Vol. 70: 201–208, 2006 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published June 23

Dynamics of viral hemorrhagic septicemia, viral erythrocytic necrosis and ichthyophoniasis in confined juvenile Pacific herring *Clupea pallasii*

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ABSTRACT: Capture of wild, juvenile herring *Clupea pallasii* from Puget Sound (Washington, USA) and confinement in laboratory tanks resulted in outbreaks of viral hemorrhagic septicemia (VHS), viral erythrocytic necrosis (VEN) and ichthyophoniasis; however, the timing and progression of the 3 diseases differed. The VHS epidemic occurred first, characterized by an initially low infection prevalence that increased quickly with confinement time, peaking at 93 to 98% after confinement for 6 d, then decreasing to negligible levels after 20 d. The VHS outbreak was followed by a VEN epidemic that, within 12 d of confinement, progressed from undetectable levels to 100% infection prevalence with >90% of erythrocytes demonstrating inclusions. The VEN epidemic persisted for 54 d, after which the study was terminated, and was characterized by severe blood dyscrasias including reduction of mean hematocrit from 42 to 6% and replacement of mature erythrocytes with circulating erythroblasts and ghost cells. All fish with ichthyophoniasis at capture died within the first 3 wk of confinement, probably as a result of the multiple stressors associated with capture, transport, confinement, and progression of concomitant viral diseases. The results illustrate the differences in disease ecology and possible synergistic effects of pathogens affecting marine fish and highlight the difficulty in ascribing a single causation to outbreaks of disease among populations of wild fishes.

KEY WORDS: Disease · Fish · Viral hemorrhagic septicemia · Viral erythrocytic necrosis · *Ichthyophonus* · Pacific herring · Wild fish

INTRODUCTION

Awareness of the effects of emerging diseases on fish and wildlife has risen over the past several years; however, our understanding of the effects of disease on marine species remains limited (Harvell et al. 2004), probably because the effects typically go unnoticed and causation is difficult to demonstrate (Scott 1988). Our understanding of the effects of disease in populations of wild marine fishes is further impaired by the frequent presence of multiple pathogens and the unavailability of immunologically naïve hosts necessary to fulfill Koch's postulates. For example, the biomass of Pacific herring *Clupea pallasii* in Prince William Sound decreased from 98 million kg in 1992 to <20 million kg in 1993 (Marty et al. 1998). Although infectious disease was proposed as a contributing factor, causation was confounded by identification of multiple etiological agents, including viral hemorrhagic septicemia virus, lymphocystis virus, *Ichthyophonus hoferi*, myxosporeans, coccidians, trematodes and cestodes (Marty et al. 1998). In southern Australia, 2 of the largest mass mortality events ever reported to involve fish were characterized by declines in sardine

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Sardinops sagax biomass from 165 000 to 37 000 tonnes in 1995 and from 147 000 to 36 000 tonnes in 1998 (Jones et al. 1997, Whittington et al. 1997, Gaughan et al. 2000, Ward et al. 2001). Although mortality was associated with herpesvirus-like particles in the gills of affected sardines (Hyatt et al. 1997, Jones et al. 1997, Gaughan et al. 2000), transmission studies and attempts to fulfill Koch's postulates were unsuccessful (Bernoth 2002).

In Puget Sound, Washington (USA), several pathogenic agents, including viral hemorrhagic septicemia virus (Hershberger et al. 1999, Kocan et al. 2001), erythrocytic necrosis virus (MacMillan & Mulcahy 1979) and *Ichthyophonus hoferi* (Kocan et al. 1999, Hershberger et al. 2002) are enzootic in populations of Pacific herring *Clupea pallasii.* Viral hemorrhagic septicemia virus (VHSV) is grouped with several other commercially and ecologically important fish viruses in the *Novirhabdovirus* genus of the family *Rhabdoviridae* (Walker et al. 2000). North American isolates belong to VHSV Genogroup IV and have been recovered almost exclusively from marine or anadromous fishes, similar to reports for the European Genogroups Ib, II and III (Snow et al. 2004). Juvenile and newly recruited Pacific herring are highly susceptible to VHSV (Kocan et al. 1997), and application of nominal stressors to wild cohorts, including capture and confinement, is often sufficient to initiate disease outbreaks (Hershberger et al. 1999, Kocan et al. 2001). Similarly, natural stressors occurring in situations where the virus is endemic among wild populations of herring may contribute to epidemics that have been reported in the eastern North Pacific (Traxler et al. 1999, Hedrick et al. 2003).

