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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?

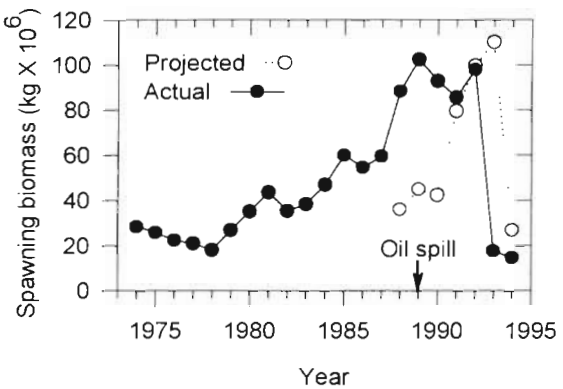

Fig. 1. *Clupea pallasi*. Biomass estimates of mature Pacific herring in Prince William Sound, Alaska. Unexploited biomass projected in the year before spawning (Projected) and estimated during spawning (Actual). Estimates were made by Fritz Funk, Alaska Department of Fish and Game, Juneau, Alaska; unpubl. 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

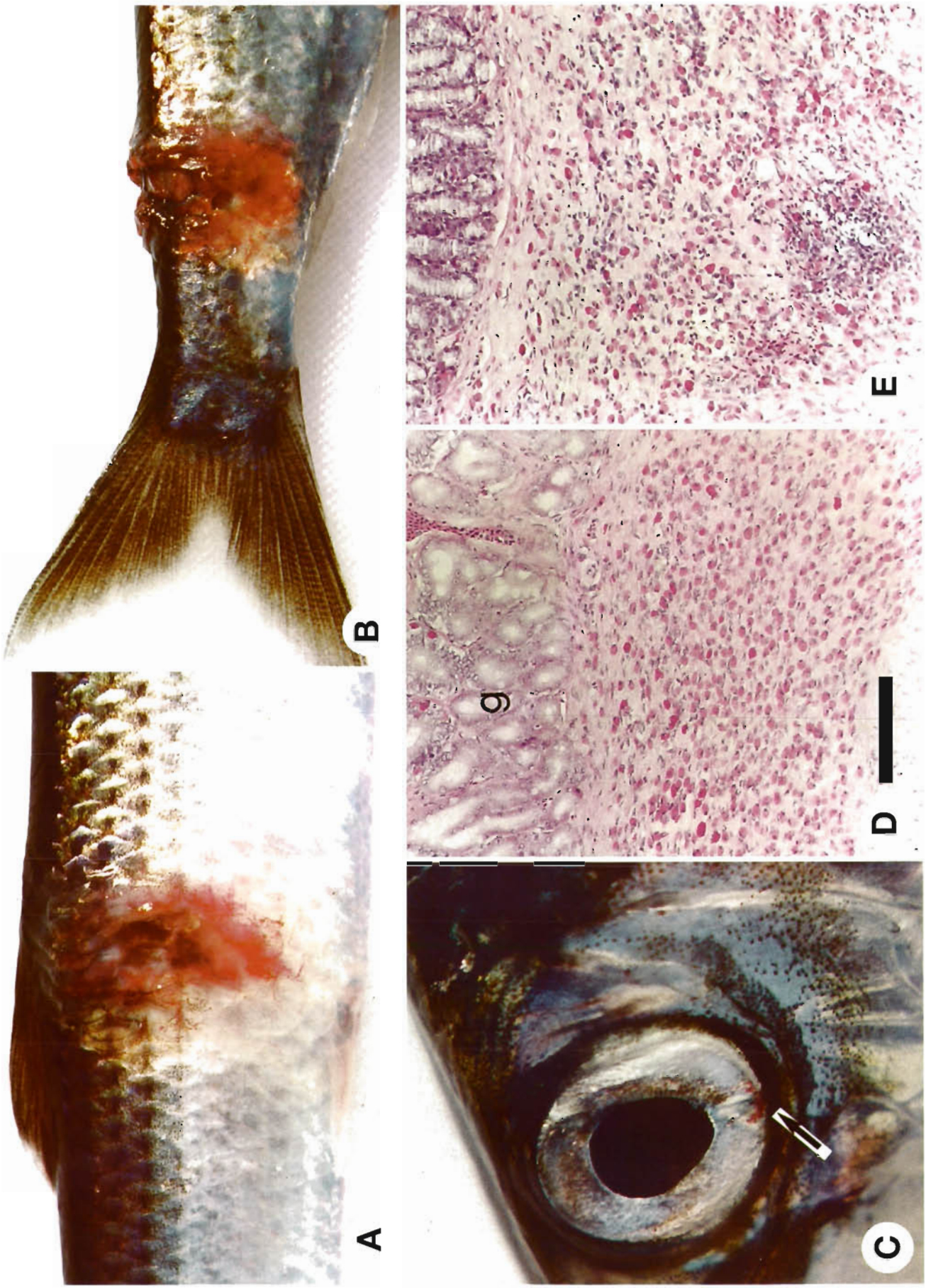
A
B
C
D
E
g

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*)	139	31	1	0	19	↑ age (0.005*)
