7±4.7	20.1±4.5*	29.0±31	21.8±3.3*
Leucocrit	0.82±0.51	0.69±0.21	0.69±0.18	0.61±0.26	0.33±0.19	1.21±0.34*	0.57±0.23	0.78±0.17

Table 4. The effects of ITP exposure on the biochemical and immunological parameters in juvenile herring. Significant differences ($p<0.05$) are shown by an *.

Parameter	0 hours		24 hours		21 days		9 weeks	
	Control	ITP	Control	ITP	Control	ITP	Control	ITP
Cortisol (ug/dl)	2.1±0.5	3.7±1.1	2.5±1.1	2.7±0.6	3.0±0.9	3.5±1.0	3.2±0.5	2.7±0.6
Lactate (mg/dl)	52.4±12.3	41.3±10.3	56.7±9.9	44.7±11.9	48.1±7.5	43.9±6.3	53.1±11.6	45.6±8.8
Glucose (mg/dl)	107.4±12.5	121.3±18.7	117.4±12.2	110.4±9.0	123.5±12.1	102.4±14.8	107.7±5.8	100.3±13.2
Hematocrit	27.2±5.2	29.5±4.8	31.2±6.2	26.7±5.6	17.1±3.1	22.2±3.0	24.5±4.9	36.1±2.7
Leucocrit	0.76±0.21	0.89±0.32	0.71±0.26	0.66±0.15	0.29±0.10	0.51±0.20	0.0.44±0.10	0.44±0.11

Experiment 3: Effects of VHSV on juvenile herring biochemistry

Juvenile herring were exposed to VHSV in a bath challenge as described previously. Blood from survivors was sampled for up to 12 weeks and analyzed for biochemical parameters which have been shown to be good indicators of 'stress' in fish. The results of this experiment are shown in Table 3. A significant stress response was seen in both control and VHSV exposed fish. This was due to the method of VHSV challenge which was stressful to herring. The most significant effect of VHSV was a reduced hematocrit which lasted up to 12 weeks in survivors, and a prolonged (up to 3 weeks) biochemical stress response. Adult fish were not used in these experiments, as later studies indicated that they were immune to VHSV under conditions in the laboratory.

Experiment 4: Effects of ITP on juvenile herring biochemistry

Juvenile herring were exposed to ITP as described previously. Blood was sampled and analyzed for the same biochemical parameters up to 9 weeks. No mortalities or signs of infection occurred in these fish. The results of this experiment are shown in Table 4. No significant stress response was seen in either control or ITP exposed fish. Adult fish were not used in these experiments, as earlier studies indicated that a high percentage of adults were already infected with this pathogen.

Objective 6: To determine the effects of oil exposure, VHSV or ITP on various components of the immune system in juvenile and adult herring.

Experiment 1: The effects of oil on juvenile herring immunology

Juvenile herring were exposed for 22 days to control, low, medium and high concentrations of an OWD as described previously. Fish were sacrificed and blood sampled via the caudal vasculature and head kidney dissected. Various immunological parameters were performed as previously described. No significant differences were seen at 22 days in hematocrit, leucocrit (Table 5) or differential white blood cell counts (Table 6). Macrophage phagocytosis was significantly depressed in all concentrations of OWD.

Table 5. Immunological parameters in juvenile herring exposed for 21 days to varying concentrations of OWD. Values means±SE of ten fish. Significant differences are denoted by * at $p<0.05$.

Parameter	Control	Low OWD	Medium OWD	High OWD
Hematocrit	26.5±2.7	27.2±2.3	26.2±2.5	25.2±2.6
Leucocrit	1.37±0.31	1.43±0.42	1.46±0.40	1.62±0.61
Macrophage (% phagocytosis)	76.3±4.7	58.4±5.6*	50.0±1.6*	53.4±2.7*

