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

Herring Disease Program

Exxon Valdez Oil Spill Trustee Council Project 16120111-K
Final Report

Paul K. Hershberger

U.S. Geological Survey
Marrowstone Marine Field Station
616 Marrowstone Point Road
Nordland, WA 98358

May 2018

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Study History: The biomass of Pacific herring (*Clupea pallasii*) in Prince William Sound, Alaska decreased from 120,000 metric tons to less than 30,000 tons following the *Exxon Valdez* Oil Spill in 1989. Cause(s) of this population decline remain unresolved; leading hypotheses include combinations of direct and indirect mortality from oil exposure, predation, competition for limited resources, and disease-related mortality. The *Exxon Valdez* Oil Spill Trustee Council launched early efforts to investigate the possible involvement of infectious and parasitic diseases in the initial population decline. These early efforts from 1994-2003 (Restoration projects 94320S, 95320S, 96162, 97162, 98162, 99328, 99462, 00462, 01462, 02462, 03462) consisted primarily of fish health surveillances lead by Dr. Gary Marty (formerly University of California Davis, currently British Columbia Ministry of Agriculture and Lands). However, larger impacts of the spill occurred in the ensuing decades since 1989, as the Prince William Sound herring population failed to recover. As a result, restoration goals shifted towards understanding the factors (including mortality from infectious and parasitic diseases) that may be contributing to the ongoing failed population recovery. Beginning in 2007, the Herring Disease Program expanded upon the early work by Dr. Marty by continuing the annual disease surveillances and providing disease data for the age-structured assessment model used by the Alaska Department of Fish and Game; however, a significant portion of the Herring Disease Program also includes controlled experimental studies intended to determine cause-and-effect disease relationships. The first phase of the Herring Disease Program (project 070819) was initiated as a 4 year project from FY 2007-2010; a one year no cost extension was granted for FY 2011. The Herring Disease Program was continued as a follow-up study (project 10100132-I) from 2010-2013, as the Herring Disease Program became an integrated project within the Prince William Sound Herring Survey Program. The integration of the Herring Disease Program within the Prince William Sound Herring Survey Program continues with this most recent project. Funding was provided during 2012-2014; as such, this report only covers the primary Herring Disease Program results from those years. Results from previous Herring Disease Program projects are provided in the earlier final reports.

13 published papers results from this project. These are provided as appendices in this report:

Appendix 1: Burge CB, Eakin CM, Friedman CS, Froelich B, Hershberger PK, Hofmann EE, Petes LE, Prager KC, Weil E, Willis EL, Ford SE, Harvell CD. 2014. Climate change influences on marine infectious diseases: implications for management and society. Annual Review of Marine Science 6:249-277. doi.org/10.1146/annurev-marine-010213-135029

Appendix 2: Conway CM, Purcell MK, Elliott DG, Hershberger PK. 2015. Detection of *Icthyphonus* by chromogenic *in situ* hybridization. Journal of Fish Diseases 38:853-857. doi:10.1111/jfd.12300

Appendix 3: Emmenegger EJ, Glenn JA, Winton JR, Batts WN, Gregg JL, Hershberger PK. 2014. Molecular identification of erythrocytic necrosis virus (ENV) from the blood of Pacific

herring (*Clupea pallasii*). Veterinary Microbiology 174:16-26. doi.org/10.1016/j.vetmic.2014.08.028

Appendix 4: Friend SE, Lovy J, Hershberger PK. 2016. Disease surveillance of Atlantic herring: molecular characterization of hepatic coccidiosis and a morphological report of a novel intestinal coccidian. Diseases of Aquatic Organisms 120: 91-107. doi: 10.3354/dao03016

Appendix 5: Gregg JL, Powers RL, Purcell MK, Friedman CS, Hershberger PK. 2016. *Ichthyophonus* parasite phylogeny based on ITS rDNA structure prediction and alignment identifies six clades, with a single dominant marine type. Diseases of Aquatic Organisms 120:125-141. doi.org/10.3354/dao03017

Appendix 6: Gregg JL, Powers RL, Purcell MK, Friedman CS, Hershberger PK. 2016. *Ichthyophonus* parasite phylogeny based on ITS rDNA structure prediction and alignment identifies six clades, with a single dominant marine type. Diseases of Aquatic Organisms 120:125-141. Supplement.

Appendix 7: Hart LM, Conway CM, Elliott DG, Hershberger PK. 2016. Persistence of external signs in Pacific herring *Clupea pallasii* Valenciennes with ichthyophoniasis. Journal of Fish Diseases 39: 429-440. doi.org/10.1111/jfd.12377

Appendix 8: Hart LM, MacKenzie A, Purcell MK, Powers RL, Hershberger PK. 2017. Optimization of a Plaque Neutralization Test (PNT) to identify the exposure history of Pacific herring to Viral Hemorrhagic Septicemia Virus (VHSV). Journal of Aquatic Animal Health 29:74-82. doi: 10.1080/08997659.2017.1285369

Appendix 9: Hart LM, Lorenzen N, Einer-Jensen K, Purcell MK, Hershberger PK. 2017. Influence of temperature on the efficacy of homologous and heterologous DNA vaccines against Viral Hemorrhagic Septicemia in Pacific herring. Journal of Aquatic Animal Health 29:121-128. doi: 10.1080/08997659.2017.1307287

Appendix 10: Hershberger PK, Hart LM, MacKenzie AH, Yanney ML, Conway CM, Elliott DG. 2015. Infecting Pacific herring with *Ichthyophonous* sp. in the laboratory. Journal of Aquatic Animal Health 27:217-221. doi.org/10.1080/08997659.2015.1095809

Appendix 11: Hershberger PK, Gregg JL, Hart LM, Moffitt S, Brenner R, Stick K, Coonradt E, Otis EO, Vollenweider JJ, Garver KA, Lovy J, Meyers TR. 2016. The parasite *Ichthyophonous* sp. in Pacific herring from the coastal NE Pacific. Journal of Fish Diseases 39: 395-410. doi:10.1111/jfd.12370

Appendix 12: Kocan R, Hart L, Lewandowski N, Hershberger P. 2014. Viability and infectivity of *Ichthyophonous* sp. in post-mortem Pacific herring, *Clupea pallasii*. Journal of Parasitology 100: 790-796. doi.org/10.1645/14-518.1

Appendix 13: Purcell MK, Pearman-Gillman S, Thompson RL, Gregg JL, Hart LM, Winton JR, Emmenegger EJ, Hershberger PK. 2016. Journal of Veterinary Diagnostic Investigation 28:382-391. DOI: 10.1177/1040638716646411

Appendix 14: Wilson A, Goldberg T, Marcquenski S, Olson W, Goetz F, Hershberger P, Hart L, Toohey-Kurth K. 2014. Development and evaluation of a blocking enzyme-linked immunosorbent assay and virus neutralization assay to detect antibodies to viral hemorrhagic septicemia virus. Clinical and Vaccine Immunology 21: 435-442. doi: 10.1128/CVI.00675-13

Abstract: This study includes annual field surveys of *Ichthyophonus*, viral hemorrhagic septicemia virus, and erythrocytic necrosis virus in adult and juvenile Pacific herring (*Clupea pallasii*) in Prince William Sound, Alaska and several reference populations in Alaska, British Columbia, and Washington. Results from controlled experimental studies with *Ichthyophonus* indicated that:

- Pacific herring could become infected after repeated feedings on simulated infected offal,
- Gross external signs of ichthyophoniasis can persist for extended periods without causing direct host mortality,
- A novel tool (chromogenic in situ hybridization) was developed to confirm the presence of *Ichthyophonus* in histological sections,
- A circulating stage of *Ichthyophonus* was detected in the blood of infected hosts,
- *Ichthyophonus* remains viable in a fish carcass for up to 4 weeks and remains infectious for at least 5 days post mortem,
- Six distinct genetic types of *Ichthyophonus* were identified in different hosts throughout the world,
- Tissue explant culture was confirmed to be more sensitive than qPCR for detecting low - intensity *Ichthyophonus* infections directly from fish tissues.

Results from controlled experimental studies with viral hemorrhagic septicemia virus indicated that:

- Cooler temperatures are more conducive to viral hemorrhagic septicemia epizootics in Pacific herring,
- A blocking ELISA was developed to detect fish antibodies to viral hemorrhagic septicemia virus,
- A more sensitive plaque neutralization test was optimized to detect herring neutralizing antibodies to viral hemorrhagic septicemia virus,
- The relative susceptibility of Pacific herring to other viral hemorrhagic septicemia virus genotypes was assessed, experimental spill-over, amplification, and spill-back was demonstrated between Atlantic salmon (*Salmo salar*) and Pacific herring,
- The efficacy of homologous and heterologous DNA vaccines against viral hemorrhagic septicemia virus was demonstrated in Pacific herring.

Controlled experimental studies with erythrocytic necrosis virus resulted in the development of a conventional PCR technique that is capable of the virus in the blood and the development of a quantitative PCR technique that is capable of detecting the virus in any herring tissues.

Key Words: disease, ENV, erythrocytic necrosis virus, ichthyophoniasis, *Ichthyophonus*, Pacific herring, VEN, VHS, viral erythrocytic necrosis, viral hemorrhagic septicemia

Project Data: *Description of data* – data were collected from field surveys and controlled experiments performed at the USGS – Marrowstone Marine Field Station. Field samples were processed at the USGS – Marrowstone Marine Field Station and the ADF&G Juneau Fish Pathology Laboratory (Case record numbers are reported in Table 1). Results from controlled laboratory experiments are maintained in notebooks at the USGS – Marrowstone Marine Field Station.

All of the data are publically available on the Alaska Ocean Observing System (AOOS) data portal (http://portal.aoos.org/gulf-of-alaska.php#metadata/fc5b0956-ef7c-49df-b261-c8e2713887fc/project).

The AOOS contact is Carol Janzen, 1007 W. 3rd Ave. #100, Anchorage, AK 99501, 907-644-6703, janzen@aoos.org, http://portal.aoos.org/gulf-of-alaska.php.

There are no limitations on the use of the data, however, it is requested that the authors be cited for any subsequent publications that reference this dataset. It is strongly recommended that careful attention be paid to the contents of the metadata file associated with these data to evaluate data set limitations or intended use.

Citation:

Hershberger, P.K. 2018. Herring Disease Program, *Exxon Valdez* Oil Spill Long-term Herring Research and Monitoring Program Final Report (*Exxon Valdez* Oil Spill Trustee Council Project 16120111-K), *Exxon Valdez* Oil Spill Trustee Council, Anchorage, Alaska.

Disclaimer:

The opinions, findings, conclusions, or recommendation expressed in this report are those of the authors and do not necessarily reflect the views of the *Exxon Valdez* Oil Spill Trustee Council. The use of trade, firm, or corporation names in is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Interior or the U.S. Geological Survey of any product or service to the exclusion of others that may be suitable.

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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 (*Clupea pallasii*) populations in Prince William Sound (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.

Ichthyophonus occurred in populations of Pacific herring throughout the NE Pacific. Infection prevalence varied with geographic location, season, and population age structure, with prevalence in adult herring from Prince William Sound ranging from 24 - 47% during 2014 – 2016; infection prevalence in juvenile cohorts was typically much lower. A review paper describing the demographic patterns detected in *Ichthyophonus* surveys throughout the NE Pacific from 2003-2013 was published. One interesting anomaly to the demographic patterns occurred in Cordova Harbor, where infection prevalence was unusually high among juvenile cohorts; follow-up studies are planned to address this anomaly. Additionally, we investigated the appearance of external ichthyophoniasis signs on the flank of juvenile herring. The characteristic open ulcers can be quite persistent on infected cohorts, but the appearance of these signs does not necessarily precede host mortality. A very small stage of *Ichthyophonus* was detected in the blood of infected fish. This parasitic life stage is most certainly involved in dissemination throughout the host tissues, and it may represent the infectious stage. We also determined that *Ichthyophonus* life stages remain viable and infectious in a dead host for extended periods; this ability of the parasite to survive both saprophytically and facultatively likely provides the parasite with a persistence strategy in the wild. We also collected *Ichthyophonus* isolates from around the world and determined that at least six distinct genotypes exist. The predominant type in the NE Pacific is common among most hosts we examined, including Pacific herring. Interestingly, a different genotype is pervasive in freshwater rainbow trout aquaculture facilities throughout the world; in all likelihood, this is freshwater genotype represents the original type species *I. hoferi*. Follow-up studies are underway to evaluate the relative virulence of the different genotypes. Finally, we confirmed that tissue explant culture is the most sensitive diagnostic technique available for detecting low-intensity *Ichthyophonus* infections; it is even more sensitive than quantitative PCR.

During the study period 2012 – 2014, VHS virus (VHSV) was detected at low prevalence and low titer in random samples of adult herring from Sheep Bay (2014). 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. An apparent VHS epizootic was detected in juvenile herring from the nearshore regions of the San Juan Islands (north Puget Sound) during 2014. Field biologists recognized lethargic juvenile herring demonstrating characteristic hemorrhages during routine beach seining exercises. Hemorrhaged individuals were high-graded from the catches, frozen at -20°C, and submitted for VHSV screening. Even using these sub-optimal handling procedures, VHSV was detected in high tissue titers among 13-27% of the samples. It is very likely that this type of small-scale VHSV epizootic - occurring in the apparent absence of any

appreciable mortality event - is quite common, yet overlooked because of a paucity of sampling efforts in wild marine fish. Laboratory studies indicated that cooler water temperatures were more conducive to VHS epizootics, resulting in greater mortality, higher viral tissue titers, and longer viral persistence in the host tissues. This inverse relationship between temperature and VHS was likely mediated by an enhanced immune response at warmer temperatures, where a robust type I interferon response was indicated by rapid and significant upregulation of the herring Mx gene. An important advancement during this funding cycle included the development and optimization of a number of techniques (ELISA, virus neutralization, and plaque neutralization – PNT) that are capable of measuring VHSV antibodies in surviving fish. The PNT was by far the most sensitive technique in Pacific herring, and future work is focusing on this technique. Further development of the PNT as a forecasting tool is currently underway to determine whether it can be employed as both a hindcasting tool capable of identifying prior VHS epizootics and a forecasting tool capable of assessing the potential for future VHS epizootics. Other studies determined the susceptibility of Pacific herring to other VHSV genotypes and assessed the effects of temperature on the efficacy of DNA vaccines against VHSV in Pacific herring.

During the study period 2012 – 2014, a low prevalence of VEN was detected in herring populations throughout the NE Pacific, including Cook Inlet (2014), Sitka (2014), and Prince William Sound (2016). These were generally low-intensity infections, with the exception of the positive fish from Cook Inlet, which demonstrated a high proportion of circulating erythroblasts and erythrocytes demonstrating VEN inclusions. Additionally, the methods for two new VEN diagnostic techniques (conventional and quantitative PCR's) were published, thereby enabling confirmation of this condition from standard tissue samples. This important advancement will enable us to diagnose the condition in the absence of blood films, which are not typically included in standard necropsy procedures.

Additional products included two additional synthesis/review papers and a description of coccidian parasites in herring. Because ichthyophoniasis is the most ecologically and economically significant disease of wild marine fishes in the world, it was included in a review paper describing the impacts of climate change on marine diseases (Burge et al. 2014; Appendix 1). As part of this exercise, all the published reports of *Ichthyophonus* impacts on wild fishes were consolidated. Additionally, we published a review of the primary diseases impacting marine fishes in the Salish Sea, including bacterial kidney disease, infectious hematopoietic necrosis, VHS, VEN, and ichthyophoniasis). Finally, we described the occurrence of two coccidian parasites in herring and the apparent paucity of *Ichthyophonus* and VHSV in Atlantic herring populations.

Status of Project Objectives:

- Provision of disease prevalence data necessary for the age structured assessment herring model **– Completed (Chapter 1)**
- Provision of disease process studies intended to investigate the seasonality of herring diseases in PWS **– Completed (Chapter 1)**
- Collection of novel disease forecasting data **– Completed (Section 3.4)**
- Production of Specific Pathogen-Free Pacific herring intended as laboratory hosts for controlled experiments intended to determine cause-and-effect disease relationships **– Completed (used in Chapters 2-4)**
- Development of a novel diagnostic technique (fluorescent in situ hybridization) intended to provide confirmatory diagnosis of *Ichthyophonus* from histology sections. **– Completed (Section 2.4)**

Detailed descriptions of the studies designed to address each of these objectives are included in the following chapters.

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

Annual infection and disease surveillance for VHSV, ENV, and *Ichthyophonus* continued during 2014-2016. The primary purpose of these efforts remains the provision of disease inputs to the PWS herring age structured assessment (ASA) model. As reference locations, herring populations outside PWS were sampled whenever opportunities arose. The three primary pathogens continue to occur throughout the NE Pacific, and the prevalence and intensity of VHSV and ENV remained low during typical endemic periods. However, indications of localized viral epizootics exist, including a likely VHS event among juvenile herring cohorts that occurred in north Puget Sound during 2014; this event was not accompanied with any observed mortality. Additionally, we investigated another case involving hemorrhaged adult herring near Craig, AK in 2015. VHSV was not detected among any of the Craig fish; rather, it is suspected that the observed lesions likely resulted from predator escapes. *Ichthyophonus* also remains endemic throughout the region; however, during typical endemic periods this parasite occurs in much higher infection prevalence than the viral agents. Unusually high *Ichthyophonus* infection prevalence and intensity were documented among juvenile herring from Cordova Harbor, and future studies are planned to investigate the causes for this anomaly.

As an attempted consolidation, survey results in the tables below indicate a summation of all results since the beginning of the Herring Disease Program in 2007. The results from 2010 – present are novel to this project.

Table 1. Results of pathogen prevalence surveys in Pacific herring. Results from 2007-2010 were reported in HDP final reports from previous years, but are also included here as a complete inventory. Results from 2011-2016 are novel to this final report. ND = no data.

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2007	PWS	St. Matthews Bay	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
	Cook Inlet	Kamishak B	May 16	A	ND[1]	32% (19/60)	ND	ND	
		Kamishak B	May 27	A	ND[1]	20% (12/59)	ND	ND	
		Kamishak B	May 27	A	ND[1]	28% (17/60)	ND	ND	
	Sitka Sound	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[2]	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]Herring lengths in Cook Inlet were recorded as standard length, not fork length.
[2]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 Sound	Beli Rock	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	Drayton Pass	Jan 15	A	144 (7)	2% (1/60)	ND	ND	MMFS #ICH 08-1
		Port Orchard[3]	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

[3]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
	Cook Inlet	Kamishak Bay	May 8	A	ND[4]	3% (2/60)	ND	ND	
		Kamishak Bay	May 21	A	ND[4]	2% (1/60)	ND	ND	
	Sitka Sound	Guide Island	Feb 15-16	A	256 (15)	40% (32/80)	ND	ND	ADF&G #09-0540
		Unknown	Mar 24-27	A	270 (19)	46% (20/44)	0% (0/44)	ND	ADF&G #09-0545 & MMFS AK 09-2
		St. John 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
		Unknown	Mar 24-27	J	175 (7)	4% (3/69)	0% (0/69)	0% (0/69)	ADF&G #09-0545 & MMFS AK 09-2
	Lynn Canal	Cohen Isl. Amalga Trench	Feb 11-12	A	203 (15)	7% (3/44)	ND	ND	ADF&G #09-0539
		Fritz Cove, Outer Pt, Lena Pt	Mar 18-19	A	ND	13% (8/60)	ND	ND	ADF&G #09-0541
		Gull Isl. & Benj. Isl. Trench	Nov 24	A	210 (14)	18% (11/60)	ND	ND	MMFS #AK09-4
		Benj. Isl. Trench & Fritz Cv.	Dec. 7	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[5]	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

[4]Herring lengths in Cook Inlet were recorded as standard length, not fork length.
[5]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%[6]	10% (6/60	ADF&G #10-0536 & MMFS # AK10-1
		Cordova Harbor	June 2-13	J	85 (12)	35% (17/49)	0% (0/49)	71% (38/48)	MMFS #AK 10-3
		Cordova Harbor	Aug 18	J	44 (3)	0% (0/18)	0% (0/54)	0% (0/17)	MMFS #AK 10-3
		Cordova Harbor	Sept 28 -Oct 7	J	50 (6)	0% (0/22)	0% (0/22)	0% (0/21)	MMFS #AK 10-3
		Simpson Bay	Nov. 3	J	73 (7)	0% (0/38)	ND	6% (2/36	MMFS #AK 10-3
		Port Fidalgo	Nov. 4	J	77 (4)	0% (0/22)	ND	5% (1/22)	MMFS #AK 10-3
		Eaglik	Nov. 5	J	90 (9)	0% (0/34)	ND	26% (8/31)	MMFS #AK 10-3
		Whale Bay	Nov 10-11	J	95 (33)	3% (2/58)	2% (1/60)	18% (10/55)	MMFS #AK 10-3
	Cook Inlet	Kamishak B	May 4	A	ND[7]	2% (1/60)	ND	ND	
		Kamishak B	May 18	A	ND[7]	3% (2/60)	ND	ND	
	Sitka Sound	Indian River	Mar 22-24	A	242 (22)	27% (16/60)	0% (0/60)	2%	MMFS #AK10-2
		Boarder / Sitka Rocks	Mar 22-24	A	209 (28)	15% (9/60)	ND	3%	MMFS #AK10-2
		Mountain Point Kruzof Island	Mar 22-24	A	241 (25)	37% (22/60)	0% (0/60)	0%	MMFS #AK10-2
	Lynn Canal	Shelter Isl	Mar 15-16	A	202 (20)	5% (3/56)	ND	ND	MMFS #AK10-4
		Bridget Cove	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)[8]	ND	ND	MMFS #PS10-1
		Hood Canal[9,10]	May 25&27	A	140 (24)	44% (43/97)	ND	ND	MMFS #PS10-1

[6]A single pooled sample containing tissues from 3 fish tested positive (n=60) for VHSV. Therefore, the prevalence was 1-3 / 60.
[7]Herring lengths in Cook Inlet were recorded as standard length, not fork length.
[8]*Ichthyophonus* prevalence was 6% (1/17) in Pacific staghorn sculpin and 78% (28/36) in American shad.
[9]Biased sample: largest fish were removed from this sample for other purposes prior to determination of *Ichthyophonus* prevalence.
[10]Sample consisted of post-spawn adult herring

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

[11]*Ichthyophonus* cultures were frozen by the airline, killing the parasite; therefore, the true population prevalence was likely greater than the reported prevalence.
[12]Both groups of fish from Gravina were from the same school; the first 30 were high graded for larger fish; the second 30 were representative of the population.
[13]Herring lengths in Cook Inlet were recorded as standard length, not fork length.

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2012	PWS	Port Gravina	Mar 28	A	218	42% (25/60)	0% (0/60)	0% (0/60)	ADF&G #12-0533 & MMFS# AK12-4
		Port Gravina	Mar 31	A	215	40% (24/60)	0% (0/60)	0% (0/60)	ADF&G #12-0533 & MMFS# AK12-4
		Fidalgo B	April 2	A	231	35% (21/60)	0% (0/60)	0% (0/60	ADF&G #12-0533 & MMFS# AK12-4
		Port Gravina	Nov 15	A	159	3% (2/60)	0% (0/60)	0% (0/60)	MMFS #AK12-8
		Simpson B	Jan 11	J	57	0% (0/28)	0% (0/60)	?	MMFS #AK12-1
		Simpson B	April	J	ND	3% (1/30)	0% (0/30)	ND	MMFS #AK 12-3
	Cook Inlet	Kamishak Bay	May 7	A	ND	2% (1/60)	ND	ND	
	Sitka Sound	N Khasiana Isl	April 3	A	232 (23)	20% (12/60)			ADF&G #12-0534 & MMFS# AK12-5
		St John Bay	April 4	A	214 (24)	32% (19/60)			ADF&G #12-0534 & MMFS# AK12-5
		Sitka breakwall	April 4	A	225 (22)	10% (6/60)			ADF&G #12-0534 & MMFS# AK12-5
	Lynn Canal	Tee Harbor	June 8	A	176 (13)	0% (0/60)	ND	ND	MMFS #AK 12-6

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2013	PWS	Port Gravina	Mar 27	J	147	3% (2/60)	0% (0/60)	0% (0/60)	ADF&G #13-0537 & MMFS# AK13-2
		Port Gravina	Mar 31	A	232	34% (20/59)	0% (0/60)	0% (0/60)	ADF&G #13-0537 & MMFS# AK13-2
		Port Gravina	April 1	A	225	32% (19/60)	0% (0/60)	0% (0/60	ADF&G #13-0537 & MMFS# AK13-2
		Lwr Herring B	Nov 9	J	93	5% (3/60)	0% (0/60)	12% (7/59)	MMFS #AK13-4
		Port Gravina	Nov 13	J	90	0% (0/39)	0% (0/39)	18% (7/39)	MMFS #AK13-4
		Cordova Hbr	Nov 20	J	70	0% (1/61)	0% (0/61)	7% (4/61)	MMFS #AK13-4
	Sitka Sound	Apple Islands	March 29	A	246 (28)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G #13-0538 MMFS #AK13-3
		Silver Bay	March 30	A	251 (16)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G #13-0538 MMFS #AK13-3
		Unknown	March 30	A	226 (26)	18% (11/60)	0% (0/60)	0% (0/60	ADF&G #13-0538 MMFS #AK13-3
	Craig	Diamond Point	Feb 20	A	214 (23)	22% (13/60)	ND	ND	MMFS #AK 13-1
	Puget Sound	Hood Canal[14]	May 19	A	171 (18)	57% (25/44)	ND	ND	MMFS #PS 13-1

[14]Hood Canal sample consisted of post-spawn herring.

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2014	PWS	Sheep B	Mar 26	A	217	24% (15/60)	2-8%[15]	0% (0/60)	ADF&G #14-0534 & MMFS #AK14-1
		Fidalgo B	Mar 28	A	218	22% (13/60)	0% (0/60)	0% (0/60)	ADF&G #14-0534 & MMFS #AK14-1
		Snug Corner	Mar 29	A	242	32% (19/60)	0% (0/60)	0% (0/60	ADF&G #14-0534 & MMFS #AK14-1
		Simpson	Nov 15-23	J	78 (12)	2% (1/60)	0% (0/60)	ND	MMFS #AK14-4
		Beartrap	Nov 16	J	70 (5)	2% (1/61)	0% (0/61)	ND	MMFS #AK14-4
		Eaglek	Nov 19	J	96 (4)	3% (2/61)	0% (0/61)	ND	MMFS #AK14-4
	Cook Inlet	Kamisha k Bay	April 30	A	ND	0% (0/60)	0% (0/60)	2% (1/60) [16]	ADF&G #14-0078 & MMFS #AK14-3
			May 13	A	ND	0% (0/60)	0% (0/59)	0% (0/60)	ADF&G #14-0078 & MMFS #AK14-3
	Sitka	Causeway	Mar 26	A	245 (26)	25% (15/60)	0% (0/60)	2% (1/60)	ADF&G #14-0533 & MMFS #AK14-2
		Middle Island	Mar 27	A	241 (31)	20% (12/59)	0% (0/60)	0% (0/60)	ADF&G #14-0533 & MMFS #AK14-2
		Inner Point	Mar 28	A	222 (20)	27% (16/60)	0% (0/60)	0% (0/60)	ADF&G #14-0533 & MMFS #AK14-2
	Puget Sound[17]	Lopez Isl.	Sept 11	J	ND	ND	27% (6/22)	ND	MMFS #PS4-1
		Waldron Isl.	Sept 12	J	ND	ND	13% (3/24)	ND	MMFS #PS4-1

[15]CPE was detected in a single pooled sample containing tissues from 5 fish after the 3rd passage; therefore, the infection prevalence was 1-5 / 60. Viral titer was below 10^1 PFU / g. VHSV was confirmed in the cell culture supernatant by nested cPCR.

[16]VEN inclusions graded at a 3+ infection severity from Fish #38.

[17]Herring samples were collected from two locations in the San Juan Islands during Chinook salmon beach seining efforts. VHSV symptoms observed included a bloody exterior, with an increase to 10% of observed fish showing these symptoms over the course of the summer. Symptomatic herring were high-graded (i.e. not a random sample), frozen -20°C, and sent to the USGS – Marrowstone for VHSV testing (plaque assay). Warm water temperatures were noted, as well as unusually large numbers of age 0+ herring and unusually low numbers of age 0+ sandlance. Positive sampled were confirmed by qPCR using VHSV-specific primers.

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Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2015	PWS	Gravina Pt	April 3	A	228 (17)	25% (15/60)	0% (0/60)	0% (0/60)	ADF&G #15-0533 & MMFS #AK15-2
		Simpson Bay	Nov 6	J	ND	2% (1/46)	0% (0/46)	ND	MMFS #AK15-4
		Lwr Herring B.	Nov 11	J	85 (5)	2% (1/54)	0% (054)	ND	MMFS #AK15-4
		E Whale Bay	Nov 12	J	89 (7)	3% (2/60)	0% (0/60)	ND	MMFS #AK15-4
	Cook Inlet	Kamishak Bay	April 27	A	ND	2% (1/60)	0% (0/60)	0% (0/60)	ADF&G #2015-0048
	Sitka	Beili Rock	Mar 20	A	239 (26)	10% (6/60)	0% (0/60)	0% (0/60)	ADF&G 15-0532 &MMFS #AK15-1
		Beili Rock	Mar 22	A	250 (22)	13% (8/60)	0% (0/60)	0% (0/60)	ADF&G 15-0532 &MMFS #AK15-1
		Beili Rock	Mar 22	A	231 (24)	20% (12/60)	0% (0/60)	0% (0/60)	ADF&G 15-0532 &MMFS #AK15-1
	Ketchican[18]	Near Craig	Dec 17	A	193 (17)	ND	0% (0/76)	ND	MMFS #AK15-5

[18]Submitted by Eric Coonradt (ADF&G): concerns of a possible disease event occurring near Craig AK. Symptoms observed included a bloody exterior, with possible causes hypothesized to include infection or predator marks. Affected individuals comprised approximately 1 out of every 100; those with obvious symptoms were removed from the larger sample and photographed (see photo below). Samples were shipped frozen to MMFS, a 5 gallon bucket of frozen herring that were stored 60 herring were stored at -20°C. VHSV was not detected.

Year	Stock	Location	Collection Date	A/J	Mean Length mm (SD)	*Ichthyophonus* Prevalence	VHSV Prevalence	VEN Prevalence	Diagnostic Lab & Ref#
2016	PWS	Red Head Pt	April 7	A	205 (28)	24% (15/60)	0% (0/60)	0% (0/60)	ADF&G #16-0539 & MMFS #AK16-2
		Knowles Head E	April 8	A	212 (22)	29% (17/59)	0% (0/60)	0% (0/60)	ADF&G #16-0539 & MMFS #AK16-2
		Snug Corner C	Mar 29	A	234 (21)	47% (28/60)	0% (0/60)	2% (1/60)	ADF&G #16-0539 & MMFS #AK16-2
		Simpson Bay	Oct 29	J	82 (4)	25% (15/60)	0% (0/60)	2% (1/60)	MMFS #AK 16-3
		Eaglek Bay	Oct 30	J	95 (5)	3% (2/60)	0% (0/60)	2% (1/60)	MMFS #AK 16-3
		Lower Herring B	Nov 2	J	96 (4)	10% (6/60)	0% (0/60)	0% (0/60)	MMFS #AK 16-3
	Sitka	S. Salsbury Anika	Mar 21	A	218 (23)	10% (6/60)	0% (0/60)	0% (0/60)	ADF&G #16-0537 & MMFS #AK16-1
	Sound	N. Crest	Mar 22	A	215 (13)	18% (11/60)	0% (0/60)	0% (0/60)	ADF&G #16-0537 & MMFS #AK16-1
		Pt. Brown	Mar 22	A	217 (24)	22% (13/60)	0% (0/60)	0% (0/60)	ADF&G #16-0537 & MMFS #AK16-1
	Puget	Dabob Bay	Feb 18	J	128	23% (28/120)	ND	ND	MMFS #PS16-1
	Sound	S. Lopez	Feb 19	J	112	0% (0/30)	ND	ND	MMFS #PS16-1
		Dallas Bank	Feb 19	J	113	2% (1/60)	ND	ND	MMFS #PS16-1
		Gulf of Georgia	Feb 24	J	121	5% (3/60)	ND	ND	MMFS #PS16-1
		Squamish Harbor	April 4	J	126	15% (9/60)	ND	ND	MMFS #PS16-1
		Dabob Bay	April 4	J	131	13% (8/60	ND	ND	MMFS #PS16-1
		Squamish Harbor	April 4	J	115	18% (9/50)	ND	ND	MMFS #PS16-1
		S. Saratoga	April 5	J	117	2% (1/60)	ND	ND	MMFS #PS16-1
		N. Saratoga	April 6	J	123	2% (1/58)	ND	ND	MMFS #PS16-1
		Oak Bay	April 5	J	143	11% (7/62)	ND	ND	MMFS #PS16-1
		E. Pt Angeles	April 13	A	164	0% (0/60)	ND	ND	MMFS #PS16-1
		Yukon Harbor	April 18	J	143	7% (4/60)	ND	ND	MMFS #PS16-1
		Nisqually / Drayton	April 20	J	146	0% (0/60)	ND	ND	MMFS #PS16-1
		Colvos Passage	April 19	J	149	3% (2/60)	ND	ND	MMFS #PS16-1
		Nisqually / Drayton	June 4	A	155	13% (8/60)	ND	ND	MMFS #PS16-1
		N Saratoga	June 8	A	172	7% (4/60)	ND	ND	MMFS #PS16-1
		Gulf of Georgia	June 15	J	126	12% (7/60)	ND	ND	MMFS #PS16-1
		Squamish Harbor	Aug 25	J	93	5% (3/60)	ND	ND	MMFS #PS16-1
		Dabob Bay	Aug 24	A	177	40% (24/60)	ND	ND	MMFS #PS16-1
		S. Lopez	Aug 29	J	91	2% (1/60)	ND	ND	MMFS #PS16-1
		N Saratoga	Aug 25	A	150	5% (3/60)	ND	ND	MMFS #PS16-1
		President Channel	Aug 16	J	89	0% (0/60)	ND	ND	MMFS #PS16-1
		President Channel	Oct 6	J	102	2% (1/60)	ND	ND	MMFS #PS16-1
		Yukon Harbor	Oct 11	A	173	5% (3/60)	ND	ND	MMFS #PS16-1
		Gulf of Georgia	Oct 6	A	173	7% (4/60	ND	ND	MMFS #PS16-1
		N Saratoga	Oct 16	A	154	7% (8/120)	ND	ND	MMFS #PS16-1
		Colvos	Oct 16	J	117	0% (0/60)	ND	ND	MMFS #PS16-1
		Oak Bay	Oct 18	J	106	15% (9/59)	ND	ND	MMFS #PS16-1
		Dabob Bay	Oct 16	J	146	53% (32/60)	ND	ND	MMFS #PS16-1
		N. Saratoga	Dec 16	A	177	13% (8/60)	ND	ND	MMFS #PS16-1
		Dallas Bank	Dec 13	J	100	2% (1/60)	ND	ND	MMFS #PS16-1

Chapter 2: Experimental Studies Involving *Ichthyophonus* spp.

2.1 The Parasite *Ichthyophonus* in Pacific Herring

A review of *Ichthyophonus* survey results since 2007 was published (Hershberger et al. 2016a). Infection prevalence in local Pacific herring stocks varied seasonally and annually, and a general pattern of increasing prevalence with host size and/or age persisted throughout the NE Pacific. An exception to this zoographic pattern occurred among a group of juvenile, age 1+ year Pacific herring from Cordova Harbor, AK in June 2010, which demonstrated an unusually high infection prevalence of 35%. Reasons for this anomaly were hypothesized to involve anthropogenic influences that resulted in locally elevated infection pressures. Inter-annual declines in infection prevalence from some populations (e.g. Lower Cook Inlet, AK; from 20-32% in 2007 to 0-3% during 2009-2013) or from the largest size cohorts of other populations (e.g. Sitka Sound, AK; from 62.5% in 2007 to 19.6% in 2013) were likely a reflection of selective mortality among the infected cohorts. All available information for *Ichthyophonus* in the NE Pacific, including broad geographic range, low host specificity, and presence in archived Pacific herring tissue samples dating to the 1980's, indicate a long-standing host-pathogen relationship.

2.2 Infecting Pacific Herring with *Ichthyophonus* in the Laboratory

The route(s) whereby Pacific herring and other planktivorous fishes become infected with *Ichthyophonus* remains unknown. Several methods for establishing *Ichthyophonus* infections in laboratory challenges were examined. *Ichthyophonus* sp. infections were most effectively established after intraperitoneal injections with suspended parasite isolates from culture or after repeated feedings with infected fish tissues (Hershberger et al 2015). Among groups that were offered infected fish tissues, infection prevalence was greater after multiple feedings (65%) than after a single feeding (5%). Additionally, among groups that were exposed to parasite suspensions prepared from culture isolates, infection prevalence was greater by intraperitoneal injection (74%) than by gastric intubation (12%); infections were not established in any experimental herring by flushing parasite suspensions over the gills. Although the consumption of infected fish tissues is not likely the primary route of *Ichthyophonus* sp. transmission in populations of wild Pacific herring, this route may contribute to abnormally high infection prevalence in areas where juvenile herring have access to infected offal.

Table 2. Prevalence of *Ichthyophonus* sp. infection in Pacific herring after single and multiple feedings with infected tissues.

Treatment Group	Infection Prevalence - Mortalities	Infection Prevalence - Survivors	Infection Prevalence - Total
Single Exposure	5.0% (1/20)	4.4% (2/45)	4.6% (3/65*)
Multiple Exposures	38% (9/24)	96% (21/22)	65% (30/46)
Negative Control	0% (0/13)	0% (0/16)	0% (0/29)

*Five additional fish were subsampled from the single-exposure treatment 1d post-exposure to assess whether *Ichthyophonus* sp. was detectable in histological sections of the stomach bolus.

2.3 Persistence of External Signs in Pacific herring *Clupea pallasii* with Ichthyophoniasis

The progression of external signs of *Ichthyophonus* infection in Pacific herring *Clupea pallasii* was highly variable and asynchronous after intraperitoneal injection with pure parasite preparations; however, external signs generally persisted through the end of the study (429 d post-exposure). Observed signs included 'sandpaper skin,' open lesions, pigmented ulcers and / or bleeding ulcers. The prevalence of external signs plateaued 35 d post-exposure and persisted in 73-79% of exposed individuals through the end of the first experiment (147 d post-exposure). Among a second group of infected herring, external signs completely resolved in only 10% of the fish after 429 d. The onset of mortality preceded the appearance of external signs (Fig. 1). Histological examination of infected skin and skeletal muscle tissues indicated an apparent affinity of the parasite for host red muscle. Host responses consisted primarily of granulomatous inflammation, fibrosis, and necrosis in the skeletal muscle and other tissues. The persistence and asynchrony of external signs and host response indicated that they were neither a precursor to host mortality nor did they provide reliable metrics for hind-casting the date of exposure. However, the long-term persistence of clinical signs in Pacific herring may be useful in ascertaining the population-level impacts of ichthyophoniasis in regularly observed populations. These results were published in Hart et al. (2016); Appendix 7.

Figure 1. Representative external signs observed in Pacific herring after treatment with *Ichthyophonus* by i.p. injection. A. No external signs. B. Large bleeding ulcers (arrow) and blood infused white tuft (arrowhead). C. Small pigmented ulcers (arrowhead) with suspected scarring (arrow) from healing lesions. D. Open lesions (arrow). E. Sandpaper skin (arrow) with pigmented nodules along complete flank. F. Mild external signs with one pigmented lesion and one small open lesion (arrows). G. Inconspicuous small black nodules (arrow).