Ichthyophonus (score combined with liver *Ichthyophonus*)						
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

[e] 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 ≤ 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

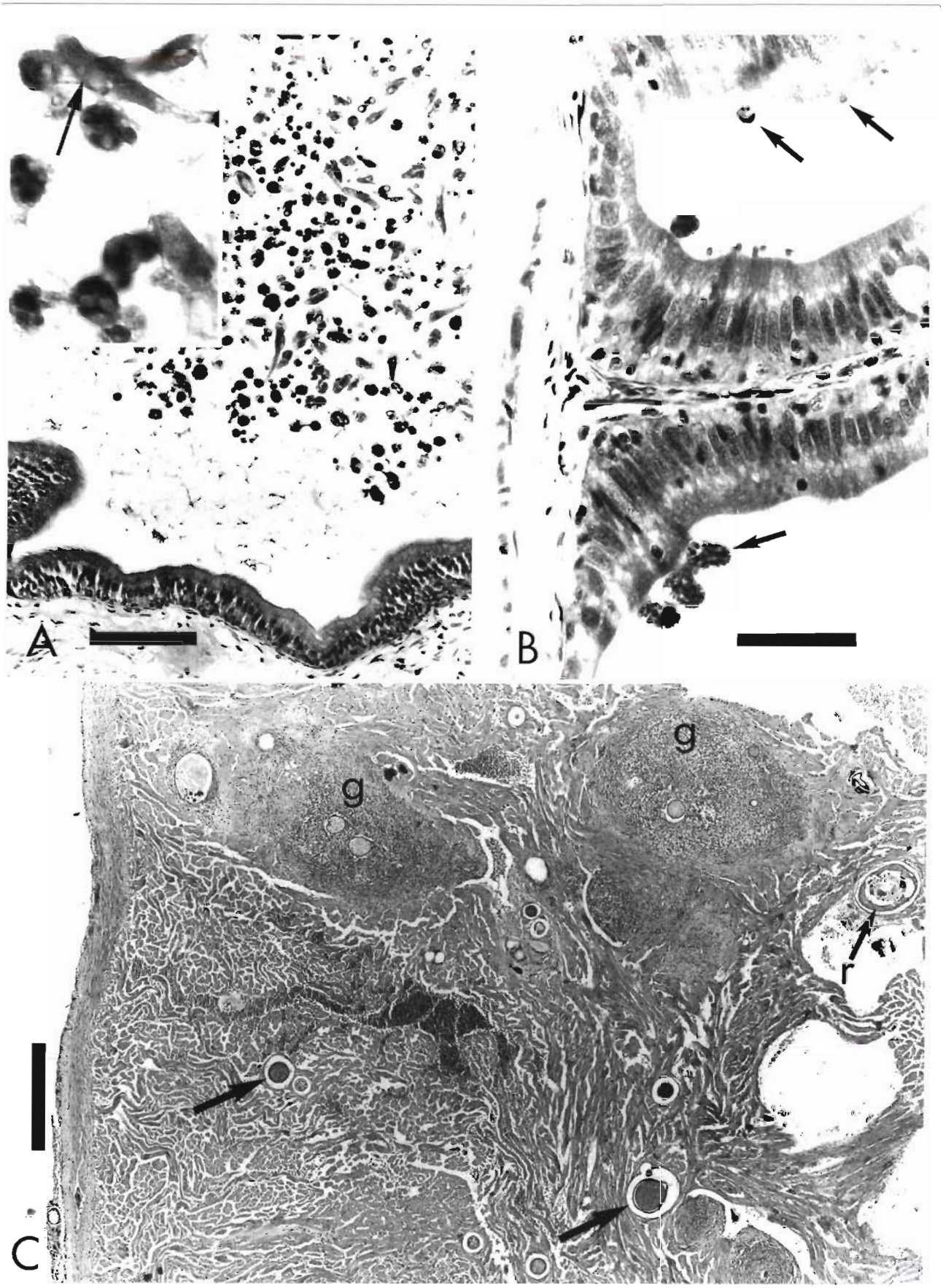
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Fig. 3. *Clupea pallasi*. Internal parasites of Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994; hematoxylin and eosin stain. (A) The myxosporean *Ceratomyxa auerbachi* in the gall bladder lumen; despite large numbers 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

