Exxon Valdez Oil Spill
Long-Term Herring Research and Monitoring Program Final Report

Herring Disease Program II

Exxon Valdez Oil Spill Trustee Council Project 21120111-E
Final Report

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Study History: The biomass of Pacific herring in Prince William Sound, Alaska decreased from 120,000 metric tons to less than 30,000 tons following the *Exxon Valdez* oil spill in 1989. Cause(s) of this population decline remain unresolved; leading hypotheses include combinations of direct and indirect mortality from oil exposure, predation, competition for limited resources, and mortality from infectious and parasitic diseases. The *Exxon Valdez* Oil Spill Trustee Council launched early efforts to investigate the possible involvement of infectious and parasitic diseases in the initial population decline. These early efforts (1994-2003) consisted primarily of fish health surveillances lead by Dr. Gary Marty (formerly U. C. Davis) - *Exxon Valdez* Oil Spill Trustee Council projects 94320S, 95320S, 96162, 99328, 99462, 00462, 01462, 02462, and 03462. These early observations were expanded (1996-1999) to include experimental work by Dr. Richard Kocan et al. that was largely directed at understanding the effects of combined exposures to oil and pathogens – projects 96162, 97162, 97162 Supp, 98162, and 99162-A. However, lingering impacts of the *Exxon Valdez* oil spill were realized in the ensuing decades since the spill, when the Prince William Sound herring population failed to recover. As a result, restoration goals shifted towards understanding the factors (including mortality from infectious and parasitic diseases) that may be contributing to the ongoing failed population recovery; in response, the Integrated Herring Restoration Plan was formed in 2007. As part of the Integrated Herring Restoration Plan, annual disease surveillances were revitalized in a Herring Disease Program to provide infection and disease information that informs the ASA model; additionally, a significant portion of the Herring Disease Program included controlled experimental studies intended to determine cause-and-effect disease relationships. The first phase of the Herring Disease Program (Project 070819) was initiated as a 4-year project from 2007-2010; a one year no cost extension was granted for 2011. The Herring Disease Program was continued as a follow-up study (Project 10100132-I) from 2010-2013. During this period (2012-2016), the Herring Disease Program was integrated into the Herring Research and Monitoring program (Project 12120111), a 20-yr program intended to dovetail with Alaska Department of Fish and Game monitoring efforts and improve predictive models of herring stocks through observations and research. This current project (Herring Disease Program II, Project 21120111-E) reflects the pathogen and disease studies performed during years 6-10 (2017-2021) of the Herring Research and Monitoring program. Annual reports for Herring Disease Program II submitted during this current 5-yr period include 17120111-E, 18120111-E, 19120111-E, and 20120111-E.

Abstract: This report summarizes the most recent 5-year period (2017-2021) of observational and experimental work performed under the Herring Disease Program, funded by the *Exxon*

Valdez Oil Spill Trustee Council. The work summarizes pathogen surveillances in wild herring from Prince Willian Sound and reference locations in Sitka Sound and Puget Sound. Herring health surveillances were expanded to include serological surveillances for neutralizing antibodies against viral hemorrhagic septicemia virus in wild herring. Experimental studies, based on the annual production and availability of specific pathogen-free Pacific herring, were performed to provide pathogen and disease prevalence data to inform the age structured assessment model, validate a novel plaque neutralization assay using wild herring, determine the effects of temperature on viral hemorrhagic septicemia virus shedding, determine the susceptibility of Pacific herring to *Vibrio*, investigate the possibility of an invertebrate host for *Ichthyophonus*, determine the causes for abnormally high *Ichthyophonus* prevalence among juvenile Pacific herring that establish temporary residency in Cordova Harbor, and determine the impacts of salinity on fish-to-fish transmission of *Ichthyophonus*. Additionally, several value-added studies were included, as they were made possible because of the herring colonies produced in this project and the professional collaborations created within the Herring Research and Monitoring program.

Key words: Disease, *Ichthyophonus*, Infection, Pacific herring, pathogen, viral erythrocytic necrosis, viral hemorrhagic septicemia

Project Data: All herring surveillance metadata resulting from this project are housed on DataOne (https://search.dataone.org/view/10.24431%2Frw1k32b) and metadata from the laboratory studies are housed on ScienceBase (https://www.sciencebase.gov/catalog/items?filter=systemType%3DData+Release&filter=browseCategory%21%3DData+Release+-+In+Progress&q=hershberger).

The data custodian is Carol Janzen, Director of Operations and Development, Alaska Ocean Observing System, 1007 W. 3rd Ave. #100, Anchorage, AK 99501, 907-644-6703. janzen@aoos.org.

Data are archived by Axiom Data Science, a Tetra Tech Company, 1016 W. 6th Ave., Anchorage, AK 99501.

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EXECUTIVE SUMMARY

This study updates annual field surveillances for *Ichthyophonus*, viral hemorrhagic septicemia virus (VHSV), and erythrocytic necrosis virus infection prevalence in adult and juvenile Pacific herring in Prince William Sound (PWS), Alaska and reference locations including Sitka Sound, Alaska and Puget Sound, Washington. Although VHSV was not detected in any herring from the spring stock assessment surveys, annually recurring viral hemorrhagic septicemia epizootics were documented in age-0 cohorts from focused hot spots throughout the coastal eastern North Pacific. Field observations were expanded to include sero-surveillances for neutralizing antibodies against VHSV in PWS and Sitka Sound. These sero-surveillances indicated that VHSV antibody assessments can be extremely useful in documenting prior virus exposures that occurred before the spring stock assessments. Further, when combined with stock assessment models, these serological assessments may provide an understanding of natural mortality factors and recruitment failures. Controlled experimental studies successfully validated an antibody assay (plaque neutralization test) using wild herring with deduced exposure histories and confirmed that these antibodies can be detected for at least three years after a single exposure to the virus. Further, we demonstrated that the duration and magnitude of VHSV shedding is inversely related to water temperature, VHSV shedding relapses appear to be coincident with seasonally deceasing water temperatures, and VHSV remains in association with herring gills for months after it can no longer be isolated from internal tissues. Additional laboratory studies indicate that herring serve as a classic host reservoir for VHSV and that fully convalesced herring continue to shed infectious VHSV for months after the virus can no longer be isolated from the internal organs. Laboratory studies further demonstrated that herring are not susceptible to disease or mortality from the ubiquitous agents *Vibrio anguillarum* or *V. ordailii*. Surveillance of PWS zooplankton by quantitative polymerase chain reaction (qPCR) found no evidence of an intermediate host for *Ichthyophonus* and laboratory studies did not support the possibility of horizontal fish-to-fish transmission in Pacific herring. Rather, emphasis on the hypothesized mode of transmission has shifted to the possibility of parasite transmission via herring ovivory on infected fish eggs. Field surveillances indicated that Cordova Harbor serves as a hot spot for *Ichthyophonus* among age-0 herring that temporarily utilize the habitat. Novel tool development and opportunities afforded by the Herring Disease Program have been leveraged to foster collaborations and extend our understanding of these pathogens beyond Prince William Sound. For example, no cost, value-added studies indicated that VHSV DNA vaccines are efficacious in Pacific herring, *Ichthyophonus* currently occurs at high infection prevalence but low infection intensity in Pacific halibut throughout the northeast Pacific; the parasite also occur in lingcod (*Ophiodon elongatus*), yelloweye rockfish (*Sebastes ruberrimus*), Pacific cod (*Gadus macrocephalus*), and black rockfish (*Sebastes melanops*) in south central Alaska; qPCR is a sensitive and specific tool that can be utilized for *Ichthyophonus* surveillance in certain situations, sockeye salmon (*Oncorhynchus nerka*) demonstrate low susceptibility to viral

hemorrhagic septicemia; exposure of herring embryos to oil increases their susceptibility to *Ichthyophonus*; exposure of early life stages of herring to oil sometimes decreases their susceptibility to viral hemorrhagic septicemia; different genetic types of *Ichthyophonus* exist in sympatry throughout the eastern North Pacific; the susceptibility of Chinook salmon (*Oncorhynchus tshawytscha*) to *Ichthyophonus* varies with the host strain; and a *Ichthyophonus* was detected in a new species (opaleye, *Girella nigricans*) from a public display aquarium

INTRODUCTION

The biomass of adult Pacific herring (*Clupea pallasii*) in Prince William Sound (PWS) collapsed from 111,000-121,000 mt in 1988-1989 to 30,000 mt in 1993; since then, the population has remained depressed, fluctuating between 10,800-32,500 mt. Consequently, the PWS herring population is currently classified as an "injured resource" that is "not recovering" (EVOSTC 2002) and commercial herring fisheries have remained severely curtailed or closed. In addition to the human economic impacts of the population decline, the prolonged ecological impacts were devastating. In marine systems, particularly upwelling-driven systems like PWS, forage fishes, including Pacific herring, represent the primary energy link in the biological community, exerting both top-down control over primary and secondary production (phytoplankton and zooplankton) and bottom-up control over higher order predators (Rice 1995, Cury et al. 2000). The critical ecological position occupied by forage fishes is equally important in bridging the flow of inorganic nutrients (mobilized by primary and secondary production) and organic nutrients (utilized by higher trophic level predators).

Definitive cause(s) of the herring population decline and failed recovery in PWS remain undetermined; however, a leading hypothesis involves chronic and epizootic mortality that result from infectious and / or parasitic diseases (Marty et al. 1998, 2003). In 1993 only 20% of the anticipated adult herring biomass appeared in the known spawning areas. Returning fish were lethargic and demonstrated external hemorrhages consistent with viral hemorrhagic septicemia (VHS). The etiological agent, VHS virus (VHSV), was later isolated from moribund individuals. Subsequently, other suspected pathogens were identified in the PWS herring population, including *Ichthyophonus hoferi*, Anisakid worms, lymphocystis virus, *Goussia* sp. (an intestinal parasite), *G. clupearum* (a liver parasite), a testicular coccidian, a myxosporean in the gall bladder, *Ortholinea orientalis*, *Ceratomyxa auerbachi*, *Gyrodactylus spp* (monogenean trematodes), branchial ciliated protozoans, a renal myxosporean, *Epitheliocystis*, gastric trematodes, intestinal trematodes, intestinal cestodes (Marty et al. 1998), and erythrocytic necrosis virus (ENV; Hershberger et al. 2009). Among the pathogens occurring in PWS herring, VHSV, *Ichthyophonus*, and ENV are considered the primary pathogens of concern because they have been associated with epizootics in populations of wild herring, pilchards, and other forage species (Meyers et al. 1986, Hershberger et al. 2009, Garver et al. 2013, Burge et al. 2014). Alternative and complementary hypotheses accounting for the herring population dynamics

include competition with pink salmon for limited resources (Deriso et al. 2008) and predation on herring populations by humpback whales and other predators.

The North American strain of VHSV (Genogroup IVa) is periodically associated with epizootics (Garver et al. 2013) in wild marine fishes, where it can be highly virulent (Kocan et al. 1997). Monospecific VHS epizootics involving wild Pacific herring were reported during 1994 in Port Fredrick (Alaska), 1993 in Prince Rupert Sound (British Columbia; Traxler and Kieser 1994, Meyers and Winton 1995), and presumably 1942 in the Strait of Georgia (British Columbia; Tester 1942). Epizootics of mixed host assemblages involving Pacific sardines (*Sardinops sagax*) and Pacific herring occurred during 1998-1999 in Queen Charlotte Strait (British Columbia) and 2001-2002 Kyuquot and Nootka Sounds (British Columbia; Hedrick et al. 2003); similar mixed assemblage VHS epizootics involving Pacific herring, Pacific hake (*Merluccius productus*), and walleye pollock (*Gadus chalcogrammus*) occurred during 1998 in Lisianski Inlet (Alaska; Meyers et al. 1999). Furthermore, capture and confinement of Pacific herring, Pacific sand lance (*Ammodytes hexapterus*), and surf smelt (*Hypomesus pretiosus*) routinely results in locally severe VHS epizootics among the confined populations (Hershberger et al. 1999, Kocan et al. 2001, Hedrick et al. 2003). As larvae (Hershberger et al. 2007) and juveniles (Kocan et al. 1997), Pacific herring are highly susceptible to VHS, with laboratory exposures resulting in 66%-100% mortality. In the wild, juvenile herring are exposed to VHSV as early as 3 months post-hatch, shortly after their metamorphosis from larvae (Kocan et al. 2001). The prevalence and severity of VHSV in confined adult herring captured for spawn-on-kelp roe fisheries decreases with age (Hershberger et al. 1999), suggesting a mechanism of adaptive immunity in adults that originates from previous exposures to the virus.

Ichthyophonus hoferi is a member of the Mesomycetozoea, a monophyletic class of protists that includes several other important pathogens (Ragan et al. 1996, Herr et al. 1999, reviewed in Mendoza et al. 2002). Currently *I. hoferi* (reviewed in McVicar 2011) and *I. irregularis* are the only two recognized species in the genus, but other species have likely been grouped with *I. hoferi* based on the plasticity of morphological characteristics (Rasmussen et al. 2010). Recent molecular phylogenetic studies indicate that distinct genetic types of the parasite exist (Criscione et al. 2002, Halos et al. 2005, Rasmussen et al. 2010, Gregg et al. 2022); therefore, the organism will be referred to generically as *Ichthyophonus* hereafter. From 1898 through the mid-1950s, six major *Ichthyophonus*-related epizootics were described in Atlantic herring (*Clupea harengus*) from the Western North Atlantic (Sindermann 1990, McVicar 2011, Burge et al. 2014). More recently, a massive *Ichthyophonus*-related epizootic killed an estimated 300 million Atlantic herring in waters around Sweden and Denmark during the early 1990's (Rahimian and Thulin 1996), and epidemiological data implicate *Ichthyophonus* as a primary factor responsible for mortality in wild Pacific herring from estuarine waters of Washington State (Hershberger et al. 2002). Unpublished reports of large *Ichthyophonus* epizootics in the waters around Iceland during the fall and winter of 2008 resulted in the capture of massive numbers of herring that were unmarketable as a result of *Ichthyophonus*-induced tissue changes.

This project was undertaken to address gaps in our understanding of the epidemiological principles governing herring diseases in PWS, with the ultimate goal of offering the basis for adaptive management strategies intended to mitigate the population-level impacts of these diseases. Early studies of known herring pathogens in PWS were conducted by Dr. Gary Marty (U. C. Davis) and provided valuable information on trends of infection prevalence and intensity since 1994. In an effort to document changes in pathogen prevalence and severity within the PWS herring population, these surveillance efforts were continued by Hershberger et al., in the form of the Herring Disease Program (HDP) from 2007 to present. The incorporation of laboratory-based manipulations and observations in the HDP has led to the realization that some of our prior assumptions of these diseases were incorrect. For example, in a typical herring population, the prevalence of VHSV generally falls below the realistic detection threshold obtained from 60-fish subsamples of a population. Even though the endemic prevalence is typically extremely low, an epizootic can occur very quickly in response to changing host and environmental conditions (reviewed in Hershberger et al. 2016). As such, the incorporation of VHSV prevalence data into the age structured assessment (ASA) model as a forecaster of future disease potential is inconsequential from an epidemiological perspective. For example, a prevalence of 0% (0/60) in a pre-spawn herring population provides no indication of whether the population previously experienced a VHS epizootic, or if an epizootic is likely to occur in the future. For this reason, we have developed a serological assay (50% plaque neutralization assay [PNT]) that is capable of determining whether herring survived previous exposure to VHSV. This knowledge is extremely important from a disease forecasting perspective because survivors of prior VHSV exposure remain refractory to the disease for a very long time; presumably for life (reviewed in Hershberger et al. 2016). In this project, we initiate serological surveillances in an effort to ultimately apply the novel PNT assay to the level of herring populations. To achieve this goal, we proceeded with a series of validation experiments intended to determine the temporal and geographic scales of serological sampling that are required to assess population herd immunity against VHS.

Although *Ichthyophonus* is one of the most significant parasites of wild marine fishes, causing recurring population-level impacts during the past century (reviewed in Burge et al. 2014), very little is known about its natural life cycle. From a disease forecasting perspective, the most important information gap involves unresolved routes of exposure and transmission to planktivorous fishes. Laboratory studies indicate that the parasite is not readily transmitted from herring-to-herring via direct contact or through the water (Gregg et al. 2012). In previous studies, we successfully established infections in herring by habituating them to the consumption of large quantities of infected fish tissues (Hershberger et al. 2015); however, the relevance of this exposure route to wild populations of Pacific herring remains questionable, as herring are generally considered planktivores. These and other results have resulted in the elevation of a hypothesis that an invertebrate, intermediate host may be involved in completing the *Ichthyophonus* life cycle. However, until recently, appropriate scientific tools did not exist for examining the possibility of an *Ichthyophonus* intermediate host. Recent work performed in the

Herring Disease Program was successful in developing novel tools (quantitative polymerase chain reaction [qPCR] and chromogenic in situ hybridization) that will be useful for assessing wild zooplankters as intermediate hosts for *Ichthyophonus*. Here we continued to assess possible natural route(s) of *Ichthyophonus* transmission by expanding laboratory studies to assess horizontal transmission, examining a particular location (Cordova Harbor) where *Ichthyophonus* infection prevalence is unusually high, and by screening common herring food items as possible intermediate hosts for the parasite.

Finally, this project continued the production and employment of specific pathogen-free (SPF) laboratory animals in a variety of controlled laboratory experiments intended to address basic epidemiological principles affecting Pacific herring.

OBJECTIVES

- Provide pathogen and disease prevalence data to inform the ASA model (Chapter 1.1)
- Contribute to novel disease modeling approaches (Chapter 1.2)
- Produce SPF Pacific herring for laboratory experiments (Chapter 2.1)
- Process new and archived herring plasma samples for indications of prior VHSV exposure (Chapter 1.2)
- Validate the novel PNT using wild herring (Chapter 2.1)
- Determine the effects of temperature on VHSV shedding (Chapter 2.2)
- Determine the susceptibility of Pacific herring to *Vibrio* (Chapter 2.3)
- Investigate the possibility of an invertebrate host for *Ichthyophonus* (Chapter 2.4)
- Determine the causes for abnormally high *Ichthyophonus* prevalence among juvenile Pacific herring that establish temporary residency in Cordova Harbor (Chapter 2.5)
- Determine the impacts of salinity on fish-to-fish transmission of *Ichthyophonus* (Chapter 2.6)

Chapter 1 Herring Health Surveillances

1.1 Pathogen Assessments

Annual infection and disease surveillance for VHSV, ENV, and *Ichthyophonus* continued during 2017-2021. The primary purpose of these efforts involved the provision of disease inputs for the PWS herring ASA model. As reference locations, herring were sampled from Sitka Sound annually and from other populations whenever opportunities arose.

Throughout the 20-year biosurveillance period of the HDP, viral hemorrhagic septicemia virus was rarely isolated from adult Pacific herring, reflecting the typical endemic phase of the infection when the virus persists covertly (Table 1). However, more focused surveillance efforts identified the presence of VHS hot spots occurring among juvenile life history stages from certain nearshore habitats (Table 2). These outbreaks sometimes recurred annually in the same temporal and spatial patterns and were characterized by infection prevalence as high as 96% with high tissue titers. Longitudinal sampling during these epizootics indicated that some were

relatively transient, represented by positive samples on a single sampling date, and others were more protracted, with positive samples occurring throughout the first 10 weeks of the juvenile life history phase. For example, recurring VHS epizootics in age-0 herring were documented from Port Angeles Harbor in 2019 and 2020 and from Hot Springs Cove (west coast of Vancouver Island) in 2018 and 2019 (Table 2). The progression of the VHS epizootic in Port Angeles Harbor was chronicled in 2020, when the virus was detected in 7% of juveniles at the time of larval metamorphosis (June 23); infection peaked at 63% from July 21 to Aug 4; and waned to undetectable levels by September 22 (Fig. 1). These results indicate that VHS epizootics in free-ranging Pacific herring are more common than previously appreciated; however, they are easily overlooked if biosurveillance efforts are not designed around times and locations with high disease potential. As an attempted consolidation, survey results in Table 1 summarize all pathogen surveillance results since inception of the HDP in 2007. The results from 2017-2021 are novel to this project.

Ichthyophonus also remained endemic in herring throughout the NE Pacific during the study period, and infection prevalence generally increased with herring size / age (Fig. 2). Interestingly, the direct relationship between infection prevalence and herring size / age appeared to break down in recent years (2017-2021); however, this observation may simply be a reflection of the small sample size among the oldest / largest cohorts. A more focused examination of *Ichthyophonus* infection prevalence in the Salish Sea during 2016 (n = 2,232 Pacific herring from 38 midwater trawls) indicated spatial heterogeneity in infection prevalence. After controlling for the positive relationship between host size and *Ichthyophonus* infection, the probability of infection was approximately 6X higher in sites from North Hood Canal than in Puget Sound and the northern Straits. Temporal changes in *Ichthyophonus* infection probability were explained by seasonal differences in fish length, owing to Pacific herring life history and movement patterns. Reasons for the spatial heterogeneity remain uncertain but may be associated with density dependent factors inherent to the boom-bust cycles that commonly occur in Clupeid populations. It is possible that this spatial heterogeneity and its unresolved causes may also account for observed differences in infection prevalence between PWS and Sitka Sound.