2.4 Detection of *Ichthyophonus* by Chromogenic in Situ Hybridization

Ichthyophonus-like organisms have been reported in amphibians, reptiles, birds and invertebrates and may have been incorrectly classified under a single type species, *I. hoferi*. Although less sensitive than other detection techniques such as explant tissue culture, histopathological examination is effective for simultaneously evaluating host response and severity of *Ichthyophonus* infections. Histological sections showing positive periodic acid-Schiff (PAS) staining of multinucleate organisms 50-250 µm in diameter can be presumptive for *Ichthyophonus*, but lack of a definitive confirmatory test may lead to misdiagnosis, particularly when the organism is not cultured. We developed a chromogenic in situ hybridization (CISH) procedure that specifically detected *Ichthyophonus* ribosomal DNA in histological sections thereby complementing the histological diagnosis by providing highly specific molecular confirmation of the observed organism (Fig. 2). A digoxigenin-labeled oligonucleotide probe was designed to target conserved portions of the 18S small subunit ribosomal gene of known *Ichthyophonus* species *I. hoferi* and *I. irregularis.* Formalin-fixed, paraffin-embedded tissues from naturally infected Chinook salmon (*Oncorhynchus tshawytscha*) and red-spotted newt (*Notophthalmus viridescens*), and experimentally infected Pacific herring, rainbow trout (*O. mykiss*) and Pacific staghorn sculpin (*Leptocottus armatus*) were analyzed by CISH and PAS staining. Probe hybridization was indicated by dark purple precipitates and correlated with the distribution and morphology of parasites observed in PAS-positive tissues and also identified *Ichthyophonus* developmental stages in the presence of PAS-positive host cells. The CISH probe hybridized with PAS-positive, *Ichthyophonus*-like organisms in all host species except the red-spotted newt, supporting the hypothesis that the organism infecting amphibians is taxonomically distinct from fish-associated *Ichthyophonus*. The CISH has utility for both diagnostic and research applications. These results are published in Conway et al. (2015); Appendix 2.

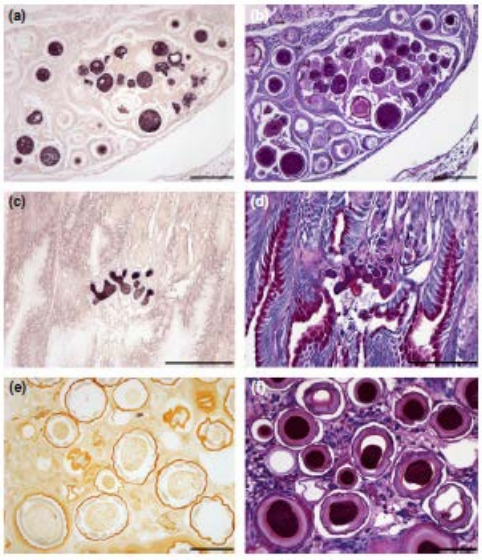

Figure 2. *Ichthyophonus* identified in (a) Pacific herring epicardial connective tissue and (c) rainbow trout stomach tunica propria by hybridization with an oligonucleotide probe targeting an *Ichthyophonus* 18s rDNA sequence. Staining with periodic acid-Schiff (PAS) revealed PAS-positive schizonts in Pacific herring (b) and amoeboid cells in rainbow trout (d). Schizonts present in red-spotted newt skeletal muscle did not hybridize with the *Ichthyophonus*-specific probe (e) (section counter-stained with Bismarck brown Y for visibility), but stained PAS-positive (f). Scale bars = 100 µm.

2.5 Identification of the Infectious Stage of *Ichthyophonus* sp. and Description of a Circulating Blood Stage

Small amoeboid cells, believed to be the infectious stage of *Ichthyophonus*, were observed in the bolus (stomach contents) and tunica propria (stomach wall) of Pacific staghorn sculpins and rainbow trout shortly after they ingested *Ichthyophonus*-infected tissues (Fig. 3)**.** By 24-48 hrs post-exposure the parasite morphed from the classically reported multi-nucleate thick walled schizonts to two distinct cell types; a larger multinucleate amoeboid cell surrounded by a narrow translucent zone and a smaller spherical cell surrounded by a "halo" and resembling a small schizont. Both cell types also appeared in the tunica propria, indicating that they had recently penetrated the columnar epithelium of the stomach. No *Ichthyophonus* pseudo-hyphae ("germination tubes") were observed in the bolus or penetrating the stomach wall. Simultaneously, *Ichthyophonus* was isolated in vitro from aortic blood, which was consistently positive from 6 -144 hrs post-exposure, then only intermittently for the next four wks. Small PAS-positive cells observed in blood cultures grew into colonies consisting of non-septate tubules (pseudo-hyphae) terminating in multinucleated knob-like apices similar to those seen in organ explant cultures. Organ explants were culture-positive every day, however typical *Ichthyophonus* schizonts were not observed histologically until 20-25 days post-exposure. From 20 to 60 days p.e. schizont diameter increased from $\leq$ 25µm to $\geq$ 82µm. Based on the data presented here, a life cycle within the piscivorous host is proposed and published in Kocan et al. (2013).

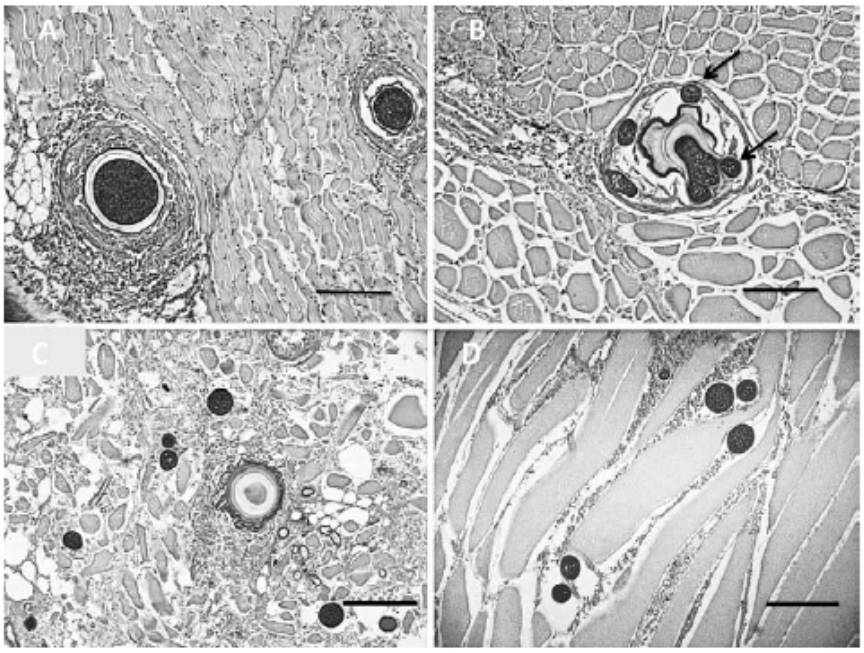

Figure 3. Transformation of *Ichthyophonus* schizonts in the stomach (bolus) of Pacific staghorn sculpins, *Leptocottus armatus*, following ingestion of infected herring tissue. (A) Normal multinuclear spherical schizonts surrounded by a granuloma (0 hr - infected homogenate). (B) Small amoeboid cells (arrows) budding from parent schizont (48 hr post consumption). (C) Empty schizont surrounded by dispersing amoeboid cells (48 hr post-consumption). (D) Amoeboid cells dispersed throughout digesting herring muscle (48 – 96 hr post consumption). (Bar - 50µm) Stain - Periodic acid-Schiff (PAS) reagent.

2.6 Viability and Infectivity of *Ichthyophonus sp.* in Post-Mortem Pacific Herring

Ichthyophonus-infected Pacific herring decomposed in flowing seawater, then serially sampled for 4 wk and examined for the presence of *Ichthyophonus* as determined by in vitro culture and single plane histology (Fig. 4). The same tissues were fed to *Ichthyophonus*-free Pacific staghorn sculpins, *Leptocottus armatus*, to determine the duration of parasite infectivity. *Ichthyophonus* sp. was viable in decomposing herring viscera and muscle for at least 4 wk post-mortem and remained infectious for sculpins for up to 5 days post-mortem. Many of the morphologic changes observed were similar to those previously reported to occur during the first 24 hr following death of the host, but also included novel forms not previously described. The significance of extended survival and progressive morphologic transformation in the post-mortem host is unknown, but it could be inferred that it has survival value for the parasite. These results are published in Kocan et al. (2014).

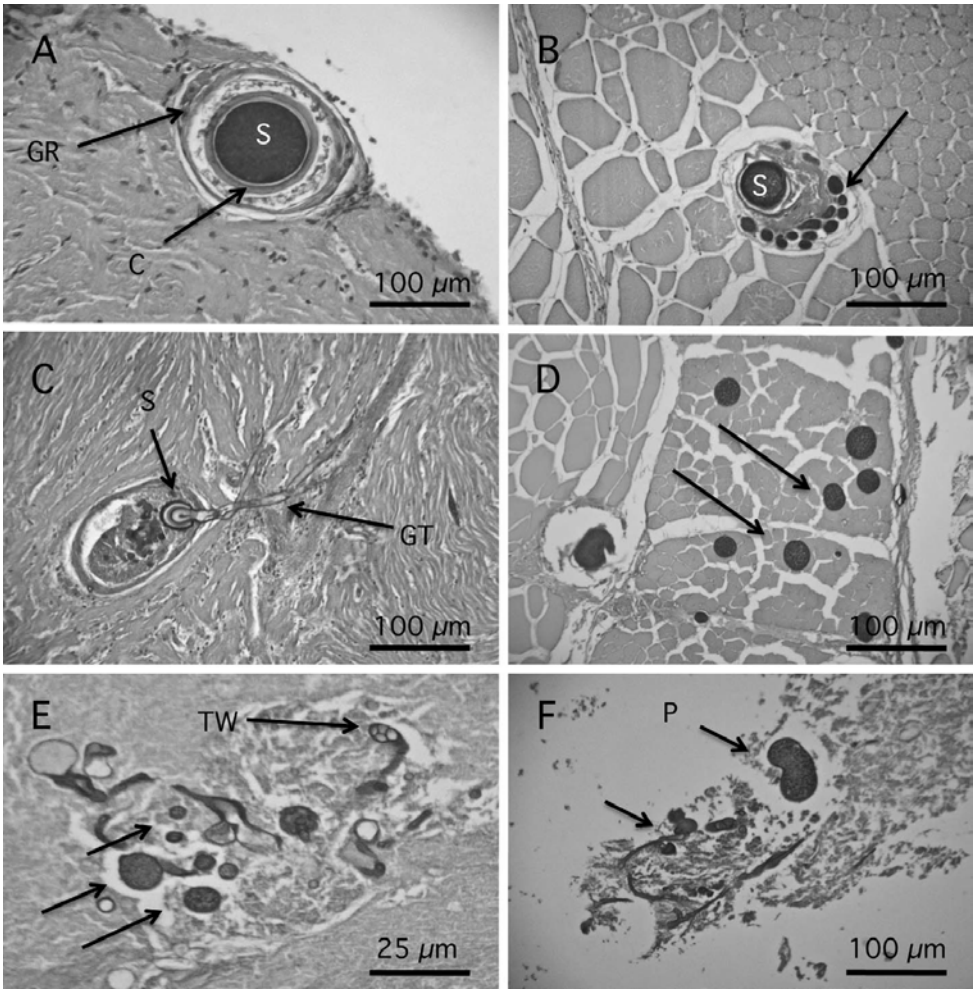

Figure 4. Periodic Acid-Schiff (PAS) stained histologic sections of post-mortem herring (*Clupea pallasii*) tissue. (**A**) (0 hr) Typical multinuclear schizont (S) with multilaminate capsule (C) and granuloma (GR). (**B**) (24 hr) Shrunken schizont (S) and multiple plasmodia (arrow). (**C**) (48 hr) Empty schizont capsule (S) in granuloma with germ tubes (GT) penetrating surrounding tissue. (**D**) (36-48 hr) Multiple un-encapsulated plasmodia (arrows) migrating into dark muscle. (**E**) (15 days) Thick walled cells in apical tip of germ tube (TW), small plasmodia (arrows), and unidentified structures. (**F**) (22 days) Un-encapsulated plasmodium (P) and unidentified structures.

2.7 *Ichthyophonus* Phylogeny Based on ITS rDNA Structure Prediction and Alignment Identifies Six Clades, with a Single Dominant Marine Type

A molecular phylogenetic study was undertaken to examine whether different genetic types of *Ichthyophonus* spp. exist. *Ichthyophonus* spp. isolates from fish hosts in the Atlantic and Pacific Oceans, several rivers, and aquaculture sites in North America, Europe, and Japan separated into six genetic clades, based on consensus sequences of the internal transcribed spacer rDNA region (Fig. 5). Species-level genetic differences were identified in each clade, however a single *Ichthyophonus* species accounted for a majority (71 of 98) of parasite isolations. This ubiquitous type occurred in 13 marine and anadromous hosts. A single *Ichthyophonus* species occurred in all samples from freshwater aquaculture, despite great geographic separation of the hosts. This ITS genotype, previously shown to be adapted to freshwater, was also encountered in a minority of the clones from two marine hosts. The remaining *Ichthyophonus* species generally occurred in a single host; however sample sizes were too small to determine if they are specialists in these species or are just rarely encountered. These results are published in Gregg et al. (2016); Appendices 5 and 6. Further research is necessary to describe phenotypic differences between the *Ichthyophonus* species identified, and more variable molecular genetic markers are necessary to understand intra-specific transmission process of the ubiquitous marine form.

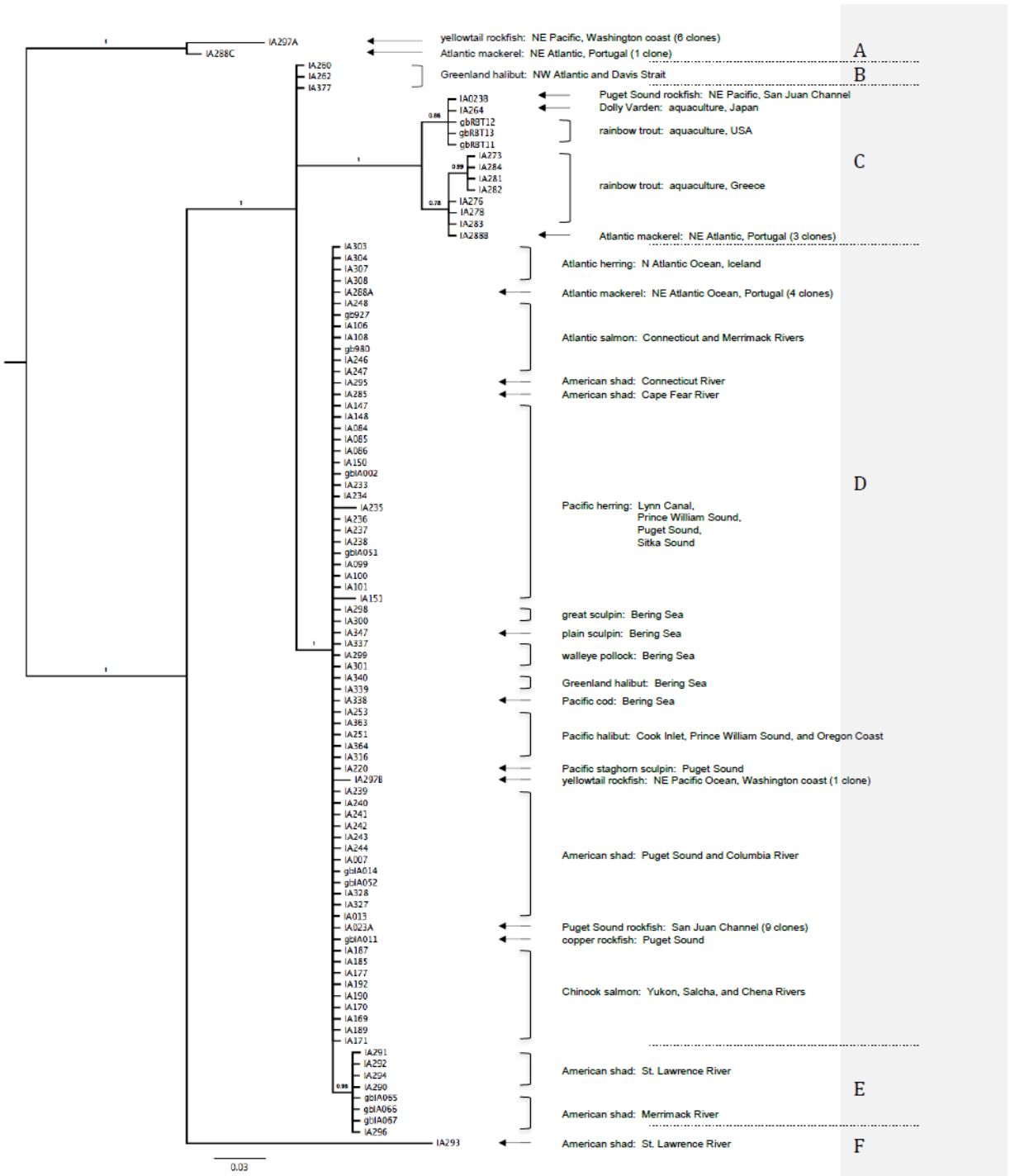

Figure 5. Phylogeny of *Ichthyophonus* based on Bayesian MCMC analysis of ITS rDNA consensus sequences under a six-partition model. Posterior probabilities are indicated above branches. Tree has an arbitrary midpoint root. Host and location of each isolate indicated right of Isolate ID. Isolates with gb prefix were obtained from GenBank. Three isolates (IA023, IA297, and IA288) contained variant haplotypes and are indicated with A, B, and C suffixes. Clades are labeled arbitrarily A through F.

2.8 Analytical and Diagnostic Performance of a qPCR Assay for *Ichthyophonus* spp. Compared to the Tissue Explant Culture 'Gold Standard'

Parasites of the genus *Ichthyophonus* infect many fish species and have a non-uniform distribution within host tissues. Due in part to this uneven distribution, there has been much debate as to whether molecular-based detection methods can be as sensitive and accurate as culture for estimating parasite prevalence in wild populations. We evaluated the analytical and diagnostic performance of an existing qPCR assay in comparison to the 'gold standard' culture method using Pacific herring with known disease history in a controlled environment (White et al. Accepted). It was determined that the assay is suitable for use on this previously untested host, and diagnostic specificity was consistently high (>98%) in both heart and liver tissues. Diagnostic sensitivity could not be fully assessed due to low infection rates in *Ichthyophonus*-inoculated fish, but our results suggest that qPCR is not as sensitive as culture under all circumstances. qPCR diagnostic sensitivity relative to culture is likely affected by the amount of sample processed, as prevalence estimates were not significantly different when the assayed sample sizes were equal. This sensitivity issue would be most problematic for fish with light infections. Although qPCR does not detect the presence of a live organism, DNA-based pathogen detection methods from preserved samples provide the opportunity for alternate testing strategies when culture is not possible.

Table 3. Diagnostic sensitivity (DSe) and specificity (DSp) of the *Ichthyophonus* qPCR test on experimentally inoculated *Ichthyophonus*-exposed (ICH) and control (PBS) *Clupea pallasii*. Cross-classified culture (C) and qPCR (Q) results are presented individually for fish heart and liver tissues at two different qPCR limits of detection for parasite DNA in fish tissue with associated DSe and DSp. Prevalence estimates are listed for each detection method with chi-square p-values from comparing each pair. Prevalence values are percentages (95% binomial proportion CI).

Inoculum/ Tank			---Number of samples---					---Infection prevalence (%)---			Diagnostic performance	
	Tissue	qPCR LOD	n	C+ Q+	C+ Q-	C- Q+	C- Q-	Culture	qPCR	P-value	DSe	DSp
PBS	Heart	≥ 3	139	0	0	0	139	0	0	-	NA	100
		≥ 1		0	0	0	139	0	0	-	NA	100
	Liver	≥ 3	139	0	0	0	139	0	0	-	NA	100
		≥ 1		0	0	0	139	0	0	-	NA	100
ICH	Heart	≥ 3	298	17	2	3	276	6.4 (4.1-9.8)	6.7 (4.4-10.1)	0.868	89.5 (69-97)	98.9 (97-100)
		≥ 1		18	1	4	275	6.4 (4.1-9.8)	7.4 (4.9-11.0)	0.627	94.7 (75-99)	98.6 (96-99)
	Liver	≥ 3	296	21	25	1	249	15.5 (11.9-20.1)	7.4 (5.0-11.0)	0.002	45.7 (32-60)	99.6 (98-100)
		≥ 1		23	23	4	246	15.5 (11.9-20.1)	9.1 (6.3-12.9)	0.018	50 (36-64)	98.4 (96-99)

Chapter 3: Experimental Studies Involving Viral Hemorrhagic Septicemia Virus (VHSV)

3.1 Principles Underlying the Epizootiology of Viral Hemorrhagic Septicemia in Pacific Herring throughout the North Pacific Ocean

A synthesis paper was published describing our current understanding of VHS epizootiology in Pacific herring (Hershberger et al.2016b). Although viral hemorrhagic septicemia virus (VHSV) typically occurs at low prevalence and intensity in natural populations of Pacific herring and other marine fishes in the NE Pacific Ocean, epizootics of the resulting disease (VHS) periodically occur, often in association with observed fish kills (Fig. 6). Here we identify a list of principles, based on a combination of field studies, controlled laboratory experiments, and previously unpublished observations, that govern the epizootiology of VHS in Pacific herring. A thorough understanding of these principles provides the basis for identifying risk factors that predispose certain marine fish populations to VHS epizootics, including the lack of population resistance, presence of chronic viral carriers in a population, copious viral shedding by infected individuals, cool water temperatures, limited water circulation patterns, and gregarious host behavioral patterns. Further, these principles were used to define the epizootiological stages of the disease in Pacific herring, including the susceptible, enzootic, disease amplification, outbreak, recovery, and refractory stages. In addition to providing a foundation for quantitatively assessing the potential risks of future VHS epizootics in Pacific herring, these principles provide insights into the epizootiology of VHS in other fish communities where susceptible species exist.

Figure 6. VHS epizootic and associated fish kill involving wild herring in Vancouver Island, B.C, Canada. Visible signs of the disease include hemorrhages along the flank of affected fish. Photo credits: Garth Traxler and Jon Richard, Department of Fisheries and Oceans, Pacific Biological Station, Canada.

3.2 Influence of Temperature on Viral Hemorrhagic Septicemia (Genogroup IVa) in Pacific Herring, *Clupea pallasii*

An inverse relationship between water temperature and susceptibility of Pacific herring to VHS was indicated by controlled exposure studies where cumulative mortalities, viral shedding rates, and viral persistence in survivors were greatest at the coolest exposure temperatures. Among groups of specific pathogen-free (SPF) Pacific herring maintained at 8, 11, and 15°C, cumulative mortality after waterborne exposure to VHSV was 78%, 40%, and 13%, respectively (Fig. 7). The prevalence of survivors with VHSV-positive tissues 25d post-exposure was 64%, 16%, and 0% (at 8, 11 and 15°C, respectively) with viral prevalence typically higher in brain tissues than in kidney/spleen tissue pools at each temperature. Similarly, geometric mean viral titers in brain tissues and kidney/spleen tissue pools decreased at higher temperatures, and kidney/spleen titers were generally 10-fold lower than those in brain tissues. This inverse relationship between temperature and VHS severity was likely mediated by an enhanced immune response at the warmer temperatures, where a robust type I interferon response was indicated by rapid and significant upregulation of the herring Mx gene. The effect of relatively small temperature differences on the susceptibility of a natural host to VHS provides insights into conditions that preface periodic VHSV epizootics in wild populations throughout the NE Pacific. These results were published in Hershberger et al. (2013a).

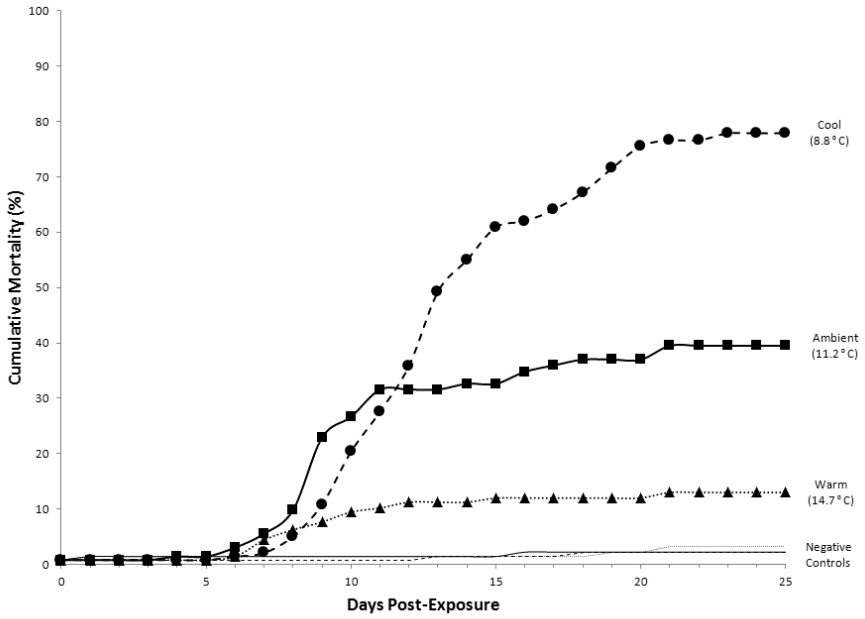

Figure 7. Effects of temperature on the cumulative mortality of VHSV exposed herring. Data points represent back-transformed percentages corresponding to the means of the arcsine-transformed proportions from triplicate tanks (31 herring/tank).

3.3. Development of a Blocking Enzyme-Linked Immunosorbent Assay and Virus Neutralization Assay to Detect Antibodies to VHSV

Detection of VHSV currently relies on virus isolation, which is lethal to fish and only indicates current infection status. A serological method is required to ascertain prior exposure. Here, we report the development of two serologic tests for VHSV that are non-lethal, rapid, and species-independent: a virus neutralization assay (VN) and a blocking enzyme-linked immunosorbent assay (ELISA). Serum was collected from 34 uninfected fish (VHS negative group) and 28 fish that survived VHS virus infection (VHS positive group). The VN did not detect neutralizing antibodies in the serum of any of the 34 VHSV negative fish, demonstrating a test specificity of 100%. Neutralizing antibodies were detected in 12 of 28 VHS positive fish, indicating a test sensitivity of 42.9%. The anti-nucleocapsid blocking ELISA detected non-neutralizing VHSV antibodies in four of the 34 fish in the VHS negative group, indicating a specificity of 88.2%. Non-neutralizing antibodies were detected in 27 of 28 VHS positive fish, indicating a sensitivity of 96.4%. Used in parallel, the VN and ELISA correctly identified all survivors of VHSV infection and unexposed fish. Our VN and ELISA are valuable tools for assessing exposure to VHSV and should improve detection and surveillance efforts for both wild and commercial fish populations. However, further testing indicated that the ELISA was only capable of detecting VHSV antibodies in Pacific herring that were hyper-immunized against VHS; the assay was incapable of detecting antibodies in survivors of lower-level exposures. Therefore, further efforts were made to develop and optimize a plaque neutralization assay (see section 3.4). These results are published in Wilson et al. (2014).

3.4 Optimization of a Plaque Neutralization Test to Identify the Exposure History of Pacific Herring to Viral Hemorrhagic Septicemia Virus.

Methods for a plaque neutralization test (PNT) were optimized for the detection and quantification of VHSV neutralizing activity in the plasma of Pacific herring. The PNT was complement-dependent, as neutralizing activity was attenuated by heat inactivation; further, neutralizing activity was mostly restored by the addition of exogenous complement from specific pathogen-free Pacific herring. Optimal methods included the overnight incubation of VHSV aliquots in serial dilutions (1:16 – 1:256) of whole test plasma containing endogenous complement. The resulting viral titers were then enumerated using a viral plaque assay in 96 well micro plates. Serum neutralizing activity was virus-specific, as plasma from VHS survivors demonstrated only negligible reactivity to infectious hematopoietic necrosis virus (IHNV), a closely-related rhabdovirus. Among Pacific herring that survived VHSV exposure, neutralizing activity was detected in the plasma as early as 37d post-exposure and peaked approximately 64 d post-exposure (Fig. 8). The onset of neutralizing activity was slightly delayed at 6.0 ^{0}C relative to warmer temperatures (8.5 and 12.0 ^{0}C); however, neutralizing activity persisted for at least 345 d post exposure in all temperature treatments. It is anticipated that this novel ability to assess VHSV neutralizing activity will enable retrospective comparisons between a priori VHS exposures and year class recruitment failures. Additionally, the optimized PNT is expected to be employed as a forecasting tool capable of identifying the potential for future VHS epizootics in wild Pacific herring populations. These results were published in Hart et al. (2017a); Appendix 8.

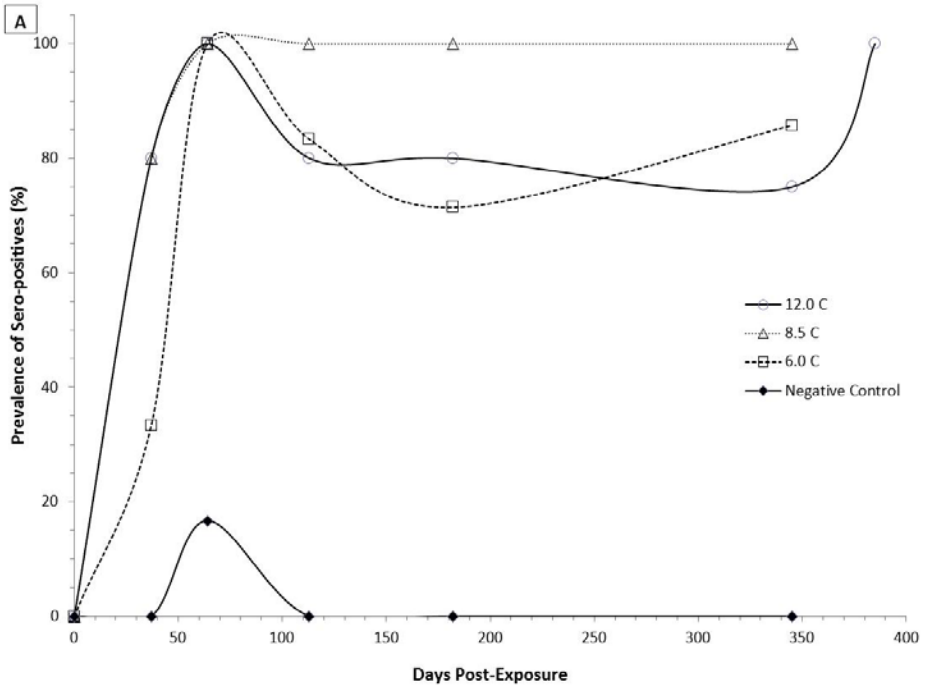

Figure 8. Kinetics of virus neutralizing activity in the plasma of VHSV survivors (n = 5 – 13 d^{-1} $group^{-1}$). Fish were considered sero-positive when neutralizing activity was detected at plasma dilutions $\geq$1:16.

3.5 Virulence of Viral Hemorrhagic Septicemia Virus (VHSV) Genotypes Ia, IVa, IVb, and IVc in Five Fish Species

The susceptibility of yellow perch (*Perca flavescens*), rainbow trout, Chinook salmon, koi (*Cyprinus carpio*), and Pacific herring to 4 strains of VHSV was assessed. Fish were challenged via intraperitoneal injection with high (1 x 10^6 plaque-forming units, PFU) and low (1 x 10^3 PFU) doses of a European strain (genotype Ia), and North American strains from the West Coast (genotype IVa), Great Lakes (genotype IVb), and the East Coast (genotype IVc). Pacific herring were exposed to the same strains, but at a single dose of 5 x 10^3 PFU / mL by immersion in static seawater. Overall, yellow perch were the most susceptible, with cumulative percent mortality (CPM) ranging from 84-100%, and 30-93% in fish injected with high and low doses (Fig. 9), respectively. Rainbow trout and Chinook salmon experienced higher mortality (47-98% CPM) after exposure to strain Ia than to other genotypes. Pacific herring were most susceptible to their endemic strain (IVa) with an average CPM of 80%, and were moderately susceptible (42-52% CPM) to the other genotypes. Koi had very low susceptibility (<5% CPM) to all 4 VHSV strains. These results are published in Emmenegger et al. (2014); Appendix 3.

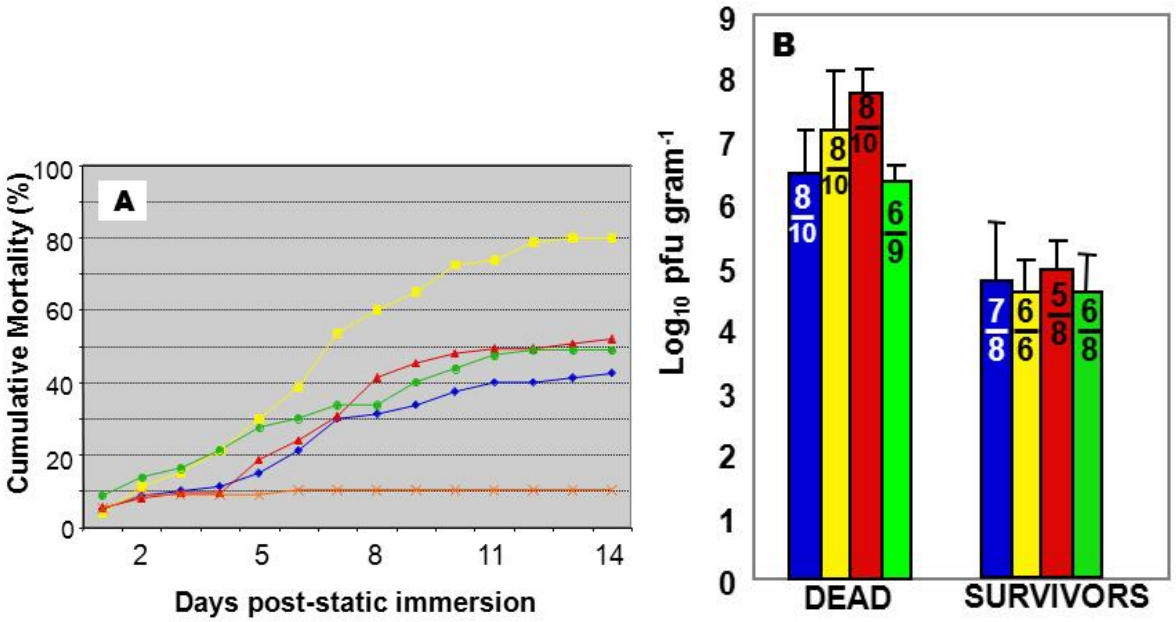

Figure 9. Susceptibility of Pacific herring to VHSV genotypes. (A) Mean Cumulative mortality of Pacific herring after immersion exposure to a dose of 5.0 x 10^3 PFU/mL of VHSV (blue - European Ia; yellow - North American West Coast IVa; red - North American Great Lakes IVb; green – North American East Coast IVc) or mock treatment (orange). (B) Virus concentrations (geo mean of virus-positive fish) in dead and surviving fish (14d post-challenge) with prevalence shown within the columns as the number of virus-positive fish / number tested. Error bars indicate SD of the virus titers.

3.6. Demonstration of Atlantic Salmon as a Host and Reservoir of Viral Hemorrhagic Septicemia Virus Type IVa

In British Columbia (BC) the farming of non-native Atlantic salmon (*Salmo salar*) in ocean net pens leads to problems with disease because salmon are exposed to pathogens occurring in the environment. VHSV is enzootic in BC and causes serious disease in wild Pacific herring, which often enter and remain in salmon netpens. Isolation of VHSV from farmed Atlantic salmon has been previously documented, but the effects on the health of farmed salmon and the wild fish sharing the environment are unknown. To determine Atlantic salmon susceptibility to VHSV, fish were infected with a mixture of VHSV isolates originating from farmed Atlantic salmon by IP-injection or through a route including immersion in virus and cohabitation with VHSV-infected Pacific herring. Transmission of virus from Atlantic salmon to Pacific herring was also tested. Measurements of disease included mortality, clinical signs, histopathology, immunohistochemistry, expression of interferon-related genes, and viral plaque assay. The results demonstrated that VHSV caused disease in Atlantic salmon infected by both methods. Fish had gross disease signs including darkening of the dorsal skin, bilateral exophthalmia, light cutaneous hemorrhage, and lethargy (Fig. 10). The virus replicated within endothelial cells causing endothelial cell necrosis leading to extensive hemorrhage in anterior kidney. Infected fish activated the type I interferon system as seen by up-regulation of genes for IFNα, Mx, and ISG15. Both IP-injected and immersion infected salmon were able to transmit the virus to SPF Pacific herring. The results demonstrate that farmed Atlantic salmon can develop clinical VHS

and virus can persist for at least 10 weeks in this host. With risks of VHS in farmed salmon and the potential for further host adaptation and spread of VHSV to native wild fishes sharing the environment, precautions should be taken to avoid the introduction of VHSV into salmon farms. These results are published in Lovy et al. (2013).

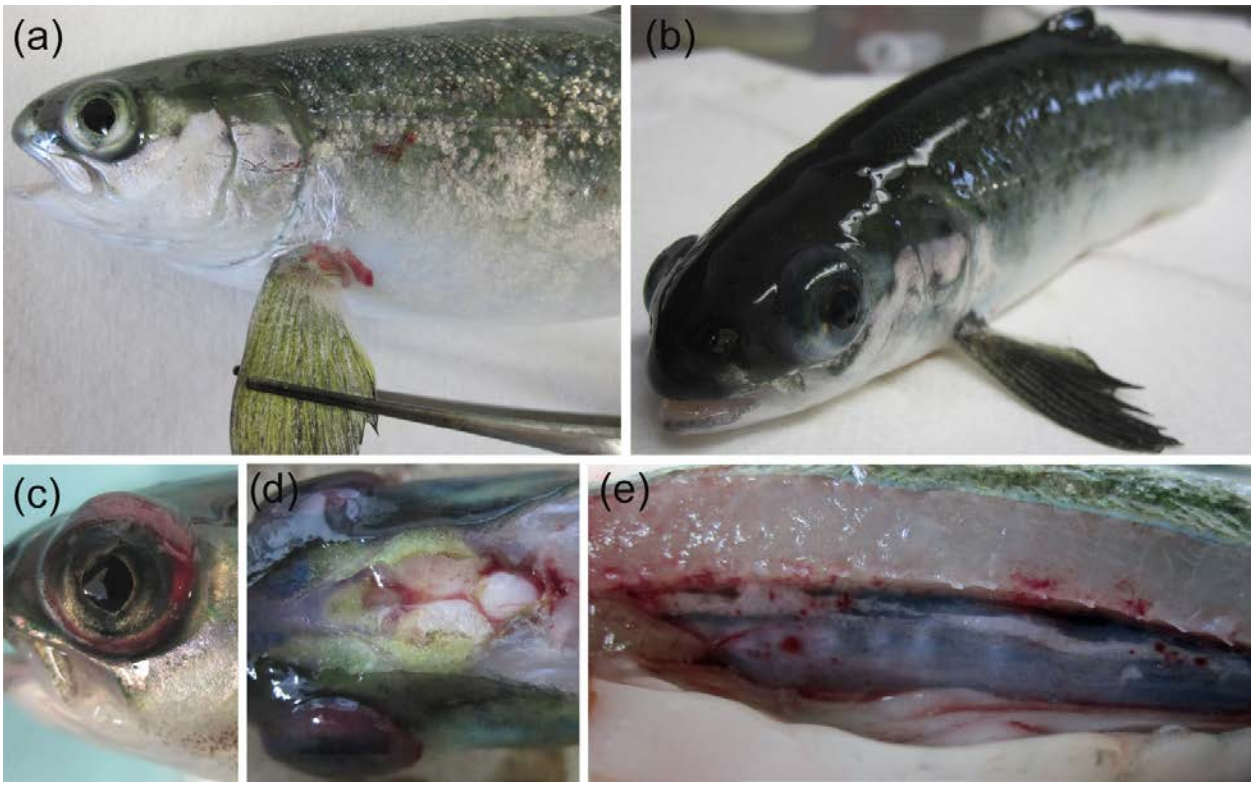

Figure 10. Gross clinical signs of VHSV in Atlantic salmon; similar signs appear in fish infected by IP-injection and immersion; shown in (a) through (d) are fish infected by immersion and (e) is a fish infected by IP-injection. Common signs included (a) skin hemorrhage frequently near the pectoral fin, (b) dark dorsal coloration and bilateral exophthalmia, (c) hemorrhage occurring within exophthalmic eyes, (d) hemorrhage in the brain cavity, and (e) hemorrhage on the swim bladder.