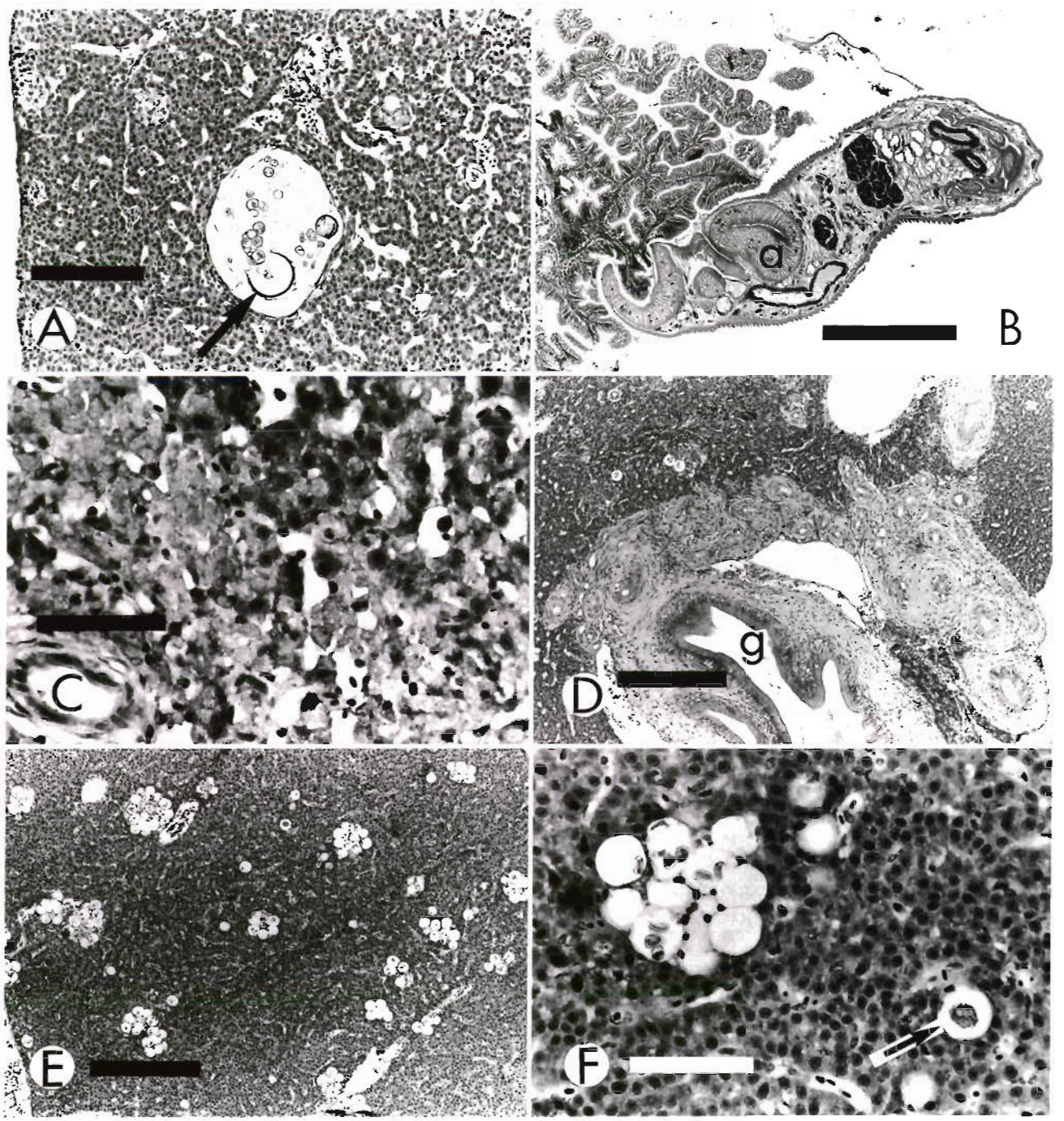