Viral erythrocytic necrosis (VEN) is characterized by the presence of viroplasmic inclusion bodies located within the cytoplasm of affected erythrocytes (reviewed in Dannevig & Thorud 1999). Although the etiology is not completely understood, primarily because of the refractory nature of established cell lines to infection by the causative agent (Evelyn & Traxler 1978), it is likely to be associated with a presumed iridovirus, referred to as erythrocytic necrosis virus (ENV). In Puget Sound and the eastern North Pacific, the condition frequently occurs in Pacific herring *Clupea pallasii* (MacMillan & Mulcahy 1979), where it has been associated with natural mortality (Meyers et al. 1986).

The genus *Ichthyophonus* is a member of the Mesomycetozoea, a monophyletic class of protozoans that includes several other pathogenic organisms (Ragan et al. 1996, Herr et al. 1999, reviewed in Mendoza et al. 2002). Currently *I. hoferi* (reviewed in McVicar 1999) and *I. irregularis* (Rand et al. 2000) are the only 2 recognized species in the genus, but other species have probably been grouped with *I. hoferi* based on the plasticity of morphological characteristics (McVicar 1999). Additional molecular phylogenetic studies are necessary to better understand the relatedness of *I. hoferi* isolates (Criscione et al. 2002, Halos et al. 2005); therefore, the organism will be referred to generically as *Ichthyophonus* hereafter in this manuscript. From 1898 through the mid-1950s, 6 major *Ichthyophonus*-related epidemics were described in Atlantic herring *Clupea harengus* from the western North Atlantic (Sindermann 1990, McVicar 1999). More recently, a massive *Ichthyophonus*-related epidemic killed an estimated 300 million Atlantic herring in marine waters around Sweden and Denmark during the early 1990s (Rahimian & Thulin 1996), and epidemiological data implicate *Ichthyophonus* as a primary factor responsible for mortality in wild Pacific herring *C. pallasii* from estuarine waters of Washington State (Hershberger et al. 2002).

One means to address the difficulties encountered with investigating the effects of disease on wild fish populations is to observe outbreaks in cohorts that are collected from the wild and confined to laboratory tanks. Although this approach introduces factors that may not occur in free-ranging populations, it provides an opportunity to observe disease dynamics resulting from natural infections with multiple pathogens. Here, we describe the comparative disease dynamics resulting from naturally-acquired infections with VHSV, ENV and *Ichthyophonus* in wild, juvenile herring held in marine aquaria.

MATERIALS AND METHODS

Collection of fish. Juvenile Age 0 Pacific herring *Clupea pallasii* were collected by dip net from 2 predator-corralled aggregations (bait balls) in Admiralty Inlet (Puget Sound, Washington) on July 30, 2003. Herring from each bait ball were transported alive to the Marrowstone Marine Station, and separated into 3 replicate 275 l tanks supplied with sand-filtered, UV-treated seawater. Herring from the first bait ball (mean fork length = 60 mm, SD = 5 mm; mean mass = 1.8 g, SD = 0.5 g; n = 30) were loaded at 187 to 235 fish $tank^{-1}$, and 10 fish d^{-1} were sampled from each tank 1, 6, 12 and 20 d post capture. Herring from the second bait ball (mean fork length = 64 mm, SD = 5 mm; mean mass = 2.2 g, SD = 0.7 g; n = 30) were loaded at 345 to 670 fish $tank^{-1}$, and 10 fish d^{-1} were sampled from each tank 1, 6, 12, 20, 28, 41 and 54 d post capture. Confined fish were fed daily with Cyclopeze™ (Argent Laboratories, Redmond, WA, USA). Dead and moribund individuals were sampled from all tanks as they appeared.