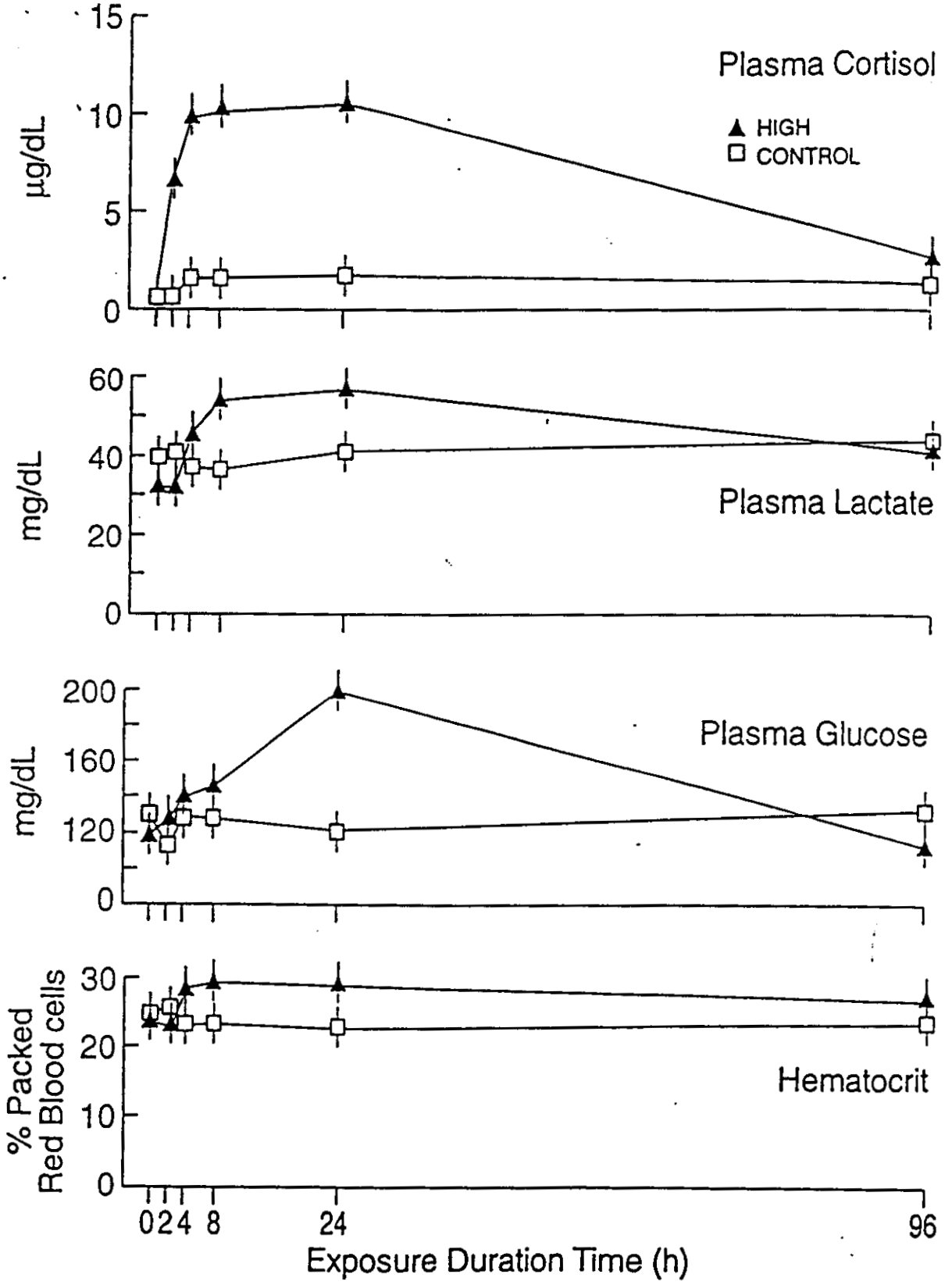


Figure 6. Effects of varying concentrations of an OWD on the plasma cortisol, lactate, glucose and hematocrit in juvenile herring before and during 2, 4, 8, 24 and 96 h exposures. Values are means±SE of three sets of pooled blood (3 fish each set). Values at 96 h are not significantly different from those at the start of the experiment at $p<0.05$.

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

OWD dose	Thrombocytes (%)	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)
Control	17.4±3.6	66.±5.1	16.0±4.4	0.6±0.3
Low	12.1±2.1	69.4±4.9	18.4±4.0	0.1±0.1
Medium	16.7±2.5	60.1±5.4	23.2±4.6	0.1±0.2
High	19.7±2.5	60.1±4.5	19.6±4.0	0.6±0.2

Experiment 2: The effects of oil on adult herring immunology

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 (Table 7). 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 was depressed at 21 days exposure to OWD, but was elevated 6 weeks following the transfer in previously OWD exposed fish.

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 8).

Experiment 3: The effects of oil and VHSV on adult 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. When sampled, measurements were made of hematocrit, leucocrit, differential white blood cell counts, lysozyme activity, macrophage phagocytosis and tissue viral loads. 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, leucocrit, macrophage phagocytosis (Figure 7),or differential white blood cell counts, 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.

Table 7. Immunological parameters in adult herring exposed to the highest concentration of OWD for 21 days followed by 6 weeks in uncontaminated water. Values means±SE of twelve fish. Significant differences are denoted by * at $p<0.05$.

Parameter	0 days		7 days		21 days		63 days	
	Control	OWD	Control	OWD	Control	OWD	Control	OWD
Hematocrit	41.3±.6.0	40.2±4.5	44.3±5.1	42.7±4.8	37.1±6.0	42.5±4.0	38.3±6.9	37.5±3.7
Leucocrit	1.17±0.26	1.10±0.21	1.11±0.31	1.06±0.15	1.67±0.36	1.11±0.35	1.70±0.27	1.0±0.67
lysozyme (ug/ml)	1.10±0.25	1.12±0.17	1.20±0.26	1.67±0.34	0.87±0.26	0.28±0.11*	0.21±0.16	0.56±0.26
Phagocytosis (yeastcells/mac)	0.20±0.06	0.21±0.06	0.23±0.11	0.21±0.09	0.34±0.06	0.18±0.04*	0.20±0.06	0.29±0.10
Respir. burst (abs/100,000 cells)	0.20±0.06	0.21±0.07	0.23±0.10	0.20±0.07	0.36±0.08	0.19±0.05*	0.20±0.03	0.27±0.03*

Table 8. Differential white blood cell counts in adult herring exposed to the highest concentration of OWD for 21 days followed by 6 weeks in uncontaminated water. Values means±SE of twelve fish. Significant differences are denoted by * at $p<0.05$.