3.7. Influence of Temperature on the Efficacy of DNA Vaccines against Viral Hemorrhagic Septicemia in Pacific Herring

Homologous and heterologous (genogroup Ia) DNA vaccines against viral hemorrhagic septicemia virus (VHSV – genogroup IVa) conferred partial protection in Pacific herring. Early protection at 2 wk post vaccination (PV) was low and occurred only at elevated temperature (12.6^0C, 189 degree days - DD), where the relative percent survival (RPS) following viral exposure was similar for the two vaccines (IVa and Ia, respectively) and higher than that of negative controls at the same temperature. Late protection at 10 wk PV was induced by both vaccines but was higher with the homologous vaccine at both 9.0^0C and 12.6°C. Virus neutralization titers were detected among 55% of all vaccinated fish at 10 wk PV. The results suggest that the immune response profile triggered by DNA vaccination of herring was similar to that reported for rainbow trout where interferon responses occur in the early days PV and

transition to adaptive response at later time points. However, the protective effect was far less prominent in herring, possibly reflecting different physiologies and or adaptations of the two fish species. These results were published in Hart et al. (2017b); Appendix 9.

Table 4. Relative percent survival (RPS) 15 d after exposure of Pacific herring to viral hemorrhagic septicemia virus. All values are relative to a negative control group that was injected with saline (9.0 ^{0}C) in lieu of vaccine.

Vaccine treatment	Temperature	RPS (%)	
		2 wk post vaccination	10 wk post vaccination
Homologous IVa	9.0°C	0.0	40.4
	12.6°C	21.9	51.1
Heterologous Ia	9.0°C	0.9	11.1
	12.6°C	22.7	27.3
Plasmid	9.0°C	0.0	2.2
	12.6°C	5.7	4.2
Saline	12.6°C	7.9	48.3

Saline controls at 10 wk post-vaccination were comporomised due a co-infections with ENV that resulted in cross-protection against VHS.

Chapter 4: Experimental Studies Involving Viral Erythrocytic Necrosis (VEN).

4.1. Molecular Identification of Erythrocytic Necrosis Virus (ENV) from the Blood of Pacific Herring

A conventional polymerase chain reaction (PCR) technique was developed to detect ENV from the blood of infected Pacific herring. Presently, VEN is diagnosed by observation of typical cytoplasmic inclusion bodies in stained blood smears from infected fish. The causative agent, ENV, is unculturable and a presumed iridovirus by electron microscopy. *In vivo* amplification of the virus in cultured Pacific herring and subsequent virus concentration, purification, DNA extraction, and high-throughput sequencing methodologies were applied to obtain genomic ENV sequences. Fragments with the highest sequence identity to the family *Iridoviridae* were used to design four sets of ENV-specific polymerase chain reaction (PCR) primers. Testing of blood and tissue samples from experimentally and wild infected Pacific herring as well as DNA extracted from other amphibian and piscine iridoviruses verified the four assays were specific to ENV. Sensitivity testing determined a limit of detection of 0.0003 ng. Preliminary phylogenetic analyses of a 1448 bp fragment of the putative DNA polymerase gene supported inclusion of ENV in a proposed sixth genus of the family *Iridoviridae* that contains other erythrocytic viruses from ectothermic hosts (Fig. 11). This study provides the first molecular evidence of ENV's inclusion within the *Iridoviridae* family and offers a conventional PCR assay as a means of rapidly surveying the ENV-status of wild and propagated Pacific herring stocks. These results are published in Emmenegger et al. (2014); Appendix 3.

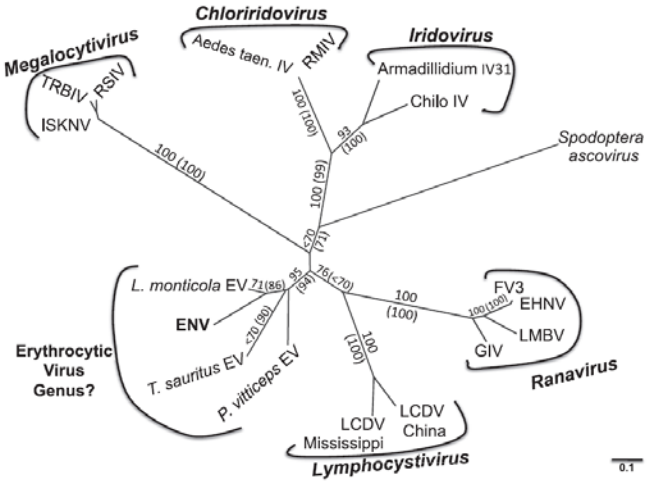

Figure 11. Maximum likelihood (ML) phylogenetic tree for ENV based on the predicted DNA dependent DNA polymerase amino acid sequences (174 - 572) of a 17 virus MUSCLE alignment. The tree was rooted to the outgroup *Spodoptera ascovirus*. ML bootstrap percent values of over 60 for the branchings from 500 re-samplings are displayed along with neighbor-joining (NJ) bootstrap percentages shown in parentheses. Erythrocytic necrosis virus (ENV) is highlighted in bold. Iridoviral genera are delineated with bracket arcs.

4.2. Identification of the Major Capsid Protein of Erythrocytic Necrosis Virus (ENV) and Development of Quantitative Real-Time PCR Assays

A highly sensitive quantitative PCR (qPCT) technique was developed that is capable of providing a quantitative assessment of ENV load in the tissues of infected Pacific herring. The technique will be capable of diagnosing ENV infections and VEN epizootics in the absence of available blood films. Phylogenetic analysis of the ENV DNA polymerase grouped ENV with other erythrocytic iridoviruses from snakes and reptiles. In the present study, we identified the gene encoding the ENV major capsid protein (MCP) and developed a quantitative PCR (qPCR) assay targeting this gene. Phylogenetic analysis of the MCP gene sequence supported the conclusion that the ENV does not group with any of the currently described iridovirus genera (Fig. 12). Because there is no information regarding genetic variation of the MCP gene across the reported host and geographic range for ENV, we also developed a second a qPCR assay for a more conserved ATPase-like gene region. The MCP and ATPase qPCR assays showed good analytical and diagnostic sensitivity and specificity based on samples from laboratory challenges of Pacific herring. The qPCR assays had similar diagnostic sensitivity and specificity as light microscopy of stained blood smears for the presence of intraerythrocytic inclusion bodies. However, the qPCR assays may detect viral DNA early in infection prior to the formation of inclusion bodies. Both qPCR assays appear suitable for viral surveillance or as a confirmatory test for ENV in Pacific herring from the Salish Sea. These results were published in Purcell et al. (2016).

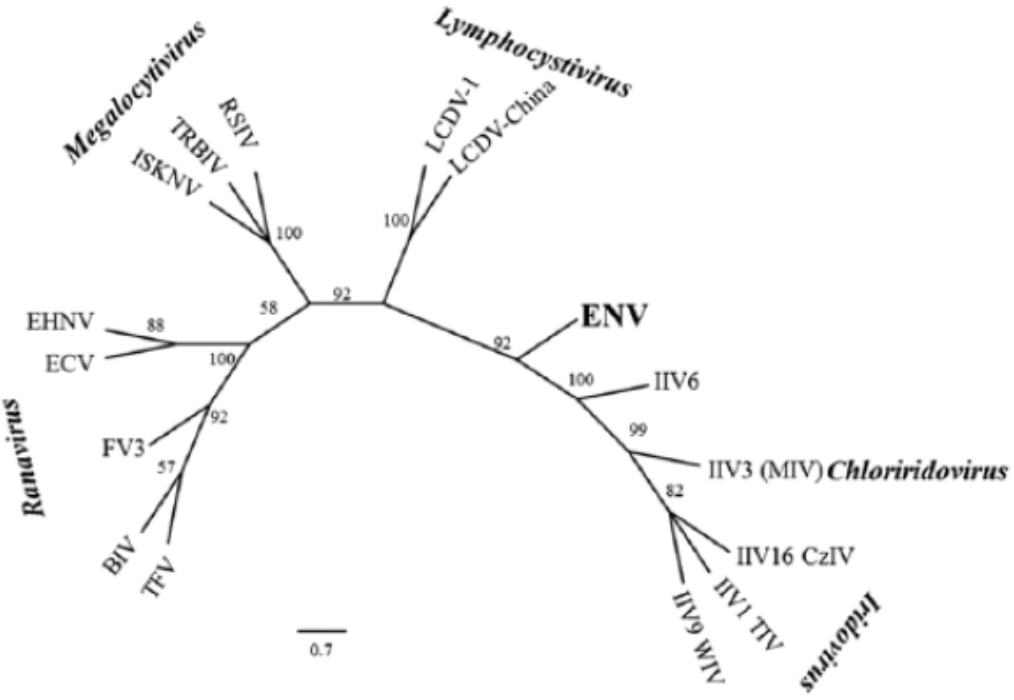

Figure 12. Relationship of erythrocytic necrosis virus (ENV) to other iridoviruses based on partial sequencing of the major capsid protein (MCP). Evolutionary relationships were inferred using the maximum likelihood with Poisson correction model. The numbers above the nodes represent bootstrap support after 1000 replicates.

Chapter 5: Additional Studies

5.1 Climate Change Influences on Marine Infectious Diseases: Implications for Management and Society

A review describing the impacts of climate change on marine diseases was published (Burge et al. 2014; Appendix 1), including a section on ichthyophoniasis (Table 5). Infectious diseases are common in marine environments, but the effects of a changing climate on marine pathogens are not well understood. Here, we focused on reviewing current knowledge about how the climate drives host-pathogen interactions and infectious disease outbreaks. Climate-related impacts on marine diseases are being documented in corals, shellfish, finfish, and humans; these impacts are less clearly linked to other organisms. Oceans and people are inextricably linked, and marine diseases can both directly and indirectly affect human health, livelihoods, and well-being. We recommended an adaptive management approach to better increase the resilience of ocean systems vulnerable to marine diseases in a changing climate. Land-based management methods of quarantining, culling, and vaccinating are not successful in the ocean; therefore, forecasting of conditions that lead to outbreaks and designing tools/approaches to affect these conditions may be the best tool to manage marine diseases.

Table 5. Reported ichthyophoniasis epizootics in wild fishes.

Time period	Location	Affected fish	Impacts	Reference(s)
1913–1914	Gulf of St. Lawrence, Canada	Atlantic herring	Dead fish "in great numbers were washed ashore on beaches or sand reefs, skirting the coast, or in quiet coves"	Cox 1914
1931–1932	Gulf of Maine	Atlantic herring	Infection prevalence reached 70% during the peak of the epizootic, then declined to 18%	Daniel 1933, Fish 1934
1940–1943	British Isles	Mackerel	Infection prevalence was as high as 100%, and the disease was described as fatal	Sproston 1944
1947	Gulf of Maine	Atlantic herring	No information	Scattergood 1948
1954–1956	Gulf of Saint Lawrence, Canada	Atlantic herring	At least half of the mature herring in the western Gulf of St. Lawrence were killed	Sindermann 1956, Tibbo & Graham 1963
1966–1970	Western North Atlantic	Yellowtail flounder	Infection prevalence was as high as 25–57%, and "there can be no question that many [affected flounder] must succumb directly to the infection"	Powles et al. 1968, Ruggieri et al. 1970
1991–1993	Eastern North Atlantic (Denmark, Sweden, Norway, and Iceland)	Atlantic herring	Total *Ichthyophonus*-induced mortality in the North Sea was estimated at 12.8–36.8%	Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997
1990s–2000s	Prince William Sound, Alaska	Pacific herring	The disease was a possible contributor to the population decline and failed recovery	Marty et al. 2010
Early 2000s	Yukon River, Alaska and Canada	Chinook salmon	The disease was a possible contributor to prespawn mortality	Kocan et al. 2004
2007	Columbia River, Washington and Oregon	American shad	Infection prevalence peaked at 72% and declined concomitantly with shad abundance	Hershberger et al. 2010
2008–2011	Iceland	Atlantic herring	Infection prevalence was as high as 70%	Oskarsson & Pálsson 2009

5.2 Infectious Diseases of Fishes in the Salish Sea

A review of the primary infectious and parasitic diseases affecting wild marine fishes in the Salish Sea was published (Hershberger et al. 2013b). As in marine regions throughout other areas of the world, fishes in the Salish Sea serve as hosts for many pathogens, including: nematodes, trematodes, protozoans, protists, bacteria, viruses, and crustaceans. Here, we review some of the better-documented infectious diseases that likely contribute to significant losses among free-ranging fishes in the Salish Sea and discuss the environmental and ecological factors that may affect the population-level impacts of disease. Demonstration of these diseases and their impacts to critical and endangered resources provides justification to expand pathogen surveillance efforts and to incorporate disease forecasting and mitigation tools into ecosystem restoration efforts.

5.3 Molecular Characterization of Hepatic Coccidiosis and a Morphological Report of a Novel Intestinal Coccidia.

Surveillance for pathogens of Atlantic herring, including VHSV, *Ichthyophonus hoferi*, and hepatic/intestinal coccidians, was conducted from 2012 to 2015 in the NW Atlantic Ocean, New Jersey, USA. Neither VHSV nor *I. hoferi* was detected from any samples. *Goussia clupearum* was found in the livers of 40-78% of adult herring in varying parasite loads; however, associated pathological changes were negligible (Fig. 13). Phylogenetic analysis placed *G. clupearum* most closely with other extraintestinal, liver coccidia from the genus *Calyptospora*, though the *G. clupearum* isolates had a unique nucleotide insertion between 604-729 bp that did not occur in any other coccidian species. *G. clupearum* oocysts from Atlantic and Pacific herring were morphologically similar, though differences occurred in oocyst dimensions. Comparison of the small subunit 18S ribosomal RNA gene of *G. clupearum* from Atlantic and Pacific herring revealed four nucleotide substitutions and two gaps in a 1749 bp region, indicating some divergence in the geographically separate populations. Intestinal coccidiosis, possibly attributed to more than one species, was described for the first time from juvenile and adult Atlantic herring. A novel intestinal coccidian species was detected based on morphological characteristics of exogenously sporulated oocysts. A unique feature in these oocysts was the presence of three long (15.1 ± 5.1 µm) spiny projections on both ends of the oocyst. The novel morphology of this coccidian led us to tentatively name this parasite *Eimeria echinata* n. sp. These results were published in Friend et al. (2016); Appendix 4.

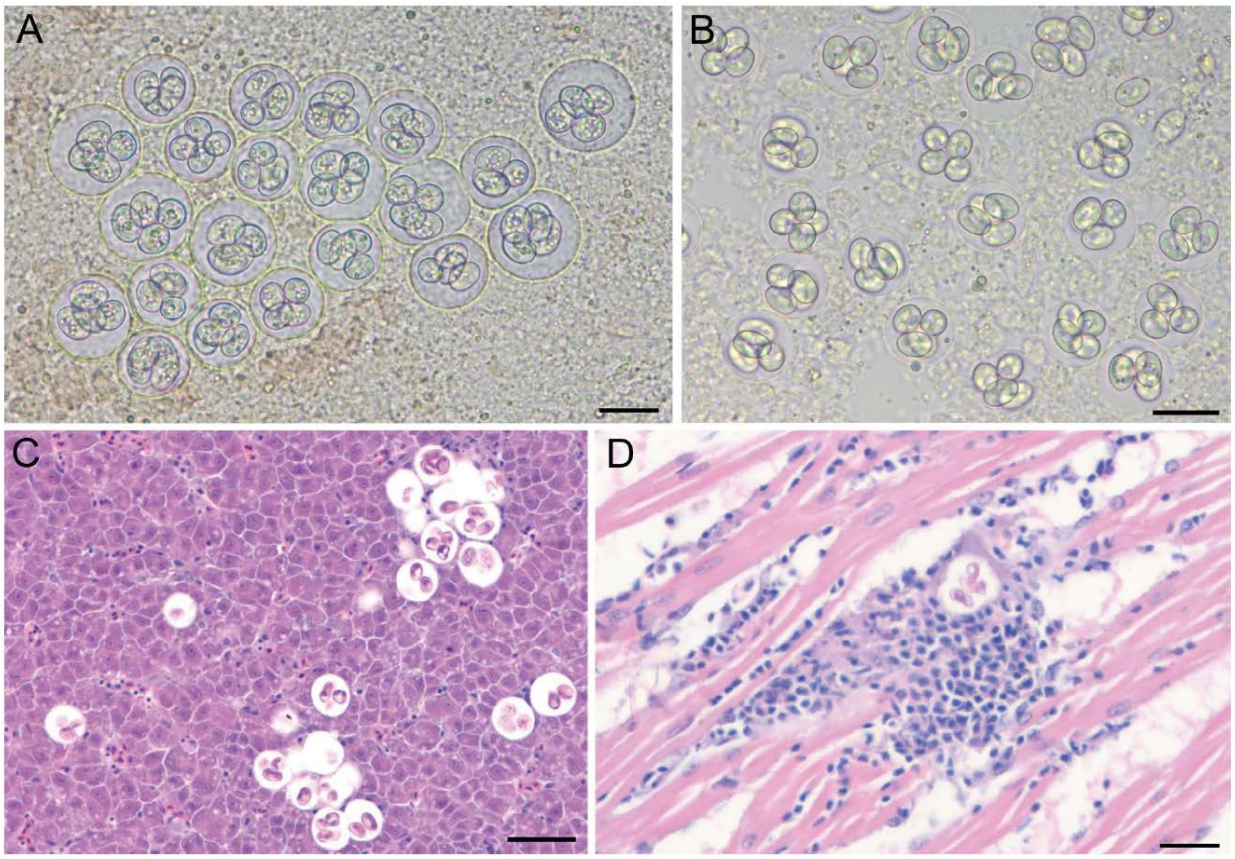

Figure 13. *Goussia clupearum,* bar=20µm (A-B) Wet mounts of fresh, homogenized liver tissue with (A) oocysts in Atlantic herring showing size variation of oocysts, and (B) oocysts in Pacific herring. (C-D) Histology of Atlantic herring stained with H&E showing (C) aggregates of oocysts in liver tissue and (D) Atlantic herring heart infected with coccidia resembling *Goussia clupearum* showing inflammatory response.

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2016: School of Aquatic and Fishery Sciences, University of Washington
Departmental Seminar (Nov 3, 2016): "Forecasting and Mitigating Disease Impacts in Wild Fishes"

2016: USGS Monthly Wildlife Disease Coordination Call
Webinar: "Current Research at the USGS – Marrowstone Marine Field Station"

2016: School of Aquatic and Fishery Sciences, University of Washington: FISH 404.
Guest Lecture and Facility Tour (April 14, 2016) "How Does Science Really Work?"

2014: Cordova Weekly Seminar Series (November 24, 2014)
Ecology of Disease in Pacific herring

2014: National Science Foundation, Research Coordination Network
Invited Presentation - Pathogen Persistence and Perpetuation Strategies in Marine Fishes: Perspectives from Pacific Herring (August 16, 2014). Friday Harbor, WA.

2014: School of Aquatic and Fishery Sciences, University of Washington: FISH 404.
Guest Lecture and Facility Tour (May 30, 2014) "How Does Science Really Work? The Frustration of Dead Ends and the Satisfaction of Tiny Advancements."

2014: Tribal Climate Change Webinar Series
Invited Webinar (May 21, 2014): Climate Change and Marine Issues
Shifting Ocean Currents and Infectious / Parasitic Diseases of Marine Fishes
Co-hosted by the Institute for Tribal Environmental Professionals – Northern Arizona University,– Pacific Northwest Tribal Climate Change Project - University of Oregon, and North Pacific Landscape Conservation Cooperative

2013: University of Southern Mississippi, Gulf Coast Research Laboratory
Invited Seminar (Nov 6)
Ecology of Diseases in Wild Marine Fishes
2013: Alaska Herring Managers Meeting
Invited Presenter (Nov 4-5)
Diseases of Pacific herring in Alaska
2013: University of Vermont (April 29)
Invited Seminars:
Biology Department: "Ecology of Infectious and Parasitic Diseases in Marine Fishes"
Experimental Program to Stimulate Competitive Research Committee: Research on Adaptation to Climate Change: "Impacts of Climate Change on Diseases of Marine and Anadromous Fishes"

Presentations at Scientific Meetings

Hershberger, P.K., L. Hart, A. MacKenzie, R, Powers, M. Purcell. January 23-27, 2017. Poster. Quantifying the potential for disease impacts to Pacific Herring. Alaska Marine Science Sympisium. Anchorage, AK.

Sitkiewicz, S., B. Harris, P. Hershberger, N. Wolf. January 23-27, 2017. Poster. Effects of the parasite *Ichthyophonus* (sp.) on groundfish growth and condition. Alaska Marine Science Symposium. Anchorage, AK.

Hart, L.M., M.K. Purcell, R.L. Powers, A.H. MacKenzie, P.K. Hershberger. June 26-30, 2016. Poster. Optimization of a plaque neutralization test capable of assessing the exposure history of Pacific herring to viral hemorrhagic septicemia virus. 2nd International Conference of Fish & Shellfish Immunology. Portland, ME.

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Elliott, D.G., C.L. McKibben, C.M. Conway, A. MacKenzie, P.K. Hershberger. September 7-11, 2015. Platform. Differential susceptibility of Yukon River and Salish Sea Chinook salmon (*Oncorhynchus tshawytscha*) stocks to *Ichthyophonus*. 17th International Conference on Diseases of Fish and Shellfish. Las Palmas de Gran Canaria, Spain.

Hart, L.M., P.K. Hershberger. August 16-20, 2015. Platform. Integration of disease information into population assessments: the case of VHS and Pacific herring. American Fisheries Society 145th Annual Meeting. Portland, OR.

Hershberger, P.K., J.L. Gregg, A.H. MacKenzie, M.L. Yanney, C. Conway, D.Elliott. June 2-4, 2015. Poster. Infecting Pacific herring (*Clupea pallasii*) with *Ichthyophonus* in the laboratory. 56th Annual Western fish Disease Workshop. Steamboat Springs, CO.

Gregg, J.L., C. Dykstra, P.K. Hershberger. November 9-14, 2014. Platform. Epizootiology of *Ichthyophonus* sp. in Pacific Halibut (*Hippoglossus stenolepis*) in the Northeast Pacific Ocean and Bering Sea. 9th International Flatfish Symposium. Cle Elum, WA.

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Garver, K.A., J. Lovy, P. K. Hershberger. August 31 – September 4, 2014. Platform. Trafficking of Viral Hemorrhagic Septicemia Virus from wild to farmed fish. 7th International Symposium on Aquatic Animal Health. Portland, OR.

Hart, L.M. C. Conway, D. Elliott, P.K. Hershberger. August 31 – September 4, 2014. Platform. A qualitative assessment of the progression of ichthyophoniasis related external signs and distribution of host response and parasite morphology in somatic tissues of Pacific herring *Clupea pallasii*. 7th International Symposium on Aquatic Animal Health. Portland, OR.

McKibben, C.L., P.K. Hershberger, M.K. Purcell, C.M. Conway, D.G. Elliott. August 31 – September 4, 2014. Poster. Influence of Temperature and Fish Stock on Progression of *Ichthyophonus* Infections in Chinook Salmon (*Oncorhynchus tshawytscha*). 7th International Symposium on Aquatic Animal Health. Portland, OR.

R.M. Kocan, P.K. Hershberger. August 31 – September 4, 2014. Platform. Impact of *Ichthyophonus* sp. on pre-spawn Yukon River Chinook salmon. 7th International Symposium on Aquatic Animal Health. Portland, OR.

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Garver, K.A., J. Lovy, P.K. Hershberger. September 2-6, 2013. Platform. Viral hemorrhagic septicemia virus in British Columbia, Canada: transmission from wild to farmed fish. 16th International Conference on Diseases of Fish and Shellfish; European Association of Fish Pathologists. Tampere, Finland.

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Appendix 1

ANNUAL REVIEWS Further
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Climate Change Influences on Marine Infectious Diseases: Implications for Management and Society

Colleen A. Burge,[1] C. Mark Eakin, Carolyn S. Friedman, Brett Froelich, Paul K. Hershberger, Eileen E. Hofmann, Laura E. Petes, Katherine C. Prager, Ernesto Weil, Bette L. Willis, Susan E. Ford, and C. Drew Harvell[1]

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Keywords

epizootics, mass mortalities, health, oceans, ocean warming

Abstract

Infectious diseases are common in marine environments, but the effects of a changing climate on marine pathogens are not well understood. Here we review current knowledge about how the climate drives host-pathogen interactions and infectious disease outbreaks. Climate-related impacts on marine diseases are being documented in corals, shellfish, finfish, and humans; these impacts are less clearly linked for other organisms. Oceans and people are inextricably linked, and marine diseases can both directly and indirectly affect human health, livelihoods, and well-being. We recommend an adaptive management approach to better increase the resilience of ocean systems vulnerable to marine diseases in a changing climate. Land-based management methods of quarantining, culling, and vaccinating are not successful in the ocean; therefore, forecasting conditions that lead to outbreaks and designing tools/approaches to influence these conditions may be the best way to manage marine disease.

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INTRODUCTION

Infectious disease: a disease caused by a transmittable agent (e.g., a virus, bacterium, parasite, fungus, alga, or prion) that infects the host tissues, leading to an identifiable illness or syndrome

Pathogen: a causative agent of disease; under certain conditions, parasites may also cause disease

Marine ecosystems are among the most valuable and heavily used natural systems worldwide (Staudinger et al. 2012) and provide critical ecosystem services, including shoreline protection, water filtration, nursery grounds, food from fisheries and aquaculture, and revenue from tourism (reviewed in Ruckelshaus et al. 2013). For example, coastal wetlands provide storm protection services estimated to be worth $23.2 billion per year in the United States alone (Costanza et al. 2008). Seafood currently provides vital jobs and ~15% of the animal protein intake for the world's population (Cooley et al. 2012). In addition to being economically and ecologically valuable, marine ecosystems and their interconnected web of organisms (e.g., from microbes to phytoplankton to zooplankton to shellfish to fish to marine mammals) are vulnerable to natural and human perturbations (Gilman et al. 2008). Marine ecosystems are influenced not only by the direct effects of stressors on populations and species but also by changes in species interactions, including competition, predation, parasitism, and mutualism (reviewed in Kordas et al. 2011). Preserving the health of marine ecosystems and managing them effectively require determining the effects of such stressors, including marine infectious disease and climate change.

Infectious diseases are important drivers within ecosystems. There are many well-documented terrestrial examples of ecosystems being reshaped by large-scale outbreaks of infectious disease, such as Dutch elm disease and chestnut blight (Anderson et al. 2004). Diseases may also impact marine ecosystems by influencing community structures, age distributions, trophic interactions, hydrodynamics, and biotic structures, as has been observed in relation to diseases in seagrasses (e.g., eelgrass wasting disease; reviewed in Burge et al. 2013), reef-building corals (Aronson & Precht 2001), oysters (Mann et al. 2009), and sea urchins (Lessios et al. 1984, Lauzon-Guay et al. 2009). Diseases have had large impacts on both cultured and wild harvests of commercially important species, such as salmon [e.g., *Ichthyophonus* infection in marine and anadromous fish (reviewed in McVicar 2011) and viral infections in Atlantic and Pacific salmon (reviewed in Kurath & Winton 2011)], abalone (e.g., withering syndrome; Friedman et al. 2000), and crustaceans (e.g., protozoan infections of natural populations and viruses in aquacultured species; reviewed in Stentiford et al. 2012). In both terrestrial and marine systems, interactions between hosts, pathogens, and the environment govern disease outbreaks, and a change in any of these components can shift the balance toward or away from a high-intensity disease state. As many host-pathogen interactions are highly vulnerable to changes in environment, climate change can alter the likelihood of disease outbreaks. Climate change has altered terrestrial agricultural disease risk (Garrett et al. 2012) and may be affecting human disease risk (Harvell et al. 2002, 2009; Lafferty et al. 2004; Baker-Austin et al. 2012). We are only beginning to understand the effects of infectious diseases in the ocean and how climate change will affect marine host-pathogen interactions, both of which are critical for informing conservation and management efforts (Harvell et al. 2009).

The effects of climate change and ocean acidification are being documented in oceans around the world (IPCC 2007, Doney et al. 2012). Here, we review aspects of climate change that could affect host-pathogen interactions, as these changes will have large impacts on disease outbreaks. Climate change is affecting ocean physical, chemical, and biological systems as well as human uses of ocean resources. Rising levels of atmospheric carbon dioxide (CO_2) are leading to increased global atmospheric and ocean temperatures; without significant near-term reductions in CO_2 levels, ocean warming is likely to continue (IPCC 2007). Increasing temperatures lead to physical impacts on ocean systems, including rising sea levels, increased ocean stratification, loss of sea ice, and altered oceanic circulation (Doney et al. 2012, Howard et al. 2013). Warming temperatures have already affected the survival, growth, reproduction, health, and phenology of marine organisms (Doney et al. 2012). For example, periods of thermal stress contribute to mass

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coral bleaching (Hoegh-Guldberg et al. 2007) and disease outbreaks (Harvell et al. 2002, 2009). Increased periodicity of anomalous thermal events is reducing the capacity for recovery between events (Baker et al. 2008, Eakin et al. 2010).

In addition to temperature-related consequences, the increased CO_2 concentrations in the atmosphere are raising CO_2 concentrations in the ocean, causing chemical reactions that reduce ocean pH, a phenomenon termed ocean acidification (Doney et al. 2009). The ocean has become 30% more acidic over the past century (Feely et al. 2004), and the rate of acidification is accelerating (Gattuso & Hansson 2011), creating conditions unparalleled in the past 300 million years (Hönisch et al. 2012). Ocean acidification directly threatens the health of many calcifying organisms, including pteropods (Fabry et al. 2009), corals (Hoegh-Guldberg et al. 2007), and oysters (Barton et al. 2012).

Physical and chemical changes associated with climate change and ocean acidification are affecting the health of marine organisms and ecosystems (Harvell et al. 2009). At least five well-characterized syndromes of corals—white syndromes in the Caribbean and Pacific, white patch disease (also known as white pox disease or *Acropora* serriatosis) of threatened acroporids in the Caribbean, white plague, black band disease, and Caribbean yellow band disease—are temperature sensitive, and disease outbreaks are increasing with warming (Harvell et al. 2009). Other marine organisms, such as abalones, fish, seagrasses, and sea urchins, are also affected by warming oceans, as are humans. Many marine organisms, including marine pathogens, are shifting their distributions poleward as ocean temperatures warm (e.g., Parmesan & Yohe 2003, Nye et al. 2009, Baker-Austin et al. 2012). Outbreaks of *Vibrio* bacterial infections affecting humans have been recently reported as far north as the Baltic Sea (Baker-Austin et al. 2012) and Alaska (Martinez-Urtaza et al. 2010). Similarly, the ranges of some protistan diseases [e.g., Dermo disease (perkinsosis) and MSX (multinucleated sphere unknown) disease of the eastern oyster, *Crassostrea virginica*] have moved further north with changing temperatures (Burreson & Ragone Calvo 1996, Ford & Tripp 1996).

Environmental changes, including temperature increases, have been linked to enhanced disease expression (reviewed in Harvell et al. 2002). Climate shifts can impair the immune response of a host and increase the frequency of disease. This is especially true for ectothermic organisms such as shellfish (Travers et al. 2009), corals (Harvell et al. 2002), and finfish (reviewed in Bowden 2008). In the US states of Oregon and Washington, the reemergence of *Vibrio tubiashii*, a bacterial pathogen of larval Pacific oysters (*Crassostrea gigas*), was linked to climate-related changes, including thermal shifts and upwelling of low-pH waters (Elston et al. 2008). Changes in host species can also increase disease frequency. For example, inbreeding of the host species could favor the parasite over the host (e.g., Dionne et al. 2007). Conversely, changes in the parasite could result in increased virulence (enhancing the parasite's ability to infect and overcome the host immune response) or increased pathogenicity (increasing clinical disease associated with infection; e.g., Martenot et al. 2011). Alternatively, changes in the host, pathogen, or environment may favor the host, thereby reducing or eliminating a particular pathogen or disease in the affected population. For example, Pacific oysters selected for resistance to summer mortality were more resistant to a subsequent herpesvirus infection (Dégremont 2011).

Here, we consider the potential consequences of climate-related factors (e.g., temperature, rainfall, storms, and increased acidity) for marine infectious disease outbreaks (see **Figure 1**) as well as the known effects of climate on disease-related mass mortality events (**Table 1**). We review (*a*) the known and hypothesized impacts of climate change on the dynamics of marine infectious disease, focusing on effects on host resistance, pathogen virulence, and interactions between host resistance and pathogen virulence; (*b*) the known and potential consequences for human society; (*c*) the tools needed to fill future knowledge gaps associated with the impacts of climate change on

Parasite: an organism (often microscopic) that is metabolically dependent on its host and typically gains energy or food from its host, thus creating a negative association

Virulence: the characteristics of a pathogen that allow it to infect, multiply, and spread within or among hosts

Pathogenicity: the ability of a pathogen to cause disease in its host

Resistance: an organism's natural tolerance to infection

Opportunistic pathogens: pathogens that can survive outside a host and are often ubiquitous in the environment and within both healthy and diseased hosts, but that are pathogenic only under specific conditions

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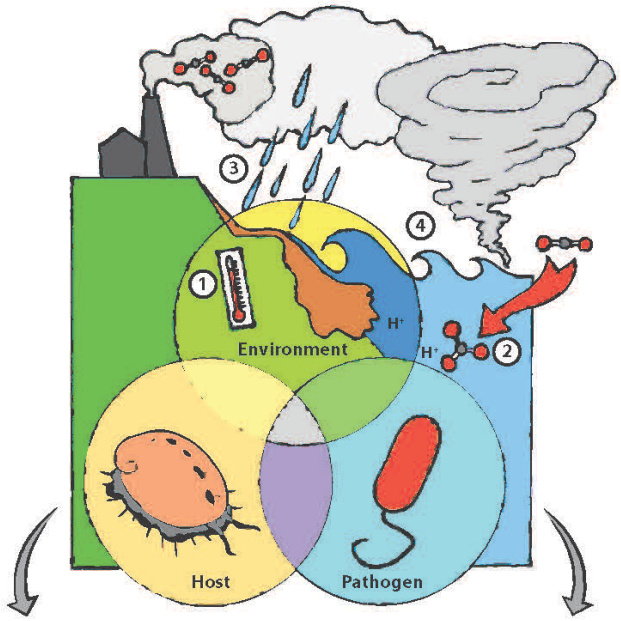

Changes in host	Climate factor	Changes in pathogen
• Increased susceptibility • Change in behavior • Invasions • Range shifts	1	• Increased activity • Change in virulence • New emergence • Invasions • Range shifts
• Increased susceptibility (?) • Change in larval survival/recruitment • Change in behavior • Reductions in diversity (?)	2 H^+ H^+ $2-$	• Change in virulence • Change in abundance
• Increased susceptibility • Nutrient stress • Salinity stress • Contaminant stress	3	• New emergence • Change in virulence • Invasions • Range shifts • Increased nutrients
• Increased susceptibility • Physical injury	4	• New emergence • Invasions • Range shifts • Mixing/resuspension of particles

Figure 1

Climate change impacts on marine host-pathogen-environment relationships. Shifts in the global environment are leading to physical ocean changes, including ① changes in temperature, ② increases in CO_2 concentrations/decreases in pH, ③ changes in precipitation (leading to changes in salinity), and ④ exposure to storms and cyclones. All of these factors are shifting the host-pathogen-environment equilibrium.

marine host-pathogen interactions; and (*d*) potential management actions to increase the resilience of ocean ecosystems, communities, and economies in the face of changes in disease. Specifically, we describe host-pathogen relationships where sufficient information exists, using case studies on the known and hypothesized effects of climate change on marine infectious diseases from invertebrate (corals, abalones, and oysters) and vertebrate (marine mammals, finfish, and humans) species.

CASE STUDIES: IMPACTS OF CLIMATE CHANGE ON MARINE HEALTH

The Decline of Coral Reef Ecosystems

Hermatypic corals have evolved in tropical oceans that in the recent past were characterized by relatively gradual changes in temperature and pH ranges. Over the past four decades, however, increasing environmental stress from rapidly changing climate-related and interacting climate-related and anthropogenic factors has disrupted the balance between hosts, agents, and the environment that underpins coral health. Disruption of coral-microbial symbioses and concomitant reduced resistance to opportunistic pathogens have been major factors in the deterioration of coral reef communities worldwide (e.g., Bruno & Selig 2007, Hoegh-Guldberg et al. 2007, Miller et al. 2009, Weil et al. 2009a, De'ath et al. 2012). Nowhere is coral reef deterioration more widespread and intense than in the wider Caribbean region, a disease hot spot characterized by frequent temperature-induced mass coral bleaching (loss of photosymbionts), the rapid emergence of a variety of new and virulent diseases, and typically higher disease prevalence than in other reef regions (reviewed in Harvell et al. 2007, Ruiz-Moreno et al. 2012). Alarming declines in coral cover have also been recorded on Indo-Pacific reefs (Bruno & Selig 2007, De'ath et al. 2012) along with the first records of several new diseases (Antonius 1999, Raymundo et al. 2003, Willis et al. 2004), highlighting the global nature of declining coral health.

Temperature-induced stress has been a key factor in mass mortalities associated with bleaching and infectious disease in coral reef organisms (Harvell et al. 1999, 2002, 2009; Hoegh-Guldberg et al. 2007; Carpenter et al. 2008; Croquer & Weil 2009a; McClanahan et al. 2009; Miller et al. 2009; Weil et al. 2009a; Rogers & Muller 2012). Significant warming of the Caribbean basin in the past 25 years (Chollet et al. 2012), including six major thermal anomalies and warmer winters, has coincided with coral bleaching events, disease emergence, and an increasing frequency of infectious disease outbreaks (Weil et al. 2009a, Eakin et al. 2010, Ruiz-Moreno et al. 2012). Similarly, on the Great Barrier Reef, high summer thermal anomalies and mild winter temperatures have been linked to outbreaks of tissue-loss coral diseases (e.g., white syndromes) (Bruno et al. 2007, Heron et al. 2010, Maynard et al. 2011). The host range and abundance of one of the most temperature- and nutrient-sensitive coral diseases, black band disease, have increased worldwide, likely reflecting the combined impacts of compromised host resistance and enhanced pathogen virulence associated with increasing seawater temperature and declining water quality (Voss & Richardson 2006; Sato et al. 2009, 2011).

