Exxon Valdez Oil Spill
Restoration Project Final Report

Prince William Sound Herring Disease Program (HDP)
Restoration Project 070819
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Study History:

The biomass of Pacific herring in Prince William Sound, AK decreased from 120,000 metric tons to less than 30,000 tons following the *Exxon Valdez* Oil Spill (EVOS) in 1989. In the ensuing decades since the spill, several impacted species have recovered; however, Pacific herring populations have yet to rebound to pre-spill biomass levels. Cause(s) of this population decline and failed recovery remain unresolved; leading hypotheses include direct mortality from toxicological impacts of the spill, predation, competition for limited resources, and disease-related mortality. Because Pacific herring are a critical forage species throughout region, with their health and abundance influencing the health and recovery of several other key species, the *Exxon Valdez* Oil Spill Trustee Council (EVOS TC) launched an effort to identify and consider feasible herring restoration goals and options for Pacific herring in Prince William Sound. Recognizing that disease can be a primary driver of herring population size and zoographics, the Herring Disease Program (HDP) was initiated as a 4 year project from FY 2007-2010; a one year no cost extension was granted for FY 2011. The HDP is a logical extension of earlier disease surveillance efforts in PWS from 1994-2003 (#94320S, 95320S, 96162, 97162, 98162, 99328, 99462, 00462, 01462, 02462, 03462) that were led by Dr. Gary Marty (formerly U.C. Davis, currently British Columbia Ministry of Agriculture and Lands). The HDP represents a continuation of these surveys from 2007 onwards, and expands upon the previous efforts by including controlled empirical studies that address cause-and-effect relationships. This report summarizes the findings from the first five years of the HDP, including results from field-based disease surveillances and laboratory-based empirical studies.

Abstract:

Surveys of pathogens in Pacific herring from 2007 – 2010 indicated that *Ichthyophonus*, viral hemorrhagic septicemia virus, and erythrocytic necrosis virus are endemic in Prince William Sound and throughout the NE Pacific. Laboratory studies with VHSV indicated that multiple herring stocks are equally susceptible to the resulting disease, Pacific herring shed copious levels of VHSV (as high at 5×10^8 plaque-forming units / day) shortly after exposure, chronic and persistent infections can occur in Pacific herring, susceptibility of Pacific herring to VHS extends to the larval life stages but not the embryonic stages, and the prior exposure history of Pacific herring to VHSV can be determined post hoc. Laboratory studies involving *Ichthyophonus* indicated that schizonts can be inactivated with chlorine and iodine solutions, the parasite can survive for extended periods in saltwater but not freshwater, a low potential exists for cross contamination between in vitro explant cultures, infectious schizonts are released from the skin surface of infected herring, schizonts are not uniformly distributed throughout the skeletal muscle of infected Pacific herring, multiple types of *Ichthyophonus* exist with different genotypes and phenotypic traits, and temperature is an important factor influencing the infectivity of *Ichthyophonus*. Additional field and laboratory studies indicated that

Ichthyophonus negatively influences the swimming performance of infected hosts and the negative impacts effects are exacerbated by increasing temperatures, American shad are an important reservoir of *Ichthyophonus* in the NE Pacific, Pacific herring are not susceptible to infectious hematopoietic necrosis (IHN), and Pacific herring will accept surgically implanted acoustic tags with negligible impacts on survival.

Key Words:

Disease, *Ichthyophonus*, Pacific herring, VEN, VHS, viral erythrocytic necrosis, viral hemorrhagic septicemia.

Project Data

Data collected from this project include a combination of laboratory-based experimental data and field-based surveillance data. Laboratory data are archived in the form of laboratory notebooks, which are maintained at the USGS Marrowstone Marine Field Station. Summaries of these data will be maintained in perpetuity in the form of peer-reviewed manuscripts that are referenced throughout this report. Permanent archives of the field surveillance data, largely including annual pathogen surveillance assessments, are made through a combination of published results in peer reviewed journals and on the AOOS workspace: http://www.aoos.org/.

Citation:

Hershberger, P.K., Elliott, D., Emmenegger, E., Hansen, J., Kocan, R., Kurath, G., LaPatra, S., Winton, J. 2011. Prince William Sound Herring Disease Program, *Exxon Valdez* Oil Spill Restoration Project Final Report (Restoration project 070819), U.S. Geological Survey, Western Fisheries Research Center, Marrowstone Marine Field Station. http://www.evostc.state.ak.us/index.cfm?FA=searchResults.projectInfo&Project_ID=1585

TABLE OF CONTENTS

Executive Summary

All available data continue to support the hypothesis that direct and indirect mortalities from infectious and parasitic diseases, including viral hemorrhagic septicemia (VHS), viral erythrocytic necrosis (VEN, and ichthyophoniasis, contribute to population-level impacts on Pacific herring populations in PWS and throughout the NE Pacific. Results from laboratory-based studies indicate that Pacific herring are highly susceptible to these diseases and exposure to the causative agents often results in host mortality. Additionally, each of these diseases is periodically associated with fish kills among Clupeid populations throughout the world.

High prevalences of *Ichthyophonus*, an internal Mesomycetozoean parasite of marine fishes, occurred in populations of Pacific herring *Clupea pallasii* throughout the NE Pacific. Infection prevalence varied with geographic location, season, and population age structure, with prevalences in adult herring from Prince William Sound ranging from 12 - 45% during 2007 - 2010. Although the geographic and host ranges of *Ichthyophonus* are extremely broad, the latitudinal range of the parasite in Pacific herring appears to end north of California (southern extreme) and south of the Bering Sea, Alaska (northern extreme). Laboratory studies indicate that *Ichthyophonus* can be highly pathogenic to Pacific herring, with exposure often resulting in rapid mortality. There is no indication that Pacific herring are able to clear the infection; therefore, a seasonal decline in *Ichthyophonus* prevalence that occurred in PWS from spring through autumn each year was likely a reflection of selective mortality among *Ichthyophonus*-infected cohorts. Several basic concepts of *Ichthyophonus* epizootiology remain unknown, most notably the route of infection for Pacific herring and other planktivorous fishes remains purely speculative. However, results from laboratory studies indicate that the parasite is more infectious to Pacific herring at cooler temperatures, suggesting that infections in wild populations may be established during periods of cool water temperatures.

During the study period 2007 – 2010, VHSV was detected in a single juvenile herring (Whale Bay, PWS, 2010) from a total sample size of 1787. This low prevalence is neither surprising nor uncommon, considering the prevalence of VHSV is generally extremely low during endemic periods (i.e. below the detection threshold of 5% prevalence with 95% confidence provided by a 60 fish sample size). However, VHS epizootics accompanied by high mortality can occur within days after these same fish become confined in high densities or subjected to limited water exchange (Hershberger et al 1999, Kocan et al 2001). Perhaps more surprising was the relative frequency of VHSV isolations that occurred during previous PWS herring disease surveillances in the 1990's, when VHSV was commonly detected at low prevalence and intensity during herring surveillance efforts. Previous VHSV isolations typically occurred among a low prevalence of the population at extremely low intensities, with viral tissue titers often detectable only after blind passage of tissues that were negative by standard viral plaque assay. Therefore, VHSV detections from the previous studies did not indicate the presence of disease epizootics; rather they confirmed that the virus was enzootic within the herring population. It should be emphasized that the kinetics of the disease resulting from VHSV are extremely rapid, and enzootic conditions can transition to epizootic conditions within a matter of days. Empirical studies performed here provide several insights into the epizootiology of VHS in Pacific herring, particularly:

- Multiple genetic stocks of Pacific herring throughout the NE Pacific are equally susceptible to VHS and survivors of the disease develop similar resistance to the disease upon re-exposure.

- Pacific herring that are diseased with VHS shed extremely high titers of VHSV into the water (as high as 10^8 PFU / d) only days after exposure to the virus. This copious viral shedding is likely an important factor for initiating epizootic cascades in Pacific herring populations, particularly under conditions of high herring density and limited water exchange.

- Pacific herring embryos are not susceptible to VHSV after waterborne exposure, likely because the egg chorion represents an impenetrable barrier to the virus. However, hatched larvae are highly susceptible to the disease; further, larval exposure to VHSV confers protection to the survivors, even after their metamorphosis to juveniles.

- Several tools, including a viral replication in excised fin tissue (VREFT) and passive immunization of naïve herring with plasma from VHS survivors, provide proof of concept that prior exposure histories of Pacific herring to VHSV can be determined post-hoc. Knowledge of herring exposure histories can potentially provide useful information for forecasting the potential for future VHS epizootics. Therefore, a high throughput technique for quantify herring herd immunity to VHS is currently being developed.

Viral erythrocytic necrosis (VEN) also occurred among herring populations in PWS and throughout the NE Pacific, with prevalence typically higher in juvenile than adult cohorts. During this study, we reported the first detection of VEN in PWS herring populations; however all available data indicate that the condition likely occurred in PWS previously but concerted surveillances for the disease were previously lacking. Additionally, we reported several VEN epizootics in juvenile Pacific herring from Puget Sound. Efforts were made to develop a confirmatory diagnostic technique for the etiological agent. Although several approaches to developing a molecular diagnostic tool were unsuccessful, we were able to develop specific molecular primers and a polymerase chain reaction (PCR) tool specific to the putative etiological agent. This breakthrough represents a significant advancement in our ability to study this disease, eliminating the need to rely on fresh blood samples for presumptive diagnosis. Confirmatory diagnosis of VEN can now be made from standard tissue samples. As an example of the utility of this technique, a fish kill of unknown etiology occurred among juvenile herring during the spring 2011; tissue samples were collected by biologist at the Prince William Sound Science Center and are currently being examined at the USGS Western Fisheries Research Center using the novel VEN primers.

List of Tables

List of Figures

Chapter 1: Surveillance of Pathogens and Diseases in Wild Herring Populations

Pathogen prevalence surveys were performed in Pacific herring populations throughout the NE Pacific, placing special emphasis on PWS herring stocks and reference stocks including Sitka Sound, Lynn Canal, and Puget Sound (Table 1). High prevalences of *Ichthyophonus* occurred in herring populations throughout the NE Pacific. Infection prevalence varied with geographic location, season, and population age structure, with prevalences in adult herring from Prince William Sound ranging from 12 - 45% during 2007-2010. Although the geographic and host range of *Ichthyophonus* are extremely broad, the latitudinal range of the parasite in Pacific herring appears to end north of California (southern extreme) and south of the Bering Sea, Alaska (northern extreme). There is no indication that Pacific herring are able to clear the infection; therefore, a seasonal decline in *Ichthyophonus* prevalence that occurred from spring through autumn each year likely reflected selective mortality among *Ichthyophonus*-infected cohorts.

Viral hemorrhagic septicemia virus (VHSV) is also endemic throughout the NE Pacific; however, from 2007-2010 the virus detected in only a single juvenile herring (from Whale Bay, PWS in 2010). This low prevalence is neither surprising nor uncommon, considering the prevalence of VHSV is generally extremely low during endemic periods (i.e. below the detection threshold of 5% prevalence with 95% confidence provided by a 60 fish sample size). However, disease epizootics accompanied by high mortality can occur within days after these same fish are confined in high concentrations or subjected to limited water exchange (Hershberger et al 1999, Kocan et al 2001). With VHSV, prevalence data are useful for identifying the presumed etiological agent during observed fish kills; however, as predictive tools, VHSV prevalence data are ineffective at forecasting disease impacts to herring populations. Therefore, the lack of detection of VHSV in wild herring does not indicate absence of the virus from herring populations; rather, it indicates that VHSV epizootics were not ongoing when the fish were sampled. When attempting to determine the potential for future VHS impacts to herring populations, a more appropriate population screening tool would employ techniques to quantify herd immunity using a serological test. For example, the susceptibility status of populations, based on the presence or absence of humoral immunity, could be determined and used to identify whether herring populations are susceptible future disease outbreaks. Proof of concept for this serological screening concept was developed during this project (ie. 2.5 and 2.6); consequently, a high-throughput diagnostic technique to detect herring antibodies to VHSV is currently being developed and validated in a newly funded EVOS TC project (#10100132I).

Viral erythrocytic necrosis (VEN) was endemic in herring populations throughout the NE Pacific, especially in juvenile cohorts. During this study, we provided the first documentation of VEN in Prince William Sound herring, documented several VEN epizootics in juvenile herring from Skagit Bay, Puget Sound, and documented a VEN epizootic in juvenile herring from Cordova Harbor during June 2010.

Table 1. Results of pathogen prevalence surveys in Pacific herring sampled from 2007-2010.

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	April 5	A	224 (17)	42% (25/60)	0% (0/60)	0% (0/60)	ADF&G #07-0540
		Simpson Bay	April 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
	Sitka	S. Cannon Island	April 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[1]	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	April 30	A	184 (13)	25% (15/60)	ND	0% (0/60)	MMFS #ICH 07-1
		Skagit Bay	April 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	June 19-20	J	116 (17)	ND	ND	38% (23/60)	MMFS #VEN Surveys
		Skagit Bay	July 24-25	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	Sept 18-20	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
		Port Townsend Bay	Oct 16	J	80 (6)	ND	ND	20% (15/75)	MMFS #VEN Surveys

[1]160 northern anchovies were also sampled from Puget Sound (Holmes Harbor) on March 11; neither VHSV nor *Ichthyophonus* was detected.

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
		?	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		Mar 5	A	262 (14)	30% (18/60)	ND	ND	MMFS #AK-081A
		N. Middle Island	March 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
			April 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[2]	Drayton Pass	Jan 15	A	144 (7)	2% (1/60)	ND	ND	MMFS #ICH 08-1
		Port Orchard (Yukon Harbor)	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	June 23-25	J	145 (24)	ND	ND	15% (8/53)	MMFS #VEN FF08
		Skagit Bay	July 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	Sept 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

[2]Four Pacific Sardines were collected from Port Orchard on March 5; none tested positive for VHSV.

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	April 13	A	217(27)	26% (16/62)	ND	ND	ADF&G #09-0543 & MMFS #AK 09-1
		Unknown Location	April 4-9	A		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
	Sitka	Guide Island	Feb 15-16	A	256 (15)	40% (32/80)	ND	ND	ADF&G #09-0540
		?	Mar 24-27	A	270 (19)	46% (20/44)	0% (0/44)	ND	ADF&G #09-0545 & MMFS AK 09-2
		St. John Babtist Bay	Mar 24-27	A	248 (23)	31% (21/67)	0% (0/67)	0% (0/67)	ADF&G #09-0545 & MMFS AK 09-2
		?	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	A		8% (5/60)	ND	ND	MMFS #AK09-4
	Puget Sound	Port Orchard (Yukon H.)	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	March 18	A	193 (20)	22% (13/60)	ND	ND	MMFS #PS 09-1
		Skagit Bay	June	J	122 (11)	ND	ND	55% (33/60)	MMFS #VEN FF09
		Skagit Bay	July	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)			17% (10/60)	MMFS #VEN FF09
	San Fran. Bay[3]	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

[3]Additional samples from San Francisco Bay included 69 longfin smelt (Jan 6-13) and 70 striped bass (May 15); none tested positive for *Ichthyophonus*.

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%[4]	10% (6/60	ADF&G #10-0536 & MMFS # AK10-1
		Cordova Harbor	June 2-13	J	84	35% (17/49)	0% (0/49)	71% (38/48)	MMFS #AK 10-3
		Cordova Harbor	Aug 18	J	35	0% (0/18)	0% (0/54)	0% (0/17)	MMFS #AK 10-3
		Cordova Harbor	Sept 28 -Oct 7	J	60	0% (0/22)	0% (0/22)	0% (0/21)	MMFS #AK 10-3
		Simpson Bay	Nov. 3	J	73	0% (0/38)	ND	6% (2/36	MMFS #AK 10-3
		Port Fidalgo	Nov. 4	J	77	0% (0/22)	ND	5% (1/22)	MMFS #AK 10-3
		Eaglik	Nov. 5	J	90	0% (0/34)	ND	26% (8/31)	MMFS #AK 10-3
		Whale Bay	Nov 10-11	J	95	3% (2/58)	2% (1/60)	18% (10/55)	MMFS #AK 10-3
	Sitka	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		Mar 15-16	A	202 (20)	5% (3/56)	ND	ND	MMFS #AK10-4
			April 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	March 23	A	171 (15)	28% (17/60)[5]	ND	ND	MMFS #PS10-1
		Hood Canal[6,7]	May 25&27	A	140 (24)	44% (43/97)	ND	ND	MMFS #PS10-1

[4] A single pooled sample containing tissues from 3 fish tested positive (n=60) for VHSV. Therefore, the prevalence was 1-3 / 60.

[5] Biased sample: largest fish were removed from this sample for other purposes prior to determination of *Ichthyophonus* prevalence.

[6] Sample consisted of post-spawn adult herring

[7] *Ichthyophonus* prevalence was 6% (1/17) in Pacific staghorn sculpin and 78% (28/36) in American shad.

Chapter 2: Empirical Studies Involving Viral Hemorrhagic Septicemia Virus and Pacific herring

The North American strain of VHSV (Genogroup IVa) is periodically associated with epizootics in wild marine species where it can be highly virulent. Monospecific VHS epidemics 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). Additionally, VHS is hypothesized to have a great effect on the decline and failed recovery of Pacific herring populations in PWS (Marty et al 2003). Epidemics of mixed host assemblages involving Pacific sardines 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 epidemics involving Pacific herring, Pacific hake, and walleye pollock occurred during 1998 in Lisianski Inlet (Alaska; Meyers et al 1999). Furthermore, capture and confinement of Pacific herring, Pacific sandlance, and surf smelt routinely results in locally severe VHS epidemics among the confined populations (Hershberger et al 1999, Kocan et al 2001, Hedrick et al 2003). In Pacific herring prevalence and severity of VHS decreases with age (Kocan et al 1997, Hershberger et al 1999, Marty et al 2003). Wild juvenile herring are exposed to VHSV as early as 3 months post-hatch, shortly after their metamorphosis from larvae (Kocan et al 2001). As juveniles, Pacific herring are highly susceptible to VHS, with laboratory exposures resulting in 66-100% mortality. 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 possible mechanism of adaptive immunity in adults that originates from previous exposures to the virus, or the onset of innate resistance with adult age.

Studies described in this chapter were designed to understand cause-and-effect epizootiological relationships between VHSV and its natural host (Pacific herring); it is our goal to integrate these relationships into forecasting tools that are capable of predicting the potential for future VHS epizootics.

2.1 Pacific Herring from Different Stocks are Similarly Susceptible to VHS

The basis of our *in vivo* laboratory model system involves the availability of Specific Pathogen-Free (SPF) Pacific herring with a known exposure history. During the course of this project, we have been successful at rearing 10,000-30,000 SPF herring each year at the Marrowstone Marine Field Station. Most of these SPF herring colonies originated from genetic stocks of Pacific herring occurring in Puget Sound, WA. To determine whether the results of VHSV exposure studies to Puget Sound herring are applicable to stocks occurring in Prince William Sound, we compared the relative VHS susceptibilities and resulting adaptive immune responses of herring from three different stocks (including PWS).

Laboratory exposures of specific-pathogen-free Pacific herring from three distinct populations (Tables 2 and 3) indicated that stock origin had no effect on susceptibility to viral hemorrhagic septicemia (VHS). All populations were highly susceptible to the disease upon initial exposure; with significantly greater ($p < 0.002$) cumulative mortalities occurring in exposed treatment groups (56.3-64.3%) than in unexposed control groups (0.8-9.0%; Figure 1). Inter-stock

differences in cumulative mortalities were not significant ($p = 0.79$). Virus load in the tissues of mortalities was 10^1 to 10^4 times higher during the acute phase of the epizootics (day 13 post-exposure) than during the recovery phase (day 30-42 post-exposure; Table 4). Survivors of the epizootics were refractory to subsequent VHS; with re-exposure of VHS survivors (Table 5) resulting in significantly less ($p < 0.00026$) cumulative mortality (1.2-4.0%) than among positive controls (38.1-64.4%); inter-stock differences in susceptibility did not occur after re-exposure ($p = 0.58$; Figure 1). The results indicate that data from experiments designed to understand the ecology of VHSV in a given stock of Pacific herring are broadly applicable to herring stocks throughout the Northeast Pacific.

Table 2. Rearing details for stocks of Pacific herring used in this study.

	Puget Sound: Holmes Harbor	Puget Sound: Cherry Point	Prince William Sound
Date of egg collection	April 3, 2008	May 5, 2008	May 1, 2008
Date of peak hatch	April 14, 2008	May 18, 2008	May 16, 2008
Month of larval metamorphosis to juveniles	July, 2008	August, 2008	August, 2008
Age at first exposure to VHSV (October 24, 2008)*	193 days	158 days	161 days

*Date of peak hatch was considered day 0 when determining herring age.

Table 3. Number of herring and mean waterborne titer of VHSV in triplicate tanks during the first challenge. Herring in the negative control treatment groups were handled similarly to those in the VHSV treatment groups, but were exposed to phosphate buffered saline (PBS) rather than VHSV.

	Puget Sound: Holmes Harbor		Puget Sound: Cherry Point		Prince William Sound	
Treatment Group	VHSV	Neg. Control	VHSV	Neg. Control	VHSV	Neg. Control
Number of herring per triplicate tank	53-58	56-59	58-59	55-58	54-57	54-61
Mean exposure titer (pfu/mL) ± 1 SD	413 ± 81	0 ± 0	593 ± 110	0 ± 0	353 ± 58	0 ± 0

Table 4. Tissue titers from mortalities and survivors during select sampling days. Numerals indicate the geometric mean VHSV titer among the positive samples (NA = Not Applicable, virus was not isolated from any mortalities), numerals in parentheses indicate ratio of positive samples / n, where 'n' = number of samples from the three replicate tanks.

	Cherry Point	Holmes Harbor	Prince William Sound
First Challenge			
VHSV treatment mortalities (day 13)	3.4×10^5 (8 / 10)	9.0×10^6 (5 / 6)	1.7×10^5 (8 / 8)
VHSV treatment mortalities (days 30-42)	4.0×10^2 (1 / 5)	5.7×10^4 (4 / 9)	1.2×10^3(1 / 8)
Negative control mortalities (days 0-42)	NA (0 / 19)	NA (0 / 2)	NA (0 / 11)
Second Challenge			
Twice – exposed VHSV treatment mortalities (days 0-18)	5.6×10^5 (1 / 6)	2.2×10^6 (3 / 5)	NA (0 / 3)
Twice – exposed VHSV treatment survivors (day 18)	4×10^3 (1 / 64)	1.2×10^3 (1 / 57)	NA (0 / 71)
Positive control mortalities (day 8)	2.6×10^6 (4 / 4)	6.8×10^6 (11 / 11)	3.9×10^6 (4 / 4)
Positive control survivors (day 18)	1.6×10^3 (6 / 30)	4×10^2 (1 / 26)	7.7×10^3 (11 / 39)
Negative control survivors + mortalities (days 0-18)	NA (0 / 15)	NA (0 / 15)	NA (0 / 20)

Table 5. Number of herring and mean waterborne titer of VHSV in triplicate tanks during the second challenge. The second VHSV exposure occurred 42 d after the first. Herring in the VHSV treatment group were exposed to VHSV during both the first second challenges. Herring in the positive (+) control treatment group were exposed to PBS during the first challenge and VHSV during the second challenge. Herring in the negative (-) control treatment group were exposed to PBS during both the first and second challenges.

	Puget Sound: Holmes Harbor			Puget Sound: Cherry Point			Prince William Sound		
Treatment Group	VHSV	+ Control	- Control	VHSV	+ Control	- Control	VHSV	+ Control	- Control
Number of herring per triplicate tank	16-24	24-25	24-25	21-25	24-25	22-26	22-26	20-27	25-27
Mean exposure titer (pfu/mL) ± 1 SD	2,620 ± 251	2,827 ± 252	0 ± 0	3,333 ± 195	2,933 ± 181	0 ± 0	2,767 ± 310	3,667 ± 231	0 ± 0

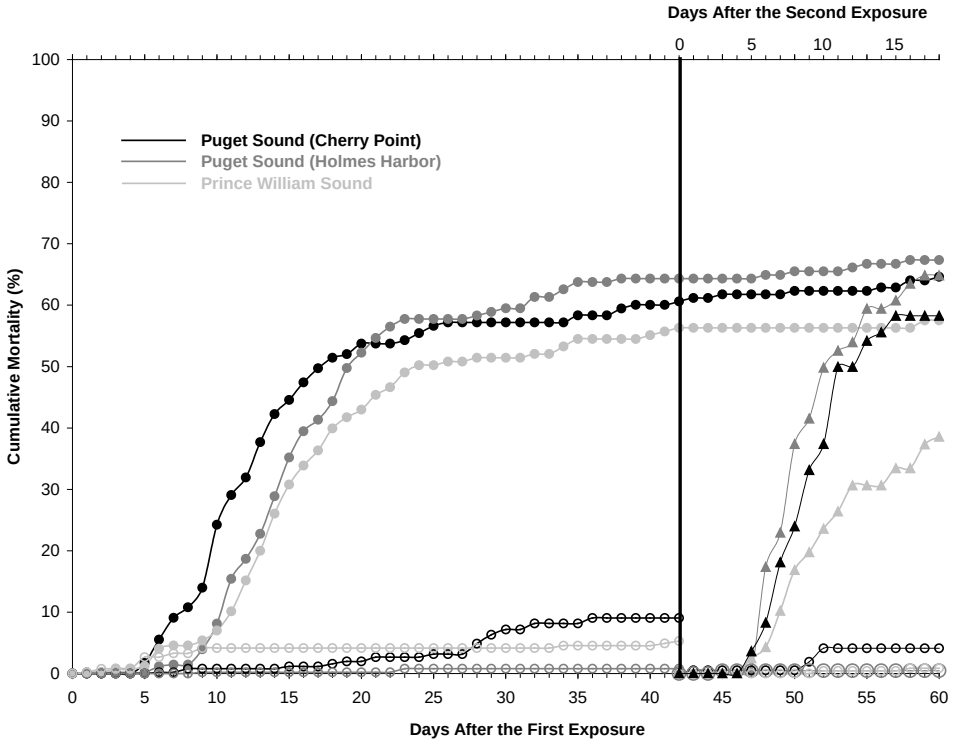


Figure 1. Relative susceptibilities of Pacific herring from three different stocks (Holmes Harbor, Cherry Point, and Prince William Sound) to VHS. Closed circles indicate treatment groups exposed to VHSV and open circles indicate negative control groups (exposed to saline). All survivors in the VHSV treatment groups from the first exposure were re-exposed to VHSV in the same tanks 42d later. All survivors in the negative control groups after 42 days were split into two groups (positive controls and negative controls) for the second exposure. Positive controls for the second exposure (closed triangles starting on day 42) were exposed to VHSV for the first time on day 42. Negative controls for the second exposure (open circles starting on day 42) were re-exposed to saline on day 42. All data points represent back-transformed percentages corresponding to the means of transformed (arc sin) proportions from triplicate tanks.

2.2 Viral Shedding from Infected Pacific herring

Factors contributing to the onset of VHS epizootics in populations of wild marine fishes are not well understood, but initial infection occurs following direct contact with exogenous, waterborne virus (Kocan et al. 2001). Previous laboratory studies demonstrated that epizootics are readily initiated in naïve stocks of Pacific herring after a 1 hr waterborne exposure to moderate levels (10^3 plaque-forming units (pfu) mL^{-1}) or greater of VHSV (Kocan et al. 1997). However, field surveys have detected only low titers of virus (5-15 pfu mL^{-1}) in samples of marine water collected in the vicinity of free-ranging schools of herring (Hershberger et al. 1999). The objectives of this study were to determine the minimum exposure thresholds required to initiate epizootics of VHS in Pacific herring and to quantify the kinetics of viral shedding from infected fish.

Outbreaks of acute VHS, accompanied by mortality and viral shedding, were initiated after waterborne exposure of herring to concentrations of VHSV as low as 10^1 plaque-forming units (pfu) mL^{-1} (Table 6). Shed virus in flow-through tanks was first detected 4-5 days post-exposure, peaked after 6-10 days, and was no longer detected after 16 days. Shedding rates, calculated from fish density, flow, and waterborne virus titer reached 1.8-5.0 $x10^8$ pfu $fish^{-1}$ day^{-1} (Table 7). Onset of viral shedding was dose-dependent and preceded initial mortality by 2 days. At 21 days, cumulative mortality in treatment groups ranged from 81-100% and was dependent not on challenge dose, but on the kinetics and level of viral shedding by infected fish in the tank (Figure 2). The results provide insights into critical virus amplification mechanism that precedes the onset of population-level VHS epizootics.

Table 6. Waterborne titer of VHSV during the initial exposure period. Numerals in series indicate the waterborne titer (pfu mL^{-1}) in each triplicate tank. '*' indicates replicates where VHS epizootics did not ensue. T_{3hr} and T_{26hr} samples were collected 2 hrs. after the water supply was resumed to the 1 and 24 hr. treatments, respectively.

	Exposure Treatments				
	1 hr exposures			24 hr exposures	
Time post-exposure	10^1 pfu mL^{-1}	10^2 pfu mL^{-1}	10^3 pfu mL^{-1}	10^1 pfu mL^{-1}	10^2 pfu mL^{-1}
0 hr	40*, 40*, 40*	300, 220, 240	2620, 2040, 3420	20, 20*, 40*	500, 400, 320
1 hr	20*, 80*, 40*	480, 380, 160	3160, 3090, 2940	60, 0*, 0*	380, 280, 220
3 hr	0*, 0*, 0*	40, 60, 40	1340, 1340, 1140		
24 hr				0, 0*, 0*	140, 0, 20
26 hr				0, 0*, 0*	0, 0, 0

Table 7. Daily VHSV shedding rates in each treatment (pfu herring^{-1} day^{-1}). Reported daily shedding rates represent the mean of 3 replicates, except treatments marked '*'. Minimum detectable shedding rate, based on assay detection threshold (20 pfu mL^{-1}), tank flow rate (3.5 L min^{-1}), number of fish in the replicates on day 0 (n≈30) and number of replicates (n=3) was 1.1 x 10^6 pfu fish^{-1} day^{-1}. Shed virus was not detected in any negative control replicate nor in replicates exposed to 10^1 pfu mL^{-1} for 1 hr.

	Exposure Treatments			
Days Post-Exposure*	10^3 pfu mL^{-1} 1 hr exposure	10^2 pfu mL^{-1}, 24 hr exposure	10^2 pfu mL^{-1}, 1 hr exposure	10^1 pfu mL^{-1}, 24 hr exposure)*
2	0	0	0	0
3	1.1 x 10^6	0	1.1 x 10^6	0
4	1.6 x 10^7	3.4 x 10^6	0	0
5	1.1 x 10^8	7.8 x 10^6	6.7 x 10^6	6.7 x 10^6
6	3.2 x 10^8	3.1 x 10^7	3.3 x 10^7	6.7 x 10^7
7	3.0 x 10^8	5.8 x 10^7	2.2 x 10^8	5.7 x 10^7
8	1.4 x 10^8	9.3 x 10^7	1.7 x 10^8	5.4 x 10^7
9	2.1 x 10^8	1.8 x 10^8	3.5 x 10^8	3.8 x 10^8
10	4.9 x 10^8	1.5 x 10^8	2.8 x 10^8	5.0 x 10^8
11	1.2 x 10^8	1.7 x 10^8	1.5 x 10^8	1.4 x 10^8
12	2.8 x 10^7	1.2 x 10^8	2.1 x 10^8	6.3 x 10^7
13	4.7 x 10^7	7.4 x 10^7	7.7 x 10^7	1.0 x 10^8
14	1.7 x 10^7	8.8 x 10^7	4.3 x 10^7	1.7 x 10^8
15	1.1 x 10^7	1.3 x 10^7	1.9 x 10^7	2.0 x 10^7
16	0	1.2 x 10^7	1.7 x 10^7	NA
17	0	0	0	NA
18	0	0	0	NA
19	0	0	0	NA
20	0	0	0	NA
21	0	0	0	NA

NA (Not Applicable): Cumulative mortality reached 100% in this tank.

*Days 0-1 post-exposure are not included to eliminate any residual waterborne challenge virus from the calculations.

** A VHS epizootic (characterized by mortality, positive tissues, and positive water titers) occurred in only 1 of the 3 replicates in this treatment. Results from this treatment indicate the calculated shedding rates from the positive replicate only.

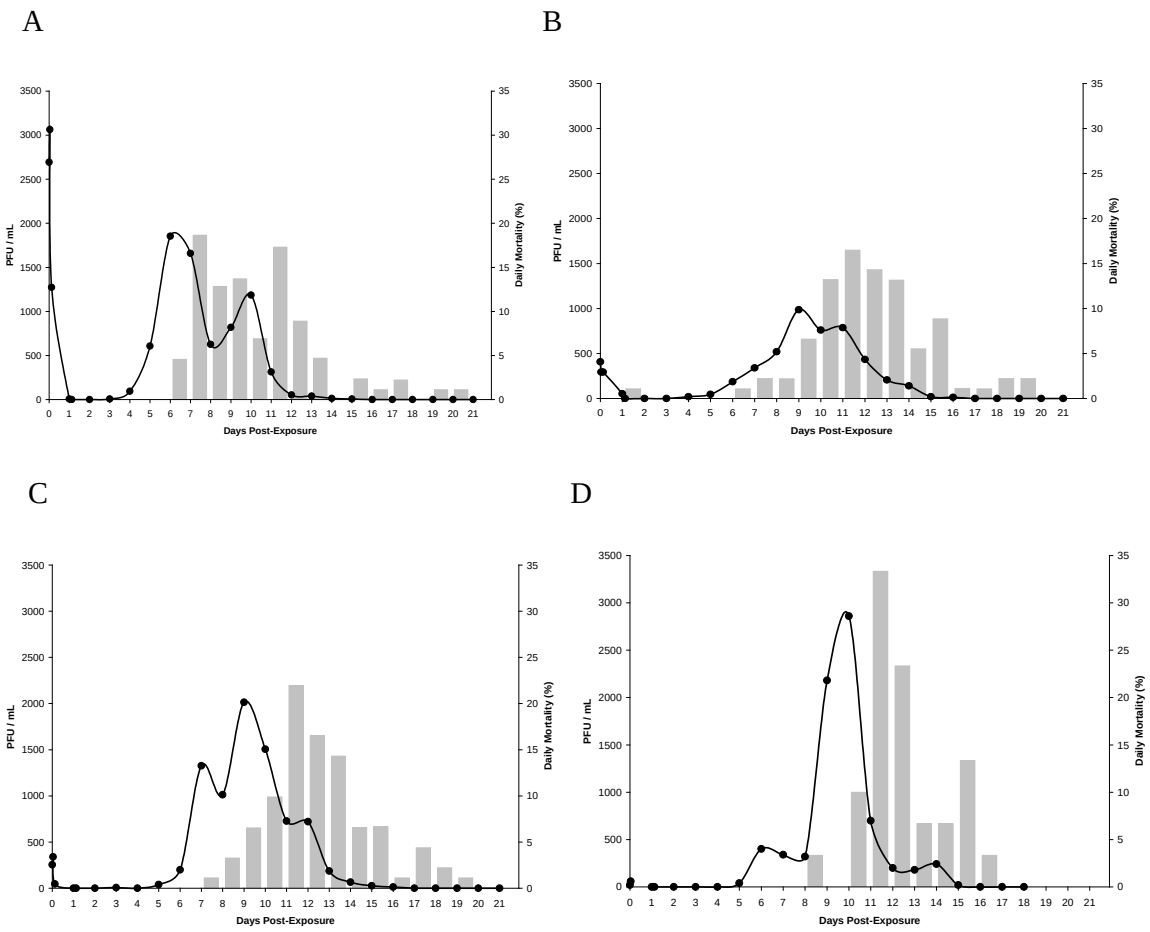


Figure 2. Waterborne titer of VHSV (line) and daily mortality (bars) in tanks of Pacific herring after exposure to virus. (A) Mean titer and daily mortality in triplicate tanks (n = 27-30 herring / tank on day 0) after exposure to 2,693-3,063 pfu mL^{-1} for 1 hr. Cumulative mortality was 95% when the experiment was terminated at 21d post-exposure. (B) Mean titer and daily mortality in triplicate tanks (n = 30-31 herring / tank on day 0) after exposure to 293-407 pfu mL^{-1} for 24 hrs. Cumulative mortality was 91% when the experiment was terminated at 21d post-exposure. (C) Mean waterborne titer and daily mortality in triplicate tanks (n = 30-31 herring / tank on day 0) after exposure to 253-340 pfu mL^{-1} for 1 hr. Cumulative mortality was 96% when the experiment was terminated at 21d post-exposure. (D) Waterborne titer and daily mortality in a single tank of herring (n=30) exposed to 23-27 pfu mL^{-1} for 24 hr. Of the three replicate tanks, a VHS epizootic occurred in only one (shown).