*Table 1. Results of pathogen prevalence surveys in Pacific herring. Results from 2007-2016 were reported in H*erring *D*isease *P*rogram *final reports from previous years but are also included here as a complete inventory. Results from 2017-2021 are novel to this final report.* A/J = Adult/Juvenile, SD = standard deviation, VHSV = viral hemorrhagic septicemia virus, VEN =viral erythrocytic necrosis, PWS = Prince William Sound, *ND = no data.*

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2007	PWS	St. Matthews Bay	Apr 5	A	224 (17)	42% (25/60)	0% (0/60)	0% (0/60)	ADF&G #07-0540
		Simpson Bay	Apr 19	J	86 (6)	15% (9/60)	0% (0/60)	17% (10/60)	ADF&G #07-0543
		Sawmill Bay	Nov 30	A	215 (21)	25% (15/60)	0% (0/60)	0% (0/60)	MMFS #PWS 07-2
		Simpson Bay	Dec 2	A	187 (13)	37% (22/60)	0% (0/60)	0% (0/60)	MMFS #PWS 07-2
	Cook Inlet	Kamishak B	May 16	A	ND[A]	32% (19/60)	ND	ND	
		Kamishak B	May 27	A	ND[A]	20% (12/59)	ND	ND	
		Kamishak B	May 27	A	ND[A]	28% (17/60)	ND	ND	
	Sitka Sound	S. Cannon Island	Apr 19	A	215 (18)	28.3% (17/60)	0% (0/60)	0% (0/60)	MMFS #VHSV07-1 & ICH07-5
	Lynn Canal		Nov 10	A	199	11% (7/61)	ND	ND	ADF&G #08-0527
	Puget Sound[B]	Johnson Point	Jan 18	A	181 (8)	7% (4/59)	ND	ND	MMFS #ICH 07-1
		Port Orchard (Yukon Harbor)	Feb 1	A	181 (11)	17% (10/60)	ND	ND	MMFS #ICH 07-1
		Skagit Bay	Feb 8	A	184 (11)	37% (22/60)	ND	ND	MMFS #ICH 07-1
		Cherry Point	Apr 30	A	184 (13)	25% (15/60)	ND	0% (0/60)	MMFS #ICH 07-1
		Skagit Bay	Apr 25-26	J	117 (25)	ND	ND	3% (2/60)	MMFS #VEN Surveys
		Skagit Bay	May 22-24	J	111 (25)	ND	ND	37% (22/60)	MMFS #VEN Surveys
		Skagit Bay	June19-20	J	116 (17)	ND	ND	38% (23/60)	MMFS #VEN Surveys
		Skagit Bay	July24-25	J	110 (25)	ND	ND	35% (27/78	MMFS #VEN Surveys
		Skagit Bay	Aug 21-22	J	112 (21)	ND	ND	25% (18/71)	MMFS #VEN Surveys
		Skagit Bay	Sept18-20	J	109 (23)	ND	ND	36% (32/92)	MMFS #VEN Surveys
		Skagit Bay	Oct 16	J	109 (14)	ND	ND	6% (4/65)	MMFS #VEN Surveys
		Skunk Bay	Jul 2	J	134 (4)	ND	ND	2% (3/170)	MMFS #VEN Surveys
		Admiralty Inlet	Aug 1	J	129 (5)	ND	ND	0% (0/60)	MMFS #VEN Surveys
		Pt. Townsend Bay	Oct 16	J	80 (6)	ND	ND	20% (15/75)	MMFS #VEN Surveys

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2008	PWS	Fish Bay	Mar 19	A	236 (27)	33% (19/58)	0% (0/45)	2% (1/60)	ADF&G #08-0541
		Evans Pt.	Mar 24	A	208 (18)	ND	0% (0/60)	ND	ADF&G #08-0541
		Unknown	Mar 17	J	141 (11)	20% (12/59)	0% (0/60)	0% (0/60)	ADF&G #08-0541
		Whale Bay	Mar 24	J	149 (22)	15% (9/60)	0% (0/60)	0% (0/59)	ADF&G #08-0541
		Port Gravina	Nov 8-12	A	197 (23)	24% (19/80)	0% (0/80)	0% (0/80)	ADF&G #09-0522
		Simpson Bay	Nov 8-12	J	65 (7)	0% (0/78)	ND	1% (1/69)	AFD&G #09-0522
	Sitka Sound	Beli Rock	Mar 5	A	262 (14)	30% (18/60)	ND	ND	MMFS #AK-081A
		N. Middle Island	Mar 26	A	249 (14)	28% (17/60)	ND	2% (1/60)	ADF&G #08-0538 & #AK08-1C
	Lynn Canal		Feb 23	A	ND	5% (3/61)	ND	ND	ADF&G #08-0527
			Apr 12	A	ND	5% (3/61)	ND	ND	ADF&G #08-0527
			May 10	A	ND	19% (11/59)	ND	ND	ADF&G #08-0527
	Puget Sound	Drayton Pass	Jan 15	A	144 (7)	2% (1/60)	ND	ND	MMFS #ICH 08-1
		Port Orchard[C]	Feb 5	A	154 (16)	7% (4/60)	ND	ND	MMFS #ICH 08-1
		Skagit Bay	Feb 2	A	176 (17)	23% (14/60)	ND	ND	MMFS #ICH 08-1
		Holmes Harbor	Mar 13	A	193 (8)	48% (29/60)	ND	ND	MMFS #ICH 08-1
		Skagit Bay	May 29	J	148 (26)	ND	ND	17% (4/23)	MMFS #VEN FF08
		Skagit Bay	Jun 23-25	J	145 (24)	ND	ND	15% (8/53)	MMFS #VEN FF08
		Skagit Bay	Jul 22	J	109 (33)	ND	ND	7% (8/111)	MMFS #VEN FF08
		Skagit Bay	Aug 19	J	93 (9)	ND	ND	0% (0/60)	MMFS #VEN FF08
		Skagit Bay	Sep 17	J	89 (12)	ND	ND	2% (1/61)	MMFS #VEN FF08
		Skagit Bay	Oct 8	J	91 (9)	ND	ND	2% (1/60)	MMFS #VEN FF08

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2009	PWS	Port Gravina	Mar 20	A	199 (15)	43% (26/60)	0% (0/60)	0% (0/60)	ADF&G #09-0543 & MMFS #AK 09-1
		Port Gravina	Mar 20	J	168 (11)	25% (15/60)	0% (0/60)	0% (0/60)	ADF&G #09-0543 & MMFS #AK 09-1
		Simpson Bay	Mar 22	J	94 (8)	13% (8/60)	0% (0/60)	5% (3/60)	ADF&G #09-0543 & MMFS #AK 09-1
		Snug Corner Cove	Apr 13	A	217(27)	26% (16/62)	ND	ND	ADF&G #09-0543 & MMFS #AK 09-1
		Unknown Location	Apr 4-9	A	ND	45% (27/60)	ND	ND	ADF&G #09-0547
		Port Gravina	Nov 15	A	179 (17)	12% (7/60)	0% (0/60)	0% (0/60)	ADF&G 10-0529 & MMFS AK 09-1B
		Elrington Pass	Nov 17	A	216 (19)	17% (10/60)	0% (0/60)	0% (0/60)	ADF&G 10-0529 & MMFS AK 09-1B
		Simpson Bay	Nov 19	J	87 (14)	5% (3/60)	0% (0/60)	3% (2/60)	ADF&G 10-0529 & MMFS AK 09-1B
		Eaglek Bay	Nov 14	J	98 (4)	3% (1/29)	0% (0/29)	16% (5/31)	ADF&G 10-0530
		Lwr. Herring Bay	Nov 16	J	99 (4)	0% (0/14)	0% (0/14)	21% (3/14)	ADF&G 10-0530
		Simpson Bay	Nov 19	J	70 (12)	5% (1/20)	0% (0/20)	0% (0/33)	ADF&G 10-0530
	Cook Inlet	Kamishak Bay	May 8	A	ND[C]	3% (2/60)	ND	ND	
		Kamishak Bay	May 21	A	ND[C]	2% (1/60)	ND	ND	
	Sitka Sound	Guide Island	Feb 15-16	A	256 (15)	40% (32/80)	ND	ND	ADF&G #09-0540
		Unknown	Mar 24-27	A	270 (19)	46% (20/44)	0% (0/44)	ND	ADF&G #09-0545 & MMFS AK 09-2
		St. John Baptist Bay	Mar 24-27	A	248 (23)	31% (21/67)	0% (0/67)	0% (0/67)	ADF&G #09-0545 & MMFS AK 09-2
		Unknown	Mar 24-27	J	175 (7)	4% (3/69)	0% (0/69)	0% (0/69)	ADF&G #09-0545 & MMFS AK 09-2
	Lynn Canal	Cohen Isl. Amalga Trench	Feb 11-12	A	203 (15)	7% (3/44)	ND	ND	ADF&G #09-0539
		Fritz Cove, Outer Pt., Lena Pt.	Mar 18-19	A	ND	13% (8/60)	ND	ND	ADF&G #09-0541
		Gull Isl. & Benj. Isl. Trench	Nov 24	A	210 (14)	18% (11/60)	ND	ND	MMFS #AK09-4
		Benj. Isl. Trench & Fritz Cv.	Dec 7-8	A	198 (23)	8% (5/60)	ND	ND	MMFS #AK09-4
	Puget Sound	Port Orchard	Feb 2	A	170 (9)	3% (2/60)	ND	ND	MMFS #PS 09-1
		Skagit Bay	Feb 2	A	166 (23)	18% (11/60)	ND	ND	MMFS #PS 09-1
		Port Gamble	Feb 12	A	169 (12)	27% (16/60)	ND	ND	MMFS #PS 09-1
		Holmes Harbor	Mar 18	A	193 (20)	22% (13/60)	ND	ND	MMFS #PS 09-1
		Skagit Bay	Jun	J	122 (11)	ND	ND	55% (33/60)	MMFS #VEN FF09
		Skagit Bay	Jul	J	125 (10)	ND	ND	32% (19/60)	MMFS #VEN FF09
		Skagit Bay	Aug 12	J	121 (18)	ND	ND	4% (2/54)	MMFS #VEN FF09
		Skagit Bay	Oct 12	J	105 (18)	ND	ND	17% (10/60)	MMFS #VEN FF09
	San Fran. Bay[D]	Pt. Chauncey	Feb 11	A	155 (15)	0% (0/81)	ND	ND	MMFS #Ich 09-3B
		Pt. Chauncey	Feb 25	A	149 (18)	0% (0/60)	ND	ND	MMFS #Ich 09-3C

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2010	PWS	Port Gravina	Mar 16	A	213 (14)	18% (11/60)	0% (0/60)	2% (1/60)	ADF&G #10-0536 & MMFS # AK10-1
		Port Fidalgo	Mar 19	A	200 (15)	23% (14/60)	0% (0/60)	3% (2/60)	ADF&G #10-0536 & MMFS # AK10-1
		Simpson Bay	Mar 20	J	109 (23)	13% (8/60)	2-5%[F]	10% (6/60	ADF&G #10-0536 & MMFS # AK10-1
		Cordova Harbor	Jun 2-13	J	85 (12)	35% (17/49)	0% (0/49)	71% (38/48)	MMFS #AK 10-3
		Cordova Harbor	Aug 18	J	44 (3)	0% (0/18)	0% (0/54)	0% (0/17)	MMFS #AK 10-3
		Cordova Harbor	Set 28 -Oct 7	J	50 (6)	0% (0/22)	0% (0/22)	0% (0/21)	MMFS #AK 10-3
		Simpson Bay	Nov 3	J	73 (7)	0% (0/38)	ND	6% (2/36	MMFS #AK 10-3
		Port Fidalgo	Nov 4	J	77 (4)	0% (0/22)	ND	5% (1/22)	MMFS #AK 10-3
		Eaglik	Nov 5	J	90 (9)	0% (0/34)	ND	26% (8/31)	MMFS #AK 10-3
		Whale Bay	Nov 10-11	J	95 (33)	3% (2/58)	2% (1/60)	18% (10/55)	MMFS #AK 10-3
	Cook Inlet	Kamishak B	May 4	A	ND[G]	2% (1/60)	ND	ND	
		Kamishak B	May 18	A	ND[G]	3% (2/60)	ND	ND	
	Sitka Sound	Indian River	Mar 22-24	A	242 (22)	27% (16/60)	0% (0/60)	2%	MMFS #AK10-2
		Boarder / Sitka Rocks	Mar 22-24	A	209 (28)	15% (9/60)	ND	3%	MMFS #AK10-2
		Mountain Point Kruzof Island	Mar 22-24	A	241 (25)	37% (22/60)	0% (0/60)	0%	MMFS #AK10-2
	Lynn Canal	Shelter Isl.	Mar 15-16	A	202 (20)	5% (3/56)	ND	ND	MMFS #AK10-4
		Bridget Cove	Apr 26	A	212 (11)	13% (5/40)	ND	ND	MMFS #AK10-4
	Puget Sound	Squaxin Pass	Jan 28	A	140 (12)	3% (2/60)	ND	ND	MMFS #PS10-1
		Holmes Harbor	Mar 23	A	171 (15)	28% (17/60)[H]	ND	ND	MMFS #PS10-1
		Hood Canal[I,J]	May 25&27	A	140 (24)	44% (43/97)	ND	ND	MMFS #PS10-1

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2011	PWS	St. Matthew's B	Apr 2	A	246	≥12%[K] (7/60)	0% (0/60)	0% (1/60)	ADF&G #11-0538 & MMFS# AK10-3
		Port Gravina	Apr 4	A	219	27% (16/60)	0% (0/60)	2% (1/60)	ADF&G #11-0538 & MMFS #AK10-3
		Hell's Hole	Apr 6	A	253	47% (28/60)	0% (0/60)	2% (61/60	ADF&G #11-0538 & MMFS #AK10-3
		Port Gravina	Nov 21	A[L]	205	63% (19/30)	0% (0/60)	3% (1/30)	ADF&G #12-0524 & MMFS #AK11-8
		Port Gravina	Nov 22	A[L]	157	13% (4/30)	0% (0/60)	0% (0/30)	ADF&G #12-0524 & MMFS #AK11-8
		Lwr Herring B	Mar 11	J	96	2% (1/59)	0% (0/60)	23% (14/60)	MMFS #AK 11-1
		Eaklek	Mar 15	J	113	5% (3/60)	0% (0/60)	2% (1/59)	MMFS #AK 11-1
		Port Fidalgo	Mar 16	J	76	10% (6/58)	0% (0/60)	13% (8/60)	MMFS #AK 11-1
		Simpson B	Oct 13	J	52	ND	0% (0/47)	ND	MMFS #AK 11-6
		Simpson B	Nov 15	J	60	ND	0% (0/60)	ND	MMFS #AK 11-9
		Whale B	Nov 20	J	83	0% (0/60)	0% (0/60)	ND	MMFS #AK 11-9
		Simpson B	Dec 13	J	60	0% (0/60)	0% (0/60)	ND	MMFS #AK 11-10
	Cook Inlet	Kamishak B	May 4	A	ND[M]	0% (0/60	ND	ND	
		Kamishak B	May 13	A	ND[M]	2% (1/60)	ND	ND	
	Sitka Sound	Bear Cove	Mar 24	J	108 (11)	2% (1/60)	63% (38/60)	3%	MMFS #AK 11-4
		Long Isl.	Mar 22	A	232 (16)	18% (11/60)	0% (0/60)	0%	MMFS #AK 11-4
		Salisbury Snd.	Apr 6	A	228 (20)	20% (12/60)	ND	ND	MMFS #AK 11-4
	Lynn Canal	Halibut Cove	Jan 12	A	ND	2% (1/60)	ND	ND	
		Amalga Tr.	Jan 28	A	ND	10% (6/60)	ND	ND	
		Amalga Tr.	Apr 9	A	ND	18% (11/60)	ND	ND	
		Auke Bay	Apr 18, Jun 4	A	202 (15)	18% (11/60)	ND	ND	
	Puget Sound	Squaxin Pass	Jan 28	A	140 (12)	3% (2/60)	ND	ND	MMFS #PS10-1
	British	Little Qualicum	Mar 17	A	189 (14)	8% (5/60)	ND	ND	MMFS #BC11-1
	Columbia,	Sydney Inlet	Mar 23	A	183 (16)	20% (12/60)	ND	ND	MMFS #BC11-1
	Canada	Prince Rupert	Mar 23	A	167 (18)	22% (13/60)	ND	ND	MMFS #BC11-1
		Kwakshua Inlet	Mar 24	A	194 (16)	27% (16/60)	ND	ND	MMFS #BC11-1
		Haida Gwaii	Mar 26	A	191 (12)	8% (5/60)	ND	ND	MMFS #BC11-1
		Haida Gwaii	Mar 30	A	192 (13)	5% (3/60)	ND	ND	MMFS #BC11-1

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2012	PWS	Port Gravina	Mar 28	A	218	42% (25/60)	0% (0/60)	0% (0/60)	ADF&G #12-0533 & MMFS# AK12-4
		Port Gravina	Mar 31	A	215	40% (24/60)	0% (0/60)	0% (0/60)	ADF&G #12-0533 & MMFS# AK12-4
		Fidalgo B	Apr 2	A	231	35% (21/60)	0% (0/60)	0% (0/60	ADF&G #12-0533 & MMFS# AK12-4
		Port Gravina	Nov 15	A	159	3% (2/60)	0% (0/60)	0% (0/60)	MMFS #AK12-8
		Simpson B	Jan 11	J	57	0% (0/28)	0% (0/60)	ND	MMFS #AK12-1
		Simpson B	Apr	J	ND	3% (1/30)	0% (0/30)	ND	MMFS #AK 12-3
	Cook Inlet	Kamishak Bay	May 7	A	ND	2% (1/60)	ND	ND	
	Sitka Sound	N. Khasiana Isl.	Apr 3	A	232 (23)	20% (12/60)	0% (0/60)	0% (0/60)	ADF&G #12-0534 & MMFS# AK12-5
		St. John Bay	Apr 4	A	214 (24)	32% (19/60)	0% (0/60)	0% (0/30)	ADF&G #12-0534 & MMFS# AK12-5
		Sitka breakwall	Apr 4	A	225 (22)	10% (6/60)	0% (0/60)	0% (0/60)	ADF&G #12-0534 & MMFS# AK12-5
	Lynn Canal	Tee Harbor	Jun 8	A	176 (13)	0% (0/60)	ND	ND	MMFS #AK 12-6
2013	PWS	Port Gravina	Mar 27	J	147	3% (2/60)	0% (0/60)	0% (0/60)	ADF&G #13-0537 & MMFS# AK13-2
		Port Gravina	Mar 31	A	232	34% (20/59)	0% (0/60)	0% (0/60)	ADF&G #13-0537 & MMFS# AK13-2
		Port Gravina	Apr 1	A	225	32% (19/60)	0% (0/60)	0% (0/60	ADF&G #13-0537 & MMFS# AK13-2
		Lwr Herring B	Nov 9	J	93	5% (3/60)	0% (0/60)	12% (7/59)	MMFS #AK13-4
		Port Gravina	Nov 13	J	90	0% (0/39)	0% (0/39)	18% (7/39)	MMFS #AK13-4
		Cordova Hbr.	Nov 20	J	70	0% (1/61)	0% (0/61)	7% (4/61)	MMFS #AK13-4
	Sitka Sound	Apple Islands	Mar 29	A	246 (28)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G #13-0538 & MMFS #AK13-3
		Silver Bay	Mar 30	A	251 (16)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G #13-0538 & MMFS #AK13-3
		Unknown	Mar 30	A	226 (26)	18% (11/60)	0% (0/60)	0% (0/60	ADF&G #13-0538 & MMFS #AK13-3
	Craig	Diamond Point	Feb 20	A	214 (23)	22% (13/60)	ND	ND	MMFS #AK 13-1
	Puget Sound	Hood Canal[M]	May 19	A	171 (18)	57% (25/44)	ND	ND	MMFS #PS 13-1

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2014	PWS	Sheep B	Mar 26	A	217	24% (15/60)	2-8%[O]	0% (0/60)	ADF&G #14-0534 & MMFS #AK14-1
		Fidalgo B	Mar 28	A	218	22% (13/60)	0% (0/60)	0% (0/60)	ADF&G #14-0534 & MMFS #AK14-1
		Snug Corner	Mar 29	A	242	32% (19/60)	0% (0/60)	0% (0/60	ADF&G #14-0534 & MMFS #AK14-1
		Simpson	Nov 15-23	J	78 (12)	2% (1/60)	0% (0/60)	ND	MMFS #AK14-4
		Beartrap	Nov 16	J	70 (5)	2% (1/61)	0% (0/61)	ND	MMFS #AK14-4
		Eaglek	Nov 19	J	96 (4)	3% (2/61)	0% (0/61)	ND	MMFS #AK14-4
	Cook Inlet	Kamishak Bay	Ap 30	A	ND	0% (0/60)	0% (0/60)	2% (1/60)[P]	ADF&G #14-0078 & MMFS #AK14-3
			May 13	A	ND	0% (0/60)	0% (0/59)	0% (0/60)	ADF&G #14-0078 & MMFS #AK14-3
	Sitka	Causeway	Mar 26	A	245 (26)	25% (15/60)	0% (0/60)	2% (1/60)	ADF&G #14-0533 & MMFS #AK14-2
		Middle Island	Mar 27	A	241 (31)	20% (12/59)	0% (0/60)	0% (0/60)	ADF&G #14-0533 & MMFS #AK14-2
		Inner Point	Mar 28	A	222 (20)	27% (16/60)	0% (0/60)	0% (0/60)	ADF&G #14-0533 & MMFS #AK14-2
	Puget Sound[Q]	Lopez Isl.	Sep 11	J	ND	ND	27% (6/22)	ND	MMFS #PS4-1
		Waldron Isl.	Sep 12	J	ND	ND	13% (3/24)	ND	MMFS #PS4-1
2015	PWS	Gravina Pt	Apr 3	A	228 (17)	25% (15/60)	0% (0/60)	0% (0/60)	ADF&G #15-0533 & MMFS #AK15-2
		Simpson Bay	Nov 6	J	ND	2% (1/46)	0% (0/46)	ND	MMFS #AK15-4
		Lwr Herring B.	Nov 11	J	85 (5)	2% (1/54)	0% (0/54)	ND	MMFS #AK15-4
		E Whale Bay	Nov 12	J	89 (7)	3% (2/60)	0% (0/60)	ND	MMFS #AK15-4
	Cook Inlet	Kamishak Bay	Apr 27	A	ND	2% (1/60)	0% (0/60)	0% (0/60)	ADF&G #2015-0048
	Sitka	Beili Rock	Mar 20	A	239 (26)	10% (6/60)	0% (0/60)	0% (0/60)	ADF&G 15-0532 &MMFS #AK15-1
		Beili Rock	Mar 22	A	250 (22)	13% (8/60)	0% (0/60)	0% (0/60)	ADF&G 15-0532 &MMFS #AK15-1
		Beili Rock	Mar 22	A	231 (24)	20% (12/60)	0% (0/60)	0% (0/60)	ADF&G 15-0532 &MMFS #AK15-1
	Ketchikan[R]	Near Craig	Dec 17	A	193 (17)	ND	0% (0/76)	ND	MMFS #AK15-5