Water quality, encompassing measures of turbidity, nutrient load, sediments, and pollutants, is a key factor in coral health that is influenced by a changing climate, with significant implications for the emergence and spread of coral diseases. Projected increases in the frequency of extreme weather events and associated increases in precipitation (Bender et al. 2010) could reduce coastal and ocean water quality through terrestrial inputs from runoff and resuspension events. Increases in terrestrial runoff lead to reduced salinity, enhanced sediments, increased human sewage pollution, and increased nutrients; this combination can compromise host resistance, as shown by links

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Table 1 Mass mortalities (>10%) of marine organisms associated with environmental correlates

Time period	Location	Host organism	Causative agent or syndrome	Environmental correlates	Source
1913–present	North Atlantic and North Pacific	Finfish	*Ichthyophonus* sp.	?	See herein; **Table 2**
1931–present	North America and Europe	Marine plant (seagrass)	*Labyrinthula* sp.	HT, salinity	Burge et al. 2013 (review)
1946–present	Gulf Coast and Northeast Atlantic	Bivalve (eastern oyster)	*Perkinsus marinus* (Dermo)	HT, salinity	See herein
1958–present	Northeast Atlantic	Bivalve (eastern oyster)	*Haplosporidium nelsoni* (MSX)	HT, high salinity	See herein
1960–present	Northeastern North America	Bivalve (hard clam)	Thraustochytrids (QPX)	HT, high salinity	Lyons et al. 2007, Burge et al. 2013 (review)
1974–present	Europe	Bivalve (flat oyster)	*Marteilia refringens*	Salinity?	Elston & Ford 2011 (review)
1975	Western United States	Echinoderm (starfish)	?	HT	Dungan et al. 1982
1979–1980	New England, United States	Marine mammal (harbor seal)	Influenza A virus	HT	Geraci et al. 1982
1979–1983	Caribbean	Corals (*Acropora* spp.)	White band disease	M-HT	Aronson & Precht 2001
1982	Central America	Octocorals	Pathogen?	HT	Guzmán & Cortés 1984
1982–1986	Australia	Gastropod (abalone)	*Perkinsus* sp.	HT	Villalba et al. 2004 (review)
1983	Caribbean	Corals	Black band disease	Seasonal	Ruetzler et al. 1983
1983	Caribbean	Echinoderm (urchin)	Bacteria?	HT	Lessios et al. 1984
1985–present	Northeast Pacific	Gastropod (abalone)	"*Candidatus* Xenohaliotis californiensis"	HT	See herein
1987–present	Florida	Marine plant (seagrass)	*Labyrinthula* sp.	HT, salinity	Robblee et al. 1991
1988	Northwestern Europe	Marine mammal (harbor seal)	Phocine distemper virus	HT	Lavigne & Schmitz 1990
1990–1992	Western Mediterranean	Marine mammal (striped dolphin)	Dolphin morbillivirus	HT	Aguilar & Raga 1993
1991–present	Worldwide	Bivalves (primarily Pacific oysters)	Oyster herpesvirus type 1	Seasonal	Martenot et al. 2011
1992	Northeastern New Zealand	Algae (kelp)	?	High turbidity	Cole & Babcock 1996
1993–present	Florida	Corals (*Acropora* spp.)	White patch disease	HT	Patterson et al. 2002
1995	Florida	Corals	White plague type II	HT	Richardson et al. 1998
1995–present	Caribbean	Octocorals (primarily sea fans)	*Aspergillus sydowii* and other fungi	HT	Burge et al. 2013 (review)
1996-present	New England, United States	Crustacea (lobster)	Epizootic shell disease	HT, pollutants?	Castro et al. 2012 (review)

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1997	Vancouver Island, Canada	Bivalve (Pacific oyster)	*Mikrocytos mackini*	LT	Bower et al. 1997
1998	Florida	Octocorals	Cyanobacteria	HT	Harvell et al. 2002
1998–1999	Central Visayas, Philippines	Corals	Porites ulcerative white spot disease	?	Raymundo et al. 2003
1998–present	France	Gastropod (abalone)	*Vibrio harveyi*	HT	Travers et al. 2009
1999–2003	Mediterranean Sea	Octocorals (gorgonian)	*Vibrio coralliilyticus*	HT	Bally & Garrabou 2007
2000	Caspian Sea	Marine mammal (seal)	Canine distemper virus	HT	Kuiken et al. 2006
2000–2002	Magnetic Island, Great Barrier Reef	Corals	Atramentous necrosis	Seasonal HT	Jones et al. 2004
2001	New England, United States	Bivalve (Eastern oyster)	*Roseovarius* oyster disease	M-HT, pollution	Ford & Tripp 1996, Boettcher et al. 2005
2002–2003	Great Barrier Reef	Corals	White syndrome	HT, mild winter temperatures	Willis et al. 2004, Bruno et al. 2007, Heron et al. 2010, Maynard et al. 2011
2003–2009	Puerto Rico	Corals	Caribbean yellow band disease, white plague disease	HT-BLE	Bruckner & Hill 2009; **Figure 2** herein
2005–2006	Virgin Islands	Corals	Multiple diseases	HT-BLE	Miller et al. 2009
2005–2006	Puerto Rico	Corals	Multiple diseases	HT-BLE	Weil et al. 2009a
2006–2007	US west coast	Bivalves (oysters)	*Vibrio tubiashii*	HT, nutrient enrichment	Elston et al. 2008
2006–2008	Pelorus Island, Great Barrier Reef	Corals	Black band disease	Seasonal HT and light	Sato et al. 2009
2008–2009	Great Barrier Reef	Corals	Atramentous necrosis	HT, low salinity, high particulate organic carbon	Haapkylä et al. 2011
2010–2011	Los Roques, Venezuela	Corals	Black band disease, white plague disease	HT-BLE	Bastidas et al. 2012
2010–2011	Curaçao	Corals	Multiple diseases	HT-BLE	E. Weil, unpublished data
2010–2011	Grenada	Corals	Multiple diseases	HT-BLE	E. Weil, unpublished data

Abbreviations: BLE, coral bleaching; HT, high temperatures; M-HT, mid-to-high temperatures; LT, low temperatures. "Seasonal" indicates that mortalities occur seasonally with maximum temperatures.

with outbreaks of the coral disease atramentous necrosis (Haapkylä et al. 2011), and can potentially increase the frequency of opportunistic pathogens (Burge et al. 2013). Severe storms also compromise host resistance through direct breakage, abrasion, and surface injuries that provide entry points for pathogens. Additionally, declining seawater pH will undermine skeletal supporting structures, further increasing the susceptibility of corals to breakage injuries. Rising temperatures, intensifying storms, and falling pH adversely affect not only adult corals but also those at earlier life-history stages (e.g., Negri et al. 2007, Albright et al. 2010), which, in combination with reduced reproductive output caused by bleaching and disease (e.g., Szmant & Gassman 1990, Weil et al. 2009b), suggests recurrent reproductive and recruitment failures that will increasingly hinder future reef recovery.

Epizootic: an unexpected increase in disease or mortality in nonhuman hosts that occurs in an unusual time or place or at an unusually high frequency

Coral epizootics illustrate how a cascade of interacting environmental and anthropogenic disturbances can dramatically alter community structure in coral reef ecosystems, leading to the need for management and restoration. Populations of two important reef-building corals (*Acropora cervicornis* and *Acropora palmata*) in the Caribbean crashed in the early 1980s (Gladfelter 1982, Aronson & Precht 2001); this was followed more recently by outbreaks of white patch disease in the Florida Keys and the Virgin Islands (Rogers & Muller 2012) that were caused by the fecal endobacterium *Serratia marcescens* and linked with human sewage in the Florida Keys (Sutherland et al. 2010). Similarly, an unknown pathogen caused mass mortality of the black sea urchin (*Diadema antillarum*) (Lessios et al. 1984), a keystone species controlling macroalgal biomass, which led to algal overgrowth of reefs and reduced space for coral recruitment. *Acropora* and *Diadema* populations experienced >90% mortality throughout their Caribbean ranges and have not recovered even 30 years after the epizootics (Weil et al. 2005). Remaining reef-building genera and other important groups have been affected by severe storms, major widespread bleaching events (in 1998, 2005, and 2010), and outbreaks of a variety of biotic diseases. The consequence has been a cascade of dramatic decreases in coral cover, increases in macroalgal cover, and changes in community structure and function (e.g., Hughes 1994; Richardson et al. 1998; Aronson & Precht 2001; Carpenter et al. 2008; Bruckner & Hill 2009; Croquer & Weil 2009a,b; McClanahan et al. 2009; Miller et al. 2009; Weil et al. 2009b) (**Figure 2**). Disease outbreaks contributed to the 2006 listing of two Caribbean coral species as threatened under the US Endangered Species Act, and to a 2012 proposal to list seven species as endangered and two additional species as threatened.

Pathogen identification and environmental correlates of coral disease remain critical knowledge gaps for understanding how coral health can be managed in the context of changing climate. Clearly, managing water quality is important for reducing environmental burdens that interact with climate change. Evidence is accumulating that marine protected areas (MPAs) with intact reef communities and reduced fishing disturbances may help maintain coral health (Page et al. 2009, Raymundo et al. 2009). Although MPAs may not protect coral reefs against climate-related threats (e.g., Miller et al. 2009, Weil et al. 2009a, Selig & Bruno 2010), MPA networks may provide important mechanisms to increase ecosystem resilience (Keller et al. 2009), potentially reseeding nearby degraded reefs. MPAs will become increasingly important for managing the growing litany of unprecedented challenges to the long-term persistence of coral reef ecosystems, which are critical for developing and developed economies that depend on the ecosystem services provided by these systems. In the case of localized coral disease outbreaks and mass coral bleaching events (which are noninfectious in nature), there is potential for early detection to prepare for vulnerabilities and implement management plans. The US National Oceanic and Atmospheric Administration (NOAA) Coral Reef Watch program provides real-time bleaching forecasts based on temperature anomalies detected as the accumulation of degree heating weeks (available at **http://coralreefwatch.noaa.gov/satellite**). Building on this program, an *Acropora* white syndrome disease forecasting algorithm was developed based on the accumulation of warm

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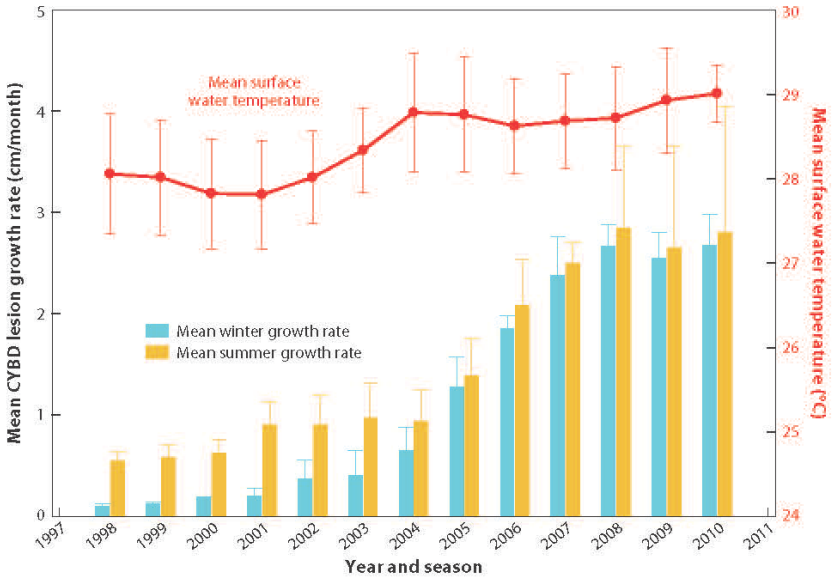

Figure 2

Seasonal (winter and summer) variability in mean linear growth rates of Caribbean yellow band disease (CYBD) lesions measured in tagged colonies of *Orbicella faveolata* in reefs off La Parguera, Puerto Rico, from 1998 to 2010. A positive ($r^2 = 0.82$) and significant ($p < 0.001$) correlation was found between mean lesion growth rates and mean yearly surface water temperatures.

temperature anomalies and the accumulated heat or cold stress from the preceding winter (Heron et al. 2010, Maynard et al. 2011). Other coral disease management actions that have been discussed include boosting coral immunity, cleaning off external signs of disease, performing phage therapy, and administering probiotics (summarized in Beeden et al. 2012).

Marine Bivalve Diseases

Much is known about the influence of diseases on marine bivalve shellfish, especially those of economic concern. Examples include bonamiasis in the European flat oyster (*Ostrea edulis*) (Grizel et al. 1988), QPX (quahog parasite unknown) disease in the eastern quahog (*Mercenaria mercenaria*) (Lyons et al. 2007), MSX and *Roseovarius* oyster disease in the eastern oyster (*C. virginica*) (Ford & Tripp 1996, Boettcher et al. 2005), and Dermo disease in a variety of molluscs worldwide (Villalba et al. 2004). The best studied of the marine bivalve hosts affected by infectious disease are oysters, which experience a disproportionately high incidence of lethal disease compared with other commercial bivalves (Ford & Tripp 1996, Ford et al. 2012). Oysters are also of ecological importance owing to the services they provide via reef habitats, benthic-pelagic coupling, and water filtration and as prey for many organisms (e.g., Grabowski & Peterson 2007). Hence, in addition to being economically devastating, oyster diseases affect overall ecosystem productivity and health.

Two diseases, both caused by protozoans, severely impact eastern oyster populations in estuarine environments along the US east and Gulf of Mexico coasts (Ford & Tripp 1996). The first,

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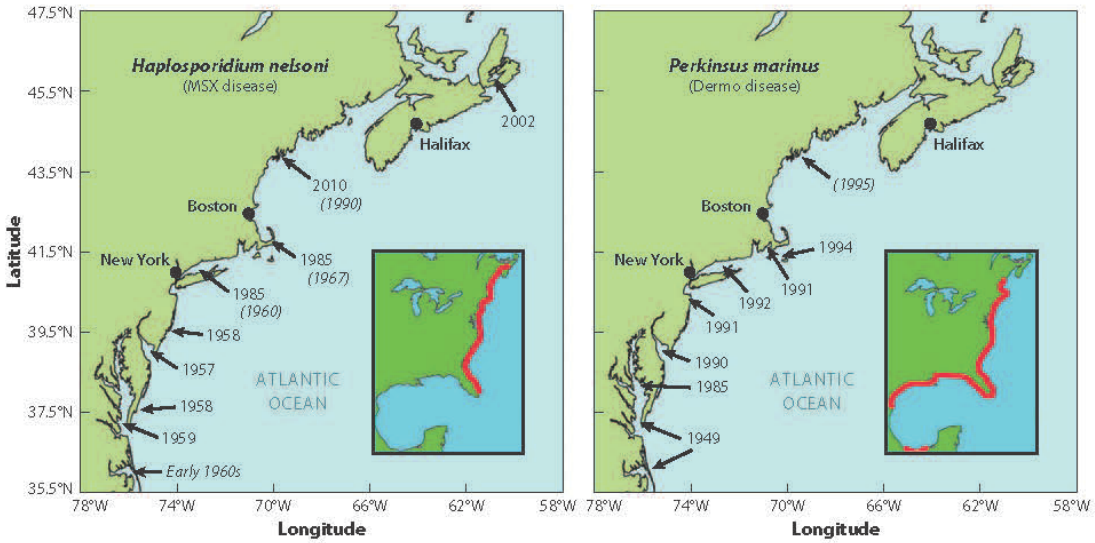

Figure 3

Range extension of oyster disease outbreaks in the northeastern United States and Canada. Years of first reported mortality are shown in roman type; when different, years in which the pathogen was first reported are shown in italic. No mortality has been associated with the northernmost report of *Perkinsus marinus* (in Maine, United States). The northward extension of *P. marinus* (Dermo disease) epizootics coincided with a pronounced winter warming period beginning in the mid-1980s, and range extension was especially pronounced between 1990 and 1992, when disease outbreaks occurred over a 500-km range from Delaware Bay, New Jersey, to southern Massachusetts. The period between 1989 and 1995 was also marked by consistently positive North Atlantic Oscillation anomalies. Insets show the parasites' entire ranges, including everywhere they have been reported.

Dermo disease, is caused by *Perkinsus marinus* (Mackin et al. 1950); it was identified in the late 1940s as the cause of extensive mortalities in the Gulf of Mexico, and was almost immediately found along the southeastern US coast and into lower Chesapeake Bay. In the mid-1980s, Dermo disease outbreaks intensified and spread northward (Figure 3), causing heavy oyster mortality as far north as Great Bay, New Hampshire. Although *P. marinus* has been found in Maine, it has not caused mortalities there (see review in Ford & Tripp 1996). The second disease, MSX disease, is caused by the protozoan *Haplosporidium nelsoni* (Haskin et al. 1966), an introduced parasite (Burreson et al. 2000). The first recorded MSX outbreak occurred in Delaware Bay, United States, in the spring of 1957 (Haskin et al. 1966), and additional devastating MSX epizootics occurred in oyster populations along the US mid-Atlantic coast and subsequently spread northward (Figure 3).

Temperature and salinity are known drivers of Dermo and MSX disease (Burreson & Ragone Calvo 1996, Ford & Tripp 1996); the prevalence and intensity of these diseases are subject to influence by cyclical climate patterns, such as the El Niño–Southern Oscillation (ENSO) and North Atlantic Oscillation, which modify regional and local temperature and rainfall (salinity) conditions. However, the relative influence of these climate cycles varies. Dermo disease prevalence and intensity in oyster populations along the Gulf of Mexico are affected by ENSO through its effect on salinity. The La Niña phase of ENSO produces warm, dry conditions throughout the Gulf of Mexico, which increases salinity and favors parasite proliferation (Powell et al. 1992, Soniat et al. 2005, Soniat et al. 2009). For oyster populations along the US mid-Atlantic coast, Dermo disease and subsequent mortality are influenced primarily by the warmer temperatures

during the positive phase of the North Atlantic Oscillation (Soniat et al. 2009, Bushek et al. 2012). For Dermo disease in eastern oysters, the prevalence and intensity along the Gulf of Mexico coast are regulated primarily by salinity, because the temperature is never low enough to inhibit *P. marinus* proliferation. Until the mid-1980s, temperature was considered the controlling factor along the east coast of the United States, restricting the parasite to waters of the southern United States for decades. Salinity does, however, exert local control on the parasite, limiting its spread into the upper reach of rivers and estuaries. Thus, the response of oysters, and possibly other organisms, to climate variability in a region is not an indicator of the response that occurs over the entire range of the species. The cyclic nature of climate cycles has been suggested as being responsible for the lack of development of resistance to Dermo disease in spite of high mortality rates and frequent epizootics (Powell et al. 2012).

Historically, Dermo disease has affected oysters along the US southeast and Gulf of Mexico coasts (Ford & Tripp 1996). However, since the early 1990s, the range over which Dermo disease occurs has expanded to include regions north of Chesapeake Bay along the US east coast (Cook et al. 1998, Ford & Smolowitz 2007). Several hypotheses have been put forward to explain this range expansion; an analysis of water temperatures showed that the northward expansion of Dermo disease was associated with long-term increases in winter water temperatures (Cook et al. 1998).

Similarly, MSX disease has been present in oyster populations along the US mid-Atlantic coast since the 1950s, but during the 1980s and 1990s it became established in populations further north along the US east coast (Haskin & Andrews 1988, Barber et al. 1997, Sunila et al. 1999, Burreson & Ford 2004), and it has now been found in populations in Canada (Burreson & Ford 2004). Environmental control of MSX disease by temperature and salinity is well documented (Ford et al. 1999, 2012). A modeling study of the effects of temperature and salinity variability on MSX-disease establishment indicated that warming winter temperatures facilitate the northward spread of MSX disease (Hofmann et al. 2001), and these results have been supported by observations (Burreson & Ford 2004). The link between the expansion of MSX disease and climate warming is not as clear as that for Dermo disease, as the MSX parasite has been present in the southeastern United States for decades without causing epizootics.

Variations in environmental conditions at seasonal, decadal, and longer-term scales interact to control the prevalence, intensity, and geographic distribution of the two primary diseases affecting eastern oyster populations. Recent studies have suggested that disease limits the ability to maintain oyster reefs because mortality rates are too high for reef accretion to occur over most of the estuarine salinity gradient where oysters exist, even in the absence of fishing (Mann & Powell 2007, Powell & Klinck 2007, Mann et al. 2009, Harding et al. 2010, Powell et al. 2012). Thus, increased mortality from disease has reduced the resiliency of oyster populations in the face of exploitation. Given the high prevalence and broad distribution of oyster diseases, management and control are difficult. Management of present-day oyster resources (through water management and fisheries management) must account for the effects of disease and environmental variations at a range of scales. For example, management of upstream freshwater input can help to manage downstream estuarine salinity and therefore disease-related oyster mortality (Petes et al. 2012). In addition, fisheries can apply adaptive management through techniques such as temporarily reducing harvest when high disease-related mortality occurs in order to ensure the long-term sustainability of oyster populations.

Management of Natural and Aquaculture Abalone Stocks

Abalones, marine vetigastropods of the genus *Haliotis*, inhabit the nearshore rocky intertidal and shallow subtidal zones and are important herbivores in many ecosystems. Their sedentary nature,

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gregarious distribution, accessible habitat, and economic value have resulted in overutilization. Declines in a number of the more than 50 *Haliotis* species worldwide are due in part to fishing pressure and disease (e.g., Hobday & Tegner 2000, Travers 2008). Significant alterations in abalone host–bacterial parasite dynamics in recent years are associated with increased seawater temperature. Climate change has been clearly linked to epizootics of two bacterial diseases: withering syndrome (WS) (Lafferty & Kuris 1993, Tissot 1995, Friedman et al. 2000, Moore et al. 2000, Braid et al. 2005, García-Esquivel et al. 2007) and vibriosis in adult European abalones (*Haliotis tuberculata*) (Travers et al. 2009).

WS is caused by the gastrointestinal rickettsial-like organism (RLO) "*Candidatus* Xenohaliotis californiensis" (Friedman et al. 2000) and leads to varying levels of disease among host species in the wild and in farms (Friedman et al. 1997, 2002, 2007; Moore et al. 2000, 2001, 2002; Tinajero et al. 2002; Wetchateng et al. 2010). WS was first observed in black abalone (*Haliotis cracherodii*) populations on the south shore of Santa Cruz Island in 1985, shortly after the strong 1982–1983 ENSO event (Tissot 1995, 2007). Subsequently, seasonal warm-water or ENSO events were associated with severe losses in farmed red abalones and enhanced clinical signs of disease in several wild abalone species (Moore et al. 2000; Césares-Martínez & Tinoco-Orta 2001; Friedman et al. 2002, 2003; Raimondi et al. 2002; Tinajero et al. 2002; García-Esquivel et al. 2007). Laboratory studies further confirmed a link between increased temperature (≥18°C), food availability, and both transmission of the WS RLO and development of clinical disease (Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005). Because of both overfishing and WS, in 2009 the black abalone was listed as endangered under the Endangered Species Act. Both the black abalone and the endangered white abalone (*Haliotis sorenseni*) are highly susceptible to WS (Friedman et al. 1997, 2002, 2007), which jeopardizes their recovery (Moore et al. 2002).

Vibriosis in European abalones is caused by the gram-negative bacillus *Vibrio harveyi* (Gauger & Gómez-Chiarri 2002, Nicolas et al. 2002). Large losses of reproductively mature abalones have coincided with thermal maxima, and the relationship between temperature and vibriosis has been demonstrated in both laboratory trials and field studies (Travers et al. 2009). In laboratory experiments, a 1°C increase in temperature (from 17°C to 18°C) resulted in an increase in losses from 0% to 80% when abalones were exposed to the bacterium during their spawning season (Travers et al. 2009). Thus, susceptibility to this pathogen is driven by both climatic factors and reproductive physiology.

These climate-driven losses have resulted in changes in abalone community structures in intertidal and subtidal habitats, reduced recruitment of abalones, closures of abalone fisheries (e.g., Leet et al. 2001, pp. 70, 89–97; CDFG 2005), and increased costs of abalone aquaculture (e.g., Friedman et al. 2003). Rock reefs historically inhabited by substantial abalone populations may undergo changes in community structure because of the absence of abalone grazing activity, further reducing recruitment. Several approaches have been proposed to alleviate losses and their associated impacts. In the United States, California developed an Abalone Recovery and Management Plan that calls for specific actions to rebuild populations of red, pink, green, white, and black abalone (CDFG 2005). Methods to reduce or eliminate WS-RLO infections have been developed that use oral dosage of oxytetracycline (100 mg per kilogram of live body weight), but given the unusually long depletion duration (extending for many months), use of this therapeutant is likely limited to valuable brood stocks or endangered species (Friedman et al. 2003, 2007). Specific guidelines to detect and limit the transfer of marine diseases have been outlined by the World Organisation for Animal Health (**http://www.oie.int**). To limit WS in California, the state prohibited movement of infected abalone outside the known distribution of the disease (C.S. Friedman, personal observation). Methods to limit the impacts of cosmopolitan pathogens such as *V. harveyi* are more difficult to develop, but Travers (2008) suggested a link between

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Table 2 Reported ichthyophoniasis epizootics in wild fishes

Time period	Location	Affected fish	Impacts	Reference(s)
1913–1914	Gulf of St. Lawrence, Canada	Atlantic herring	Dead fish "in great numbers were washed ashore on beaches or sand reefs, skirting the coast, or in quiet coves"	Cox 1914
1931–1932	Gulf of Maine	Atlantic herring	Infection prevalence reached 70% during the peak of the epizootic, then declined to 18%	Daniel 1933, Fish 1934
1940–1943	British Isles	Mackerel	Infection prevalence was as high as 100%, and the disease was described as fatal	Sproston 1944
1947	Gulf of Maine	Atlantic herring	No information	Scattergood 1948
1954–1956	Gulf of Saint Lawrence, Canada	Atlantic herring	At least half of the mature herring in the western Gulf of St. Lawrence were killed	Sindermann 1956, Tibbo & Graham 1963
1966–1970	Western North Atlantic	Yellowtail flounder	Infection prevalence was as high as 25–57%, and "there can be no question that many [affected flounder] must succumb directly to the infection"	Powles et al. 1968, Ruggieri et al. 1970
1991–1993	Eastern North Atlantic (Denmark, Sweden, Norway, and Iceland)	Atlantic herring	Total *Ichthyophonus*-induced mortality in the North Sea was estimated at 12.8–36.8%	Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997
1990s–2000s	Prince William Sound, Alaska	Pacific herring	The disease was a possible contributor to the population decline and failed recovery	Marty et al. 2010
Early 2000s	Yukon River, Alaska and Canada	Chinook salmon	The disease was a possible contributor to prespawn mortality	Kocan et al. 2004
2007	Columbia River, Washington and Oregon	American shad	Infection prevalence peaked at 72% and declined concomitantly with shad abundance	Hershberger et al. 2010

the pathogenicity of this bacterium and a possible plasmid; thus, limiting transfers of animals harboring the pathogen is recommended.

Ichthyophoniasis in Marine and Anadromous Finfishes

The impacts of diseases on wild marine fishes are extremely difficult to enumerate, owing largely to the challenges of studying highly mobile organisms in large systems where direct observation is not feasible. Epizootics in marine finfishes are periodically reported, primarily in response to the appearance of massive fish kills that wash ashore or plug the nets of commercial fishers. Examples include herpesvirus disease in south Australia (Murray et al. 2003), viral hemorrhagic septicemia in the northeast Pacific (Meyers et al. 1999), and *Ichthyophonus* disease (ichthyophoniasis) throughout the Northern Hemisphere (reviewed in McVicar 2011). Of these diseases, ichthyophoniasis is arguably the most economically and ecologically significant, based on the well-documented magnitude, distribution, frequency, and effects of recurring epizootics (**Table 2**).

In addition to its population-level effects on marine fish resources (**Table 2**), ichthyophoniasis affects human societies by reducing the market value of fish. For example, epizootics in yellowtail flounder (*Limanda ferruginea*), European plaice (*Pleuronectes platessa*), alewife (*Alosa pseudoharengus*),

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and mackerel (*Scomber scombrus*) from the Nova Scotian shelf resulted in unsightly and aromatic lesions in the skeletal muscles of infected fish, rendering the affected fillets unmarketable (reviewed in McVicar 2011). This clinical manifestation of the disease also affected commercial and subsistence fishers along the Yukon River (Kocan et al. 2004), leading to the culling of chinook salmon prior to human consumption, which further damaged remaining fish stocks as additional fishing effort was employed.

The linkage between ichthyophoniasis epizootics and climate change remains speculative, owing largely to issues of geographical and ecological scale as well as observational difficulties in quantifying disease impacts on wild marine fishes; however, both empirical and field observations suggest that such a linkage exists. The direct effects of temperature manipulations on ichthyophoniasis disease kinetics are well demonstrated. In *Ichthyophonus*-exposed rainbow trout, infection prevalence and cumulative mortality increase with temperature (Okamoto et al. 1987), as do the negative impacts of ichthyophoniasis on host swimming performance (Kocan et al. 2006, 2009). Field observations during the Yukon River epizootic revealed that elevated river temperatures likely contributed to disease progression (Kocan et al. 2006), and the most heavily diseased individuals were seen at the end of migration (Zuray et al. 2012).

The indirect effects of climate change are likely more important determinants of ichthyophoniasis. For example, the most parsimonious hypothesis accounting for natural routes of infection in planktivorous hosts involves consumption of an infectious-stage parasite through an intermediate host (Gregg et al. 2012); however, neither the infectious stage nor the intermediate host has been identified. Climate changes that affect ocean circulation patterns, nutrient regimes, and temperatures will likely alter zooplankton assemblages and the abundance and bloom dynamics of this proposed intermediate host.

Managing ichthyophoniasis epizootics in marine fishes involves understanding the climate-driven variables that influence the disease, including host physiological state, temperature, and natural route(s) of exposure and transmission. Ichthyophoniasis surveillance, combined with stock assessment surveys, is critical for understanding long-term infection and disease trends in affected populations. Once understood, these metrics can be developed into predictive tools to forecast the epizootics and then implemented as part of management strategies intended to mitigate disease impacts. For example, if an intermediate host for *Ichthyophonus* is identified, then its relative abundance may provide some predictive capability for future epizootics. A more pragmatic example involves options specific to the Yukon River, where water temperatures may be incorporated into adaptive fishery management strategies. Additional strategies may incorporate projected mortality from ichthyophoniasis into an effective population size. For example, management decisions for chinook salmon are based on in-season sonar counts and other indices near the mouth of the river, which are then used to designate proportions of the population for harvest and escapement in the United States and Canada. A more conservative approach to meeting escapement goals may involve allocating harvest and escapement quotas based on an effective population size that is calculated using the current population assessments minus any projected prespawn mortality from ichthyophoniasis. Interannual variability in projected prespawn mortality would likely be affected by climate-driven changes that directly and indirectly influence ichthyophoniasis.

Noncholera *Vibrio* Diseases in Humans

Human infections caused by the marine bacteria *Vibrio vulnificus* and *Vibrio parahaemolyticus* are typically acquired through either ingestion of undercooked seafood or infection of existing wounds (Tantillo et al. 2004, Faruque & Nair 2006, Oliver 2006). Clinical signs from *Vibrio* infection can

present in as little as 7 hours, and the case fatality rate is greater than 50% (Oliver 2006). Disease can present as gastroenteritis or fulminating wound infections but can quickly progress to primary septicemia (Iida et al. 2006, Oliver 2006). Warm (20–30°C), mesohaline (<5–30‰) waters are the most hospitable for growth of pathogenic *Vibrio* bacteria (Tantillo et al. 2004). Because these bacteria are most prolific in warmer water, the vast majority of infections (~85%) occur between May and October, and infections rarely occur in regions with consistently low water temperatures (Kaysner et al. 1990, Rippey 1994, Oliver 2006). As global temperatures rise, the geographic and seasonal ranges of these bacteria may expand, along with the potential for infection as the relative numbers of *Vibrio* bacteria increase (Huq et al. 2005, Baker-Austin et al. 2010, Vezzulli et al. 2012). Storms or other extreme climatic events can further broaden the geographic range of these pathogens and lead to outbreaks of *Vibrio* disease (Lejeusne et al. 2010). Hurricanes, for example, can create storm surges that carry *Vibrio* bacteria, increasing the risk of human exposure, and can generate rain events that freshen full-strength marine waters, providing a more hospitable environment for these mesohaline bacteria. ENSO events can produce warmer conditions that potentially increase *Vibrio* abundance and can generate flooding that extends the boundaries of *Vibrio*-abundant waters (Harvell et al. 1999, Baker-Austin et al. 2010, Martinez-Urtaza et al. 2010).

Pathogenic *Vibrio* bacteria pose a significant human health risk. In the United States alone, there are approximately 4,600 cases of *Vibrio* infection each year, of which approximately 90 are *V. vulnificus* cases and 4,500 are *V. parahaemolyticus* cases (CDC 2009a,b). The consequences of climatological effects for pathogenic *Vibrio* infection risk can be either immediate (e.g., following intense storms) or long term (driven by increases in annual mean temperature and rainfall). Immediately after Hurricane Katrina struck the United States in 2005, 22 cases of *V. vulnificus* were reported, including 5 deaths (CDC 2005, Baker-Austin et al. 2010). The chronic effects of warming temperatures have been observed in northern Europe, where an unusually warm summer in 1994 coincided with the first reports of *V. vulnificus* infections in Germany along the Baltic Sea (Hoyer et al. 1995, Boer et al. 2012). Since those initial infections, cases of *Vibrio* disease have been repeatedly documented in the Baltic Sea, where water temperatures have increased markedly over the past several decades (Boer et al. 2012).

Historically, abnormally high temperatures in the Baltic Sea area coincide with unusually large numbers of reported *Vibrio* cases (Baker-Austin et al. 2012) (**Figure 4**). Furthermore, long-term sea-surface temperature increases are linked with elevated *Vibrio* infection risk. Waters warmed by approximately 0.5°C per century in the Baltic Sea from 1854 to 2010, but this rate increased to 5°C per century from 1980 to 2010 (Baker-Austin et al. 2012). Modeling efforts indicated that *Vibrio* illnesses increased by 1.93 times for every 1°C increase in annual maximum water temperature (Baker-Austin et al. 2012).

Because these infections can be acquired through ingestion as well as through wound infection, tackling the growing problem of *Vibrio* infection must also include addressing risks associated with seafood consumption. Most of the ingestion cases are the result of undercooked shellfish, such as oysters, which are often purposely consumed raw. Reducing the risk of infection requires a combination of effective seafood industry regulation and improved consumer awareness, particularly for at-risk populations (e.g., immunocompromised individuals). In the United States, *Vibrio* controls are in place that include a maximum time to refrigeration from harvest, or postharvest processing to reduce bacterial populations. Postharvest processing methods such as flash freezing and pasteurization are available for oysters and shellfish and provide safe seafood products for consumers, but these techniques kill the oysters in the process, leading many consumers to reduce their consumption of the product (Muth et al. 2000). As a newer alternative, it has been suggested that oysters be relayed to a high-salinity site for depuration of harmful *V. vulnificus* bacteria, which

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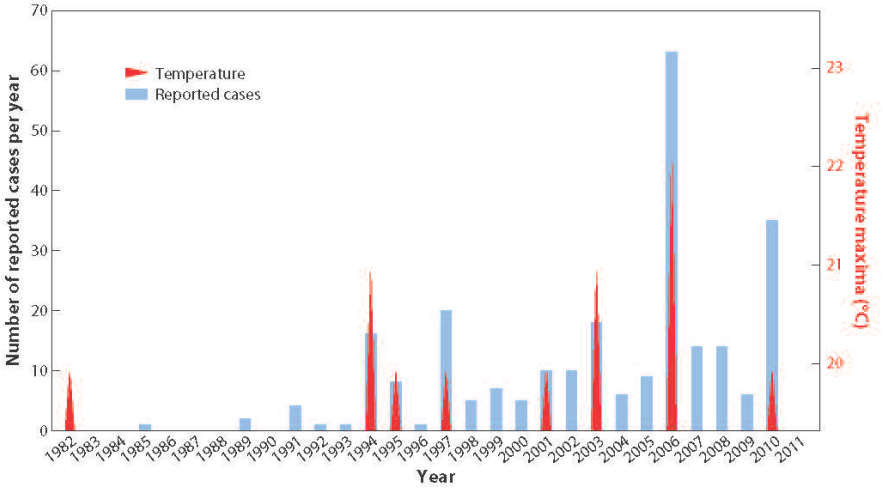

Figure 4

Number of reported *Vibrio* cases in the Baltic Sea area from 1982 to 2010. Figure provided by Craig Baker-Austin.

leaves the product both raw and alive (Audemard et al. 2011). Management of wound infections is also difficult, as elevated temperature is usually a driver of increased recreational water activity, exposing even more people to risk (Baker-Austin et al. 2012). Education of at-risk populations and the development of more accurate predictive models of abundance could mitigate wound-associated infections (B. Froehlich, J. Bowen, R. Gonzalez & R. Noble, manuscript in review).

Marine Mammal Diseases

Climate change is clearly influencing infectious disease dynamics in the marine environment; however, no studies have shown a definitive causal relationship between any components of climate change and increases in infectious disease among marine mammals. This is due in large part to a lack of sufficient data and to the likely indirect nature of climate change's impact on these diseases. Climate change could potentially affect the incidence or prevalence of infection, the frequency or magnitude of epizootics, and/or the severity or presence of clinical disease in infected individuals. There are a number of potential proposed mechanisms by which this might occur. Ocean warming can change haul-out patterns (Lavigne & Schmitz 1990), thus changing pathogen transmission risk because of shifts in host density, duration at haul-outs, and/or contact with terrestrial hosts. Temperature changes can shift species' ranges, richness (Walther et al. 2002, Gilg et al. 2012), or assemblages (Benson et al. 2002), potentially changing the risk of contact between susceptible individuals and pathogen reservoirs. Climate change may shift the distribution and/or abundance of food sources, causing poor nutrition and immunosuppression (Latshaw 1991, Van Loveren et al. 2000). Changes in sea-surface temperature and salinity can alter pathogen survival (Kelly & Stroh 1988) by modifying pathogen persistence or range. Shifts in land-use or rainfall patterns can increase terrestrial pathogen flow to the coastal zone, increasing marine mammal exposure (Shapiro et al. 2010). And, finally, warming can affect pathogen evolution (Yan & Wu 2011),

potentially resulting in strains better adapted to infecting and persisting in marine mammals. However, attribution is difficult, as many effects on these diseases that could be attributed to climate change could also be attributed to changes in nonclimatic factors.

Although there is a lack of definitive evidence for the impacts of climate change on marine mammal infectious disease, insight can be gained from associations with climate variability and extreme weather and climate events. In addition, the potential effects of climate change on these diseases have been discussed for avian influenza in harbor seals (*Phoca vitulina*) (Geraci et al. 1982); *Toxoplasma gondii* in polar bears (*Ursus maritimus*), phocids, and cetaceans (Jensen et al. 2010); and *V. parahaemolyticus* in sea otters (*Enhydra lutris*) (Burek et al. 2008), among other examples. Abnormal climate events have also been associated with morbillivirus (canine distemper virus, phocine distemper virus, and cetacean morbillivirus) epizootics and mass mortalities: A 1988 phocine distemper virus outbreak in harbor seals in northern Europe was associated with unseasonably warm temperatures (LaVigne & Schmitz 1990); a 1990–1992 cetacean morbillivirus event in the western Mediterranean that affected multiple cetacean species, with the greatest impact on striped dolphins (*Stenella coeruleoalba*), was associated with high winter sea-surface temperatures, low rainfall, and reduced prey availability (Domingo et al. 1992, Aguilar & Raga 1993, Aguilar & Borrell 1994); and a 2000 canine distemper virus epizootic in Caspian seals (*Pusa caspica*) in the Caspian Sea was associated with warm temperatures and early disappearance of ice cover (Kuiken et al. 2006).