2.3. Chronic and Persistent Manifestations of VHS in Pacific herring

An apparent paradox exists when considering the persistence of VHSV in natural populations of Pacific herring where it is thought that high VHSV shedding rates from infected individuals contribute to virus perpetuation through a process of continuous transmission from infected to uninfected individuals (Kocan et al. 2001, Hershberger et al. 2010). This means of perpetuation can persist only within a narrow range of R_0's, the average number of infected individuals resulting from a single infected host (Ostfeld 2009). R_0 values much greater than one would

result in an epizootic cascade followed by a paucity of susceptible hosts, and R_0 values much below this level would eventually result in virus attrition due to ineffective transmission; in either case the pathogen would eventually be extirpated from the host population. Recognizing the unlikelihood that this narrow R_0 range persists for extended periods in dynamic natural systems like the NE Pacific, it is hypothesized that alternative or complementary VHSV perpetuation strategies exist in wild populations of Pacific herring. While only acute manifestations of VHS have been reported, lower levels of mortality are difficult to observe in the ocean and the occurrence of sub-acute forms of the disease could easily go unnoticed. Here, we report the results of long-term laboratory studies of VHSV-exposed Pacific herring that resulted in atypical disease manifestations that were characterized by tempered and prolonged mortality.

Chronic VHSV infections were established in a laboratory stock of Pacific herring when held in a large-volume tank supplied with pathogen-free seawater at temperatures ranging from 6.8 to 11.6 °C. The infections were characterized by viral persistence for extended periods and near-background levels of host mortality (Figure 3). Infectious virus was recovered from mortalities occurring up to 167 d post-exposure and was detected in normal-appearing herring for as long as 224 d following initial challenge. Geometric mean viral titers were generally as high as or higher in brain tissues than in pools of kidney and spleen tissues with overall prevalence of infection being higher in the brain (Table 8). Upon re-exposure to VHSV in a standard laboratory challenge, negligible mortality occurred among groups of herring that were either chronically infected or fully recovered, indicating that survival from chronic manifestations conferred protection against future disease (Figures 4&5). However, some survivors of chronic VHS infections were capable of replicating virus upon re-exposure (Table 9). Recognition of sub-acute forms of VHS in Pacific herring, analogous to those known to occur in cultured rainbow trout (*Oncorhynchus mykiss*) in Europe (Smail 1999), offer an alternative means by which the virus may be maintained in populations without the need for exclusive reliance on transmission of acute disease within a narrow R_0 range.

Table 8. Recovery of VHSV from the tissues of dead Pacific herring *Clupea pallasii*. Calculation of geometric mean VHSV tissue titers were calculated from positive tissues only.

			Kidney / Spleen		Brain	
Weeks post-exposure	**Total mortalities**	**Subsampled mortalities**	**% positive**	**Geometric mean titer (pfu / g)**	**% positive**	**Geometric mean titer (pfu / g)**
1	6	ND	ND	ND	ND	ND
2	60	23	100%	3.3×10^7	100%	2.2×10^7
3	163	8	100%	$>4.0 \times 10^4$	100%	$>4.0 \times 10^4$
4-5	199	ND	ND	ND	ND	ND
6	47	3	67%	1.8×10^4	100% [A]	5.3×10^4
7	55	23	52%	2.2×10^4	87% [B]	2.1×10^5
8	128	2	0%	NA	100% [C]	9.4×10^4
9-15	332	ND	ND	ND	ND	ND
16	55	29	14% [D]	1.4×10^6	17% [C]	9.1×10^5
17	28	25	40% [D]	1.8×10^5	40% [A]	1.3×10^5
18	29	5	0%	NA	20% [A]	8.0×10^2
19	35	18	28% [D]	4.9×10^5	22%	7.9×10^6
20	43	37	19%	6.4×10^5	24% [C]	4.2×10^5
21	39	21	24% [D]	1.6×10^4	24% [A]	2.2×10^5
22	47	28	18%	4.8×10^4	18%	3.3×10^4
23	57	25	8% [D]	4.0×10^4	24% [E]	6.1×10^4
24	57	24	4% [D]	1.2×10^3	8% [C]	7.1×10^3
25+	433	199	0%	NA	0%	NA

ND (No Data): Subsamples of dead herring were not assayed for VHSV during these weeks.
NA (Not Applicable): None of the subsampled mortalities tested positive for VHSV.
[A] VHSV was detected in the brain but not the kidney / spleen pools from 1 fish
[B] VHSV was detected in the brain but not the kidney / spleen pools from 7 fish
[C] VHSV was detected in the brain but not the kidney / spleen pools from 2 fish
[D] VHSV was detected in kidney / spleen pool but not the brain from 1 fish
[E] VHSV was detected in the brain but not the kidney / spleen pools from 4 fish

Table 9. Detection of VHSV in tissue pools from daily mortalities and survivors after re-exposure of persistently-infected (second VHSV exposure occurred 106 d after the initial exposure) and recovered (second VHSV exposure occurred 343d after the initial exposure) Pacific herring. All daily mortalities were pooled by replicate tank, and all survivors at the end of the experiments were pooled by replicate tank. Tissues pools from each fish consisted of kidney, spleen, and brain. Numerals indicate the number of VHSV-positive tissue pools / total number of tissue pools.

		Herring re-exposed (106 d after initial exposure) while undergoing persistent VHSV infections	Herring re-exposed (343 d after the initial exposure) after recovery from persistent VHSV infections
Re-exposed treatment	Mortalities	17/25	5/8
	Survivors	2/3	0/3
Once-exposed control	Mortalities	6/16	0/4
	Survivors	0/3	0/3
Positive control	Mortalities	29/36	22/22
	Survivors	2/3	0/3
Negative control	Mortalities	0/16	0/3
	Survivors	0/3	0/3

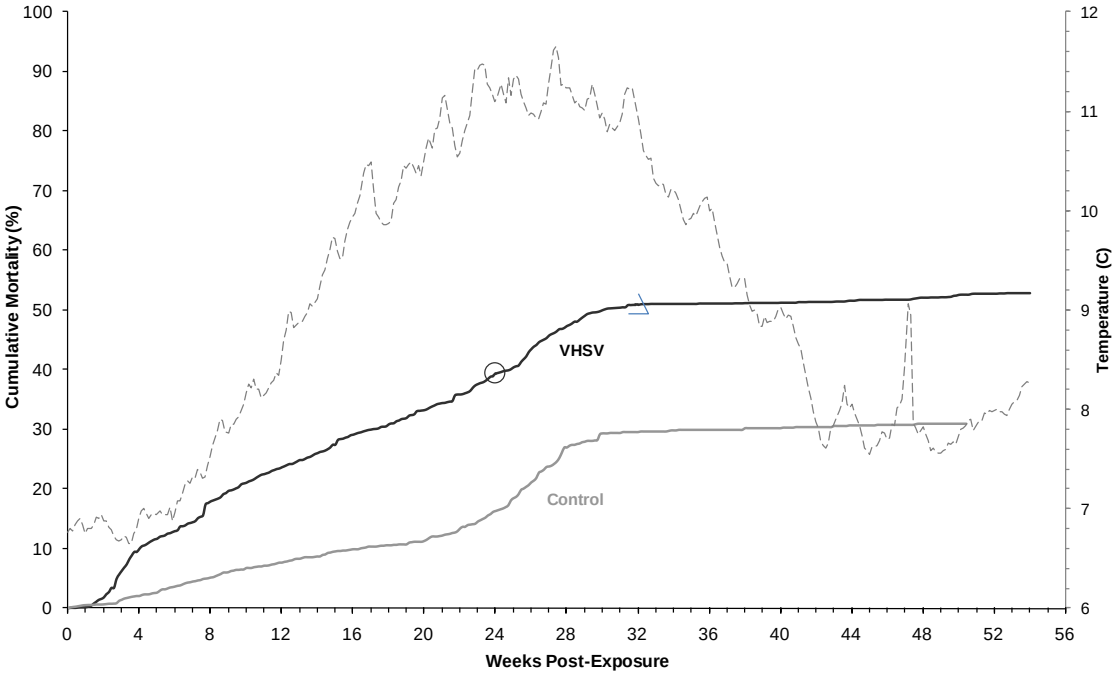


Figure 3. Chronic mortality following exposure of a colony of Pacific herring *Clupea pallasii* to VHSV in a large tank. Circle indicates the occurrence of the last VHSV-positive mortality (167d post-exposure). Triangle indicates the last day when positive survivors were known to exist in the exposed colony (224 d post-exposure). Dashed line indicates the daily ambient temperature in the tanks, reported as the daily mean of temperatures that were logged every 30 min.

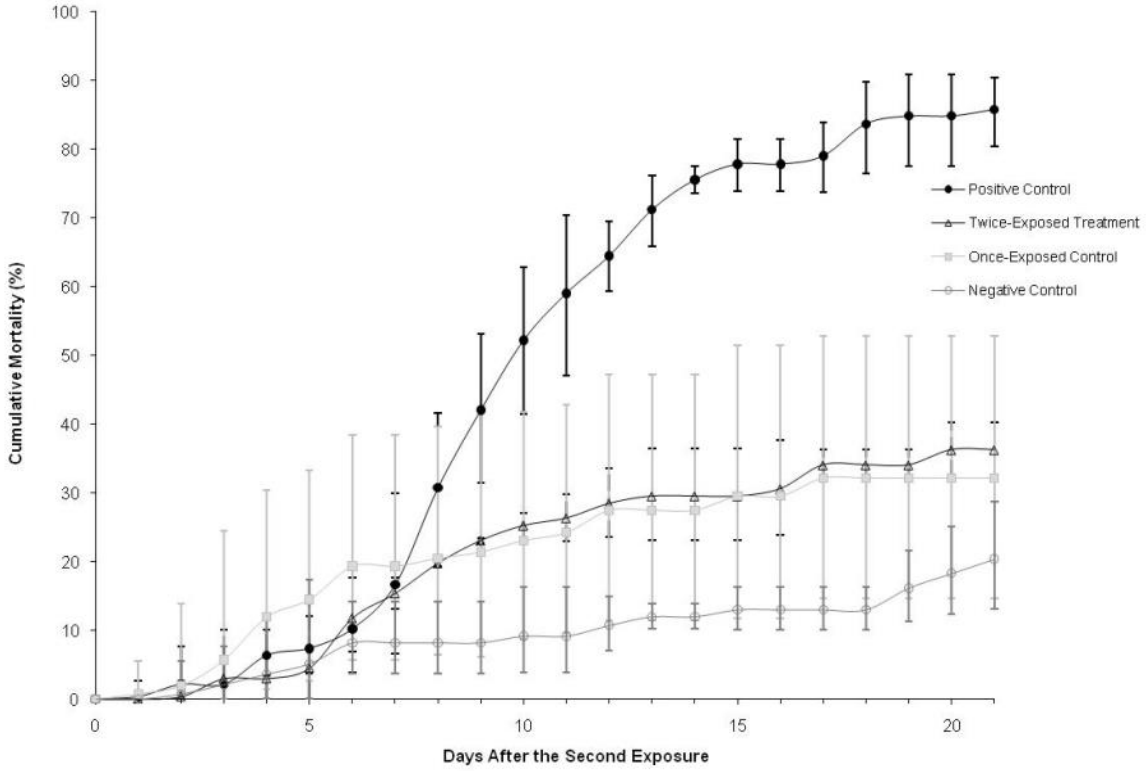


Figure 4. Susceptibility of chronically-infected Pacific herring *Clupea pallasii* to acute VHS. All data points represent back-transformed percentages corresponding to the means of arc-sin transformed proportions from triplicate tanks; error bars indicate 2 SD from the mean.

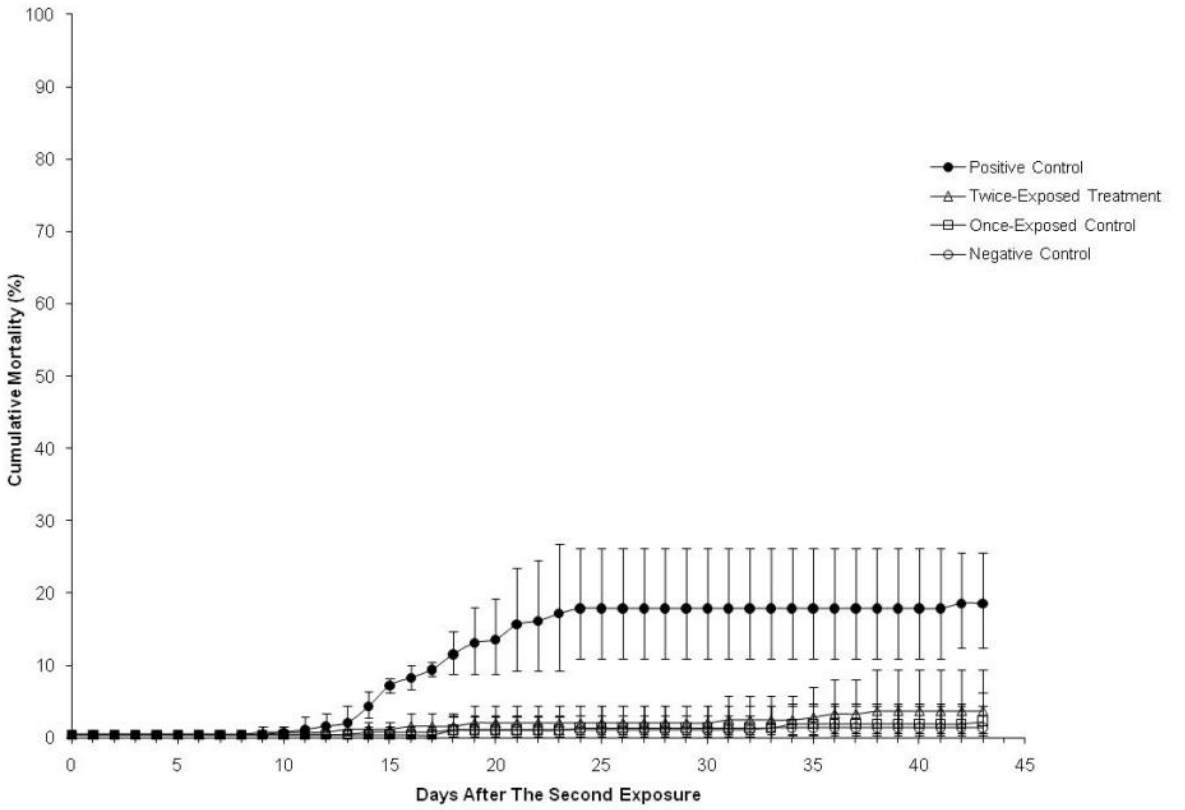


Figure 5. Susceptibility of Pacific herring *Clupea pallasii* to VHS after recovering from chronic infections. All data points represent back-transformed percentages corresponding to the means of arc-sin transformed proportions from triplicate tanks.

2.4 Pacific herring embryos are not susceptible to VHS

During initial PWS Herring Steering Committee meetings, some discussions focused on supplementation as a possible option for restoring PWS herring populations through the intensive culture and release of YOY herring juveniles. Much of the discussion focused on the utilization of herring confinement facilities such as floating net pens and barges where herring could be intensively reared from eggs to juveniles. Immediate disease concerns with the intensive culture of herring involved the high likelihood of VHS outbreaks in the cultured herring followed by the spill-back of amplified VHSV to sympatric wild populations. Alternatively, if exposure to VHSV was prevented in the rearing facilities, then large numbers of susceptible cohorts would be released into the wild population, effectively overwhelming any herd immunity in the wild population. One possible solution to these disease issues involved the implementation of mass immunization strategies for the cultured herring. Due to biocontainment issues, mass immunization procedures involving the exposure of herring larvae and juveniles to live VHSV would be impractical. However, biosecurity and biocontainment of VHSV would be much easier to control through exposure of fertilized herring embryos (eggs) to live virus. Therefore, this study was performed to address the following objectives:

1) To determine whether herring embryos are susceptible to waterborne VHSV and;
2) To determine whether herring juveniles that survived VHSV exposure as embryos demonstrate adaptive resistance to the disease upon re-exposure.

Herring embryos were not susceptible to VHSV. Replicate swatches (n = 9) containing fertilized herring eggs were immersed in waterborne VHSV at each of 4 exposure levels (0, 77, 710, and 2,127 PFU / mL) for 1 hr. As a positive virus control, a group of SPF herring larvae were also exposed to waterborne VHSV (2,260PFU / mL). Subsamples of the eggs were collected on days 0 (shortly after exposure) 4, and 8d post-exposure; none tested positive for VHSV by plaque assay. Additionally, newly hatch larvae were sampled as they appeared; again, none tested positive for VHSV by plaque assay. The only VHSV-positive samples in this experiment involved the positive controls (ie. juvenile SPF herring that were exposed to VHSV to demonstrate virus viability). These results indicated that herring embryos did not replicate VHSV. The results are not surprising because VHSV exposure to fish embryos is likely prevented by impervious barriers in the chorion.

Similarly, egg exposure to waterborne VHSV did not result in adaptive protection to the resulting juveniles that survived the embryonic exposure. A group of fertilized herring eggs was split in half; one half was exposed to waterborne VHSV (1,400 PFU / mL for 1 hr); the other was exposed to saline and served as unexposed controls. Eggs were hatched and larvae were reared through metamorphosis. The resulting juvenile herring (123d post-hatch) were separated into triplicate 270L tanks (45 juvenile herring / tank) for each of four treatments (Figure 6): Cumulative mortality among the treatment group (59%) was similar to that of the positive control group (50%; Figure 6), indicating that exposure of eggs to VHSV did not confer protection to survivors after their metamorphosis to juveniles. Therefore, exposure of herring eggs to viable VHSV is not a viable method of immunizing large numbers of herring against VHS in proposed enhancement facilities.

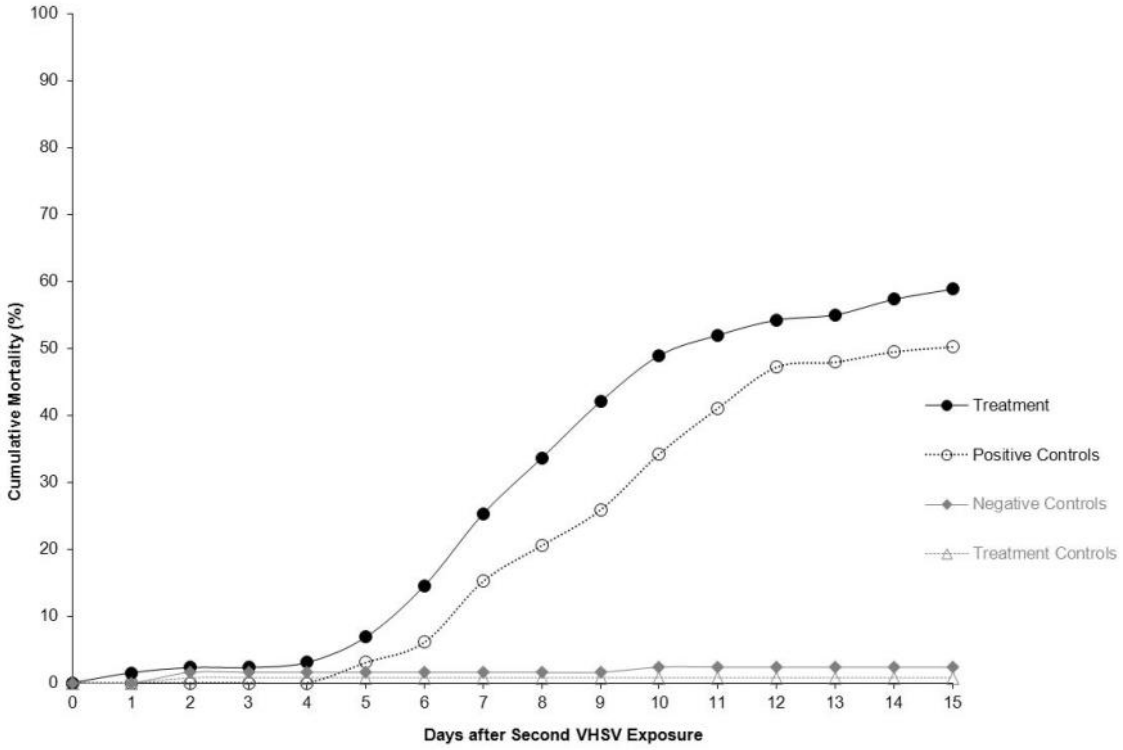


Figure 6: Cumulative mean mortality after re-exposure of juvenile herring to VHSV. The treatment group consisted of VHSV-exposed juveniles that were previously exposed to VHSV as eggs. Positive controls consisted of VHSV-exposed juvenile herring that were exposed to saline as eggs. Negative controls remained naïve to VHSV (i.e. never exposed to VHSV). Treatment controls were exposed to VHSV as eggs, but exposed to saline as juveniles.

2.5 Larval Pacific herring are highly susceptible to VHS

The effects of VHS epizootics on juvenile and adult Pacific herring are easily visualized as fish kill events; however, susceptibility of pre-metamorphosed larval herring to VHS have been uninvestigated. The objectives of this study were:

1) To determine the susceptibility of Pacific herring early life history stages to VHSV during the approximate 3 month developmental period occurring between hatch and larval metamorphosis to juveniles and;
2) To determine whether metamorphosed Pacific herring juveniles that survived a VHS epizootic as larvae demonstrate protection against subsequent exposure to VHSV.

Pacific herring were susceptible to waterborne challenge with viral hemorrhagic septicemia virus (VHSV) throughout their early life history stages (Table 10); with significantly greater ($p < 0.001$) cumulative mortalities occurring among VHSV-exposed groups of age 9 d, 44 d, 54 d, and 76 d larvae than among respective control groups (Figures 7-10). Similarly, among age 89 d and 1+ yr post-metamorphosed juveniles, cumulative mortality was significantly greater ($p < 0.0001$) in VHSV - exposed groups than in respective control groups (Figures 11-12). Additionally, VHSV was recovered from the mortalities in exposed treatment groups (Table 11).

Larval exposure to VHSV conferred partial protection to the survivors after their metamorphosis to juveniles because cumulative mortalities were significantly less ($p < 0.025$) among juvenile groups that survived a VHS epidemic as larvae than among groups that were previously naïve to VHSV (Figures 13-15). Although mortality was significantly less in the treatment groups than in the positive controls, VHSV was detected in the mortalities from both groups (Table 12). Magnitude of the protection, measured as relative percent survival, was a direct function of larval age at first exposure and was likely a reflection of gradual developmental onset of immunocompetence. These results indicate the potential for easily overlooked VHS epizootics among wild larvae in regions where the virus is endemic and emphasize the importance of early life history stages of marine fishes in influencing ecological disease processes.

Table 10. Details of the VHSV challenges performed in this study.

Age at 1st VHSV exposure	Treatment	VHSV exposure titer at 1st challenge (PFU / mL)	n	Age at 2nd VHSV exposure	VHSV exposure titer at 2nd challenge (PFU / mL)	n (3 rep's)
9 d (larvae)	VHSV	1.2-1.7 x 10^4	351-571 (5 rep's)	ND	ND	ND
9 d (larvae)	Neg. Control	0	290-506 (5 rep's)	ND	ND	ND
44 d (larvae)	VHSV	3.0 x 10^4	1,272	129 d	2.3 x 10^4	59-60
44 d (larvae)	Neg. Control	0	1,250	129 d	0	49-55
44 d (larvae)	Pos. Control	NA	NA	129 d	1.8 x 10^4	60-62
54 d (larvae)	VHSV	5.6 x 10^4	377	139 d	0.6 x 10^4	47-49
54 d (larvae)	Neg. Control	0	448	139 d	0	47-50
54 d (larvae)	Pos. Control	NA	NA	139 d	1.5 x 10^4	47-49
76 d (larvae)	VHSV	3.6 x 10^4	271	161 d	2.6 x 10^4	33-34
76 d (larvae)	Neg. Control	0	337	161 d	0	33-34
76 d (larvae)	Pos. Control	NA	NA	161 d	1.9 x 10^4	32-34
89 d (juv.)	VHSV	4.3 x 10^4	560	174 d	1.4 x 10^4	49-52
89 d (juv.)	Neg. Control	0	359	174 d	0	49-50
89 d (juv.)	Pos. Control	NA	NA	174 d	3.8 x 10^4	49-50
1+ yr (juv.)	VHSV	1.2 – 2.1 x 10^4	34-36 (5 rep's)	ND	ND	ND
1+ yr (juv.)	Neg. Control	0	32-35 (5 rep's)	ND	ND	ND

NA: Positive controls were not applicable for the first VHSV exposure.
ND: Survivors of the first VHSV exposure were not reared for a second exposure.

Table 11. Isolation of VHSV from Pacific herring mortalities after initial exposure to the virus. VHSV was not recovered from mortalities in any of the respective negative control groups.

Days Post-Exposure	9 d larvae[1]	44 d larvae[2]	54 d larvae[2]	76 d larvae[2]	89 d juveniles[2]	1+ yr juveniles[1]
1	0%	ND[4]	ND[4]	ND[4]	ND[4]	0%
2	0%	Positive	Negative	Negative	Positive	20%
3	20%	ND[4]	ND[4]	ND[4]	ND[4]	0%
4	60%	ND[4]	ND[4]	ND[4]	ND[4]	80%
5	60%	Positive	Positive	Positive	Positive	80%
6	80%	Positive	Positive	Positive	Positive	100%
7	100%	Positive	Positive	Positive	Positive	100%
8	100%	Positive	Positive	Positive	Positive	100%
9	100%	Positive	Positive	Positive	Positive	100%
10	100%	Positive	Positive	Positive	Positive	100%
11	80%	Positive	Positive	ND[4]	Positive	80%
12	100%	ND[4]	ND[4]	ND[4]	ND[4]	60%
13	100%	ND[4]	ND[4]	ND[4]	ND[4]	20%
14	100%	Positive	Positive	Positive	Positive	20%
15	100%	ND[4]	ND[4]	ND[4]	ND[4]	60%
16	100%	Positive	Negative	ND[4]	Positive	0%
17	100%	Positive	Negative	ND[4]	ND[4]	20%
18	80%	Positive	Negative	Positive	Positive	0%
19	100%	Positive	Positive	ND[4]	ND[4]	0%
20	80%	ND[4]	ND[4]	ND[4]	ND[4]	20%
21	60%	Positive	ND[4]	ND[4]	Positive	0%
22	NA[3]	Negative	Negative	Negative	ND[4]	NA[3]
23	NA[3]	Positive	Positive	Negative	ND[4]	NA[3]
24	NA[3]	Negative	ND[4]	ND[4]	ND[4]	NA[3]
26	NA[3]	Negative	ND[4]	ND[4]	ND[4]	NA[3]
28	NA[3]	Negative	Negative	ND[4]	ND[4]	NA[3]
34	NA[3]	ND[4]	Negative	ND[4]	Negative	NA[3]
35	NA[3]	ND[4]	ND[4]	Positive	ND[4]	NA[3]
36	NA[3]	ND[4]	Positive	ND[4]	ND[4]	NA[3]
38	NA[3]	Negative	Negative	Negative	ND[4]	NA[3]
41	NA[3]	Negative	Negative	ND[4]	ND[4]	NA[3]
42	NA[3]	Negative	ND[4]	ND[4]	ND[4]	NA[3]
46	NA[3]	ND[4]	Negative	ND[4]	ND[4]	NA[3]

[1]VHSV prevalence in mortality pools from each of 5 replicate tanks.

[2]VHSV presence in mortality pools from the single tank.

[3]Not Applicable: experiment was terminated on day 21.

[4]Mortalities were not assayed for VHSV on these days or on days not included in the table.

Table 12. Median tissue titers (PFU g^{-1}) after the second exposure to VHSV. Median VHSV tissue titers were calculated from the combined mortalities in all 3 replicate tanks. Groups of negative controls were exposed to PBS during both challenges, positive controls were exposed to PBS during the first challenge and VHSV during the second challenge. Experimental groups were exposed to VHSV during both challenges.

Days after second exposure[1]	First exposed as 44 d larvae			First exposed as 54 d larvae			First exposed as 76 d larvae			First exposed as 89 d juveniles		
	Neg. Control	**Pos. Control**	**Treatment (n)**	**Neg. Control**	**Pos. Control**	**Treatment (n)**	**Neg. Control**	**Pos. Control**	**Treatment (n)**	**Neg. Control**	**Pos. Control**	**Treatment (n)**
1	0 (7)	0 (2)	0 (2)	0 (2)	$10^{2.2}$ (4)	0 (0)	0 (1)	0 (1)	0 (0)	0 (0)	0 (3)	0 (2)
2	0 (0)	0 (1)	0 (0)	0 (1)	$10^{4.3}$ (1)	0 (1)	0 (0)	0 (1)	0 (1)	0 (2)	0 (0)	0 (0)
3	0 (0)	$>10^{8.0}$ (3)	$>10^{8.0}$ (3)	0 (0)	$>10^{8.0}$ (1)	0 (0)	0 (0)	$10^{7.5}$ (2)	0 (0)	0 (2)	0 (0)	0 (0)
4	0 (0)	$>10^{8.0}$ (36)	$10^{7.3}$ (8)	0 (0)	$10^{7.2}$ (13)	$10^{7.3}$ (2)	0 (0)	$10^{7.3}$ (9)	$10^{7.5}$ (2)	0 (0)	0 (0)	$>10^{8.0}$ (2)
5	0 (1)	$10^{6.6}$ (81)	$10^{6.3}$ (30)	0 (0)	$10^{7.1}$ (42)	$10^{6.4}$ (11)	0 (1)	$10^{7.2}$ (27)	$10^{6.5}$ (10)	0 (1)	$10^{7.2}$ (11)	$10^{6.1}$ (7)
6	0 (0)	$10^{6.2}$ (27)	$10^{5.8}$ (36)	0 (0)	$10^{7.1}$ (40)	$10^{5.4}$ (16)	0 (0)	$10^{6.5}$ (27)	$10^{5.4}$ (14)	0 (0)	$10^{7.1}$ (28)	$10^{5.3}$ (14)
7	0 (0)	$10^{6.2}$ (14)	$10^{5.2}$ (31)	0 (0)	$10^{6.2}$ (21)	$10^{6.1}$ (18)	0 (1)	$10^{6.2}$ (16)	$10^{6.6}$ (12)	0 (0)	$10^{6.8}$ (47)	$10^{6.2}$ (14)
8	0 (0)	$10^{5.4}$ (8)	$10^{5.2}$ (15)	0 (0)	$10^{6.1}$ (10)	$10^{5.4}$ (9)	0 (0)	$10^{5.2}$ (7)	$10^{5.5}$ (8)	0 (0)	$10^{5.3}$ (28)	$10^{5.2}$ (16)
9	0 (0)	$10^{6.3}$ (2)	$10^{5.1}$ (5)	0 (0)	$10^{5.5}$ (3)	$10^{5.1}$ (11)	0 (0)	0 (1)	$10^{5.3}$ (5))	0 (1)	$10^{5.2}$ (11)	$10^{6.1}$ (7)
10	0 (0)	0 (0)	$10^{5.7}$ (1)	0 (0)	0 (0)	$10^{5.2}$ (4)	0 (0)	$10^{5.3}$ (3)	$10^{5.2}$ (4)	0 (0)	$10^{5.1}$ (2)	$10^{4.3}$ (5)
11	0 (0)	0 (0)	$10^{4.6}$ (2)	0 (0)	0 (0)	$10^{5.2}$ (9)	0 (0)	0 (0)	$10^{5.1}$ (5)	0 (1)	$10^{5.2}$ (1)	$10^{4.4}$ (4)
12	0 (0)	$10^{5.2}$ (1)	$10^{5.3}$ (2)	0 (0)	0 (0)	$10^{6.0}$ (2)	0 (1)	$10^{4.1}$ (1)	$10^{6.0}$ (2)	0 (0)	$10^{4.5}$ (1)	$10^{4.3}$ (5)
13	0 (0)	0 (0)	$10^{4.8}$ (1)	0 (0)	$10^{6.7}$ (1)	$10^{5.2}$ (3)	0 (0)	$10^{5.2}$ (1)	0 (0)	0 (0)	$10^{5.0}$ (2)	0 (0)
14	0 (0)	$10^{4.1}$ (2)	$10^{4.0}$ (1)	0 (0)	$10^{6.2}$ (3)	$10^{4.3}$ (3)	0 (0)	0 (0)	$10^{4.6}$ (1)	0 (1)	0 (0)	$10^{4.8}$ (2)
15	0 (0)	0 (0)	0 (0)	0 (0)	$10^{6.2}$ (1)	$10^{4.1}$ (3)	0 (0)	0 (0)	$10^{5.2}$ (1)	0 (0)	$10^{5.2}$ (2)	$10^{4.3}$ (2)
16	0 (0)	$10^{5.2}$ (1)	0 (0)	0 (1)	0 (0)	$10^{5.2}$ (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	$10^{3.5}$ (1)
17	0 (0)	0 (0)	$10^{4.2}$ (1)	0 (0)	0 (0)	$10^{4.2}$ (1)	0 (0)	0 (0)	$10^{4.2}$ (2)	0 (0)	0 (0)	0 (1)

[1] Second exposure to VHSV occurred 85 d after the first; larvae that survived the first exposure metamorphosed to juveniles before the second exposure.

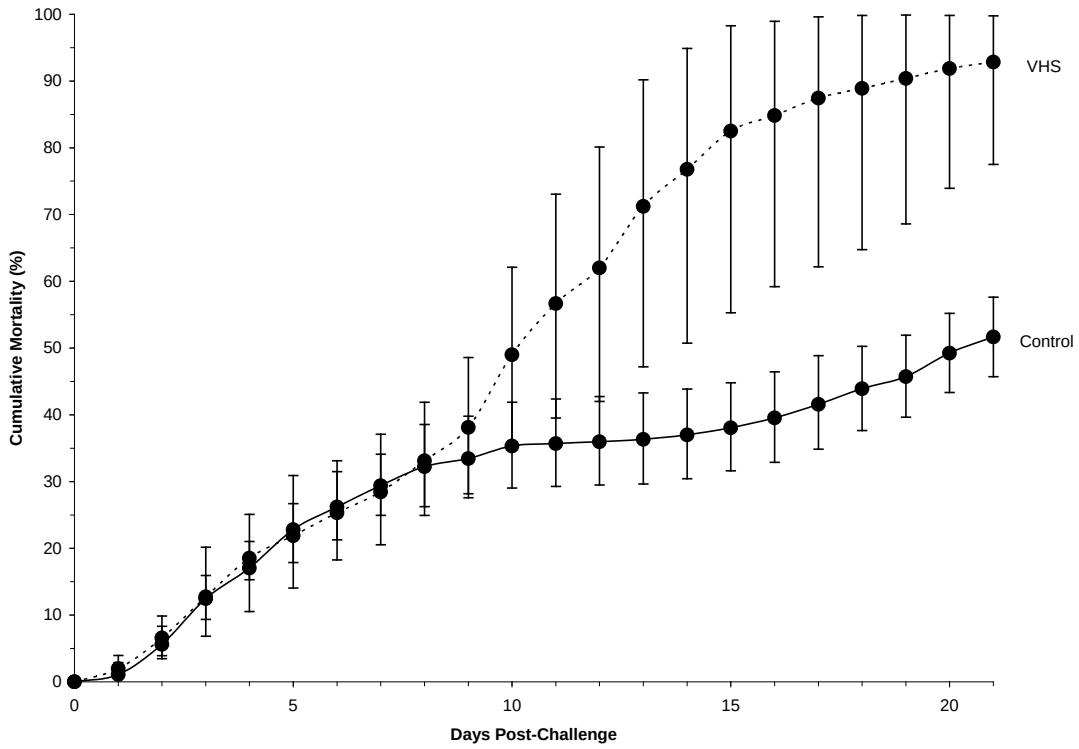


Figure 7. Cumulative mortality after exposure of age 9 d Pacific herring larvae to VHSV (VHS) or to HBSS (Control). Graphed data represent the percentages corresponding to the means of arc sin transformed mortality in 5 replicate aquaria (290-571 larvae / replicate tank). Error bars indicate 2 standard deviations from the mean.