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

Experiment 4: The effects of VHSV on juvenile herring immunology

Juvenile herring were exposed to VHSV as described in methods. Fish were sampled before the challenge (0 hours) and 7 and 14 days post VHSV challenge and assayed for hematocrit, leucocrit, macrophage phagocytosis and lysozyme. The results are shown in Table 10. Reductions in hematocrit by VHSV are not significant, however, lysozyme levels were elevated in VHSV exposed fish.

Table 10. The effects of VHSV on aspects of juvenile herring immunology. Significant effects are denoted by an • at $p<0.05$.

Parameter	0 hours		7 days		14 days	
	Control	VHSV	Control	VHSV	Control	VHSV
Hematocrit	19.9±3.9	134±5.9	22.0±4.8	15.8±4.2	23.6±4.7	16.1±4.0
Leucocrit	1.67±0.54	1.86±0.38	2.11±0.73	1.14±0.18*	1.23±0.11	1.40±0.33
Macrophage (yeast/macro)	5.0±0.4	5.4±1.1	7.6±1.1	9.5±1.3	14.5±2.8	10.7±1.2
Lysozyme (ug/ml)	134.3±64.8	108.0±3.0	48.7±11.9	125.7±48.6*	58.3±4.8	120.5±48.6*

Experiment 5: The effects of ITP on juvenile herring immunology

See Objective 5, Experiment 4.

Objective 7: To determine the effects of oil exposure on disease resistance in juvenile and adult herring.

Experiment 1: The effects of oil on juvenile herring disease resistance to *Vibrio anguillarum*

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

The results of the disease challenge are shown in Figure 8. This figure shows cumulative mortality in herring exposed to *Vibrio anguillarum* following an exposure to varying concentrations of OWD for 22 days. The lowest mortalities through 6 weeks occurred in the highest OWD concentration.

Table 9. Differential white blood cell counts for adult herring exposed to oil or VHSV or a combination of the two stressors. No significant differences were seen at $p<0.05$.

Cell type	2 weeks		4 weeks		6 weeks		8 weeks	
	Control	OWD	Control	OWD	Control	OWD	Control	OWD
Lymphocytes	32.1±2.8	27.6±6.7	32.8±5.5	27.1±4.9	34.5±5.1	30.2±4.7	29.6±7.6	27.8±4.0
Basophils	0.14±0.05	0.15±0.04	0.12±0.03	0.14±0.06	0.11±0.04	0.11±0.05	0.14±0.04	0.13±0.03
Neutrophils	18.7±3.4	22.4±3.7	25.6±4.3	23.3±3.9	19.5±3.7	22.7±5.1	25.6±3.7	24.1±2.4
Thrombocytes	48.9±4.8	49.5±5.8	41.2±6.3	50.84±7.7	45.6±6.0	46.8±5.7	55.6±5.1	52.3±6.9
Monocytes	0.2±0.1	0.3±0.2	0.3±0.2	0.3±0.1	0.2±0.1	0.2±0.2	0.3±0.2	0.3±0.1
Eosinophils	0	0	0	0	0	0	0	0

Cell type	2 weeks		4 weeks		6 weeks		8 weeks	
	VHSV	VHSV/OWD	VHSV	VHSV/OWD	VHSV	VHSV/OWD	VHSV	VHSV/OWD
Lymphocytes	30.5±4.5	28.9±4.1	34.5±6.9	27.5±6.0	29.6±2.5	30.7±4.9	32.1±5.2	30.9±5.8
Basophils	0.10±0.06	0.14±0.04	0.11±0.04	0.10±0.03	0.13±0.06	0.12±0.04	0.13±0.04	0.11±0.06
Neutrophils	13.5±7.3	22.3±5.7	21.5±3.6	24.1±5.3	21.9±4.1	19.0±5.5	23.3±4.2	23.5±3.8
Thrombocytes	55.6±6.8	48.5±6.6	43.7±5.3	48.2±5.9	48.2±4.4	49.9±5.8	44.3±4.2	45.2±5/1
Monocytes	0.3±0.1	0.2±0.1	0.2±0.1	0.1±0.1	0.2±0.1	0.3±0.2	0.2±0.2	0.3±0.1
Eosinophils	0	0	0	0	0	0	0	0