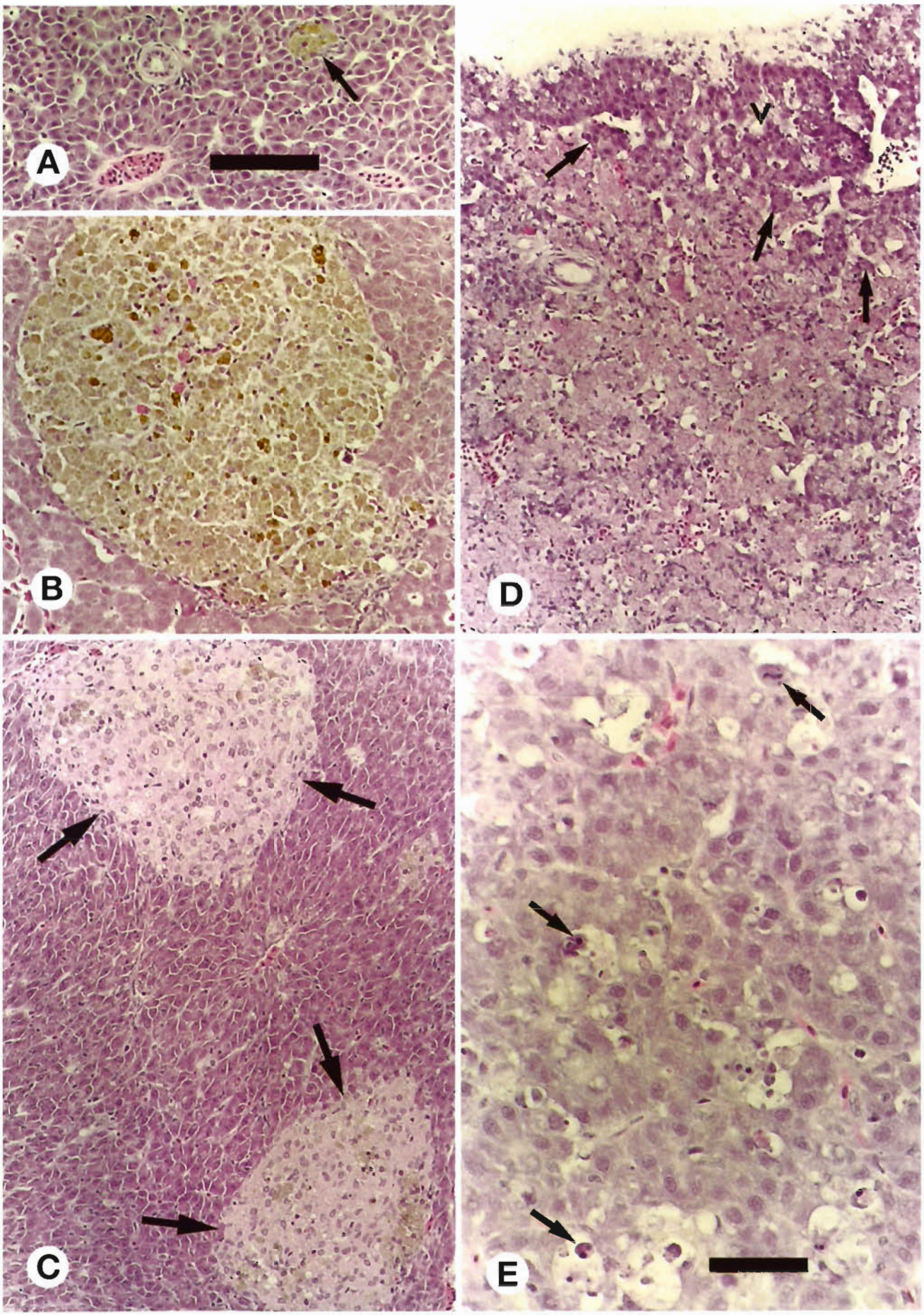