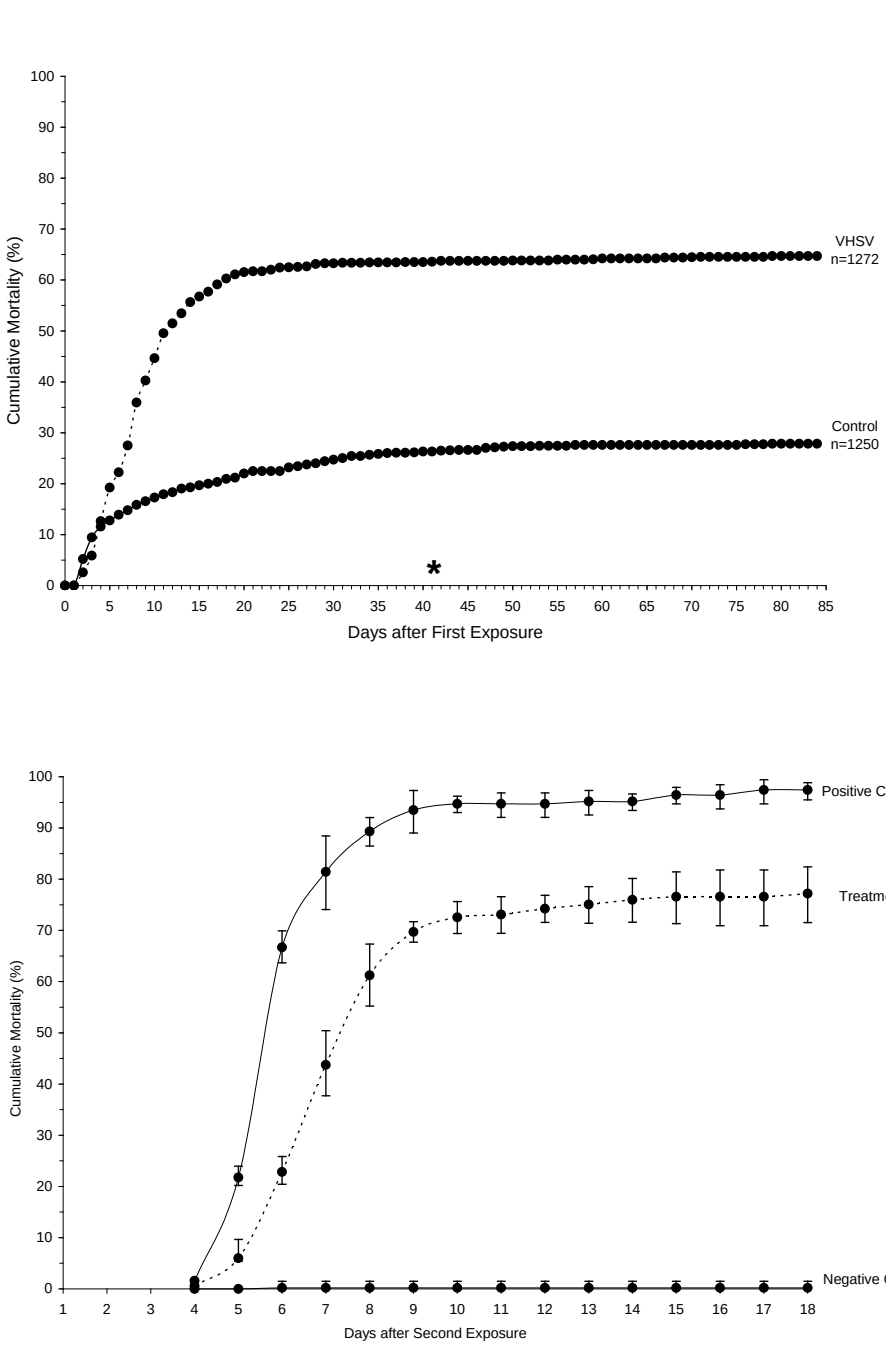


Figure 8 (A&B). Cumulative mortality after first exposure of age 44 d larvae (A) and second exposure of surviving age 129 d juveniles (B) to VHSV. "*" indicates approximate date when metamorphosis to juveniles was complete. Treatment groups were exposed to VHSV during both challenges, negative controls were exposed to HBSS during both challenges, and positive controls were exposed to HBSS during the first challenge and VHSV during the second. Graphed data represent cumulative mortalities in each tank (A), or percentages corresponding to the means of arc sin transformed mortality in 3 replicate tanks (B). Error bars indicate 2 standard deviations from the mean.

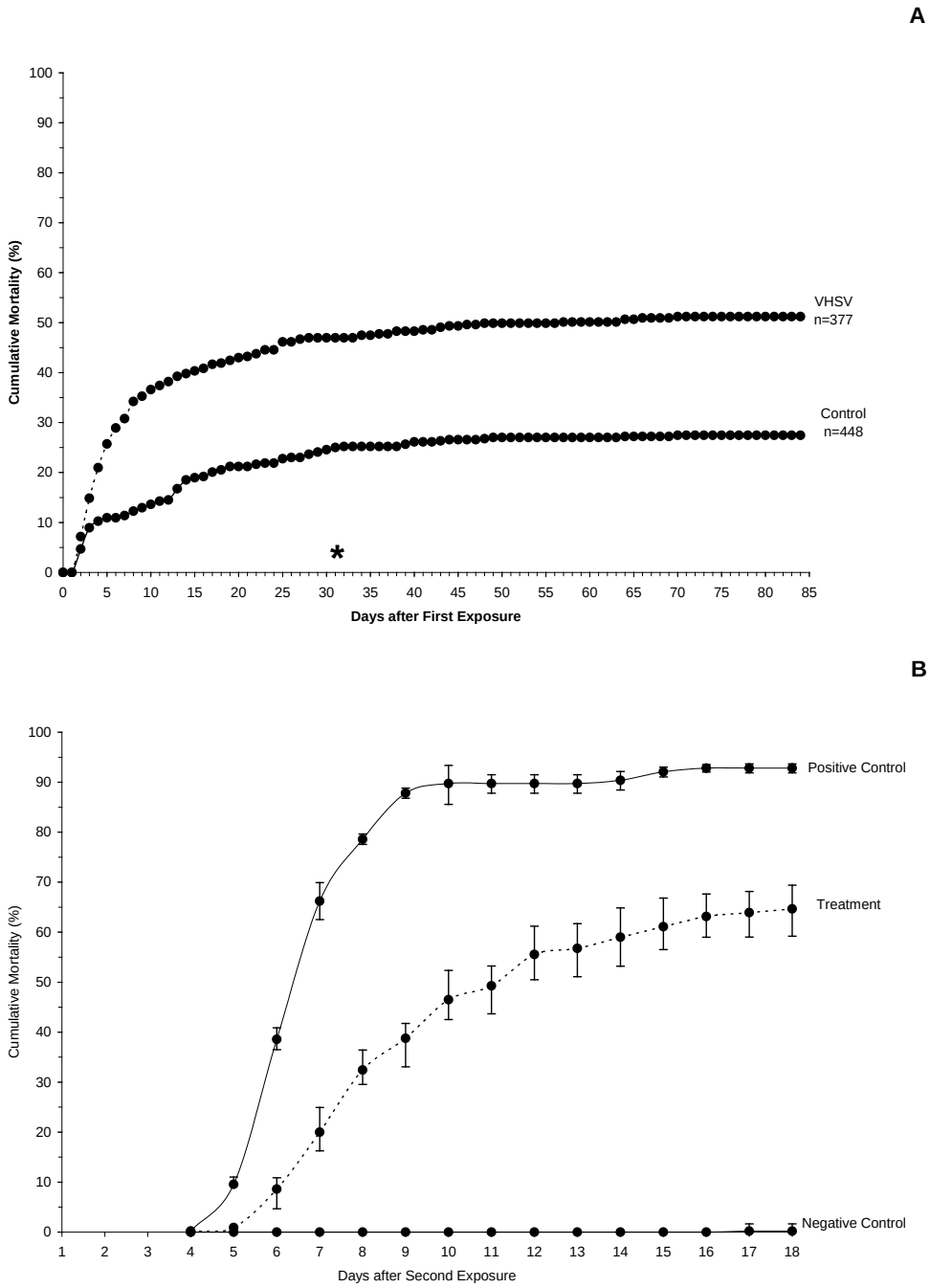


Figure 9 (A&B). Cumulative mortality after first exposure of age 54 d larvae (A) and second exposure of surviving age 139 d juveniles (B) to VHSV. "*" indicates approximate date when metamorphosis to juveniles was complete. Treatment groups were exposed to VHSV during both challenges, negative controls were exposed to HBSS during both challenges, and positive controls were exposed to HBSS during the first challenge and VHSV during the second. Graphed data represent cumulative mortalities in each tank (A), or percentages corresponding to the means of arc sin transformed mortality in 3 replicate tanks (B). Error bars indicate 2 standard deviations from the mean.

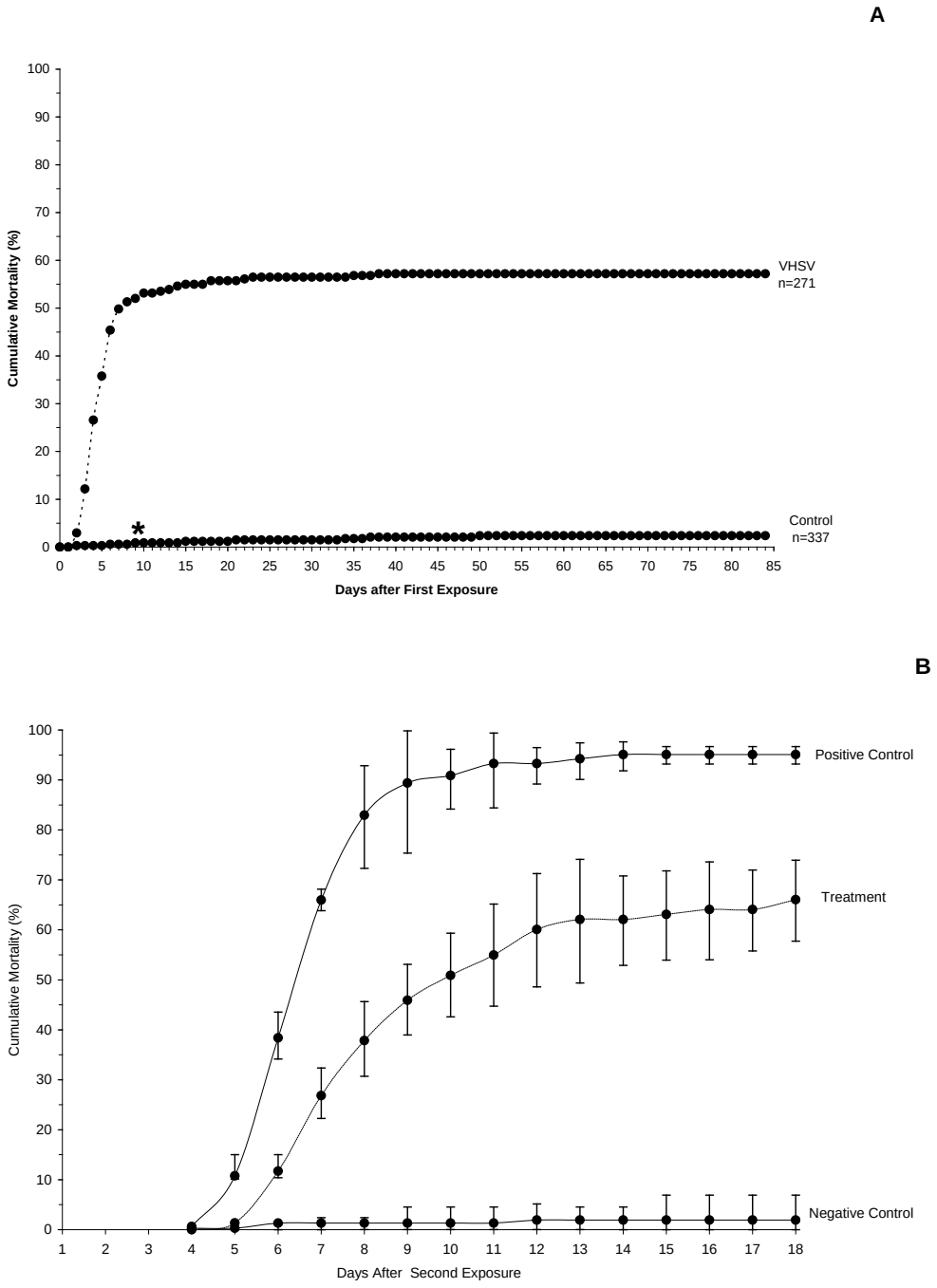


Figure 10 (A&B). Cumulative mortality after first exposure of age 76 d larvae (A) and second exposure of surviving age 161 d juveniles (B) to VHSV. "*" indicates approximate date when metamorphosis to juveniles was complete. Treatment groups were exposed to VHSV during both challenges, negative controls were exposed to HBSS during both challenges, and positive controls were exposed to HBSS during the first challenge and VHSV during the second. Graphed data represent cumulative mortalities in each tank (A), or percentages corresponding to the means of arc sin transformed mortality in 3 replicate tanks (B). Error bars indicate 2 standard deviations from the mean.

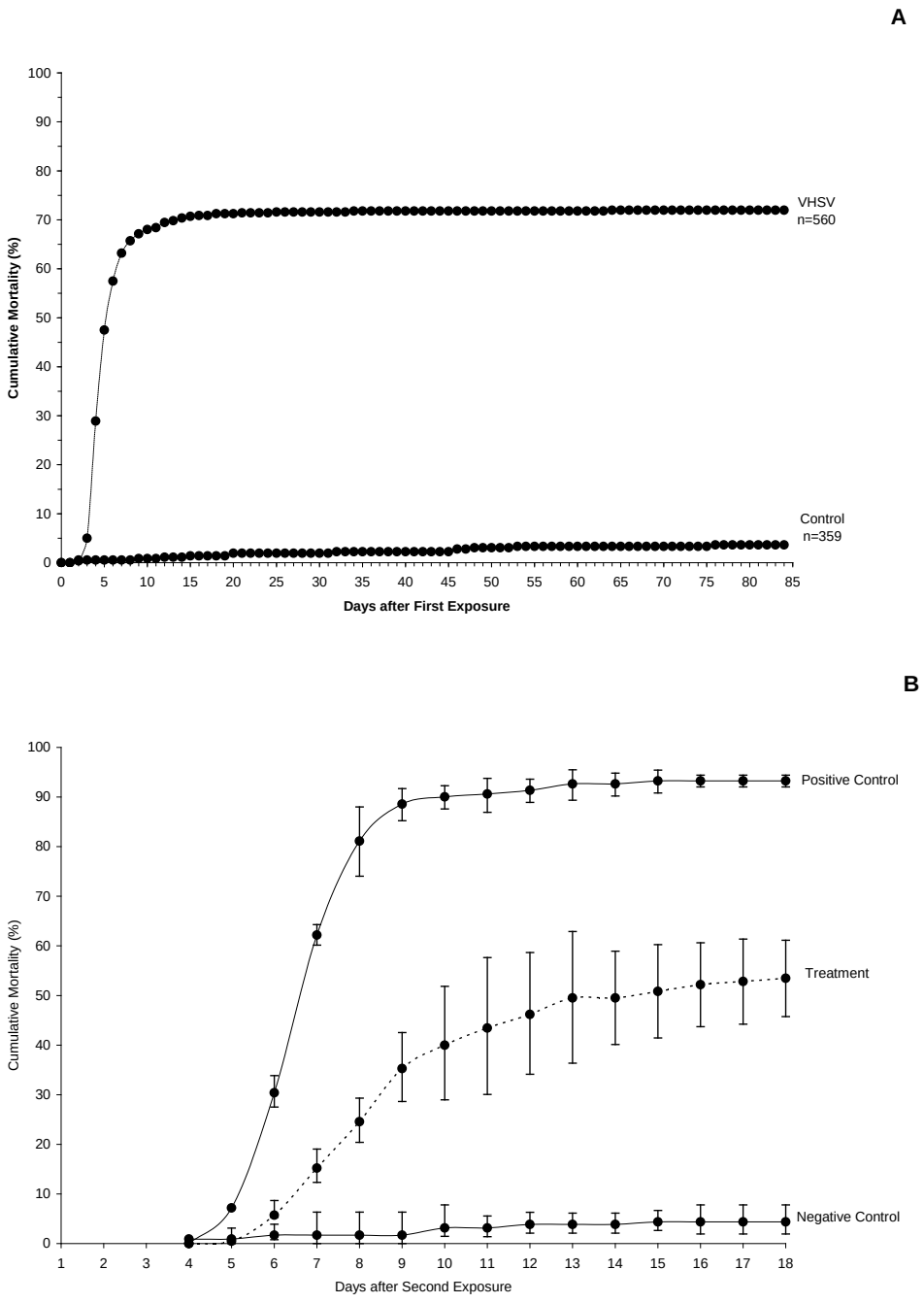


Figure 11 (A&B). Cumulative mortality after first exposure of age 89 d juveniles (A) and second exposure of surviving age 129 d juveniles (B) to VHSV. "*" indicates approximate date when metamorphosis to juveniles was complete. Treatment groups were exposed to VHSV during both challenges, negative controls were exposed to HBSS during both challenges, and positive controls were exposed to HBSS during the first challenge and VHSV during the second. Graphed data represent cumulative mortalities in each tank (A), or percentages corresponding to the means of arc sin transformed mortality in 3 replicate tanks (B). Error bars indicate 2 standard deviations from the mean.

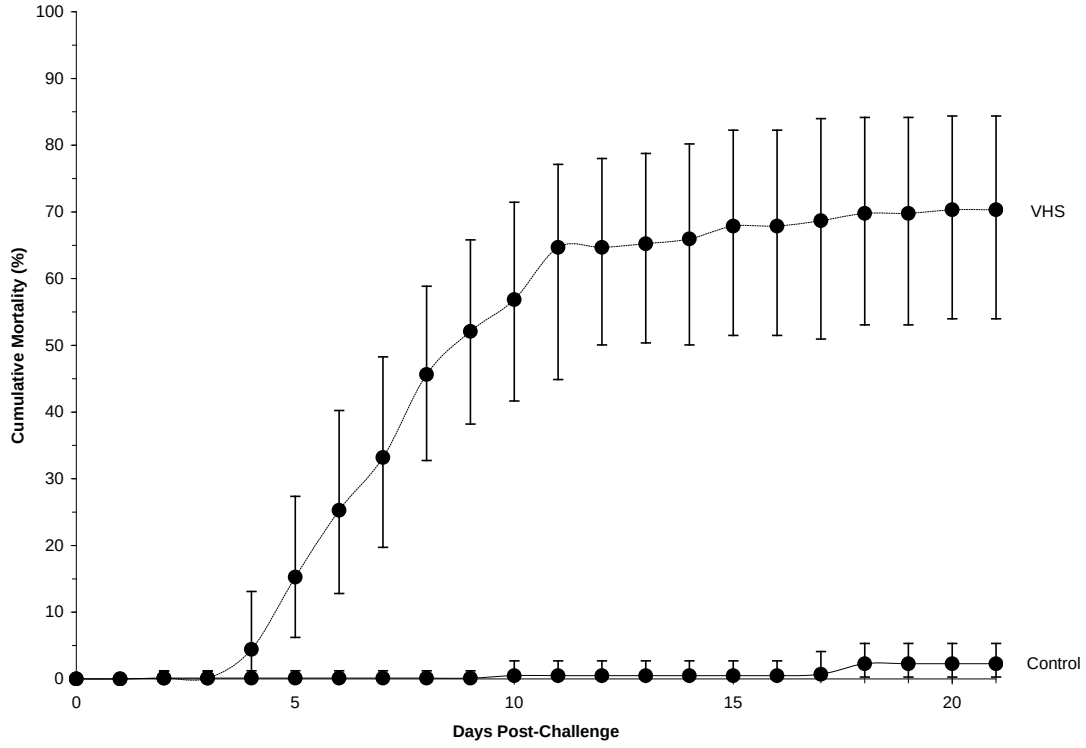


Figure 12. Cumulative mortality after exposure of age 1+ yr Pacific herring juveniles to VHSV. Graphed data represent the percentages corresponding to the means of arc sin transformed mortality in 5 replicate tanks. Error bars indicate 2 standard deviations from the mean.

2.6 Development of Predictive Tools to Forecast VHS Epizootics in Pacific herring

From a resource management perspective, identification of the potential for future VHS epizootics represents a critical first step towards developing adaptive disease management strategies. One approach to forecasting disease potential involves identification of the susceptibility status of wild fish populations. For example, the susceptibility of wild Pacific herring to VHS generally decreases with age (Hershberger et al. 1999, Marty et al. 2003), presumably because survivors of prior exposure develop acquired immunity to the disease (Kocan et al. 2001). This adaptive mechanism is supported by empirical studies in the laboratory, demonstrating that previously naïve Pacific herring are highly susceptible to VHS (Kocan et al. 1997); however, those that survived prior exposure are refractory to the disease, even upon re-exposure to high levels of VHSV (Hershberger et al. 2007; Hershberger et al. 2010b). Therefore, knowledge of the prior exposure history of a wild population to VHSV would be insightful for identifying the potential for future disease epizootics, whereby disease potential would be considered high among populations predominated by previously naïve or susceptible individuals and low among those predominated by previously-exposed or refractory individuals.

Identification of the susceptibility status of wild populations to VHS may be accomplished by assessing specific immunological indicators of exposure. For example, rainbow trout (*Oncorhynchus mykiss*) that survive VHS mount an adaptive immune response, the humoral component of which can be quantified by the detection of neutralizing antibodies in the serum using a complement-dependent plaque neutralization assay (Olesen and Jorgensen 1986, Jorgensen et al. 1991, Olesen et al. 1991, Ahne and Jorgensen 1993). Unfortunately, extensive efforts to adapt this plaque neutralization test to the VHSV / Pacific herring system have been largely unsuccessful, resulting in little or no *in vitro* neutralization of virus from the plasma of herring survivors that are known to be refractory to the disease (LaPatra and Hershberger unpublished data).

In lieu of an effective plaque neutralization assay, this study was performed to evaluate alternative techniques that may prove useful in identifying the exposure history and resulting disease potential in wild herring populations. Techniques included 1) adaptation and optimization of an in vitro Viral Replication in Excised Fin tissues (VREFT) assay that utilizes fin clips and 2) investigation into the feasibility of an in vivo passive immunization assay that utilizes plasma from wild herring.

Procedures for a VREFT assay were adapted to Pacific herring (*Clupea pallasii)* and optimized to both reduce processing time and provide the greatest resolution between naïve herring and those previously exposed to VHSV. The optimized procedures included removal of the left pectoral fin from a euthanized fish (Figure 13), inoculation of the fin with $>10^5$ plaque forming units (PFU) mL^{-1} VHSV for 1 h (Figure 14), rinsing the fin in fresh medium six times to remove unadsorbed virions, incubation of the fin in fresh medium for 4 d (Figure 15), and enumeration of the viral titer in a sample of the incubation medium by plaque assay (Figure 16). The optimized VREFT assay was effective at identifying the prior exposure history of laboratory-reared Pacific herring to VHSV (Figure 17). The geometric mean VREFT value was significantly greater ($P < 0.01$) among naïve herring (1.2×10^3 PFU mL^{-1}) than among groups that survived exposure to VHSV ($1.0\text{-}2.9 \times 10^2$ PFU mL^{-1}); additionally, the proportion of cultures with no detectable virus was significantly greater ($P = 0.0002$) among fish that survived exposure to VHSV (39 - 47 %) than among naïve fish (3.3 %; Figure 18). The optimized VREFT assay demonstrates proof-of-concept that the VHSV exposure history of herring can be identified a posteriori, however, laboratory costs and processing time likely prevent application of this technique as a viable high-throughput population screening tool.

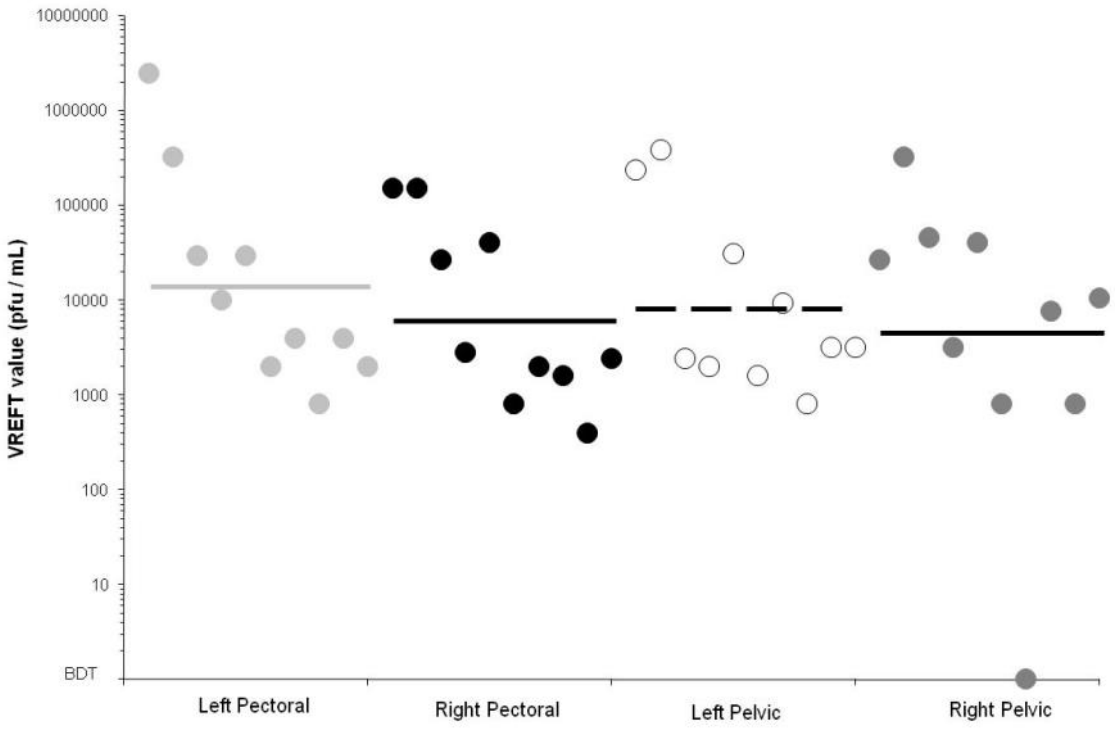


Figure 13. VREFT values from each of four fins removed from individual fish. Data points indicate VREFT values for individual fin cultures, and horizontal lines indicate geometric mean titers for each fin treatment. Samples with VREFT values below the detection threshold (400 PFU mL^{-1}) were assigned BDT.

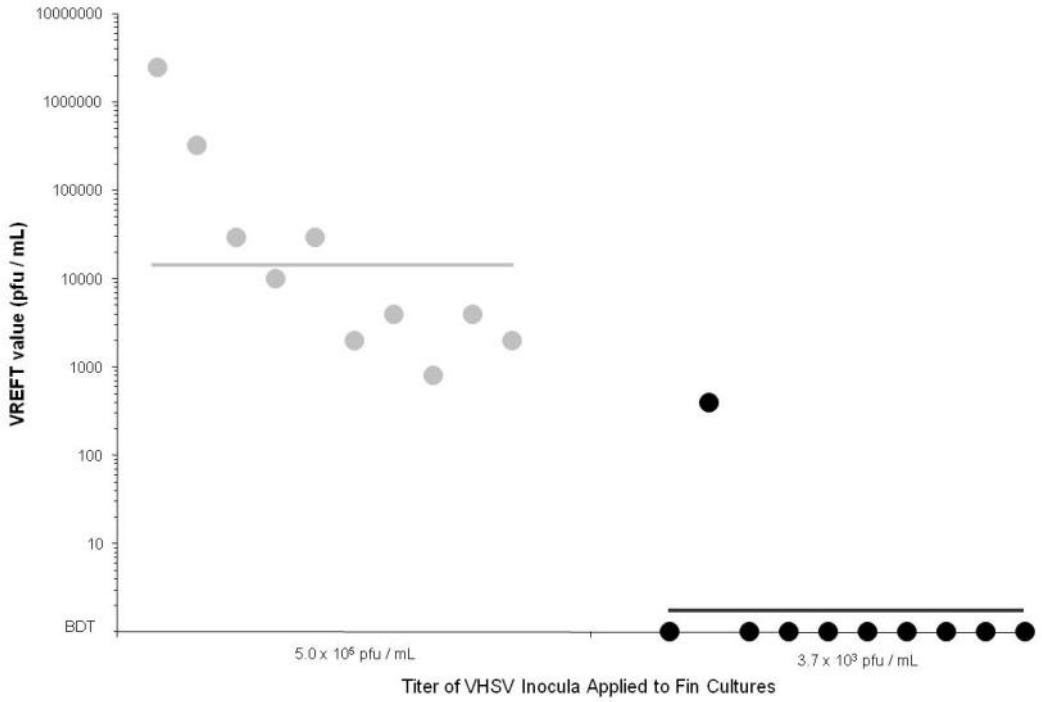


Figure 14. VREFT values resulting from exposure of fin explant cultures to different VHSV inocula (5.0 x 10^5 PFU mL^{-1} and 3.7 x 10^3 PFU mL^{-1}). Circles indicate results from the individual replicates, and horizontal lines indicate geometric means for the two treatment groups. Samples with VREFT values below the detection threshold (400 PFU mL^{-1}) were assigned BDT.

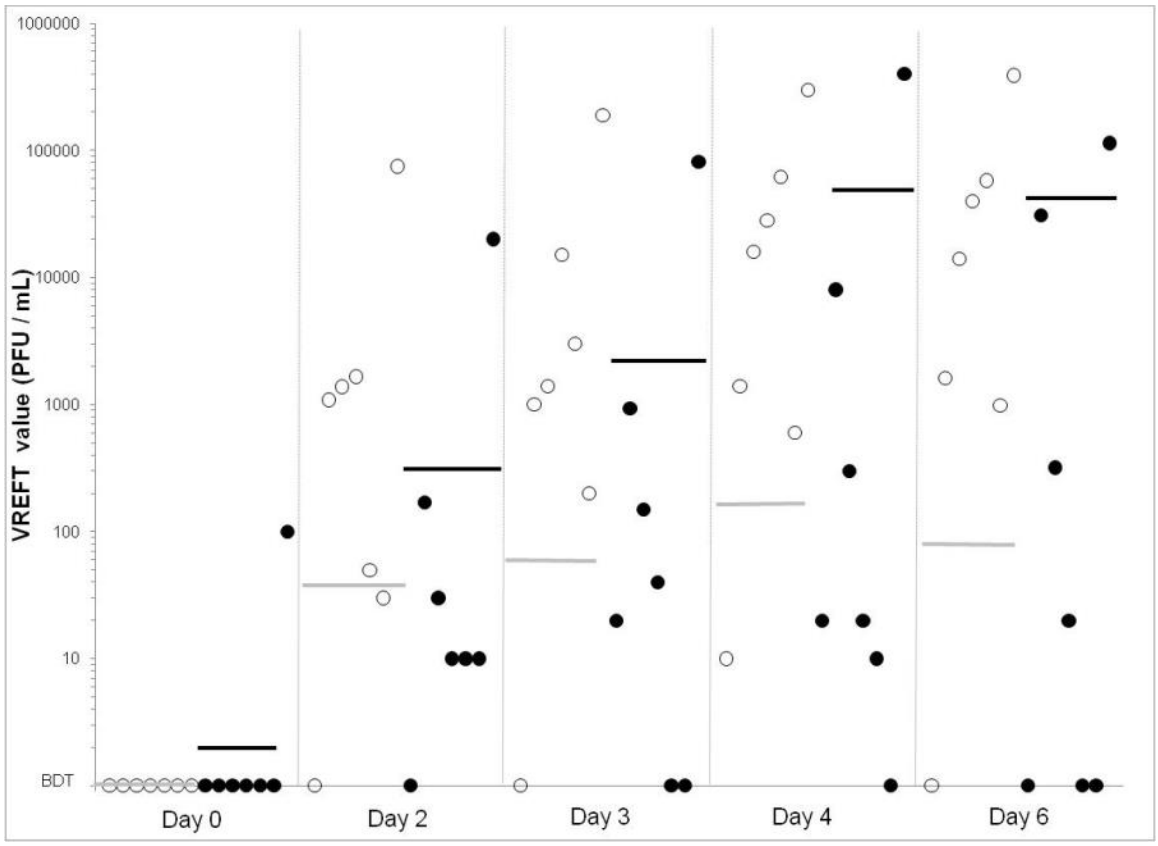


Figure 15. VREFT values in fin explant cultures on consecutive days after inoculation with VHSV. Open circles correspond to fins excised from naïve fish (n=7). Closed circles correspond to fins excised from fish previously exposed to VHSV (n=7). Samples where no virus was detected were assigned 'below the detection threshold' (BDT, <10 PFU mL^{-1}). Horizontal lines (grey and black) indicate the daily geometric mean from the respective treatments. Day-0 samples were collected after the viral inocula were rinsed from the cultures.

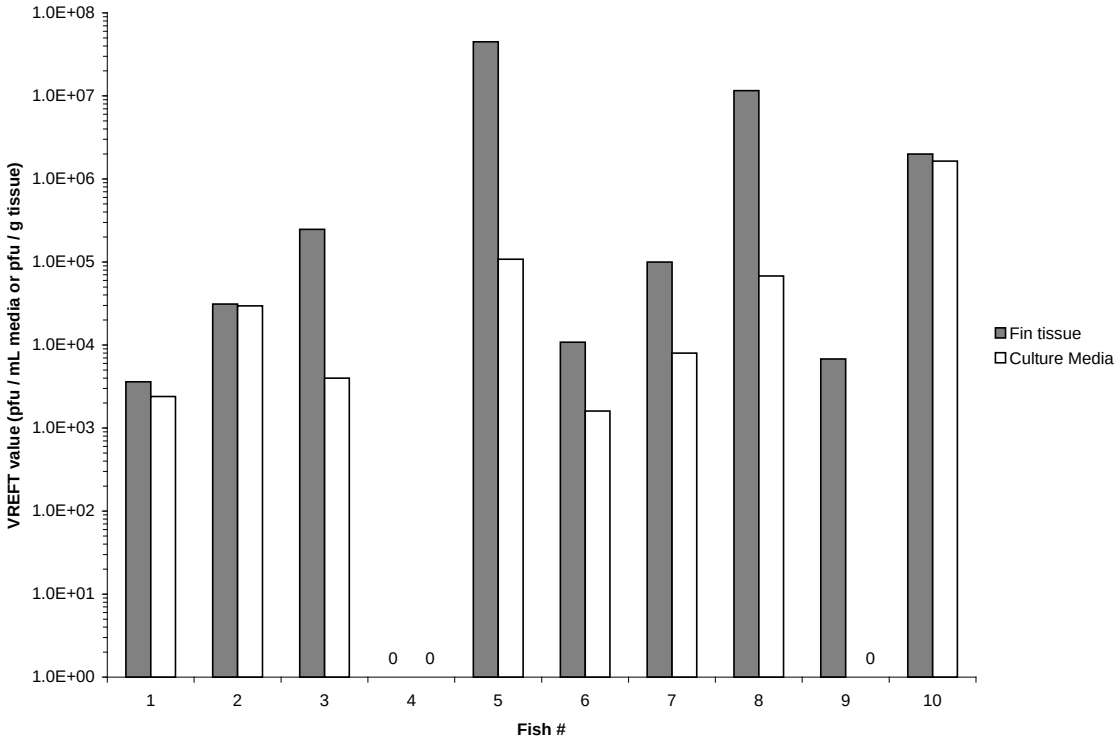


Figure 16. VREFT values in the fin tissues vs culture medium.