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2016	PWS	Red Head Pt.	Apr 7	A	205 (28)	24% (15/60)	0% (0/60)	0% (0/60)	ADF&G #16-0539 & MMFS #AK16-2
		Knowles Head E	Apr 8	A	212 (22)	29% (17/59)	0% (0/60)	0% (0/60)	ADF&G #16-0539 & MMFS #AK16-2
		Snug Corner C	Mar 29	A	234 (21)	47% (28/60)	0% (0/60)	2% (1/60)	ADF&G #16-0539 & MMFS #AK16-2
		Simpson Bay	Oct 29	J	82 (4)	25% (15/60)	0% (0/60)	2% (1/60)	MMFS #AK 16-3
		Eaglek Bay	Oct 30	J	95 (5)	3% (2/60)	0% (0/60)	2% (1/60)	MMFS #AK 16-3
		Lower Herring B	Nov 2	J	96 (4)	10% (6/60)	0% (0/60)	0% (0/60)	MMFS #AK 16-3
	Sitka	S. Salsbury Anika	Mar 21	A	218 (23)	10% (6/60)	0% (0/60)	0% (0/60)	ADF&G #16-0537 & MMFS #AK16-1
	Sound	N. Crest	Mar 22	A	215 (13)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G #16-0537 & MMFS #AK16-1
		Pt. Brown	Mar 22	A	217 (24)	22% (13/60)	0% (0/60)	0% (0/60)	ADF&G #16-0537 & MMFS #AK16-1
	Puget	Dabob Bay	Feb 10	J	129 (9.5)	23% (28/120)	ND	ND	MMFS #PS16-1
	Sound	S. Lopez	Feb 17	J	112 (7.6)	0% (0/30)	ND	ND	MMFS #PS16-1
		Dallas Bank	Feb 19	J	114 (8.5)	2% (1/60)	ND	ND	MMFS #PS16-1
		Strait of Georgia	Feb 23	J	121 (12)	5% (3/60)	ND	ND	MMFS #PS16-1
		Squamish Harbor	Apr 4	J	131 (14)	15% (9/60)	ND	ND	MMFS #PS16-1
		Dabob Bay	Apr 4	A	169 (12)	13% (8/60	ND	ND	MMFS #PS16-1
		Squamish Harbor	Apr 4	A	157 (17)	18% (9/50)	ND	ND	MMFS #PS16-1
		S. Saratoga	Apr 5	J	131 (7.3)	2% (1/60)	ND	ND	MMFS #PS16-1
		N. Saratoga	Apr 6	A	156 (17)	2% (1/58)	ND	ND	MMFS #PS16-1
		Oak Bay	Apr 5	J	143 (11)	11% (7/62)	ND	ND	MMFS #PS16-1
		E. Pt Angeles	Apr 13	J	126 (11)	0% (0/60)	ND	ND	MMFS #PS16-1
		Yukon Harbor	Apr 18	J	136 (11)	7% (4/60)	ND	ND	MMFS #PS16-1
		Nisqually	Apr 20	J	151 (10)	0% (0/60)	ND	ND	MMFS #PS16-1
		Colvos Passage	Apr 19	J	142 (9.0)	3% (2/60)	ND	ND	MMFS #PS16-1
		Nisqually	Jun 1	J	155 (10)	13% (8/60)	ND	ND	MMFS #PS16-1
		N. Saratoga	Jun 8	A	172 (18)	7% (4/60)	ND	ND	MMFS #PS16-1
		Strait of Georgia	Jun 13	J	126 (3.9)	12% (7/60)	ND	ND	MMFS #PS16-1
		Squamish Harbor	Aug 24	J	90.0 (6.6)	5% (3/60)	ND	ND	MMFS #PS16-1
		Dabob Bay	Aug 24	A	177 (7.3)	40% (24/60)	ND	ND	MMFS #PS16-1
		S. Lopez	Aug 26	J	91 (4.5)	2% (1/60)	ND	ND	MMFS #PS16-1
		N. Saratoga	Aug 25	A	150 (4.8)	5% (3/60)	ND	ND	MMFS #PS16-1
		President Channel	Aug 27	J	89 (6.7)	0% (0/60)	ND	ND	MMFS #PS16-1
		President Channel	Oct 4	J	102 (3.6)	2% (1/60)	ND	ND	MMFS #PS16-1
		Yukon Harbor	Oct 11	A	173 (6.9)	5% (3/60)	ND	ND	MMFS #PS16-1
		Strait of Georgia	Oct 5	A	173 (15)	7% (4/60	ND	ND	MMFS #PS16-1
		N. Saratoga	Oct 12	J	130 (24)	3% (2/60)	ND	ND	MMFS #PS16-1
		N. Saratoga	Oct 12	J	178 (11)	10% (6/60)	ND	ND	MMFS #PS16-1
		Colvos	Oct 11	J	117 (5.5)	0% (0/60)	ND	ND	MMFS #PS16-1

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2016		Oak Bay	Oct 18	J	106 (8.0)	15% (9/59)	ND	ND	MMFS #PS16-1
		Dabob Bay	Oct 19	A	179 (12)	53% (32/60)	ND	ND	MMFS #PS16-1
		N. Saratoga	Dec 7	A	177 (11)	13% (8/60)	ND	ND	MMFS #PS16-1
		Dallas Bank	Dec 13	J	100 (7.7)	2% (1/60)	ND	ND	MMFS #PS16-1
2017	PWS	Port Gravina	Apr 7	A	195 (14)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G # 17-0537 & MMFS #AK 17-2
		Rocky Bay	Apr 10	mix	140 (46)	10% (6/60)	0% (0/60)	0% (0/60)	ADF&G # 17-0537 & MMFS #AK 17-2
		Port Fidalgo	Apr 10	A	191 (14)	23% (14/60)	0% (0/60)	0% (0/60)	ADF&G # 17-0537 & MMFS #AK 17-2
	Sitka	Unknown	Mar 24	A	221 (14)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G #17-0536 & MMFS #AK 17-1
		S. Magoun Isl.	Mar 25	A	225 (15)	15% (9/60)	0% (0/60)	0% (0/60)	ADF&G #17-0536 & MMFS #AK 17-1
		Unknown	Mar 25	A	225 (16)	8% (5/60)	0% (0/60)	0% (0/60)	ADF&G #17-0536 & MMFS# AK 17-1
2018	PWS	Hells Hole	Apr 10-11	mix	152 (36)	10% (6/59)	0% (0/60)	0% (0/60)	ADF&G # 18-0543 & MMFS #AK 18-2
		Cedar Bay	Apr 12	A	201 (15)	22% (13/59	0% (0/60)	0% (0/60)	ADF&G # 18-0543 & MMFS #AK 18-2
		Rocky Bay	Apr 13	A	204 (16)	8.3% (5/60)	0% (0/60)	0% (0/60)	ADF&G # 18-0543 & MMFS #AK 18-2
	Sitka	Guide Island	Mar 22	A	221 (15)	21% (12/56)	0% (0/56)	0% (0/56)	ADF&G # 18-0539 & MMFS #AK 18-1
		Unknown	Mar 23	A	215 (17)	18% (9/49)	0% (0/49)	0% (0/49)	ADF&G # 18-0539 & MMFS #AK 18-1
		Kruzof Island	Mar 23	A	224 (23)	24% (17/72)	0% (0/72)	0% (0/72)	ADF&G # 18-0539 & MMFS #AK 18-1
	P. Sound	Squaxin Pass	Feb 8	A	177 (13)	3% (2/60)	ND	ND	MMFS #PS 18-1
2019	PWS	Hawkins / Double Bluff	Apr 5	A	176 (16)	18% (11/60)	0% (0/60)	2% (3/176)[S]	ADF&G # 19-0543 & MMFS #AK 19-2
		Canoe Pass	Apr 6	mix	157 (28)	18% (11/60)	0% (0/60)	2% (3/176)[S]	ADF&G # 19-0543 & MMFS #AK 19-2
		Windy Bay	Apr 6	A	179 (13)	20% (12/59)	0% (0/60)	2% (3/176)[S]	ADF&G # 19-0543 & MMFS #AK 19-2
	Sitka	Kristoff	Mar 25	A	204 (17)	8% (5/60)	0% (0/60)	0% (0/60	ADF&G # 19-0540 & MMFS #AK 19-1
		Kristoff	Mar 26	A	197 (17)	19% (11/59)	0% (0/60)	0% (0/60	ADF&G # 19-0540 & MMFS #AK 19-1
		Whitestone Narrows	Mar 27	A	204 (18)	15% (9/60)	0% (0/60)	0% (0/60	ADF&G # 19-0540 & MMFS #AK 19-1
	P. Sound	Vashon Island[T]	Jan 24-25	J	121 (7.0)	0% (0/30)	0% (0//60)	ND[U]	MMFS PS #19-1
2020	PWS	Canoe Pass	Apr 8	A	213 (13)	19% (25/130)	0% (0/130)	ND[U]	MMFS #AK 20-2
		Double Bay	Apr 10	A	223 (13)	12% (7/58)	0% (0/70)	ND[U]	MMFS #AK 20-2
	Sitka	Kruzof	Mar 31	A	215 (14)	18% (11/60)	0% (0/60)	ND[U]	MMFS #AK 20-1
		Low Island	Apr 1	A	215 (14)	18% (11/60)	0% (0/60)	ND[U]	MMFS #AK 20-1
		Silver Bay	Apr 2	A	204 (10)	28% (17/60)	0% (0/60)	ND[U]	MMFS #AK 20-1
	P. Sound	Squaxin Pass	Jan 27	A	159 (9.1)	0% (0/27)	0% (0/27)	0% (0/27)	MMFS #PS 20-1
		Port Orchard	Jan 29	A	169 (7.8)	3% (1/30)	0% (0/30)	0% (0/30)	MMFS #PS 20-1
		Semiahmoon B.	Feb 18	A	161 (10)	17% (5/30)	0% (0/30)	0% (0/30)	MMFS #PS 20-1

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2021	PWS	Hell's Hole	Apr 2	A	219 (15)	17% (10/60)	ND[U]	ND[U]	MMFS # AK 21-2
		Hell's Hole	Apr 3	A	215 (14)	20% (12/60)	ND[U]	ND[U]	MMFS # AK 21-2
		Red Head	Apr 11	mix	160 (19)	22% (13/60)	ND[U]	ND[U]	MMFS # AK 21-2
	Sitka	Hayward St.	Mar 29	A	220 (15)	24% (14/58)	0% (0/60)	ND[U]	MMFS #AK 21-1
		Lisanski Pt.	Mar 30	A	219 (14)	20% (12/59)	0% (0/60	ND[U]	MMFS #AK 21-1
		Deep Inlet	Mar 30	A	222 (20)	17% (10/59)	0% (0/60)	ND[U]	MMFS #AK 21-1

[A]Herring lengths in Cook Inlet (2007) were recorded as standard length, not fork length.
[B]160 northern anchovies (2007) were also sampled from Puget Sound (Holmes Harbor) on March 11; neither VHSV nor *Ichthyophonus* was detected.
[C]Four Pacific Sardines were collected from Port Orchard on March 5, 2008; none tested positive for VHSV.
[D]Herring lengths in Cook Inlet (2009) were recorded as standard length, not fork length.
[E]Additional samples from San Francisco Bay (2009) included 69 longfin smelt (Jan 6-13) and 70 striped bass (May 15); none tested positive for *Ichthyophonus.*
[F]A single pooled (2010) sample containing tissues from 3 fish tested positive (n=60) for VHSV. Therefore, the prevalence was 1-3 / 60.
[G]Herring lengths in Cook Inlet (2010) were recorded as standard length, not fork length.
[H]*Ichthyophonus* prevalence was 6% (1/17) in Pacific staghorn sculpin and 78% (28/36) in American shad in 2010.
[I]Biased sample in 2010: largest fish were removed from this sample for other purposes prior to determination of *Ichthyophonus* prevalence.
[J]Sample consisted of post-spawn adult herring (2010).
[K]*Ichthyophonus* cultures were frozen by the airline (2011), killing the parasite; therefore, the true population prevalence was likely greater than the reported prevalence.
[L]Both groups of fish from Gravina (2011) were from the same school; the first 30 were high-graded for larger fish; the second 30 were representative of the population.
[M]Herring lengths in Cook Inlet (2011) were recorded as standard length, not fork length.
[N]Hood Canal (2013) sample consisted of post-spawn herring.
[O]Cytopathic effects were detected in a single pooled sample (2014) containing tissues from 5 fish after the 3rd passage; therefore, the infection prevalence was 1-5 / 60. Viral titer was below 10^1 PFU / g. VHSV was confirmed in the cell culture supernatant by nested cPCR.
[P]VEN inclusions (2014) graded at a 3+ infection severity from Fish #38.
[Q]Herring samples were collected from two locations in the San Juan Islands (2014) during Chinook salmon beach seining efforts. VHSV symptoms observed included a bloody exterior, with an increase to 10% of observed fish showing these symptoms over the course of the summer. Symptomatic herring were high-graded (i.e., not a random sample), frozen -20°C, and sent to the USGS – Marrowstone for VHSV testing (plaque assay). Warm water temperatures were noted, as well as unusually large numbers of age 0+ herring and unusually low numbers of age 0+ sand lance. Positive samples were confirmed by qPCR using VHSV-specific primers.
[R]Submitted by Eric Coonradt (ADF&G) in 2015: concerns of a possible disease event occurring near Craig, AK. External signs included a bloody exterior, with possible causes hypothesized to include infection or predator marks. Affected individuals comprised approximately 1 out of every 100; those with obvious symptoms were removed from the larger sample and photographed (see photo below). Samples were shipped frozen to MMFS (Marrowstone Marine Field Station), a 5-gallon bucket of frozen herring that were stored 60 herring were stored at -20°C. VHSV was not detected.
[S]All 3 VEN-positive samples from 2019 had low densities of intraerythrocytic inclusions (<10% of red cells demonstrated viral inclusion bodies).
[T]2019 Vashon Island Fish were collected from a fish kill event.
[U]VHSV and / or VEN samples were not collected in 2020 and 2021 due to COVID limitations.

Table 2. Viral hemorrhagic septicemia (VHS) virus survey results from Pacific herring during atypical epizootic periods. SD = standard deviation, NA = data Not Available.

Region	Year	Stock	Collection Site	Collection Date	Mean fork length mm (SD)	VHS virus Prevalence
AK, USA	2011	Sitka Sound	Bear Cove Bay	March 24	108 (11)	63% (38/60)
BC, Canada	2018	W. Vancouver Island	Hot Springs Cove	June 24	NA[A]	85% (22/26)
	2019	W. Vancouver Island	Hot Springs Cove	June 27	NA[B]	96% (29/30)
WA, USA	2014	Puget Sound	Lopez Island	Sept 11	NA[A]	27% (6/22)
		Puget Sound	Waldron Island	Sept 12	NA[A]	13% (3/24)
	2018	Puget Sound	Pt. Angeles Harbor	Sept 18 – Nov 5	67 (8.0) – 78 (7.4)	Fig. 1
	2019	Puget Sound	Pt. Ludlow Harbor	July 25 – Sept 25	52 (2.4) - 78 (6.8)	Not Shown
		Puget Sound	Pt. Angeles Harbor	July 23 – Sept 24	64 (6.7) – 76 (7.0)	Fig. 1
	2020	Puget Sound	Pt. Angeles Harbor	June 23 – Oct 20	37 (2.0) – 63 (8.3)	Fig. 1

[A]Photographs suggest 50mm-60 mm length.

[B]Pacific herring were sampled from two locations in the north Puget Sound, WA region (San Juan Islands). External signs of VHS included hemorrhaging along the flank in a small proportion of individuals, increasing to approximately 10% of the population throughout the summer. Samples from 2014 were not randomly collected; rather, individuals demonstrating external signs of VHS were selected, frozen at -20°C, and submitted for laboratory diagnostics.

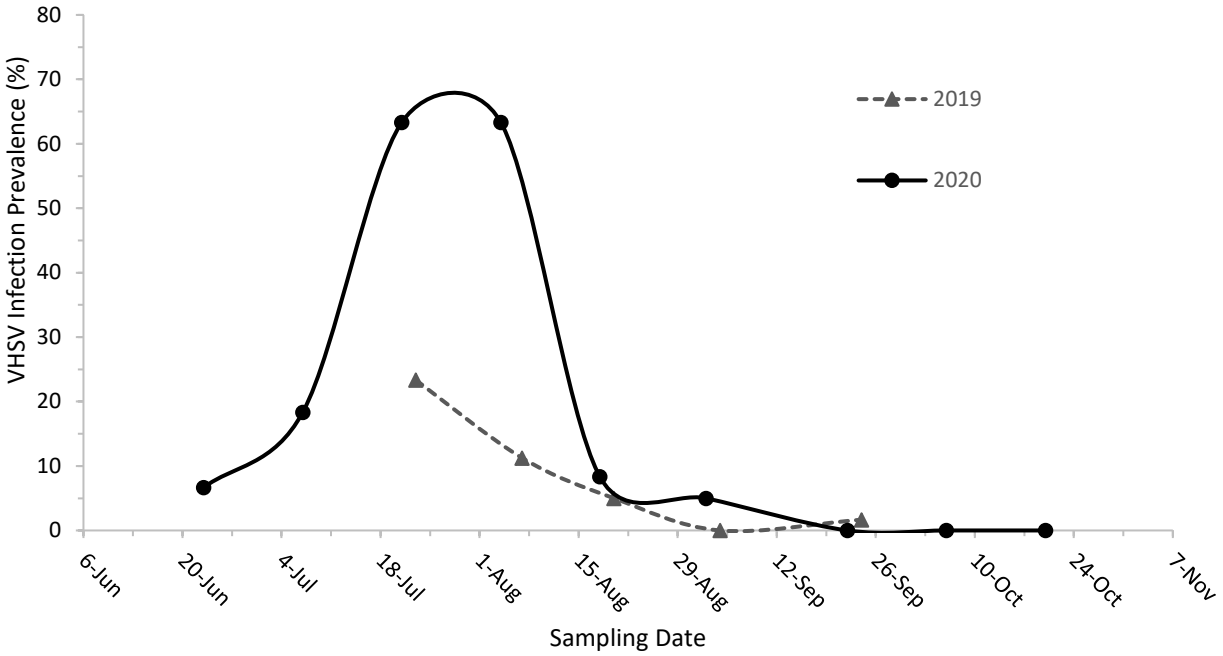

Figure 1. Progression of viral hemorrhagic septicemia virus (VHSV) epizootics in age-0 Pacific herring from Port Angeles Harbor, Puget Sound, WA (2019-2020).

A

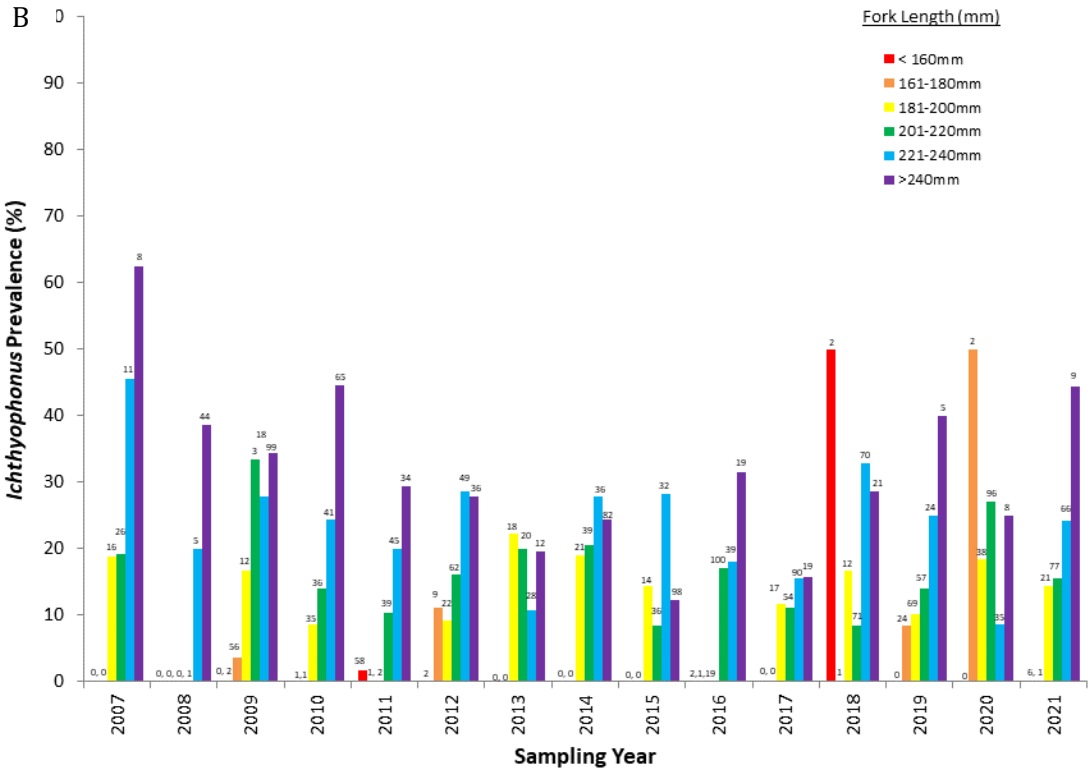

Figure 2. Prevalence of Ichthyophonus herring in each herring size class from Prince William Sound (A) and Sitka Sound (B). Numerals above the bars indicate sample size (n).

Finally, *Ichthyophonus* surveys in spawning walleye pollock were performed from the Shelikof Strait, Alaska region during March 7-14, 2020, aboard a National Oceanic and Atmospheric Administration stock assessment cruise through the generous contribution of Dave McGowan. *Ichthyophonus* infection prevalence, based on tissue explant culture, was 42% (25/59) in livers and 52% (28/54) in eggs. These relatively high prevalence in pollock eggs supports the hypothesis that herring predation on walleye pollock eggs reflects a plausible mechanism for *Ichthyophonus* transmission to Pacific herring; further these results provide the foundation for pursuing additional studies with herring and pollock eggs in PWS.

1.2 VHSV Neutralizing Antibody Assessments.

Neutralizing antibodies to VHSV varied annually in herring from both PWS and Sitka Sound. The prevalence of seropositives in each population decreased dramatically between 2018 and 2019 and remained low thereafter (Fig. 3). This decrease is coincident with the recruitment of the very large 2016 year class into the spawning stock. The prevalence of seropositives in PWS generally increased with herring age, until the 2019 shift, after which the prevalence of detectable antibodies decreased dramatically in the oldest (age-5+) cohorts, as was evidenced by robust sample sizes from 2017-2021 (Fig. 4). These data were provided to Trevor Branch (University of Washington) and integrated into a novel modelling approach that involved the incorporation of serological data into stock assessment models (Trochta et al. 2022).