Infection assays. Sampled fish were euthanized in 1 mg ml^{-1} tricaine methane sulfonate (MS 222) and screened for prevalence and intensity of VEN, VHS and ichthyophoniasis. For VEN, blood films were made from a severed caudal peduncle, air dried, fixed in 100% methanol, and stained with 7.5% Giemsa in phosphate buffer (pH 6.9). The prevalence and intensity of ENV infections were determined by examining 200 blood cells under oil immersion at 1000× magnification for presence of intracytoplasmic inclusions (Fig. 1). Mean diameter of the cytoplasmic inclusions was measured with a micrometer (n = 300). Differential blood cell counts (n = 200), including mature erythrocytes, circulating erythroblasts, and leukocytes, were recorded from all blood films. Additional blood from each sampled fish was collected in heparinized capillary tubes and centrifuged for determination of hematocrit.

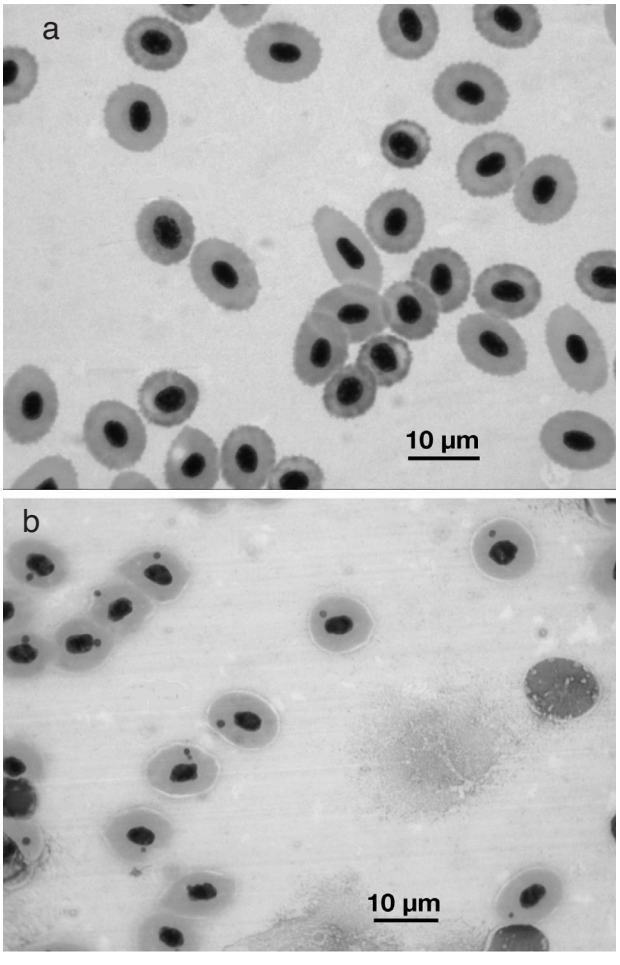

Fig. 1. *Clupea pallasii.* Giemsa-stained blood films from (a) laboratory-reared VEN-negative herring, demonstrating normal, healthy erythrocytes, and (b) confined, wild herring, demonstrating heavy infection with VEN (note inclusion bodies in cytoplasm of affected erythrocytes and presence of erythrocytic ghost cells)

Gross ichthyophoniasis was identified by the presence of black, melanophorous ulcers on the skin and/or white nodular lesions on internal organs. *Ichthyophonus* infection was confirmed by explant culture of heart tissues in Tris-buffered Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 100 µg ml^{-1} gentamycin. Cultures were incubated at 12°C and examined microscopically (40× magnification) for presence of *Ichthyophonus* after 7 d, with a final examination after 14 d if not already deemed positive.