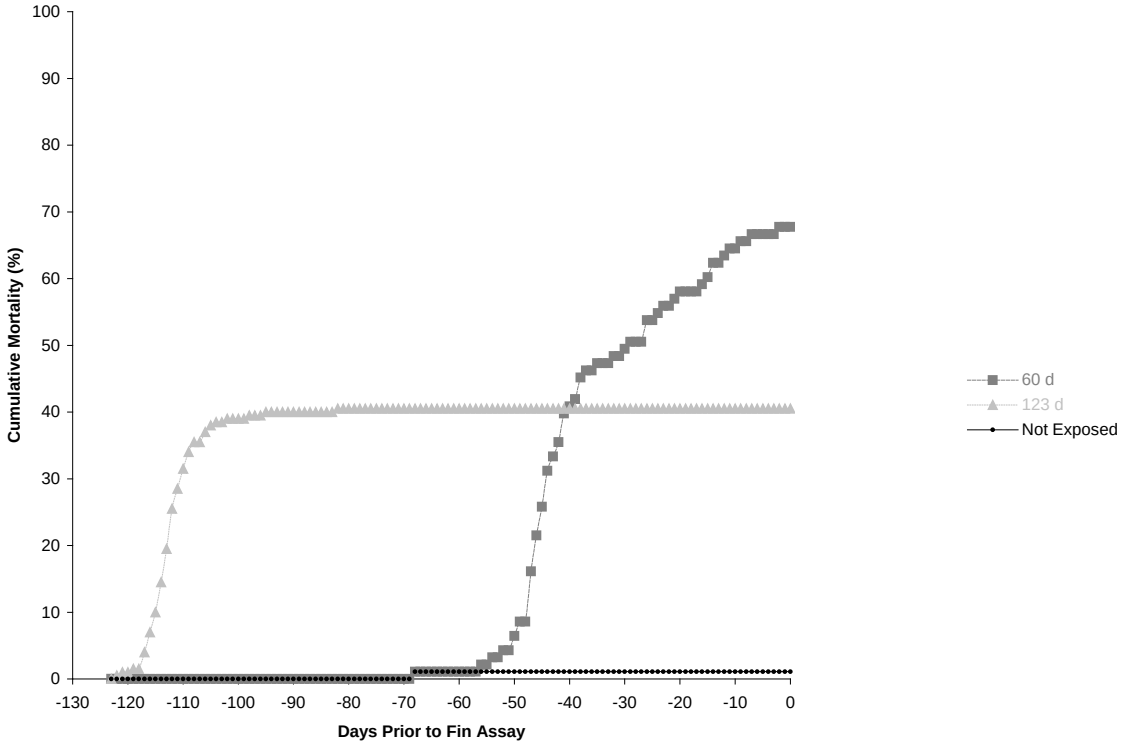


Figure 17. Mortality in the three groups of SPF herring after exposure to VHSV (10^3 PFU mL^{-1} for 1 h). Legend indicates how many days prior to the VREFT procedure that VHSV exposure occurred; fish were euthanized and fins were harvested for VREFT assay on Day-0.

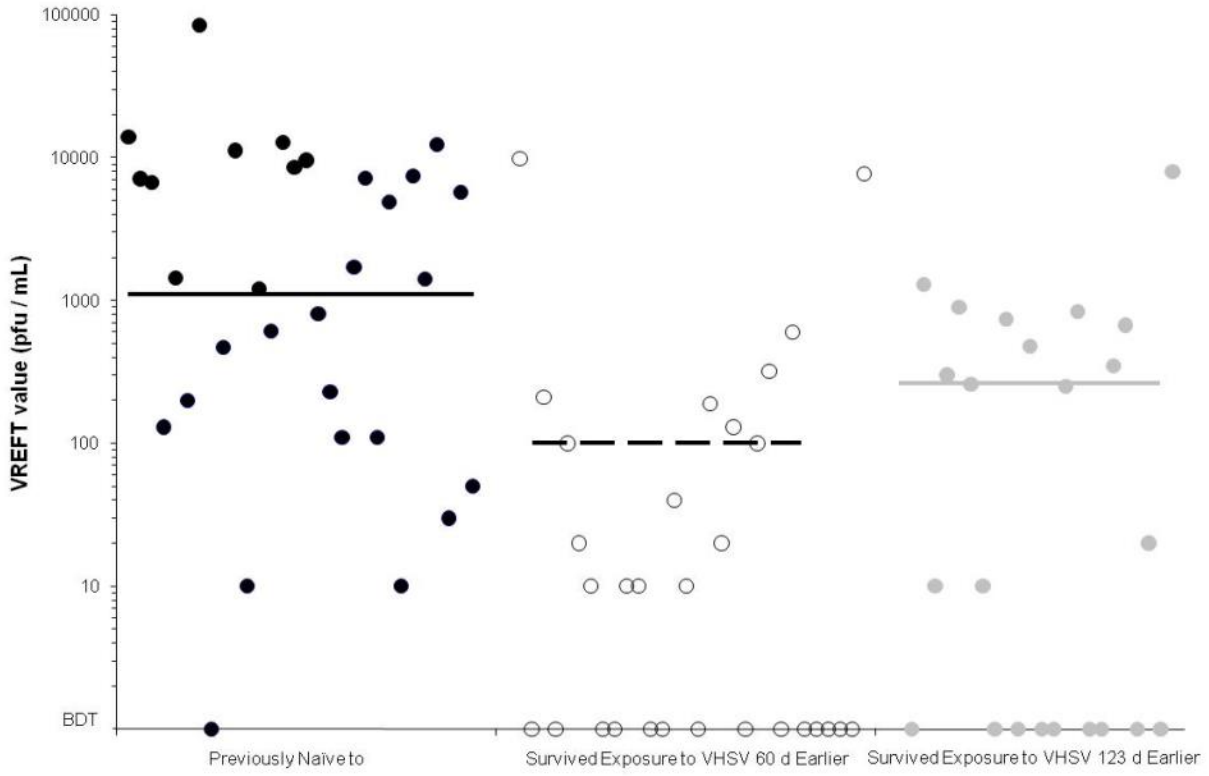


Figure 18. VREFT values from three groups of herring with different VHSV exposure histories. Data points indicate VREFT values for the individual replicates and horizontal lines (solid black, dashed black, and solid gray) indicate the geometric means for each of the three treatment groups. Samples with VREFT values below the detection threshold (10 PFU mL^{-1}) were assigned BDT.

The plasma of Pacific herring surviving laboratory-induced viral hemorrhagic septicemia (VHS) epizootics contained humoral substances that, when injected into naïve animals, conferred passive immunity against the disease. Among VHSV-exposed groups, injection of donor plasma from VHS survivors resulted in significantly greater ($P < 0.025$) survival (50%; Figure 19) and significantly lower ($P < 0.001$) tissue titers (1.5×10^5 plaque-forming units (PFU) g^{-1}; Table 13) than did plasma from VHSV-naïve donors (6% survival and 3.7×10^6 PFU g^{-1}). Additionally, the magnitude of the protective immune response increased during a 4-month period post-exposure, with plasma from 123 d (931 degree day) VHS survivors providing greater protection than that of 60 d (409 degree day) VHS survivors (Figure 20). These results provide further proof-of-concept that the VHSV exposure history of herring populations can be determined post hoc. Although this passive immunization technique remains too laborious and costly as a high-throughput diagnostic technique for quantifying herd immunity, the success of this technique provides justification for attempted development of an Enzyme-Linked Immunosorbent Assay to detect herring antibodies to VHSV. This ELISA development is currently underway under EVOST TC Project # 10100123-I.

Table 13. Geometric mean viral tissue titers after exposure of passively immunized groups to VHSV.

Source of Plasma	Plasma Dilution	Geometric mean VHSV tissue titer (PFU g^{-1})	Geometric SD
123d VHS survivors	0-fold	$2.0 \times 10^{4(*\dagger)}$	4.2×10^{1}
	10-fold	$5.1 \times 10^{5(*\dagger)}$	1.0×10^{2}
	50-fold	3.1×10^{6}	4.2×10^{1}
60d VHS survivors	0-fold	$7.3 \times 10^{5(*)}$	5.1×10^{1}
	10-fold	8.6×10^{6}	2.2×10^{1}
	50-fold	1.3×10^{7}	1.6×10^{1}
VHSV-naïve individuals (Positive Control)	0-fold	6.3×10^{6}	2.8×10^{1}

* Indicates titers in treatment groups that were significantly lower ($P < 0.05$) than in the positive control.

†Indicates titers that were significantly lower ($P < 0.01$) in the 123 d group than in the corresponding dilution from the 60 d group.

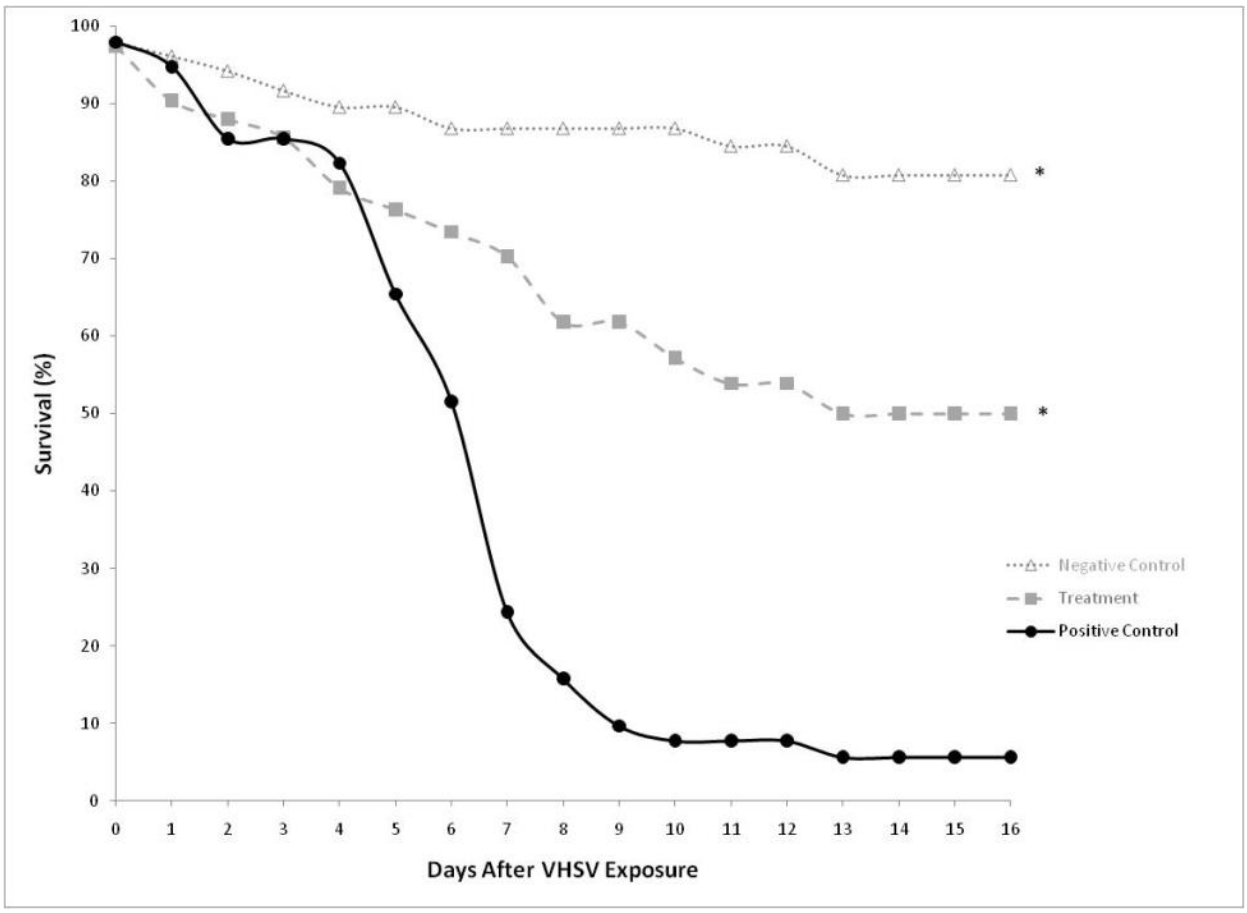
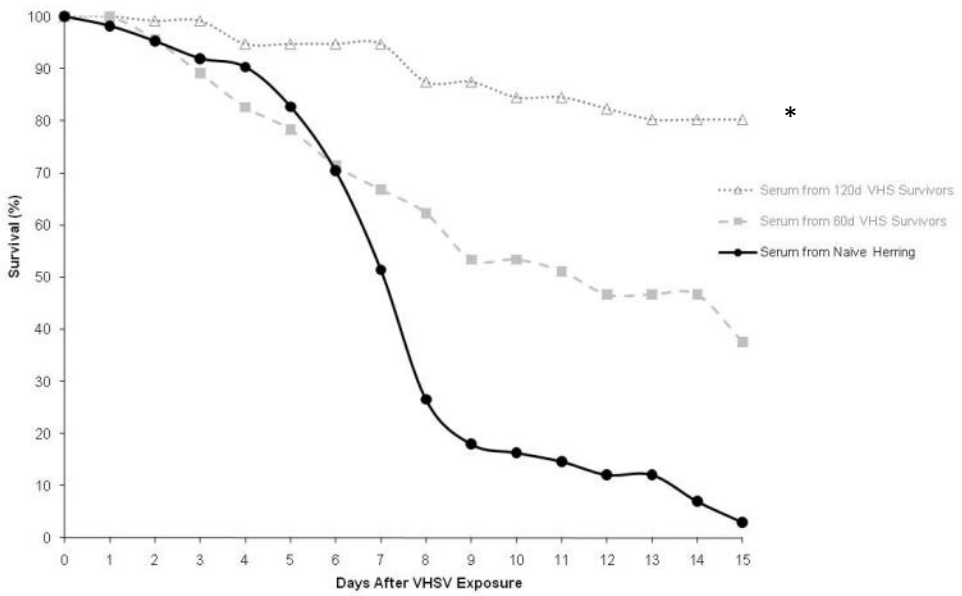
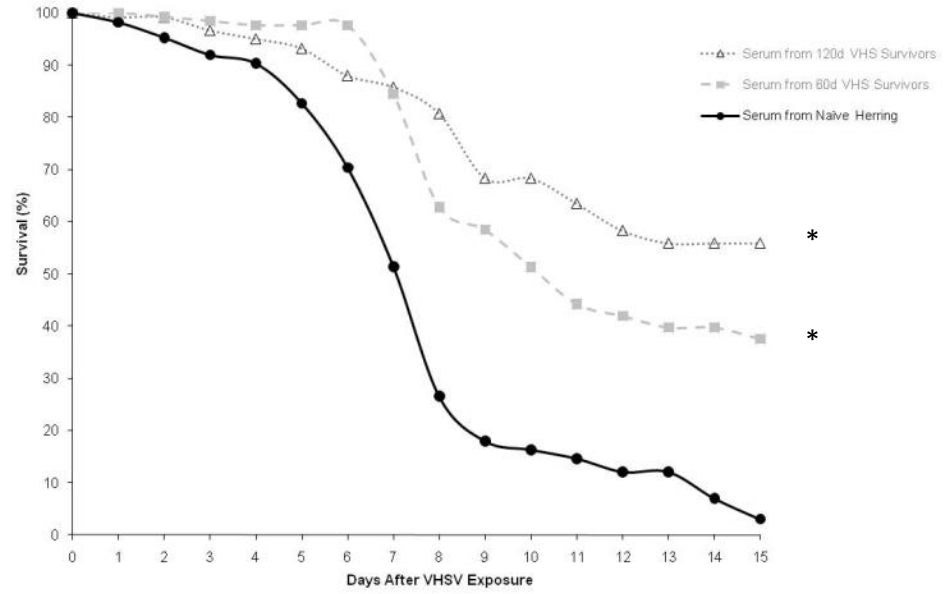


Figure 19. Survival after passively-immunized Pacific herring were exposed to VHSV. Positive controls consisted of SPF Pacific herring that were passively immunized with 50µL of undiluted plasma from previously-naïve herring then injected with VHSV. Treatments consisted of SPF Pacific herring that were passively immunized with plasma from VHS survivors (approximately 28 months post-exposure), then injected with VHSV. Negative controls consisted of SPF Pacific herring that were passively immunized using plasma from VHS survivors, then injected with saline. Data points represent back-transformed percentages corresponding to the means of arc sin transformed proportions from triplicate tanks (n = 10-13 herring / tank). '*' indicates significantly greater survival than in positive controls.

A


Survival (%)
Days After VHSV Exposure
Serum from 120d VHS Survivors
Serum from 60d VHS Survivors
Serum from Naive Herring
*

B


Survival (%)
Days After VHSV Exposure
Serum from 120d VHS Survivors
Serum from 60d VHS Survivors
Serum from Naive Herring
*
*

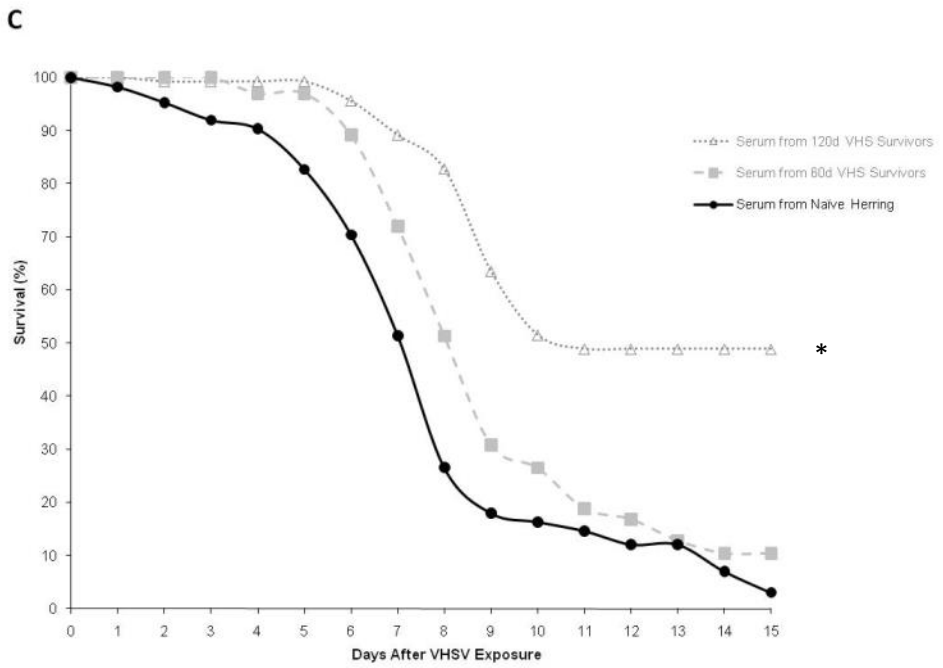


Figure 20 (A-C). Survival after VHSV exposure among Pacific herring that were passively immunized with 0-fold (A), 10-fold dilutions (B), or 50-fold dilutions (C) of plasma from donors collected from surviving fish at 60 and 123 days post-exposure to VHSV. Data points represent back-transformed percentages corresponding to the means of arc sin transformed proportions from triplicate tanks (n = 12-16 herring / tank). '*' indicates treatments where survival was significantly greater than that of herring injected with donor plasma from VHSV-naïve herring.

Chapter 3: Empirical Studies Involving *Ichthyophonus* and Pacific herring

Ichthyophonus disease (sometimes referred to as ichthyophoniasis, *Ichthyosporidium* disease, and ichthyosporidiosis) is a systemic granulomatous disease caused by the Mesomycetozoean parasite *Ichthyophonus* spp. A lack of distinguishing morphological characteristics and incomplete species descriptions of the causative agent have resulted in nomenclature inconsistencies within the genus; to avoid further confusion, the organism(s) should be referred to generically as *Ichthyophonus* until molecular phylogenetic studies provide an objective basis for speciation.

The geographic range of ichthyophoniasis is generally considered to include marine waters throughout the world; as such, ichthyophoniasis is one of the most widespread diseases of fish. Ecological and economic impacts of ichthyophoniasis are most commonly reported in wild marine fishes (eg. reviewed in McVicar 1999, Kocan et al 2004, Marty et al 2010, Hershberger et al 2010c); however, the disease is periodically reported from cultured marine and freshwater species (Gustafson and Rucker 1956, Doriere and Degrange 1960, Erickson 1965, Miyazaki and Kubota 1977, Anonymous 1991, Athanassopoulu 1992, Franco-Sierra 1997, Gavryuseva 2007) where its origin is often traced to the feeding of un-processed tissues from infected marine fishes.

Although reports are relatively rare, *Ichthyophonus* infections are periodically documented in free-ranging freshwater fishes (eg. Schmidt-Posthaus & Wahli 2002)

Basic epizootiological understanding of the disease remains limited because the predominance of scientific reports consist of case histories and responses to observed epizootics rather than controlled empirical studies. Transmission in piscivorous and scavenger hosts likely occurs through consumption of infected prey (Kocan et al 1999). Additionally, horizontal fish-to-fish transmission occurs in some species, including cultured rainbow trout (Gustafson & Rucker 1956). The route of transmission for planktivorous hosts, including Clupeids, remains unclear; laboratory studies have repeatedly failed to establish infections through horizontal transmission, feeding with food containing *Ichthyophonus* schizonts, or by direct intubation of *Ichthyophonus* schizonts into the stomach of Pacific herring (Hershberger & Gregg unpublished data). Repeated feeding of captive, Atlantic herring with *Ichthyophonus*-spiked mussel and liver tissues resulted in low prevalence of infection (1/25 and 3/25); however, a natural route of infection has not been demonstrated. Spiking invertebrate colonies with *Ichthyophonus* resulted in attachment of the parasite to copepod appendages and invasion of parasite germination tubes into the body of a single *Calanus finmarchicus*; however, feeding of these invertebrates to Atlantic herring did not establish infection (Sindermann and Scattergood 1954). Further research is needed to understand the possible involvement of intermediate hosts and other natural routes of infection.

3.1 Inactivation of *Ichthyophonus* schizonts with halogens

In vitro studies were performed to identify biocontainment procedures that are effective at inactivating *Ichthyophonus*. Chlorine and iodine solutions were effective at inactivating *Ichthyophonus* schizonts *in vitro*. Inactivation in seawater increased directly with halogen concentration and exposure duration, with significant differences ($p < 0.05$) from controls occurring at all chlorine concentrations and exposure durations tested (1.5 - 13.3 ppm for 1-60 min; Figure 21) and at most iodine concentrations and exposure durations tested (1.2 ppm for 60 min and 5.9 - 10.7 ppm for 1-60 min; Figure 22). However, 10-fold reductions in schizont viability occurred only after exposure to halogen solutions at higher concentrations and / or longer durations (13 ppm total chlorine for 1-60 min, 5.9 ppm total iodine for 60 min, and 10.7 ppm total iodine for 1-60 min). Inactivation efficacy was greater when halogen solutions were prepared in freshwater (Figures 23 & 24), presumably because of combined effects of halogen-induced inactivation and general schizont instability in freshwater.

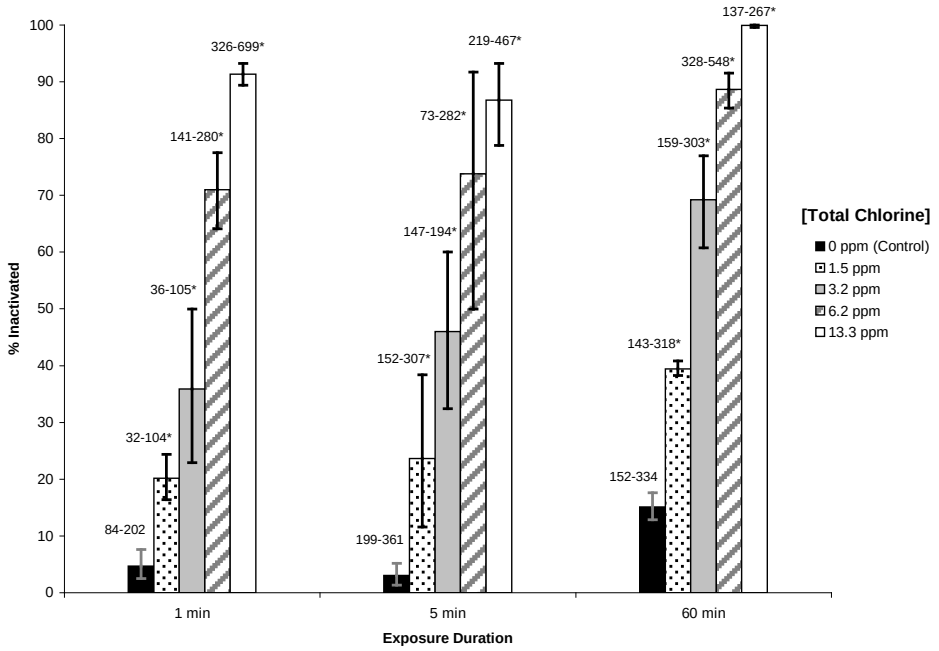


Figure 21. Inactivation of *Ichthyophonus* schizonts in chlorine / seawater (30‰) solutions. Bars indicate the percentages corresponding to the means of arc sine transformed proportions for the three replicates; error bars indicate 2 SD from the mean. Numerals above the bars indicate the number of schizonts per replicate (n = 3), and "*" indicates treatments where % inactivation was significantly greater ($p \leq 0.05$) than that in controls.

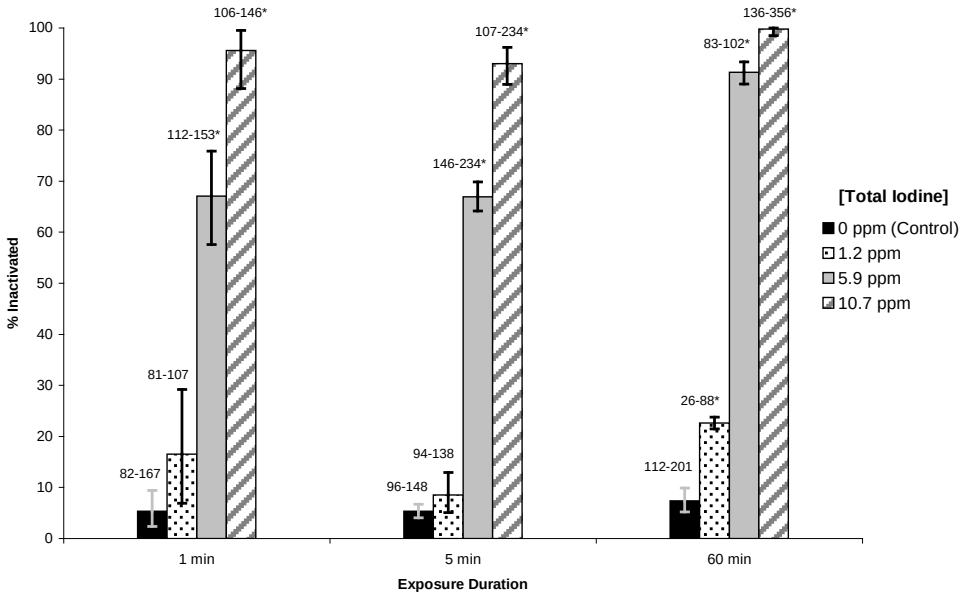


Figure 22. Inactivation of *Ichthyophonus* schizonts in iodine / seawater (0‰) solutions. Bars indicate the percentages corresponding to the means of arc sine transformed proportions for the three replicates; error bars indicate 2 SD from the mean. Numerals above the bars indicate the number of schizonts per replicate (n = 3), and "*" indicates treatments where % inactivation was significantly greater ($p \leq 0.05$) than that in controls.

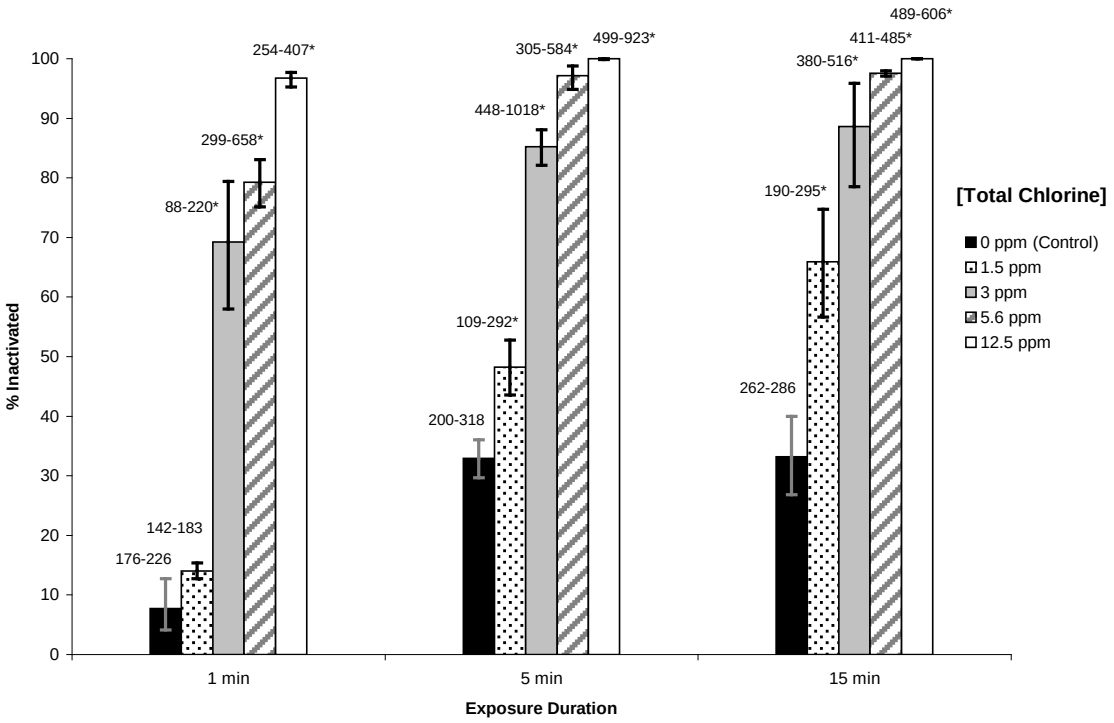


Figure 23. Inactivation of *Ichthyophonus* schizonts in chlorine / freshwater (0‰) solutions. Bars indicate the percentages corresponding to the means of arc sine transformed proportions for the three replicates; error bars indicate 2 SD from the mean. Numerals above the bars indicate the number of schizonts per replicate (n = 3), and "*" indicates treatments where % inactivation was significantly greater ($p \leq 0.05$) than that in controls.

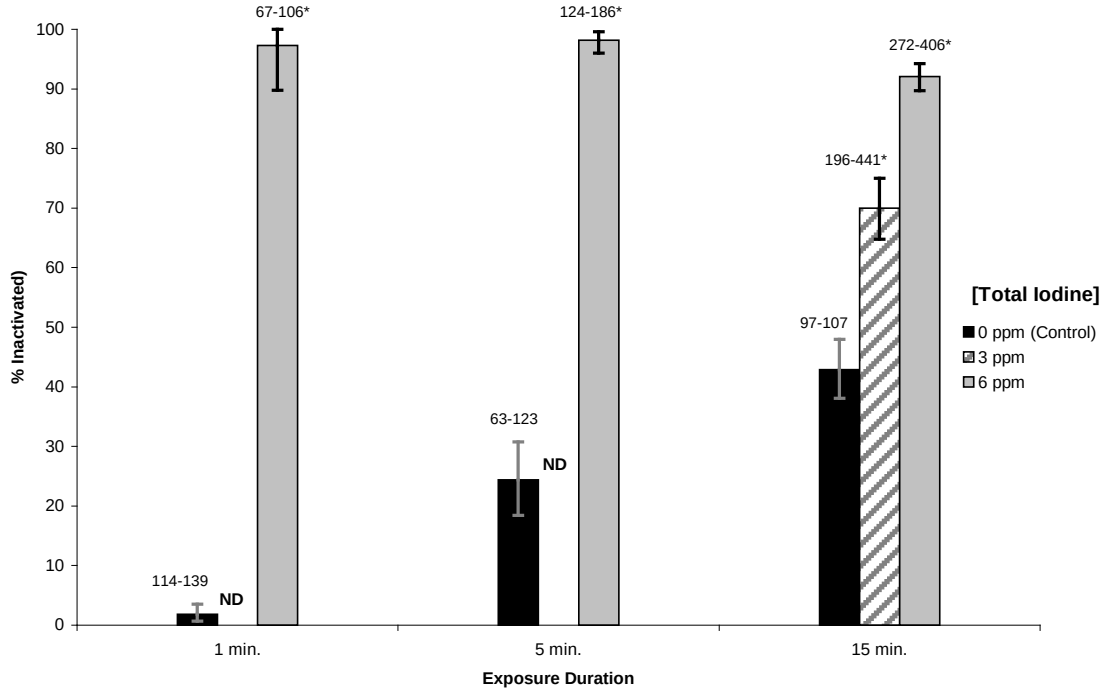


Figure 24. Inactivation of *Ichthyophonus* schizonts in iodine / freshwater (0‰) solutions. Bars indicate the percentages corresponding to the means of arc sine transformed proportions for the three replicates; error bars indicate 2 SD from the mean. Numerals above the bars indicate the number of schizonts per replicate (n = 3), and "*" indicates treatments where % inactivation was significantly greater ($p \leq 0.05$) than that in controls. "ND" indicates treatments that were not tested.

3.2 Facultative Survival of *Ichthyophonus* in Fresh and Saltwater

In vitro viability of *Ichthyophonus* schizonts in seawater and freshwater corresponded with the water type of the host from which the schizonts were isolated. Among *Ichthyophonus* schizonts from both marine and freshwater fish hosts (Pacific herring, *Clupea pallasii* and rainbow trout, *Oncorhynchus mykiss*; respectively), viability was significantly greater ($p < 0.05$) after incubation in seawater than in freshwater at all time points from 1-60 minutes post-immersion; however, magnitude of the schizont tolerances to water-type differed with host origin. *Ichthyophonus* adaptation to its host environment was indicated by greater seawater tolerance of schizonts from the marine host and greater freshwater tolerance of schizonts from the sympatric fish host (Figure 25-27). Prolonged aqueous survival of *Ichthyophonus* schizonts in the absence of a host provides insight into routes of transmission, particularly among planktivorous fishes, and should be taken into consideration when designing strategies to dispose of infected fish carcasses and tissues.

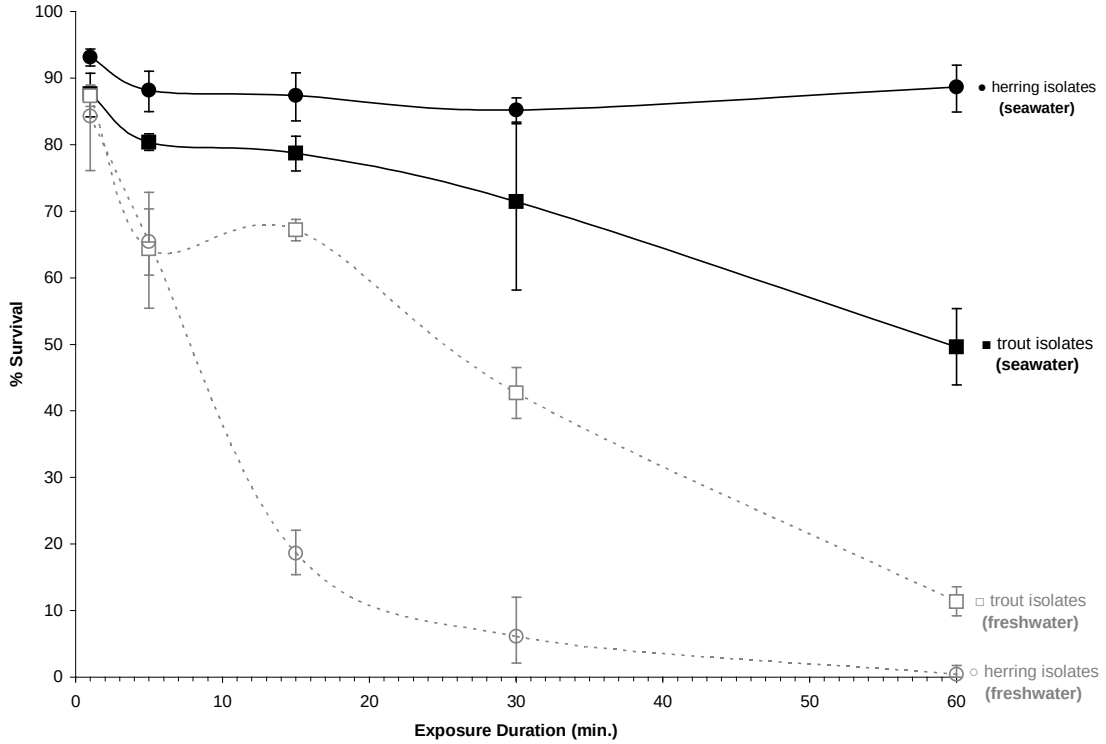


Figure 25. Survival of *Ichthyophonus* schizonts in seawater and freshwater from Trial 1. Data points indicate the percentages corresponding to the means of arc sin - transformed proportions for the replicates (n=3); 87 - 501 schizonts from each replicate were analyzed each sampling day. Error bars indicate 2 SD from the mean. Mean survival in duplicate control groups (not exposed to seawater or freshwater) was 87.6% (trout isolates) and 93.3% (herring isolates).