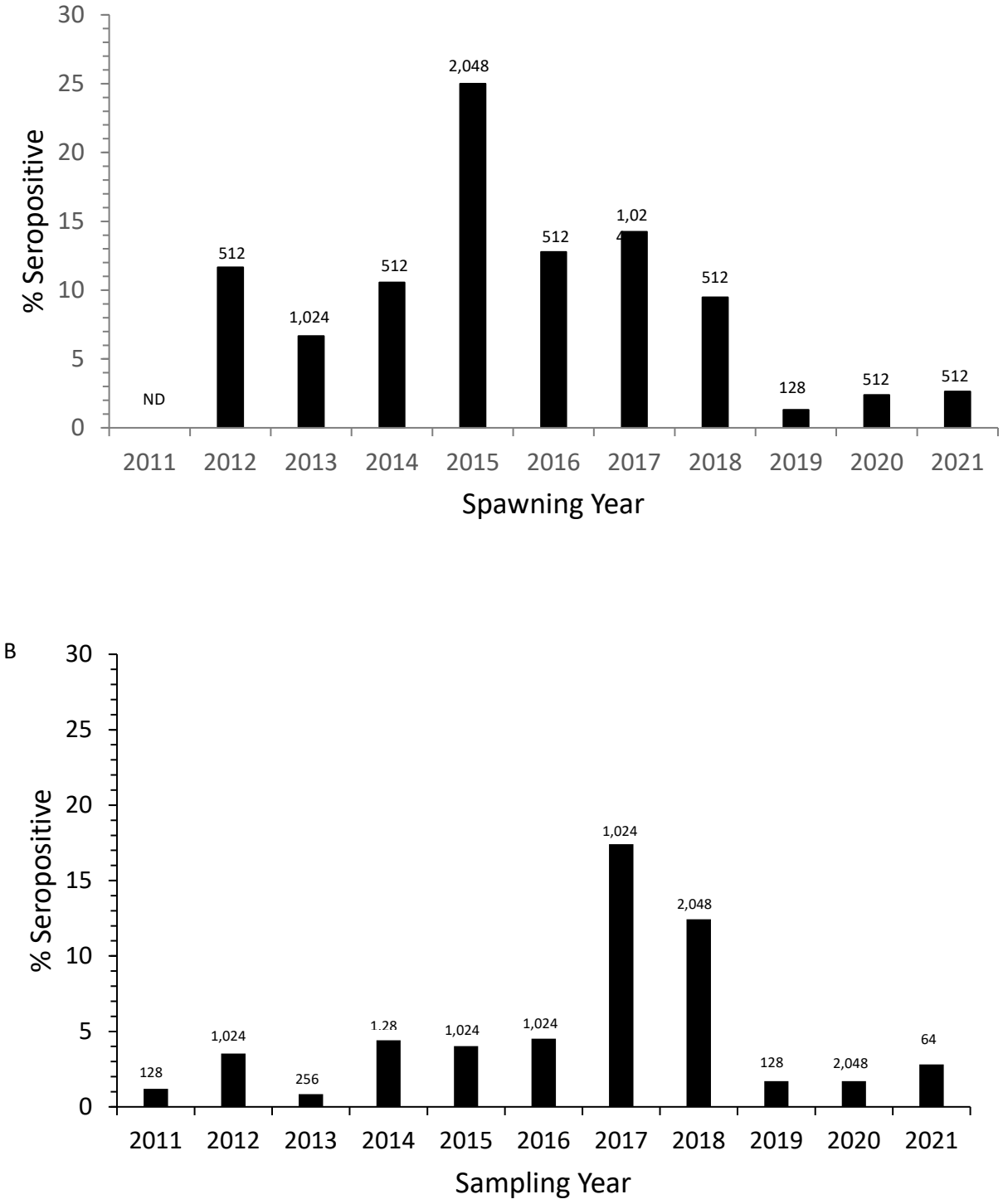

Figure 3. Annual prevalence of viral hemorrhagic septicemia virus neutralizing antibodies in herring from Prince William Sound (A) and Sitka Sound (B). ND = No Data; plasma samples were not collected from Prince William Sound in 2011. Numerals above the bars indicate the median neutralizing titer in seropositives, reported as the reciprocal 50% inhibitory dilution – ID_{50} (titer range: 64 - 2,048).

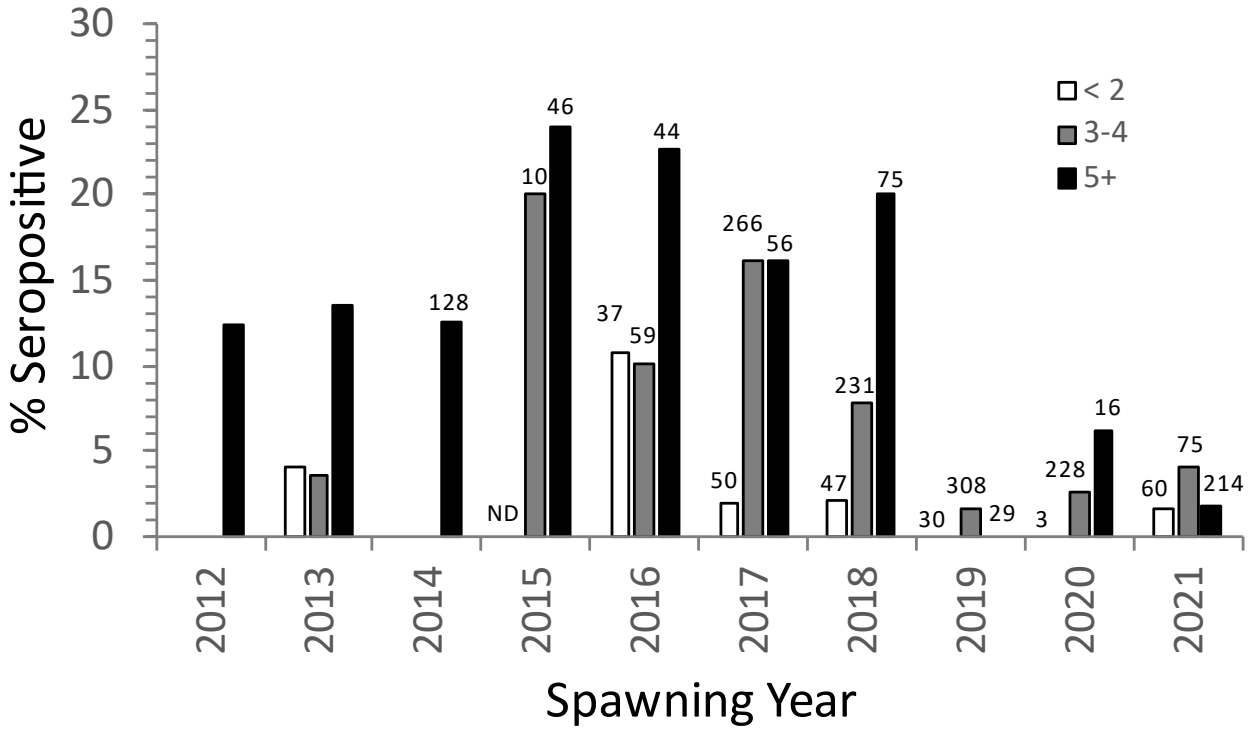

Figure 4. Inter-annual changes in prevalence of viral hemorrhagic septicemia virus neutralizing antibodies in Prince William Sound herring age classes. Legend indicates herring age (yr), determined by Alaska Department of Fish and Game - Cordova from scales. Numerals above the bars indicate sample size (n).

CHAPTER 2 LABORATORY AND FIELD STUDIES

2.1 Validate the novel plaque neutralization assay using wild herring

The detailed laboratory methods for the plaque neutralization test (PNT) were optimized to achieve the highest diagnostic sensitivity possible. The PNT was complement-dependent, as neutralizing activity was attenuated by heat inactivation; further, neutralizing activity was restored and enhanced by the addition of exogenous complement from specific pathogen-free Pacific herring (Table 3). Optimal methods included the overnight incubation of VHSV aliquots in serial dilutions (starting at 1:16) of whole test plasma containing endogenous complement. The resulting viral titers were then enumerated using a viral plaque assay in 96 well micro plates. Serum neutralizing activity was virus-specific, as plasma from VHS survivors demonstrated only negligible reactivity to infectious hematopoietic necrosis virus (IHNV), a closely related rhabdovirus. Among Pacific herring that survived VHSV exposure, neutralizing activity was detected in the plasma as early as 37 d post-exposure and peaked approximately 64 d post-exposure. The onset of neutralizing activity was slightly delayed at 6.0 ºC relative to warmer temperatures (8.5 ºC and 12.0 ºC); however, neutralizing activity persisted for at least 345 d post exposure in all temperature treatments. It is anticipated that this novel ability to assess VHSV neutralizing activity will enable retrospective comparisons between a priori VHS exposures and year class recruitment failures. Additionally, the optimized PNT is expected to be employed as a forecasting tool capable of identifying the potential for future VHS epizootics in wild Pacific Herring populations.

Table 3. Sensitivity of the plaque neutralization test increases after addition of exogenous complement. Note the increase in detectable neutralization activity among viral hemorrhagic septicemia survivors (treatment) after the addition of exogenous complement (Heat-Inactivated + Complement column) compared to reliance on endogenous complement (Whole Plasma column).

Fish Treatment	Plasma Treatment		
	Whole Plasma (Endogenous Complement)	Heat-Inactivated (all Complement Removed)	Heat-Inactivated + Exogenous Complement
Neg. Control	0	0	0
Neg. Control	0	0	0
Neg. Control	0	0	0
Neg. Control	0	0	0
Neg. Control	0	0	0
Neg. Control	0	0	32
Neg. Control	0	0	0

Fish Treatment	Plasma Treatment		
	Whole Plasma (Endogenous Complement)	Heat-Inactivated (all Complement Removed)	Heat-Inactivated + Exogenous Complement
Neg. Control	0	0	0
Neg. Control	0	0	0
Neg. Control	0	0	0
Treatment	64	0	256
Treatment	128	0	2048
Treatment	16	0	16
Treatment	64	0	0
Treatment	256	0	2048
Treatment	256	0	2048
Treatment	256	0	1024
Treatment	64	0	0
Treatment	128	0	16
Treatment	0	0	256
Treatment	32	0	256
Treatment	0	0	0
Treatment	32	0	64
Treatment	256	0	128
Treatment	512	0	2048
Treatment	256	0	1024
Treatment	256	0	2048
Treatment	128	0	1024
Treatment	32	0	2048
Treatment	16	0	0

For the neutralizing antibody assay to functionally apply to the population level, it is important to understand the kinetics of antibody production and persistence. For example, if antibodies are detectable for at least 1-year post-exposure, then annual herring surveillances should be adequate to detect whether VHSV exposures occurred since the previous annual stock assessment. A long-term study to evaluate the persistence of detectable VHSV antibodies was completed. Briefly, specific pathogen-free (SPF) Pacific herring were exposed to VHSV during a single waterborne challenge and neutralizing antibodies were assessed at monthly intervals after exposure. Fish were subsampled at monthly intervals post-exposure and assessed for the presence of detectable

antibodies. The experiment was terminated approximately three years after the herring were exposed to VHSV. Neutralizing antibodies were detected as early as 28 d post-exposure and persisted throughout the duration of the 3 yr study (Fig. 5).

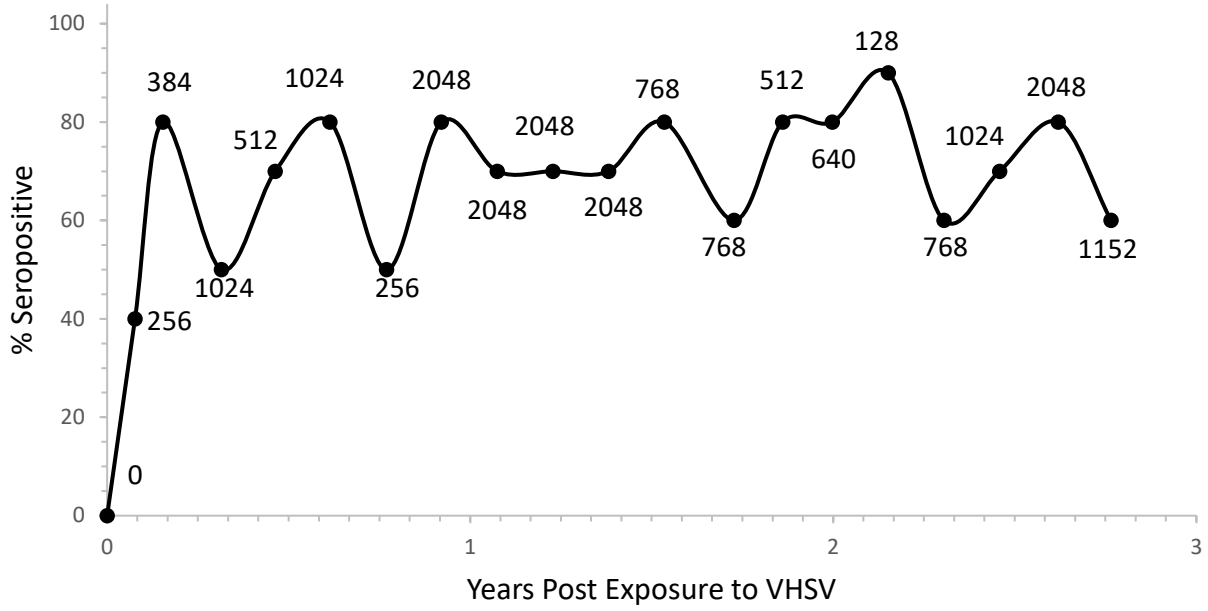

Figure 5. Persistence of detectable neutralizing antibodies in herring that survived viral hemorrhagic septicemia virus (VHSV) exposure. Numerals associated with each data point indicate the median antibody titer among seropositive individuals, reported as the reciprocal 50% inhibitory dilution – ID50 (titer range: 64 - 2,048). Sample size (n) = 10 fish at each subsampling interval. None of the specific pathogen-free negative controls (n = 10 / sampling date) tested positive on any of the subsampling dates (data not shown). Note: these results reflect the novel (optimized) plaque neutralization methods.

After optimizing the antibody assay and demonstrating its ability to detect antibodies for extended periods post-exposure in the laboratory, the next step was to demonstrate the usefulness of the assay in wild herring by determining whether the assay correctly identified their deduced prior exposure histories. Exposure histories were deduced using a unique principle involving VHS and Pacific herring, whereby wild herring confined into net pens or laboratory tanks often (but not always) experience VHS epizootics. It can be deduced that any group of herring experiencing a VHS epizootic under these conditions was previously naïve to VHSV and did not possess herd immunity at the time of capture. In contrast, any group of herring failing to undergo a VHS epizootic under these confinement conditions most likely survived prior exposure and was demonstrating herd immunity. Utilizing this principle, we repeatedly collected groups of juvenile Pacific herring from various locations, transported them alive to the laboratory, and subsampled their VHSV antibody levels at the time of capture. Their exposure histories were

deduced by confining them into laboratory tanks and observing whether a VHS epizootic ensued. If an epizootic did not ensue, it could be inferred that either:

1) The population survived prior exposure and was refractory to the disease (in possession of herd immunity), or

2) The population was never previously exposed, remained susceptible, and had no herd immunity; however, viral carriers were not present among the captured individuals, so exposure to VHSV did not occur in the tanks. This possibility was eliminated by further exposing these groups to known amounts of VHSV under controlled conditions.

Susceptible Groups: It was concluded that six of these herring groups were largely naïve to VHSV at time of capture and did not demonstrate herd immunity when they were collected because classic VHS epizootics (characterized by mortalities accompanied by high VHSV prevalence and tissue titers) ensued after their confinement into the tanks (Table 4). The day 0 antibody profiles generally support this conclusion, as seropositives at the time of capture were typically low (≤3%).

Refractory Groups: It was concluded that three groups survived prior exposure to VHSV and demonstrated herd immunity when they were collected because VHS epizootics did not ensue after their confinement into laboratory tanks, nor did they occur after subsequent exposure to known amounts of virus (Table 4). The Day 0 antibody profiles generally supported this conclusion, as seropositives at the time of capture were much higher (26%-33%) than in the susceptible groups.

Ambiguous Group: One group of susceptible herring (Protection Island, Oct 4, 2018) returned relatively high antibody levels (27%), indicating that they survived prior exposure (Table 4). However, laboratory confinement indicated that these fish were still susceptible when they were introduced to the laboratory. This ambiguity may reflect a mixed school demonstrating differing exposure histories. However, the samples are currently being re-processed to assess whether a sampling mistake may have occurred.

Table 4. Comparison of viral hemorrhagic septicemia virus (VHSV) neutralizing antibody results between the original and improved methods in the Plaque Neutralization Test.

Collection Location	Collection Date	VHSV exposure history[1]	Seropositives (%)
Admiralty Inlet	Aug 2, 2018	Naive	0% (0/29)
Admiralty Inlet	Aug 7, 2018	Naive	0% (0/29)
Admiralty Inlet	Aug 21, 2018	Naive	0% (0/24)
Admiralty Inlet	Aug 29, 2018	Naive	0% (0/30)
Admiralty Inlet	Sept 11, 2019	Naive	0% (0/30)
Pt. Angeles Harbor	Sept 18, 2018	Prior Exposure	31% (8/26)
Protection Island	Oct 4, 2018	Ambiguous[3]	27% (8/30)
Pt. Angeles Harbor	Oct 9, 2018	Prior Exposure	26% (7/27)
St. of Juan de Fuca	Oct 11, 2018	Naive	3% (1/30)
Pt. Angeles Harbor	Nov 5, 2018	Prior Exposure	33% (10/30)

[1]VHSV exposure history was deduced by observing whether the sampled herring school experienced a VHS epizootic after transport and confinement into laboratory tanks. Those that experienced an epizootic were assigned "Previously Naïve" status, as they were susceptible to the disease at time of capture and antibody levels were generally low / undetectable. Those that did not experience an epizootic were deduced to have evidence of "Prior Exposure" to VHSV, as they were not susceptible to the disease, and neutralizing antibodies were detected.

[2]Antibody titer is reported as the inverse serum dilution required to neutralize 50% of VHSV in the antibody neutralization test. A larger numeral indicates a higher titer of neutralizing antibodies.

[3]The exposure history of this sample is ambiguous, as antibodies were detected at the time of collection (indicating prior exposure), yet some of the fish died from VHS in the laboratory (indicating they were naïve).

2.2 Demonstration of Pacific herring as a reservoir for VHSV

Processes that allow VHSV to persist in the marine environment remain enigmatic, owing largely to the presence of covert and cryptic infections in marine fishes during typical sub-epizootic periods. As such, marine host reservoirs for VHSV have not been fully demonstrated, nor have the mechanism(s) by which infected hosts contribute to virus perpetuation and transmission. Here, we demonstrated that, after surviving VHS, convalesced Pacific herring continue to shed virus at a low rate for extended periods. Viral shedding from VHS survivors occurred at longer durations in cooler water and a recurrence of viral shedding among fully recovered individuals coincided with seasonal temperature declines (Fig. 6). Further, exposure of previously naïve conspecific sentinels to this shed virus can result in infections for at least 6 months after cessation of overt disease (Fig. 7, Table 5). Transmission potential from viral

shedding was not necessarily dependent on the magnitude of the disease outbreak, as prolonged transmission occurred from two groups of donor herring that experienced cumulative mortalities of 4% and 29%. The results further suggest that the virus persists in association with the gills of fully recovered individuals (Table 6) and long-term viral shedding or shedding relapses are related to cooler or decreasing water temperatures. These results provide support for a new VHSV perpetuation paradigm in the marine environment, whereby the virus can be maintained in convalesced survivors and trafficked from these carriers to sympatric susceptible individuals.

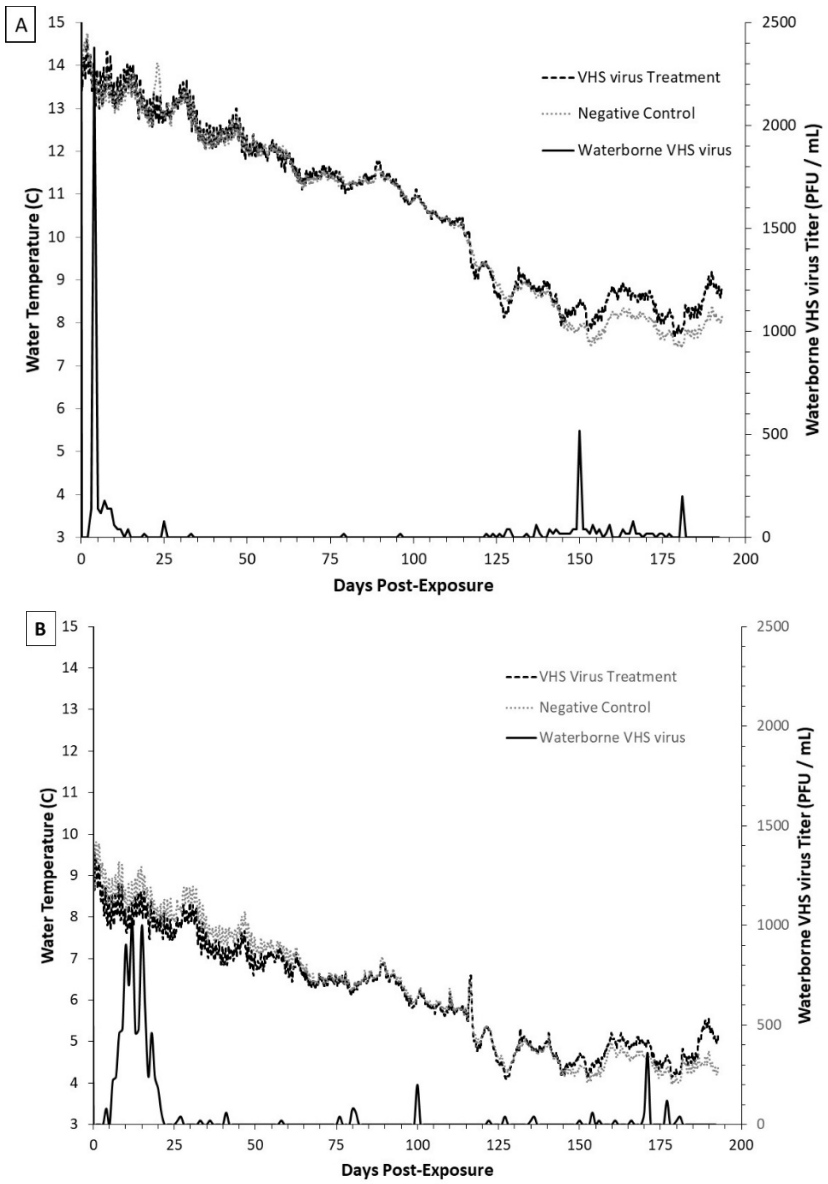

Figure 6. Daily profiles of water temperature and waterborne viral hemorrhagic septicemia (VHS) virus titers in the donor colonies for the ambient (A) and chilled (B) treatments. Temperature profiles are displayed for both donor colonies (VHS virus treatment and negative control). Water samples were collected daily and analyzed by viral plaque assay. Waterborne VHS virus titers are displayed for the VHS virus treatments only; waterborne virus was not detected in the negative controls on any sampling day.