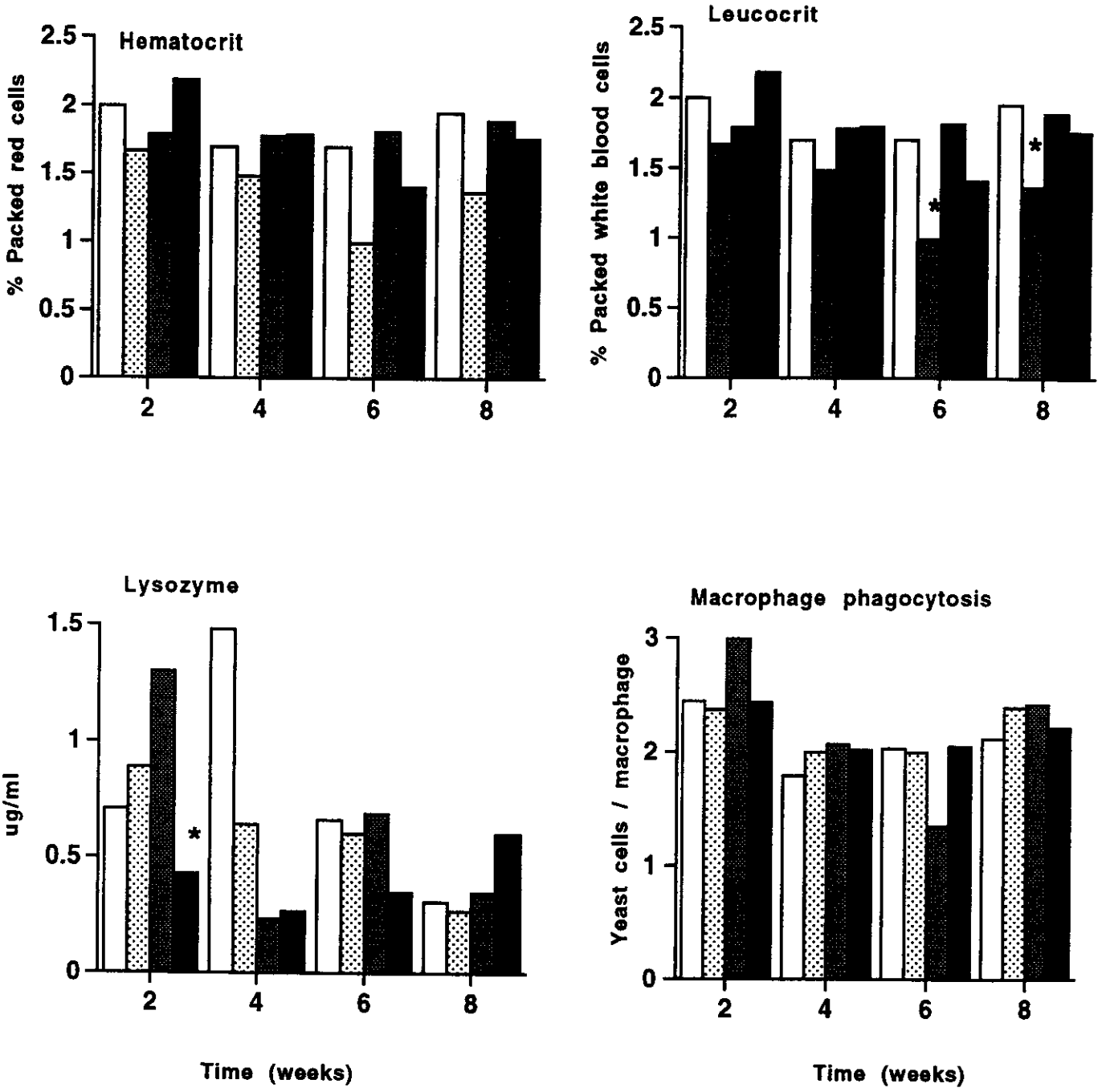


Figure 7. Effects of a high concentration of OWD, VHSV or combination on hematocrit, leucocrit, lysozyme and macrophage phagocytosis in adult herring. Control (□), OWD (▨), VHSV (■), or VHSV + OWD (■). Significant differences at $p<0.05$ are denoted by an *.