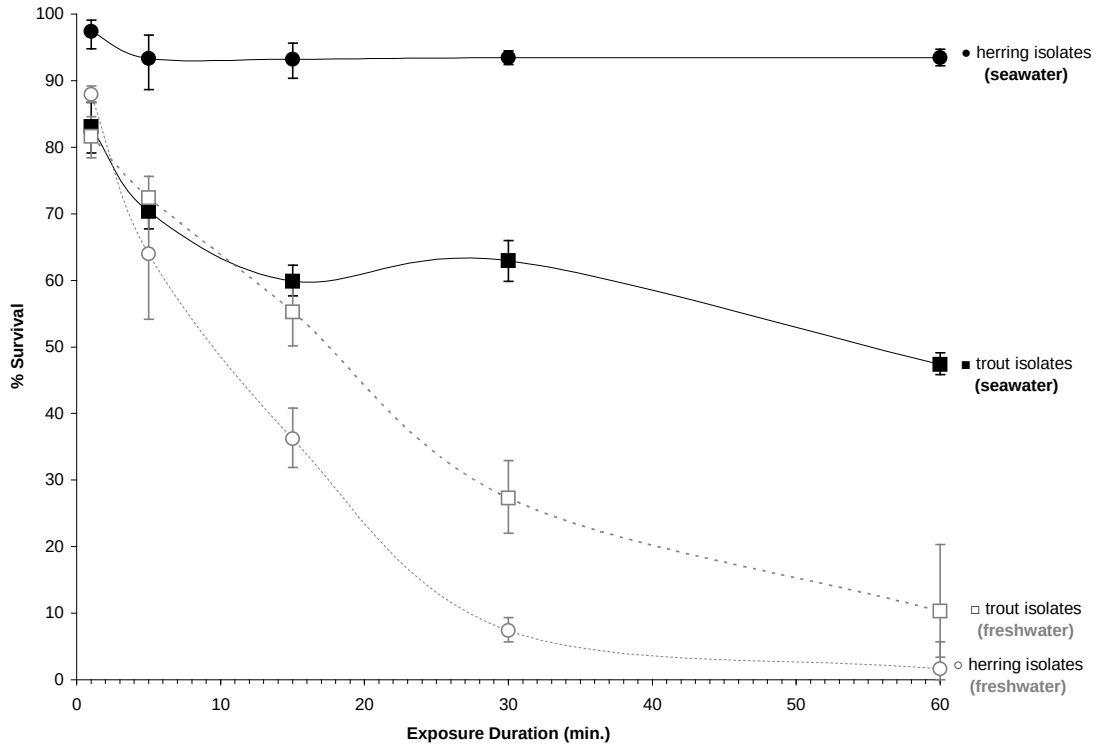


Figure 26. Survival of *Ichthyophonus* schizonts in seawater and freshwater from Trial 2. Data points indicate the percentages corresponding to the means of arc sin - transformed proportions for the replicates (n=3); 101 - 676 schizonts from each replicate were analyzed each sampling day. Error bars indicate 2 SD from the mean. Mean survival in duplicate control groups (not exposed to seawater or freshwater) was 80.56% (trout isolates) and 99.98% (herring isolates).

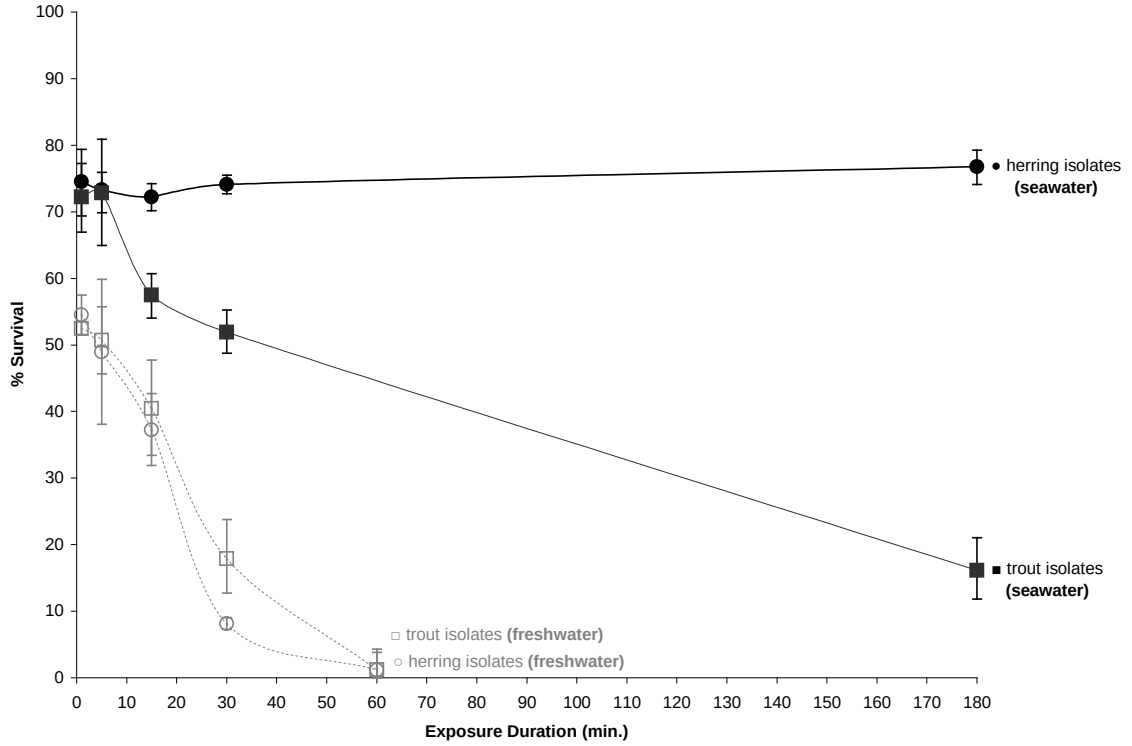


Figure 27. Survival of *Ichthyophonus* schizonts in seawater and freshwater from Trial 3. Data points indicate the percentages corresponding to the means of arc sin - transformed proportions for the replicates (n=3); 42 - 286 schizonts from each replicate were analyzed each sampling day. Error bars indicate 2 SD from the mean. Mean survival in triplicate control groups (not exposed to seawater or freshwater) was 71.92% (trout isolates) and 86.8% (herring isolates).

3.2 Low potential exists for cross-contamination of *Ichthyophonus* samples

In vitro culture of pathogens has been used for over a century to identify the causative organisms for a variety of diseases (Koch 1881, Bass & Johns 1912, Dobell & Laidlaw 1926, Diamond 1960, Kocan 1969). For *in vitro* culture to correctly identify pathogenic organisms and / or determine infection prevalence, the parasite should be isolated in pure culture with a high degree of certainty that cross-contamination of cultures has not occurred. Currently, *in vitro* culture of tissue explants represents the 'old standard' diagnostic technique for the detection of *Ichthyophonus* infections in fishes (Hershberger In Press). Experiments were performed to determine the potential for cross-contamination of *Ichthyophonus* cultures under field conditions, which can often be sub-optimal for ensuring aseptic techniques.

To evaluate the effect of instrument disinfection, heart tissue was excised and cultured from 48 rainbow trout that were collected from a raceways know to contain *Ichthyophonus*. One set of tissues was collected using aseptic technique and by disinfecting the dissection tools between fish; a paired set of samples was collected without the implementation of disinfection procedures between fish. Infection prevalence was 70.8% when disinfected instruments were used and 72.9% when contaminated instruments were used. The one additional positive culture was

detected in the group initiated with contaminated instruments, which was preceded by an *Ichthyophonus*-infected fish, suggesting, but not confirming, the possibility of cross contamination.

In a second experiment, explant cultures were initiated from heart tissue obtained from 24 rainbow trout. Twelve fish were experimentally exposed to *Ichthyophonus* by cohabitation with known infected fish for 15 months, and 12 were specific-pathogen-free (SPF). The entire heart was aseptically removed first from each of the 12 SPF fish followed by the *Ichthyophonus*-exposed fish. The specimens were arranged such that an *Ichthyophonus*-exposed tissue was followed by SPF-negative tissue. In the first sampling, disinfected instruments (forceps and scalpel) were used between each heart. In a second sampling contaminated instruments (not cleaned in any way) were used between each heart. No difference in the number of *Ichthyophonus*-positive fish was observed when disinfected and contaminated instruments were compared. A total of 75% (9/12) of the experimentally exposed fish cultured positive for *Ichthyophonus*, while none of the SPF fish cultured positive.

Based on the findings presented here, we find it highly improbable that *Ichthyophonus* cultures could be cross-contaminated if minimal effort is made to disinfect instruments between fish. The inability of *Ichthyophonus* cells to contaminate instruments is likely related to their large size and close association with the host tissue.

3.3 Infectious cells released from epidermal ulcers of Pacific herring with *Ichthyophonus*

A common clinical sign of ichthyophoniasis in herring and trout is “sandpaper” skin, a roughening of the epidermis characterized by the appearance of small papules followed by ulceration and sloughing of the epithelium (Figure 28); early investigators hypothesized that these ulcers might be a means of transmitting the parasite, *Ichthyophonus* sp., without the necessity of ingesting an infected host. We examined the cells associated with the epidermal lesions and confirmed they were viable *Ichthyophonus* sp. cells (Figures 29-31) that were readily released from the skin into the mucous layer and ultimately into the aquatic environment. The released cells were infectious when injected into the body cavity of specific pathogen-free herring, supporting our hypothesis that different mechanisms for transmission occur in carnivorous and planktivorous hosts.

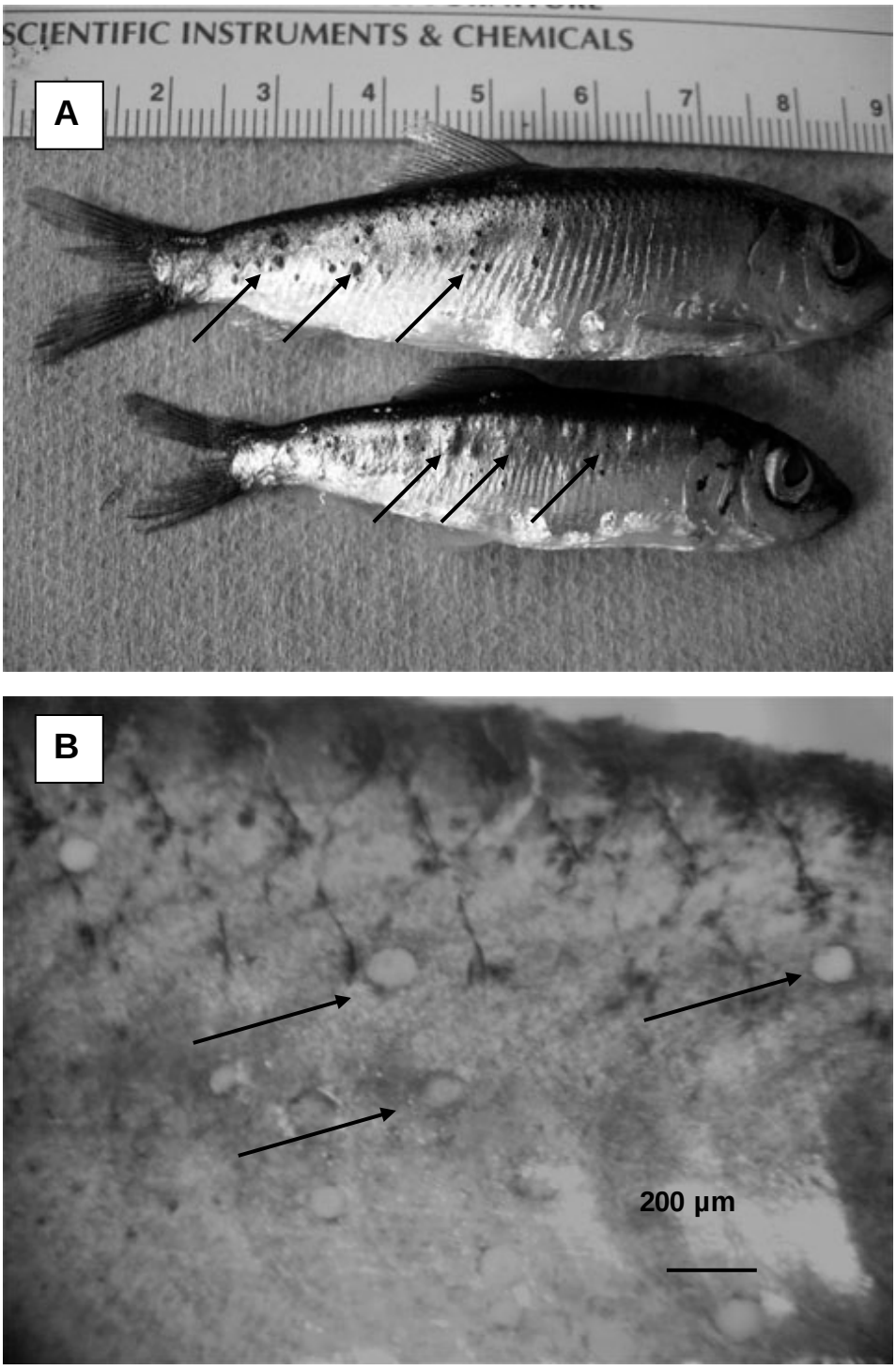


Figure 28. A. Open dermal ulcers (upper fish) and papules (lower fish) in Pacific herring caused by *Ichthyophonus*. B. Magnified view (5X) of ulcers showing whitish parasite cell(s) protruding through ulcerated skin.

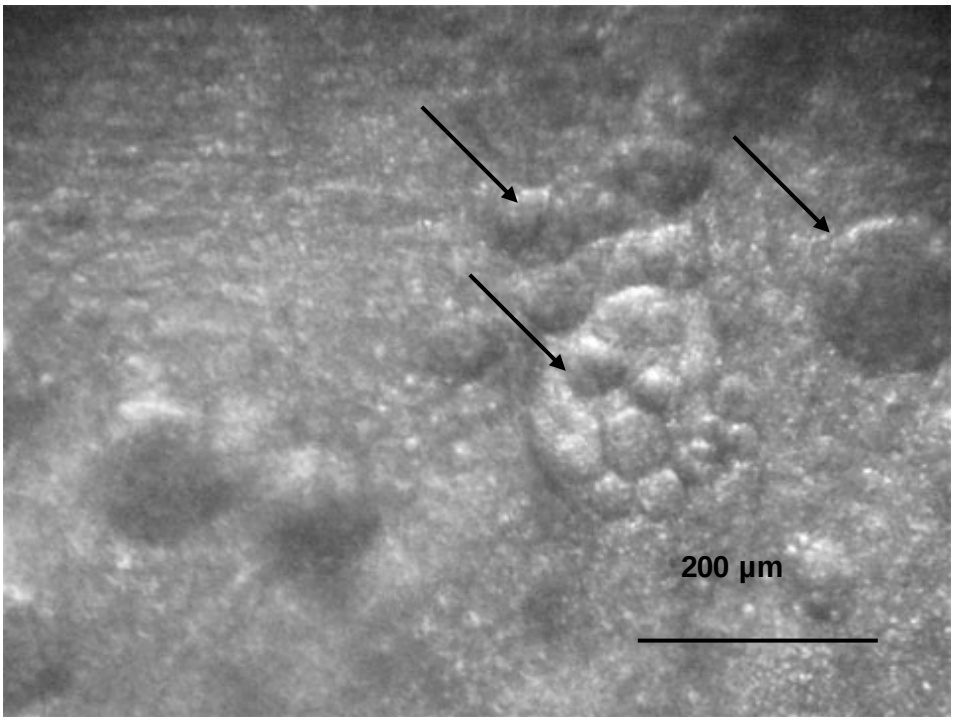


Figure 29. High magnification (40X) of epidermal ulcers showing PAS-positive multinucleate cells (arrows) protruding into mucous layer. Note difference in size range of different cells.

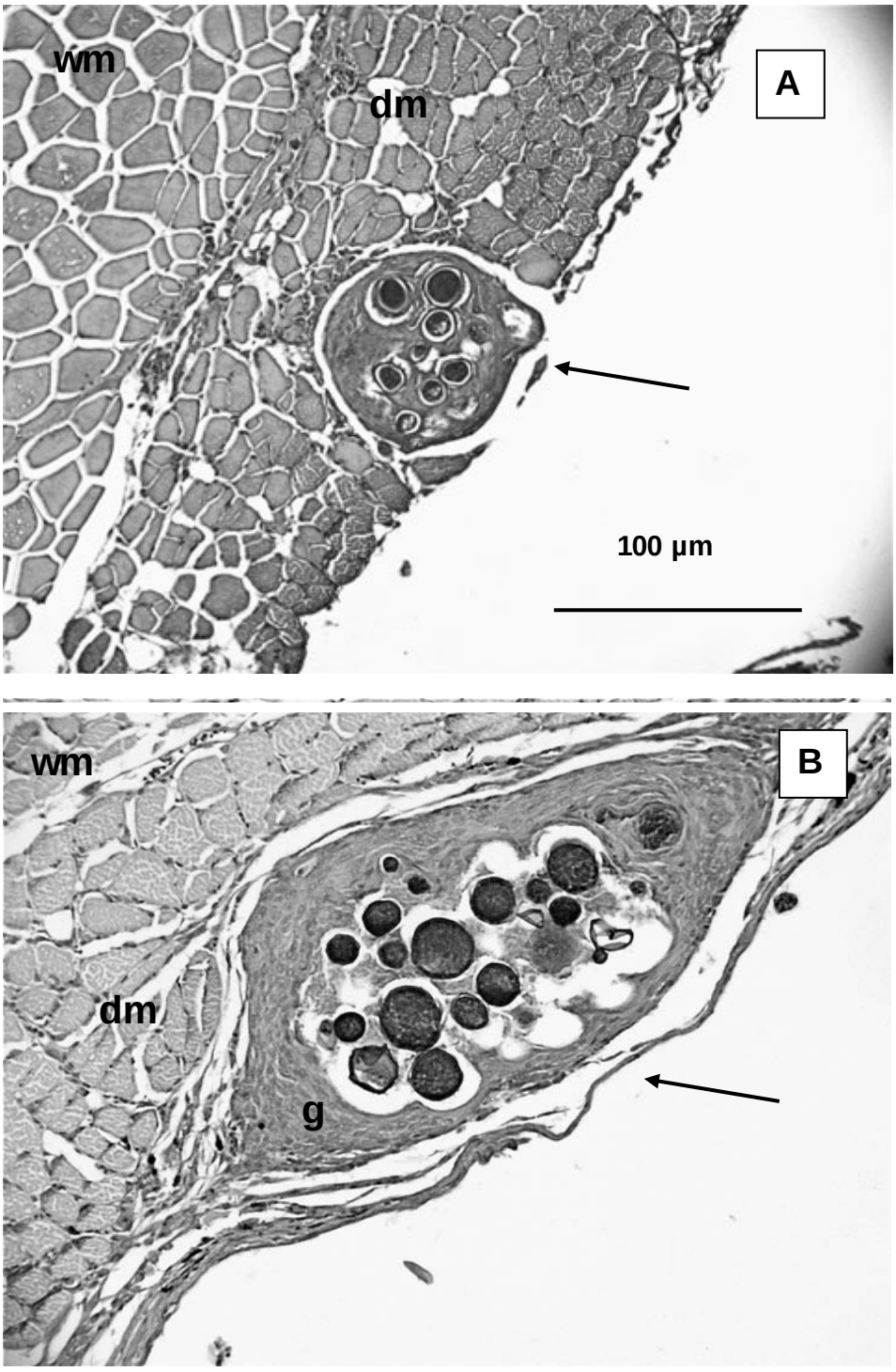


Figure 30. A. Histologic section of herring muscle/skin showing PAS-positive multinucleate cell protruding through epithelium. Note disintegration of the epithelium (arrow). B. Section of herring muscle and skin showing PAS-positive cells within a large granuloma (host cells) and disintegration of the epithelium (arrow) adjacent to the parasite. wm – white muscle; dm – dark muscle; g – granuloma

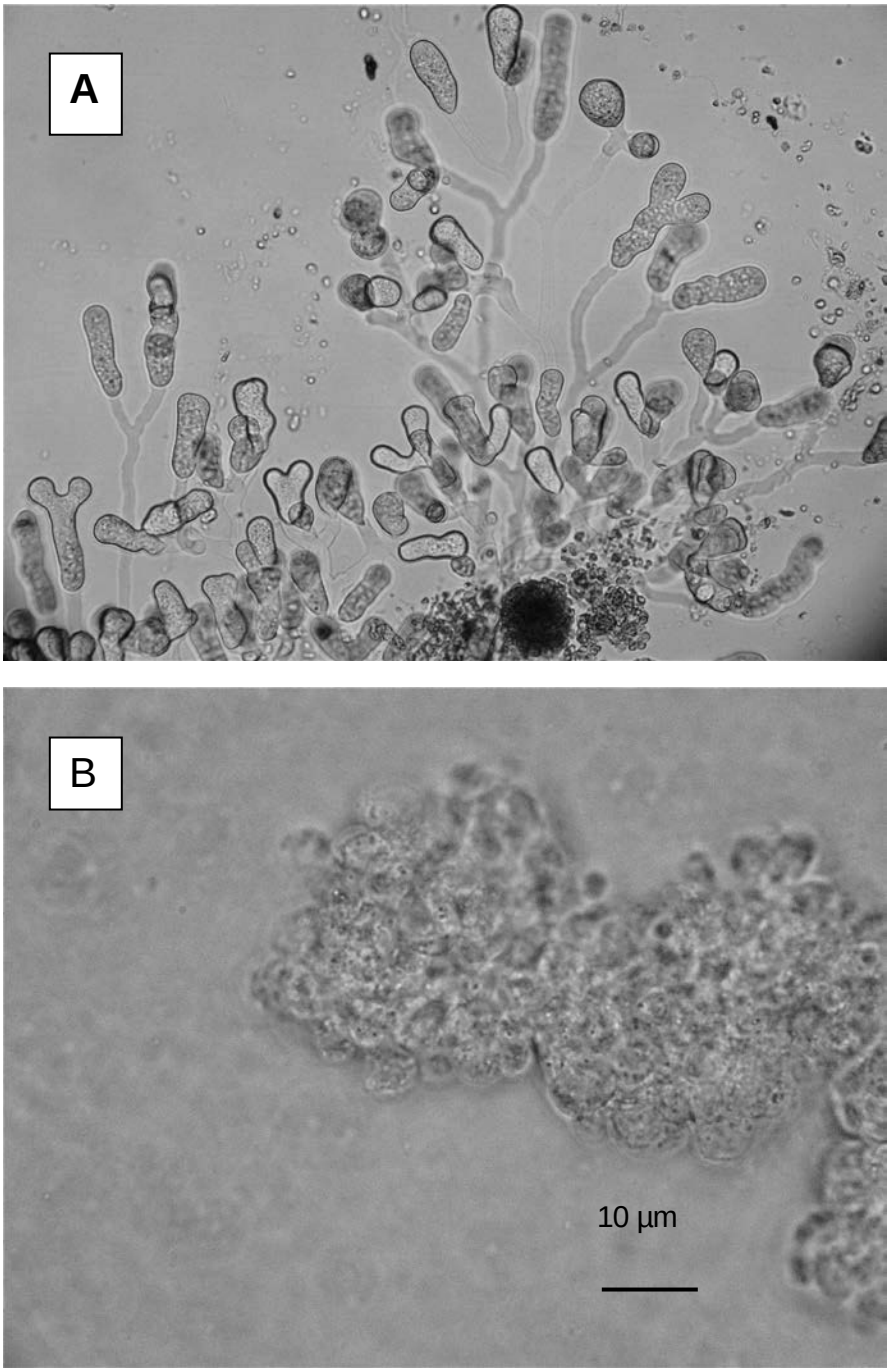


Figure 31. In vitro growth of *Ichthyophonus* from mucous scrapings occurred only in low pH medium (pH 3.5). 4B Cluster of cells from ruptured multinucleate cell released from an epidermal ulcer of an *Ichthyophonus*-infected Pacific herring. These masses were observed germinating in culture. (100X).

3.4 *Ichthyophonus* schizonts are not uniformly distributed throughout the skeletal muscle of infected Pacific herring

Since its discovery, several different techniques have been employed to detect and identify *Ichthyophonus* spp. in infected hosts; these included macroscopic observation, microscopic examination of squash preparations, histological evaluation, in vitro culture and molecular techniques. Studies that used more than one diagnostic method often reported significantly different results for each method. For example, standard histological evaluation detected only 47-59% of low-level and 84-92% of high-level *Ichthyophonus* infections in Chinook salmon when compared with in vitro culture of the same tissues from the same individuals (Table 14), while other studies on different species reported the same disparity. We observed non-random distributions of both ulcers on the skin surface and the parasite in three-dimensionally-visualized tissue sections (Tables 15-16, Figure 32-34), providing a possible explanation for the disparate sensitivities of the commonly-used diagnostic techniques. Based on experimental evidence and a review of the existing peer-reviewed literature we have concluded that in vitro culture should be considered the preferred diagnostic technique for determining infection prevalence of *Ichthyophonus*, particularly when the exposure history of the population is not known.

Table 14. Correspondence between confirmed *Ichthyophonus*-infected tissues and single plane histology.

	Year	N[1]	Histology% positive	Correspondence
Early infections	2002	15	7	47
	2003	27	16	59
	Total	42	23	55
Advanced infections	2002	25	23	92
	2003	37	31	84
	Total	62	54	87
	Grand total 2002+2003	104	77	74

[1]Number of *Ichthyophonus*-infected samples confirmed by in vitro culture.

Table 15. Mean and range of epidermal ulcer density from *Ichthyophonus*-infected Pacific herring (left side)

	Females (n=41)			Males (n =28)			Total (n = 69)		
Sector	**Mean**	**Low**	**High**	**Mean**	**Low**	**High**	**Mean**	**Low**	**High**
1	7.0	0	28.0	1.2	0	7.0	4.1	0	28.0
2	10.6	0	42.0	6.7	0	56.0	8.6	0	56.0
3	5.3	0	14.0	1.4	0	10.0	3.3	0	14.0
4	20.8	0	52.0	3.5	0	13.0	12.1	0	52.0
5	37.3	0	104.0	15.3	0	95.0	26.3	0	104.0
6	7.2	0	25.0	3.4	0	21.0	5.3	0	25.0
Mean	14.7	0.0	44.2	5.2	0.0	33.7	9.97	0.0	38.9

Table 16. Mean and range of *Ichthyophonus* schizont density (cells/gram) in herring skeletal muscle (left side)

	Females (n=41)			Males (n =28)			Total (n = 69)		
Sector	**Mean**	**Low**	**High**	**Mean**	**Low**	**High**	**Mean**	**Low**	**High**
1	199	0	1092	242	0	668	212	0	1092
2	110	7	460	25	0	62	85	0	460
3	300	73	3622	82	0	496	704	0	3622
4	168	2	349	86	0	509	123	0	349
5	94	0	287	98	0	445	96	0	277
6	89	0	186	21	0	100	58	0	186
Mean	160	14	999	92	0	380	213	0.0	998

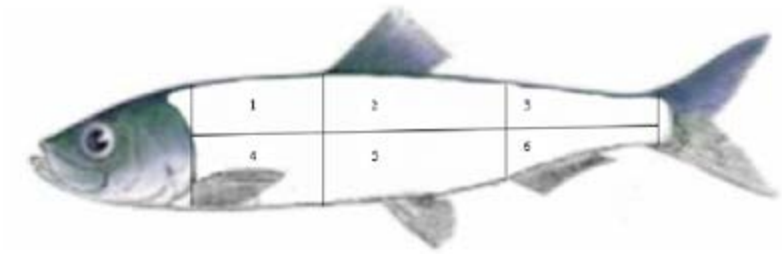


Figure 32. Sector numbering system used for evaluating epidermal ulcers and *Ichthyophonus* cells in underlying skeletal muscle.

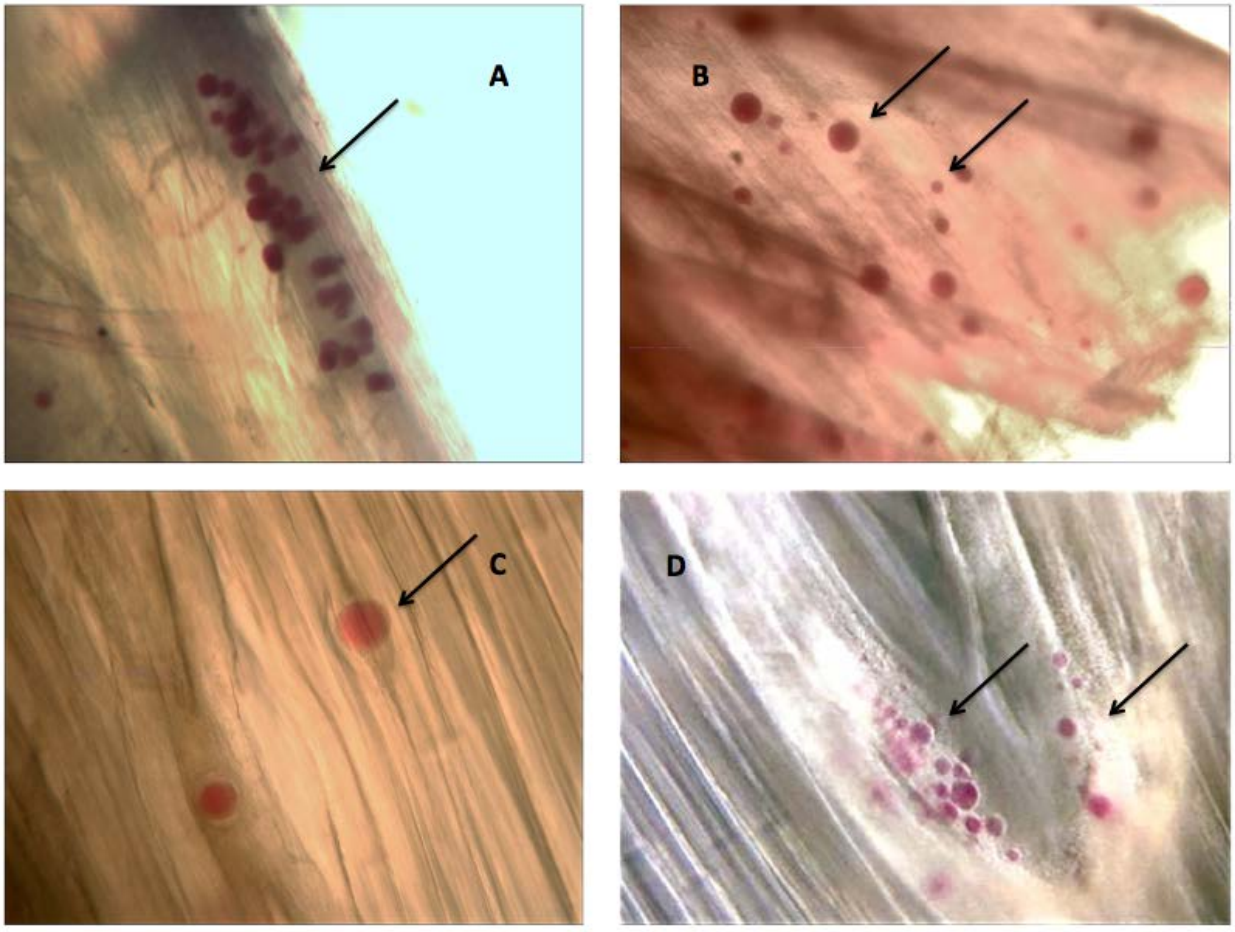


Figure 33. 3-dimentional view of *Ichthyophonus* schizonts in situ from different Pacific herring.
A- Clustering of similar size schizonts in the dark muscle (arrow). B- random distribution of various size schizonts throughout white muscle (arrows). C- Low-density of mature schizonts. D- Clustering of various sized schizonts in white muscle. Schizont diameter: 42 µm to 117 µm (stain – PAS following clearing)

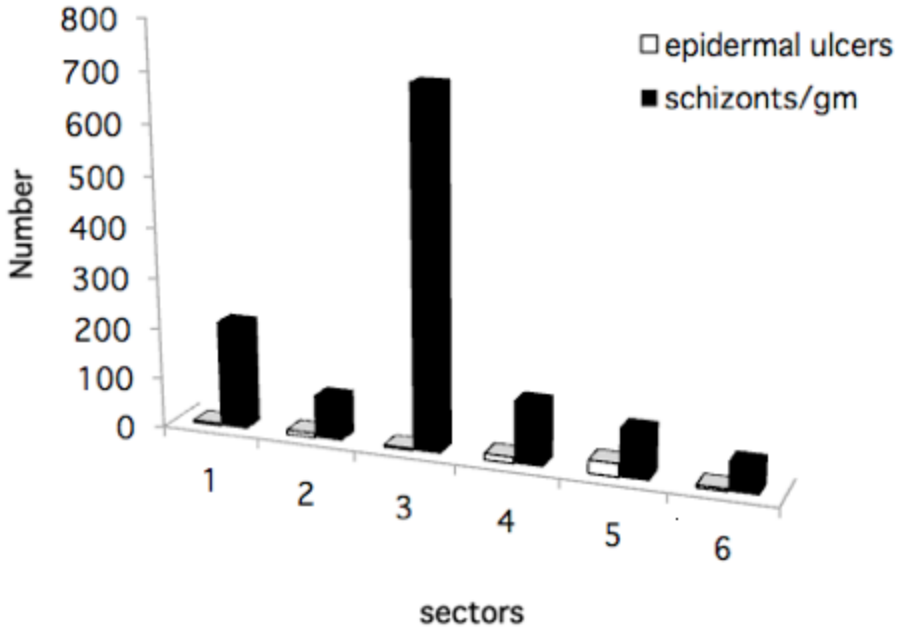


Figure 34. Mean number of epidermal ulcers compared with parasite density per gram of skeletal muscle for each of six sectors. (Only fish with visible external ulcers used for comparison).

3.5 Multiple Genetic and Phenotypic Types of *Ichthyophonus* Exist

Ichthyophonus is most commonly associated with marine fish hosts but the parasite also occurs in some portions of the freshwater rainbow trout (*Oncorhynchus mykiss*) aquaculture industry in Idaho, USA. It is not certain how the parasite was introduced into rainbow trout culture but it may be associated with the historical practice of feeding raw, ground common carp (*Cyprinus carpio*) caught by commercial fisherman. Here we report a major genetic division between west coast freshwater and marine isolates of the *Ichthyophonus hoferi* (Figure 35). Sequence differences were not detected in two regions of the highly conserved small subunit (18s) rDNA gene; however, nucleotide variation was seen in internal transcribed spacer loci (ITS1 and ITS2), both within and among the isolates. Intra-isolate variation ranged from 2.4 to 7.6 nucleotides over a region consisting of approximately 740 bp (Table 17). Majority consensus sequences from marine / anadromous hosts differed in only 0-3 nucleotides (99.6-100% nucleotide identity) while those derived from freshwater rainbow trout had no nucleotide substitutions relative to each other. However, the consensus sequences from freshwater trout differed from those of marine / anadromous hosts by 13-16 nucleotides (97.8 - 98.2% nucleotide identity; Table 18).