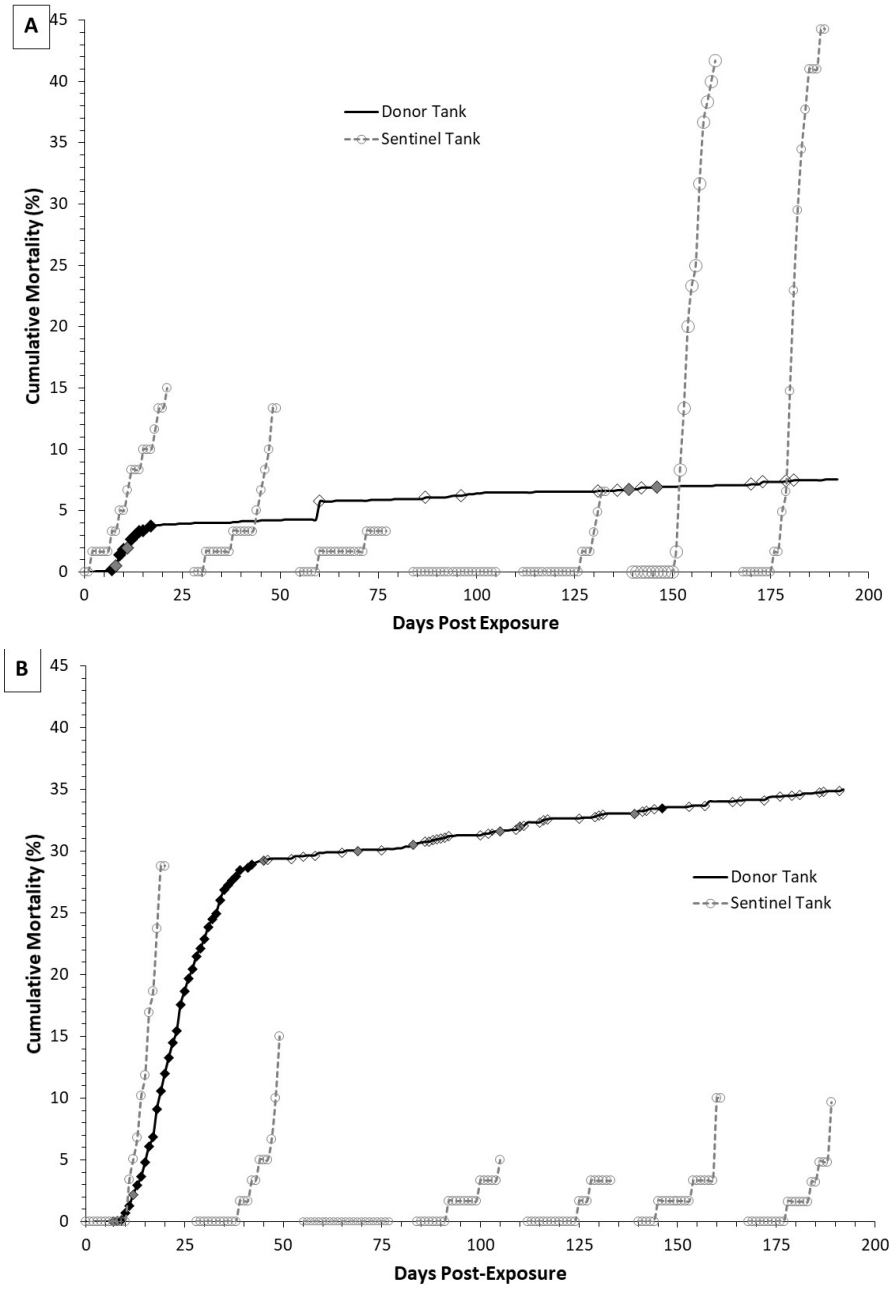

Figure 7. Cumulative mortalities in the viral hemorrhagic septicemia (VHS) virus treatments at ambient (A) and chilled (B) temperatures. The mortality anomaly in the ambient treatment (A) on 60 d was caused by a low oxygen event after a disruption in seawater supply. Diamonds indicate days when tissues from at least one mortality in the donor tank tested positive for VHS virus, including kidney / spleen homogenates by plaque assay (black diamonds), gills by RT-qPCR (open diamonds), or both tissues (gray diamonds). Results for the negative control groups are not displayed, as cumulative mortalities in the donor colonies were 1.3% (ambient) and 0.4% (chilled), and cumulative mortalities in the sentinel control groups were 0%-10%. VHS virus was not detected in any mortalities or survivors in the negative control groups (donor or sentinels) by RT-qPCR or plaque assay.

Table 5. Viral hemorrhagic virus (VHSV) prevalence in kidney / spleen homogenates (as determined by plaque assay) in Pacific herring sentinels exposed to effluent water from the VHSV donor colonies. Each sentinel tanks contained 58-62 herring during each exposure period; infection prevalence reflects the summation of results from mortalities and survivors. Results for sentinels exposed to effluent from the negative control colonies are not displayed, as none (neither mortalities nor survivors) tested positive for VHSV.

	Percent VHSV positive sentinels	
Period of exposure to effluent water from donor tank	Ambient Treatment	Chilled Treatment
1 - 21 d	15%	64%
28 - 49 d	10%	44%
55 - 77 d	0%	12%*
84 - 105 d	40%*	97%
112 - 133 d	65%	37%
140 - 161 d	73%	52%
168 - 189 d	67%	63%

*Indicates groups where VHSV positive samples occurred only in survivors; positives in all other groups reflect a combination of survivors and mortalities.

Table 6. Prevalence of viral hemorrhagic septicemia virus (VHSV) in kidney / spleen homogenates (as determined by plaque assay) and gills (as determined by reverse transcriptase quantitative polymerase chain reaction [RT-qPCR]) from donor herring in the VHSV treatment groups. Results for negative controls are not displayed; none (neither mortalities nor survivors) tested positive for VHSV from either tissue type.

	Ambient Treatment	
	Kidney / spleen (cell culture)	Gills (RT-qPCR)
Mortalities during the acute disease phase (0-17 d post-exposure)	83% (44/53) *	75% (3/4)
Mortalities during the post-acute phase (17-192 d post-exposure)	4% (2/49) *	68% (15/22)
Survivors at the end of the experiment (192 d post-exposure)	0% (0/145)	40% (2/5)
	Chilled Treatment	
Mortalities during the acute disease phase (0-45 d post-exposure)	85% (252/297) *	100% (5/5)
Mortalities during the post-acute phase (45-192 d post-exposure)	7% (6/88) *	82% (43/52)
Survivors at the end of the experiment (192 d post-exposure)	0% (0/150)	50% (5/10)

*Designates results from all mortalities; results not designated with an asterisk reflect subsamples of mortalities.

2.3 Pacific herring are not susceptible to vibriosis from *Vibrio anguillarum* or *V. ordalii* under laboratory conditions

The ubiquity of *Vibrio* spp. throughout the coastal marine waters of the Pacific Northwest of North America raises questions about the susceptibility of native marine fishes, including Pacific herring. Early reports of *Vibrio*-like disease (Rucker et al. 1954, Walford 1958) and *Vibrio* sp. isolations (Pacha and Kiehn 1969) in Pacific herring remain questionable because both occurred while the classification of vibrios was still developing and prior to the availability of techniques capable of discerning viral etiologies. This study was performed to address these uncertainties by determining the susceptibility of Pacific herring to vibriosis caused by strains of *V. anguillarum* and *V. ordalii*.

Pacific herring were not susceptible to vibriosis from either *V. anguillarum* or *V. ordalii*. Cumulative mortalities among Vibrio-exposed groups (3.3% and 5.0%, respectively) were similar ($P > 0.50$; ANOVA) to that of negative controls (10%; Fig. 8. Gross signs of vibriosis

were not observed on any dead or surviving Pacific herring. Vibrio-like colonies grew from kidney inoculations in 0% (n = 2) of mortalities exposed to *V. ordalii*, 50% (n = 2) of mortalities exposed to *V. anguillarum*, and 0% (n = 6) of mortalities exposed to Phosphate Buffered Saline. The bacterial isolate from the mortality in the *V. anguillarum* treatment was not further identified.

Chinook salmon were highly susceptible to vibriosis caused by *V. anguillarum* and *V. ordalii* (Fig. 8). Cumulative mortalities among Vibrio-exposed groups (96.7% and 60.0%, respectively) were significantly greater ($P < 0.001$; ANOVA, Dunnett's Test) than that of the PBS-exposed negative controls (3.3%). Mortalities in both *Vibrio* spp. groups demonstrated classic signs of vibriosis, including hemorrhagic ulcerations of the skin and underlying musculature, exopthalmia, and distention of the body cavity (Fig. 9). *Vibrio*-like colonies grew from kidney inoculations in 69% (n = 36) of mortalities exposed to *V. ordalii*, 100% (n = 58) of mortalities exposed to *V. anguillarum*, and 0% (n = 2) of mortalities exposed to PBS. Using the sorbitol test, *V. anguillarum* was confirmed in all subsamples from the *V. anguillarum* treatment (n =4). Isolates from the *V. ordalii* group were not further identified; however, none (n = 7) produced a positive sorbitol test.

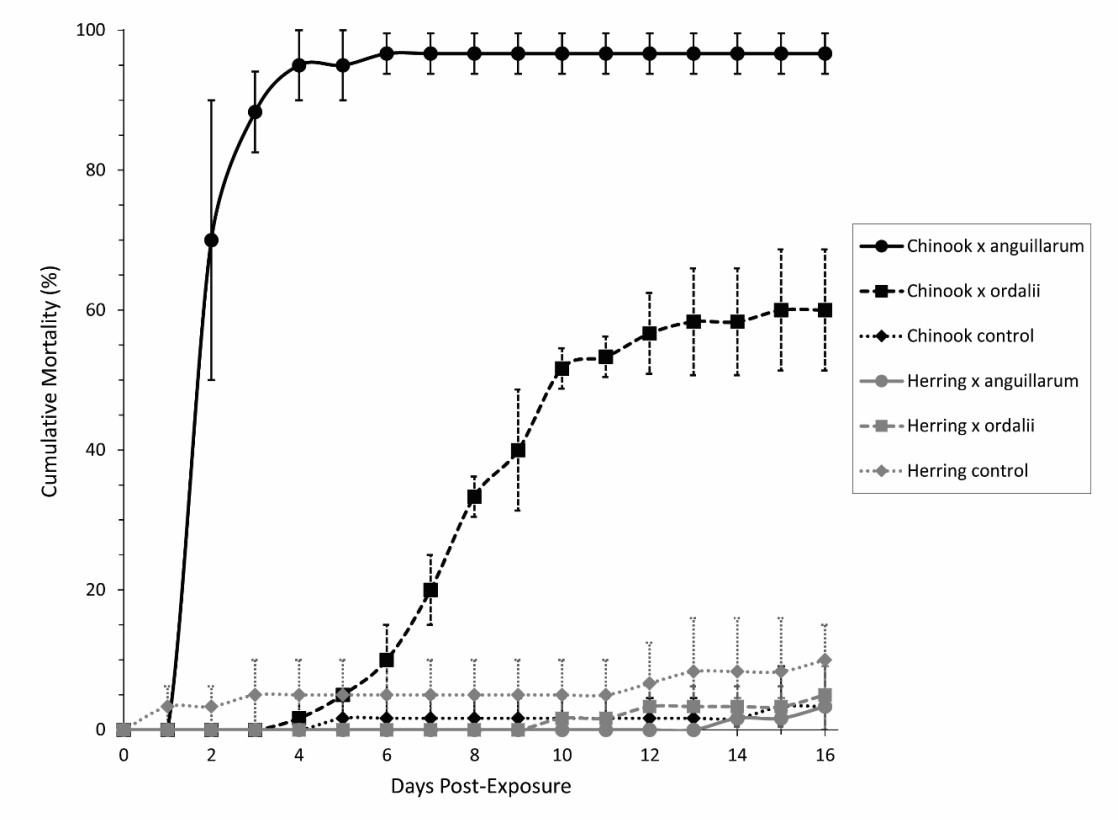

Figure 8. Cumulative mortality after exposure of Chinook salmon and Pacific herring to V. anguillarum and V. ordalii. Data points indicate the mean mortality from triplicate tanks; error bars indicate ±1 SD from the mean.

Figure 9. Vibrio anguillarum-exposed Chinook salmon demonstrating external signs of vibriosis.

2.4 Inability to demonstrate fish-to-fish transmission of *Ichthyophonus* in Pacific herring

Transmission of *Ichthyophonus* in clupeid hosts has been a subject of speculation since the earliest reported epizootics, but neither the mode of infection in herring nor the life cycle of *Ichthyophonus* spp. are adequately described. Captive reared SPF sentinel herring (age-1+) were cohabitated with *Ichthyophonus*-infected donor herring under four simulated environmental conditions: 1) ambient seawater, 2) ambient seawater with low salinity events, 3) chilled seawater, and 4) chilled seawater with low salinity events. Ambient seawater temperature ranged between 13.3 ˚C (24 Sept 2019) and 7.9 ˚C (16 Jan 2020) over the 5-month exposures, and chilled seawater temperatures ranged between 9.5 ˚C (6 Sept 2019) and 3.7 ˚C (15 Jan 2020) (Fig. 10. Mean temperature difference between treatments was 3.48 ˚C (SD = 0.23 ˚C). Seawater salinity in all tanks remained in a narrow range, 27.9 ppt to 32.3 ppt during the study, except during induced low salinity events which ranged from 11.7 ppt to 13.5 ppt minimum concentration (Fig. 11).

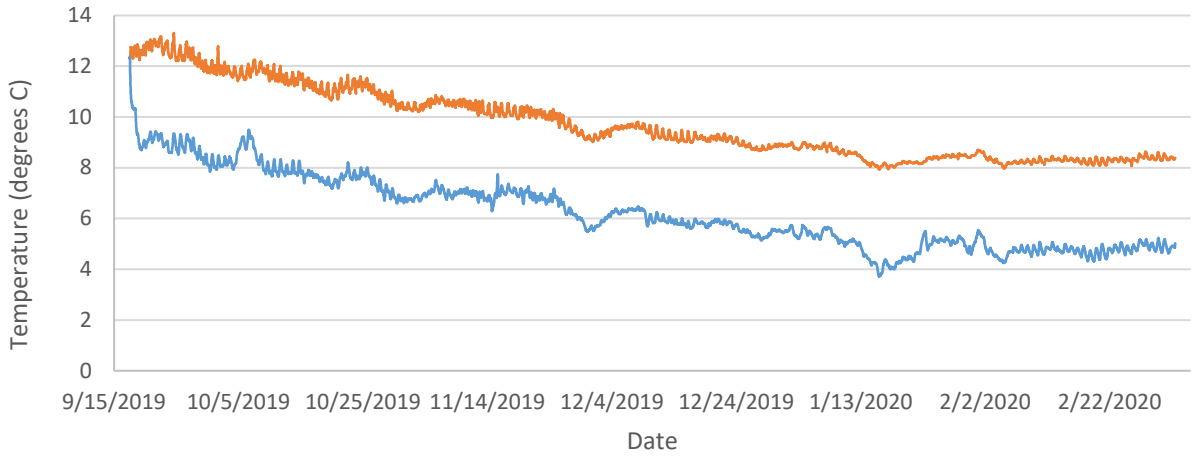

Figure 10. Seawater temperatures recorded from ambient (orange line) and chilled (blue line) treatments over the course of the Ichthyophonus transmission study. Data are means of three tanks per treatment collected at 15 min intervals.

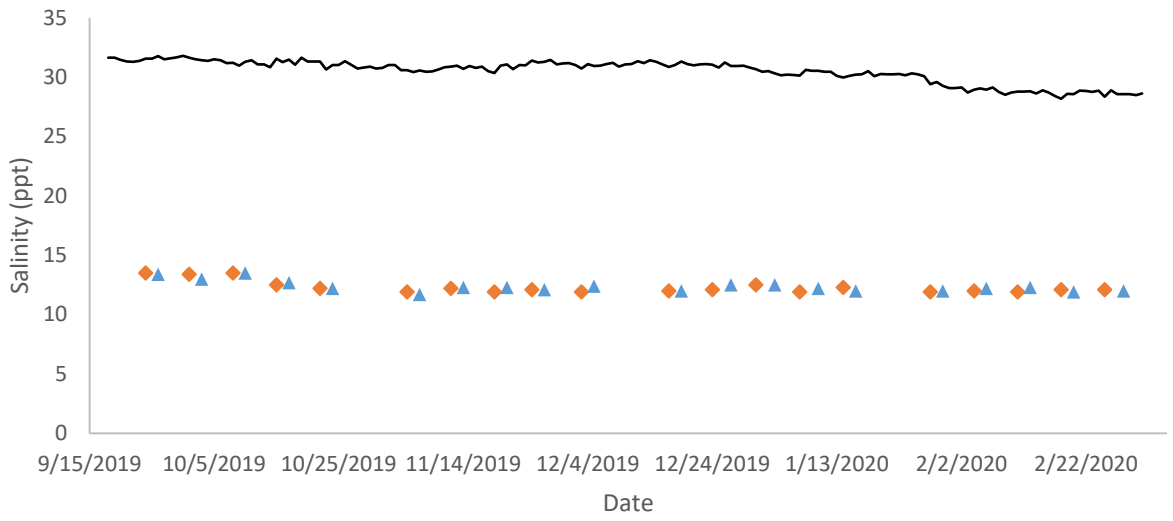

Figure 11. Mean seawater salinity readings (black line). Lowest salinity recorded during the ≈8 hr low salinity events is indicated by orange diamonds (ambient treatment) and blue triangles (chilled treatment).

Donor herring were injected with approximately 2,407 ± 1,574 SD *Ichthyophonus* schizonts and large meronts plus thousands of smaller parasite cells in each 0.2 ml dose. These inoculations successfully established infections in 73% of donor herring, mean fork length (FL) = 148.9 ± 11 (SD) mm and mean weight = 33.9 ± 8.9 (SD) g. Infections progressed to disease in the donor herring and resulted in 20% to 40% mortality (Table 7). Mortalities began on the first day of sentinel cohabitation (7 days after donor injections) and continued at a low rate throughout the duration of the study. No infections were detected in long-term or short-term sentinels in any treatment.

The results of this study corroborate those of earlier studies in finding no support for fish-to-fish transmission of *Ichthyophonus* between Pacific herring. There are a wide range of conditions that herring experience in the wild that cannot be reproduced in the laboratory, and it is impossible to prove a negative. However, in this experiment and others, we have now cohabitated hundreds of herring under infection pressures (fish densities, infection intensities, etc.) that are much higher than those typically experienced in the wild, and we have been unable to confirm fish-to-fish transmission of *Ichthyophonus* in Pacific herring.

Future investigations into possible *Ichthyophonus* transmission routes, including possible transmission via predation on infected pollock eggs, may be helpful for elucidating other potential epizootic mechanisms in this system.

Table 7. Count of herring mortalities (dead) and survivors (live) from each treatment in this study. Donors were exposed to Ichthyophonus by IP injection, sentinels were Specific Pathogen Free herring. Number of Ichthyophonus-positive fish from each group indicated in parentheses. Overall percent infected for donors, long-term sentinels, and short-term sentinels indicated.

	Donors			Long-Term Sentinels[a]			Short-Term Sentinels[b]		
Treatment Group	Dead	Live	% infected	Dead	Live	% infected	Dead	Live	% infected
Control Ambient Seawater	0(0)	49 (0)	0%	0(0)	12(0)	0%	0(0) 0(0) 0(0) 0(0)	12(0) 12(0) 12(0) 12(0)	0%
Ichthyophonus Ambient Seawater	21(20)	29(19)	78%	0(0)	12(0)	0%	1(0) 0(0) 0(0) 0(0)	11(0) 12(0) 12(0) 12(0)	0%
Ichthyophonus Ambient Seawater with low salinity events	14(10)	36(23)	66%	1(0)	11(0)	0%	2(0) 0(0) 0(0) 0(0)	10(0) 12(0) 12(0) 12(0)	0%
Control Chilled Seawater	2(0)	48(0)	0%	1(0)	11(0)	0%	0(0) 0(0) 0(0) 0(0)	12(0) 12(0) 12(0) 12(0)	0%
Ichthyophonus Chilled Seawater	16(16)	34(22)	76%	1(0)	11(0)	0%	1(0) 0(0) 0(0) 0(0)	11(0) 12(0) 12(0) 12(0)	0%
Ichthyophonus Chilled Seawater with low salinity events	16(16)	34(20)	72%	0(0)	12(0)	0%	0(0) 0(0) 0(0) 0(0)	12(0) 12(0) 12(0) 12(0)	0%

[a]Long-term sentinels were cohabitated with donors for entire 24 weeks of study.
[b]Short-term sentinels were replaced every 6 weeks.