Obtaining data on the incidence and prevalence of infection in wild, free-ranging populations is logistically difficult; therefore, evaluating changes in long-term disease dynamics in marine mammal populations and identifying the role of climate change in these shifts can be quite difficult and is often impossible with the data currently available. To better understand the role of climate change in marine mammal infectious disease dynamics, improved in-depth baseline data acquired through specific and directed long-term marine health programs are needed. In addition, identifying sources of pathogen spillover can assist in mediation efforts. For example, protection and restoration of wetlands can reduce coastal contamination with pathogens that are transported in contaminated runoff, such as *T. gondii* (Shapiro et al. 2010). Vaccination, population control, and restriction of the movement of domestic dog populations in proximity to phocid haul-out sites may reduce the potential for transmission of pathogens such as canine distemper virus to susceptible marine mammals.

FINAL THOUGHTS

Key Knowledge Needs for Improving Understanding and Management of the Impacts of Climate Change on Marine Diseases

Although our ability to detect links between climate change/variability and marine infectious disease outbreaks has improved, the effects on most marine host-pathogen interactions are still poorly known. Knowledge gaps still exist regarding causative agents and transmission dynamics, and we need improved diagnostic methods and management strategies. Increasing outbreaks in some taxa are expected with climate change based on changes in physiological state for the host species (stressed or immunocompromised) and/or microbes (increased growth and virulence). The vulnerability and responses of marine organisms to climate change are highly variable, and certain species—including marine calcifiers, cold-adapted species, and rare, endemic, threatened, and endangered species—are particularly at risk from climate change (Howard et al. 2013), with potential implications for stress that could exacerbate sensitivity to disease. In contrast, species

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with a high physiological tolerance for changes in environmental conditions will likely experience fewer climate-related effects (Howard et al. 2013), and less sensitivity could possibly translate into lower disease risk. For example, as discussed above, both tropical and temperate species (i.e., tropical corals and temperate abalones) may be highly endangered by climate-driven synergisms.

There are several reasons that more is known about certain host-pathogen-environment interactions compared with others in marine systems. Some of these systems may be particularly sensitive to climate variability and change and therefore respond strongly. Some systems may be more easily monitored (e.g., slow-moving or sessile taxa and taxa that show clear and distinguishable signs of infection) and therefore have more baseline data available. Commercial species (e.g., oysters) and those with human health consequences (e.g., *Vibrio* in seafood and water) are often well monitored owing to their direct economic and societal consequences. Some of the host-pathogen interactions described above (e.g., between abalones and WS RLO/*V. harveyi*, between oysters and Dermo/MSX disease, and between elkhorn coral and *Serratia marcescens*) could serve as sentinel host-pathogen systems, given that each of these diseases is relatively well studied and the hosts have a large latitudinal range.

In contrast, there are far fewer examples of climate-related effects on diseases in more mobile marine organisms (e.g., fish, crustaceans, and marine mammals) and in species with a low direct impact on economies and human health. Improved disease surveillance, particularly when linked with environmental/climate monitoring, is needed to improve understanding, early warning, and management of diseases in marine systems under climate change. We suggest that, for these taxa, disease detection is a problem that calls for new and better long-term health and population monitoring and forecasting. The most effective management strategy for mitigating disease involves developing adaptive disease management strategies. These strategies should be based on sound science, including long-term climatic and organismal monitoring, experimental work to test the effects of climatic stressors on host-pathogen interactions, and forecasts and decision-support tools to inform management. The sidebar (Key Science Needs for Marine Health in the Face of Climate Change) identifies several ways that we can improve our ability to manage climate-related impacts on marine diseases.

KEY SCIENCE NEEDS FOR MARINE HEALTH IN THE FACE OF CLIMATE CHANGE

- Collect long-term data to understand host-pathogen interactions, including monitoring to understand how individual syndromes are affected by extreme events, climate variability, and climate change
- Develop sensitive and specific diagnostic tools, especially those that can be broadly applied and perhaps used in autonomous monitoring systems
- Develop new monitoring protocols to better understand the effects of disease in mobile vertebrates
- Identify the relative proportion of host-pathogen interactions affected by climate variability and climate change
- Improve understanding of the effects of climate change and ocean acidification on host-pathogen interactions across multiple life-history stages of the host (i.e., larval versus adult)
- Develop decision-support tools to improve the integration of climate-related disease considerations into marine ecosystem management (e.g., marine protected area design)
- Develop modeling tools to advance understanding and prediction of epizootics under different climate scenarios
- Enhance collaborations between natural resource managers, culturists, and researchers
- Increase public awareness of marine diseases and their consequences for people

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Challenges and Opportunities for Management of Marine Diseases Under Climate Change

Above, we discussed the impacts of climate variability and change on marine diseases and articulated potential management actions that could reduce disease risk. Management of marine diseases is difficult, as little is known about which management actions could be successfully applied in the ocean, particularly given that standard terrestrial strategies (e.g., culling, quarantining, and vaccinating) are not widely applicable. In addition to the strategies outlined in the case studies above, we suggest the following strategies to reduce climate-related disease risk:

- When possible, reduce exposure to and impacts of marine diseases by reducing nonclimatic stressors, such as coastal pollution (e.g., resulting from poor sewage management), habitat loss, translocation of pathogens, and overharvesting
- Improve forecasting programs to identify sensitive times and places for disease outbreaks, and improve monitoring and detection as a positive feedback to enhance the accuracy of forecasting programs (e.g., the integration of *Acropora* white syndrome into the Coral Reef Watch program; Maynard et al. 2011)
- Improve understanding of factors that contribute to disease resistance in marine organisms
- Encourage sharing of best practices and lessons learned, creating a community of practice among managers/culturists who control, manage, and/or respond to marine diseases

There is very little effort under way to integrate climate-related consequences for marine diseases into management approaches, such as ecosystem-based management, MPA design and implementation, and fisheries management. Factoring climatic and disease considerations into management will be key to ensuring the sustainability of ocean ecosystems, and the benefits they provide to people, for generations to come.

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Appendix 2

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Short Communication

Detection of *Ichthyophonus* by chromogenic *in situ* hybridization

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Keywords: Ichthyophonus, mesomycetozoea, ribosomal small subunit DNA.

Ichthyophonus hoferi (Plehn & Mulsow 1911) is a protistan parasite in the class Mesomycetozoea that infects a large range of marine and freshwater fish (Mendoza, Taylor & Ajello 2002; McVicar 2011). The broad host and geographic range, which includes both fresh and marine waters of the Northern and Southern Hemispheres, combined with a lack of distinguishing morphological characteristics, have prompted speculation that *Ichthyophonus*-like organisms in multiple species of fish, as well as reptiles, amphibians, birds and invertebrates, may have been incorrectly classified under a single type species *I. hoferi* (McVicar 2011). At present, only two species, *I. hoferi* and *I. irregularis*, are currently recognized within the genus (Rand *et al.* 2000; Mendoza *et al.* 2002). Investigations of ribosomal DNA sequence variation have begun to clarify relationships among *Ichthyophonus* types (Criscione *et al.* 2002; Rasmussen *et al.* 2010). Here, we will use the term *Ichthyophonus* to broadly represent all members of the genus regardless of species/subspecies.

Ichthyophonus disease, or ichthyophoniasis, can result in negative impacts to fisheries by causing recurring epizootics, and declines in affected populations (reviewed in Burge *et al.* 2014) and by creating skeletal muscle lesions in affected hosts, thereby reducing product quality (reviewed in McVicar 2011). Because the parasite may reside in both skeletal and cardiac muscle, infection can have significant sublethal effects on growth, condition, reproductive capacity, energy and swimming stamina (Kocan *et al.* 2006; Kramer-Schadt, Holst & Skagen 2010; Vollenweider *et al.* 2011).

Detection of *Ichthyophonus* infections typically involves observation of visible signs, including white nodules in the heart, liver, spleen or kidney, combined with culture of explant tissue or microscopic visualization in tissue squash preparations (Kocan, Dolan & Hershberger 2011; Hershberger 2012). Although typically less sensitive than other techniques, histopathology is also widely used and is effective for evaluating *Ichthyophonus* infections because disease severity and host response can be assessed simultaneously (Kocan *et al.* 2011). Positive periodic acid-Schiff (PAS) staining of spherical multinucleate organisms 10–250 μm in diameter can be presumptive for *Ichthyophonus,* but should not be considered confirmatory because a number of PAS-positive organisms occur in this size range (Hershberger 2012). The lack of a definitive *Ichthyophonus* confirmatory test for histopathological evaluation may lead to misdiagnosis, particularly when the organism cannot be cultured due to lack of material, contamination or inability of the organism to propagate. Infections with fungal organisms may be misidentified as *Ichthyophonus* due to similar morphological characteristics and histopathological presentation (Sprotson 1944; Maureen Purcell and Paul Hershberger, unpublished results). Conventional and quantitative polymerase chain reaction (PCR)-based confirmation can be

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Journal of Fish Diseases

effective for confirming moderate to heavy infections (Whipps *et al.* 2006; White *et al.* 2013), but false-negative results can occur as the parasite is focally distributed and may not be detected if only a small tissue amount is analysed. Additionally, several researchers have reported *Ichthyophonus*-like organisms in amphibians (McVicar 2011), but it is unclear whether these organisms represent the same species found in fish because they are not culturable and cannot be detected using *Ichthyophonus*-specific PCR tests (Hershberger & Purcell, unpublished results). *In situ* hybridization is a method that complements histological diagnosis by providing highly specific molecular confirmation of the observed organism in preserved tissue. Here, we report the development of a chromogenic *in situ* hybridization (CISH) procedure that specifically detected *Ichthyophonus* ribosomal DNA in histological sections.

Laboratory-reared, specific-pathogen-free Pacific herring, *Clupea pallasii* Valenciennes, (Hershberger *et al.* 2007) were infected with *Ichthyophonus* by intraperitoneal injection. Infected fish and non-infected controls were killed by an overdose of buffered tricaine methanesulfonate (MS-222, Argent Chemical Laboratories) at various days post-injection, and the heart and liver removed. A portion of the tissue was fixed in 10% neutral-buffered formalin (NBF) for 24 or 48 h, transferred to 70% ethanol and then processed and embedded in paraffin wax by conventional histological methods. Formalin-fixed, paraffin-embedded tissues collected from naturally infected Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and red-spotted newt, *Notophthalmus viridescens* (Rafinesque), and experimentally infected rainbow trout, *O. mykiss* (Walbaum), and Pacific staghorn sculpin, *Leptocottus armatus* Girard, were also analysed in this study; those tissues were fixed using various methods.

An *Ichthyophonus*-specific oligonucleotide probe was designed to target conserved portions of the 18S small subunit (SSU) ribosomal gene of known *Ichthyophonus* species, including *I. hoferi* (GenBank accession numbers FJ869836, GQ370781, AF467785, U43712, GQ370802, AF467786) and *I. irregularis* (AF232303); the probe contained nucleotide mismatches to prevent hybridization with closely related mesomycetozoan species (*Rhinosporidium seeberi*, AF118851; *Dermocystidium* sp., AF533950; *Paramoebidium* sp., AY336708; *Amoebidium parasiticum*, Y19155). The digoxigenin (DIG)-labelled probe (5′-/5DigN/GCC TTC GAG AAG AAG AAA CTG -3′) was commercially synthesized (Integrated DNA Technologies, Inc.).

The *Ichthyophonus* CISH hybridization protocol was modified from previously reported methods (McCarthy, Urquhart & Bricknell 2008; Marcino 2013). All steps were performed at room temperature (22–25 °C) unless otherwise specified (Table 1). Briefly, 5-μm tissue sections were adhered to positively charged glass slides (Colorfrost® Plus; Fisher Scientific), dried and dewaxed. Tissues were then rehydrated through a graded ethanol series followed by deionized water. Sections were equilibrated in phosphate-buffered saline and then permeabilized with proteinase K in a humid chamber at 37 °C for 40 min. Proteolysis was stopped by immersing sections in 0.2% glycine, followed by immersion in glacial acetic acid and washes in Tris-buffered saline and 5× SET buffer. A hybridization chamber (HybriWell-FL; Sigma-Aldrich) was applied to each slide and filled with pre-warmed (42 °C) hybridization solution before incubating in a humid chamber at 42 °C for 90 min. The hybridization solution was removed and replaced with the *Ichthyophonus* probe diluted to 6 ng μL^{-1} in 42 °C hybridization solution and incubated in a humid chamber at 42 °C overnight (18–20 h). Hybridization chambers were removed, and slides immersed in 42 °C 0.2× SET and then in Buffer 1. Sections were then immersed in blocking solution for 1 h. New hybridization chambers were applied to slides, filled with diluted anti-DIG-alkaline phosphatase conjugate solution and then incubated in a humid chamber for 3 h. Sections were then washed in Buffer 1, followed by Buffer 2. The colour development solution was prepared, and sections were immediately immersed and incubated for 2 h in the dark, followed by washing in TE buffer and then deionized water. Bleaching of endogenous melanin was performed after the CISH procedure; sections were immersed in 0.25% potassium permanganate for 15 min, rinsed in deionized water, immersed in 5% oxalic acid for 5 min and rinsed again in deionized water. Slides were mounted with aqueous mounting medium and glass coverslips. Serial sections were stained by PAS following standard methods (Carson 1997).

Hybridization of the DIG-labelled probe to *Ichthyophonus* nucleic acid was indicated by dark purple precipitates in schizonts and other developmental stages (Figs 1a and c) and

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Table 1 Chromogenic *in situ* hybridization protocol for the detection of *Ichthyophonus* in formalin-fixed, paraffin-embedded tissues[a]

In situ Hybridization Step	Reagent	Time
Dewaxing and Rehydration	Clear-Rite™ 3 (ThermoFisher Scientific)	2x, 5 min
	100% and 95% ethanol	2x, 3 min
	80%, 70%, 50% and 30% ethanol	1x, 3 min
	Deionized water	3x, 1 min
	Phosphate-buffered saline (PBS): 0.014 M, pH 7.4	5 min
Permeabilization	Proteinase K (Sigma-Aldrich P2308): 25 μg mL^{-a} in 37 °C PBS Incubate in a humid chamber	40 min at 37°C
	0.2% glycine (w/v in PBS)	2x, 5 min
	Glacial acetic acid	30 s
	Tris-buffered saline (TBS): 0.05 M Tris, 0.15 M NaCl; pH 7.6	5 min
	5× SET buffer: 0.75 M NaCl, 0.0064 M EDTA, 0.10 M Tris; pH 8.0	2x, 5 min
Prehybridization	Hybridization solution (42 °C): 5× SET, 0.020% [w/v] bovine serum albumin, 0.025% [w/v] sodium dodecyl sulphate Apply HybriWell-FL™ hybridization chamber (Sigma-Aldrich), add 200 μL of 42 °C hybridization solution. Incubate in a humid chamber	90 min at 42°C
Hybridization	Dilute probe to 6 ng μL^{-1} in 42 °C hybridization solution Replace hybridization solution with 200 μL of diluted probe Incubate in a humid chamber	18–20 h at 42°C
Post-hybridization washes	Remove hybridization chamber 42 °C 0.2× SET	2x, 5 min
	Buffer 1: 0.10 M Tris, 0.15 M NaCl; pH 7.5	5 min
Hybridization detection	Blocking solution: Buffer 1, 0.3% Triton X®-100, 2% normal sheep serum (NSS). Incubate in a plastic 5-slide mailer	1 h
	Anti-DIG-alkaline phosphatase (AP) conjugate solution (Roche Applied Science) diluted 1:500 in Buffer 1, 0.3% Triton X®-100 and 1% NSS. Apply a new hybridization chamber; add 200 μL of conjugate solution. Incubate in a humid chamber	3 h
	Remove hybridization chamber Buffer 1	2x, 5 min
	Buffer 2: 0.10 M Tris, 0.10 M NaCl, 0.05 M $MgCl_2$; pH 9.5	2x, 5 min
	Colour development solution: Buffer 2, 0.45% NBT [v/v] 0.35% BCIP [v/v], 1% levamisole (w/v) 24 mg mL^{-1} Prepare immediately before use; incubate in a plastic 5-slide mailer	2 h in the dark
	TE buffer: 0.01 M Tris pH 8.0, 0.001 M EDTA	5 min
	Deionized water	2x, 3 min
Melanin bleach	0.25% potassium permanganate	15 min
	Deionized water	30 s
	5% oxalic acid	5 min
	Deionized water	30 s
Mount	Mount coverglass with Faramount aqueous mounting medium (Dako)	

[a]Section tissues at 4–5 μm and mount on positively charged glass slides. All steps are performed at room temperature (22–25 °C) unless otherwise stated. The number of solution changes is noted by 'x'.

correlated with the distribution and morphology of cells observed in PAS-stained tissues (Figs 1b and d). Further, the CISH procedures were effective at identifying *Ichthyophonus* developmental stages in locations where the parasite was in the presence of normal host tissues staining PAS positive (Fig. 1d). No hybridization signals or evidence of *Ichthyophonus* infection were detected in specific-pathogen-free Pacific herring control tissues by CISH or PAS (photographs not shown). *Ichthyophonus* nucleic acid was successfully detected in Pacific herring (Fig. 1a), rainbow trout (Fig. 1c), Pacific staghorn sculpin and Chinook salmon (photographs not shown). Genetic typing of *Ichthyophonus* from the North American West Coast indicates that *Ichthyophonus* from freshwater rainbow trout is a genetically distinct type compared with the more broadly distributed type infecting marine and anadromous fish species in the region (Rasmussen *et al.* 2010). We observed no detectable difference in CISH hybridization quality between *Ichthyophonus* genetic types or between laboratory-challenged and naturally occurring infections (photographs not shown). An *Ichthyophonus*-like, PAS-positive organism in red-spotted newt tissues (Fig. 1f) did not hybridize with the *Ichthyophonus* probe (Fig. 1e). This result supports the hypothesis that the organism infecting amphibians is taxonomically distinct from fish-associated *Ichthyophonus*.

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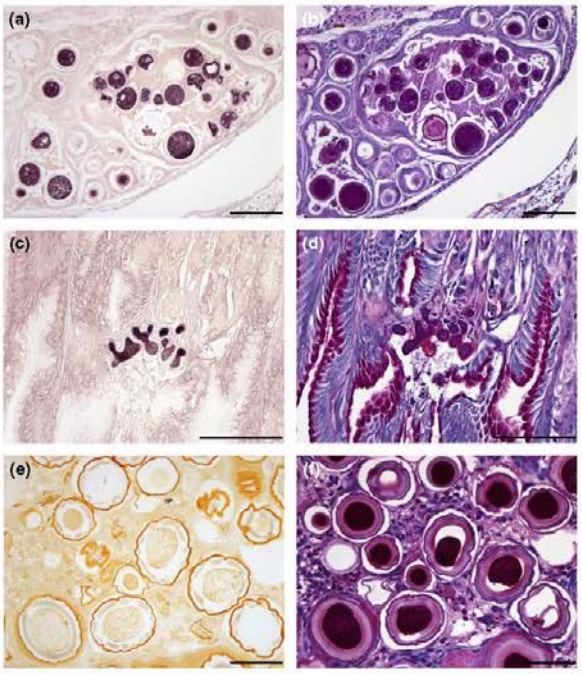

Figure 1 Comparisons between the newly developed chromogenic *in situ* hybridization (CISH) procedure and conventional periodic acid-Schiff (PAS) staining for *Ichthyophonus* detection in histological sections. *Ichthyophonus* schizonts and other developmental stages were easily identified by both CISH and PAS in an epicardial granuloma in Pacific herring (a and b, respectively). CISH was effective at identifying *Ichthyophonus* stages in rainbow trout stomach tunica propria (c), where the parasite was in the presence of PAS-positive host tissues (d). Parasitic stages in the skeletal muscle of red-spotted newt, previously identified as *Ichthyophonus*, failed to hybridize with the *Ichthyophonus*-specific probe (e) (section counter-stained with Bismarck brown Y for visibility), but stained PAS positive (f). Scale bars = 100 μm.

There was no difference in dewaxing efficiency between Clear-Rite™ 3 (ThermoFisher Scientific) and xylene; however, several schizonts detached from slides when the total dewaxing time exceeded 10 min. The addition of conventional denaturation and annealing steps did not improve the sensitivity of *Ichthyophonus* nucleic acid detection; however, incorporation of these steps did result in increased degradation of fish tissues and parasite schizonts. Several different counter stains were tested during development of the CISH protocol, but these stains did not improve visualization of cellular morphology. However, melanin bleaching was required to confidently distinguish positive CISH staining from tissue melanin.

In summary, we developed a CISH assay capable of detecting *Ichthyophonus* nucleic acid in standard histology sections. The assay has utility for both diagnostic and research applications. The CISH assay can be used to confirm histological diagnosis of *Ichthyophonus* or be readily applied to archival material. There remain many unanswered questions regarding the *Ichthyophonus* life cycle and transmission routes. For example, although *Ichthyophonus* is typically observed as 10- to 250-μm-diameter schizonts in tissues of live fishes, the parasitic life stages can be extremely pleomorphic *in vitro* and additional life stages have been reported *in vivo* (Kocan, LaPatra & Hershberger 2013), raising the possibility that small cryptic stages of the parasite have been overlooked when non-specific histological stains are used. If this is the case, the CISH assay may be useful for tracking sequential dissemination of *Ichthyophonus* throughout fish tissues following laboratory exposure. Additionally, the CISH assay may prove beneficial in ongoing studies intended to identify non-piscine intermediate hosts for the parasite.

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U.S. Geological Survey. All animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Western Fisheries Research Center. The use of trade, firm or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of the Interior or the U.S. Geological Survey of any product or service to the exclusion of others that may be suitable.

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Appendix 3

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Molecular identification of erythrocytic necrosis virus (ENV) from the blood of Pacific herring (*Clupea pallasii*)

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ABSTRACT

Viral erythrocytic necrosis (VEN) is a condition affecting the red blood cells of more than 20 species of marine and anadromous fishes in the North Atlantic and North Pacific Oceans. Among populations of Pacific herring (*Clupea pallasii*) on the west coast of North America the disease causes anemia and elevated mortality in periodic epizootics. Presently, VEN is diagnosed by observation of typical cytoplasmic inclusion bodies in stained blood smears from infected fish. The causative agent, erythrocytic necrosis virus (ENV), is unculturable and a presumed iridovirus by electron microscopy. *In vivo* amplification of the virus in pathogen-free laboratory stocks of Pacific herring with subsequent virus concentration, purification, DNA extraction, and high-throughput sequencing were used to obtain genomic ENV sequences. Fragments with the highest sequence identity to the family *Iridoviridae* were used to design four sets of ENV-specific polymerase chain reaction (PCR) primers. Testing of blood and tissue samples from experimentally and wild infected Pacific herring as well as DNA extracted from other amphibian and piscine iridoviruses verified the assays were specific to ENV with a limit of detection of 0.0003 ng. Preliminary phylogenetic analyses of a 1448 bp fragment of the putative DNA polymerase gene supported inclusion of ENV in a proposed sixth genus of the family *Iridoviridae* that contains other erythrocytic viruses from ectothermic hosts. This study provides the first molecular evidence of ENV's inclusion within the *Iridoviridae* family and offers conventional PCR assays as a means of rapidly surveying the ENV-status of wild and propagated Pacific herring stocks.

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1. Introduction

Viral erythrocytic necrosis (VEN), also historically known as piscine erythrocytic necrosis, is a disease that can cause severe blood abnormalities and has been reported in >20 species of marine and anadromous fish throughout both the Atlantic and Pacific Oceans (Nicholson and Reno, 1981; Smail, 1982; Wolf, 1988; Dannevig and Thorud, 1999). The severity of VEN can range from a benign infection to a highly fatal disease characterized by extreme anemia with associated osmoregulatory difficulties, loss of stamina, and greater susceptibility to secondary infections or environmental stressors (MacMillan et al., 1980; Nicholson and Reno, 1981; Meyers et al., 1986; Haney et al., 1992; Dannevig and Thorud, 1999). Incidents of VEN were initially reported in the late 1960s in a variety

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of fish species from the Atlantic coast of North America, such as longhorn sculpin *Myoxocephalus octodecemspinosus*, Atlantic seasnail *Liparis atlanticus*, Atlantic herring *Clupea harengus*, and Atlantic cod *Gadus morhua* (Laird and Bullock, 1969; Walker and Sherburne, 1977; Reno et al., 1978a; Reno et al., 1985). Natural hosts from the Pacific coast of North America include coho *Oncorhynchus kisutch*, Chinook *O. tshawytscha*, chum *O. keta*, pink *O. gorbuscha* salmon and Pacific herring *Clupea pallasii* (Evelyn and Traxler, 1978; MacMillan and Mulcahy, 1979; Rohovec and Amandi, 1981; Hershberger et al., 2013). Disease outbreaks periodically occur in populations of Pacific herring, particularly juveniles, throughout coastal regions of the NE Pacific Ocean in both cultured and wild fish (Meyers et al., 1986; Hershberger et al., 2006, 2009). Laboratory challenges of chum salmon, pink salmon, and Pacific herring with blood or tissue homogenates from VEN-affected fish have shown these species to be highly susceptible to infection and have reproduced essential features of the disease (Evelyn and Traxler, 1978; MacMillan and Mulcahy, 1979; Haney et al., 1992; Glenn et al., 2012).

The disease is currently diagnosed by microscopic examination of Giemsa-stained blood smears for the presence of typical inclusion bodies within the cytoplasm of infected erythrocytes (AFS-FHS, 2012). Electron microscopy of affected erythrocytes reveals the presence of icosahedral virions, whose reported size and morphology can vary with host species or geographic region (Smail, 1982; Wolf, 1988; Dannevig and Thorud, 1999). Although first described more than 50 years ago, the virus, termed erythrocytic necrosis virus (ENV), has proven refractory to isolation in cell culture and has never been molecularly characterized, although it is presumed to be an iridovirus based on ultrastructural features (Walker and Sherburne, 1977; Reno et al., 1978b; Reno and Nicholson, 1980).

Iridoviruses are large (120–300 nm) icosahedral particles with a genome of up to ~212 kb of double-stranded DNA (Williams et al., 2005; Eaton et al., 2010). There are currently five genera within the family *Iridoviridae* that infect a variety of invertebrate and cold-blooded vertebrate host species (Chinchar et al., 2009; Jancovich et al., 2012). Viruses of the *Iridovirus* and *Chloriridovirus* genera primarily infect insects, *Megalocytivirus* and *Lymphocystivirus* species are associated with fish hosts, and viruses of *Ranavirus* genus are known to cause disease in amphibians, reptiles, and finfish (Chinchar et al., 2011; Jancovich et al., 2012; Kurita and Nakajima, 2012). Many of the iridoviruses infecting cold-blooded vertebrates are significant pathogens that have been propagated in cell culture and are thus well characterized; however a subset of iridoviruses, including ENV, that infect the erythrocytes of cold-blooded vertebrates have often failed to propagate in established cell lines (Gruia-Gray et al., 1989; Wolf, 1988).

Various high-throughput sequencing technologies are now being used in virus discovery and for deciphering the etiology of novel viral pathogens (Delwart, 2007; Radford et al., 2012; Mokili et al., 2012; Bzhalava and Dillner, 2013; Belák et al., 2013). Improvements in the procedures used to obtain viral nucleic acids for high-throughput sequencing from blood or tissue samples containing culture-resistant viruses have further advanced the fields of virus discovery and viral metagenomics (Breitbart and Rohwer, 2005; Thurber et al., 2009; Hall et al., 2014). The objectives of this project were to obtain sufficient amounts of genomic ENV DNA to molecularly characterize the virus using next generation sequencing methods, develop a diagnostic polymerase chain reaction (PCR) assay for detecting ENV, and perform a preliminary phylogenetic evaluation to explore the relationship of ENV to other iridoviruses.

2. Materials and methods

2.1. Fish (Pacific herring)

Test animals consisted of specific pathogen-free (SPF) Pacific herring (Beaulaurier et al., 2012) that were reared at USGS Marrowstone Marine Field Station (Nordland, WA). The protocols for experimental use of live animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Western Fisheries Research Center under the guidelines provided by the Guide for the Care and Use of Laboratory Animals (NRC, 201 1).

2.2. In vivo amplification of ENV by serial inoculations

Six serial inoculations of SPF Pacific herring, henceforth referred to as herring, were conducted to maximize the concentration of ENV in infected blood for downstream molecular analyses. *In vivo* passage of the virus was accomplished by injecting each group of herring with pooled blood collected from survivors of the previous inoculation (Fig. 1). Fish were held in ambient seawater for the entire inoculation series with temperatures ranging from 9.7 to 13.2 °C. Injections occurred every 7–10 days, as this incubation period results in the highest viral titer in herring erythrocytes (Glenn et al., 2012). All herring used in the first five passages were 3 years of age. Larger herring 6 years of age were utilized in the last transfer to maximize the final blood volume drawn from the ENV-infected survivors. For the initial infection, kidney tissues from four ENV-positive herring stored at −80 °C were thawed, pooled, diluted 1:4 in phosphate buffered saline (PBS), and homogenized in a Stomacher (Seward Lab Systems, West Sussex, UK). In the first passages, five herring were anesthetized using a solution of tricaine methanesulfonate (MS-222, 60 mg l^{-1}) buffered with sodium bicarbonate (SB, 300 mg l^{-1}) and each fish received a 200 μl intraperitoneal (IP) injection of ENV-infected kidney homogenate. Fish were held for 7 days at which time survivors were euthanized by immersion in water containing 240 mg l^{-1} of MS-222 and 1.2 g l^{-1} SB. Pooled blood from three survivors was harvested and used the same day to inject 10 more fish for the second passage (Fig. 1). This series was repeated four more times. The final pooled whole blood (27 ml) withdrawn from the survivors of sixth passage was centrifuged at 1000× *g* for 10 min. The plasma was removed and the remaining volume of red blood cells (RBCs) was pooled and suspended in 0.2% NaCl at a 1:2 ratio to lyse the RBCs. The blood sample was placed on ice and transported to Western Fisheries Research Center (WFRC) in Seattle, WA for same day virus purification.

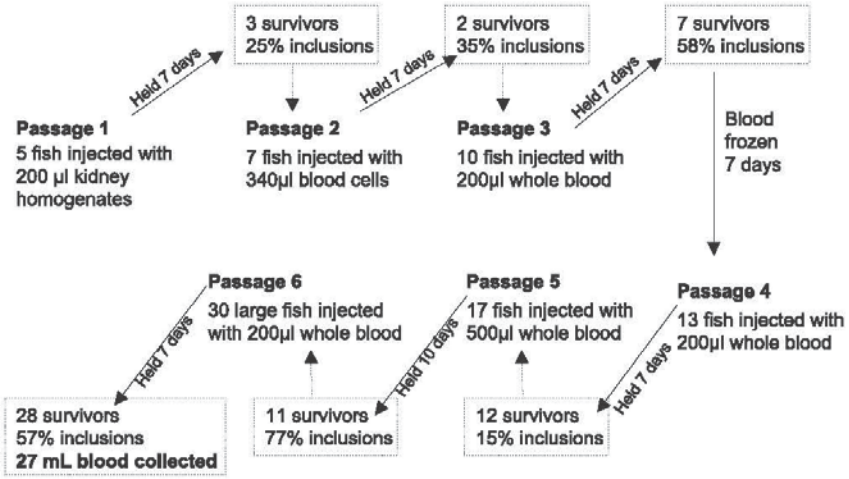

Fig. 1. *In vivo* amplification of ENV in Pacific herring experiment schematic. Six virus passages in herring were performed at 7-to 10-day intervals. The number of herring receiving intraperitoneal injections, and inoculate tissue type containing erythrocytic necrosis virus (ENV), and injection volume is listed under each inoculation group. Listed above the arrows is the number of days fish were held after exposure to virus. The number of survivors sacrificed and average percent prevalence of inclusions in the red blood cells of the survivors for each inoculation group are displayed within the rectangles.

A subsample of blood from survivors of each transfer in the series was smeared onto a glass slide. Blood films were Giemsa stained as previously described by Glenn et al. (2012) and inclusion body concentrations for each donor fish were determined. Average percent prevalence of inclusions in RBCs of the survivors for each challenge was calculated (Fig. 1).

2.3. Virus purification and DNA extraction

Many erythrocytes in the lysate, as examined by light microscopy, were still intact after arrival at the WFRC virology laboratory thus the blood suspension was gently sonicated for 10 seconds in a SoniPrep 150 (MSE Ltd, London, UK) at 50% power output (75 W), examined again by light microscopy, and an additional sonication was performed for 30 seconds to ensure complete lysis. Cellular debris was pelleted by low speed centrifugation at 1000× *g* for 10 min at 4 °C. Clarified lysate was loaded onto a three-layer density gradient (1.2 g cm^{-3}, 1.5 g cm^{-3}, and 1.7 g cm^{3}) of cesium chloride (CsCl) in polyallomer tubes (Beckman Coulter Inc., Brea, CA, USA). Ultracentrifugation of CsCl gradients for 2.5 h at 25,000 rpm (82,700× g) was used to concentrate the virus at the interface between the 1.2 g cm^{-3} and 1.5 g cm^{-3} CsCl layers. Six 1.0 ml fractions were collected with an 18-gauge hypodermic needle and syringe, and two were stored at 4 °C for later processing.

Four 1.0 ml fractions of semi-purified virus immediately underwent DNAse digestion (1 U DNase I/µl virus) for 3 hr at 37 °C in order to reduce the amount of host DNA and maximize the ratio of capsid-protected viral DNA. Subsequent DNA extraction of the DNase-treated gradient fractions followed procedures outlined by Thurber et al. (2009). Purified total DNA extracted from each ENV fraction was suspended in 50 µl molecular-grade water. Absorbance was measured on a spectrophotometer (NanoDrop ND1000; Thermo Scientific, Waltham, MA, USA) to assess DNA quantity and quality. A PCR assay for acidic ribosomal phosphoprotein (ARP) gene (Hansen et al., 2012) was used to determine the extent of host DNA contamination in the semi-purified virus fraction.

2.4. Sequencing

One sample, presumed to contain the highest concentration of ENV genomic DNA, was sent to the Purdue University Genomics Core Facility (West Lafayette, IN) for high-throughput sequencing on a GS-FLX 454 sequencer (Roche) using titanium chemistry as originally described by Margulies et al. (2005). The sample was run on 1/8 of a PicoTiterPlate (PTP) to generate a 3Kb-paired end sequence library. Sequence reads were aligned and assembled into contigs using GS *De Novo* Assembler (Roche), and used in various NCBI BLAST database searches (Altschul et al., 1997; Zhang et al., 2000). An additional tblastx screening against iridovirus genome sequences was also performed. Sequence data from the first pass genome assembly, BLAST searches, and Blast2Go annotation was uploaded to a secure web site and acquired by WFRC researchers. The sequences with the highest identity scores in the iridovirus tblastx alignments were selected as having the highest potential of being authentic ENV genomic sequence.

2.5. Primer sequence selection for conventional PCR assay

Sequence contigs that were identified as iridovirus-like homologs were further evaluated by a similarity BLAST search of the NCBI nr (non-redundant protein) database (Altschul et al., 1997; Zhang et al., 2000). Homologs with the highest scoring (bit-score) hits, *e*-values of 10^{-3} or below, a suggested threshold used in other viral metagenomic studies (Delwart, 2007; Lysholm et al., 2012),

containing presumptive conserved gene domains, and matching either fish or erythrocytic iridoviruses were used in designing sequence primers for diagnostic PCR assays. Primer sequence pairs from the selected iridovirus-like homologs were designed using MacVector (version 7.2.3) Primer3 algorithm (Accelrys Inc., San Diego, CA, USA) for use in a standard PCR assay. Reagents for the optimized conventional PCR included 5 μl each of 10× GeneAmp buffer, 25 mM $MgCl_2$, 2.5 mM dNTP mixture, and 0.5 μl of AmpliTaq (Life Technologies, Carlsbad, CA, USA) along with 50 pmol of each forward and minus primer. Nuclease free water and 1–10 ng μl^{-1} of target DNA were added to a final volume of 50 μl for a single reaction that was run on a PTC-100 thermocycler (Bio-Rad, Hercules, CA, USA) beginning with a 2 min start up at 95 °C, then cycling parameters of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C repeated 29 times, and ending with 7 min at 72 °C and holding at 4 °C.

2.6. Specificity and sensitivity testing of PCR assay

All DNA samples used as templates for testing the ENV-PCR assay were extracted from herring blood or tissues (kidney and spleen) with QIAamp DNA mini kits (Qiagen Inc., Hilden, Germany) following kit instructions. Initial PCR testing of the candidate ENV primer pairs used an ENV-negative DNA sample taken from pooled kidney and spleen of a SPF juvenile herring sampled in 2009 and an ENV-positive DNA harvested from the spleen of an ENV experimental-infected juvenile herring sampled in 2010. Primer sets that generated no products, amplification products of the wrong size, or that produced multiple bands were not selected for further evaluation. The final four candidate primer sets A–D (Table 1) were tested with an expanded panel of 10 DNA templates extracted from blood, spleen, or spleen/kidney pools of 3 SPF (ENV-negative) and 7 ENV-positive herring sampled between 2004 and 2010. DNA from frog virus 3 (FV3), the type species of the *Ranavirus* genus, harvested from FV-3 infected fathead minnow cells following culture techniques detailed by (Mao et al., 1997), was included as a non-specific iridoviral DNA template. Additional PCR specificity testing of the ENV primer sets using DNA extracted from six other iridoviruses (Table 2) was performed using the 55 °C annealing temperature and the same previously described PCR parameters, but annealing temperatures of 45 °C and 50 °C were also tested.

2.7. Confirmation of PCR amplicon sequences

To confirm the contig regions amplified by PCR were authentic ENV sequences, amplification products from conventional PCR using the four primer sets were purified with StrataPrep PCR purification kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced using BigDye chemistry on a 3130 Genetic Analyzer (Life Technologies). The generated nucleotide sequence fragments were edited with Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI, USA) and compared to the original corresponding ENV contig sequences using MacVector (MacVector Inc., Cary, NC, USA) or Sequencher alignment software.

2.8. Phylogenetic analyses

The 00037 contig sequence was selected for phylogenetic analysis as it encoded a portion of the DNA-dependent DNA polymerase gene of ENV. For this contig, the entire sequence was confirmed as above using multiple primers to generate amplicons from DNA extracted from the purified ENV-positive blood sample (data not shown). Open reading frame analyses and translation of the ENV contig 00037 nucleotide sequence used MacVector protein analysis software. Homologous amino acid sequences of ENV and 16 other iridovirus polymerases, representing all five genera, were aligned by two different methods; MUSCLE (Edgar, 2004) and ClustalW (Thompson et al., 1994). *Spodoptera ascovirus*, a member of the *Ascoviridae*

Table 1
Four PCR primer sets (A, B, C, and D) based on sequences of irido-like virus contigs generated from 454 sequencing of an ENV-enriched blood sample from VEN diseased Pacific herring.

Primer set	Primer name	Sequence 5′-3′	Product size (bp)	Contig identifier	Contig length (bp)	GenBank accession number[a]	Iridovirus homolog[b]	Putative conserved gene[c]
A	A2 A1	TTTACCTGCACAATATAAATCTC TTTTTTGGACGGGTGCTGG	278	00094	1000	KJ730210	LCDV (fish)	ATPase
B	B13 B10	TTTAAATTCTTAAAAACTACAG AAAGATTTCTTGATTGCTTCTC	266	00037	1448	KJ756347	TsEV (snake) LEV (lizard)	DNA Polymerase
C	C4 C2	AGATTGTTTACACAGAGTGATC CATTCAACAACCTTTCCCATTCC	378	00016	1970	KJ756345	LCDV (fish)	RNRS
D	D8 D10	GGTGATAAATTTGCGTCGTTT TGTCAAACATGGCTTCTCGAAG	552	00007	2913	KJ756346	GIV (fish) LCDV (fish)	RNA Polymerase

[a] Direct PCR amplification of contig 00037 sequence, used in phylogenetic analyses, revealed three sequence discrepancies. Two adenine (A) insertions are present in the 454 generated 0037 contig sequence at positions 1392 and 1396. At position 1442 the PCR amplified sequence had an ambiguous base read of W (A or thymine [T]), whereas the 454 contig sequence was listed as an 'A'. All three nucleotide discrepancies occurred outside of the open reading frame of amino acid sequence used for phylogenetic analyses and a notation of these differences was included in the 00037 contig sequence Genbank submission.