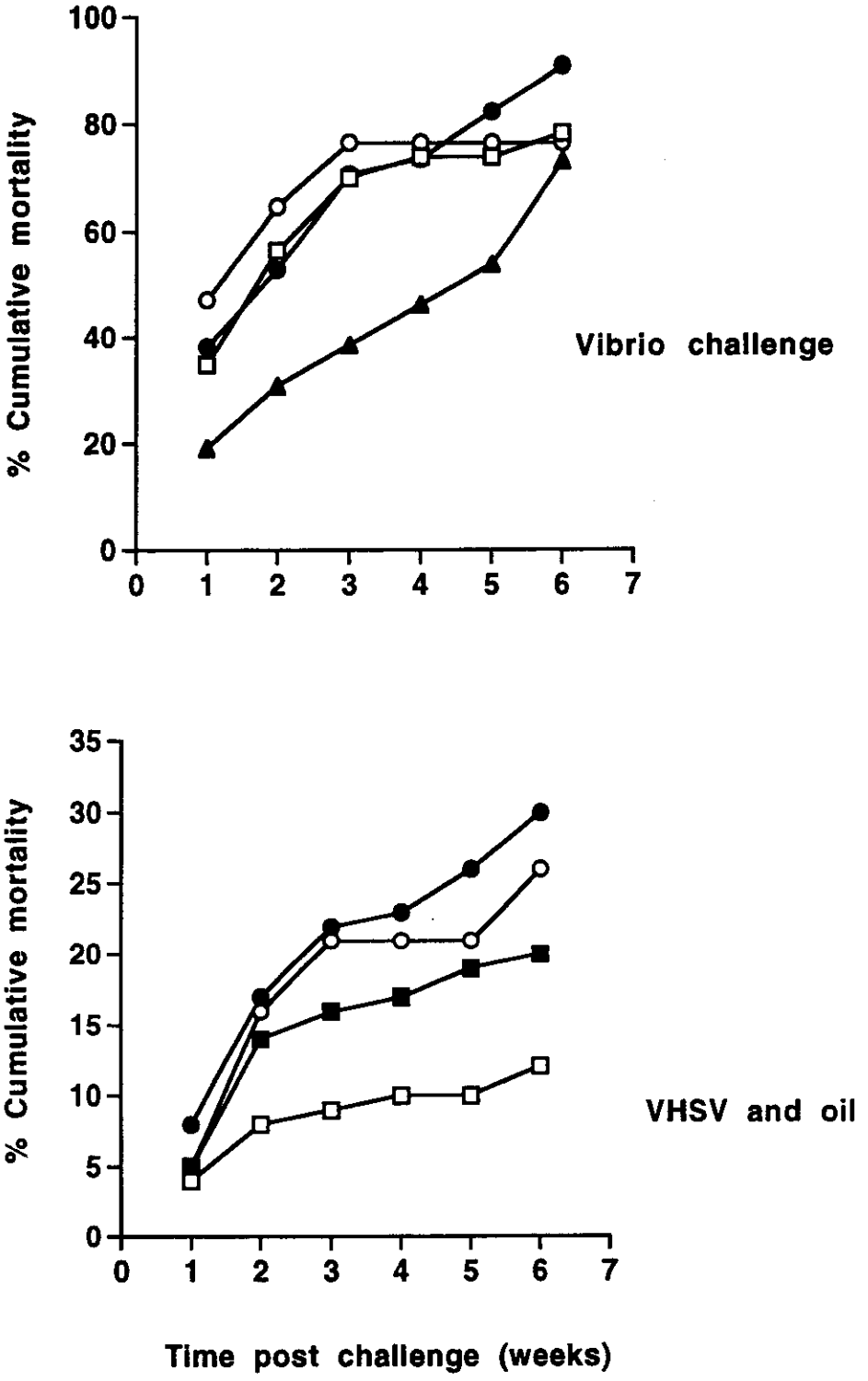


Figure 8. Top: The cumulative percent mortality in juvenile herring exposed to varying concentrations, control (-□-), low (-●-), medium (-○-) and high (-▲-) of an OWD of oil and the marine pathogen *Vibrio anguillarum*. Values are means of duplicate tanks. Significant differences were seen at $p<0.05$ in the high concentration. Bottom: The cumulative percent mortality in juvenile herring exposed to OWD (-○-), VHSV (-■-) or a combination (-●-). Significantly higher mortalities occurred in fish exposed to stressors.

Experiment 2: The effects of oil on juvenile herring disease resistance to VHSV

Juvenile herring were challenged with VHSV as described previously for adult fish following exposure to a high concentration of OWD for 21 days. The results of these experiments were highly variable, and no dose-response effect of OWD exposure on herring mortality due to VHSV could be determined. Both OWD and VHSV were acutely toxic to juvenile herring, however, both stressors together resulted in much higher mortalities (Figure 8).

Experiment 3: The effects of oil on adult herring disease resistance

Adult Pacific herring were exposed to control, low, medium and high concentrations of an OWD as described above for 28 days. Herring were exposed to VHSV titres as outlined by Kocan (1997) and monitored for signs of VHSV and mortality for up to 8 weeks. No signs of VHSV infection were noted, and no mortality occurred in these fish.

Objective 8: To determine the effects of oil exposure and VHSV on the swimming performance and exercise recovery of juvenile and adult Pacific herring.

Experiment 1: The effects of oil on adult herring swimming and exercise recovery

Adult herring were exposed to OWD for either 4 or 22 days to determine effects on swimming performance and recovery of fish from exercise. OWD in this experiment resulted in significant mortalities which are shown in Table 11. 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 (Table 12). 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 swimming'. Figure 9 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 baseline levels following exercise.

Table 11. The effects of swimming on mortality in adult herring and critical swimming speeds in fish dosed with OWD. No significant differences were seen between control and OWD-exposed fish at $p<0.05$.

Dose OWD	Post swim mortality		Critical swimming speed	
	4 days	22 days	4 days	22 days
Control	30±10	30±0	5.8±2.7	4.8±1.7
Low	30±10	40±30	6.1±2.6	5.3±1.5
Medium	50±10	40±0	4.7±1.6	5.2±1.4
High	60±20	40±20	4.6±1.8	5.3±2.0