Prevalence and intensity of VHSV infections were determined by plaque assay (Batts & Winton 1989). Briefly, whole herring bodies (minus head, tail and heart) were homogenized in Tris-buffered Eagle's MEM containing 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 100 µg ml^{-1} gentamycin, and 2.5 µg ml^{-1} amphotericin B. Serial 10-fold dilutions of the homogenates were plated on monolayer cultures of *epithelioma papulosum cyprini* (EPC) cells pretreated with polyethylene glycol, overlayed with methylcellulose, incubated at 15°C for 7 d, and fixed/stained with a mixture of formalin and crystal violet. Virus titers were expressed as plaque forming units (pfu) g^{-1} tissue. Isolated virus from 15 infected fish was confirmed as VHSV using the polymerase chain reaction and VHSV-specific primers (Einer-Jensen et al. 1995). Fish were considered diseased with VHS when whole body tissue titers exceeded 10^4 pfu g^{-1}.

Challenge of pathogen-free herring. To confirm that erythrocytic inclusions were not a sign of late stage VHS in recovering individuals, specific-pathogen-free (SPF) herring were challenged with VHSV, and blood from the survivors was screened for erythrocytic inclusion bodies. To produce SPF test individuals, naturally spawned herring eggs adhering to submerged macrophytes were collected from Discovery Bay (Straight of Juan de Fuca, Washington) and transferred to laboratory tanks, supplied with double sand-filtered, particle-filtered, and double ultraviolet irradiated seawater, at the Marrowstone Marine Station. After hatching, larvae were fed a combination of laboratory-reared rotifers *Brachionus plicatilis* and recently-hatched *Artemia* sp. enriched with Super Selco® (INVE Aquaculture), Protein HUFA (Salt Creek), and Advanta Excel Feed (Aquatic Ecosystems). After approximately 60 d, larvae were weaned to frozen Cyclopeze™. Larvae metamorphosed to juveniles 70 to 90 d post-hatch, and were maintained on Cyclopeze™ until completion of these studies.

At 89 d post-hatch, 40 SPF juvenile herring were exposed to an isolate of VHSV (North American strain, Genogroup IV, Isolate No. 99-292 supplied by Garth Traxler) obtained in 1999 from Atlantic salmon *Salmo salar* cultured near Vancouver Island, Canada. The

SPF juvenile herring were infected by waterborne exposure to 4220 pfu ml^{-1} in aquarium water for 1 h, transferred to flow-through aquaria supplied with sand-filtered, UV-irradiated seawater, and monitored daily for mortality. Control cohorts (n = 40) were exposed to Hanks' buffered saline solution (HBSS), transferred to a separate tank, and observed similarly. Dead and moribund fish were sampled from each tank as they appeared. Surviving herring were euthanized with an overdose of MS-222 after 22 d and sampled for the presence of erythrocytic inclusion bodies. Blood films were prepared and processed as described above.

Electron microscopy of erythrocytic inclusions. Transmission electron micrographs of blood cells were prepared from fish demonstrating severe VEN. Heavily infected fish were obtained by capturing an additional 345 juvenile herring by dip net from a bait ball in Admiralty Inlet on July 1, 2004, and transporting them to a 275 l tank supplied with flow-through seawater, where they underwent natural VHS and VEN epidemics similar to those described in the 2003 studies. Dead and moribund fish were collected from the tank daily. After 21 d of laboratory confinement, 15 herring were euthanized by an overdose of MS-222 and blood films were screened for VEN as described previously. Additional blood from all euthanized fish was drawn in heparinized capillary tubes, fixed in 4% glutaraldehyde (diluted with one-third strength seawater) for 1 h, washed 3 times in one-third seawater, then post-fixed in 1% osmium tetroxide. Fixed blood samples corresponding to heavily infected blood films were then processed for transmission electron microscopy (Bozzola & Russell 1992) and visualized with a Hitachi H-7100 transmission electron microscope.