[b] Iridovirus homolog (table name abbreviation, GenBank accession no.): lymphocystis disease virus (LCDV) – China isolate (AY380826), *Thamnophis sauritus* erythrocytic virus (TsEV, EF608450), *Lacerta monicola* erythrocytic virus (LEV, HQ123319), and Grouper iridovirus (GIV, AY666015).

[c] Adenosine triphosphatase (ATPase), ribonucleotide reductase small subunit (RNRS), DNA-dependent DNA polymerase (DNA polymerase), and DNA-dependent RNA polymerase (RNA polymerase).

Table 2
Iridovirus isolates used for specificity testing of erythrocytic necrosis virus (ENV) conventional PCR assay.

Virus[a]	Iridovirus genus	Animal host		Year of virus isolation
		Common name	Latin name	
Largemouth Bass virus (LMBV)	*Ranavirus*	Largemouth bass	*Micropterus salmoides*	2001
Infectious spleen & kidney necrosis virus (ISKNV)	*Megalocytivirus*	Black molly	*Poecilia sphenops*	2009
Red sea bream iridovirus (RSIV)	*Megalocytivirus*	Florida pompano	*Trachinotus carolinus*	2010
Ambystoma tigrinum virus (ATV)	*Ranavirus*	Tiger salamander	*Ambystoma tigrinum*	2003
Lymphocystis disease virus (LCDV)	*Lymphocystivirus*	Koran angelfish	*Pomacanthus semicirculatus*	2010
Rana catesbeiana virus (RCV-Z)	*Ranavirus*	American bullfrog	*Rana catesbeiana*	2002
Frog virus 3 (FV3)	*Ranavirus*	Northern leopard frog	*Lithobates pipiens*	1965

[a] Virus isolate source: LMBV (also known as *Santee-Cooper ranavirus*), ATV, and RCV-Z provided by Scott LaPatra of Clears Springs Trout Inc. (Buhl, Idaho). ISKNV, RSIV, and LCDV provided by Thomas Waltzek of University of Florida College of Veterinary Medicine. FV3 isolate retrieved from Western Fisheries Research Center (Seattle, WA) −80 °C virology stock freezer, and sub-cultured in a fish cell line in 2007. FV3 isolate tested in first validation PCR assay screening.

family which is thought to be closely related to *Iridoviridae* (Stasiak et al., 2003), served as an outgroup in the phylogenetic analyses. Maximum likelihood (ML) and neighbor-joining (NJ) trees using 500 replicate re-samplings, with both amino acid sequence alignments, were constructed using the MEGA 5.5.2 program (Tamura et al., 2011; Hall, 2013). Whelan and Goldman (WAG + G + I, 2001) model was the highest ranked amino acid substitution model, one with the lowest Bayesian information criterion (BIC) score, for both alignments was selected as the best substitution pattern to generate the ML trees. The ML tree with the highest log likelihood with all positions less than 95% site coverage eliminated and utilizing a partial deletion gap treatment was presented. Suitability of aa alignment data for NJ phylogenetic analyses was confirmed by computing the overall mean distances (average evolutionary divergence over all sequence pairs) for both MUSCLE and ClustalW alignments. NJ tree evolutionary distances were calculated using the Poisson correction method (Zuckerkandl and Pauling, 1965); all ambiguous positions were removed for each sequence pair, and a pairwise deletion approach for missing data.

3. Results

3.1. In vivo ENV amplification and viral DNA sample

The prevalence of erythrocytic inclusion bodies in survivors from the 6 successive virus passages averaged 25%, 35%, 58%, 15%, 77%, and 57%, respectively (Fig. 1). From the final sixth transfer, pooled whole blood obtained from 28 surviving virus-exposed herring was processed to produce fractions with the highest possible proportion of viral DNA. While all gradient fractions contained herring host DNA by PCR screening (data not shown), one fraction was selected for 454 sequencing based on its higher DNA concentration (2312 ng/μl) and quality (optical density [OD] 260/280 ratio of 1.91 and OD 260/230 ratio of 2.05).

3.2. 454 Sequence output and PCR primer selection

Total number of bases, outputted from 454 sequencing of the DNA extracted from the virus-enriched blood sample, was 37,894,903 of which 13.1% were assembled into contigs, 77.3% were singletons, and 2.1% were repeats. After the first standard sequence assembly, 551 contigs were generated with the largest contig containing 4,126 bases. Due to large gaps between sequences a full genome alignment was not obtained. A second assembly that allowed sequence read breaks across contigs performed better and created 2698 contigs with the largest contig again containing 4126 bases. There were 81 contigs that shared various levels of identity with known iridovirus genomic sequences. Primer sets for a PCR assay were designed from eight of these iridovirus-like contigs that met one or more of the following criteria: low e-values (ranging from 6.7e-62 to 9.0e-93), homology with sequences from a fish or eythrocytic iridovirus, and containing orthologous sequences from conserved iridovirus genes.

3.3. Specificity and sensitivity testing of ENV PCR assays

Four of the eight primer pairs initially screened by PCR with a single sample of ENV-positive DNA produced bands of correct molecular size with little background (Table 1). Further confirmatory testing of the four primer sets (A, B, C, and D) using a panel of 3 negative and 7 ENV-positive DNA templates showed that the assay was ENV specific (Fig. S1). The primer sets A, B, and D produced no amplification products from DNA extracted from healthy herring; however primer set C produced some background banding patterns. Primer set D correctly detected all DNA samples tested ($n = 7$) from ENV-positive fish and the remaining primer sets A, B, and C detected $n = 6/7$, 5/7, and 5/7 of the ENV-positive samples respectively. Contig sequences 00094, 00037, 00016, and 00007 used to construct primer sets A, B, C, and D, respectively, were cataloged in Genbank (Table 1). One PCR product from each primer set (A, B, C, and D) was shown by conventional sequencing to be identical to the target region within the specific ENV contig. The no template PCR controls were all negative and none of the four ENV primer pairs produced target bands from the frog virus 3 (FV3) DNA. Primer set A (F2/R1) generated a weak band product (~800 bp) from the FV3 template, but this was much larger than the ENV target amplification product of 278 bp. Similarly, no PCR products

were generated from any of the six additional iridovirus isolates used to further test specificity of the PCR at the standard 55 °C annealing temperature (Table 2, Fig. S2). There were some non-specific binding products generated at lower annealing temperatures of 45 °C and 50 °C, but the bands were not of the expected size (data not shown). Sensitivity testing determined that all ENV primer sets could detect template at 1000-fold dilution of a 30 ng starting concentration. Primer pair D8/D10 (set D) was the most sensitive, with product still being amplified at the highest tested dilution of 10^{-5} (Fig. 2).

3.4. Amino acid alignments and phylogenetic analyses

Direct PCR amplification of DNA purified from the concentrated ENV DNA sample and subsequent sequencing with conventional methods generated multiple amplification products in both directions that were contiguous and the overlapping sequence fragments confirmed that no contaminating DNA sequence was present in the sequence of the 00037 ENV contig used in the phylogenetic analyses. Comparisons between the sequence of the ENV 00037 contig and that generated by conventional methods revealed three sequence differences near the 3′ end of the 1448 nucleotide sequence. Two adenine (A) insertions are present in the 454 generated contig sequence at positions 1392 and 1396, and an ambiguous base read of W (A or thymine [T]) at position 1442 in the PCR amplification sequence rather than an 'A' in the 454 contig sequence. However all three nucleotide discrepancies occurred outside of the open reading frame used for phylogenetic analyses. It is unknown whether the sequence variation is due to PCR amplification errors, 454 sequencing/contig assembly inaccuracies, or possible ENV population variability, thus a notation regarding these discrepancies are included in the Genbank submission for contig 00037 (Table 1). Translation in the sense direction from position 3 to 1447 of contig 00037 nucleotide sequence produced the longest protein fragment of 433 amino acids with homology to a portion of the putative iridovirus DNA polymerase.

Both ClustalW and MUSCLE amino acid alignments of polymerase sequences of 00037 contig, 16 representative iridoviruses, and a single ascovirus had average *p*-distances of <1.0 confirming the reliability of the configurations and suitability of subsequent phylogenetic analyses with latter alignment presented in Fig. 3. The amino acid MUSCLE alignment of 18 sequences had a total of 597 positions with no gap sequence lengths ranging from 142 to 572 amino acids with all ambiguous positions removed from analyses in estimating evolutionary divergence. *L. monticola* erythrocytic virus (EV) sequence contained five undetermined amino acids at positions 59–63; due to poor quality sequence reads (Alves de Matos et al., 2011). Phylogenetic analyses based on the MUSCLE alignment produced final data sets of 92 and 597 positions for ML and NJ methods respectively. Both phylogenetic analysis methods inferred that ENV was most closely associated with *L. monticola* EV, *Thamnophis sauritus* EV, and *Pogona vitticeps* EV (Fig. 4). Both ML and NJ trees had the same topology and groupings of iridovirus genera. Within the erythrocytic virus cluster, ENV and *L. monticola* EV had higher similarity to each other than to *T. sauritus* EV or *P. vitticeps* EV.

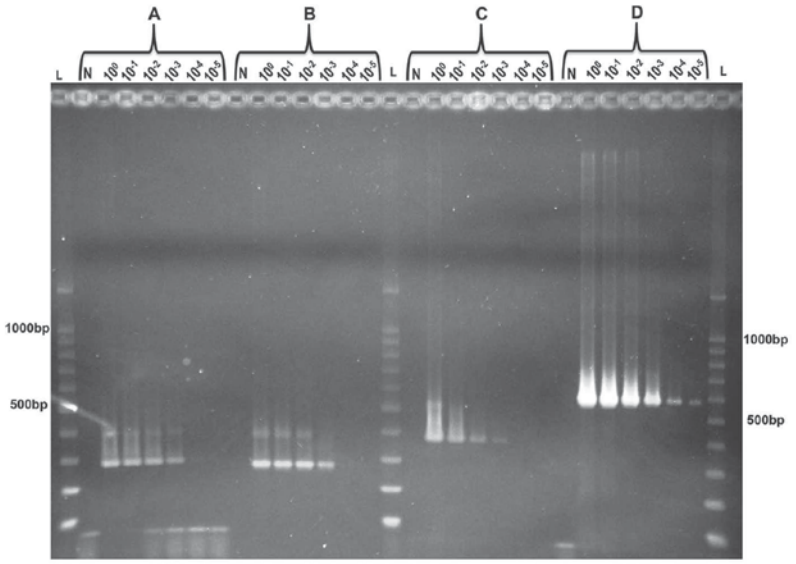

Fig. 2. PCR sensitivity testing of four ENV primer sets ((A) primer pair A2/A1 with a 278 bp product; (B) primer pair B13/B10 with a 266 bp product; C, primer pair C4/C2 with a 378 bp product; (D) primer pair D8/D10 with a 552 bp product). Lane designations listed at the top of each well on the gel are as follows: 100 base pair ladder (L), no template negative (N) PCR control, and a ten-fold series dilution (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) of 30 ng ENV DNA to a final concentration of 0.0003 ng. All ENV primer sets could detect the template at 1000-fold dilution. Primer set D had the highest limit of detection of 0.0003 ng.

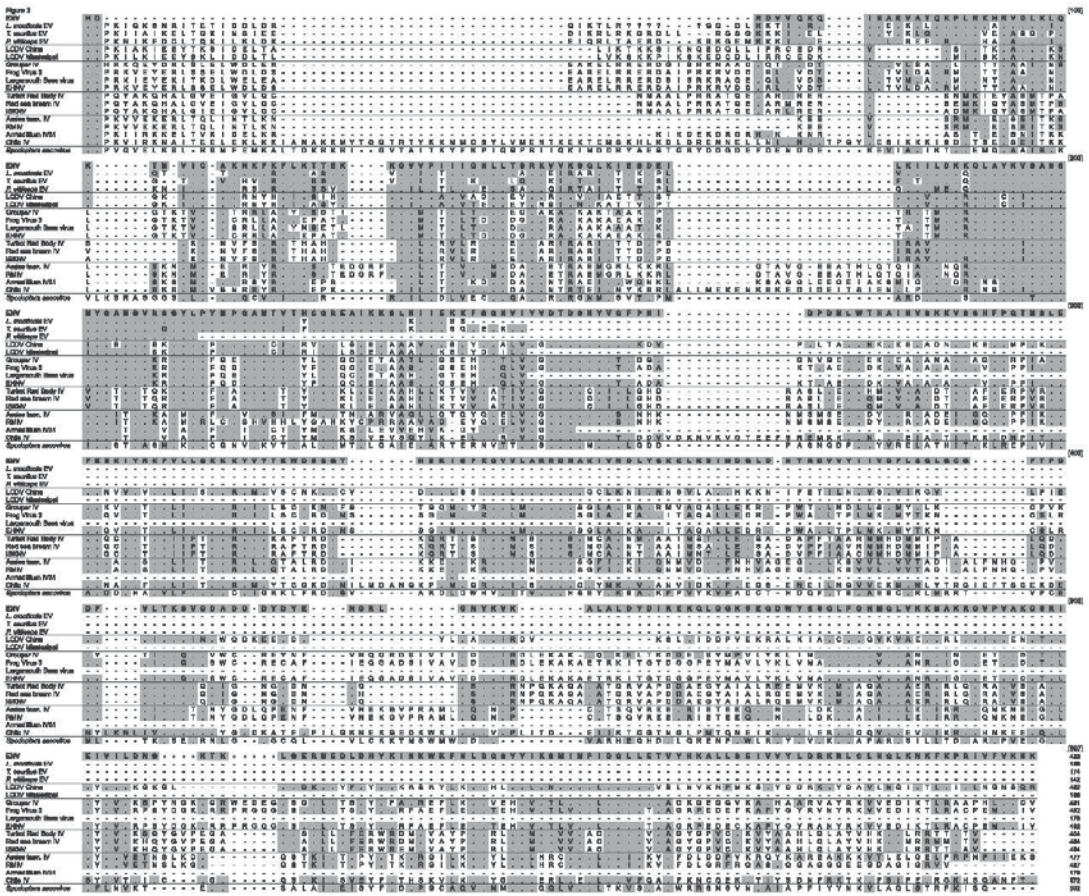

Fig. 3. MUSCLE alignment of deduced partial amino acid sequences of DNA-dependent DNA polymerases of representative iridoviruses and erythrocytic necrosis virus (ENV in bold, KJ756347) contig sequence (00037) from this study. GenBank accession numbers of the iridoviruses: *Lacerta monticola* (lizard) erythrocytic virus (EV, HQ123319), *Thamnophis sauritus* (snake) EV (EF608450), *Pogona vitticeps* (central bearded dragon) EV (KF767871), Lymphocystis disease virus (LCDV) Mississippi (DQ159939), LCDV China (AY380826), Grouper iridovirus (GIV, AY666015), Frog virus 3 (FV3, AY548484), Largemouth Bass virus also known as *Santee-Cooper ranavirus* (LMBV, ABA41591), Epizootic hematopoietic necrosis virus (EHNV, FJ433873), Turbot reddish body iridovirus (Turbot reddish body IV or TRBIV, GQ273492), Red Sea bream iridovirus (Red Sea bream IV or RSIV, AB007366), Infectious spleen and kidney necrosis virus (ISKNV, FN429981), Aedes taeniorhynchus iridescent virus (Aedes taen. IV, NC 008187), Regular mosquito iridescent virus (RMIV, CAC84133), Armadillidium vulgare iridescent virus (Armadillidium IV31, CAC19196), and Chilo iridescent virus (Chilo IV, AF303741). *Spodoptera ascovirus* (AAC54632) served as the outgroup. *L. monticola* EV aa sequence contained five undetermined amino acids at positions 59–63. Amino acids identical (dots) to ENV and conserved substitutions (shaded) are listed below the ENV aa sequence. Lines separate iridoviral genera. Sequence lengths ranged from 142 to 572 aa with a total of 597 positions in the alignment.

4. Discussion

Three (*Lymphocystivirus, Megalocytivirus, and Ranavirus*) of the five genera within the *Iridoviridae* family include viruses that infect finfish from both marine and fresh water environments (Chinchar et al., 2011). Although lymphocystis disease virus (LCDV) was the first identified fish iridovirus, it has not yet been associated with large-scale fish epizootics; whereas, devastating disease events linked to megalocytiviruses and ranaviruses have emerged on a global scale in wide range of host species including fish (Whittington et al., 2010; Subramaniam et al., 2012; Chinchar and Waltzek, 2014). Recent PCR assays have been developed to better diagnose and study fish diseases associated with some iridoviral infections (Gias et al., 2011; Jaramillo et al., 2012). The more highly conserved nucleotide sequences of viral genes coding for adenosine triphosphatase (ATPase), ribonucleotide reductase small subunit (RNRS), major capsid protein (MCP), and DNA-dependent DNA polymerase have successfully been used to design sets of degenerate PCR primers for the initial characterization of novel fish iridoviruses of the *Ranavirus* and *Megalocytivirus* genera (Oshima et al., 1996; Mao et al., 1999; Go et al., 2006; Hanson et al., 2006; Holopainen et al., 2009).

Our own attempts using these same conserved primers (Mao et al., 1997; Hanson et al., 2006), rapid amplification

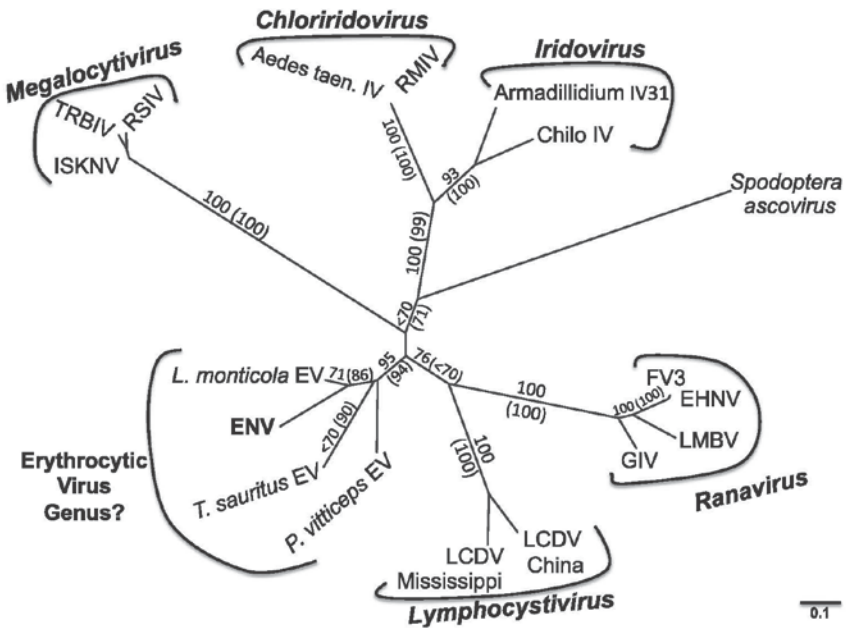

Fig. 4. A maximum likelihood (ML) phylogenetic tree based on the predicted DNA dependent DNA polymerase amino acid sequences (142 – 572) of an 18 virus MUSCLE alignment. The tree was rooted to the outgroup *Spodoptera ascovirus*. ML bootstrap percent values of over 70 for the branches from 500 re-samplings are displayed along with neighbor-joining (NJ) bootstrap percentages shown in parentheses. Erythrocytic necrosis virus (ENV) is highlighted in bold. Iridoviral genera are delineated with bracket arcs. GenBank accession numbers and name abbreviations of the viruses are presented in Fig. 3.

of cDNA ends (RACE) kits with random primers, or other universal virus and iridovirus degenerate primers (Oshima et al., 1996; Mao et al., 1999; Go et al., 2006; Hanson et al., 2006; Holopainen et al., 2009; Nanda et al., 2008) in PCR-based assays did not yield any virus-related amplification products. Other molecular strategies we employed in attempts to obtain authentic ENV genomic sequence included screening extracts of DNA or RNA on a pan-viral microarray (Wang et al., 2002) and subtractive hybridization (Diatchenko et al., 1996) likewise did not produce any ENV-specific sequences. In addition, we attempted *in vitro* cultivation of ENV in a cell line derived from larval Pacific herring (Ganassin et al., 1999) without evidence of ENV replication, a phenomenon that frequently occurs with erythrocytic viruses. Thus, an *in vivo* virus amplification system to generate high titer ENV blood samples and a subsequent viral metagenomic approach for sample analysis were used to provide ENV-specific sequence fragments.

The initial sequence-based corroboration of ENV's taxonomic placement was based on BLAST sequence comparisons. High homology of the generated sequence contigs in concordance with the previously described ultrastructural characteristics confirmed the iridovirus nature of ENV. However residual host DNA contamination and *de novo* contig assembly difficulties prevented compilation of a full-length ENV genome. Because fish red blood cells are nucleated, a large amount of host genetic material remained in our extracted preparations and the proportion of viral DNA may have been lower than desired. Longer DNase treatments and other recently refined virus enrichments methods (Hall et al., 2014) for future purification of ENV infected tissues could be utilized to decrease host DNA sample contamination and increase viral DNA concentration. Another limitation of ENV contig assembly was the lack of highly similar viral reference sequences available in DNA databases to facilitate binning or sorting of large high-throughput data sets (Hurwitz and Sullivan, 2013; Martínez Martínez et al., 2014). The absence of a closely related iridovirus sequence made it impossible to position the smaller ENV contigs onto a longer reference genome sequence. Furthermore some of the contigs may have included chimeric assemblages; ENV sequence intermingled with herring host or other exogenous sequences, making it unfeasible to assemble contiguous ENV sequence fragments with confidence. Assembly algorithms continue to be improved and utilization of multiple assembly programs would assist ENV contig sequence construction (Bzhalava and Dillner, 2013). Transcriptome subtraction, a sequence-based technique where reference host sequences are bioinformatically subtracted from the sequenced sample containing both host and viral transcripts (Weber et al., 2002), has been used to molecularly identify virus pathogens present in very low concentrations (Bexfield and Kellam, 2011). Pacific herring genome sequencing projects are underway (Roberts et al., 2012) and transcriptome subtraction of herring host sequences from the next generation ENV sequence data sets could provide a means of obtaining a higher ratio of authentic ENV sequence

fragments. Despite the presence of herring host DNA in the sample used for sequencing, an estimated 2–4× coverage of the ENV genome was obtained, which was sufficient to substantiate inclusion of ENV within the *Iridoviridae* family.

Results from our PCR testing demonstrated the potential use of the assays as confirmatory tests after an initial VEN diagnosis based on the observation of typical inclusions in blood films. The assays can also be used as primary diagnostic screening tools in suspect cases when blood films are not available. Our PCR assays targeted ENV-DNA from infected Pacific herring found in Puget Sound of North America and these fish frequently break with VEN disease less than a few weeks after capture from the wild (Hershberger et al., 2006). PCR screening of wild juveniles initially after collection and testing of progeny fry newly hatched from collected eggs that were naturally fertilized would facilitate rearing of ENV-free herring stocks in captivity. The lack of ENV reference tissues from other fish species in the Pacific and Atlantic Oceans prevented broader specificity testing of the PCR assays. Future testing of ENV samples of salmonids and other fish species in the Pacific Northwest along with samples from ENV-infected fish species from the Atlantic Ocean would determine the assays' applicability to identify ENV in multiple fish species and detect novel ENV subtypes.

The first evaluation of ENV's phylogenetic relationship to other iridoviruses was based on contig 00037, a homolog to a region of the DNA dependent DNA polymerase. This contig was selected for analysis due to the conserved nature of the polymerase gene and the greater availability of both complete and partial polymerase sequences from other fish and erythrocytic iridoviruses. Preliminary phylogenetic analyses of the putative DNA polymerase gene supported inclusion of ENV in a proposed sixth genus of the family *Iridoviridae*, which contains three other erythrocytic viruses, *Thamnophis sauritus* erythrocytic virus (EV), *Lacerta monticola* EV, and *Pogona vitticeps* EV from ectothermic snake, lizard, and bearded dragon hosts respectively (Wellehan et al., 2008; Alves de Matos et al., 2011; Grosset et al., 2014). Our phylogenetic assessment and those from the three previous studies strongly supported segregation of these erythrocytic viruses into a separate clade. In addition, our phylogenetic iterations consistently placed fish ENV and the lizard *L. monticola* EV together, though branch lengths were shorter and bootstrap value support was borderline (Fig. 4; ML 71% and NJ 86%). Partial sequence phylogenies are valuable introductory determinants of virus relatedness; however a complete ENV genome sequence or other larger core iridovirus gene sequences of ENV and other squamate EVs are needed to further confirm this taxonomic classification. The proposed molecular association of these erythrocytic viruses from such diverse hosts (snakes, reptiles, bearded dragons, and fish) may indicate that for these viruses the cellular host type, e.g. nucleated red blood cell, is a greater factor influencing relatedness than its animal host species.

In conclusion, ENV has been identified molecularly and confirmed to be an iridovirus. Our conventional PCR-assays can be used as diagnostic tools and provide a cost-effective means of rapidly surveying the ENV-status of wild and propagated Pacific herring stocks. Additionally, if the PCR assays can amplify sequences of similar erythrocytic iridoviruses, the generated sequence data could aid in determining the extent of diversity of these erythrocytic viruses in other host species and geographic regions, and ultimately provide greater phylogenetic resolution. ENV appears to belong to a unique genus in the family *Iridoviridae* that includes other related erythrocytic viruses of ectothermic hosts (Wellehan et al., 2008; Alves de Matos et al., 2011; Grosset et al., 2014). Until now only iridoviruses from the *Ranavirus* genus have been reported to infect cold-blooded vertebrates from three taxa (Chinchar et al., 2011; Chinchar and Waltzek, 2014). Similarly, the phylogenetic grouping of lizard, snake, bearded dragon, and fish EVs suggests these erythrocytic iridoviruses also infect a wide range of ectothermic vertebrate hosts and may be more widespread than once thought. In the future, it will be important to determine if a specific erythrocytic iridovirus can infect multiple ectothermic species from different taxa.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.08.028.

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Appendix 4

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DISEASES OF AQUATIC ORGANISMS
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Disease surveillance of Atlantic herring: molecular characterization of hepatic coccidiosis and a morphological report of a novel intestinal coccidian

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ABSTRACT: Surveillance for pathogens of Atlantic herring, including viral hemorrhagic septicemia virus (VHSV), *Ichthyophonus hoferi*, and hepatic and intestinal coccidians, was conducted from 2012 to 2016 in the NW Atlantic Ocean, New Jersey, USA. Neither VHSV nor *I. hoferi* was detected in any sample. *Goussia clupearum* was found in the livers of 40 to 78% of adult herring in varying parasite loads; however, associated pathological changes were negligible. Phylogenetic analysis based on small subunit 18S rRNA gene sequences placed *G. clupearum* most closely with other extraintestinal liver coccidia from the genus *Calyptospora*, though the *G. clupearum* isolates had a unique nucleotide insertion between 604 and 729 bp that did not occur in any other coccidian species. *G. clupearum* oocysts from Atlantic and Pacific herring were morphologically similar, though differences occurred in oocyst dimensions. Comparison of *G. clupearum* genetic sequences from Atlantic and Pacific herring revealed 4 nucleotide substitutions and 2 gaps in a 1749 bp region, indicating some divergence in the geographically separate populations. Pacific *G. clupearum* oocysts were not directly infective, suggesting that a heteroxenous life cycle is likely. Intestinal coccidiosis was described for the first time from juvenile and adult Atlantic herring. A novel intestinal coccidian species was detected based on morphological characteristics of exogenously sporulated oocysts. A unique feature in these oocysts was the presence of 3 long (15.1 ± 5.1 µm, mean ±SD) spiny projections on both ends of the oocyst. The novel morphology of this coccidian led us to tentatively name this parasite *G. echinata* n. sp.

KEY WORDS: Atlantic herring · *Clupea harengus* · Coccidiosis · *Goussia clupearum* · *Goussia echinata*

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INTRODUCTION

The Atlantic herring *Clupea harengus* is an economically and ecologically important species, with populations ranging from the subarctic to temperate zones of the Atlantic Ocean. This migratory species is present in New Jersey waters in the winter and early spring, retreating to spawning grounds in the Gulf of Maine and Georges Bank during the summer and fall (Hay et al. 2001). Atlantic herring juveniles can also be sporadically found in New Jersey coastal waters throughout the summers, based on New Jersey Division of Fish and Wildlife (NJDFW) ocean trawl and Delaware Bay trawl survey results (unpubl.). Herring are primarily planktivores and represent a significant source of forage for marine mammals, sea birds, and large predatory fish. As such, they play a vital role in the transferring of nutrients to higher trophic levels. Additionally, the Atlantic herring is an important commercial species, representing the fifth largest marine fishery by weight worldwide, with over 1.8 million tons harvested in 2012 (FAO 2014). Though

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current populations appear stable, large population declines in the 1970s, largely attributed to overfishing, led to stock collapses across the Atlantic, with some areas experiencing declines greater than 98% (Melvin & Stephenson 2007, Dickey-Collas et al. 2010, Richardson et al. 2011). The scale of the declines and slow recovery in some areas indicate that other factors may be involved in the historic fisheries collapses observed in the 1970s (Richardson et al. 2011). The apparent vulnerability of Atlantic herring to population declines highlights the need to identify factors influencing population dynamics. Though overfishing likely exerts the greatest pressure, other factors such as disease should be considered for understanding the biology of this species.

The impacts of diseases and parasites in clupeid species have been a focus of study in some populations. Two pathogens, viral hemorrhagic septicemia virus (VHSV) and *Ichthyophonus hoferi*, a fungal-like protist belonging to the class Mesomycetozoea, are of particular concern in both Atlantic and Pacific herring populations. Both VHSV and *I. hoferi* have been implicated as major causes of mortality in Pacific herring *C. pallasii* following stock collapse in Prince William Sound, Alaska, USA, after the 'Exxon Valdez' oil spill (Marty et al. 1998, 2010). Though many species of fish are known to be susceptible to VHSV, the 4 genotypes (I to IV) do exhibit some degree of host and geographic specificity (Einer-Jensen et al. 2004, Skall et al. 2005, Emmenegger et al. 2013). VHSV types I to III are considered endemic in Europe, affecting both marine and freshwater fish (Einer-Jensen et al. 2004, Pierce & Stepien 2012), with genotype Ib found at a relatively high prevalence in Atlantic herring from the Norwegian spring-spawning stock (Johansen et al. 2013). In North America, genotype IVa was first detected in the Pacific Northwest in 1988 in cultured salmon, though now it has been isolated from various marine fish species (Meyers & Winton 1995, Pierce & Stepien 2012), including Pacific herring (Meyers et al. 1994, Meyers & Winton 1995, Marty et al. 1998). In 2005, VHSV was isolated during an epizootic event from fish in Lake Ontario and later determined to be a novel genotype, IVb (Elsayed et al. 2006). Later testing revealed strain IVb was present in fish across the Great Lakes. Genotype IVc, found in brackish water fishes from the Atlantic coastal regions of Canada (Gagné et al. 2007), is lesser known, and information is lacking on its prevalence and impacts to marine fish, including herring in the western Atlantic Ocean. Though viral hemorrhagic septicemia has not been found in New Jersey waters, the known susceptibility and migratory life history of Atlantic herring make them a possible reservoir of the virus. Likewise, infection by *I. hoferi*, which stimulates granulomatous inflammation with necrosis in multiple organs, contributes to mortality and population declines in both Atlantic and Pacific herring. Multiple mass mortality events attributed to *I. hoferi* have been reported in Atlantic herring from both sides of the North Atlantic (Sindermann & Chenoweth 1993, Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997). Several of these epizootics have been associated with steep declines in landings of Atlantic herring (Sindermann 1990, Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997, Burge et al. 2014). Given the economic and ecological importance of Atlantic herring, as well as the known susceptibility of this species to VHSV and *I. hoferi*, disease surveillance is important for assessing potential threats to herring populations. An intention of the present study was to determine if these pathogens could be detected off the coast of New Jersey.

A group of apicomplexan parasites, the coccidia, are known to cause both intestinal and extraintestinal infections in marine and freshwater fish. Despite being common parasites of teleosts, little information is available on their biology and diversity and the severity of infection in fish hosts. Because coccidiosis is often assumed to cause little pathology in wild fish, the impacts of intestinal and extraintestinal infections may be grossly underestimated (Dyková & Lom 1981). Observations of mortalities caused by coccidiosis may be more common in cultured fish; *Goussia kuehae* was implicated as the cause for mortality in cultured Asian bass *Lates calcarifer* (Gibson-Kueh et al. 2011, Székely et al. 2013), and *Cryptosporidium molnari* causes severe lesions and mortality in cultured gilthead sea bream *Sparus aurata* (Alvarez-Pellitero & Sitjà-Bobadilla 2002, Sitjà-Bobadilla & Alvarez-Pellitero 2003). However, coccidian infections have been reported to reduce body condition (Morrison & Hawkins 1984, Abollo et al. 2001) and cause heavy infections in wild fish populations (Abollo et al. 2001, Lovy & Friend 2015). Reports of coccidia in Atlantic herring are limited to *G. clupearum* from the liver and *Eimeria sardinae*, which infects the testes (Morrison & Hawkins 1984, MacKenzie 1987, Morrison & Marryatt 1990). Though neither of these parasites is considered a serious pathogen of herring, both can elicit well-developed innate immune responses; this is reported to be more pronounced in *G. clupearum* infections, where phagocytes may occur in large numbers (Morrison & Hawkins 1984). Intestinal coccidiosis is unknown from Atlantic herring, though an undescribed coccidian species from the pyloric cecum of Pacific herring has been reported by histology (Marty et al.

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1998, 2010). Although the prevalence of this intestinal coccidian was high, up to 100% in some populations (Marty et al. 2010), infection intensity was low, with no associated inflammation or lesions (Marty et al. 1998). Intestinal coccidiosis by *G. ameliae* has been described in another clupeid, the alewife *Alosa pseudoharengus*, at high prevalence (up to 92% in some populations) and with heavy infections associated with epithelial necrosis and sloughing of intestinal cells (Lovy & Friend 2015).

With information on intestinal coccidiosis in Atlantic herring lacking, an intention of the present study was to document and describe intestinal coccidians present in this species. Additionally, though *G. clupearum* is unlikely to cause population level declines, its common occurrence in the livers of Atlantic and Pacific herring, as much as 89% reported in some populations of Atlantic herring (Morrison & Hawkins 1984) and 84% in Pacific herring (Marty et al. 2010), as well as reports of hepatic histopathological changes (Costa & MacKenzie 1994), led us to provide a more thorough assessment of *G. clupearum* in Atlantic herring from New Jersey, USA. Additional objectives included assessing morphologic and molecular differences between *G. clupearum* from Atlantic and Pacific herring, determining the phylogenetic placement of this parasite in relation to other coccidians, and assessing the infectivity of *G. clupearum* isolates to Pacific herring.

MATERIALS AND METHODS

Fish collection and sampling

Juvenile Atlantic herring were collected in October 2012 from the Delaware Bay Juvenile Finfish Trawl Survey conducted by the Bureau of Marine Fisheries, NJDFW (project led by B. Neilan and J. Hearon). Briefly, fish were collected by (~5 m) 16 ft otter trawl towed for 10 min behind a 42 ft (~13 m) research vessel, the R/V 'Zephyrus'. Fish were dissected and fixed in 10% neutral-buffered formalin (NBF) in the field and transported back to the Fish Pathology Laboratory in Oxford, New Jersey, for routine histological processing (Table 1).

Adult Atlantic herring (total length 25.0 ± 2.1 cm, mean ± SD) were collected aboard the R/V 'Seawolf' from waters off the coast of New Jersey as part of an ongoing Ocean Stock Assessment project conducted by the Bureau of Marine Fisheries, NJDFW (project led by G. Hinks and L. Barry). Briefly, fish were collected by 30 m otter trawl towed behind the vessel. Fish were stored on ice and transported from the port at Point Pleasant, New Jersey, to the Fish Pathology Laboratory in Oxford, New Jersey, in the evening and either refrigerated (4°C) for next-day processing or frozen (−20 to −80°C) for future virology sampling. Sampling dates and details are provided in Table 1.

Histopathology

Internal organs including the gastrointestinal tract were collected from 10 juvenile Atlantic herring and fixed in 10% NBF in October 2012. Livers, hearts, and internal viscera from adult Atlantic herring were collected and fixed in 10% NBF from 2013 to 2015 (Table 1). Tissues were routinely processed for histology and embedded in paraffin wax, and 4 µm sections were cut and stained with hematoxylin and eosin. For surveillance of *Ichthyophonus*, heart tissue was cut in half and embedded in wax, and 2 serial

Table 1. Atlantic herring sampling details from 2012 to 2016. Histo.: routine histological processing; NBF: neutral-buffered formalin; C.C.: cell culture on *Epithelioma papulosum* cyprinid cells; RT-PCR: real-time reverse transcription polymerase chain reaction

Date	No. of samples	Testing method	Storage condition	Organs sampled	Age	Location
Oct 2012	10	Histo.	NBF	Internal viscera	Juvenile	Delaware Bay
Apr 2013	160	C.C.	−20°C	Brain, spleen, kidney	Adult	Atlantic Ocean
	40	Histo.	NBF	Liver, heart		
Jan 2014	120	C.C.	−80°C	Brain, spleen, kidney	Adult	Atlantic Ocean
	60	RT-PCR	−80°C	Brain, kidney		
	60	Histo.	NBF	Heart		
Jan 2015	120	C.C./RT-PCR	−80°C	Brain, kidney	Adult	Atlantic Ocean
	60	Histo.	NBF	Heart		
	20	Histo.	NBF	Liver		
May 2015	4	C.C./RT-PCR	4°C	Brain, kidney, gills	Adult	Atlantic Ocean
	20	Histo.	NBF	Internal viscera		
Jan 2016	120	C.C./RT-PCR	−40°C	Brain, kidney, gills	Adult	Atlantic Ocean

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sections about 100 µm apart were examined with a Nikon Eclipse E600 light microscope for the presence of schizonts or other stages of the parasite. For surveillance of coccidian infections, liver tissue was examined for the presence of *Goussia clupearum*, and the anterior intestine (October 2012 and May 2015 only) was screened for intestinal coccidiosis. Photographs were taken with a Jenoptik ProgRes Speed XT core 3 microscope-mounted digital camera.

Fresh liver and intestinal coccidia collection

In January 2015, 60 freshly captured Atlantic herring were analyzed for the presence of *G. clupearum*. The fish were first measured by taking total body length (BL, cm) and body weight (BW, g), from which the Fulton's K index ($K = BW/BL^3 \times 100$) was used to calculate the condition factor of fish. Whole livers were dissected and homogenized, and wet mounts were prepared for analysis by light microscopy. To identify the infection intensity with the coccidian, the total number of oocysts was counted in 4 fields of view under 200× magnification. Linear regression analysis was then used to determine if an association existed between fish condition and intensity of *G. clupearum* in the liver. Measurements were made directly from digital images of fresh parasites using the Jenoptik imaging software; measurements taken included oocyst diameter and sporocyst length and width. Additionally, the sporocyst length/width relationship was calculated. Samples containing the highest infection intensity were selected for molecular analysis. These were diluted in phosphate-buffered saline (PBS) and filtered through gauze to remove host liver tissue. Samples were then centrifuged, the supernatant was removed, and the pellet was frozen at −80°C for future molecular analysis. For molecular and morphological comparison of *G. clupearum* from this study to the respective parasite in Pacific herring, liver samples were collected from 30 pre-spawn adult Pacific herring (fork length 228 ± 26 mm), collected from Sitka Sound, Alaska, on March 22, 2015. Tissues were stored on ice and processed within 48 h for *G. clupearum* as described for Atlantic herring samples earlier in this paragraph. A 2-tailed t-test was used to compare parasite dimensions between the Atlantic and Pacific *G. clupearum*.