Fig. 4. *Clupea pallasi*. Microscopic lesions in the liver and stomach of Pacific herring sampled from Prince William Sound, Alaska, during spawning, 1994; hematoxylin and eosin stain. (A) Liver with a ruptured *Ichthyophonus* resting spore (arrow) that has released several multinucleate endospores; 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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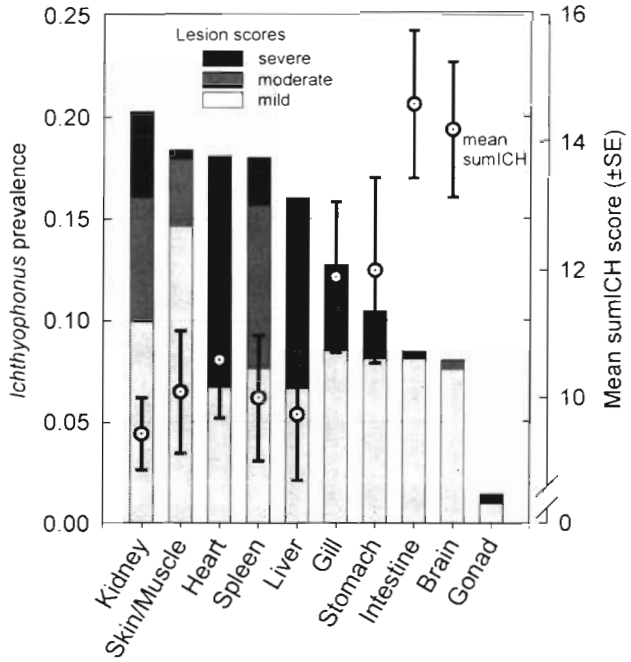