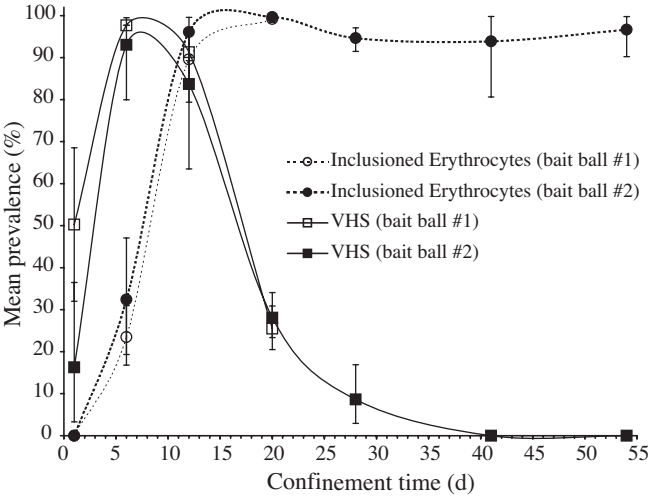

Fig. 2. *Clupea pallasii*. Progression of VHS and VEN in confined herring. Fish were considered diseased with VHS when tissue titers exceeded 10^4 pfu g^{-1}. VEN inclusions were detected in erythrocytes of all fish from Day 6 until end of study. Data represent proportions corresponding to means of arcsine-transformed prevalence in 3 replicate tanks; prevalence in each replicate was determined by sampling 10 fish $tank^{-1}$ on each sampling date. (Means ± 2 SD; 95% confidence intervals)

RESULTS

Dynamics of VHS, VEN and ichthyophoniasis

Laboratory confinement of wild, juvenile herring resulted in rapid progression of a VHS epidemic (Fig. 2), characterized by an initially low disease prevalence that increased quickly, peaking at 93 to 98% after confinement for 6 d. Prevalence of VHS remained at 84 to 91% through 12 d, before eventually dropping to negligible levels. Median VHSV tissue titers responded similarly, increasing with confinement time from 3×10^3 pfu g^{-1} after 1 d to 3.6×10^6 pfu g^{-1} after 6 d, then decreasing to 1.4×10^6, pfu g^{-1} after 12 d, 4×10^2 pfu g^{-1} after 20 d, and 0 pfu g^{-1} thereafter.

An outbreak of VEN followed the VHS epidemic and persisted through a period of 54 d at which time the study was terminated (Fig. 2). Mean prevalence of erythrocytes bearing inclusions was 0% at 1 d, but increased to 23–32% after 6 d and 90–96% after 12 d. High levels of VEN, characterized by cytoplasmic inclusions appearing in >90% of erythrocytes, persisted through 54 d, at which time the study was terminated. Intraerythrocytic inclusions occurred in both mature and immature erythrocytes and averaged 1.56 µm in diameter (n = 300, SD = 0.60).

Blood changes associated with the viral epidemics included decreased numbers of cells in circulation and altered cell assemblages. Mean hematocrit decreased steadily with increasing confinement time from 44 to 45% at 1 d to 6% at 54 d, when the study was terminated (Fig. 3). Anemia in fish that survived for 54 d was further characterized by pale, white gills and was associated with lethargic swimming behavior. Prevalence of circulating erythroblasts declined from 6–9% at 1 d to 1–2% after 6 d, and then increased steadily until the end of the study, at which time 78% of all circulating blood cells were erythroblasts (Fig. 3). Throughout this period a large proportion of erythrocytic ghost cells appeared in circulation, probably representing the vanishing population of mature erythrocytes that were overwhelmed by ENV replication.