Table 17. *Ichthyophonus* isolates, host species, location, year, GenBank numbers and characteristics of the ITS rDNA gene regions.[1]

Isolate ID	Host Species	Location	Year	18S GenBank # (region A/ region B)	ITS region Genbank #	# of ITS PCR clones[1]	Size of ITS region (bp)[2]	ITS Intra-isolate variation
IA2	Pacific Herring	Puget Sound, WA	2005	GQ370767/ GQ370788	GQ402831-GQ402842	12	727	4.6 (0.006)
IA113	Pacific herring	Puget Sound, WA	2008	GQ370766/ GQ370787	GQ402907-GQ402916	10	730	3.8 (0.005)
IA51	Pacific Herring	Sitka, AK	2007	GQ370773/ GQ370794	GQ402872-GQ402886	15	727	5.9 (0.008)
IA6	American Shad	Puget Sound, WA	2005	GQ370768/ GQ370789	GQ402843-GQ402854	12	727	5.7 (0.008)
IA52	American Shad	Columbia River, WA	2007	GQ370774/ GQ370795	GQ402887-GQ402899	13	731	3.5 (0.005)
IA14	American Shad	Columbia River, WA	2005	GQ370770/ GQ370791	GQ402860-GQ402871	12	733	4.8 (0.006)
IA11	Copper Rockfish	Puget Sound, WA	2006	GQ370769/ GQ370790	GQ402855-GQ402859	5	729	7.6 (0.010)
RBT48	Rainbow Trout	Hagerman Valley, ID	2007	GQ370772/ GQ370793	GQ402995-GQ403008	14	738	3.8 (0.005)
RBT27	Rainbow Trout	Hagerman Valley, ID	2007	GQ370771/ GQ370792	GQ402982-GQ402994	13	739	3.6 (0.005)
RBT11	Rainbow Trout	Hagerman Valley, ID	2008	GQ370776/ GQ370801	GQ402928-GQ402937	10	739	3.7 (0.005)
RBT12	Rainbow Trout	Hagerman Valley, ID	2008	GQ370777/ GQ370797	GQ402938-GQ402947	10	740	2.5 (0.003)
RBT13	Rainbow Trout	Hagerman Valley, ID	2008	GQ370778/ GQ370798	GQ402948-GQ402963	16	738	2.4 (0.003)
RBT15	Rainbow Trout	Hagerman Valley, ID	2008	GQ370779/ GQ370799	GQ402964-GQ402969	6	740	5.1 (0.007)
RBT16	Rainbow Trout	Hagerman Valley, ID	2008	GQ370780/ GQ370800	GQ402970-GQ402981	12	738	3.2 (0.004)
RBT2[4]	Rainbow Trout	Hagerman Valley, ID	2008	GQ370775/ GQ370796	GQ402917-GQ402927	11	738	4.1 (0.006)

[1] A single band of the ITS rDNA gene regions (ITS-5.8s-ITS2) was PCR amplified, cloned and multiple PCR clones were sequenced per isolate.
[2]The average number of nucleotides in the ITS1-5.8s-ITS2 rDNA gene regions for an individual isolate.
[3]Average number of nucleotide changes and p-distances among the PCR clones for each isolate spanning ITS1-5.8s-ITS2 regions.
[4]*Icthyophonus* was not cultured from this sample and molecular analysis was performed directly on the infected tissue.

Table 18. Pairwise differences among representative *Ichthyophonus* isolates for the ITS rDNA regions (ITS1-5.8s-ITS2). The p-distance is shown above the diagonal and number of nucleotide differences (out of 637 aligned nucleotides) is shown below the diagonal.

	IA2 (herring)[1]	IA11 (rockfish)	RBT2 (trout)[2]	*A. parasiticum*
IA2 (herring)	-	0.005	0.020	0.190
IA11 (rockfish)	3	-	0.025	0.190
RBT2 (trout)	13	16	-	0.193
A. parasiticum	121	119	123	-

[1]The IA2, IA113 (herring), IA51 (herring), IA6 (shad), IA52 (shad) and IA14 (shad) majority consensus sequences had no nucleotide substitutions relative to each other; IA2 is shown as the representative.
[2]The majority consensus sequences of all rainbow trout isolates (RBT2, RBT48, RBT27, RBT11, RBT12, RBT13, RBT15 and RBT16) had no nucleotide substitutions relative to each other; RBT2 is shown as the representative.

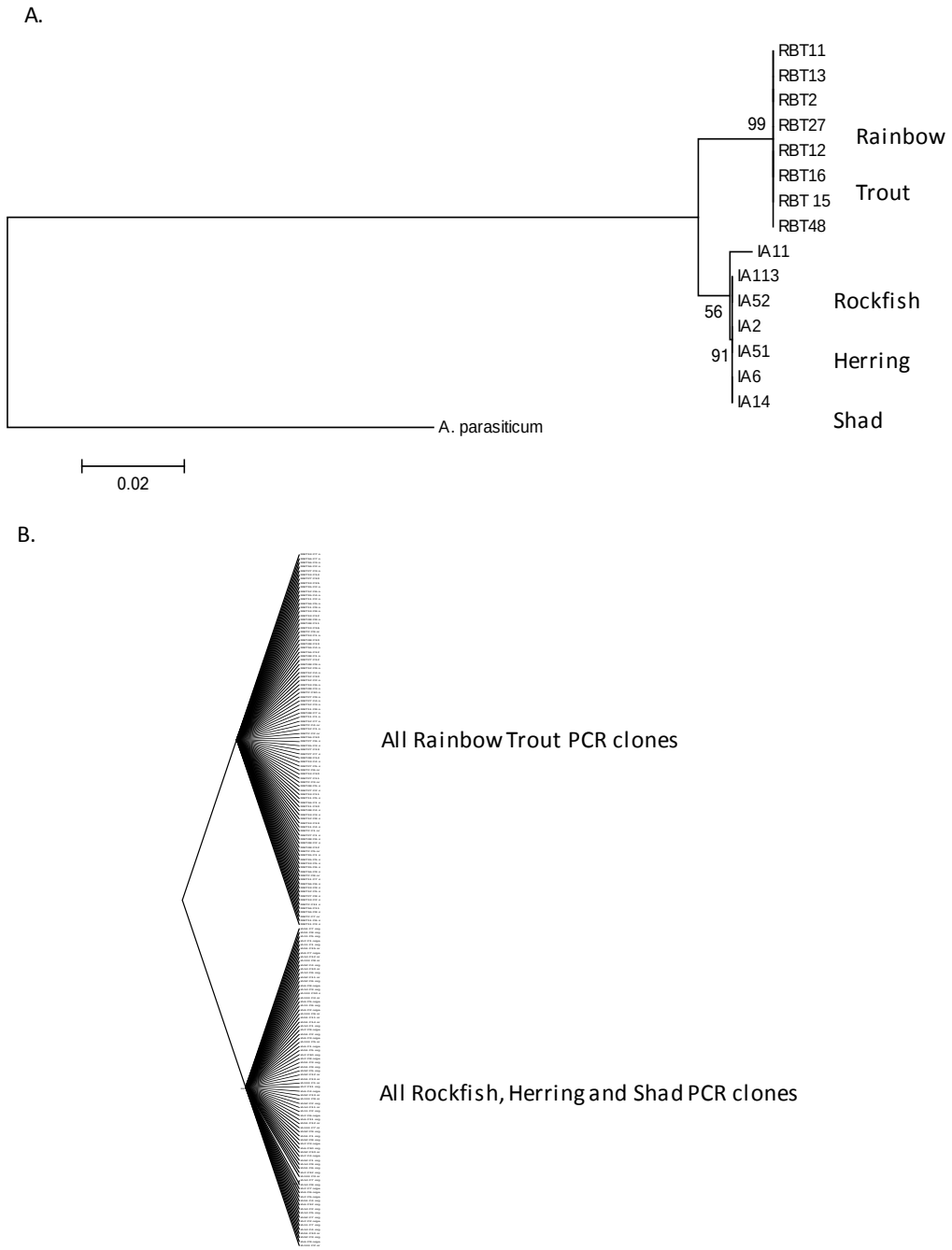


Figure 35. A. Evolutionary relationships of *Ichthyophonus* isolates based on the majority consensus sequence of the ITS region (ITS1-5.8s-ITS2) inferred using the neighbor-joining method (1000 bootstrap iterations). Similar tree topology was obtained using the maximum parsimony method (not shown). Isolates are derived from rainbow trout (RBT-11, 13, 2, 27, 12 16, 15 and 48), Pacific herring (IA2, IA113 and IA51), American shad (IA6, IA52 and IA14), and Copper rockfish (IA11). *Amoebidium parasiticum* (AY388646) was used as an outgroup. B. Evolutionary relationships of the individual PCR clones inferred using the neighbour-joining method (1000 bootstrap iterations). The tree was condensed using a bootstrap cut-off value of 70%.

3.6 Effects of Temperature on *Ichthyophonus* Infections

The effects of temperature and *Ichthyophonus* infection were examined in juvenile Pacific herring (*Clupea pallasii*) undergoing simulated overwinter fast (Table 19). In addition to defining parameters for a herring bioenergetics model (discussed in Vollenweider et al final EVOSTC project report and Vollenweider et al 2011), these experiments provide new insights into factors influencing the infectivity and virulence of the parasite *Ichthyophonus*. In groups of fish with established disease, temperature variation had little effect on disease outcome, with *Ichthyophonus* mortality outpacing that resulting from starvation alone at all temperatures (Figure 36). In newly infected fish, temperature variation significantly changed the mortality trajectory of the disease. Both elevated and lowered temperatures suppressed disease related mortality relative to ambient treatments (Figure 37). When parasite exposure dose decreased, an inverse relationship between infection prevalence and temperature occurred (Figure 38). These findings suggest interplay between temperature optima for parasite growth and host immune function, and have implications for our understanding of how *Ichthyophonus* infections are established in wild fish populations.

Table 19. Summary data for 3 *Ichthyophonus* experiments conducted in this study. Pacific herring in experiments 1 and 2 fasted during challenge, herring in experiment 3 fasted for 56 d prior to challenge and then fed to satiation during challenge.

Experiment			Herring			*Ichthyophonus*		Temperature (°C)[1]		
#	Disease State	Duration (d)	Age (d)	FL (mm)	Weight (g)	Schizonts[2] per inoculation	Dose (schizonts g^{-1})[3]	Low	Amb	High
1	Diseased: 19 d post-exposure	80	164	44	1.0	174	174	6.7	9.3	12.3
2	Infected: 1 d post-exposure	127	241	70	3.6	167	46	5.6	7.9	12.4
3	Infected: 1 d post-exposure	54	431	92 (100)[d]	6.5 (9.2)[4]	112	17 (12)[d]	9.3	12.0	15.3

[1]Temperature reported is mean of temperature recordings made every 30 minutes during course of challenge.
[2]Schizonts are multinucleate spherical bodies from in vitro Ichthyophonus cultures, number reported is mean of three 50 µl samples.
[3]Dose = mean number of schizonts divided by mean weight of fish.
[4]Values in parentheses are from group of herring that did not go through pre-challenge fast in Experiment 3.

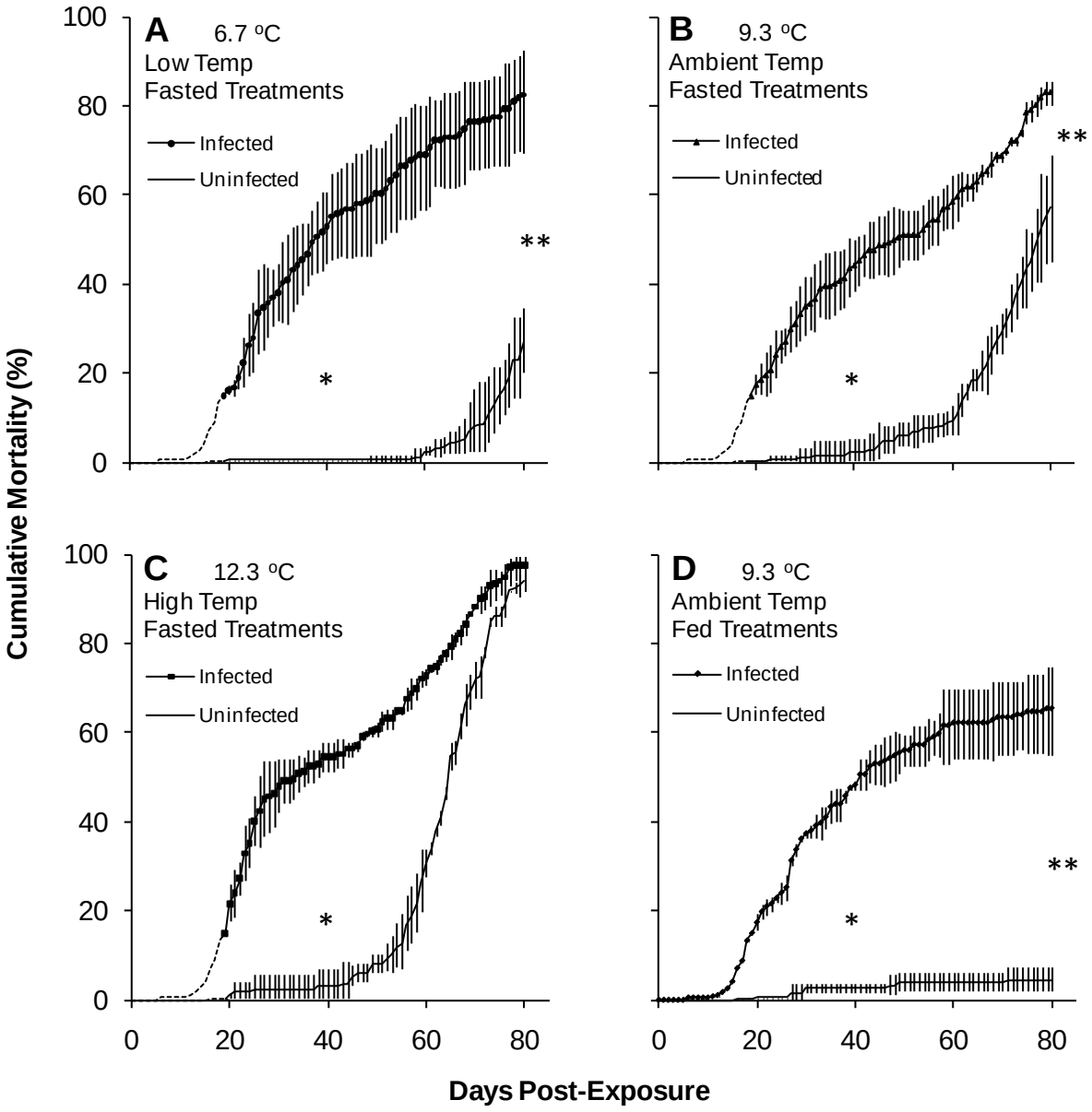


Figure 36. Cumulative mortality of diseased (ichthyophoniasis) and control Pacific herring in three temperature treatments. Data in A, B, and C are from fish undergoing simulated over-winter fast. Data in D are from fed treatments. Temperature adjustments were made 19 days after inoculation with *Ichthyophonus* schizonts. Data are means of 3 replicate tanks in each treatment. Error bars are one SD above and below the mean. Means and SD were calculated from arcsine transformed data. Mortality data prior to day 19 are from the pools of infected and control fish prior to separation into treatment. Significant differences between infected and uninfected groups (Tukey multiple comparisons) indicated by * and ** for midpoint and end of challenge respectively.

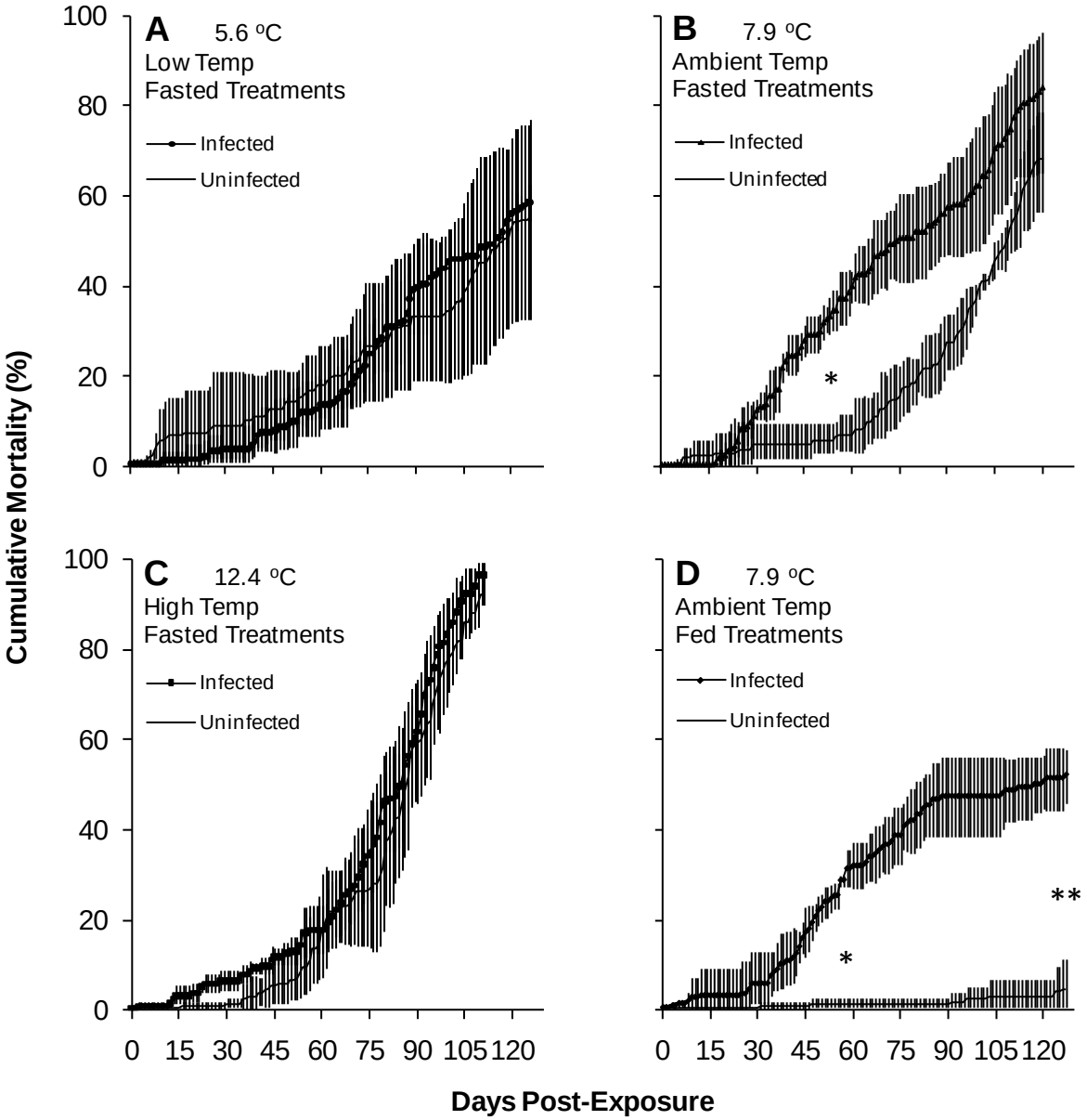


Figure 37. Cumulative mortality infected (*Ichthyophonus*) and control Pacific herring in three temperature treatments. Data in A, B, and C are from fish undergoing simulated over winter fast. Data in D are from fed treatments. *Ichthyophonus* inoculation occurred on day-0, temperature adjustments on day-1. Data are means of 3 replicate tanks in each treatment. Error bars are one SD above and below the mean. Means and SD were calculated from arcsine transformed data. Significant differences between infected and uninfected groups (Tukey multiple comparisons) indicated by * and ** for midpoint and end of challenge respectively.

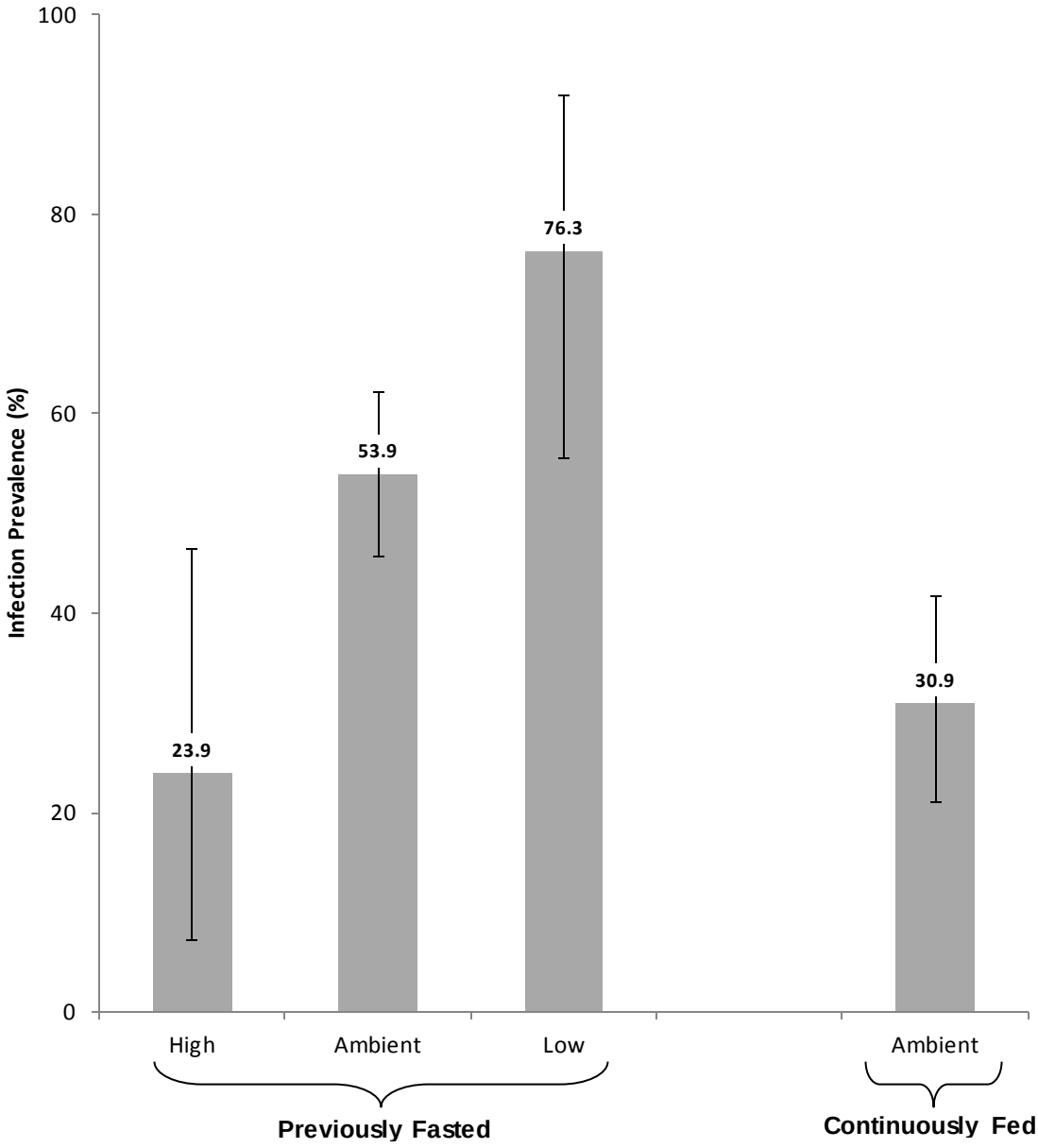


Figure 38. *Ichthyophonus* infection prevalence in groups of Pacific herring held at three temperatures. Data are means of arcsin transformed data from 3 replicate tanks, error bars are one SD above and below the mean. Mean temperatures were 9.3, 12.0, and 15.3°C for Low, Ambient, and High treatments respectively. Pre-challenge fast lasted 56 days, after which fish were inoculated with *Ichthyophonus*, separated to treatments, and fed to satiation daily. ANOVA and Tukey test for multiple comparisons indicates that High Temp treatment and Continuously Fed treatments are significantly different from Low Temp treatment.

Chapter 4: Empirical Studies Involving Erythrocytic Necrosis Virus and Pacific herring

Viral erythrocytic necrosis (VEN), first described by Laird and Bullock (1969) as piscine erythrocytic necrosis (PEN), is a disease of marine fish characterized by erythrocytic degeneration and the presence of cytoplasmic inclusion bodies (viroplasms) in circulating erythrocytes (Evelyn and Traxler 1978; Reno *et al.* 1978). Reports of similar conditions in amphibians and reptiles, combined with reports of VEN in fishes from around the world, suggest that the reported cases are likely caused by a group of related etiological agents. For a long time, the inclusions were suspected to be caused by blood parasites, but evidence of a viral aetiology was first reported in reptiles (Stehbens and Johnston 1966). The viral nature of the disease in fish was later demonstrated by electron microscopy studies indicating association of icosahedral virus particles within affected erythrocytes (Appy *et al.* 1976; Walker and Sherburne 1977). The associated virus has been referred to as erythrocyte necrosis virus (ENV) (Haney *et al.* 1992), but fulfillment of Koch's Postulates is currently precluded by the refractory nature of the virus to established cell lines.

The disease is not usually associated with high mortality, but it has been reported to occur epizootically in Pacific herring (Meyers *et al.* 1986). Transmission experiments have verified the infectious nature of the disease. Blood smears from affected fish reveal a single inclusion body in the cytoplasm of erythrocytes, visible by light microscopy. A virus belonging to the icosahedral cytoplasmic deoxyribovirus (ICDV) group and presumptively classified as an iridovirus has been associated with the inclusion bodies (Appy *et al.* 1976, Reno *et al.* 1978).

4.1 VEN in juvenile Pacific herring

Epizootics of viral erythrocytic necrosis (VEN) occurred among juvenile Pacific herring in Skagit Bay, Puget Sound, WA (Figure 39) during 2005 - 2007 and were characterized by high prevalences and intensities of cytoplasmic inclusion bodies within circulating erythrocytes. Prevalence of VEN peaked at 67% during the first epizootic in October, 2005, after which prevalence waned to 0% by August of 2006 (Figure 40). A second VEN epizootic occurred throughout the summer of 2007, and was characterized by disease initiation and perpetuation in the 1+ yr, 2006 age class, followed by involvement of the 0+ yr, 2007 age class cohorts shortly after their larval metamorphosis to juveniles (Table 20). The disease was detected in other populations of juvenile herring throughout Puget Sound and Prince William Sound, AK where prevalences and intensities typically did not correspond to those observed in Skagit Bay (Table 21). The persistence and recurrence of VEN epizootics indicates that the disease is likely common among juvenile herring throughout the eastern North Pacific, and although population-level impacts likely occur, they are typically covert and not easily detected.

Table 20. Prevalence of VEN in each herring age cohort from Skagit Bay. All adult herring ≥ 2 years old were combined into the adult category. 'ND' indicates no data; none of the sampled herring were members of that particular age cohort. 'NA' indicates not applicable; the age cohort was either not yet born or larval metamorphosis to juveniles was not yet complete at the time of sampling.

	Age Cohort (Birth Year)									
	2004		2005		2006		2007		Age 2+ yr Adults	
Sampling Month	**VEN Prevalence (n)**	**Length Bracket (mm)**	**VEN Prevalence (n)**	**Length Bracket (mm)**	**VEN Prevalence (n)**	**Length Bracket (mm)**	**VEN Prevalence (n)**	**Length Bracket (mm)**	**VEN Prevalence (n)**	**Length Bracket (mm)**
2005 Sept.	0% (13)	115-150	48% (36)	85-110	NA		NA		36% (11)	155-180
Oct.	33% (3)	120-125	65% (49)	75-105	NA		NA		ND	
2006 May	Recruited to adults		33% (9)	85-140	NA		NA		7% (41)	155-200
June	Recruited to adults		28% (18)	125-145	NA		NA		30% (27)	150-205
July	Recruited to adults		43% (7)	115-150	4% (48)	45-100	NA		0% (5)	160-215
Aug.	Recruited to adults		ND		0% (60)	75-115	NA		ND	
Sept.	Recruited to adults		ND		0% (60)	75-120	NA		0% (60)	170-220
Oct.	Recruited to adults		ND		0% (60)	75-105	NA		ND	
2007 April	Recruited to adults		Recruited to adults		4% (56)	85-125	NA		0% (4)	195-210
May	Recruited to adults		Recruited to adults		35% (57)	90-120	NA		40% (5)	175-210
June	Recruited to adults		Recruited to adults		37% (60)	100-140	NA		50% (2)	185-200
July	Recruited to adults		Recruited to adults		43% (60)	100-145	0% (17)	40-85	100 (1)	185
Aug.	Recruited to adults		Recruited to adults		35% (48)	115-140	4.3% (23)	65-105	ND	
Sept.	Recruited to adults		Recruited to adults		35% (43)	115-150	33% (48)	70-110	100 (1)	180
Oct.	Recruited to adults		Recruited to adults		17% (6)	135-160	5.1% (59)	80-120	ND	

Table 21. Prevalence of VEN in Pacific herring from locations other than Skagit Bay.

Location	Collection Date	Mean length (SD) mm	n	VEN Prevalence %
Prince William Sound	4/5/07	224 (17)	60	0
Prince William Sound	4/19/07	86 (6)	60	17
Puget Sound: Cherry Point	4/30/07	184 (13)	60	0
Puget Sound: Skunk Bay	7/2/07	131 (4)	170	1.8
Puget Sound: Admiralty Inlet	8/1/07	129 (5)	60	0
Puget Sound: Port Townsend Bay	10/16/07	80 (6)	75	20

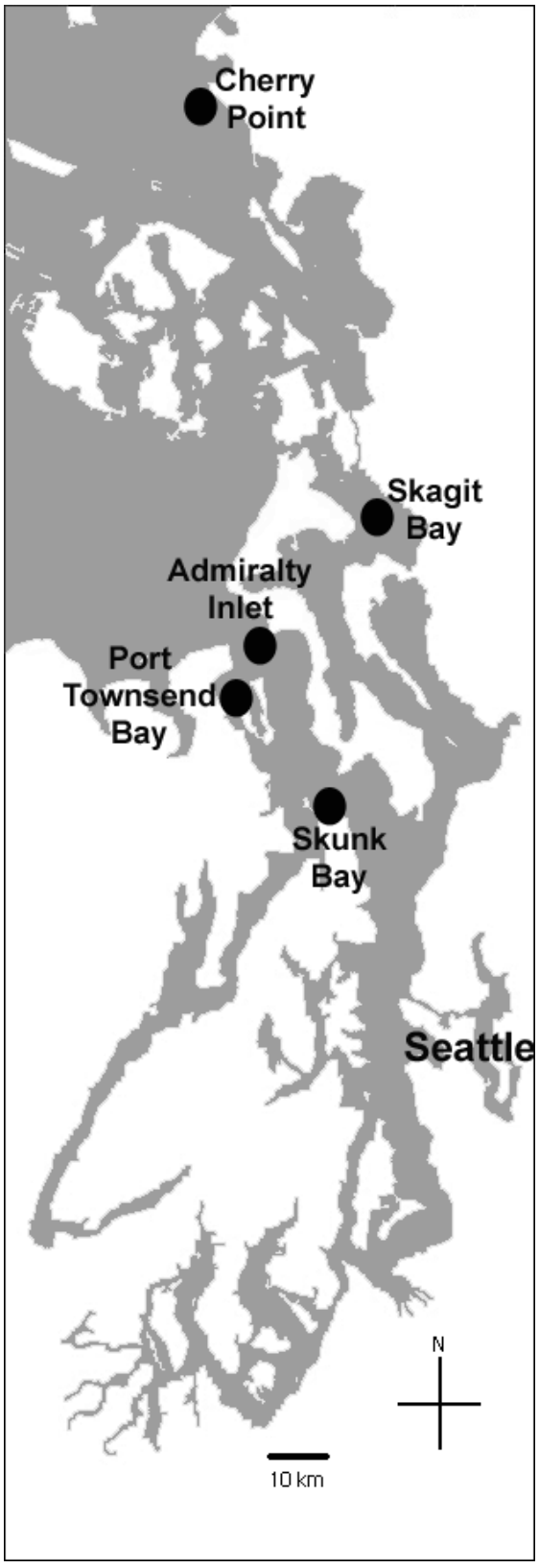


Figure 39. Sampling locations in Puget Sound, WA.

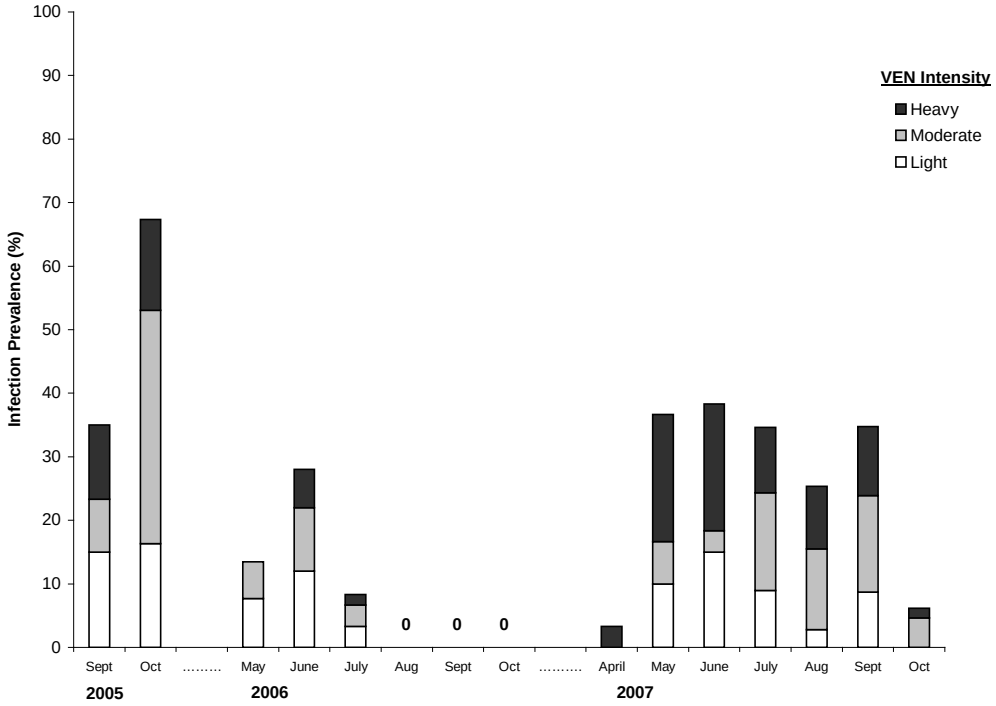


Figure 40. Temporal pattern in VEN prevalence and intensity among Skagit Bay herring, irrespective of age. Infection intensity was determined as the percent of erythrocytes demonstrating inclusions; light (1 - 33%), moderate (34 - 66%), or heavy (67 - 100%).

4.2 Kinetics of Viral Load and VEN Progression in Pacific Herring

To better understand the kinetics of ENV-infection, we followed the course of the disease after specific pathogen-free Pacific herring (*Clupea pallasii*) were exposed to the putative iridovirus and we demonstrated that the prevalence of erythrocytic inclusion bodies increased from 0% at 0-4 days post-exposure, to 94% after 28 days (Figures 41 & 42). The kinetics of viremia throughout this period were be evaluated by observation of virions in paired blood and spleen/kidney samples using transmission electron microscopy (Figures 43 & 44). Inclusion body concentration in circulating red blood cells (RBC) increased throughout the infection, while viral load with in circulating RBCs peaked around 2 weeks post exposure. However viral load in tissue-borne blood cells of the the kidney and spleen peaked between days 14 and 28, indicating the tissue with the highest viral concentration shifts during the course of the infection and that viral load does not correlate with inclusion body concentration. The results of this experiment described the relationship between viral load and cytoplasmic inclusion bodies during an ENV infection and provided information for the optimal timing in collection of blood samples containing the highest concentration of virus during artificial infections of Pacific herring.