2.5 Investigate the possibility of an invertebrate host for *Ichthyophonus*

Ethanol-preserved zooplankton from PWS (provided by Dr. Rob Campbell, PWS Science Center) were screened for *Ichthyophonus* by qPCR using specific primers; however, did not test positive. This lack of positives forced us to re-evaluate our hypothesis that the parasite may be transmitted through plankton. Rather, we learned that at certain times of the year, herring eat copious amounts of fish eggs. It has long been recognized that walleye pollock and other piscivorous fishes become infected with *Ichthyophonus* through the consumption of infected prey items, including Pacific herring. However, it is unknown how the parasite cycles back from these predators to planktivorous fishes, like Pacific herring. Because of this uncertainty, the fate of *Ichthyophonus* in these predatory fishes has been considered a life cycle dead end, whereby the parasite life history is presumed to be terminated and an alternative (unknown) cycle exists to transmit the parasite back to herring. However, herring are often large consumers of pollock eggs; for example, herring consumed 11.4% of all pollock eggs spawned the Sea of Okhotsk (Gorbatenko et al. 2012). Work during the current study demonstrated that *Ichthyophonus* was detected on the eggs in >50% of female pollock in Shelikof Strait. Additionally, we have observed herring stomachs that were gorged with herring eggs during spawning events (Fig. 12), and we have isolated the parasite in from herring eggs as well. Having investigated several other potential routes of *Ichthyophonus* transmission to herring without success, including fish-to-fish transmission and intermediate / paratenic hosts, the ovivory hypothesis remains the most well-supported and parsimonious transmission hypothesis and will be investigated in the future.

Figure 12. Pacific herring that was feeding on herring eggs.

2.6 *Ichthyophonus* in Pacific herring from Cordova Harbor

Catrin Wendt defended her M.S. thesis (Wendt 2020) at the University of Washington, School of Aquatic and Fishery Sciences, titled "*Ichthyophonus* in Pacific herring: Investigating a transmission hot spot." She determined that the prevalence of *Ichthyophonus* in age-0 herring rapidly increases in Cordova Harbor during the spring (Fig. 13). The cause of this increased prevalence was not determined, but may involve one of the following:

1. An exodus of healthy herring from the harbor during the spring, leaving the infected herring behind if they are too sick to participate in the outmigration.
2. Increased infection pressures resulting from offal discharges when the fish processing plants become active in the spring.
3. Other disease co-factors that may occur in Cordova Harbor, including exposure to biofouling contaminants (e.g., tributyltin or copper-based paints), limited food availability, and temperature differences.

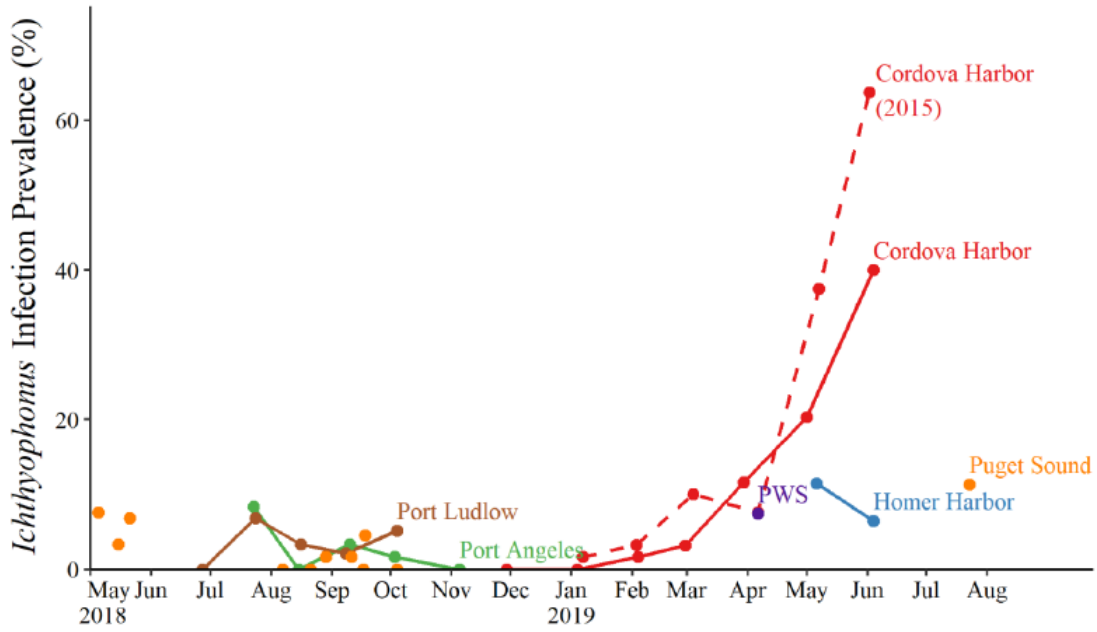

Figure 13. Ichthyophonus infection prevalence in age-0 herring from various locations throughout the NE Pacific, including Cordova Harbor, throughout the year (May 2018 – Aug 2019).

CHAPTER 3 ADDITIONAL VALUE-ADDED STUDIES

Novel tool development and opportunities afforded by the Herring Disease Program (HDP) have been leveraged to foster collaborations and provide value-added to the broader field of marine ecosystem health. These no-cost collaborations were made possible largely by the availability of the specific pathogen-free (SPF) Pacific herring that were produced in the HDP and from the pathogen isolates obtained during HDP biosurveillances.

3.1 Influence of temperature on the efficacy of homologous and heterologous DNA vaccines against VHS

Homologous and heterologous (genogroup Ia) DNA vaccines against viral hemorrhagic septicemia virus (VHSV – genogroup IVa) conferred partial protection in Pacific herring. Early protection at 2 week post vaccination (PV) was low and occurred only at elevated temperature (12.6ºC, 189 degree days [DD]), where the relative percent survival (RPS) following viral exposure was similar for the two vaccines (IVa and Ia, respectively) and higher than that of negative controls at the same temperature. Late protection at 10 week PV was induced by both vaccines but was higher with the homologous vaccine at both 9.0ºC and 12.6°C. Virus neutralization titers were detected among 55% of all vaccinated fish at 10 week PV. The results suggest that the immune response profile triggered by DNA vaccination of herring was similar to that reported for rainbow trout *Oncorhynchus mykiss* (Lorenzen and LaPatra 2005) where interferon responses occur in the early days PV and transition to adaptive response at later time points. However, the protective effect was far less prominent in herring, possibly reflecting different physiologies and or adaptations of the two fish species.

3.2 High prevalence and low intensity *Ichthyophonus* infections in Pacific halibut

Ichthyophonus occurred at high prevalence but low intensity in Pacific halibut (*Hippoglossus stenolepis*) throughout the west coast of North America, ranging from coastal Oregon to the Bering Sea (Fig. 14). Infection prevalence in adults was variable on spatial and temporal scales, with the lowest prevalence typically occurring on the edges of the geographic range and highest prevalence consistently occurring inside PWS (58%-77%). Additionally, intra-annual differences occurred at Albatross - Portlock, Alaska (71% vs 32% within 2012) and inter-annual differences occurred along coastal Oregon (50% vs 12% from 2012 – 2015). The infection prevalence was influenced by host age, increasing from $\leq$ 3% among the youngest cohorts ($\leq$ age 6) to 39%-54% among age 9-17 cohorts, then decreasing to 27% among the oldest (age 18+) cohorts (Fig. 15). There was little indication of significant disease impacts to Pacific halibut, as the intensity of infection was uniformly low, and length-at-age was similar between infected and uninfected cohorts. These results suggest that *Ichthyophonus* in Pacific halibut currently represents a stable parasite-host paradigm in the North Pacific.

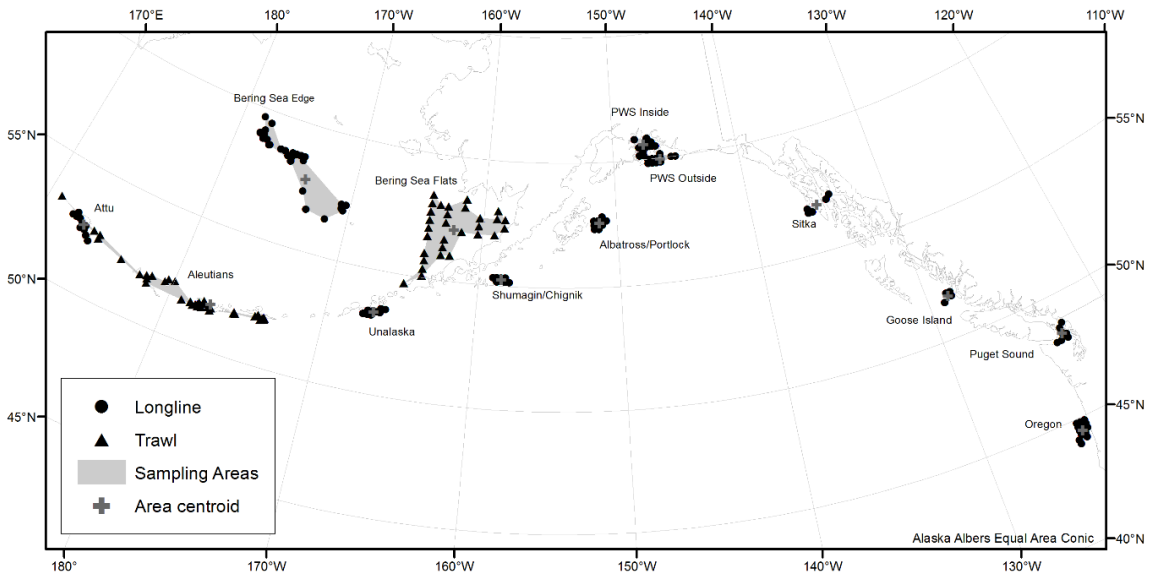

Figure 10. Locations of Pacific halibut sampling sites representing each sampling location in the eastern North Pacific Ocean and Bering Sea.

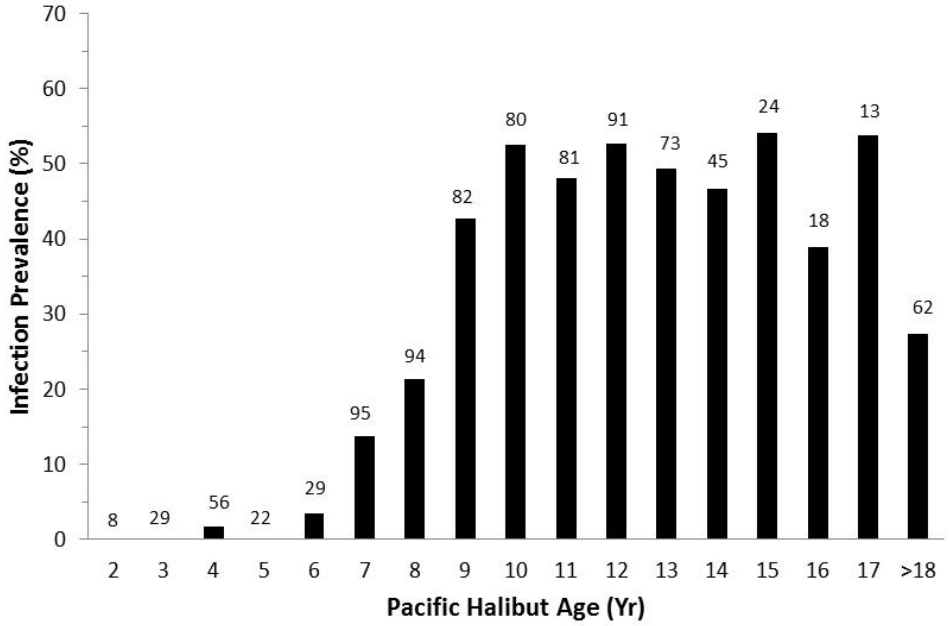

Figure 11. Prevalence of Ichthyophonus infection in each age class of Pacific halibut. Data include samples from 10 setline locations and 2 trawl locations sampled during 2012. Numerals above the bars indicate sample sizes.

3.3 *Ichthyophonus* in sport-caught groundfishes from southcentral Alaska

This report of *Ichthyophonus* in common sport-caught fishes throughout the marine waters of southcentral Alaska represents the first documentation of natural *Ichthyophonus* infections in lingcod (*Ophiodon elongates*), yelloweye rockfish (*Sebastes ruberrimus*), and Pacific cod (*Gadus macrocephalus*). Additionally, the known geographic range of *Ichthyophonus* in black rockfish (*Sebastes melanops*) has been expanded northward to include southcentral Alaska. Among all species surveyed, the infection prevalence was highest (35%, n = 334) in Pacific halibut (*Hippoglossus stenolepis*). There were no gross indications of high-level infections or clinically diseased individuals. These results support the hypothesis that under typical conditions, *Ichthyophonus* can occur at high infection prevalence accompanied with low infection intensities among a variety of fishes throughout the eastern North Pacific Ocean, including southcentral Alaska.

3.4 Analytical and diagnostic performance of a qPCR assay for *Ichthyophonus* spp. compared to the tissue explant culture 'gold standard'

Due in part to the uneven distribution of *Ichthyophonus* throughout host tissues, the comparative sensitivity and accuracy of using molecular-based detection methods versus culture to estimate parasite prevalence in wild populations is under debate. We evaluated the analytical and diagnostic performance of an existing qPCR assay in comparison to the 'gold standard' culture method using Pacific herring with known exposure history in a controlled environment. We determined that the assay is suitable for use in this host, and diagnostic specificity was consistently high (>98%) in both heart and liver tissues. Diagnostic sensitivity could not be fully assessed due to low infection rates in *Ichthyophonus*-inoculated fish, but our results suggest that qPCR is not as sensitive as culture under all circumstances. Diagnostic sensitivity of qPCR relative to culture is likely affected by the amount of sample processed. The prevalence values estimated by the two methods were not significantly different when sample amounts were equal (heart tissue), but when assayed sample amounts were unequal (liver tissue), the culture method detected a significantly higher prevalence of the parasite than qPCR. The culture method can accommodate a larger piece of tissue than the qPCR method, however, culture of liver also detected significantly more *Ichthyophonus* infections than culture of heart, suggesting that the density and distribution of parasites in tissues also plays a role in assay sensitivity. This sensitivity issue would be most problematic for fish with light infections. Although qPCR does not detect the presence of a live organism, DNA-based pathogen detection methods provide the opportunity for alternate testing strategies when culture is not possible.

3.5 Low susceptibility of sockeye salmon (*Oncorhynchus nerka*) to viral hemorrhagic septicemia virus genotype IVa

VHSV genotype IVa is an endemic pathogen to the marine waters of BC, with numerous marine fishes being susceptible to infection and disease, including Atlantic salmon (*Salmo salar*) reared in open net-pen aquaculture. The susceptibility of Atlantic salmon and sockeye salmon to VHSV-IVa was evaluated using exposure routes including injection, static immersion, and

cohabitation with diseased Pacific herring. Exposed fish were monitored for mortality and external pathology, mortalities were tested by cell culture, and live fish were regularly sampled and screened for infection. Among injected sockeye, VHSV was detected in one mortality (n = 195) and two sub-sampled fish (n = 30), whereas sockeye exposed by immersion and cohabitation did not experience mortality nor was systemic infection indicated by tissue screening. Injection and cohabitation exposure routes confirmed the susceptibility of Atlantic salmon to VHSV. Neither sockeye nor Atlantic salmon surviving the cohabitation served as a reservoir of VHSV, but Pacific herring did. The results suggest that VHSV-IVa poses low risk to sockeye salmon under natural routes of exposure.

3.6 Increased Herring Susceptibility to *Ichthyophonus* after embryonic exposure to PAHs

As a no-cost contribution to support the herring genetics project (principal investigator [PI] Whitehead, project 20170115), an exposure study was performed to evaluate the susceptibility of herring to *Ichthyophonus* after surviving embryonic exposure to oil. Briefly, groups of Pacific herring were exposed to polycyclic aromatic hydrocarbons (PAHs) as embryos and raised under specific pathogen-free conditions through metamorphosis to juveniles at the U.S. Geological Survey (USGS) Marrowstone Marine Field Station. After metamorphosis, groups of previously oiled and unoiled SPF herring were injected with *Ichthyophonus*. After *Ichthyophonus* exposure, mortality was slightly higher among the group that survived oil exposure (Fig. 16).

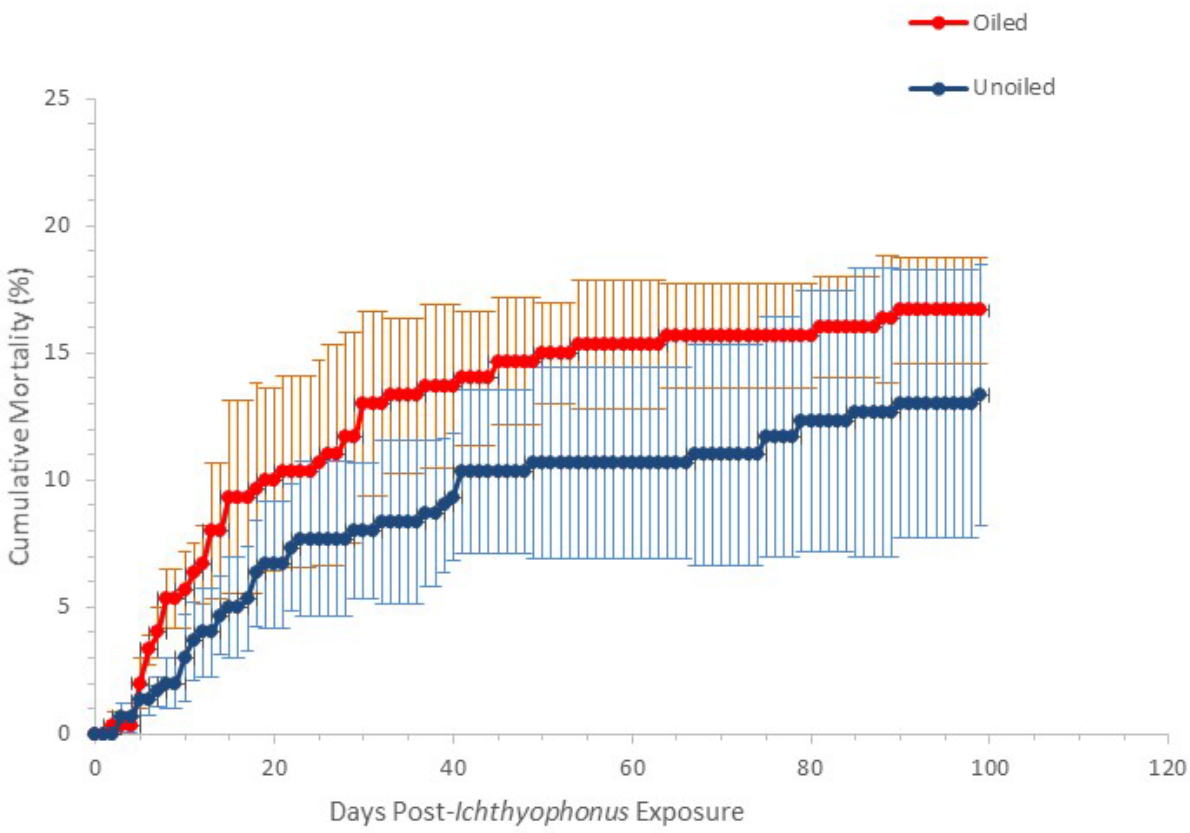

Figure 12. Cumulative mortality among two groups of Specific Pathogen Free herring (previously oiled and unoiled) after exposure to Ichthyophonus by inter-coelomic injection. Mortality in both negative control groups (exposed to saline in lieu of Ichthyophonus) was negligible (data not shown). Each data point represents mean cumulative mortality from each of 3 tanks (n = 62 herring / tank), and error bars indicate 2 SD from the mean.

3.7 Decreased Herring susceptibility to VHS after surviving larval exposure to PAHs

As a no-cost contribution to support the herring genetics project (PI Whitehead, project 20170115), a series of experiments was performed to evaluate the susceptibility of herring to VHSV after surviving larval exposure to oil.

First Test: Herring Larvae were Highly Susceptible to Direct Effects of Oil Exposure:

Herring embryos were collected from wild spawn in Puget Sound and transferred to the USGS Marrowstone Marine Field Station for grow-out. On the day of hatch, larvae were transferred to replicate tanks (n = 4 tanks / treatment: 1,000 larvae / tank) for each of three treatments (unoiled, exposure to low oil concentrations, and exposure to high oil concentrations). Groups were exposed to Alaska North Slope oil for 20 consecutive days, after which the oil generator was

turned off and the larvae were raised through metamorphosis to juveniles. Total PAH concentrations were assessed from the exposure water and the herring tissues (results pending). A clear dose response occurred in larval survival to metamorphosis (Fig. 17) and fish mass at 85 d post-hatch (Fig. 18).