Heavy mortality during the first week of captivity was not enumerated because it is likely to have resulted from the combined effects of capture/handling/transport stress and early stage VHS. However mortality declined to 35–52% during the second

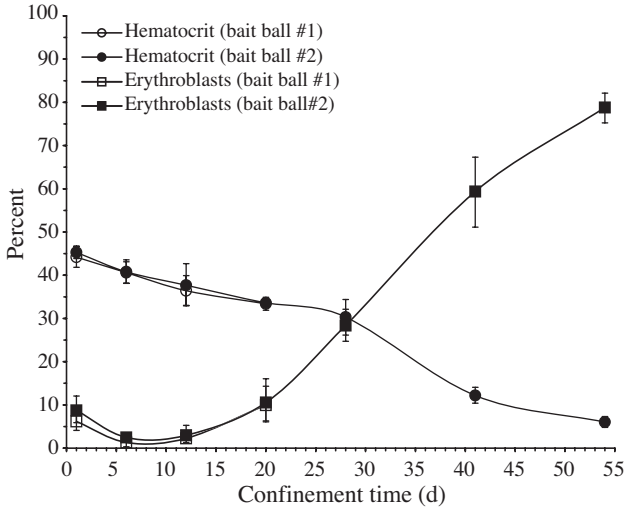

Fig. 3. *Clupea pallasii.* Blood changes during VHS and VEN epidemics. Erythroblast curves indicate percentage of immature erythrocytes among all blood cells. Data represent proportions corresponding to means of arcsine-transformed percentages in 3 replicate tanks; percent in each replicate was determined by sampling 10 fish tank^{-1} each sampling date. (Means ± 2 SD; 95% confidence intervals)

week, and remained steady at 31 to 35% thereafter. All mortality among fish naturally infected with *Ichthyophonus* occurred within 3 wk of confinement. Prevalence of ichthyophoniasis in the dead fish declined from 2.3% after 1 wk to 0% after 4 wk (Fig. 4). No further fish with ichthyophoniasis remained in the tanks after 20 d, when the final 2 live fish demonstrating gross signs were removed during random sampling for VHS and VEN.

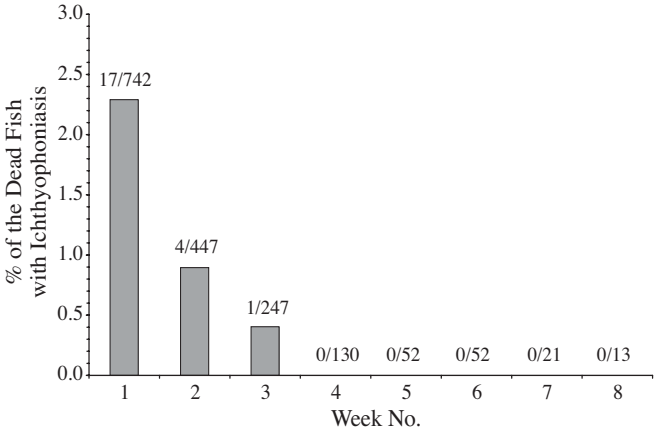

Fig. 4. *Clupea pallasii.* Ichthyophoniasis in fish that died during 54 d laboratory confinement. Fish were considered diseased when visible signs (black spots) were apparent on skin and *Ichthyophonus* was isolated from explant cultures. Data represent combined mortalities in all 6 replicates from both bait balls

Virology of erythrocytic inclusions

Erythrocytic inclusion bodies occurring after confinement of wild, juvenile herring were typical of those described for VEN, and challenges of specific-pathogen-free herring demonstrated that the observed erythrocytic inclusions were not an unreported sign of VHS. Among specific-pathogen-free, laboratory-reared herring challenged with VHSV, a rapid epidemic ensued, characterized by weekly mortalities of 78, 22 and 0% after Weeks 1 to 3, respectively. During the first week after challenge, VHSV tissue titers were $>6 \times 10^7$ pfu g^{-1} in pools of tissues from dead fish. However, erythrocytic inclusions did not occur in any (0 of 7) of the fish that survived for 22 d post-challenge, when the study was terminated. This contrasted with juvenile wild herring for which, 22 d after laboratory confinement and subsequent VHS outbreak, erythrocytic inclusions occurred in >90% of erythrocytes among surviving cohorts (Fig. 2). Among SPF negative controls exposed to HBSS, cumulative mortality was 0%, VHSV was not isolated, and erythrocytic inclusions were not detected in any survivors after 22 d (0 of 10).