Fig. 6. *Clupea pallasi*. Sample prevalence of *Ichthyophonus* lesion scores in various organs compared with mean sum-*Ichthyophonus* (mean sumICH) score for each organ. Lesions were scored as none (0), mild (1), moderate (2), or severe (3), and the sumICH score was calculated for each fish by adding 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).

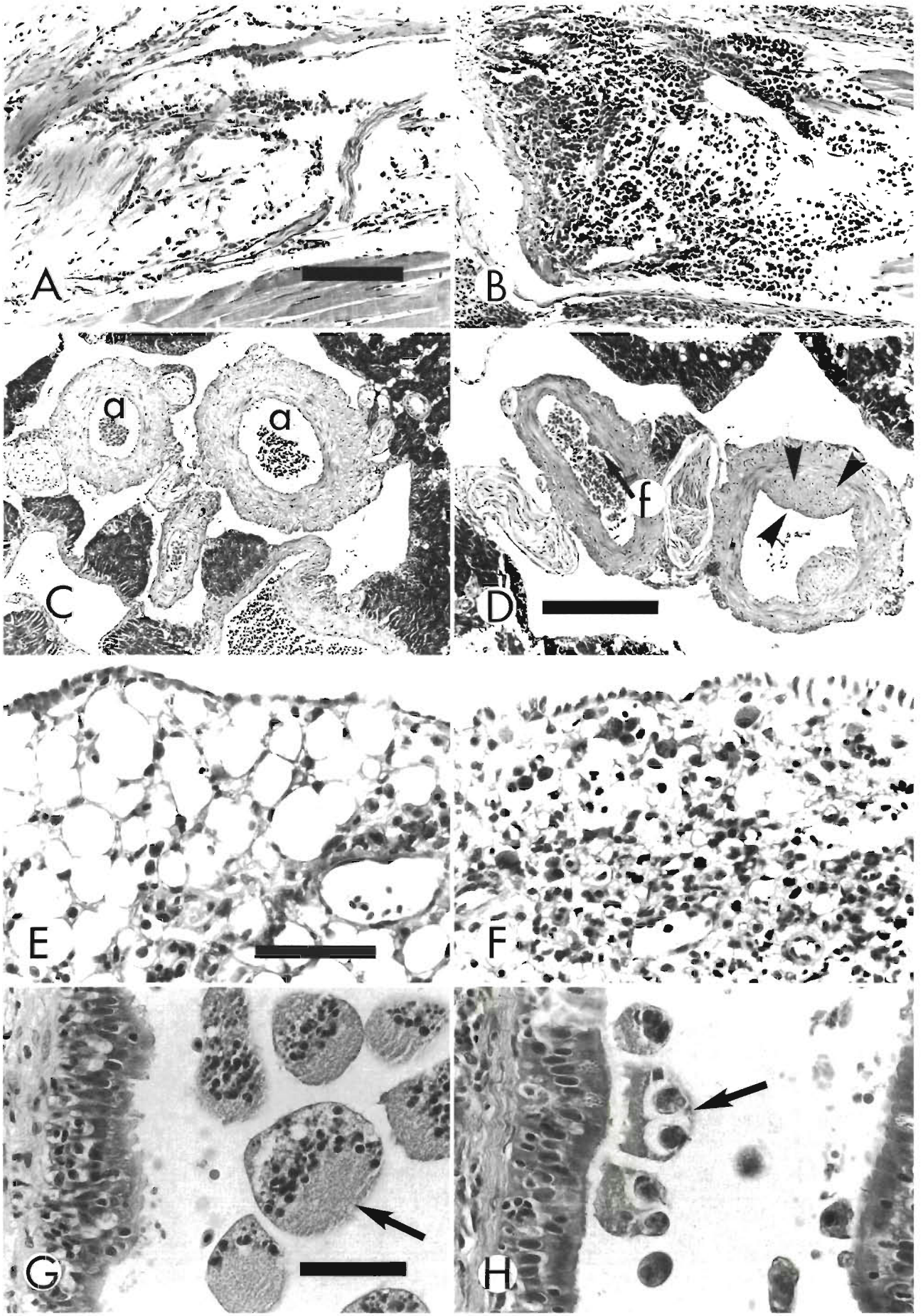
Focal intimal hyperplasia of arteriolar walls was relatively common and was scored in sections of intestine, skin and skeletal muscle, and spleen. In the intestine only, this lesion was significantly associated with VHSV infection (Table 2). Normal arteries and arterioles had a smooth intimal surface 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*