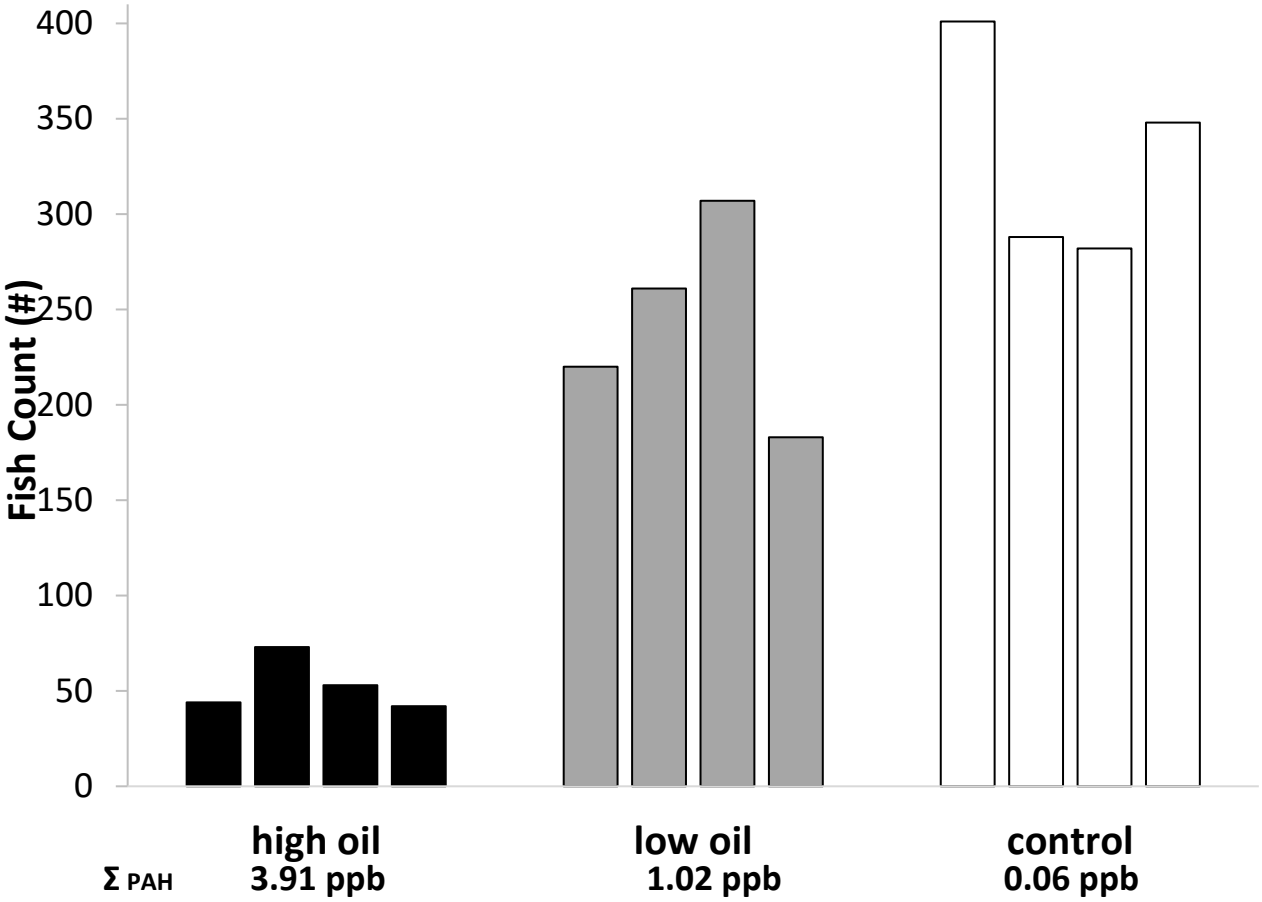

Figure 13. Larval survival after exposure to polycyclic aromatic hydrocarbons (PAHs). Each exposure was performed in 4 replicate tanks (indicated by the treatment bars), with each tank loaded with 1,000 newly-hatched larvae. Fish counts were made 85 d post hatch, after larvae were mostly metamorphosed to juveniles. Note: development was retarded in the high PAH treatment group, where most individuals were not yet metamorphosed 85 d post-hatch.

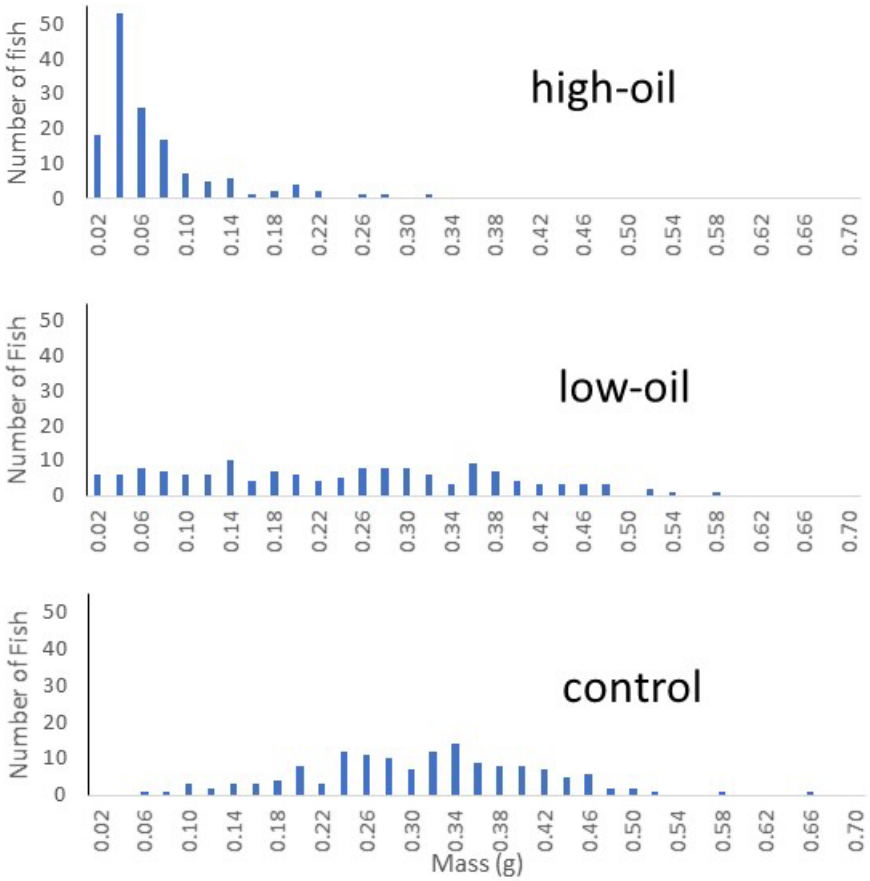

Figure 14. Frequency of wet weights among 85 d post-hatch juveniles in each of the polycyclic aromatic hydrocarbon (PAH) exposure treatments. Note: development was retarded in the high PAH treatment group, where most individuals were not yet metamorphosed 85 d post-hatch.

Second Test (Pilot Study): Decreased Susceptibility of Juveniles that Survived Larval PAH Exposure:

After metamorphosis, the high oil group was terminated because too few fish survived through metamorphosis to justify further experimentation. Equal numbers of fish from the remaining treatments (unoiled and low oil) were transferred to larger tanks where survival and growth assessments continued through the juvenile phase; these fish were used for an expanded VHSV study to assess VHS susceptibility. Additional fish from both groups (unoiled and oiled) remained and were used in a pilot study to assess whether larval exposure to PAHs impacts the susceptibility of juvenile herring to VHS. Pilot results indicated that the onset of VHS mortality was delayed among juvenile herring and 21 d cumulative mortality was lower among herring that survived larval exposure to PAHs than unoiled herring (Fig. 19).

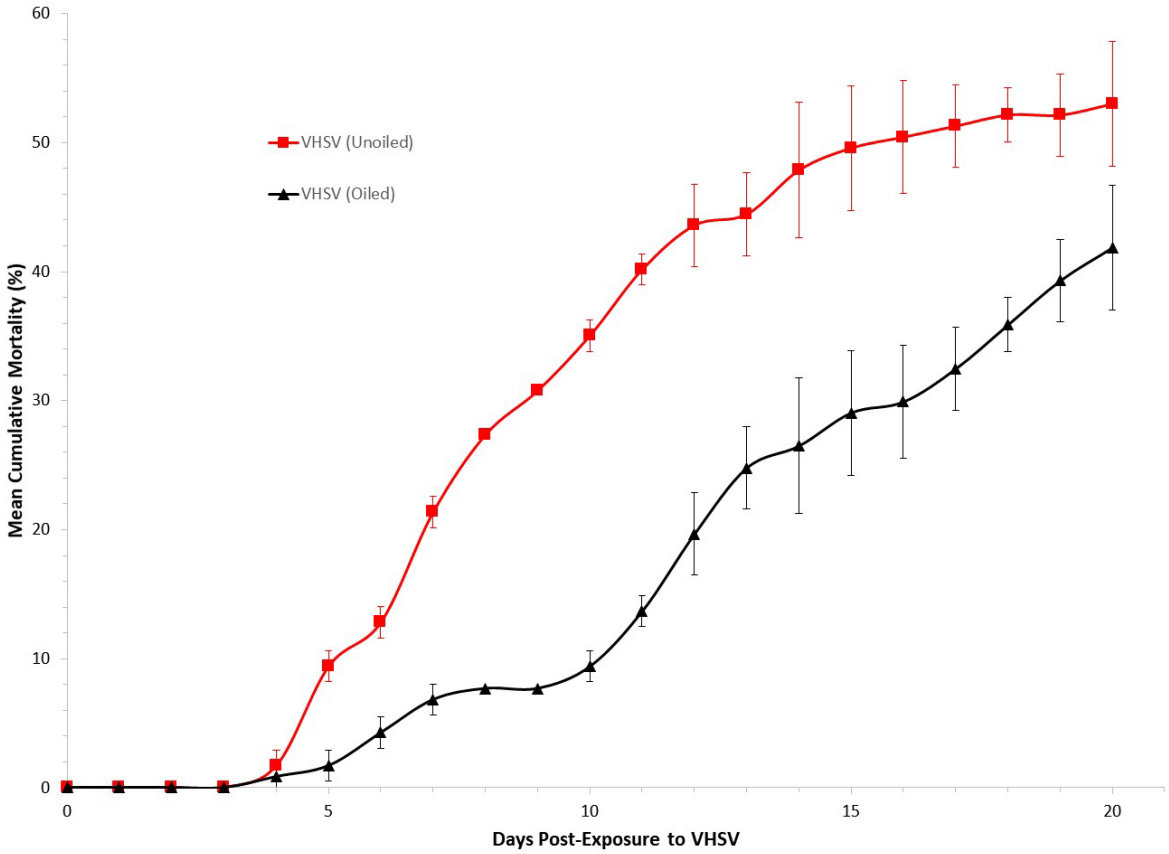

Figure 15. Pilot Study to examine the effects of larval polycyclic aromatic hydrocarbon exposure on the subsequent susceptibility of juvenile survivors to viral hemorrhagic septicemia virus (VHSV). Each data point represents mean cumulative mortality from each of 3 tanks (n = 39 herring / tank), and error bars indicate 2 SD. Mean cumulative mortalities in negative controls (exposed to saline in lieu of VHSV) for both treatments were ≤ 3.2% (results not shown).

Third Test (Expanded Study): Decreased Susceptibility of Juveniles that Survived Larval PAH Exposure:

An expanded study was performed using more robust sample sizes (n = 71-72 herring / tank x 3 replicate tanks / treatment), which permitted periodic subsampling for transcriptomics. Experimental animals were evenly represented by the original grow out tanks (e.g., Fig. 20). Fish were periodically sampled from each replicate to assess differences in the herring transcriptome between the various treatments (results pending). Additionally, genomic samples were collected to assess whether certain herring genotypes may be more susceptible to VHS (results pending). Mortality results were similar to those in the pilot study and were characterized by lower cumulative VHS mortalities among juveniles that survived larval exposure to PAHs than among unoiled cohorts (Fig. 20).

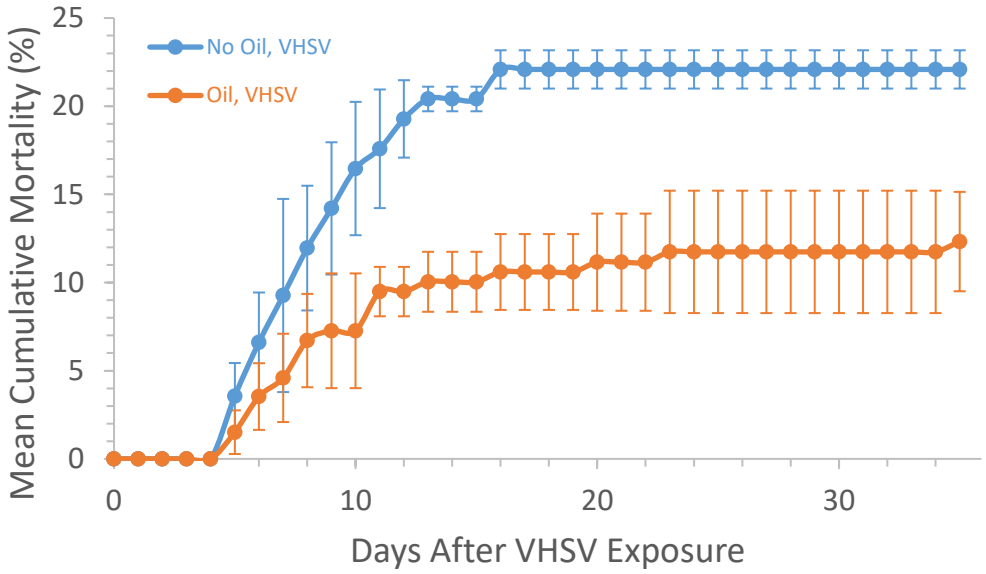

Figure 16. Expanded study to examine the effects of larval polycyclic aromatic hydrocarbon exposure on the subsequent susceptibility of juvenile survivors to viral hemorrhagic septicemia virus (VHSV). Each data point represents mean cumulative mortality from each of 3 tanks (n = 72 herring / tank), and error bars indicate 2 SD. Mean cumulative mortalities in negative controls (exposed to saline in lieu of VHSV) for both treatments were < 3.3% (results not shown).

Fourth Test: Ability of Oil-Exposed Survivors to Mount a protective Immune Response to VHSV:

In considering possible reasons for the VHS epizootic that occurred in PWS during the early 1990s, some have suggested that early life stage exposures of herring to PAHs compromised their ability to mount an adaptive (antibody) response to VHSV. If this were the case, then any prior exposures to VHS virus would not have resulted in the development of disease resistance in the form of herd immunity. As such, new recruits from the 1989-year class would have all been susceptible to VHS in 1992 - 1993, regardless of their prior exposure histories to VHSV.

To test this hypothesis, survivors of the prior VHSV exposure (third test) were re-distributed to new replicates (n = 3 replicate tanks / treatment, with each tank containing 20-21 herring) and re-exposed to VHSV 59 d after the initial virus exposure to assess whether larval exposure to PAHs impacted their ability to mount an adaptive immune response to VHSV. Cumulative mortality results provided no phenotypic indication that larval exposure to PAHs impacted the ability of juvenile survivors to mount a protective response after surviving VHSV exposure (Fig. 21).

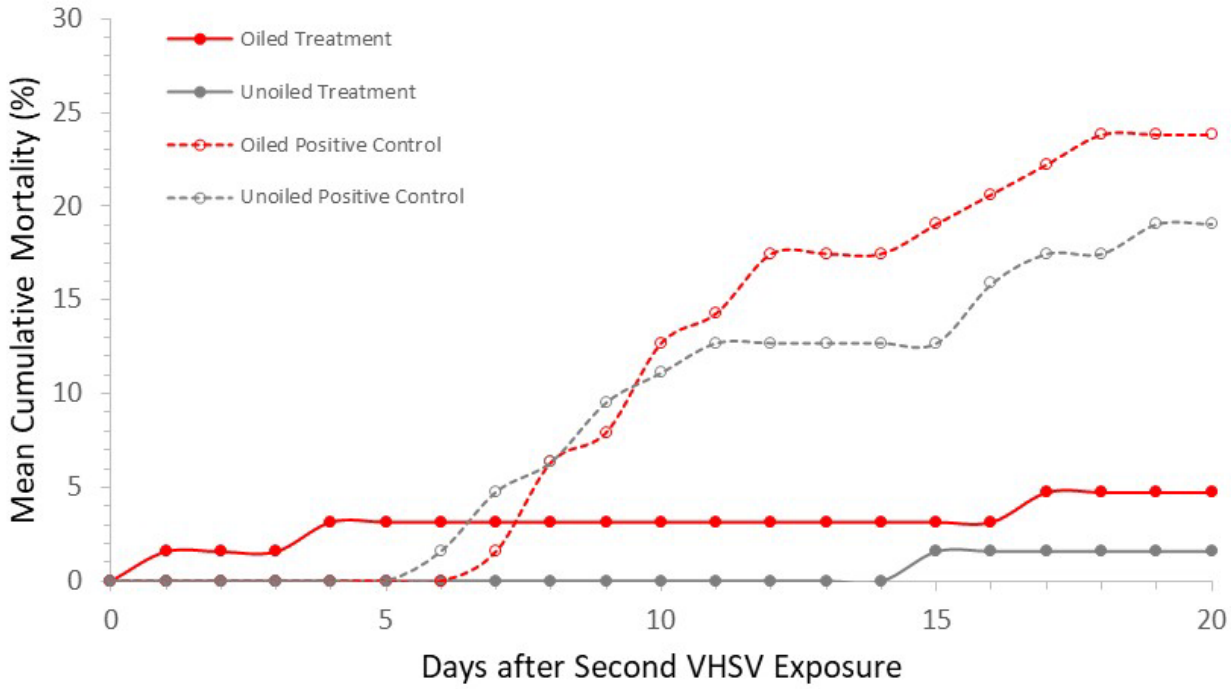

Figure 17. Ability of oiled survivors to mount a protective immune response against viral hemorrhagic septicemia virus (VHSV). Experimental fish were survivors from the previous experiment. Treatment groups were exposed to VHSV twice (first exposure depicted in Fig. 20 and second exposure depicted here). Positive Controls were exposed to saline in the first experiment and VHSV in this second experiment. Two sets of negative controls were included: 1) Groups that survived VHSV exposure in the first experiment that were exposed to saline in this experiment and 2) Groups that were exposed to saline in both experiments (mean cumulative mortalities < 1.6% for both groups; data not shown).

3.8 *Ichthyophonus* phylogenetics

The longstanding parasite species name *Ichthyophonus hoferi* has been applied to multiple parasite types due to historical assumptions of low biodiversity, and a lack of morphological structures that can be used to differentiate new species. Molecular methods are a logical next step in the description of these parasites, but markers used to date have been too conserved to resolve species boundaries. Here we used mitochondrial encoded cytochrome-c oxidase (MTCO1) gene sequences and phylogenic analysis to compare *Ichthyophonus* isolates from several marine and anadromous fish hosts. The resulting phylogeny (Fig. 22) displays lineage separation among isolates, and possible host/niche segregation not previously described. The parasite type that infects Pacific herring, Atlantic herring *Clupea harengus*, Atlantic salmon *Salmo salar*, and Pacific staghorn sculpin *Oligocottus maculosus* (Clade A) is different from that which infects Chinook salmon *Oncorhynchus tshawytscha,* walleye pollock *Gadus chalcogrammus*, Greenland halibut *Reinhardtius hippoglossoides*, and Pacific halibut *Hippoglossus stenolepsis* (Clade B).

This separation suggests that clupeids and other forage species may not be a source of *Ichthyophonus* infection for the larger predators. MTCO1 sequences confirmed the presence of a more divergent form of *Ichthyophonus* isolated from American shad *Alosa sapidissima* in rivers of Eastern North America (Clade C), while American shad introduced to the Pacific Ocean are infected with the same parasite that infects Pacific herring (Clade A). Currently there are no consensus criteria for delimiting species within *Ichthyophonidae*, but MTCO1 sequences hold promise as a potential species identifying marker and useful epizootiological tool.

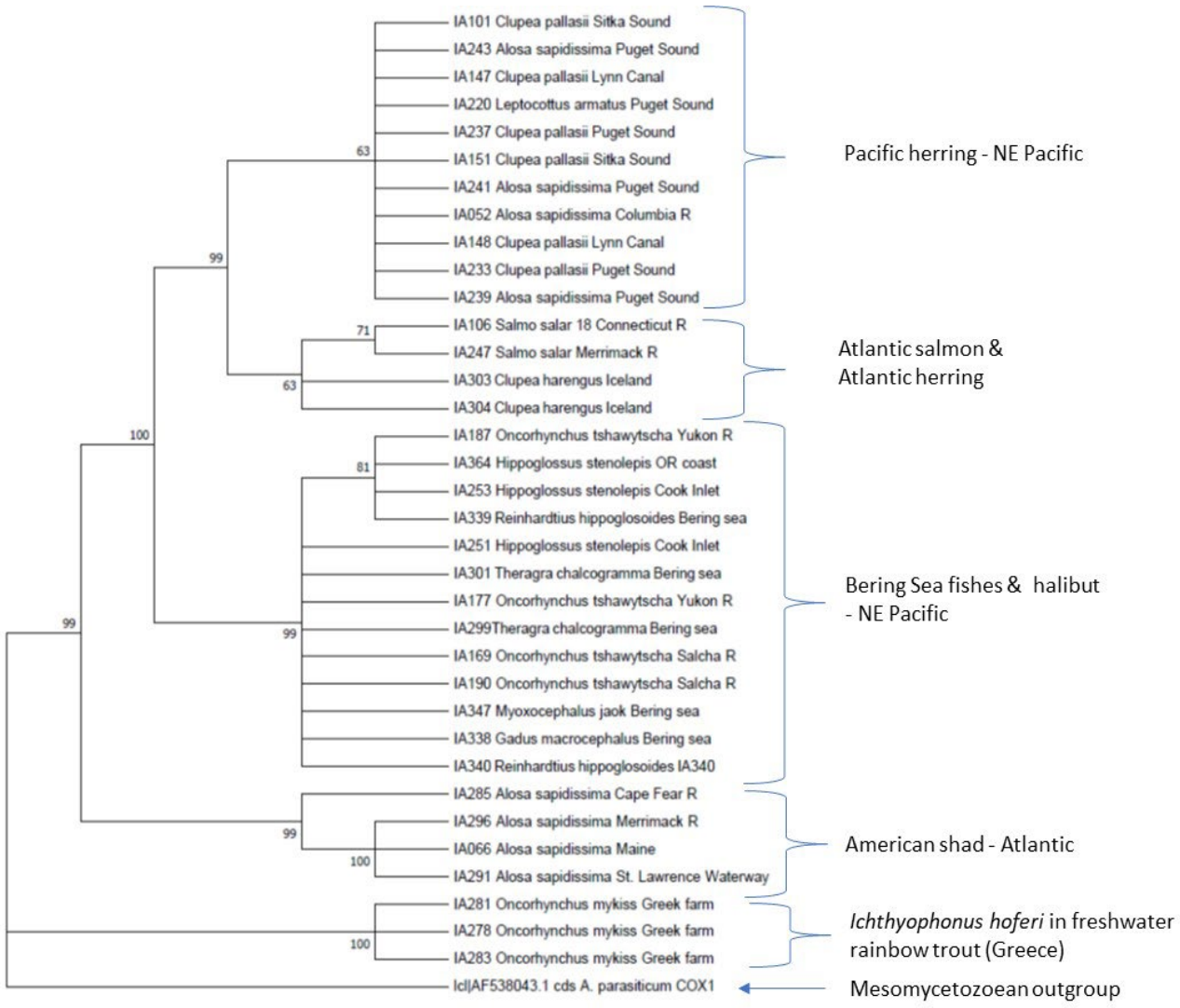

Figure 18. Ichthyophonus phylogenetics based on 1191 bases in the COX-1 gene. Values on the branches indicate percentages of bootstrap support, indicating the percentage of times the same branch occurred in repeated phylogenetic constructions.