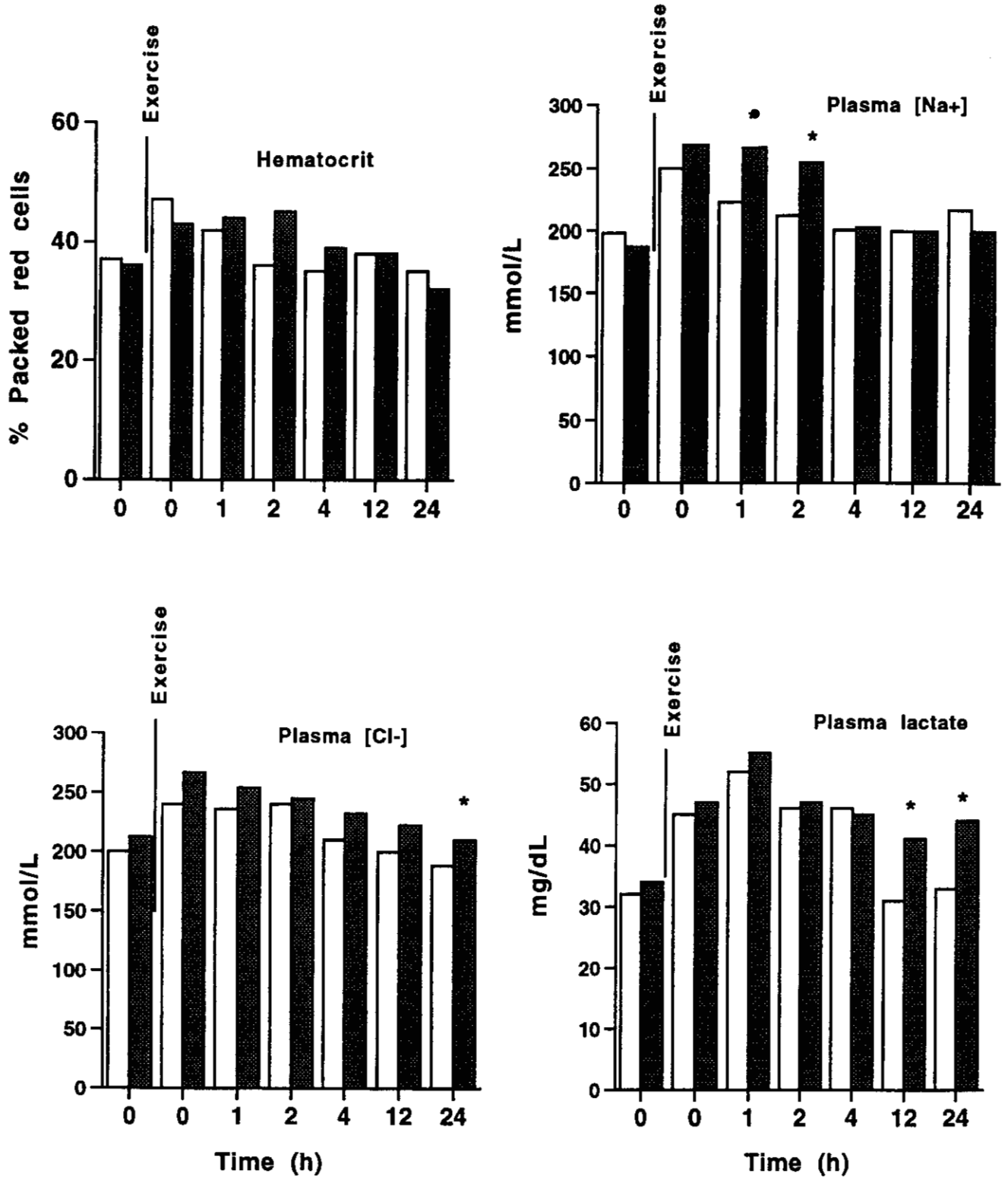


Figure 9. Effects of varying concentrations of an OWD of oil on adult herring hematocrit, plasma lactate, plasma chloride and sodium concentrations following 6 minutes of burst swimming. Controls (□) and OWD (■). Values are means±SE of three fish. Significant differences from control fish at $p<0.05$ 24 h following exercise are denoted by an * at $p<0.05$.