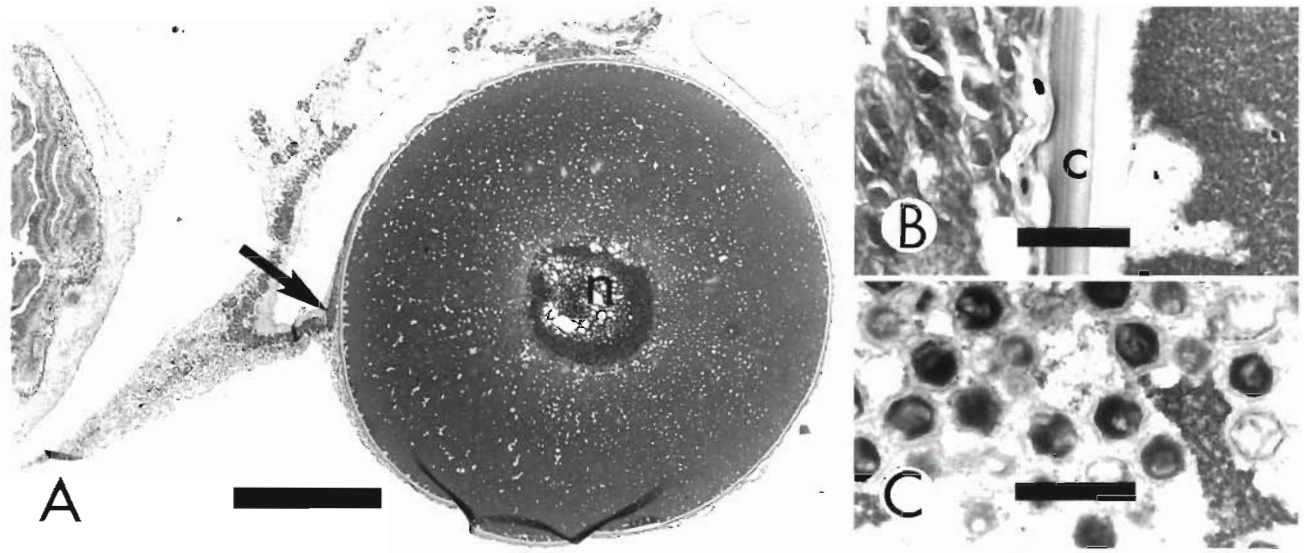

Fig. 8. *Clupea pallasi*. Lymphocystis virus in a spawning Pacific herring from Prince William Sound, Alaska, 1994. (A) intestine (left) and hypertrophied fibroblast expanding intestinal mesenteries (arrow); note hypertrophic fibroblast nucleus (n); arrow = margin of hypertrophic fibroblast and area 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.

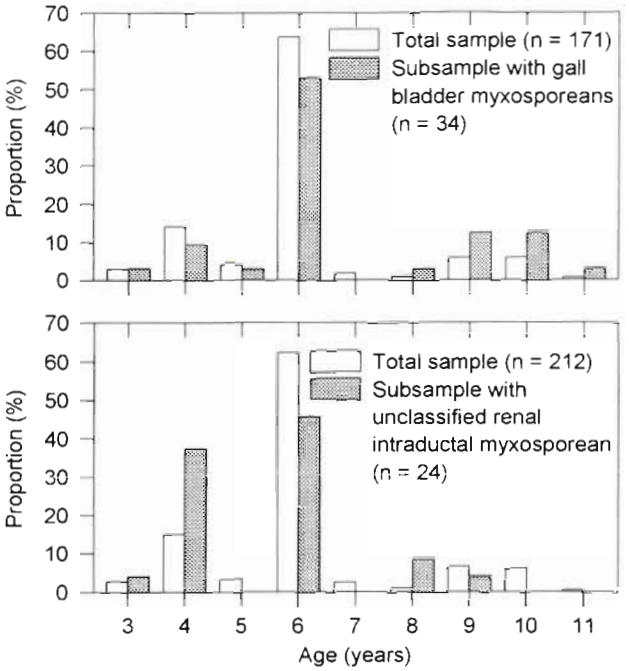

Fig. 9. *Clupea pallasi*. Age composition of Pacific herring with common parasites compared with the age composition of the sample. Pacific herring were sampled from Prince William Sound, Alaska, during spawning, 1994. Top: intraluminal gall bladder myxosporean (*Ceratomyxa auerbachi*). Bottom: unclassified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

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