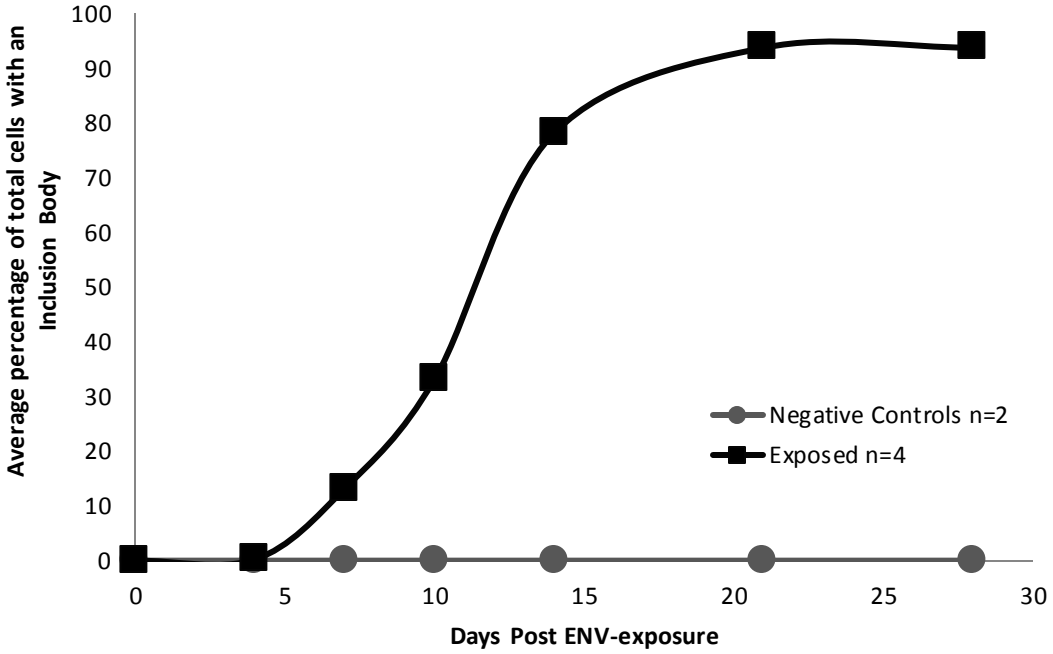


Figure 41. -Average percentages of erythroid cells with inclusion bodies (IB) observed on Geimsa-stained blood smears via light microscopy. Four exposed fish were sampled per time point (days: 1, 4, 7, 10, 14, 21, and 28). A single cell with an IB was first observed in 1 out of 4 fish sampled on day 4.

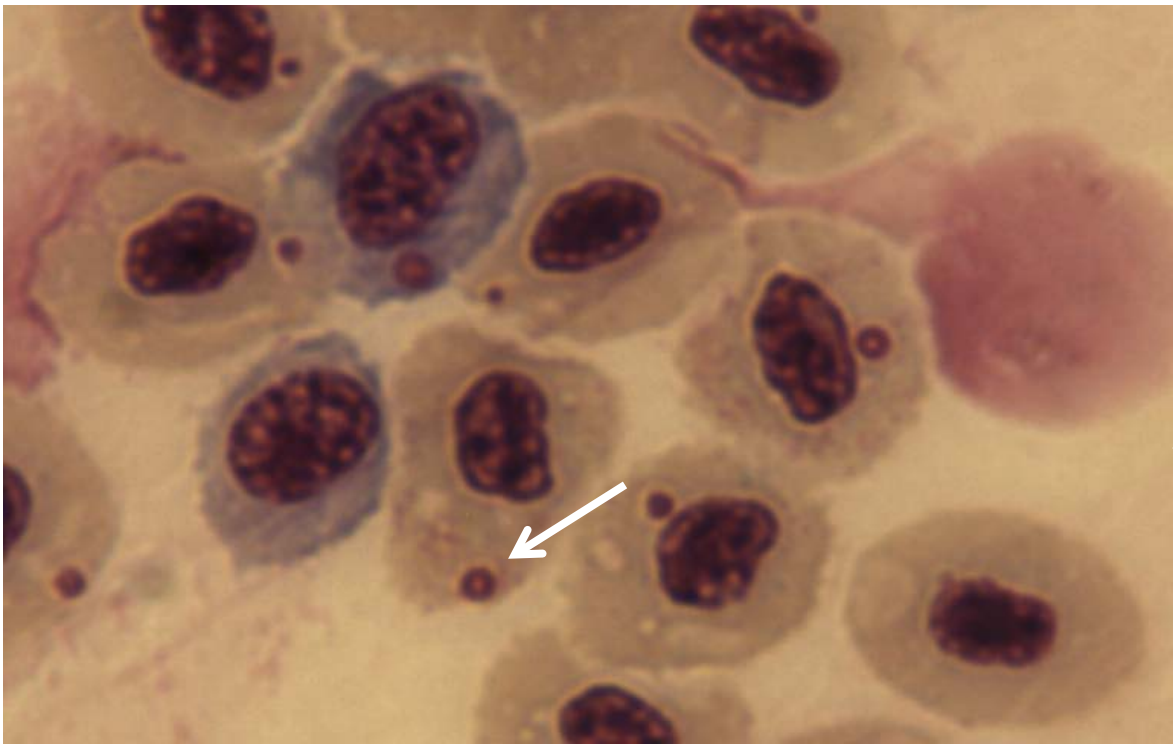


Figure 42. -Geimsa stain of erythrocytic necrosis virus (ENV) infected erythrocytes and erythroblasts. Cytoplasmic inclusion bodies in some of the infected cells are indicated by the arrow.

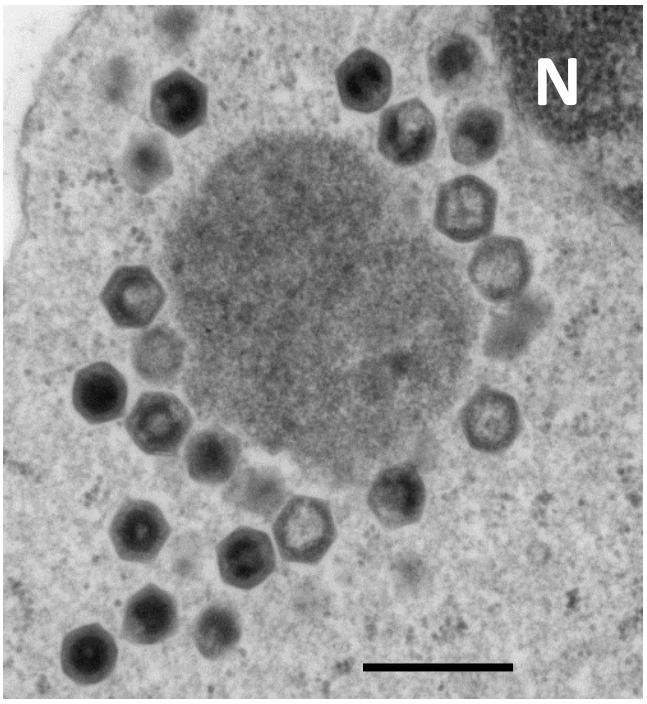


Figure 43. Electron micrograph of an ENV inclusion body (IB) surrounded by virions in the cytoplasm of a mature erythrocyte from a day 7 post-exposure blood sample. Virions are approximately 190nm in diameter. Nucleus (N) of the infected erythrocyte is present in the upper right corner. Bar represents 500nm.

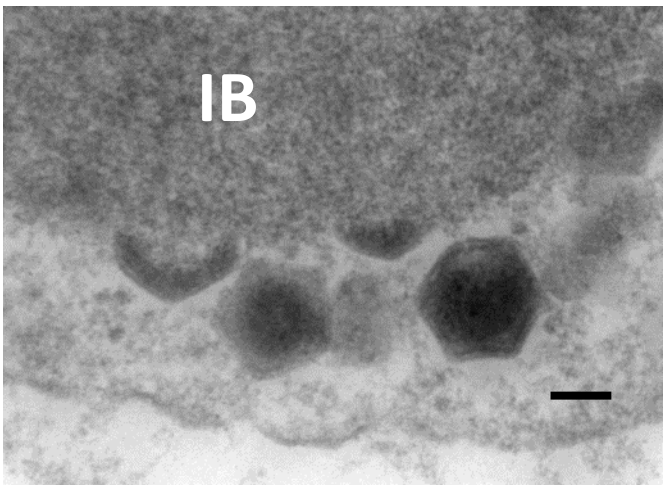


Figure 44. Electron micrograph of an ENV inclusion body (IB) with possible budding virions from a blood sample on day 7 post-exposure. Bar represents 100nm.

4.3 Development of a Confirmatory Diagnostic Test for ENV

Because of the intractability of ENV to established cell lines, confirmatory diagnostic tests for the presumed etiological agent are lacking; currently the condition is recognized by the detection of cytoplasmic inclusion bodies in the erythrocytes of affected fishes. Logistical difficulties inherent to working with fresh blood render this diagnostic test impractical for field surveillances. The objective of this study was to develop a confirmatory, molecular-based, diagnostic technique that was capable of detecting VEN in blood and tissue samples from infected fishes.

Initial strategies to characterize ENV included generating a set of nucleotide primers that could be used for PCR screening of blood and tissues from infected herring. We designed multiple primer sets that bind to conserved or degenerate viral gene sequences, in particular targeting fish iridoviruses. The Genebank sequences comparisons from the PCR sequence products we generated had no convincing alignments or homologs. A second strategy for molecular characterization included testing RNA or DNA extracted from pooled ENV-infected herring kidney and spleen samples on a ViroChip that contained sequence homologs to hundreds of virus families. This technique had successfully generated novel sequence fragments for a previously uncharacterized fish stickleback iridovirus (personal communication K. Garver Dept. of Fisheries & Oceans, British Columbia, Canada). No conclusive matches to any virus family were found for our ENV samples. Explanations for the failure of these initial attempts at molecular characterization likely involved low viral titers in the samples and / or background interference from host nucleic acid that interfered with the binding of viral DNA.

In order to increase the viral load and decrease host nucleic acid background we next employed a subtractive hybridization strategy. This technique used two blood samples from the same herring; one prior to infection (negative) and another that was collected after the herring was infected with ENV. The host nucleic acid was "subtracted" from the positive sample by hybridization and the target ENV cDNA sample remained. This cDNA was then cloned and sequenced. After removal of host nucleic acid, the target sample lacked sufficient concentrations of viral nucleic acids to successfully clone the cDNA. We also repeated the subtractive hybridization technique with ENV-infected kidney and spleen tissues, which also generated low concentrations of viral nucleic acid.

In an effort to generate higher titer virus samples we attempted *in vitro* propagation of ENV in a cell line developed from newly hatched Pacific herring larvae (PHL). However no cytopathic effect was observed when ENV-infected blood or tissue homogenates were cultured on the PHL cell line even after frequent blind-passes.

In lieu of an effective *in vitro* virus propagation model, ENV was propagated and concentrated *in vivo* by serial passage of infected blood through SPF Pacific herring. Using this technique, we obtained 27 mL of heavily infected herring blood. The pooled whole blood was then processed through a cesium chloride gradient to obtain the fraction expected to contain iridoviruses. This fraction was then submitted for parallel sequencing, a high throughput sequencing method that produces many small DNA fragments. We obtained authentic ENV genomic sequence fragments, which were used to develop a rapid diagnostic PCR assay.

PCR-assay Development

The suspected VEN raw sequence fragments that aligned with other iridoviruses from the lymphocystivirus and ranavirus genera were used to design a series of eight primer sets. After initial PCR screening, we selected four primer sets VEN 1, 2, 3, and 4, with base pair product sizes of 277, 265, 377, and 552 respectively, for further validation. DNA extracted from both VEN-negative and VEN-positive Pacific herring sampled from 2002 to 2009 were screened. Negative controls (ENV-negative, SPF herring) generated no PCR products with primer sets 1, 2, and 4, while primer set 3 produced some background banding patterns (Figure 45). PCR synthesis of the ENV-positive DNA samples consistently generated products of expected size for 5/7 samples screened using all four primer sets. PCR products were produced for the two remaining ENV-positive DNA samples with primer sets 1 and 4, but the bands had weaker intensity suggesting that these samples contained less viral DNA (Figure 45).

The VEN primer sets were also tested for specificity and sensitivity. DNA from six viruses (largemouth bass virus, infectious spleen and kidney necrosis virus, red sea bream virus, axolotl virus, lymphocystis disease virus, and rana catesbeiana virus), representing three different iridovirus genera (ranavirus, megalocytivirus, lymphocystivirus) infecting fishes, frogs, and salamanders, did not generate any PCR products when using the VEN primer sets (Figure 46). A ten-fold dilution series of 30.0 ng of total DNA extracted from VEN-positive samples indicated that the PCR detection limit was 1:1000 for VEN primers sets 1, 2, and 3. Primer set 4 had the highest sensitivity with PCR products still being generated at the 1:100,000 dilution (i.e. 0.00003 ng). This PCR assay provides a rapid method for screening wild Pacific herring for VEN; the tool is now considered ready for field implementation.

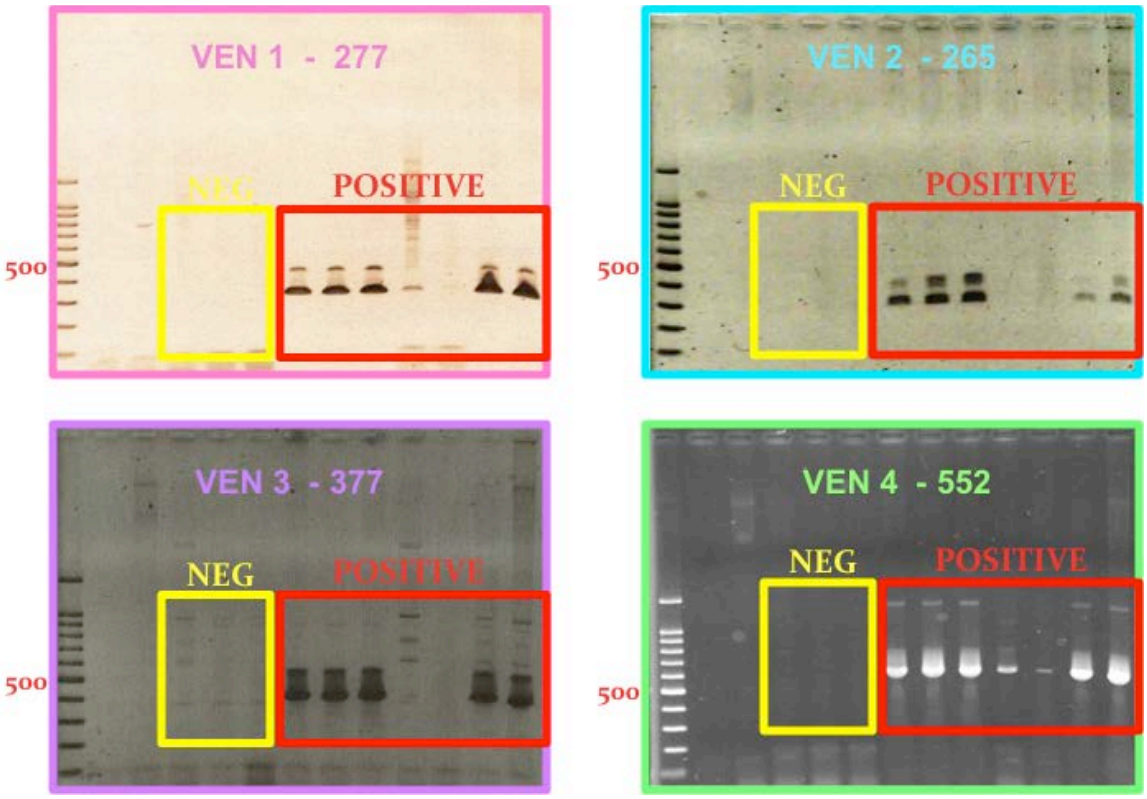


Figure 45. Testing the ENV diagnostic PCR assay with the four VEN primer sets with DNA extracted from VEN-negative and VEN-positive Pacific herring sampled from 2002 – 2009.

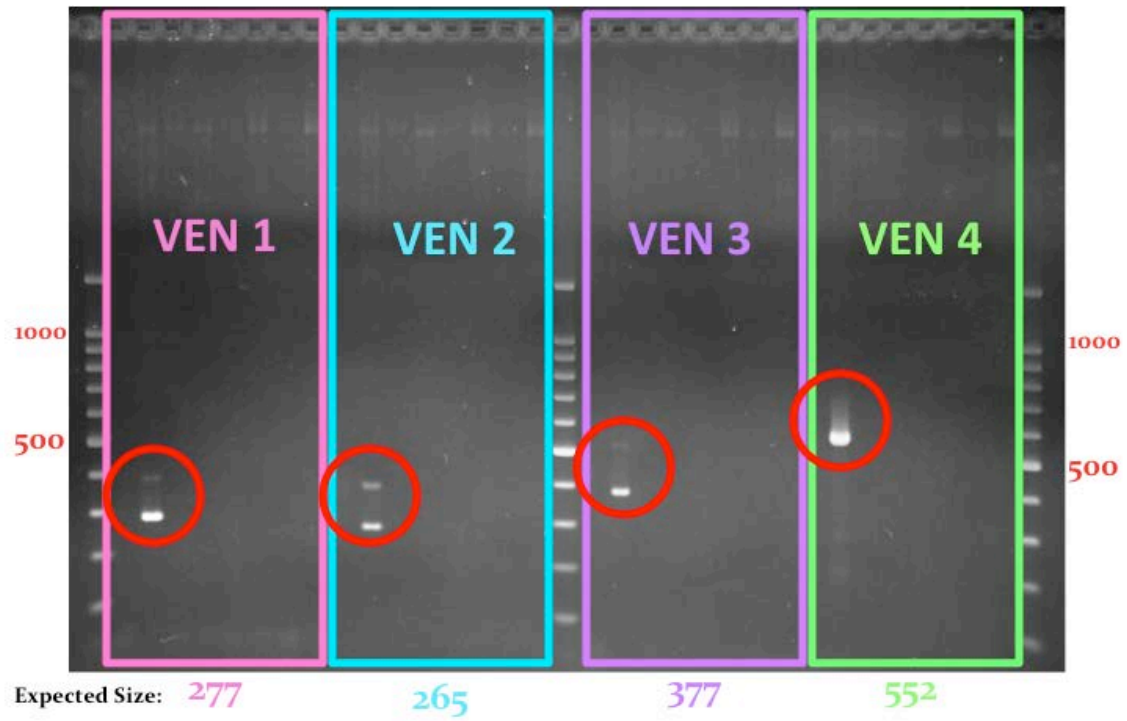


Figure 46. Specificity testing of ENV PCR assay with VEN primer sets 1, 2, 3, and 4 against six other iridoviruses and ENV. PCR products/bands of the expected size were only generated from VEN-positive DNA samples displayed within the red circles.

Chapter 5: Additional Empirical Studies

The availability of SPF herring and other resources provided by this project enabled us to address and respond to some additional research questions that arose throughout the course of this project. These additional studies were performed at no additional cost to EVOSTC Project #070819.

5.1 Effects of temperature on disease progression and swimming stamina in *Ichthyophonus*-infected rainbow trout (*Oncorhynchus mykiss*)

Although *Ichthyophonus* infections often results in acute mortality to susceptible hosts, some infected hosts survive as chronic carriers. Sublethal effects of the infection to these chonic carriers remain a focus of our investigations. Particularly, previous studies have indicated that *Ichthyophonus* infections in rainbow trout result in decreased swimming performance compared to uninfected cohorts (Kocan et al 2006). We were interested in expanding these swimming performance studies to the Pacific herring / *Ichthyophonus* model, but we encountered host behavioral issues that prevented reliable swimming of Pacific herring in the respirometer (swim chamber). Therefore, the Kocan et al (2006) results were expanded using the rainbow trout / *Ichthyophonus* model as a surrogate for Pacific herring. Specifically, we investigated the effects of temperature on the swimming performance of an *Ichthyophonus*-infected host.

Rainbow trout (*Oncorhynchus mykiss*) were infected with *Ichthyophonus sp*. and held at 10 °C, 15 °C and 20 °C for 28 days to monitor mortality and disease progression. Infected fish demonstrated more rapid onset of disease, higher parasite load, more severe host tissue reaction and reduced mean-day-to-death at 20 °C (Table 22). In a second experiment, *Ichthyophonus*-infected fish were reared at 15 °C for 16 weeks then subjected to forced swimming at 10 °C, 15 °C and 20 °C. Stamina improved significantly with increased temperature in uninfected fish; however, this was not observed for infected fish (Figures 47 & 48). The difference in performance between infected and uninfected fish became significant at 15 °C ($P = 0.02$) and highly significant at 20 °C ($P = 0.005$). These results demonstrate the effects of higher temperature on the progression and severity of ichthyophoniasis as well as on swimming stamina, a critical fitness trait of salmonids, and have implications for changes in the ecology of fish diseases in the face of global warming.

Table 22. Percent mortality and mean-day-to-death for groups of *Ichthyophonus*-infected rainbow trout reared at three temperatures. Fish were held at 15 °C for 7 days before moving groups of 40 infected and control fish to 10 °C, 15 °C and 20 °C for 28 days.

	Percent total mortality[1]			Mean-day-to-death[2]		
	10 °C	**15 °C**	**20 °C**	**10 °C**	**15 °C**	**20 °C**
Infected	35%	35%	15%	21.6	13.4	10.7
Control	0	0	0	0	0	0

[1] Percent mortality occurring during the 28 days that fish were held at each temperature.
[2] Calculation for mean-day-to-death includes rearing for an initial 7 days at 15 °C.

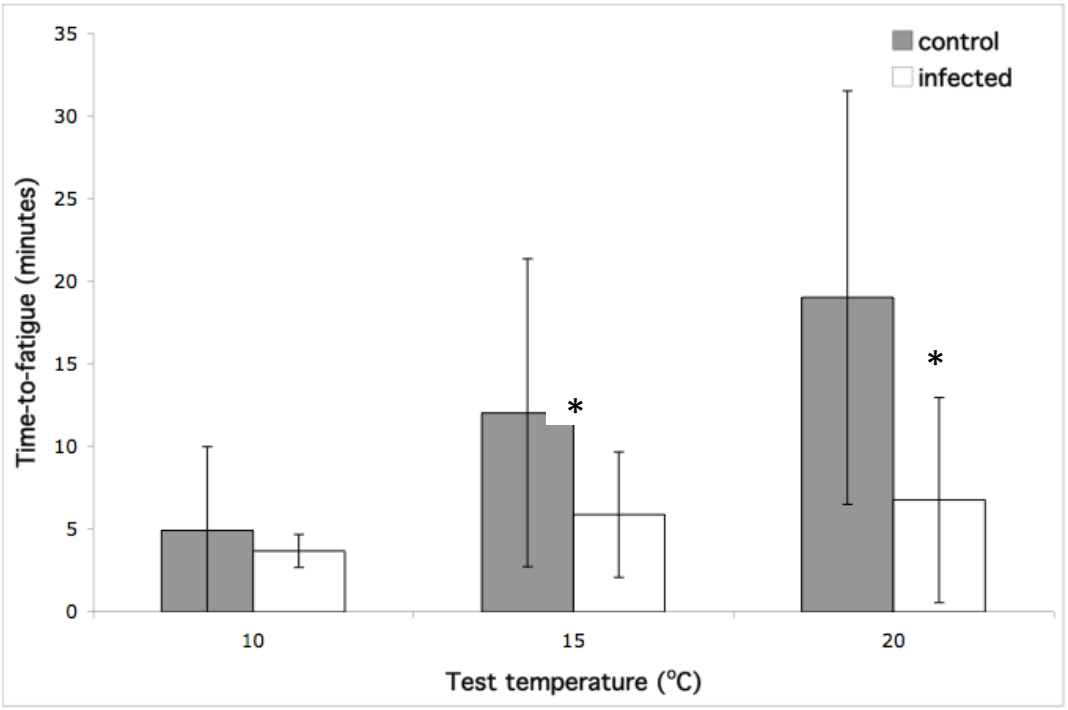


Figure 47. Effect of temperature on the swimming performance (time-to-fatigue) of *Ichthyophonus*-infected and uninfected rainbow trout. Increase in stamina significant (AOVA; $P = 0.007$) for controls but not for infected fish ($P = 0.26$). Bars = 1 SD of the mean, * = significant difference in performance between infected and control fish (paired t-Test).

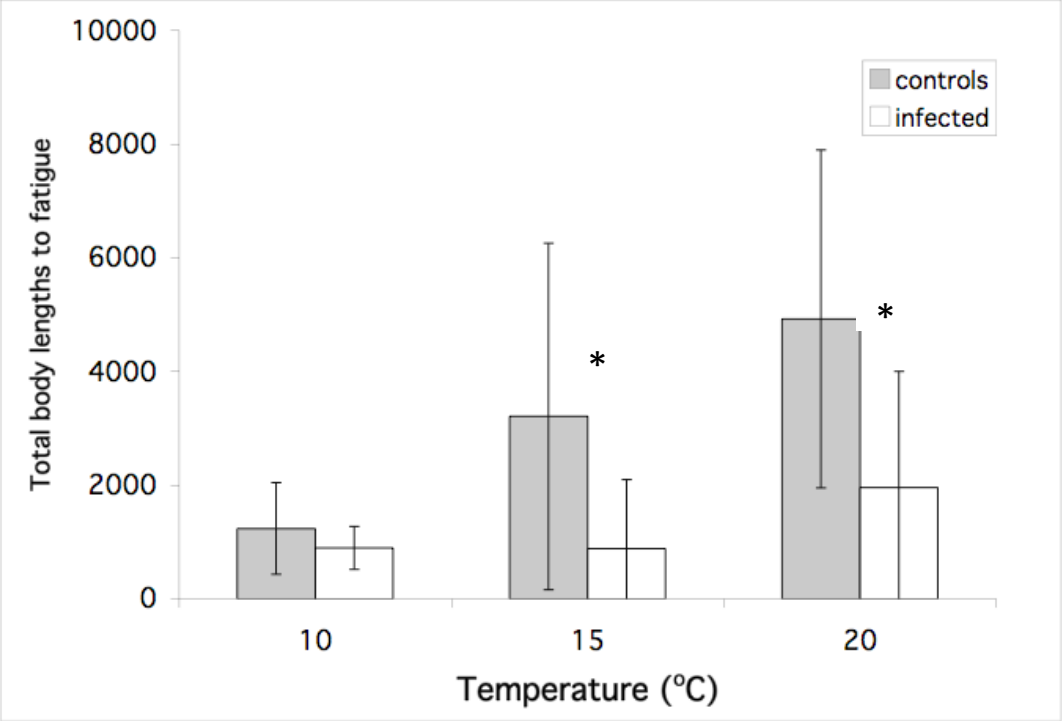


Figure 48. Effect of temperature on swimming performance of *Ichthyophonus*-infected and uninfected rainbow trout normalized for body length. Increase in stamina significant (AOVA; $P = 0.01$) for controls but not for infected fish ($P = 0.16$). Bars = 1 SD of the mean, * = significant difference between infected and control fish (paired t-Test).

5.2 Amplification and transport of an endemic fish disease (ichthyophoniasis) by an introduced Clupeid (American shad)

The introduction of American shad from the Atlantic to the Pacific coast of North America in the late 1800's and the subsequent population expansion in the 1980's resulted in the amplification of *Ichthyophonus*. Sequence analysis of the ribosomal DNA gene complex (small subunit and internal transcribed spacer regions; Table 23, Figure 49) and *Ichthyophonus* epidemiological characteristics indicate a low probability that *Ichthyophonus* was co-introduced with American shad from the Atlantic; rather, *Ichthyophonus* was likely endemic to marine areas of the Pacific region and amplified by the expanding population of a highly susceptible host species (Figure 50). The migratory life history of shad resulted in the transport of amplified *Ichthyophonus* from its endemic region in the NE Pacific to the Columbia River watershed. An *Ichthyophonus* epizootic occurred among American shad in the Columbia River during 2007, when infection prevalence was 72% (Table 24) and 57% of the infections were scored as moderate or heavy intensities. The epizootic occurred near the record peak of shad biomass in the Columbia River, and corresponded to an influx of 1,595 mt of infected shad tissues into the Columbia River

(Figure 51). A high potential for parasite spillback and the establishment of a freshwater *Ichthyophonus* life cycle in the Columbia River results from currently elevated infection pressures, broad host range, plasticity in *Ichthyophonus* life history stages, and precedents for establishment of the parasite in other freshwater systems. The results raise questions regarding the risk for sympatric salmonids and the role of *Ichthyophonus* as a population-limiting factor affecting American shad in the Columbia River.

Table 23. *Ichthyophonus* isolates from American shad including GenBank numbers and characteristics of the partial ITS rDNA gene region used for phylogenetic analysis.

Isolate ID	Location	Year	ITS region Genbank # (PCR clones[1])	# of PCR clones[1]	Size of ITS region (bp)	ITS intra-isolate variation[3]
IA6[2]	Puget Sound, WA	2005	GQ402843-GQ402854	12	631	5.1
IA52[2]	Columbia River, WA	2007	GQ402887-GQ402899	13	632	3.2
IA14[2]	Columbia River, WA	2005	GQ402860-GQ402871	12	632	4.3
IA65	Merrimack River, MA	2008	GU059890-GU059892 GU059901-GU059903	6	641	1.3
IA66	Merrimack River, MA	2008	GU059893-GU059900	8	643	1.2
IA67	Merrimack River, MA	2008	GU146052-GU146058	7	642	1.0

[1]A single band of the ITS rDNA gene regions (ITS-5.8s-ITS2) was PCR amplified, cloned and multiple PCR clones were sequenced per isolate.
[2]ITS sequencing of the Puget Sound and Columbia River isolates have been previously reported elsewhere (Rasmussen et al. (accepted).
[3]Average number of nucleotide substitutions using the partial ITS1, 5.8s and partial ITS2 sequence.

Table 24. Sampling details and *Ichthyophonus* prevalence. ND = No Data.

	Sampling Date	Sampling Location	Length, mm (mean ± SD)	Weight, g (mean ± SD)	*Ichthyophonus* prevalence
Shad life history stage					
Outmigrating YOY shad	Sept 13, 2007	Columbia R. watershed (RKM 235)	67 ± 7	3 ± 1	0% (0/60)
	Oct 15, 2007	Columbia R. watershed (RKM 235)	79 ± 8	5 ± 2	0% (0/60)
Atypical freshwater holdover juvenile shad	Oct 15-26, 2007	Columbia R. watershed (RKM 235)	212 ± 19	72 ± 20	0% (0/62)
Typical marine phase juvenile shad	May 14, 2004	Puget Sound, WA (Cherry Pt.)	200 ± 6	99 ± 12	60% (6/10)
	April 28, 2005	Puget Sound, WA (Hood Canal)	205 ± 24	ND	33% (2/6)
Pre-spawn adult shad[1]	June 8 – July 13, 2007	Columbia R. watershed (RKM 235)	390 ± 39	860 ± 282	72% (145/201)
Post-spawn adult shad	July 12 – Aug 8, 2007	Columbia R. watershed (RKM 235)	386 ± 29	557 ± 124	70% (102/145)
Effect of freshwater migration					
Adult, pre-spawn shad	July 10, 2007	Columbia R. watershed (RKM 493)	358 ± 27	ND	58% (35/60)
Interannual variability					
Adult, pre-spawn shad[2]	June 18 – July 10, 2008	Columbia R. watershed (RKM 235)	ND	ND	49% (28/57)
Adult, pre-spawn shad[2]	June 2 – 25, 2009	Columbia R. watershed (RKM 235)	ND	ND	37% (22/60)
Sympatric species					
Smallmouth bass	June 30, 2007	Columbia R. watershed (RKM 235)	277 ± 49	376 ± 232	0% (0/14)
Northern pikeminnow	July 23-25, 2007	Columbia R. watershed (RKM 293)	343 ± 47	515 ± 241	0% (0/73)
White sturgeon	June 19, 2007	Columbia R. watershed (RKM 54)	ND[3]	ND	0% (0/12)
Adult Spring Chinook[2]	Aug 15-22, 2007	Columbia R. watershed (RKM 243)	780 ± 81	ND	4% (4/90)
Adult Fall Chinook[2]	Sept 17-24, 2007	Columbia R. watershed (RKM 266)	791 ± 136	ND	0% (0/101)

[1]Group intended for direct comparisons with the 'effect of freshwater migration" and "interannual variability."
[2]Fish tissues processed for virological and bacteriological analysis.
[3]Lengths were not recorded from the white sturgeon; however, all were sampled from the recreational fishery and fell within the legal slot harvest size of 966 – 1372 mm.

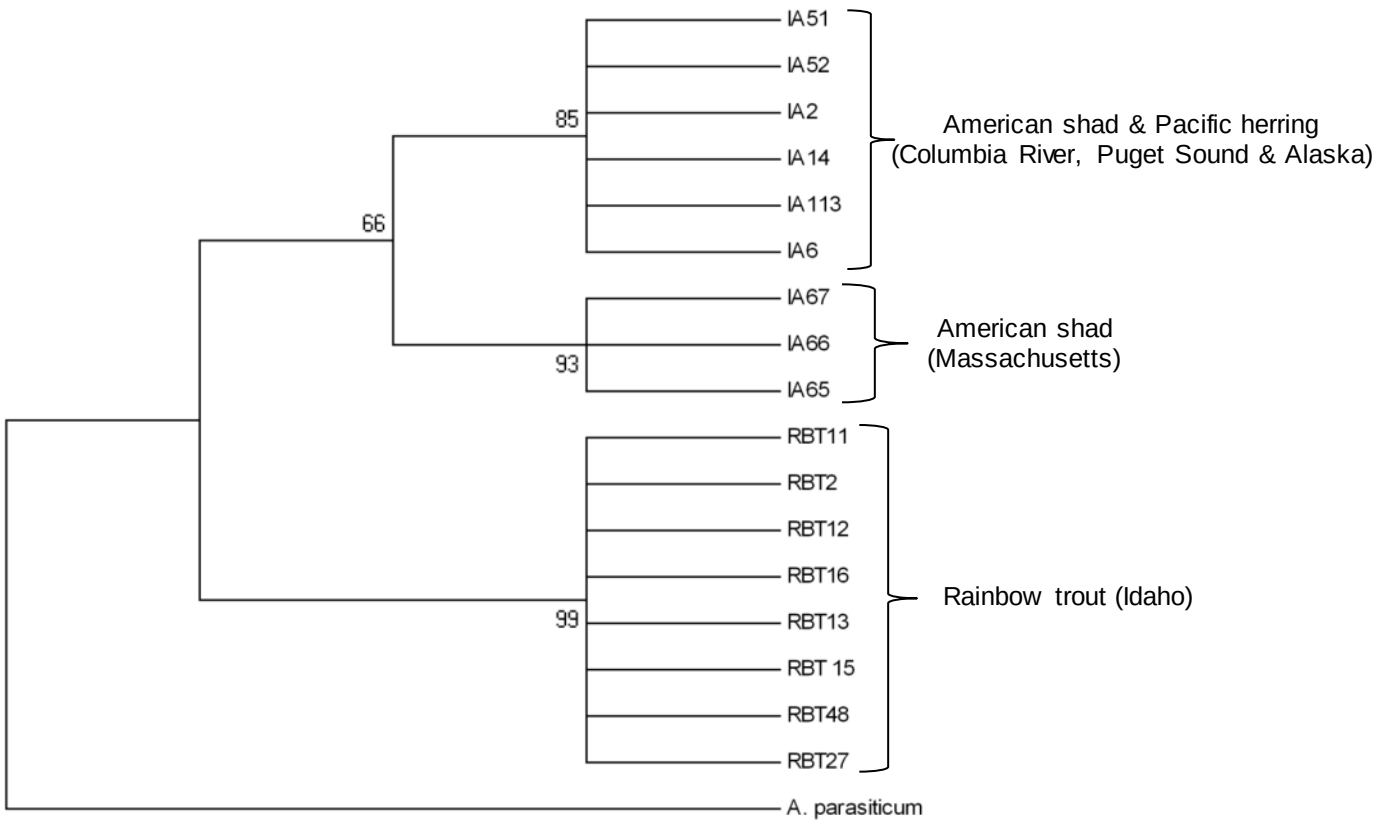


Figure 49. Relationship of *Ichthyophonus* isolates based on the majority consensus sequence of the ITS region (partial ITS1, 5.8s and partial ITS2) inferred using the neighbor-joining method (distances calculated using Kimura 2 parameter method). Similar tree topology was obtained using the maximum parsimony method. Isolates are derived from American shad from Merrimack River, MA (IA65, IA66, IA67; see Table 2) and compared to sequences previously reported by Rasmussen et al. (2010).