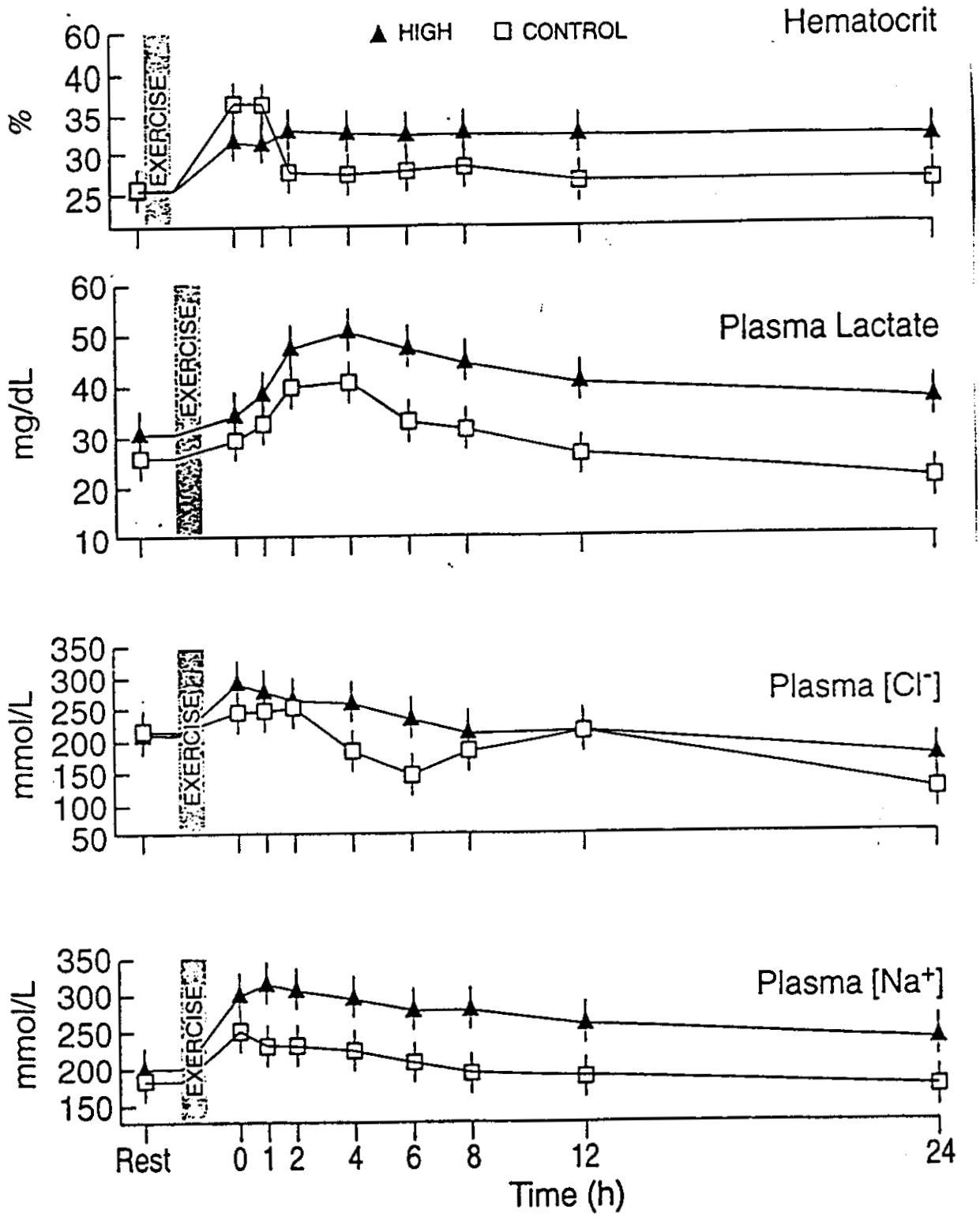


Figure 10. Effects of varying concentrations of an OWD of oil on juvenile herring hematocrit, plasma lactate, plasma chloride and sodium concentrations following 6 minutes of burst swimming. Values are means±SE of three fish. Significant differences from control fish at $p<0.05$ 24 h following exercise is found in hematocrit, plasma lactate and plasma [Na^+].

Experiment 2: The effects of oil on juvenile herring swimming and exercise recovery

Juvenile herring were exposed to OWD for 24 or 96 h to determine effects on swimming performance and recovery of fish from exercise. OWD in this experiment resulted in significant mortalities. The effects of sublethal OWD exposure on herring swimming performance as measure by critical swimming speed (Ucrit) can be seen in Table 12. Significant effects on fish swimming were seen only in the medium and high OWD concentrations at 96 h. Due to the high mortalities in fish which had been forced to swim, a separate experiment was set up to determine OWD effects on the recovery of herring from 'burst swimming' following a 96 h exposure. Figure 10 shows the effects of exercise on hematocrit, plasma lactate and [Cl-] and [Na+]. Exposure of fish to high doses of OWD caused more of a disturbance in parameters measured and appeared to inhibit a return of these values to baseline following exercise.

Table 12. The effects of oil on the swimming performance of juvenile herring. Values are critical swimming speeds. Fish were exposed to OWD for 24 or 96 hours. Significant differences at p,0.05 are denoted by an *.

Dose OWD	Critical swimming speed (body lengths/s)	
	24 hours	96 hours
Control	5.4±0.6	5.2±0.6
Low	5.6±0.4	4.9±0.3
Medium	5.0±0.5	4.7±0.4*
High	4.8±0.5	4.1±0.3*

Objective 9: Describe the effects of density on Pacific herring biochemistry and immunology.

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 experiments, the effects of fish stocking density on baseline biochemical measures and differential white blood cell counts were made. Measurements included plasma cortisol, lactate, glucose, hematocrit, and leucocrit, and were performed as described previously. Densities were 12, 50 and 80 fish per 200L.

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 13). There were no effects of stocking density on white blood cell differential counts are shown in Table 14.

Table 13. The effects of stocking density on various baseline levels of hematological and biochemical parameters in juvenile herring. Values are means±SE for 6 fish.

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
Lysozyme (U/ml)	4.3±6.0	10.4±6.0	12.2±6.2

Table 14. The effects of fish stocking density on differential white blood cell counts in wild Pacific herring which had undergone a natural epizootic of VHSV. There were no significant differences in differential white blood cell counts between fish stocked at higher densities compared to the lowest density at a significance level of $p<0.05$.

Cell type	Percent of total white blood cell population		
	Low density	Medium density	High density
Lymphocytes	59.9±6.3	42.3±4.3	35.±4.0
Neutrophils	18.5±6.1	9.4±2.7	1.0±0.0
Macrophages	1.1±0.5	0.7±0.5	0.1±0.1
Monocytes	0.1±0.1	0.0±0	0.0±0.0
Thrombocytes	19.8±3.8	47.9±4.7	59.4±4.1
Eosinophils	0.0±0.0	0.1±0.1	0.0±0.0

Objective 10: To determine the effects of oil, VHSV and ITP on herring egg survival

Adult herring were spawned and fertilized artificially in the laboratory. Eggs were placed into plexiglass chambers on nylon mesh and submerged in tanks containing either varying doses of OWD, VHSV, or ITP as described in previous experiments. No effect of either VHSV or ITP was found on hatching success which exceeded 93% in all cases. In a separate experiment, eggs were exposed to OWD for either 24, 96 or 168 hours and then raised in uncontaminated water. Exposure of eggs to oil decreased survival in a dose dependent manner. The effects of oil on herring egg survival is shown in Table 15.