3.9 Differential susceptibility of Yukon River and Salish Sea stocks of Chinook salmon *Oncorhynchus tshawytscha* to ichthyophoniasis

Under controlled laboratory challenges, juvenile Chinook salmon from a Yukon River stock were more susceptible to ichthyophoniasis than were those from a Salish Sea stock. After feeding with tissues from infected Pacific herring, Chinook salmon from both stocks became infected. The infection was persistent and progressive in Yukon River stock fish, where infections sometimes progressed to mortality and histological examinations revealed parasite dissemination and proliferation throughout the host tissues. However, infections were largely transient in Salish Sea-origin fish, where host mortalities were rare, and parasite stages were largely cleared from most tissues after 3-4 weeks. Susceptibility differences were evidenced by greater cumulative mortality, infection prevalence, parasite density, proportion of fish demonstrating a cellular response, and intensity of the cellular response among fish from the Yukon River stock. These observed differences between Chinook salmon stocks were consistent when parasite exposures occurred in both fresh water and seawater (Fig. 23). These results support the hypothesis that a longer-standing host-pathogen relationship, resulting in decreased disease susceptibility, exists among Salish Sea Chinook salmon than among Yukon River conspecifics.

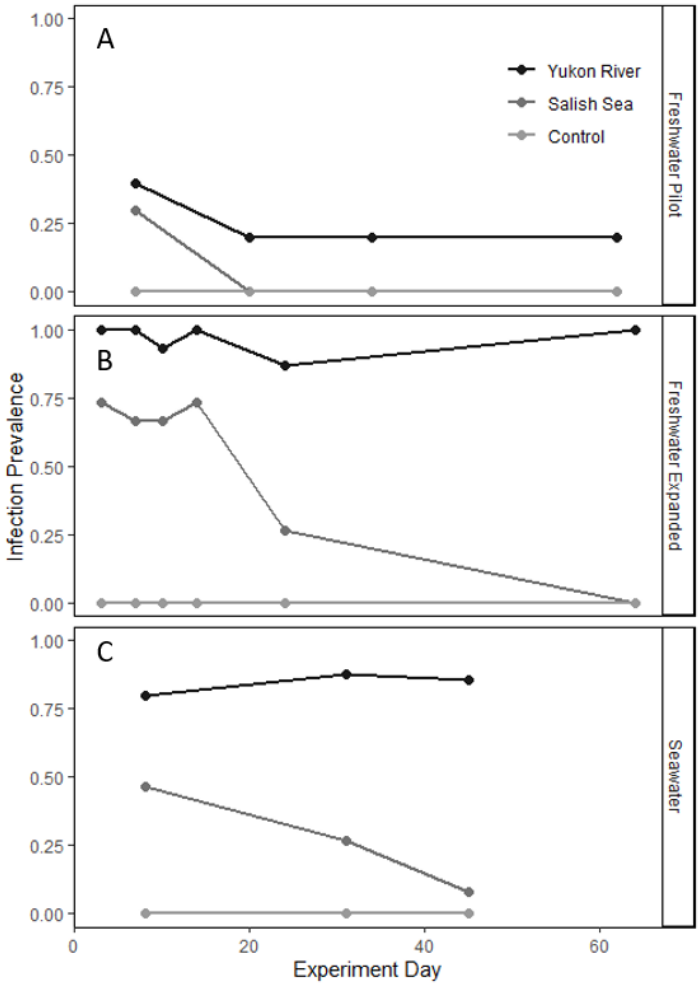

Figure 19. Ichthyophonus infection prevalence in heart and liver tissues cultured from subsampled survivors from the freshwater pilot (A), freshwater expanded (B), and seawater (C) exposure studies. For the seawater experiment, the only experiment in which separate cultures were made from heart and liver tissues, results were considered positive if one or both tissues tested positive for Ichthyophonus. Ichthyophonus was not detected in any unexposed fish from the negative control groups. Sample sizes were as follows (Freshwater pilot: n = 10 on d 7, 10 and 34 and n=15 on d 64; Freshwater expanded: n = 15, except on day 64 when n = 12 for Yukon River exposed fish and n = 6 for Salish Sea exposed fish; Seawater: n = 15-17 on days 8 and 31 and n = 24-28 on day 45).

3.10 *Ichthyophonus* in Opaleye (*Girella nigricans*)

Over a 3-year period, 17 wild-caught opaleye (*Girella nigricans*) housed in a public display aquarium were found dead without premonitory signs. Grossly, four animals had pinpoint brown/black foci on coelomic adipose tissue. Histologically, the liver, spleen, heart and posterior kidney had mesomycetozoan granulomas in all cases; other organs were less commonly infected. Four opaleye had goiter; additional substantial lesions were not identified. Granulomas

surrounded melanized debris, leukocytes, and mesomycetozoa, represented by folded membranes (collapsed schizont wall), intact schizonts (50- to >200-µm-diameter with a multilaminate membrane), plasmodia (budding from schizont or free in tissue), or rarely germinal tubes (Fig. 24). *Ichthyophonus* was grown from fresh, unfrozen tissues in tissue explant broth cultures of the heart, liver, and/or spleen. PCR using 18S rDNA primers returned a 1730 BP region, the sequence of which aligned most closely with *I. hoferi*, which is often associated with freshwater aquaculture fishes (Fig. 25).

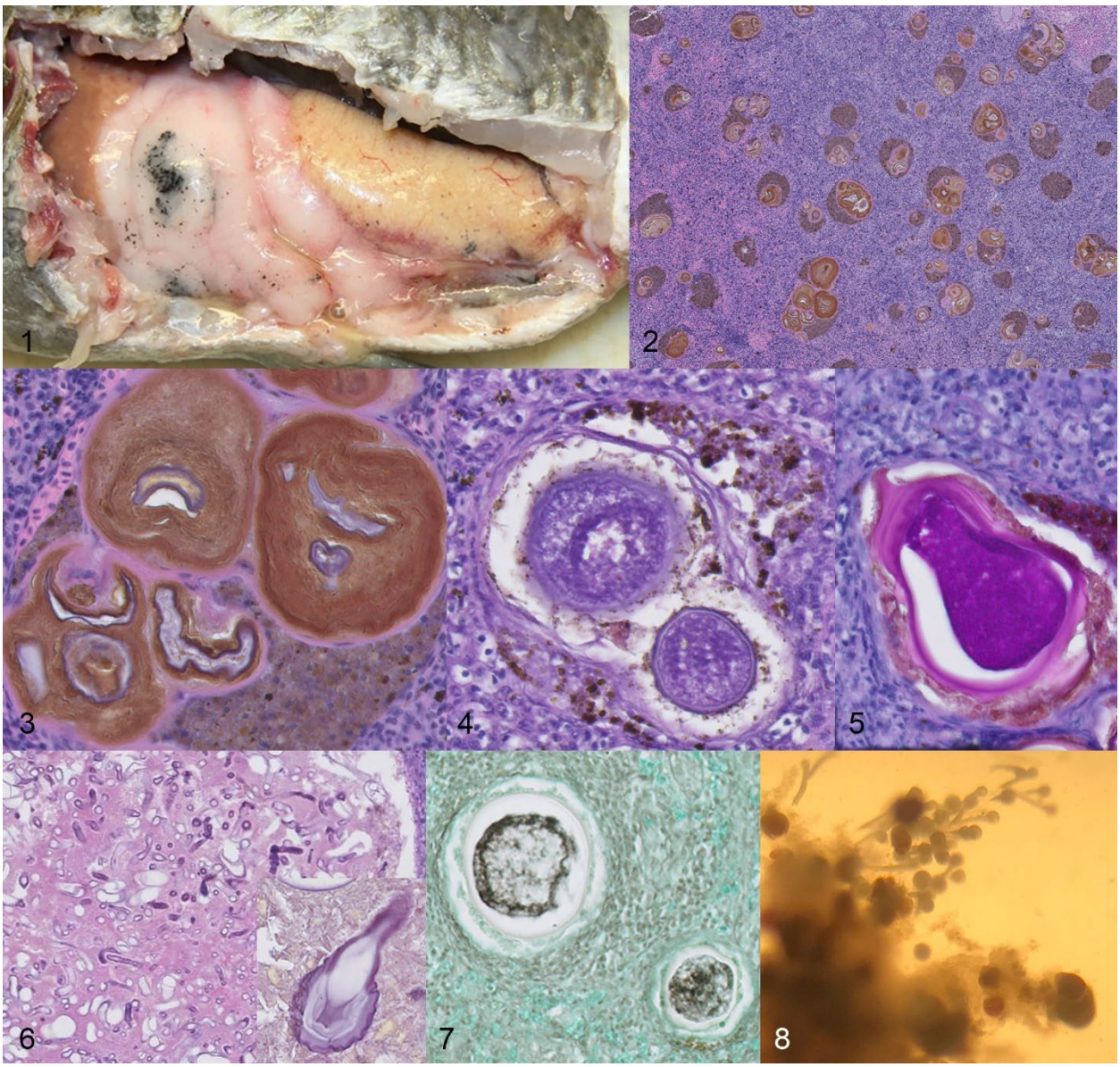

*Figure 20. Ichthyophoniasis in opaleye (Girella nigricans). **1**. coelom, case No. 5. Pinpoint black foci confirmed histologically as Ichthyophonus granulomas are throughout the coelomic adipose tissue and ovaries. **2**. spleen, case No. 17. Approximately 30%-40% of the splenic parenchyma is expanded by pigmented granulomas containing folded schizont walls. **3**. spleen, case No 17. Higher magnification of figure 2, showing folded membranes*

*representing walls of ruptured schizonts. **4**. spleen, case No. 7. Two intact schizonts in a pigmented granuloma have a multilaminate wall and numerous internal merozoites. **5**. spleen, case No. 6. Plasmodia budding from the schizont have a "flask shape." **6**. spleen, case No. 4. A large granuloma contains numerous hyphae that lack septae and have non-parallel walls. **7**. spleen, case No. 6. Internal merozoites of a schizont are argyrophilic.. **8**. tissue explant culture, spleen, case No. 11. On the culture plate, there is abundant growth typical of Ichthyophonus, which includes round schizonts with branching germ tubes.*

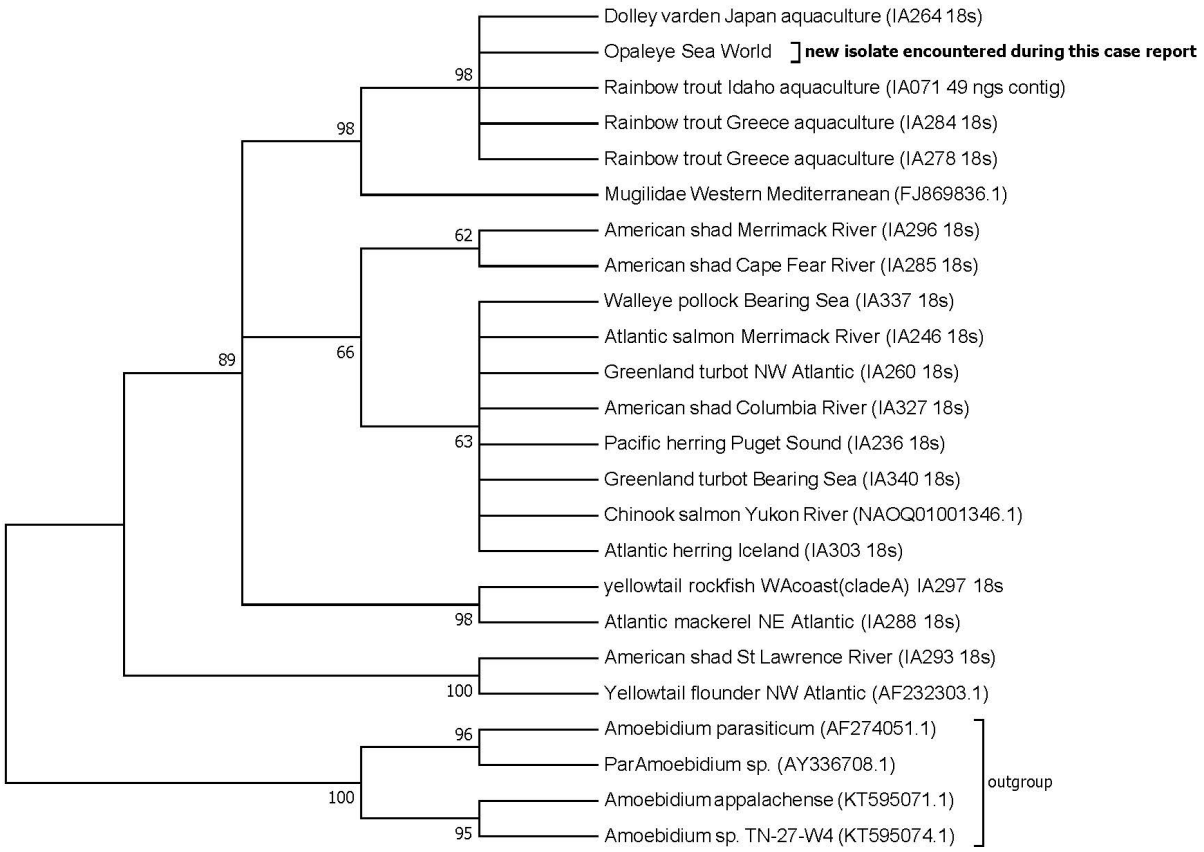

Figure 21. Phylogeny of Ichthyophonus isolates from case No. 13 (Opaleye SeaWorld 199-2), based on rDNA SSU sequences. Gene tree inferred using Maximum Likelihood methods on a GTR-I model with 1000 bootstrap replicates in the program MEGA7 (Kumer et al. 2016). Host and host capture location indicated at the end of each branch for Ichthyophonus isolates but not for Amoebidium spp. included as outgroup. GenBank accession numbers, or other unique ID provided in parentheses.

CONCLUSIONS

- *Ichthyophonus* infections remained endemic in Pacific herring throughout the NE Pacific during the study period, with infection prevalence in PWS ranging from 10%-23% in 2017, 8.3%-22% in 2018, 18%-20% in 2019, 12%-19% in 2020, and 17%-22% in 2022. Infection prevalence of the parasite generally increased with herring size / age.
- VHSV was not isolated from per-spawn Pacific herring during the study period; however, evidence of annual exposures to the virus was provided by the serological surveillances. Serological surveillance indicated a change in VHSV exposures from 2018 to 2019, when the proportion of fish with detectable levels of neutralizing antibodies decreased from 9.1%-1.3% in PWS and from 12%-1.7% in Sitka Sound. Thereafter, the seropositive rate remained low throughout the duration of the study period. The prevalence of neutralizing antibodies generally increased with herring size / age each year.
- VHS epizootics were documented annually from juvenile herring aggregations in the same geographic locations, indicating that these epizootics occur routinely if the appropriate environmental conditions are present.
- The plaque neutralization test (neutralizing antibody assay) was validated using wild herring, where it is generally effective correctly identifying group of herring with deduced exposure histories. Neutralizing antibodies are detectable as long as 3 years after herring survived a single exposure to VHSV.
- Laboratory studies demonstrated that Pacific herring satisfy all the requirements to serve as a natural reservoir for VHSV.
- VHSV shedding is higher at cooler temperatures, and it appears as though shedding may relapse as the seasonal water temperatures decrease.
- Fully convalesced herring continue to shed infectious virus at low levels for months after the virus can no longer be isolated from the kidney / spleen tissues, further qPCR results indicated that the virus remains in association with the gills of fully recovered herring for months after it can no longer be isolated from the internal tissues.
- Pacific herring did not demonstrate susceptibility to vibriosis in controlled laboratory exposures.
- There was no evidence of a planktonic intermediate host for *Ichthyophonus*, and the parasite could not be transferred to Pacific herring through cohabitation. Future investigations will focus on the determining whether transmission is the result of ovivory on infected fish eggs.
- High prevalence of *Ichthyophonus* infections occurs in juvenile Pacific herring from Cordova Harbor and the prevalence increases dramatically from April to June.
- Homologous and heterologous DNA vaccines are effective at immunizing Pacific herring against VHSV.
- qPCR using *Ichthyophonus*-specific primers was highly specific and sensitive for detecting *Ichthyophonus*, indicating that this diagnostic tool may be appropriate for certain field applications.
- Herring susceptibility to *Ichthyophonus* is increased after embryonic exposure to PAHs.

- Herring early life stage exposures to PAHs sometimes lead to decreased susceptibility to VHS.
- Different genetic types of *Ichthyophonus* occur in sympatry in fishes from the NE Pacific.

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Publicly available datasets

All herring surveillance metadata resulting from this project are housed on DataOne (https://search.dataone.org/view/10.24431%2Frw1k32b) and metadata from the laboratory studies are housed on ScienceBase (https://www.sciencebase.gov/catalog/items?filter=systemType%3DData+Release&filter=browseCategory%21%3DData+Release+-+In+Progress&q=hershberger).

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Cypher, A. D., P. **Hershberger**, N. Scholz, and J. P. Incardona. 2019. Larval cardiotoxicity and juvenile performance are likely contributors to the delayed fishery collapse of Pacific herring after the *Exxon Valdez* oil spill. Poster presentation, Society for Integrative & Comparative Biology Annual Meeting. Tampa, FL. January.

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Groner, M., E. Bravo, C. Conway, J. Gregg, P. **Hershberger**. 2019. A quantitative histological index to differentiate between endemic and epidemic ichtyophoniasis in Pacific herring. Poster presentation, Alaska Marine Science Symposium. Anchorage, AK. January.

Groner, M. L., E. Bravo-Mendoza, C. M. Conway, A. H. MacKenzie, J. L. Gregg, and P. K. **Hershberger**. 2021. Epidemiology of ichthyophoniasis in Pacific herring in Sitka Sound and Prince William Sound from 2007 – 2018. Virtual platform presentation, Alaska Marine Science Symposium. Anchorage, AK. January.

Hershberger, P. K., L. Hart, A. MacKenzie, R, Powers, and M. Purcell. 2017. Quantifying the potential for disease impacts to Pacific Herring. Poster presentation. Alaska Marine Science Symposium. Anchorage, AK. January.

Hershberger, P. K., A. H. MacKenzie, J. L. Gregg, R. Powers, and M. K. Purcell. 2020. Long term shedding of viral hemorrhagic septicemia virus from Pacific herring. Poster presentation. Alaska Marine Science Symposium. Anchorage, AK. January.

Hershberger, P. K., A. H. MacKenzie, J. L. Gregg, M. D. Wilmot, R. Powers, and M. K. Purcell. 2017. Long term shedding of viral hemorrhagic septicemia virus from Pacific herring. Virtual platform presentation. 58th Western Fish Disease Workshop. Suquamish, WA. June.

MacKenzie, A. H., J. L. Gregg, M. D. Wilmot, T. Sandell, D. Lowry, and P. K. **Hershberger**. 2017. Temporal and spatial patterns of *Ichthyophonus* in Pacific herring throughout the southern Salish Sea. Poster presentation. 58th Western Fish Disease Workshop. Suquamish, WA. June.

Mena, A. J., J. St. Ledger, A. MacKenzie, J. Gregg, M. Purcell, W. Batts, P. **Hershberger**, and E. E. B. LaDouceur. 2020. *Ichthyophonus* sp. infection in opaleye (*Girella nigricans*). Poster presentation. International Aquatic Animal Medicine Conference. Tampa, FL. May.

Sitkiewicz, S., B. Harris, P. **Hershberger**, and N. Wolf. 2017. Impacts of the Parasite *Ichthyophonus* (sp.) on Groundfish Growth and Condition. Poster presentation. Joint Meeting of the American Fisheries Society, Alaska Chapter American Water Resources Association, Alaska Section. Fairbanks, AK. March.

Sitkiewicz, S., B. Harris, P. **Hershberger**, and N. Wolf. 2017. Effects of the parasite *Ichthyophonus* (sp.) on groundfish growth and condition. Poster presentation. Alaska Marine Science Symposium. Anchorage, AK. January.

Sitkiewiz, S. E., B. P. Harris, P. K. **Hershberger**, and N. Wolf. 2017. Effects of the parasite *Ichthyophonus* on groundfish growth and condition. Poster presentation. 58th Western Fish Disease Workshop. Suquamish, WA. June.

Sitkiewicz, S., P. **Hershberger**, N. Wolf, and B. Harris. 2018. Effects of the parasite *Ichthyophonus* (spp.) on Pacific halibut (*Hippoglossus stenolepis*) growth and condition. Poster presentation. Alaska Marine Science Symposium. Anchorage, AK. January.

Stinson, M. E., B. C. Hall, B. C. Stewart, and P. K. **Hershberger.** 2017. Validation of improved *Listonella* (*Vibrio*) *anguillarum* vaccine in coho salmon. Poster presentation. 58th Western Fish Disease Workshop. Suquamish, WA. June.

Trochta, J., M. Groner, P. **Hershberger**, and T. Branch. 2021. Using antibody data to inform viral infection history and improve estimates of survival in fisheries stock assessment models. Virtual platform presentation. Alaska Marine Science Symposium. Anchorage, AK. January.

Wendt, C., P. **Hershberger**, and C. Wood. 2019. Patterns of *Ichthyophonus* sp. infection in age zero Pacific herring. Poster presentation. Alaska Marine Science Symposium. Anchorage, AK. January.

Outreach

Hershberger, P. K. 2020. Marine diseases. Guest panel member. Marine and Coastal Science Seminar, Western Washington University. June 1.

Hershberger, P. K. 2019. Principals of l hemorrhagic septicemia virus. Guest lecture in FHL 568. Ecology of Infectious Marine Disease. University of Washington, Friday Harbor Laboratories. June 27-28.

Hershberger, P. K. 2018. Causes of Pacific herring mortality: A disease perspective. Annual Science Night invited talk. Prince William Sound Regional Citizens Advisory Council. December 6.

Hershberger, P. K. 2018. The ecology of disease in marine fishes: Insights from Pacific herring. Invited seminar. NOAA – Northwest Fisheries Science Center, Monster Seminar Jam. May 24.

Hershberger, P. K. 2018. Salish Sea marine survival project, an update on Puget Sound Research. Invited talk. Puget Sound Steelhead Advisory Group, Lynwood, WA. January 18.

Hershberger, P. K. 2017. Long term shedding of VHS virus from Pacific herring: Demonstration of a marine reservoir host. Invited talk. Washington State Disease Co-Managers Meeting, Olympia, WA. July 5-6.