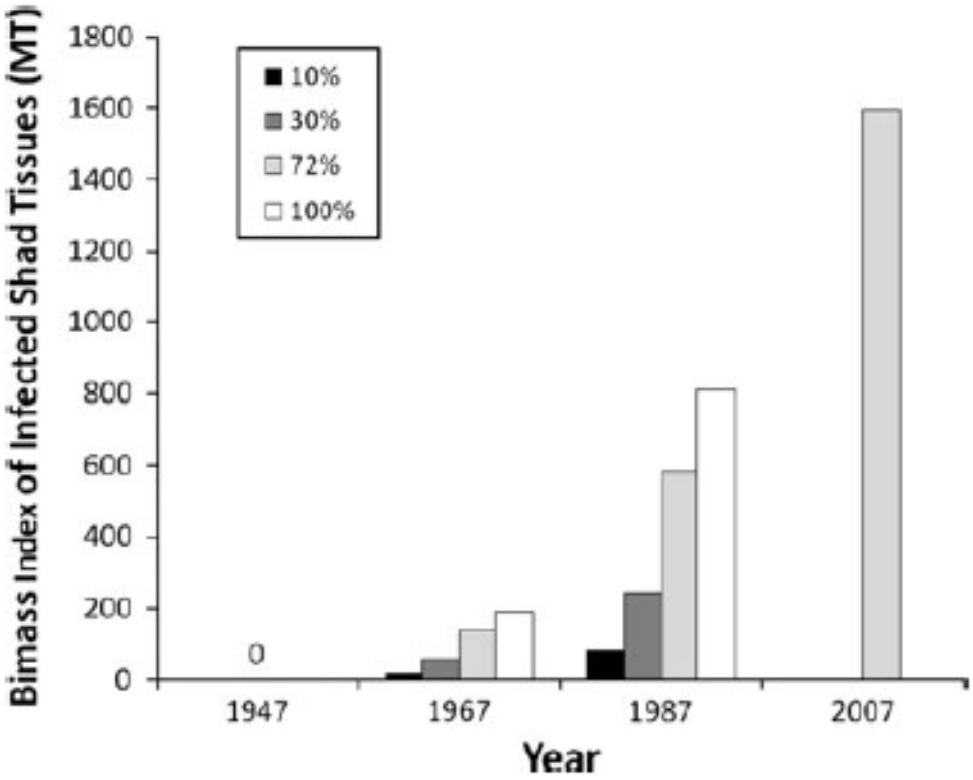


Figure 50. Emergence of *Ichthyophonus* in the Columbia River, indicated by the temporal increase in Biomass Index of infected shad tissues at the Bonneville Dam fish ladder (RKM 235). Because *Ichthyophonus* prevalence values prior to 2007 are unknown, the historical biomass index (prior to 2007) was calculated based on a range (10-100%) of presumed prevalences (indicated in the legend).

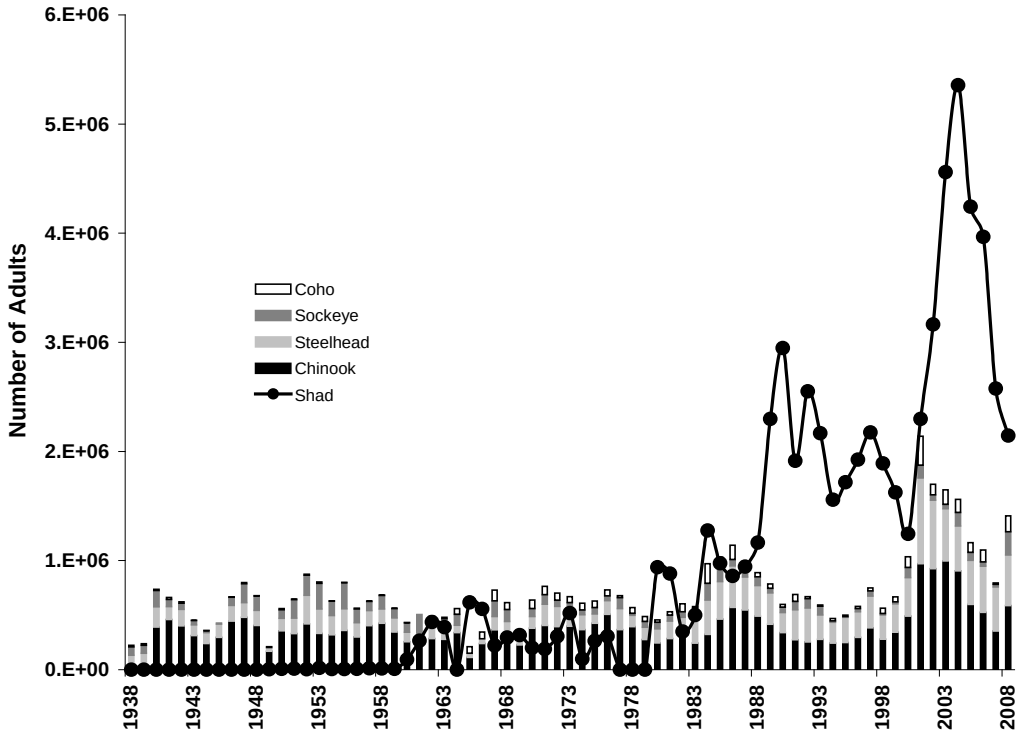


Figure 51. Adult anadromous fish counts at the Bonneville Dam fish ladder (RKM 235). Data from Columbia River Data Access in Real Time (DART; http://www.cbr.washington.edu/cgi-bin/dart/dart?report=adult_annual_rpt&format=standard&year=2001&access_time=Monday%2C+10-Aug-2009+16%3A37%3A58+PDT&proj=BON&run=+&startdate=1%2F1&enddate=12%2F31).

5.3 Pacific herring are not susceptible to Infectious Hematopoietic Necrosis

Infectious Hematopoietic Necrosis Virus (IHNV) is a rhabdovirus, endemic to salmonids throughout the NE Pacific, that is closely related to VHSV. IHNV is a recurring problem in Atlantic salmon net pens in British Columbia and in wild sockeye populations throught the NE Pacific. Because of geographical range overlaps and phylogenetic relatedness with VHSV, questions regarding the possible role(s) of Pacific herring as a natural reservoir for IHNV have persisted for decades. In this study we investigated the susceptibility of Pacific herring to IHNV.

Specific pathogen-free (SPF) larval and juvenile Pacific herring *Clupea pallasii* were not susceptible to infection by infectious hematopoietic necrosis virus (IHNV) by waterborne immersion in 10^{3-4} plaque forming units (pfu) of virus mL^{-1}. Cumulative mortalities among exposed groups (Table 25) were not significantly different from those of negative control groups (Figure 52). After waterborne exposure, IHNV was transiently recovered from the tissues of larvae but absent in tissues of juveniles (Table 26). Additionally, no evidence of viral shedding was detected in the tank water containing exposed juveniles. After intraperitoneal (IP) injection of IHNV in juvenile herring with 10^3 pfu, IHNV was recovered from the tissues of sub-sampled individuals for only the first 5d post-exposure (Table 27). The lack of susceptibility to overt disease and transient levels of IHNV in the tissues of exposed fish indicate that Pacific herring do not likely serve a major epizootiological role in perpetuation of IHNV among free-ranging sockeye salmon *Oncorhynchus nerka* and farmed Atlantic salmon *Salmo salar* in the NE Pacific.

Table 25. Mean exposure titer and sample size N (number of fish) per tank for Pacific herring treated with IHNV or MEM (negative control) in herring susceptibility experiments. For each treatment there were 5 replicate tanks. pfu: plaque forming units.

Age	9 day		57 day		63 day		1 year	
	IHNV	**Control**	**IHNV**	**Control**	**IHNV**	**Control**	**IHNV**	**Control**
Water titer (To) pfu mL^{-1} ± SD	$2.4x10^3$	0	$4.0x10^3$	0	$3.5x10^3$	0	$2.3x10^3$	0
N $tank^{-1}$	332 - 537	290 - 506	97 - 140	76 - 124	8 - 15	3 - 9	33 - 36	32 - 35

Table 26. IHNV in the tissues (whole bodies) of larval and juvenile Pacific herring that were exposed to IHNV or MEM (negative control) by immersion challenge.

IHNV EXPOSED MORTALITIES												
Age	**9 day larvae**			**57 day larvae**			**63 day larvae**			**1+ year juveniles**		
Days after waterborne exposure	Inclusive Number of Fish	pooled samples	IHNV positive	Inclusive Number of Fish	pooled samples	IHNV positive	Inclusive Number of Fish	pooled samples	IHNV positive	Inclusive Number of Fish	pooled samples	IHNV positive
1	112	5	0	62	60	0	4	4	0			
2	106	6	0	18	18	0						
3	59	5	0	23	23	0						
4	61	5	0	18	18	0						
5	74	5	1	28	28	4	3	3	0			
6	41	5	3	17	16	3	1	1	1			
7	33	5	2	9	9	0	1	1	0			
8	34	5	1	14	12	7						
9	25	5	1	12	12	0						
10	30	5	3	12	11	0	2	2	0			
11	8	8	2	7	7	7						
12	25	5	2	12	12	0	2	2	0			
13	20	7	5	10	10	0						
14	58	5	2	5	5	0	1	1	0			
15	34	4	0	13	13	0	1	1	0			
16	59	5	2	5	5	0						
17	62	5	2	11	11	0	1	1	0			
18	42	5	1	6	6	0						
19												
20	55	5	2	10	10	0	2	2	0			
21	31	5	1	9	9	0				2	2	0
IHNV EXPOSED SURVIVORS												
21	560	20	4	45	45	0	22	22	0	172	40	0
LIVE SUBSAMPLED NEGATIVE CONTROLS												
2	87	7	0	33	13	0	1	1	0	1	1	0
15	23	5	0	5	5	0						
18	46	5	0	2	2	0						
20	69	7	0	12	12	0	1	1	0	1	1	0

Table 27. IHNV titers in the tissues (kidney-spleen and flank) of age 1+ yr juvenile herring injected with IHNV or PBS (negative control) and sub-sampled on four separate days post-injection. pfu: Plaque forming units.

IHNV INJECTED				
Day post-injection	**Number of fish tested**	**Number of fish IHNV positive**	**IHNV titer in kidney-spleen pools (pfu g^{-1})**	**IHNV titer in flank (pfu g^{-1})**
3	5	4	$8.8x10^{4}$	$4.8x10^{3}$
			$4x10^{7}$	$1.5x10^{5}$
			$4x10^{2}$	0
			$2.4x10^{3}$	0
5	5	3	0	$3.7x10^{4}$
			$1x10^{5}$	$8.0x10^{4}$
			0	$1.6x10^{4}$
12	5	0	0	0
21	1	0	0	0
NEGATIVE CONTROLS				
3	3	0	0	0
5	5	0	0	0
12	3	0	0	0
21	2	0	0	0

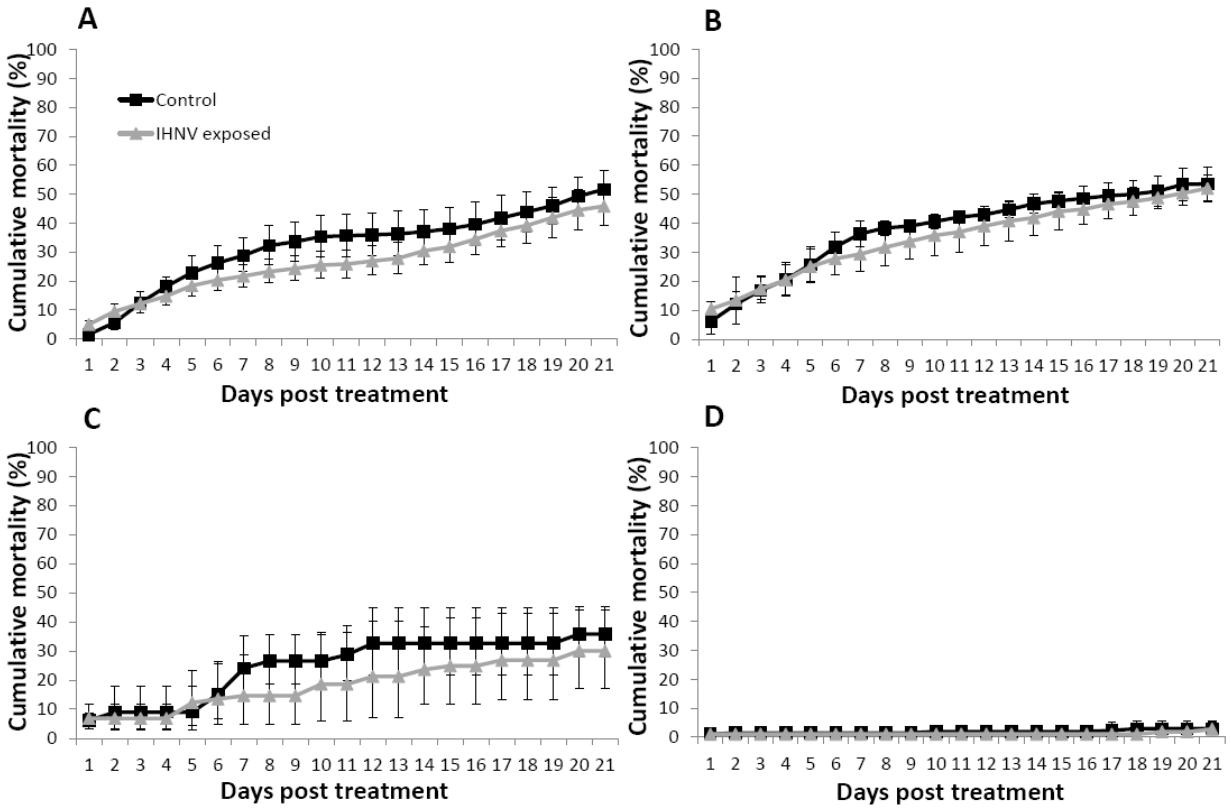


Figure 52. Susceptibility of Pacific herring to IHNV. A. Age 9d larvae. B. Age 56d larvae. C. Age 63d larvae. D. Age 1+ yr juveniles. Percent mortality represents the back-transformed mean of the transformed data from the five replicates for each group. Error bars represent one standard deviation from the mean.

5.4 Pacific herring will accept surgically-inserted acoustic tags

Information gaps in our understanding of Pacific herring residence and migration patterns throughout the NE Pacific have been limited in part by our inability to track individuals and populations with acoustic tags. For example, a prevailing belief that Pacific herring are 'delicate' and too sensitive to survive surgical implantation of acoustic tags has resulted in resistance to apply modern tagging technologies to Pacific herring. In this study, we examined the feasibility of implanting acoustic tags in adult Pacific herring as a method of monitoring their movements. Two sizes of dummy tags were surgically implanted (9 mm diam x 21 mm length, 1.6 g, n=50) and (7 mm diam x 18 mm length, 0.7 g, n=50) in adult Pacific herring ranging from 165 to 215 mm FL and 41.6 to 142.6 g (Tables 28 & 29). After 135 days, the fish in this study experienced relatively low mortality (4%) and tag shedding (4%) rates (Table 30). Furthermore, their growth was not significantly different than those in two control groups. Pacific herring appear to be amenable to acoustic tag implantation, given careful handling and surgical procedures.

Table 28. Pacific herring lengths and weights in the four treatment groups on the first day of the acoustic transmitter implantation experiment. There were no significant differences in mean lengths or weights among the four treatment groups.

		Fork length (mm)		Weight (g)	
Treatment	**n**	**range**	**mean ± SD**	**range**	**mean ± SD**
Anesthesia	25	175–210	194 ± 8.7	71.7–124.2	94.7 ± 12.58
Incision and anesthesia	24	165–211	192 ± 11.0	60.1–117.4	91.5 ± 16.55
Vemco V7-1L dummy tag	50	168–215	193 ± 10.1	41.6–128.8	93.4 ± 17.78
Vemco V9-6L dummy tag	50	169–215	194 ± 8.3	55.6–142.6	95.1 ± 14.49
All treatments	149	165–215	194 ± 9.4	41.6–142.6	93.8 ± 15.60

Table 29. Pacific herring lengths and weights in the four treatment groups on the last day of the acoustic transmitter implantation experiment. There were no significant differences in mean lengths or weights among the four treatment groups.

		Fork length (mm)		Weight (g)	
Treatment	**n**	**range**	**mean ± SD**	**range**	**mean ± SD**
No incision evident	27	182–212	198 ± 7.6	77.6–123.7	103.2 ± 14.44
Incision evident, no tag	26	166–217	195 ± 12.3	54.1–128.1	96.6 ± 19.51
Vemco V7-1L dummy tag	47	180–217	198 ± 8.7	55.2–141.0	100.4 ± 16.83
Vemco V9-6L dummy tag	45	181–220	197 ± 7.3	69.7–155.1	100.7 ± 15.23
All treatments	145	166–220	197 ± 1.4	54.1–155.1	100.2 ± 2.72

Table 30. Mortality and acoustic tag shedding events by Pacific herring in the acoustic transmitter implantation experiment. Vemco V7-1L dummy tag mortalities occurred 10 and 25 days post-surgery while the extruded tag was found on the bottom of the holding tank 39 days post-surgery. Vemco V9-6L dummy tag mortalities occurred 9 and 15 days post-surgery while extruded tags were found on the bottom of the holding tank 51 (n=2) and 53 days post-surgery.

Treatment	Sample Size	Mortalities	Extrusions
Anesthesia	25	0	NA
Incision and anesthesia	24	0	NA
Vemco V7-1L dummy tag	50	2	1
Vemco V9-6L dummy tag	50	2	3

List of Scientific Products:

Peer reviewed scientific publications resulting from this project include:

Hershberger, P.K., J.L. Gregg, C.A. Grady, S.E. LaPatra, and J.R. Winton. *In Press*. Passive immunization of Pacific herring *Clupea pallasii* against viral hemorrhagic septicemia. Journal of Aquatic Animal Health.

Kocan R., H. Dolan, and P. Hershberger. 2011. Diagnostic methodology is critical for accurately determining the prevalence of *Ichthyophonus* infections in wild fish populations. Journal of Parasitology 97: 344-348.

Gregg J., J. Vollenweider, C. Grady, R. Heintz, and P. Hershberger. 2011. Effects of environmental temperature on the dynamics of ichthyophoniasis in juvenile Pacific herring (*Clupea pallasii*). Journal of Parasitology Research, vol.2011, Article ID 563412, 9pp. doi: 10.1155/2011/563412.

Vollenweider, J.J., J.L. Gregg, R.A. Heintz, and P.K. Hershberger. 2011. Energetic cost of *Ichthyophonus* infection in juvenile Pacific herring (*Clupea pallasii*). Journal of Parasitology Research, vol. 2011, Article ID 926812, 10 pp. doi:1155/2011/926812.

Hart L., G.S. Traxler, K.A. Garver, J. Richard, J.L. Gregg, C.A. Grady, G. Kurath, and P.K. Hershberger. 2011. Larval and juvenile Pacific herring *Clupea pallasii* are not susceptible to infectious hematopoietic necrosis under laboratory conditions. Diseases of Aquatic Organisms 93: 105-110.

Grady, C.A., J.L. Gregg, R.M. Collins, and P.K. Hershberger. 2011. Viral Replication in Excised Fin Tissues (VREFT) corresponds with prior exposure of Pacific herring, *Clupea pallasii* (Valenciennes), to *viral haemorrhagic septicaemia virus* (VHSV). Journal of Fish Diseases 34: 34-12.

Hershberger P.K., J.L. Gregg, C.A. Grady, L. Taylor, and J.R. Winton. 2010. Chronic and persistent viral hemorrhagic septicemia virus infections in Pacific herring. Diseases of Aquatic Organisms 93: 43-49.

Hershberger, P.K., B.K. van der Leeuw, J.L. Gregg, C.A. Grady, K. Lujan, S. Gutenberger, M. Purcell, J.C. Woodson, J.R. Winton, and M. Parsley. 2010. Amplification and transport of an endemic fish disease by an invasive species. Biological Invasions 12: 3665-3675.

Seitz, A.C., B.L. Norcross, J.C. Payne, A. Kagley, B. Meloy, J.L. Gregg, and P.K. Hershberger. 2010. Feasibility of Surgically Implanting Acoustic Tags in Pacific Herring. Transactions of the American Fisheries Society 139: 1288-1291.

Kocan, R.M. , J.L. Gregg, and P.K. Hershberger. 2010. Release of infectious cells from epidermal ulcers in *Ichthyophonus* sp. – infected Pacific herring (*Clupea pallasii*): evidence for multiple mechanisms of transmission. Journal of Parasitology 96: 348-352.

Hershberger P.K., J.L. Gregg, C.A. Grady, R.M. Collins, and J.R. Winton. 2010. Susceptibility of three stocks of Pacific herring to viral hemorrhagic septicemia. Journal of Aquatic Animal Health 22: 1-7.

Hershberger P., J. Gregg, C. Grady, R. Collins, and J. Winton. 2010. Kinetics of viral shedding provide insights into the epidemiology of viral hemorrhagic septicemia in Pacific herring. Marine Ecology Progress Series 400: 187-193.

Rasmussen, C., M.K. Purcell, J.L. Gregg, S.E. LaPatra, J.R. Winton, and P.K. Hershberger. 2010. Sequencing of the internal transcribed spacer (ITS) region reveals a novel clade of *Ichthyophonus* sp. from rainbow trout. Diseases of Aquatic Organisms 89: 179-183.

Kocan, R., P. Hershberger, G. Sanders, and J. Winton. 2009. Effects of temperature on disease progression and swimming stamina in *Ichthyophonus*-infected rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 32: 835-843.

Hershberger, P.K., N.E. Elder, C.A. Grady, J.L. Gregg, C.A. Pacheco, C. Greene, C. Rice, and T.R. Meyers. 2009. Recurring viral erythrocytic necrosis (VEN) in juvenile Pacific herring from Puget Sound, WA, USA. Journal of Aquatic Animal Health 29: 1-7.

Hershberger, P.K., C.A. Pacheco, and J.L. Gregg. 2008. Inactivation of *Ichthyophonus* Spores Using Sodium Hypochlorite and Polyvinyl Pyrrolidone Iodine (PVPI). Journal of Fish Diseases 31: 853-858.

Hershberger, P.K., C.A. Pacheco, J.L. Gregg, M. Purcell, and S.E. LaPatra. 2008. Differential survival of *Ichthyophonus* isolates indicates parasite adaptation to its host environment. Journal of Parasitology 94: 1055-1059.

LaPatra, S., R. Kocan, and P. Hershberger. 2008. Potential for cross-contamination of *in vitro* explant cultures initiated from *Ichthyophonus* - infected rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 31: 317-320.

Hershberger, P.K., C.A. Pacheco, and J.L. Gregg. 2008. Inactivation of *Ichthyophonus* Spores Using Sodium Hypochlorite and Polyvinyl Pyrrolidone Iodine (PVPI). Journal of Fish Diseases 31: 853-858.

Hershberger, P.K., C.A. Pacheco, J.L. Gregg, M. Purcell, and S.E. LaPatra. 2008. Differential survival of *Ichthyophonus* isolates indicates parasite adaptation to its host environment. Journal of Parasitology 94: 1055-1059.

LaPatra, S., R. Kocan, and P. Hershberger. 2008. Potential for cross-contamination of *in vitro* explant cultures initiated from *Ichthyophonus* - infected rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 31: 317-320.

Hershberger P.K., J. Gregg, C. Pacheco, J. Winton, J. Richard, and G. Traxler. 2007. Larval Pacific herring, *Clupea pallasii* (Valenciennes), are highly susceptible to viral hemorrhagic septicemia and survivors are partially protected after their metamorphosis to juveniles. Journal of Fish Diseases 30: 445-458.

Professional presentations resulting from this project include:

Invited Seminars:

2010: School of Aquatic and Fishery Sciences, University of Washington
Guest Lecture and Facility Tour "What can we do about Diseases in Wild Marine Fishes?"

2010: Peninsula College: Fisheries Technology Program
Guest lecture. "Influences of the Physical Environment on Diseases in Wild Fish Populations."

2010: Herring Conservation and Research Society
Invited Seminar and Workshop Leader "Herring Diseases in the NE Pacific; Implications for Fisheries Management."

2009: School of Aquatic and Fishery Sciences, University of Washington
Invited Seminar "Ecology of Diseases in Wild Fish Populations."

2009: School of Microbiology, Oregon State University: MB499.
Guest Lecture "Ecology of Disease in Wild Fish Populations."

2008: School of Aquatic and Fishery Sciences, University of Washington: FISH 404.
Guest Lecture and Facility Tour "Disease Ecology Research at the Marrowstone Marine Station."

2008: Bodega Bay Marine Laboratories, University of California, Davis.
Invited Seminar: "Ecology of Disease in Marine Fishes from the Eastern North Pacific."
2007: WA State Seminar Series (Olympia, WA)
Invited seminar: "Disease Ecology in Puget Sound Fishes."

Presentations at Scientific Meetings

Vollenweider, J.J., J. Gregg, R.A. Heintz, and P.K. Hershberger. September 13-16, 2010. *Platform*. Impaired compensatory growth following disease exposure in fasting herring. American Fisheries Society 140th Annual Meeting. Pittsburgh, PA.

Hershberger, P.K., J.L. Gregg, M.K. Purcell, C.A. Grady, J.C. Woodson, and J.R. Winton. September 5-9, 2010. Platform. Development of tools to forecast the potential for viral hemorrhagic septicemia epizootics in wild fish populations. Sixth International Symposium on Aquatic Animal Health. Tampa, FL. (Presented).

Gregg, J.L., J.J. Vollenweider, C.A. Grady, R.A. Heintz, and P.K. Hershberger. September 5-9, 2010. Effects of environmental temperature on the kinetics of ichthyophoniasis in juvenile Pacific herring (*Clupea pallasii*). Sixth International Symposium on Aquatic Animal Health. Tampa, FL.

Emmenegger, E.J., J.A. Glenn, W.N. Batts, C.A. Grady, J.L. Gregg, S.E. Roon, J.R. Winton, and P.K. Hershberger. September 5-9, 2010. Platform. Molecular characterization and infection kinetics of erythrocytic necrosis virus (ENV) in Pacific herring. Sixth International Symposium on Aquatic Animal Health. Tampa, FL.

Hershberger, P.K., J.L. Gregg, C.A. Grady, L. Taylor, and J.R. Winton. June 22-24, 2010. Platform. Chronic and persistent viral hemorrhagic septicemia virus infections in Pacific herring *Clupea pallasii*. Western Fish Disease Workshop. Corvallis, OR. (Presented).

Hershberger, P.K., J.L. Gregg, C.A. Grady, L. Taylor, S.E. Roon, and J.R. Winton. June 22-24, 2010. Platform. Low doses of viral hemorrhagic septicemia virus (VHSV) are infectious for Pacific herring *Clupea pallasii*. Western Fish Disease Workshop. Corvallis, OR.

VanderPol, J.A., J.L. Gregg, C.A. Grady, S. Roon, J.R. Winton, E.J. Emmenegger, and P.K. Hershberger. June 22-24, 2010. Platform . Kinetics of viral load and erythrocytic inclusion body formation among Pacific herring with viral erythrocytic necrosis (VEN). Western Fish Disease Workshop. Corvallis, OR.

Hart, L.M., G.S. Traxler, K.A. Garver, J. Richard, J.L. Gregg, C.A. Grady, G. Kurath, and P.K. Hershberger. June 22-24, 2010. Platform. Susceptibility of larval and juvenile Pacific herring *Clupea pallasii* to infectious hematopoietic necrosis virus. Western Fish Disease Workshop. Corvallis, OR.

Woodson, J.C., M.K. Purcell, S.M. Badil, C.A. Grady, J.L. Gregg, J.D. Hansen, E.S. Bromage, and P.K. Hershberger. June 22-24, 2010. Platform. Development of immunological tools for the study of disease in Pacific herring *Clupea pallasii*. Western Fish Disease Workshop. Corvallis, OR.

Hershberger, P.K., C. Rasmussen, M.K. Purcell, J.L. Gregg, S.E. LaPatra, and J.R. Winton. March, 2010. Poster. Genetic Characterization of the Internal Transcribed Spacer (ITS) Region Reveals Two Major Genetic Lineages of *Ichthyophonus hoferi*. USGS – Genomics and Genomics Symposium. Reston, VA.

Hershberger, P.K., C. Grady, J. Gregg, R. Collins, and J.R. Winton. January 18-22, 2010. Poster. Herring fin explant cultures provide a reliable indication of host exposure history

to viral hemorrhagic septicemia virus. Alaska Marine Science Symposium. Anchorage, AK. (Presented).

Cox, M.K., J.J. Vollenweider, R.A. Heintz, and P.K. Hershberger. January 18-22, 2010. Poster. Bioenergetic models for Pacific herring; create or borrow. Alaska Marine Science Symposium. Anchorage, AK.

Vollenweider, J.J., J. Gregg, R.A. Heintz, and P.K. Hershberger. January 18-22, 2010. Platform. The energetic toll of disease and starvation on overwintering juvenile herring. Alaska Marine Science Symposium. Anchorage, AK.

Grady, C.A., J.L. Gregg, R.M. Collins, and P.K. Hershberger. June 7-10, 2009. Platform. Optimization of an in vitro technique to replicate viral hemorrhagic septicemia virus (VHSV) in herring fin explant cultures. 50th Western Fish Disease Workshop and AFS Fish Health Section Annual Meeting. Park City, UT.

Rasmussen, C., M.K. Purcell, J.L. Gregg, S.E. LaPatra, J.R. Winton, and P.K. Hershberger. June 7-10, 2009. Poster. Sequencing of the internal transcribed spacer (ITS) regions indicate genetic heterogeneity between freshwater and marine Ichthyophonus sp. isolates. 50th Western Fish Disease Workshop and AFS Fish Health Section Annual Meeting . Park City, UT.

Seitz, A., J. Gregg, P. Hershberger, A. Kagley, B. Meloy, and J. Payne. April 20-23, 2009. Platform. A Pilot study of mortality and tag loss in captive Puget Sound herring surgically implanted with acoustic tags. 2009 Annual General Meeting of the Washington - British Columbia Chapter of American Fisheries Society. Shelton, WA.

Hershberger, P.K. January 29-30, 2009. Platform. Impacts of diseases to wild fish populations and the exacerbating effects of temperature. USGS – Climate Change, Natural Resources, and Coastal Management: A Workshop on the Coastal Ecosystems of California, Oregon, and Washington. San Francisco, CA, (Presented).

Hershberger, P.K., J.L. Gregg, C.A. Grady, and R.M. Collins. January 19-22, 2009. Platform. Virus shedding after exposure to viral hemorrhagic septicemia virus (VHSV). Alaska Marine Science Symposium. Anchorage, AK. (Presented).

Winton, J., D. Elliott, P. Hershberger, G. Kurath, M. Purcell. November 5-7, 2008. Platform. The Ecology and population effects of disease – bacterial kidney disease and viral hemorrhagic septicemia as examples from the Great Lakes and Pacific Northwest. Great Lakes Fishery Commission: Understanding the relationships between ecosystem dysfunction and fish health in the Great Lakes. Ann Arbor, MI.

Hershberger, P.K. October 15-17, 2008. Platform. Disease impacts on populations of wild marine fishes. USGS Interdisciplinary Microbiology Workshop. Estes Park, CO. (Presented).

Hershberger P., J. Gregg, C. Pacheco, J. Winton, J. Richard, and G. Traxler. August 26-29, 2008. Poster. Larval Herring are highly susceptible to VHS and survivors are partially protected after their metamorphosis to juveniles. Linking Herring: Herring; Linking Biology, Ecology, and Status of Populations in the Context of Changing Environments. Galway, Ireland. (Presented).

Hershberger, P.K., N.E. Elder, C.A. Grady, J.L. Gregg, C.A. Pacheco, C. Greene, C. Rice, and T.R. Meyers. August 26-29, 2008. *Platform*. Viral Erythrocytic Necrosis (VEN) Epizootics in juvenile Pacific herring from Puget Sound, WA, USA. Linking Herring: Herring; Linking Biology, Ecology, and Status of Populations in the Context of Changing Environments. Galway, Ireland. (Presented).

Hershberger, P.K., J.L. Gregg, C.A. Grady, and R.M. Collins. June 23-25, 2008. Platform. Virus Shedding from Pacific Herring after Exposure to Viral Hemorrhagic Septicemia Virus (VHSV). AFS - 49th Annual Western Fish Disease Workshop. Ocean Shores, WA. (Presented).

Hershberger, P.K., B.K. van der Leeuw, J.L. Gregg, C.A. Grady, K. Lujan, S. Gutenberger, and J.H. Petersen, M.J. Parsley. June 23-25, 2008. Platform. Emergence of *Ichthyophonus hoferi* in the Columbia River by American Shad. AFS - 49th Annual Western Fish Disease Workshop. Ocean Shores, WA. (Presented).

Hershberger, P.K. June 23-25, 2008. Platform. Recurring Viral Erythrocytic Necrosis Epizootics in Juvenile Pacific Herring from Puget Sound. AFS - 49th Annual Western Fish Disease Workshop. Ocean Shores, WA. (Presented).

Garver, K., G. Traxler, P. Hershberger, and S. LaPatra. June 23-25, 2008. Platform. VHSV in Farmed and Wild Fish in the Marine Waters of the Pacific Northwest. AFS - 49th Annual Western Fish Disease Workshop. Ocean Shores, WA.

Kraus, D., P. Hershberger, C. Grady, J. Gregg, J. Winton, and J. Hansen. June 23-25, 2008. Platform. Analysis of Immune Regulated Genes in Pacific Herring Challenged with Viral Hemorrhagic Septicemia Virus. AFS - 49th Annual Western Fish Disease Workshop. Ocean Shores, WA.

Hershberger, P.K., C.A. Pacheco, J.L. Gregg, M.K. Purcell, and S.E. LaPatra. June 23-25, 2008. Poster. Differential Survival of *Ichthyophonus* Isolates Indicates Parasite Adaptation to its Host Environment. AFS - 49th Annual Western Fish Disease Workshop. Ocean Shores, WA. (Presented).

Hershberger, P.K., C.A. Pacheco, and J.L. Gregg. June 23-25, 2008. Poster. Inactivation of *Ichthyophonus* Spores using Sodium Hypochlorite and Polyvinyl Pyrrolidone Iodine. AFS - 49th Annual Western Fish Disease Workshop. Ocean Shores, WA. (Presented).

Hershberger, P.K. May 19-22, 2008. Platform. Emergence of *Ichthyophonus* in Fishes from the Eastern North Pacific. Native American Fish and Wildlife Society National Meeting. Yakima, WA (Presented).

Hershberger, P.K., B.K. van der Leeuw, J.L. Gregg, C.A. Grady, K. Lujan, S. Gutenberger, J.H. Petersen, and M.J. Parsley. May 5-8, 2008. Platform Emergence of *Ichthyophonus hoferi* in the Columbia River by American shad. Western Division, American Fisheries Society. Portland, OR. (Presented).

Hershberger, P.K. and D. Fagergren. March 26, 2008. Forage Fish Health and Changes. South Sound Science Symposium. Tacoma, WA. Invited Speaker.

Hershberger, P.K., N.E. Elder, C.A. Grady, J.L. Gregg, C.A. Pacheco, C. Greene, C. Rice, and T.R. Meyers. March 4-6, 2008. Platform. Recurring viral erythrocytic necrosis (VEN) epizootics in juvenile Pacific herring from Puget Sound. Fish Health / Disease Ecology Symposium Organizer and Session Chair. Annual Meeting: North Pacific International Chapter - American Fisheries Society. Bellingham, WA. (Presented, Session Organizer and Moderator).

Gregg, J., M. Meyers, C. Grady, J. Word, and P. Hershberger. March 4-6, 2008. *Ichthyophonus* in fishes from the northeastern Pacific Ocean. Annual Meeting: North Pacific International Chapter - American Fisheries Society. Bellingham, WA (Session Organizer and Moderator).

Kocan, R.M., P.K. Hershberger, and J.R. Winton. March 4-6, 2008. Platform. Cardiac Failure: A possible mechanism to explain poor migratory performance in Ichthyophonus-

infected Chinook salmon. Annual Meeting: North Pacific International Chapter - American Fisheries Society. Bellingham, WA (Session Organizer and Moderator).

Hershberger et al. January 2008. Larval Herring are highly susceptible to VHS and survivors are partially protected after their metamorphosis to juveniles. Platform. Alaska Marine Science Symposium. Anchorage, AK. (Presented).

Hershberger, P.K. October 24-25, 2007. Platform. Disease Issues in Populations of Marine Fishes: Potential Impacts of Climate Change. 4th Annual Northwest Tribal Water Rights Conference on Climate Change. Shelton, WA (Presented).

Hershberger P.K., J.L. Gregg, and J.R. Winton. March 26-29, 2007. Platform. Impacts of Disease to Wild Fish Populations with Special Reference to the Salish Sea Region. Georgia Basin / Puget Sound Research Conference. Vancouver, B.C. (Presented).

Gregg J., C. Pacheco, M. Myers, and P. Hershberger. March 26-29, 2007. Platform. Prevalence of *Ichthyophonus* in copper rockfish (*Sebastes cayurinus*) from Puget Sound, WA. Georgia Basin / Puget Sound Research Conference. Vancouver, B.C.

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