Transmission electron micrographs of inclusioned erythrocytes demonstrated the presence of icosahedral-shaped virions with an ultrastructure typical of that described for erythrocytic necrosis virus (Dannevig & Thorud 1999). Virions averaged 125.4 nm in diameter (n = 10) and had an electron-dense core (Fig. 5). The virions were not necessarily associated with the cytoplasmic inclusions, generally appearing in clusters or singly throughout the cytoplasm; however, virion size and shape was similar to that reported for Atlantic herring *Clupea harengus* affected with VEN (Reno et al. 1978).

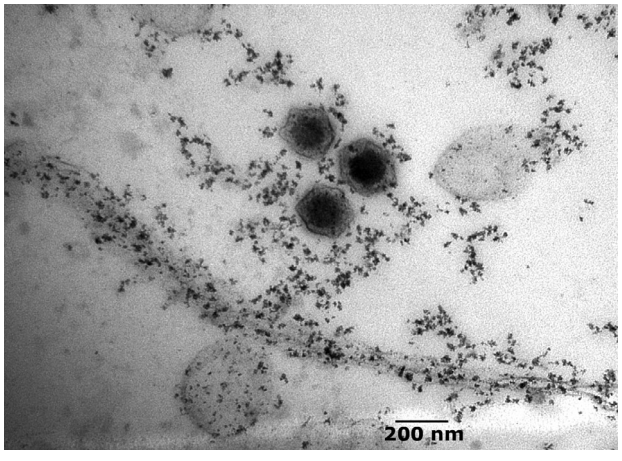

Fig. 5. *Clupea pallasii.* Transmission electron micrograph of virions within erythrocytes demonstrating cytoplasmic inclusions. Note icosahedral shape and electron-dense cores typical of iridoviruses

DISCUSSION

Capture and laboratory confinement of wild, juvenile herring resulted in progression of 3 infectious diseases, namely VHS, VEN and ichthyophoniasis; however, the timing and progression of the 3 diseases differed, reflecting differences in essential epidemiological factors such as the level of carriers in the captured population, virulence of the causative agent, mode of transmission and rapidity of spread. The acute rhabdoviral disease VHS was observed initially and probably resulted from infections that spread among susceptible individuals quickly after capture from a relatively few active carriers. For example, Kocan et al. (2001) demonstrated that within minutes after capture and confinement of wild, juvenile Pacific herring in static transport tanks, shedding of endogenous virus by a small percentage of individuals results in a concentration of waterborne VHSV sufficient to initiate lethal infections in naïve Pacific herring (Kocan et al. 1997). Although the herring used in our studies were not assayed for VHS immediately after capture, the prevalence and intensity of infection in these populations at any given time point are typically very low (Hershberger et al. 1999, Kocan et al. 2001). Susceptible fish that became infected in confinement either died from VHS or mounted a protective immune response, clearing the infection and surviving as individuals that were later refractory to re-infection (Kocan et al. 2001).