The presence of intestinal coccidians was assessed from 30 Atlantic herring (January 2015 collection). Wet mounts of intestinal mucus were analyzed using a Zeiss Stemi 2000C stereomicroscope; samples containing unsporulated coccidia were transferred to 15 ml conical tubes containing 5 ml of seawater supplemented with 200 U penicillin ml^{-1}, 200 µg streptomycin ml^{-1}, and 0.5 µg amphotericin B ml^{-1} (Lonza). Samples were incubated in the dark at room temperature for 48 h, after which they were examined for sporulation of oocysts using light microscopy. Length and width were measured from fresh preparations of sporulated oocysts and sporocysts using Jenoptik imaging software, and the length/width relationship was calculated.

VHSV virological analysis

Because of the timing of fish collection, samples for cell culture and real-time reverse transcription polymerase chain reaction (RT-PCR) were collected from previously frozen fish, with the exception of May 2015, when fish were refrigerated overnight and sampled the next day. For dissection of the organ pools from frozen fish, fish and organs were maintained frozen during dissection of brain and head kidney. The organs were aseptically removed, placed into Whirlpak bags, and transported frozen to the New Jersey Department of Agriculture Animal Health Diagnostic Laboratory (Ewing, New Jersey). The tissue pools included 5 fish pools of brain, head kidney, and spleen; in May 2015 and January 2016, gill was also included in the pooled tissue sample (Table 1). Tissue pools were thawed, homogenized, routinely processed for viral isolation on epithelioma papulosum cyprinid (EPC) cells, and observed for cytopathic effect (CPE) at 15°C for 14 d. Lacking CPE, a blind passage was performed after 14 d by re-inoculating newly seeded EPC cells and incubating for an additional 14 d.

Samples were tested using real-time RT-PCR from 2014 to 2016 using 2 different methods. Briefly, in 2014, using methods adapted from Phelps et al. (2012), a small sample of the tissue homogenate (see previous paragraph) was reserved for RNA extraction using a MagMAX Total RNA Isolation Kit (Ambion) with automated sample processing using a KingFisher Flex (Ambion). RT-PCR reactions were run in 20 µl reaction volumes consisting of 5 µl extracted RNA, 5 µl of 4X TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems), 1µl each of forward and reverse primers (10 µM each), 1 µl probe (5 µM), and 7 µl RT-PCR grade water. Primers (Invitrogen) and a probe (Applied Biosystems) specific to the N-gene were used: the VHSV N-gene forward (5′-ATG AGG CAG GTG TCG GAG G-3′), VHSV N-gene reverse (5′-TGT AGT AGG ACT CTC CCA GCA TCC-3′),

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and MGB N-gene probe (5′-FAM-TAC GCC ATC ATG ATG AGT-MGBNFQ-3′). RT-PCR was run on an ABI 7500 qPCR thermocycler (Applied Biosystems) under the following conditions: reverse transcription at 50°C for 5 min, reverse transcriptase inactivation and initial denaturation at 95°C for 20 s, and 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. In 2015 and 2016, testing was conducted by New Jersey Department of Agriculture staff using protocols provided by the US Department of Agriculture National Veterinary Services Laboratories published by Jonstrup et al. (2013) and evaluated by Warg et al. (2014). Future RT-PCR testing for VHSV will utilize the methods adopted in 2015.

G. clupearum molecular analysis

DNA extraction, polymerase chain reaction, and DNA sequencing

DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) and a QIAcube (Qiagen) according to the manufacturer's instructions. Portions of the small subunit 18S rDNA (ssrDNA) were amplified in 50 µl volumes using 3 µl of DNA template and a final concentration of 1X polymerase chain reaction (PCR) buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer (primer details provided in the next paragraph), and 1.25 U *Taq* polymerase (Invitrogen). PCRs were performed on a Veriti thermocycler (Applied Biosystems) with initial denaturation at 95°C for 3 min, followed by 35 amplification cycles at 94°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 75 s. A final extension was run at 72°C for 7 min. The amplified products were visualized with ultraviolet light on a 1.2% agarose E-gel (Invitrogen) containing ethidium bromide. Samples containing a single product at approximately 1000 bp size were purified with ExoSAP-IT (Affymetrix) and diluted to 3 ng of DNA μl^{-1} with molecular-grade water. Sequencing was performed in both directions using a 5 µM final concentration of the amplification primers. DNA sequencing was completed by GENEWIZ using ABI BigDye version 3.1 and run on an ABI 3730xl DNA analyzer (Applied Biosystems).

Initially, 2 sets of primers specific for coccidia were used for each sample: the 18E (5′-CTG GTT GAT CCT GCC AGT) forward and Coc2r (5′-CTT TCG CAG TAG TTC GTC) reverse primers (Whipps et al. 2012) and the Coc1f (5′-GAT TAA TAG GGA CAG TTG) forward and 18R (5′-CTA CGG AAA CCT TGT TAC G) reverse primers (C. M. Whipps pers. comm.). The Coc1f/18R primers amplified and yielded a single product around 1000 bp, which was selected for sequencing. However, the 18E/Coc2r primers were unsuccessful in amplifying the target region. A new reverse primer, Gclup2r (5′-AGG AGA AGT CGG AGA GAC G), was designed based on the sequence results of the Coc1f/18r region and used with the 18E forward primer. The annealing temperature was modified from 50 to 54°C, but all other cycling conditions remained the same. This primer set amplified and yielded a single product containing approximately 1000 bp. PCR, product visualization, purification, and sequencing were then performed as described in the previous paragraph.

Sequence alignment and phylogenetic analysis

DNA sequence chromatograms were visually inspected and edited using Chromas Lite version 2.1 and aligned using the BioEdit version 7.2.5 sequence alignment editor (Hall 1999). After alignment with ClustalW, a consensus sequence was generated and checked against all known sequences from the GenBank DNA database using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Sequences were confirmed to be most closely related to other known coccidian species. Phylogenetic analyses were performed using MEGA7 (Kumar et al. 2016). The evolutionary history was inferred by using the maximum likelihood method based on the general time-reversible model (Nei & Kumar 2000). The tree with the highest log likelihood (−5569.0826) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 0.2330]). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 37.2419% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were 1356 positions in the final dataset.

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Direct infectivity of *G. clupearum* to Pacific herring

A controlled experiment was performed to investigate the direct infectivity of *G. clupearum* oocysts to Pacific herring. Experimental inocula consisted of *G. clupearum* oocysts obtained from the livers of wild Pacific herring. Livers were screened by microscope squash preparations from 30 wild-captured Pacific herring, which were euthanized with an overdose of MS-222. Heavily infected livers were pooled and homogenized in Whirl-pak bags, followed by dilution with PBS. The diluted liver homogenate was filtered through cheesecloth packed into a syringe to remove large tissue debris. The sample was allowed to settle overnight at 4°C, and the supernatant was replaced with fresh PBS. The oocyst preparation was used within 72 h. Prior to infection of fish, oocyst counts were made by re-suspending in PBS and using a hemocytometer to estimate the number of sporocysts. The final preparation was diluted to contain at least 5000 sporocysts ml^{-1}. Experimental animals, consisting of age 0 yr specific pathogen-free Pacific herring (Hershberger et al. 2007), were maintained in 260 l circular tanks supplied with single-pass, filtered, and UV-irradiated seawater. The experimental fish were each exposed to about 500 *G. clupearum* sporocysts within a 100 µl suspension by gastric gavage (n = 27); negative controls (n = 60) were exposed to PBS in lieu of parasite suspensions. Gastric gavage was done by syringe with soft flexible tubing attached; fish were lightly anesthetized with MS-222, intubated with 100 µl of suspension into the stomach, and transferred to the research tank to recover. Following exposures, fish were maintained on a diet of commercial pellets (Bio-Olympic). Fish were fed to satiation every 2 to 3 d. Mortalities were sampled daily, and survivors were subsampled at 21, 43, and 77 d post-exposure (n = 4 d^{-1}). Liver squash preparations from all sampled herring were examined microscopically (200× magnification) for the presence of *G. clupearum* oocysts.

RESULTS

VHSV and *Ichthyophonus hoferi* surveillance in Atlantic herring

Throughout the course of this study (April 2013 to January 2016), VHSV was not detected by either cell culture (n = 524) or RT-PCR (n = 304). Likewise, *I. hoferi* was not detected in hearts by histology (n = 160).

Goussia clupearum in Atlantic herring

G. clupearum occurred in 78% (47 of 60) of fresh preparations from liver tissue sampled in January 2015. The intensity of infection ranged from light to severe, with the heaviest being over 800 oocysts observed in 4 fields of view at 200× magnification. Severe infection, defined as having over 300 oocysts in 4 microscope fields under 200× magnification, occurred in 10% of the sampled fish. No association was seen between infection intensity and fish condition factor *K*. The sporulated oocysts were spherical, with a smooth, thin wall and variation in size (range = 20.1–31.3 µm) (Fig. 1A). Oocysts contained 4 ellipsoidal sporocysts which each contained 2 sporozoites and abundant sporocyst residuum. Measurements for sporulated oocysts and sporocysts are summarized in Table 2.

Table 2. Measurements of oocyst and sporocyst diameter, length, width, and length/width (L/W) relationship ± standard deviation (SD) in *Goussia clupearum* from Atlantic and Pacific herring and *G. echinata* from Atlantic herring with spine length. na: not applicable

Goussia spp.	Oocyst diameter or length (µm) ± SD (range)	Oocyst width (µm) ± SD (range)	Oocyst L/W (µm) ± SD (range)	Sporocyst length (µm) ± SD (range)	Sporocyst width (µm) ± SD (range)	Sporocyst L/W (µm) ± SD (range)	Spine length (µm) ± SD (range)
G. clupearum (Atlantic)	25.4 ± 2.4 (20.1–31.3) (diameter), n = 85	na	na	11.7 ± 0.9 (9.6–14.2), n = 100	8.4 ± 0.8 (6.9–10.8), n = 100	1.4 ± 0.1 (1.0–1.7), n = 100	na
G. clupearum (Pacific)	21.1 ± 1.1 (18.4–25.4) (diameter), n = 85	na	na	10.7 ± 0.8 (8.7–12.9), n = 100	7.6 ± 0.4 (6.4–8.6), (n = 100)	1.4 ± 0.1 (1.1–1.7), n = 100	na
G. echinata n. sp.	18.7 ± 0.5 (18.0–19.3) (length), n = 6	11.1 ± 0.9 (9.4–11.7), n = 6	1.7 ± 0.2 (1.5–2.0), n = 6	9.2 ± 0.9 (7.8–11.1), n = 13	4.1 ± 0.5 (2.9–4.8), n = 13	2.3 ± 0.5 (1.9–3.8), n = 13	15.1 ± 5.1 (2.9–20.8), n = 19

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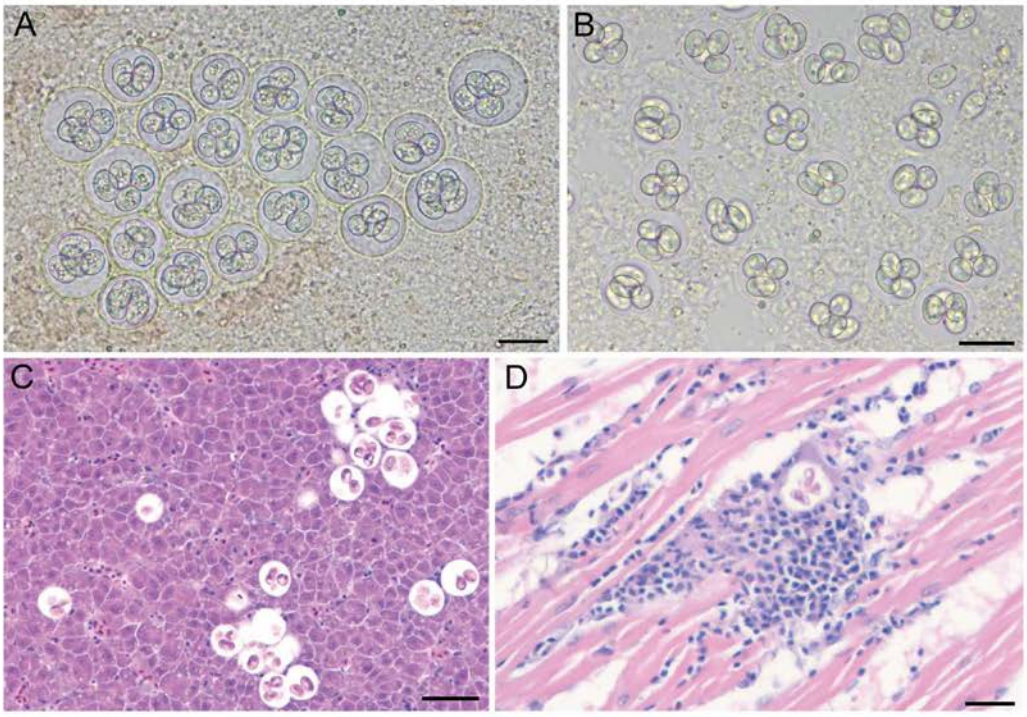

Fig. 1. *Goussia clupearum*. Scale bars = 20 µm. Wet mounts of fresh homogenized liver tissue with (A) oocysts in Atlantic herring showing size variation of oocysts and (B) oocysts in Pacific herring. (C,D) Histology of Atlantic herring stained with hematoxylin and eosin (H&E) showing (C) aggregates of oocysts in liver tissue and (D) Atlantic herring heart infected with coccidia resembling *G. clupearum* showing inflammatory response

Histological assessment of liver sections indicated prevalences of 40% (April 2013), 75% (January 2015), and 55% (May 2015). Oocysts frequently occurred in aggregates (Fig. 1C), often surrounded by a yellow-brown cellular matrix, resembling pigmented macrophage aggregates. No notable lesions were observed in the hepatocytes, and no significant inflammatory response was observed, even in cases of heavy infection. In 2 fish, hearts that were screened for *I. hoferi* using histology had light infection with a coccidian consistent with *G. clupearum*. These oocysts were surrounded by multiple layers of inflammatory cells (Fig. 1D).

G. clupearum measurements from Atlantic and Pacific herring

The *G. clupearum* oocysts observed in fresh wet mounts of Atlantic and Pacific herring liver homogenates were similar in shape and morphology (Table 2, Fig. 1A,B). However, the mean oocyst diameter was significantly different in the Atlantic and Pacific herring ($p < 0.001$), with the range of dimensions indicating that the oocysts from Pacific herring were slightly smaller. The sporocyst morphology appeared similar between clupeid hosts; however, the length and width of sporocysts from Pacific herring were again slightly smaller ($p < 0.001$) (Table 2). The sporocyst length/width ratio was not significantly different between the host species.

Sequencing and phylogenetic analysis of *G. clupearum* from Atlantic and Pacific herring

Analysis of the small subunit 18S rRNA gene in *G. clupearum* from Atlantic herring yielded a 1757 bp consensus DNA sequence, deposited in GenBank under accession number KT025255. For Pacific herring, a

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Table 3. Nucleotide differences observed between small subunit 18S rDNA sequences of *Goussia clupearum* from Atlantic and Pacific herring based on alignment to Atlantic sequence and number of nucleotides

Position	Atlantic	Pacific
180	C	T
249	A	G
649	T	A
656	G	A
719	T	–
1350	T	–

1749 bp consensus DNA sequence was generated and deposited under accession number KT025256. Comparison of the 2 sequences, based on alignment of 1749 nucleotides, demonstrated significant identity (99.6%). Differences in the 2 sequences included 4 nucleotide substitutions and 2 gaps (Table 3).

When the *G. clupearum* sequences were compared to other coccidia using BLAST (NCBI), the closest identities were with coccidia in the *Calyptospora* genus, including *C. funduli, C. spinosa*, and *C. serrasalmi*. The closest match was with *C. funduli* (accession number GU479670.1), which had 92% query cover and 94% identity. When aligned to all coccidia, the query coverage never exceeded 92% because *G. clupearum* had an approximately 124-nucleotide insertion that did not occur in any other coccidian species. This insertion occurred from nucleotide positions 605 to 729, based on the 1757 nucleotide sequence from Atlantic herring *G. clupearum* (KT025255). A search for similarities to this 124-nucleotide sequence did not reveal any significant similarities using BLAST. Three of the nucleotide differences between the Pacific and Atlantic *G. clupearum* occurred within this unique 124-nucleotide region. Phyloge-

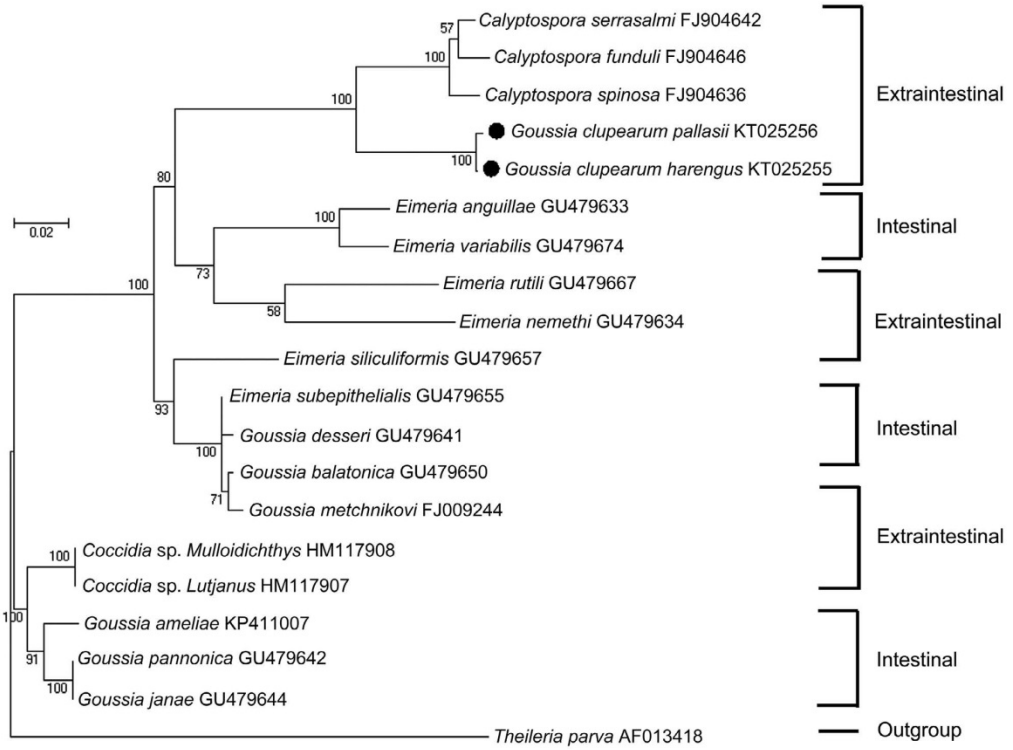

Fig. 2. Phylogenetic tree constructed using the criterion of maximum likelihood analysis based on variation in the small subunit rRNA gene from 21 sequences obtained from GenBank and 2 sequences from this study (*Goussia clupearum*, denoted by ●). *G. clupearum* groups most closely with other extraintestinal liver coccidia of fish and is distinct from intestinal fish coccidia. *Theileria parva* was used as an outgroup to root the tree. Branch lengths shown here are proportional to the number of substitutions (as shown by the scale bar). Bootstrap support is represented by the numbers at the internal nodes

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netic analysis demonstrated that *G. clupearum* grouped most closely to other extraintestinal coccidians within the *Calyptospora* genus (Fig. 2).

Direct infectivity of *G. clupearum* to Pacific herring

G. clupearum was not detected in the livers of any Pacific herring that were exposed to parasite suspensions by gastric gavage, including 4 mortalities (3, 9, 19, and 34 d post-exposure) and 12 subsampled survivors (4 each at 21, 43, and 76 d post-exposure). Similarly, *G. clupearum* was not detected in any negative controls (mortalities or subsampled survivors).

Intestinal coccidiosis in Atlantic herring

In October 2012, 10 juvenile Atlantic herring were collected and screened by histology for signs of parasites and diseases. In 6 of the fish, a coccidian parasite was observed attached to the intestinal epithelial cells within the pyloric ceca (Fig. 3), with microgametocytes and macrogamonts found in an epicellular position within the epithelium (Fig. 3).

Because intestinal coccidiosis had not been previously described in Atlantic herring, additional anterior intestine and pyloric cecum samples were subsequently examined from 30 adult herring, collected in January 2015. Because of the timing and location of collection, fresh wet mounts could not be prepared until nearly 24 h after fish were collected. At the time of wet mount preparation, the digestive tract had some post-mortem changes related to autolysis. Unsporulated oocysts were identified in one sample of intestinal mucus. After incubation for 48 h, the oval unsporulated oocysts had fully sporulated. Oocysts were oval, with a smooth, thin wall and no oocyst residuum, micropyle, or polar granules visible under light microscopy (Fig. 4). Within the oocyst were 4 ellipsoidal sporocysts, each containing 2 sporozoites and plentiful sporocyst residuum. Stieda bodies were not observed under light microscopy. The most notable and unexpected feature of these oocysts was the presence of 6 variably long spines projecting from the wall of the oocyst, with 3 spines on each pole of the oocyst (Figs. 4 & 5). The spines contained a narrow lumen, which appeared to be continuous with the oocyst wall. The length of the spines varied from 2.9 to 20.8 µm, with an average length of 15.1 µm (Table 2). The novel morphology of this coccidian led us to tentatively name this parasite *G. echinata* n. sp., as described in the next subsection. The sample was saved for molecular analysis. However, attempts to amplify the DNA using the previously reported coccidian primers were unsuccessful. This may be related to the relatively small numbers of oocysts in the sample preparation, or it is also possible that the currently used coccidian primers were not compatible with this species.

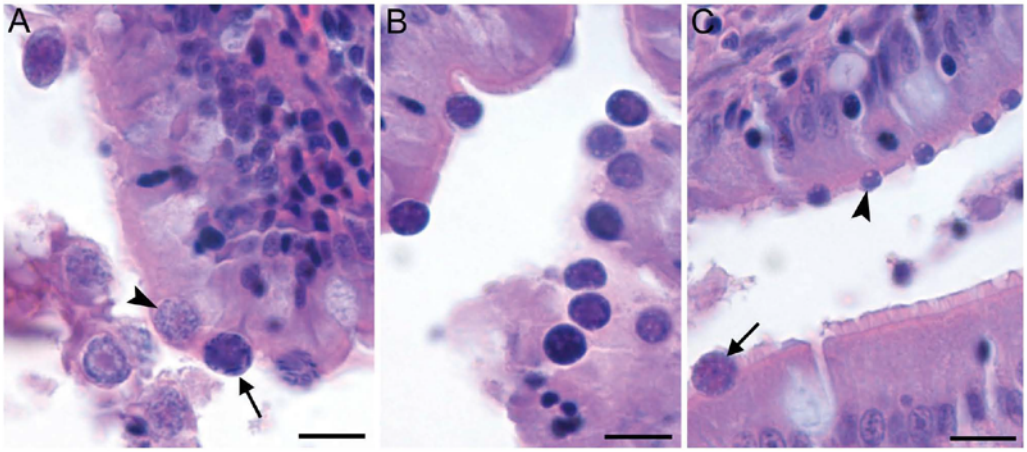

Fig. 3. Histology of various stages of coccidian infection in juvenile Atlantic herring within the pyloric ceca, stained with hematoxylin and eosin (H&E); scale bars = 10 µm. (A) Epicellular position of microgametocyte (arrow) and macrogamont (arrowhead), (B) macrogamonts embedded in the epithelium, and (C) early developmental stages (arrowhead) and macrogamont (arrow) within the brush border of the epithelium

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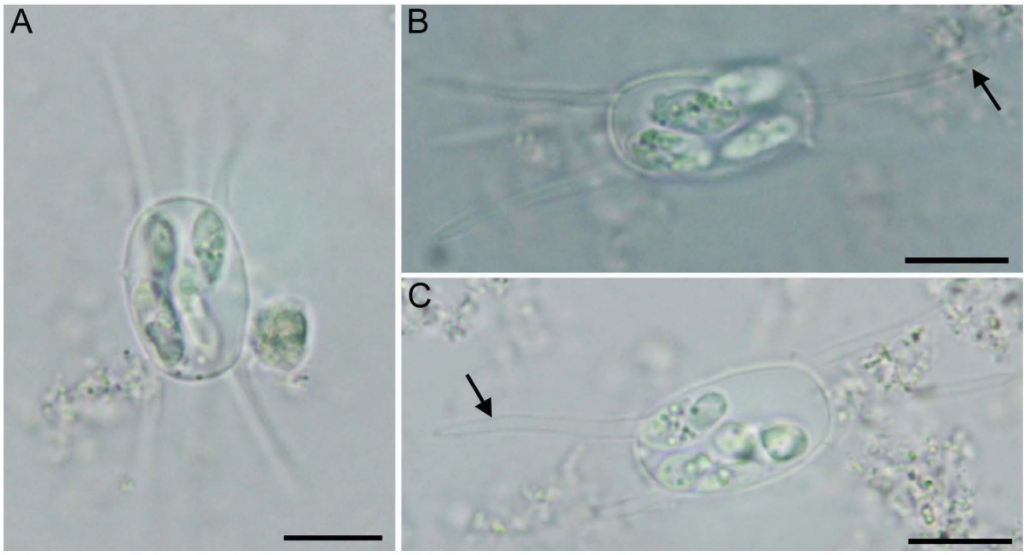

Fig. 4. *Goussia echinata* n. sp. from the intestine of Atlantic herring. Scale bars = 10 µm. (A–C) Wet mounts of sporulated oocysts showing smooth oocyst wall with long, spiny projection (arrows). Oocysts contain 4 ellipsoidal sporocysts

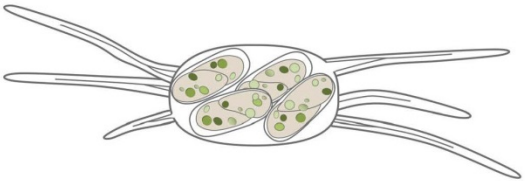

Fig. 5. Diagram of sporulated oocyst of *Goussia echinata* n. sp. in Atlantic herring. Scale bar = 5 µm

In May 2015, 20 adult Atlantic herring were collected and preserved in 10% NBF for additional histological observations of the anterior intestine and pyloric ceca. During this time, only 4 fish could be collected for fresh examination of intestinal mucus. Of the 20 fish collected for histology, 11 contained coccidia in the pyloric ceca (55% prevalence). The coccidian infection was very light, with no associated lesions in the intestinal epithelium (Fig. 6A–E). Because of the light infection intensity in these fish and limited availability of fresh fish (n = 4), fresh preparations of the parasite were unsuccessful. In histology, only epicellular coccidia were observed in the anterior intestine of all adult herring sampled. It is possible that 2 different species of epicellular coccidians exist in the tissue. The macrogamonts in the adult herring gut histology were significantly elongated (Fig. 6C–E), which corresponds more with the elongated sporulated oocysts seen in fresh preparations from adult fish sampled in January 2015 (Fig. 4), whereas those observed in the juvenile herring gut histology from 2012 were mainly spherical (Fig. 3). Considering the likelihood that 2 species of coccidians are infecting the intestine of herring, the histology and wet mount preparations of the coccidian could not be definitively linked.

Taxonomic description

Goussia echinata n. sp. (Figs. 4A–C & 5)

Type host: Atlantic herring *Clupea harengus*

Other host: Unknown

Type locality: NW Atlantic Ocean, New Jersey, USA (40° N, 74° W)

Other localities: Unknown

Site of infection: Anterior intestine, pyloric cecum

Prevalence: Unknown

Intensity: Unknown

Phototypes: Catalogued at the NJDFW Fish Pathology Laboratory, Oxford, New Jersey 07863, USA

Parasite description: Epicellular coccidian with exogenous sporulation. Unsporulated oocysts from fresh preparations were ovoid, with a smooth wall and no projections or spines. Sporulation occurred exoge-

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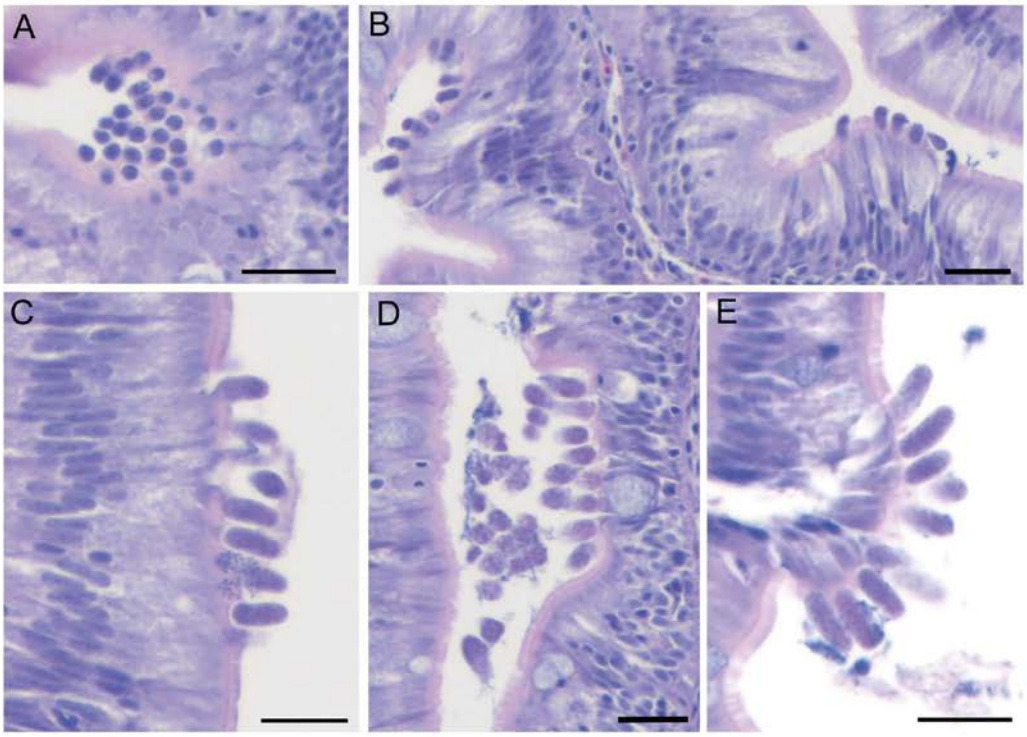

Fig. 6. Histology of coccidian infection in adult Atlantic herring within the pyloric ceca, stained with hematoxylin and eosin (H & E); scale bars = 20 µm. (A–E) Various stages of coccidia infection showing (C–E) elongated macrogamonts and (D) indentation of the epithelium, with little disruption of the brush border

nously within 48 h; oocysts were ovoid, with a smooth, thin wall. Each end (pole) of the oocyst bears 3 spine-like projections with an average length of 15.1 µm; however, the length was highly variable (2.9–20.8 µm). A lumen occurred within the spine-like projections. Oocysts (excluding spine length) measured 18.7 × 11.1 µm (18.0–19.3 × 9.4–11.7) (n = 6); length/width relationship 1.7 (1.5–2.0) (n = 6). Oocyst residuum, micropyle, or polar granules absent. Four ellipsoidal sporocysts, measuring 9.2 × 4.1 (7.8–11.1 × 2.9–4.8) (n = 13), each contained 2 sporozoites and plentiful sporocyst residuum. A Stieda body was not observed under light microscopy. A line drawing of the sporulated oocyst is shown in Fig. 5; measurements are summarized in Table 2.

Pathology: Epicellular infection of gastrointestinal epithelial cells, with no apparent lesions.

Etymology: Name is derived from the Latin word *echinatus*, meaning spiny or prickly, and refers to the unique spiny projections that occur in this species.

DISCUSSION

This is the first study to provide genetic information on *Goussia clupearum* in Atlantic and Pacific herring to clarify their taxonomic position and to understand the relatedness of the 2 similar parasite species. The small differences in the nucleotide sequence reported here between *G. clupearum* in Atlantic and Pacific herring indicate some divergence between the parasites in the 2 geographically separated host species, though they are mostly conserved with 99.6% identity. Less-conserved genes, such as the internal transcribed spacer (ITS) gene (White et al. 1990, Barta et al. 1998), would likely aid in determining additional details in the divergence within these species. Though the small changes in the ssrDNA of *G. clupearum* are notable between the 2 host herring species, additional sampling from different populations within these hosts is necessary to establish whether the level of variation observed here is at the

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host species level or also observed across wider geographical areas within a host species range.

Further work on liver *Goussia* sp. from outside the clupeid genus would aid in determining variations within these liver coccidians in marine fish. From this study, the similar tissue tropisms, parasite morphology, relatively conserved ssrDNA, and common hosts within the genus *Clupea* support these organisms as the same species. An unexpected finding from this study was the unique 124-nucleotide sequence insertion in positions 605 to 729, found only in this coccidian species. Despite the unknown relevance of the 124-nucleotide insertion, which does not align with any coccidian species, the remaining portion of the sequence aligns with and shows greatest identity with other extraintestinal liver coccidia from the genus *Calyptospora*. This is consistent with the findings of Molnár et al. (2012), who have previously shown that the coccidia infecting intestinal sites evolved separately from those occupying extraintestinal tissues. Our analysis places *G. clupearum* more distantly from other *Goussia* species, which mainly represent intestinal and epicellular species. Rosenthal et al. (2016) present evidence that parasites presently assigned to the genus *Goussia* comprise at least 3 distinct evolutionary lineages and represent significant phylogenetic diversity. They further suggest that revision of the taxonomic nomenclature for the myriad species currently ascribed to *Goussia* may be warranted. Our phylogenetic analysis has a small sample size (21 nucleotide sequences) for elucidating evolutionary relationships but does demonstrate that the extraintestinal *Calyptospora* genus and *G. clupearum* grouped separately from other intestinal species, though other extraintestinal species were interspersed within intestinal species. The findings of this study suggest that currently classified *G. clupearum* is most closely related to extraintestinal species in the *Calyptospora* genus rather than the predominantly intestinal and epicellular species most frequently placed within the genus *Goussia*. More work on the evolutionary diversity of fish coccidia is needed to elucidate the relationships between different groups.

Life cycle characteristics may also provide clues to taxonomic structure. It has previously been proposed that the genus *Calyptospora* can be distinguished from other coccidians by their heteroxenous life cycle (Overstreet et al. 1984). A heteroxenous life cycle has been demonstrated in *C. funduli*, which requires a developmental period in the gut basal cells of the grass shrimp *Palaemonetes pugio* prior to transmission to the definitive host, the Gulf killifish *Fundulus grandis* (Fournie & Overstreet 1983, Fournie et al. 2000, Whipps et al. 2012). Our results on the infectivity of *G. clupearum* suggest that transmission is not direct and that passage through an intermediate host is likely. This life cycle characteristic is also supported given the close phylogenetic placement of *G. clupearum* to the *Calyptospora*. However, it is also possible that sporocysts introduced through gastric gavage in this study were either no longer viable or that the challenge period was not sufficiently long enough (77 d) to detect the coccidian oocysts in the liver. Further studies on transmission are needed to elucidate the life cycle characteristics of *G. clupearum*.

Although many coccidia display strong host specificity (Molnár et al. 2005), *G. clupearum* is reported to parasitize a range of clupeid, scombroid, and gadoid hosts (Costa & MacKenzie 1994, Abollo et al. 2001). Within those hosts, differences in morphology have been found, particularly in oocyst diameter and sporocyst length and width (MacKenzie 1979, Lom & Dyková 1992, Abollo et al. 2001, Azevedo 2001). The oocyst and sporocyst dimensions described in this study correlate to ranges summarized by Abollo et al. (2001), with the large range in oocyst diameter we observed in Atlantic herring most similar to a *Goussia* sp. reported from the liver of blue whiting *Micromesistius poutassou* (MacKenzie 1979). Several studies of *G. clupearum* and similar liver coccidia have struggled with confidently assigning a species name based on morphological criteria alone (Costa & MacKenzie 1994, Abollo et al. 2001). Morphometric data from sporulated oocysts and sporocysts are commonly used to identify morphologically similar coccidian species. However, in the absence of molecular information, it is difficult to determine by morphology alone whether coccidia reported from different hosts truly represent different species or may be conspecific. Molnár et al. (2005) devised cross-infection experiments, which demonstrated strict host specificity for the gut coccidia *G. carpelli* in common carp *Cyprinus carpio*. It has been suggested that intestinal coccidia display a higher degree of host specificity than liver coccidia in fish (Abollo et al. 2001). We hope that the availability of reliable primers for the ssrDNA for *G. clupearum* and the present ease and affordability of sequencing technology might lead other researchers to apply molecular comparisons to any questionable isolates.

G. clupearum is reported as a parasite specific to the liver parenchyma of fish, though other *Goussia* species have been reported to be less tissue specific (Lom & Dyková 1992). In the current study, little pathology in terms of cell damage, lesions, and in-

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flammation was associated with infection in the liver. Studies have found that hepatic coccidiosis is a major factor contributing to poor condition in some hosts (MacKenzie 1981, Abollo et al. 2001, Gestal & Azevedo 2005). We did not observe this effect in the current study. However, with regard to hepatic coccidiosis caused by *G. clupearum*, some differences in effect of body condition seem to be related to the host in question. Costa & MacKenzie (1994) suggest that *G. clupearum* is not a serious pathogen in herring but is so in other species such as blue whiting. It has been hypothesized that intense infections are likely to stress hosts (Morrison & Hawkins 1984). Abollo et al. (2001) suggest that the liver is the preferred target organ but that infections may spread to other organs in cases of chronic infection. In the current study, we observed sporulated oocysts consistent with *G. clupearum* in low numbers in the hearts of 2 Atlantic herring. Interestingly, these oocysts induced an inflammatory response, which was not seen in the liver of infected fish. Though we cannot rule out the possibility of this being a different coccidian species, it is possible that the presence of *G. clupearum* in this non-target organ elicits an inflammatory reaction, whereas immune responses may be better modulated by the parasite in the liver.

This study documented intestinal coccidiosis in Atlantic herring for the first time. Juvenile Atlantic herring (October 2012) had heavier infection intensities observed in histological sections than did adult fish (May 2015), though infection prevalence was similar, 60 and 55% in juveniles and adults, respectively. Similar observations have been noted with Pacific herring populations, with young-of-the-year fish having heavier infections and prevalence compared to adult fish (J. Lovy pers. obs.). Given the differences in season of observation, geographic location, age of fish, and macrogamont morphology, it is possible that these are 2 different intestinal coccidia. However, the oblong macrogamonts observed in May 2015 likely correspond to the elongated sporulated oocysts of *G. echinata* n. sp. observed in January 2015 from the same geographic location and population. A fascinating finding in the present study was the unique morphology of *G. echinata* n. sp. sporulated oocysts. The presence of 6 long, spine-like projections from the oocyst wall is unlike any other coccidia reported from fish and rare in any reported coccidian. Several coccidia of turtles have similar but much smaller conical projections. *Eimeria stylosa* in red-eared sliders *Trachemys scripta elegans* possesses 2 projections on one end and 3 on the opposite side of its ovoid oocyst wall; however, some variation was observed in the number of projections present. These projections were 4.0 µm on average (McAllister & Upton 1989). *E. jirkamoraveci*, also from turtles, has 3 blunt, knob-like projections at one end, approximately 1 to 1.5 µm long (Široký et al. 2006). *E. mitraria*, with similar, smaller ornate projections, is found in multiple species of turtles from geographically distant regions and has been suggested to represent a morphotype rather than a species (Široký & Modrý 2006). *G. echinata* n. sp., described in the current study, had significantly longer spiny projections than the short, knob-like or conical projections reported from the coccidia of turtles (described in this section). The purpose of the unique long projections found in the coccidial oocyst of this species is currently unknown, though they are likely adaptive traits benefitting the parasite in successfully infecting their hosts. Myxosporean actinospore appendages are hypothesized to help increase buoyancy for better dispersal and may serve to extend suspension time in the water column, facilitating transmission to a host (Kallert et al. 2015). It is possible that the long, spine-like appendages of *G. echinata* n. sp. serve a similar function, though further work on the life cycle of this parasite will be important in fully understanding the function of this unique adaptation. Unfortunately, we were not able to provide genetics to help clarify the taxonomic position of this unique coccidian species, which should be addressed in the future. We have assigned this species into the genus *Goussia* because of its epicellular nature in the intestine, apparent lack of a Stieda body, and requirement for exogenous sporulation, which are characteristics found most frequently in *Goussia* sp. In general, for coccidia in fish, considerable effort is required to better define the taxonomy of these abundant and diverse parasites. As more molecular sequences are obtained for fish coccidia, they will aid in understanding the taxonomic diversity and distribution of these parasites in fish hosts. Additionally, for *G. echinata* n. sp., more work is needed to establish the seasonal prevalence, intensity, and pathology associated with this intestinal coccidian.