Table 15. The effect of varying doses of oil on the survival of herring eggs. Significant differences between control and OWD-exposed eggs at $p<0.05$ is denoted by an *.

Dose of OWD	Percent survival to hatch		
	24h exposure	96 hour exposure	168 hour exposure
Control	93±4	90±5	90±5
Low	94±5	81±8	71±2*
Medium	93±3	76±7*	69±7*
High	79±5*	75±6*	46±8*

Discussion

The overall purpose of the present study was to determine the potential effects of three stressors, viral hemorrhagic septicemia virus (VHSV), *Ichthyophonus hoferi* (ITP) and oil, or some combination, on various aspects of herring fitness or health to begin to assess their role in population declines and recovery in Prince William Sound (PWS).

This section of the project also had 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 PWS are discussed in Section I of this final report. Considerable effort was placed into ensuring that the plasma chemistry analysis methods used were appropriate for herring plasma.

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 or VHS 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 than juvenile fish in most parameters when exposed to OWD. 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. Both juvenile and adult herring exposed to OWD exhibited the 'classical' stress response when exposed, however, the response was transient. 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 were 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 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 that have potential as biomonitoring tools of population recovery include plasma cortisol (if herring are sampled immediately), plasma lactate and plasma glucose.

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 juvenile and adult Pacific herring to oil resulted in effects on specific components of their immune system. Exposures in these experiments were longterm in nature to better mimic conditions which may have occurred during the oil spill, and effects on herring immune systems only occurred under these exposure durations. 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. However, these effects were not always consistent between adult and juvenile fish, nor with different exposure durations or sampling times, indicating that other factors play an important role in modulating the response of the immune system to stressors. Alterations in immune parameters may not return to preexposure values up to seven weeks following transfer to uncontaminated water which indicates that stressor-induced damage may be longterm in herring. As bioindicators of affected immune systems, only macrophage function was consistently altered by OWD. Other measures were highly variable in herring and not useful as biomonitoring tools unless large sample sizes were used, which is different from many other fish species in which these parameters occupy a much more narrow range in which changes are more easily identified.

These studies also indicate that the prediction of stressor effects on disease resistance based upon the measurement of immune system components is difficult. For example, in the disease challenge experiments it was shown that exposure to OWD decreased the susceptibility of herring to the bacteria V. anguillarum, but increased their susceptibility to the virus VHSV. Disease challenge experiments also point out a substantial difference between adult herring and juveniles with respect to their susceptibility to VHSV. Juvenile fish were very sensitive to the effects of VHSV both lethally and sublethally. However, when adult fish were exposed to 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 (Dr. T. Williams, Simon Fraser University, pers. comm.), therefore, VHSV or similar disease outbreaks may only occur during spawning or other very stressful events. The impacts of ITP exposure on herring is difficult to determine based on the results of this study. No effects were seen in adult or juvenile herring after exposure to the fungus. Moreover, no signs of the disease ever manifested itself over the time course of any experiment. Again, the effects of this pathogen, like VHSV, may only occur when fish are stressed under unusual circumstances such as spawning events.

The effects of OWD on swimming performance was age-dependent with juvenile fish being affected more so than adults. Although the concentrations of hydrocarbons in these studies did not result in the mortality of adult herring, significant effects on both juvenile and adult 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. Significant alterations in biochemistry are known to occur during 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.

The survivability of herring eggs was also affected by exposure. This work support previous field research into the survivability of herring eggs and the post hatch survival of larval herring (Kocan et al. 1996; McGurk and Brown 1996). In those studies, egg percent hatch was lower when eggs were collected from a site in PWS previously oiled by the EVOS. This reduced survival of herring eggs exposed to oil may be a significant contributor to reductions in herring in PWS seen in later years.

The results from the reproduction experiments, coupled with the fitness studies with juveniles and adults, indicates that the sensitivity of herring to at least two of the stressors (oil and VHSV) decreases with age. The experiments with density and its effects on stressor modulation indicate that abiotic factors also play a major role in determining the responses of herring to stressors. These two significant conclusions indicate that the prediction of effects to potential stressors may be difficult due to confounding factors, although general statements can be addressed.

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, swimming performance and immunology were found. The time frame for the inducement of effects is shortest for biochemical effects, followed by swimming performance and finally immune system effects. The reverse is also true, the slowest recovery of disturbance usually occurs in the immune system, followed by swimming and then biochemistry. These studies also indicate that although stressors affect various components of the herring immune system, it is difficult to predict the resulting effect on disease resistance to different pathogens. Biological factors such as age of fish and abiotic factors such as

density play important modulating roles in the response of herring to stressors such as contaminants and disease. The results of these studies partly 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.

Acknowledgements

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. Rosemarie Ganassin for her work on immunological assay development for the project. Several technicians including Joanne Scherba and Keith Tierney are also thanked.

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