The peak in VEN followed that of VHS, and unlike VHS where the outbreak subsided rapidly after the peak, the resulting chronic viremia persisted for 54 d, at which time the study was terminated. Similar prolonged viremias of 3 to 4 mo occur in Atlantic cod infected with ENV (Reno et al. 1986). Intracellular infection of erythrocytes may offer protection from the host immune system and explain the prolonged viremia associated with ENV or other viruses with tropism for erythropoietic cells (Emmons 1985). While a nominally protective immune response sufficient to clear or suppress natural infections is likely to develop after exposure to ENV (MacMillan & Mulcahy 1979), this response would be absent among naïve fish or might be overwhelmed in previously exposed individuals confronted with a high level of virus that probably occurs from viral shedding and concentration in laboratory tanks. As with VHSV, the initial carrier rate for ENV among wild fish is probably low, but sufficient to initiate waterborne infections that spread and intensify among captive individuals in a progressive, but less acute, manner. The resulting VEN then manifests as a suite of severe blood dyscrasias (Haney et al. 1992) including erythrocytopenia, possible lymphopenia, erythroblastosis and reduced hematocrits that can result in direct mortality, especially in situations requiring high levels of respiratory activity; however, predisposition to secondary pathogens or increased sensitivity to environmental perturbations are more typically observed (MacMillan et al. 1989).

Unlike the acute disease VHS or the more chronic condition VEN, the early mortality of *Ichthyophonus*-infected cohorts probably resulted from pre-existing, chronic infections that did not spread within the confined population, but which were activated or exacerbated by the multiple stressors associated with capture, transport, confinement and progression of concomitant viral diseases. For example, elevated levels of circulating glucocorticoids occur after fish capture and handling (Mazur & Iwama 1993), and application of high levels of corticosteroids to fish chronically infected with *Ichthyophonus* results in progression to overt disease and mortality (Perry et al. 2004). Additionally, the health of infected cohorts was probably compromised from the onset, because *Ichthyophonus*-infected fish demonstrate reduced fitness compared to uninfected groups (Tierney & Farrell 2004), probably reducing their probability of resisting or recovering from viral diseases.

Our results indicate the need for precaution when ascribing causation for epizootics in populations of wild Pacific herring. For example, episodic herring kills caused by VHS occur periodically in the eastern North Pacific (Meyers et al. 1999, Traxler et al. 1999, Hedrick et al. 2003). However, if a delay of several days occurs prior to investigation of a VHS-induced epidemic among herring, the survivors may have cleared the primary agent and a secondary outbreak of VEN may be inappropriately identified as the cause of the epizootic. This principle, although general for disease ecology, is likely to be host- and pathogen-specific. For example, VHS is endemic to populations of Pacific sandlance *Ammodytes hexapterus* in Puget Sound, where the epidemiology is very similar to that of juvenile herring (Kocan et al. 2001); however, confined wild Pacific sandlances do not develop erythrocytic inclusion bodies typical of VEN (P. Hershberger unpubl. data) like those observed in Pacific herring.

The results of this study illustrate the differences in disease ecology and possible synergistic effects of pathogens affecting marine fish as well as the impact of other stressors on the disease process. For example, we hypothesize that such synergistic effects may begin to explain historical reports of massive mortalities associated with *Ichthyophonus*, a pathogen that can persist in a host for long periods without initiating rapid disease or mortality. If a high percentage of a population were chronically infected with *Ichthyophonus*, as is currently the case with herring populations in the eastern North Pacific (Hershberger et al. 2002, Jones &

Dawe 2002), and exposure of these populations to multiple natural stressors and/or other endemic pathogens results in increased mortality among the *Ichthyophonus*-infected cohorts, as in our laboratory experiment, then the resulting increase in mortality may represent a major epidemic similar to those reported in Atlantic herring populations (Sindermann 1990, Rahimian & Thulin 1996, McVicar 1999).

Acknowledgements. The authors acknowledge D. Elliott for interpretation of cellular degeneration in blood film preparations. G. Traxler and J. Richard provided VHSV isolates for the laboratory challenge studies. Preparation of samples for transmission electron microscopy was provided by C. Conway. Technical assistance was provided by Richard Hitchcock and pilot studies with VEN were performed by J. Hemplemann.

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Editorial responsibility: Jo-Ann Leong, Kaneohe, Hawaii, USA

Submitted: October 6, 2005; Accepted: February 6, 2006
Proofs received from author(s): June 12, 2006