Pathogen surveillance did not detect VHSV in any Atlantic herring sampled from New Jersey waters between 2013 and 2016. This should not be misinterpreted as an absence of the virus in the waters of New Jersey. Random samples of Pacific herring typically fail to return positive results (P. K. Hershberger unpubl.), even though the species is highly susceptible (Kocan et al. 1997) and epizootics occur frequently (Garver et al. 2013). Furthermore, VHSV-positive

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samples from Pacific herring are often not observed until after the capture and confinement of previously negative individuals (Hershberger et al. 1999, Lovy et al. 2013). A similar exacerbating factor may be required for the expression of VHSV in Atlantic herring populations, where low prevalence has previously been reported in North Sea, Baltic, and UK waters (Mortensen et al. 1999, King et al. 2001, Brudeseth & Evensen 2002, Dixon et al. 2003, Matejusova et al. 2010). However, recent work by Johansen et al. (2013) investigating Atlantic herring from the Norwegian spring-spawning stock found prevalence as high as 69% when gill tissue was included in real-time RT-PCR analyses, versus a 33% prevalence found in pooled brain, kidney, and spleen. This high prevalence, in the absence of disease signs, may indicate a passive carrier status rather than an active infection. Further explanation of the failure to detect VHSV may lie in the ability of fish to mount an immune response to the virus; Pacific herring surviving acute and sub-acute infections have demonstrated an adaptive immune response, which confers protection following re-exposure (Kocan et al. 2001, Hershberger et al. 2010b,c). Measuring the immune status of wild populations may help assess whether previous exposure has occurred and a present cycle of herd immunity may be protecting herring from epizootic events. Though VHSV has never been detected in New Jersey waters, strain IVc has been isolated from brackish water fishes from the Atlantic coastal regions of Canada (Gagné et al. 2007). As VHSV is typically found in coldwater environments, it is possible that the warm ocean currents associated with the Gulf Stream may contribute to the apparent lack of detections from New Jersey waters (Hershberger et al. 2013). The migratory behavior of Atlantic herring and their potential to harbor the virus actively or passively make this species a possible reservoir of VHSV in the North American Atlantic coast, and continued surveillance for this virus should identify if these populations may serve as a reservoir for the virus.

Perhaps more surprisingly, *I. hoferi* was not detected in any Atlantic herring during the current surveillance, even though the resulting disease has been responsible for epizootics on both sides of the Atlantic Ocean. Atlantic herring is recognized as a commonly infected species in the North Atlantic, and infection with *I. hoferi* is hypothesized to be an important factor limiting population growth (Sindermann 1990). Sindermann (1963) estimated that the Atlantic herring stock was reduced by at least 50% during a 1954–1956 outbreak of *I. hoferi* in the Gulf of St. Lawrence, whereas the Atlantic herring around Denmark may have experienced mass mortality of 36% during an epizootic in 1991 (Mellergaard & Spanggaard 1997). Although *I. hoferi* was not detected in any samples from the current study, ongoing disease surveillance efforts are necessary for revealing possible threats to Atlantic herring populations. The absence of *I. hoferi* that we report in Atlantic herring is different from the pattern currently observed in populations of Pacific herring, where the parasite is typically observed in high prevalence, especially among older and larger age cohorts (Hershberger et al. 2016). Causes for these striking differences in infection prevalence between the 2 herring species remain uncertain but could be related to immunity or absence of the parasite. An analogous pattern occurs in American shad *Alosa sapidissima*, another clupeid, where a high prevalence of *I. hoferi* occurs in the NE Pacific (Hershberger et al. 2010a) and a low prevalence occurs along the Atlantic coast of North America (Gregg et al. 2016).

Detection of fish epizootics in the marine environment is typically limited to massive mortality events because quick dispersal by ocean currents, consumption by scavengers, and the vast size of marine water bodies prevent observations of dead fish. Diseases which produce chronic morbidity may lead to population declines that can easily go unnoticed. Monitoring important fish species for pathogens is useful for identifying diseases with the potential for population-level impacts.

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Appendix 5

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Ichthyophonus parasite phylogeny based on ITS rDNA structure prediction and alignment identifies six clades, with a single dominant marine type

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ABSTRACT: Despite their widespread, global impact in both wild and cultured fishes, little is known of the diversity, transmission patterns, and phylogeography of parasites generally identified as *Ichthyophonus*. This study constructed a phylogeny based on the structural alignment of internal transcribed spacer (ITS) rDNA sequences to compare *Ichthyophonus* isolates from fish hosts in the Atlantic and Pacific oceans, and several rivers and aquaculture sites in North America, Europe, and Japan. Structure of the *Ichthyophonus* ITS1–5.8S–ITS2 transcript exhibited several homologies with other eukaryotes, and 6 distinct clades were identified within *Ichthyophonus*. A single clade contained a majority (71 of 98) of parasite isolations. This ubiquitous *Ichthyophonus* type occurred in 13 marine and anadromous hosts and was associated with epizootics in Atlantic herring, Chinook salmon, and American shad. A second clade contained all isolates from aquaculture, despite great geographic separation of the freshwater hosts. Each of the 4 remaining clades contained isolates from single host species. This study is the first to evaluate the genetic relationships among *Ichthyophonus* species across a significant portion of their host and geographic range. Additionally, parasite infection prevalence is reported in 16 fish species.

KEY WORDS: *Ichthyophonus* · Parasite phylogenetics · Internal transcribed spacer · RNA secondary structure prediction · rDNA · Herring · Salmon · Halibut · Shad · Rainbow trout

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INTRODUCTION

Parasites in the genus *Ichthyophonus* impact fish populations, fisheries, and aquaculture across a wide geographic range. Infections have been reported in over 145 fish species, from the Barents Sea, AK, to the southern tip of Africa, in Atlantic and Pacific oceans, and in freshwater on 6 continents (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d120p125_supp.pdf). Impacts in wild populations vary from acute, mass-mortality events, as occur in Atlantic herring *Clupea harengus* (Cox 1916, Fish 1934, Rahimian & Thulin 1996) and Chinook salmon *Oncorhynchus tshawytscha* (Kocan et al. 2004), to chronic epizootics that change population structure without causing synchronous fish kills. Evidence suggests the latter is true for populations of European plaice *Pleuronectes platessa* (McVicar 1981), Pacific herring *Clupea pallasii* (Hershberger et al. 2002, Marty et al. 2003), and American shad *Alosa sapidissima* (Hershberger et al. 2010), and likely occurs in many less-studied populations. In addition to culling individuals from commercially exploited populations, *Ichthyophonus* spp. destroy or reduce the value of fillets in haddock (McVicar 1979), Chinook salmon (Kocan et al. 2004), Atlantic mackerel

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Scomber scombrus (Sproston 1944) and walleye pollock *Gadus chalcogrammus* (White et al. 2014).

First identified as a problem in the culture of brown trout *Salmo trutta* and brook trout *Salvelinus fontinalis* in Germany at the end of the 19th century (von Hofer 1893), *Ichthyophonus* spp. have subsequently emerged, and persist today, in aquaculture around the globe. The parasites are reported in cultured salmonids (Rucker & Gustafson 1953, Ono et al. 1966, Slocombe 1980, Athanassopoulou 1992), carangids (Fujiya 1976, Egusa 1983), cichlids (El-Ghany & El-Ashram 2008, Shawer et al. 2011), cyprinids (Reichenbach-Klinke 1954, Prabhuji & Sinha 2009), sparids (Athanassopoulou 1992, Franco-Sierra et al. 1997), moronids (Sitja-Bobadilla & Alvarez-Pellitero 1990, Franco-Sierra et al. 1997) and mugilids (Paperna 1986, Franco-Sierra et al. 1997). As in wild populations, effects in aquaculture range from significant acute mortality (Rucker & Gustafson 1953) to chronic low level mortality and destruction of fillets (Erickson 1965, Miyazaki & Jo 1985, Franco-Sierra et al. 1997).

Despite their widespread impact, little is known about the diversity, transmission patterns or phylogeography of *Ichthyophonus* spp. The internal, histozoic parasites are difficult to differentiate under microscopic examination due to highly plastic morphology *in vivo* (Sproston 1944) and *in vitro* (Okamoto et al. 1985, Spanggaard et al. 1994). Interspecific transmission demonstrated in the laboratory (Gustafson & Rucker 1956, McVicar & McLay 1985) made it convenient to label them generalists, and for a century, descriptions of these parasites were lumped into a single specific name, *I. hoferi* (Plehn & Mulsow 1911), with uncertain placement within Fungi. The fungal classification was held in common use until 1996 when 2 groups of researchers used 18S rDNA sequences to determine that *Ichthyophonus* spp. are not a fungi, but rather, single-celled relatives of choanoflagellates and metazoans (Ragan et al. 1996, Spanggaard et al. 1996). Subsequently, *Ichthyophonus* was placed in a newly erected class, Mesomycetozoea, with other single-celled eukaryotes, primarily parasites of aquatic species (Mendoza et al. 2002). At lower taxonomic levels, molecular genetic studies, based on both 18S and internal transcribed spacer (ITS) rDNA regions, identified novel *Ichthyophonus* types in 5 of the 6 new hosts examined (Rand et al. 2000, Criscione et al. 2002, Hershberger et al. 2010, Rasmussen et al. 2010) with a single new species formally described (Rand et al. 2000).

The generalizations applied to *Ichthyophonus* and lack of information regarding parasite strain distribution are problematic for a parasite that is globally distributed. These information gaps severely limit our understanding of the processes that lead to epizootics. Historically, outbreaks of ichthyophoniasis were attributed to changes in host population or environment, and the role of parasite distribution, adaptation, and strain differences were generally ignored. The primary aim of this study was to gain an understanding of *Ichthyophonus* species richness by constructing a new ITS rDNA-based phylogeny using parasite isolates from several hosts and regions, and to identify any phylogeographic patterns that occur in the distribution of the parasite. Secondarily, we report infection prevalence data for several of the host species from which the parasites were isolated.

MATERIALS AND METHODS

Sample collection

During 2010 and 2011, 2215 fish from 16 known and potential host species were sampled opportunistically from academic, state, provincial, and federal fishery research surveys occurring in Canada and the USA (see Table 1). Sampling occurred along the Pacific and Atlantic Coasts of North America, off shore, and in coastal bays and rivers. Target sample number per species per location was 60 individuals, allowing for 95% confidence of detection where apparent prevalence was ≥5% (Dohoo et al. 2009). In some cases, this sample number was not met. Data are reported for all samples where n ≥ 50 and for smaller samples when parasites were detected.

Ichthyophonus infections were detected by explant culture of heart tissue, except in the case of yellowtail rockfish where liver tissue was cultured. A small piece (approx. 0.5 cm^3) of tissue was aseptically removed from each carcass and placed in a 15 ml tube containing 6 ml of Eagle's minimum essential medium (MEM), buffered to pH 7.8 with Tris, and supplemented with fetal bovine serum (5% v/v), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}), and gentamycin (100 µg ml^{-1}). Cultures were kept cool during transport to the US Geological Survey Marrowstone Marine Field Station (Nordland, WA, USA) where they were incubated at 15°C and examined microscopically (40× magnification) for the presence of *Ichthyophonus* schizonts and/or hyphae. Generally, each sample was examined twice, after 7 and 14 d incubation. However, these times were adjusted to account for duration of sampling and

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transit time. Questionable cultures were examined a third time after 21 d, and medium was exchanged in tubes that became turbid due to host tissue autolysis. Prevalence of infection (%) was calculated as: 100 × number of positive cultures per sample/total number cultures in sample. A sample included all individuals of a single species from a given location.

rDNA sequencing

Sequences of the rDNA ITS region (i.e. ITS1–5.8S–ITS2) were generated for 81 *Ichthyophonus* isolates from 17 host species. These isolates came from a subset of tissue explant cultures obtained from the prevalence survey (n = 39), fresh cultures collected during ongoing epizootics (n = 13), and material that had been archived at cooperating laboratories (n = 29) (see Table 2). Host tissue not consumed by parasites was removed from cultures, and medium was exchanged every 5 to 10 d. In many cultures, hydrochloric acid, HEPES buffer (Gibco®, Life Technologies) and glucose were added to the MEM to lower pH (ca. 3.5) and stimulate parasite growth following the method of Spanggaard et al. (1994). When sufficient parasite material was present, samples were preserved in 100% ethanol. DNA was extracted from preserved cultures using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's instructions, with the following modifications: tissue lysis buffer and Proteinase K volumes were increased 2-fold (360 µl and 40 µl, respectively), beads (lysing matrix D; MP Biomedicals) were added to the extraction tubes, and tissues were homogenized with a Fast-Prep®-24 bead mill (MP Biomedical) for 40 s prior to digestion. PCR amplification of the ITS1–5.8S–ITS2 locus was performed as previously described (Hershberger et al. 2010, Rasmussen et al. 2010) using primers that annealed on the 18S–ITS1 and ITS2–28S boundaries (Out ITS1-F: 5′-GCG GAA GGA TCA TTA CCA AAT AAC G; and Out-ITS2-R: 5′-GCC TGA GTT GAG GTC AAA TTT, respectively). Multiple clones were sequenced from each isolate to account for possible intra-isolate genetic variation. PCR products from the above reactions were purified using the QIAquick PCR purification protocol (Qiagen) and cloned with the Topo-TA Cloning Kit for Sequencing (Invitrogen). Cloned inserts were amplified by PCR, insert length was verified by gel electrophoresis, and inserts of the correct size were sequenced in the forward and reverse direction using stock vector primers (i.e. M13 Forward-21, M13 Reverse-29) at the high-throughput genomics unit at the University of Washington (htSEQ). Forward and reverse sequences from each clone were combined, chromatographs were visually inspected, and ambiguous base calls were edited manually using Sequencher Software (version 5.0, Gene Codes). A subset of clones (n = 93) was re-sequenced to clarify ambiguous chromatographs that followed a polyadenylation region at the 3′ end of ITS1. A forward primer that annealed at the 3′ end of the 5.8S sequence (IH.ITS_Seq_F_Int: 5′-ACA ACT TTT AAC GGT GGA TCT C) and a reverse primer that annealed in the 3′ region of ITS2 (IH.ITS_Seq_R_Int: 5′-CCG TGA ACT TCA TTT ATT CCA CAT) were used in addition to the M13 primers during this second sequencing run. These 4-sequence sets were combined and edited as described above. All newly generated clone sequences (n = 723) were combined with 137 ITS rDNA clone sequences from 13 isolates previously deposited in GenBank (Hershberger et al. 2010, Rasmussen et al. 2010). Clone sequences were aligned with Clustal and average evolutionary distance (*p*-distance) over sequence pairs within each isolate was calculated using MEGA5 (Tamura et al. 2011) (see Table 2).

Consensus ITS sequences were produced from clone sequences of each isolate by majority rule, except in cases where intra-isolate variation indicated divergent haplotypes were present in 1 isolate. Multi-haplotype isolates were identified by large intra-isolate *p*-distance (>0.01) and confirmed by estimating neighbor-joining phylogenies in MEGA5 using all the clone sequences from a given host species and consensus sequences from a 'backbone tree' made up of isolates from Chinook salmon (n = 1), Pacific herring (n = 1), Greenland turbot (n = 2), Dolly Varden (n = 1), rainbow trout (n = 3), American shad (n = 3) and yellowtail rockfish (n = 1). When all clones of an isolate formed a polytomy in 1 clade, a single majority consensus sequence was used for that isolate. When clones from an isolate diverged into more than 1 well supported clade, these sequences were grouped accordingly and multiple haplotypes from that isolate were considered in subsequent structural alignment and phylogenetic inference.

Alignment

The secondary structure of the ITS rRNA transcript was used to aid alignment of *Ichthyophonus* isolate consensus sequences. An iterative process utilizing thermodynamic optimization (Zuker & Stiegler 1981, Mathews et al. 1999) and a phylogenetic comparative

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method (Noller & Woese 1981, Mai & Coleman 1997) were used to predict secondary structure. ITS consensus sequences were first aligned, with primer sequences in place, using Clustal in MEGA5, and then divided into 3 sequences corresponding to constituent molecules (i.e. ITS1, 5.8S, and ITS2) with the 5′ end of 5.8S set at 5′-UUU AGA CAA CUU UUA ACG-3′ and the 3′ end at 5′-CAU GCC UGG UUG AGU GUC-3′ following Vaughn et al. (1984) and Gottschling & Plotner (2004). Structure was predicted for the 3 molecules separately using the MFOLD web server (Zuker 2003). Default folding parameters were employed with no constraints on bases. Optimal and suboptimal structures were examined to identify locally conserved, thermodynamically stable features. Structure prediction was repeated with constraints to account for the connection of the 3 molecules, ITS2 interaction with flanking regions, and 5.8S–28S hybridization (Vaughn et al. 1984) that occurs during formation of the ribosome large subunit. The 5′ end of an *Ichthyophonus* 28S sequence (GenBank acc. no. AY026370) was joined to the 3′ end of each ITS2 sequence, and locations of 5.8S–28S hybridizations were identified by alignment of 5.8S sequences with reversed 28S transcript using Sequencher. These 5.8S regions were forced to remain unpaired during the second MFOLD run. Sequences with optimal dot-bracket structural annotation from the 3 molecules were then re-combined in the program 4SALE (Seibel et al. 2006, 2008) and the entire alignment was manually edited using the phylogenetic method described by Mai & Coleman (1997). Homology with structures from related taxa, published in the ITS2 (Koetschan et al. 2010) and RFAM (Burge et al. 2013) databases, was considered during manual editing. Structural homology across isolates was given priority over sequence alignment, while Clustal alignment was retained in large unpaired regions. The complete resulting structures were visualized in 4SALE and VARNA (Darty et al. 2009). The final structural alignment was trimmed to remove primer and 28S sequences prior to model selection.

Phylogenetic inference

To account for presumed heterogeneity of molecular evolution across regions and local structures (Brandley et al. 2005, Brown & Lemmon 2007), the data were analyzed under 4 partition strategies: complete sequence (no partitioning), partitioned by stem and loop (2 partitions), partitioned by region (i.e. ITS1, 5.8S, and ITS2; 3 partitions) and partitioned by stem and loop within regions (6 partitions). Nucleotide substitution model selection was implemented in jModelTest2 (Posada 2008, Darriba et al. 2012) for the entire structural alignment, for the individual molecules, and for paired and unpaired regions. From an initial BIONJ topology (Gascuel 1997), likelihood scores were calculated based on 24 candidate models (reviewed by Posada & Crandall 2001). Akaike's information criterion (AIC) was used to select the model that best fit sequence data (see Table S2 in the Supplement at www.int-res.com/articles/suppl/d120p125_supp.pdf) (Posada & Buckley 2004).

Gene trees were estimated using Bayesian Metropolis-coupled Markov chain Monte Carlo (MC^3) methods in MrBayes (Ronquist & Huelsenbeck 2003). Duplicate MC^3s of 2×10^7 generations were run for each partition scheme with parameter sampling every 2×10^4 generation. The first quarter of samples were discarded as 'burn-in'. Heating values for the 4 Markov chains in each run were left at default values. During the partitioned analyses a nucleotide substitution (*nst*) value was set for each partition, and rate parameters were allowed to vary among partitions. MrBayes parameters *revmat*, *tratio*, *statefreq*, *shape*, and *pinvar* were unlinked across partitions. In stem partitions the doublet model (nucmodel = doublet) was used to account for correlation of paired bases (Schöniger & von Haeseler 1994), other partitions were assigned the standard 4 by 4 model. The exponential branch length prior was decreased to 0.01 to avoid entrapment of Markov chains in unrealistic 'long tree' parameter space, which can occur in partitioned models (Brown et al. 2010, Marshall 2010). MC^3 results were visualized using the program Tracer (Rambaut & Drummond 2007) to verify a plateau in the plot of likelihood ($-\ln L$) over generation time and to check for abnormal distributions in estimated parameters. To determine which partitioning scheme best fit the data, Bayes factors were calculated for pairwise comparison of phylogenies on the estimated harmonic means of likelihoods from the *sump* command in MrBayes (Kass & Raftery 1995, Nylander et al. 2004). Differences between major clades of the final (i.e. best fit) phylogeny were characterized by calculating pairwise percent nucleotide differences for ITS1 and ITS2 sequences, and by identifying compensatory base pair changes (CBCs) present in paired regions.

In order to relate our findings to previous work based solely on 18S rDNA sequences, a subset of isolates representing distinct ITS clades was selected for 18S sequencing. Forward and reverse primers (18S-82F and 18S-1520R) from Takishita et al. (2005) were

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used for PCR amplification of a region approximately 1590 bp long. The amplicon was direct sequenced in the forward and reverse directions using the above primers and a second set that annealed near its center (ICH1F and ICH4R; Criscione et al. 2002). Sequences were generated with the ABI BigDye® Terminator v1.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer (Applied Biosystems). The 4 segments from each isolate were combined and ambiguous base calls were manually edited using the Sequencher program. These sequences were combined with GenBank sequences representing *Ichthyophonus* isolates from 7 host species (Ragan et al. 1996, Rand et al. 2000, Criscione et al. 2002, Hershberger et al. 2010). Alignment, model testing, and phylogenetic analysis followed that of ITS sequences, except that no secondary structure or partitioning were incorporated into models, and an 18S sequence from *Amoebidium parasiticum* (GenBank acc. no. Y19155) was included as an outgroup.

RESULTS

Infection prevalence

Ichthyophonus was widespread in the NE Pacific, occurring in 10 of the 13 host species surveyed from the Bering Sea to the coast of Washington (Table 1). Among positive hosts, prevalence ranged from 3.3 to 73.7%. More fish were sampled in the NW Atlantic, but from fewer host species. *Ichthyophonus* was detected in American shad and Greenland halibut, but at relatively low prevalence, 0.007 and 6.75% respectively.

ITS sequence and structure

Clone sequences produced for the *Ichthyophonus* rDNA ITS region were 645 to 684 nt long with a mean G+C content of 35.4%. Both ITS1 (220 to 235 nt) and ITS2 (263 to 288 nt) sequences varied in length by 15 nt, while 5.8S sequences (161 to 162 nt) varied by only 1 nt. Intra-isolate *p*-distance was of similar magnitude and small (i.e. <0.01) in all isolates except Atlantic mackerel (3 haplotypes), yellowtail rockfish (2 haplotypes), Puget Sound rockfish (2 haplotypes) and copper rockfish (1 haplotype) (Table 2).

Structures predicted for the ITS rRNA transcript of *Ichthyophonus* are relatively conserved and contained many features common among eukaryote taxa (Fig. 1). ITS1 formed an open central loop of 92 to 102 unpaired bases with 4 hairpin helices. Helices I, II, III, and IV contained 5 to 6, 9 to 10, 22 to 26, and 5 to 6 paired bases, respectively. Sequence variation in ITS1 primarily took the form of indels that occurred in unpaired regions of the central loop and in the bulged bases and terminal loop of Helix III. Substitutions did occur in the paired region of Helix I, where 2 to 5 CBCs occur between isolates. A single CBC (G-C/T-A) occurred in Helix II and 1 hemi-CBC occurred in Helix III (G-C/G-U).

The 5.8S secondary structure was completely conserved except for a single indel at the 5′ end of 2 isolates. The structure was similar to the universal model proposed by Vaughn et al. (1984). A dichotomous branched structure formed by 3 helices (Fig. 1: B, C, and D) and 2 hairpin helices (Fig. 1: E and F) extended from a 63 nt unpaired region. Three G/A transitions were present in the dichotomous helical structure, 2 occurred in bulged bases and 1 contributed to a hemi-CBC (G-U/A-U) in Helix D. One T/C transition occurred in an unpaired region. The sites of putative 28S hybridization that occurred at the 3′ end and Region A of 5.8S were 100% conserved, and showed high fidelity in both location and sequence to these sites described in other eukaryotes (Vaughn et al. 1984, Keller et al. 2009).

ITS2 formed a closed loop of 47 to 50 unpaired bases, terminating on 5′ and 3′ ends at the site of 5.8S–28S hybridization. As is typical in Eukaryota (Coleman 2007), 4 helices were present with the third being the longest. In *Ichthyophonus*, Helices I′, II′, III′, and IV′ contained 3, 15 to 22, 42 to 49, and 13 base pairings, respectively. Sequences of the central loop and the proximal paired bases were highly conserved. The majority of polymorphisms occurred in bulged bases and distal paired bases of Helices II′ and IV′. Other features conserved within Eukaryota were also present in the ITS2 structure of *Ichthyophonus*, including a pyrimidine-pyrimidine mismatch near the base of Helix II′, a UGGN motif 5′ to the apex of Helix III′, and a highly conserved segment (40 nt) on the 5′ side of Helix III′ (Schultz et al. 2005, Coleman 2007, Hamilton et al. 2010).

Phylogenetics

The final structural alignment of *Ichthyophonus* ITS sequence was 719 nt in length with primer sequences removed. MC^3 for all partitioning strategies reached stationarity by the end of burn-in. The 6-partition model resulted in the highest likelihood

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Table 1. *Ichthyophonus* infection prevalence in 16 host species from the NW Atlantic and NE Pacific regions. Capture method, length (mean ± SD), weight (mean ± SD) and age data (mean [range]) of host are given when available. Prevalence (Prev.) of infection was determined by microscopic examination of explant heart cultures (see 'Materials and methods' for details). fl = fork length, tl = total length, YOY = young of the year, NAFO = North Atlantic Fisheries Organization

Species Collection locations	Capture method	Length (cm)	Weight (g)	Age (yr)	Infected/n	Prev. (%)
NE Pacific						
American shad *Alosa sapidissima*						
Puget Sound	Trawl[a]	20.8 ± 2.6 fl	104 ± 27	–	28/38	73.7
Columbia River, WA	Trap[b]	34.5 ± 4.4 fl	567 ± 243	–	17/60	28.3
Arctic cod *Boreogadus saida*						
Bering Sea	Trawl[c]	17.2 ± 1.5 tl	34 ± 9	–	0/58	0
Capelin *Mallotus villosus*						
Bering Sea	Trawl[c]	14.7 ± 1.1 tl	20 ± 5	–	0/61	0
Great sculpin *Myoxocephalus polyacanthocephalus*						
Bering Sea	Trawl[c]	52.6 ± 10.4 tl	2742 ± 1640	–	4/65	6.2
Greenland halibut *Reinhardtius hippoglossoides*						
Bering Sea	Trawl[c]	56.5 ± 20.4 tl	2617 ± 3036	–	5/28	17.9
Pacific cod *Gadus macrocephalus*						
Bering Sea	Trawl[c]	67.5 ± 8.6 tl	3509 ± 1528	–	2/56	3.6
Pacific halibut *Hippoglossus stenolepis*						
Cook Inlet	Hook & line[d]	85.8 ± 17.6 tl	–	–	30/60	50.0
Pacific herring *Clupea pallasii*						
Bering Sea	Trawl[c]	29.7 ± 1.8 tl	253 ± 52	–	0/64	0
Puget Sound	Trawl[a]	14 ± 2 fl	28 ± 14	–	45/156	28.8
Pacific staghorn sculpin *Leptocottus armatus*						
Puget Sound	Trawl[a]	18 ± 2.9 tl	91 ± 39	–	1/17	5.9
Plain sculpin *Myoxocephalus jaok*						
Bering Sea	Trawl[c]	47 ± 7.2 tl	1340 ± 594	–	4/34	11.8
Walleye pollock *Gadus chalcogrammus*						
Bering Sea	Trawl[c]	65 ± 9.7 tl	1907 ± 850	–	6/57	10.5
Yellowfin sole *Limanda aspera*						
Bering Sea	Trawl[c]	26.5 ± 6.9 tl	259 ± 166	–	0/59	0
Yellowtail rockfish *Sebastes flavidus*						
WA Coast	Hook & line[e]	–	–	–	1/30	3.3
NW Atlantic						
American shad *Alosa sapidissima*						
St. Lawrence River, QC	Gillnet[f]	49.8 ± 2.3 fl	1612 ± 238	–	5/60	8.3
Merrimack River, MA	Trap[g]	43.5 ± 4.4 fl	1195 ± 392	–	1/60	1.7
Connecticut River, MA	Trap[g]	48.2 ± 5 tl	1154 ± 413	–	0/60	0
Connecticut River, CT	Gillnet[h]	45.6 ± 3 fl	–	–	1/65	1.5
Nanticoke River, MD&DE	Electro[i,j]	44.8 ± 3.8 tl	991 ± 309	4.4 [3–6]	0/55	0
Patuxent River, MD	Electro[i]	44.2 ± 3.6 tl	–	4.1 [3–7]	0/60	0
Potomac River, DC	Gillnet[k]	46.9 ± 2.4 tl	1192 ± 235	–	0/53	0
Rappahannock River, VA	Gillnet[l]	47.5 ± 2.4 tl	1350 ± 219	5.5 [4–7]	0/60	0
York River, VA	Gillnet[l]	48.1 ± 2.4 tl	1377 ± 237	5.6 [4–9]	0/60	0
James River, VA	Gillnet[l]	48.1 ± 2.1 tl	1369 ± 176	5.4 [4–7]	0/60	0
Albemarle Sound, NC	Gillnet[m]	45.8 ± 4.5 tl	1116 ± 373	–	0/53	0
Roanoke River, NC	Electro[n]	44.4 ± 4.7 tl	686 ± 237	–	0/60	0
Tar River, NC	Electro[n]	45.1 ± 4.1 tl	820 ± 267	–	0/61	0
Cape Fear River, NC	Electro[n]	45.3 ± 3.9 tl	999 ± 355	–	1/60	1.7
Santee River, SC	Electro/Trap[o]	49.3 ± 2.9 tl	1400 ± 259	4.1 [3–5]	0/60	0
Savannah River, GA	Electro[p]	46.7 ± 3.8 tl	999 ± 290	5.4 [4–7]	0/58	0
Altamaha River, GA	Gillnet[p]	45.9 ± 3.1 tl	1143 ± 276	5.1 [4–7]	0/60	0
St. Johns River, FL	Electro[q]	42.7 ± 4.7 tl	752 ± 268	4.4 [2–7]	0/72	0
Bay anchovy *Anchoa mitchilli*						
Chesapeake Bay	Trawl[l]	–	1	YOY	0/60	0
Croaker *Micropogonias undulatus*						
Chesapeake Bay	Gillnet[l]	24.2 ± 3 tl	200 ± 89	–	0/60	0
Greenland halibut *Reinhardtius hippoglossoides*						
Davis Strait, NAFO area 0B	Trawl[r]	49.9 ± 7.2 tl	–	–	4/59	6.8
Newfoundland, NAFO area 3K	Trawl[r]	43.1 ± 8 tl	739 ± 450	–	4/60	6.7
Spot *Leiostomus xanthurus*						
Chesapeake Bay	Gillnet/Trawl[l]	14.7 ± 5.4 tl	68 ± 64	–	0/56	0

[a]Washington Department of Fish and Wildlife, [b]US Fish and Wildlife Service, [c]US National Marine Fisheries Service, [d]Alaska Department of Fish and Game, [e]US Geological Survey, [f]Ministry of Natural Resources and Wildlife Quebec, [g]Massachusetts Division of Fisheries and Wildlife, [h]Connecticut Department of Energy and Environmental Protection, [i]Maryland Department of Natural Resources, [j]Delaware Department of Natural Resources and Environmental Control, [k]District Department of Ecology, [l]Virginia Institute of Marine Science, [m]North Carolina Department of Environment and Natural Resources, [n]North Carolina Wildlife Resource Commission, [o]South Carolina Department of Natural Resources, [p]Georgia Department of Natural Resources, [q]Florida Fish and Wildlife Conservation Commission, [r]Department of Fisheries and Oceans Canada

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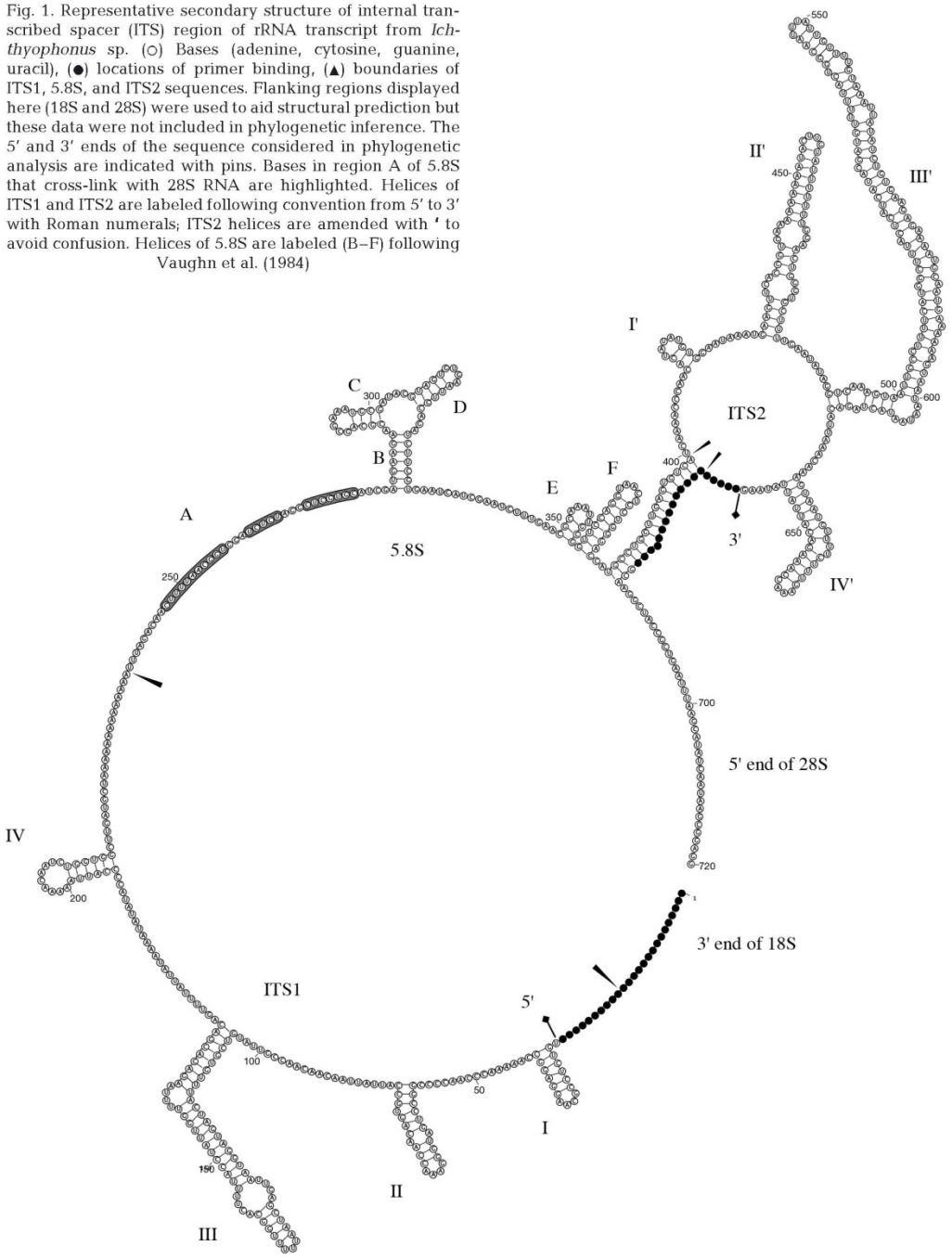

Fig. 1. Representative secondary structure of internal transcribed spacer (ITS) region of rRNA transcript from *Ichthyophonus* sp. (○) Bases (adenine, cytosine, guanine, uracil), (●) locations of primer binding, (▲) boundaries of ITS1, 5.8S, and ITS2 sequences. Flanking regions displayed here (18S and 28S) were used to aid structural prediction but these data were not included in phylogenetic inference. The 5′ and 3′ ends of the sequence considered in phylogenetic analysis are indicated with pins. Bases in region A of 5.8S that cross-link with 28S RNA are highlighted. Helices of ITS1 and ITS2 are labeled following convention from 5′ to 3′ with Roman numerals; ITS2 helices are amended with ′ to avoid confusion. Helices of 5.8S are labeled (B–F) following Vaughn et al. (1984)

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Table 2. Summary data for 94 *Ichthyophonus* isolates used in phylogenetic comparison. Novel isolates were obtained from the prevalence survey of this study, and from material archived by several cooperating laboratories (see footnotes). Number of clone sequences is indicated. Isolates with clone sequences previously deposited in GenBank have the prefix 'gb' on the isolate ID. Average evolutionary divergence over sequence pairs within isolates (*p*-distance) was calculated using MEGA5 (see 'Materials and methods'), with SE estimated from 500 bootstrap samples

Host species / Isolate ID	Collection location	Year	No. of clones	*p*-distance Mean	*p*-distance SE	GenBank acc. no(s). ITS	GenBank acc. no(s). 18S
American shad							
IA285	Cape Fear River, North Carolina, USA	2011	15	0.001	0.0004	KF987552–KF987566	
IA013	Columbia River, Oregon, USA[a]	2005	8	0.004	0.0014	KF987093–KF987100	
gb IA014	Columbia River, Oregon, USA	2005	12	0.006	0.0017	GQ402860–GQ402871	GQ370770, GQ370791
gb IA052	Columbia River, Oregon, USA	2007	13	0.004	0.0015	GQ402887–GQ402899	GQ370774, GQ370795
IA327	Columbia River, Oregon, USA	2011	8	0.003	0.0014	KF987730–KF987737	
IA328	Columbia River, Oregon, USA	2011	7	0.005	0.0018	KF987738–KF987744	
IA295	Connecticut River, Connecticut, USA	2011	8	0.003	0.0013	KF987639–KF987646	
IA296	Merrimack River, Massachusetts, USA	2011	6	0.002	0.0011	KF987647–KF987652	
gb IA065	Merrimack River, Massachusetts, USA	2008	6	0.003	0.0013	GU059890–92, GU059901–03	GQ370782, GQ370803
gb IA066	Merrimack River, Massachusetts, USA	2008	8	0.002	0.0011	GU059893–GU059900	GQ370783, GQ370804
gb IA067	Merrimack River, Massachusetts, USA	2008	14	0.005	0.0014	GQ402900–06, GU146052–58	GQ370781, GQ370802
IA007	Puget Sound, Washington, USA[a]	2005	8	0.007	0.0020	KF987085–KF987092	
IA239	Puget Sound, Washington, USA	2010	8	0.002	0.0014	KF987364–KF987371	
IA240	Puget Sound, Washington, USA	2010	8	0.004	0.0017	KF987372–KF987379	
IA241	Puget Sound, Washington, USA	2010	14	0.002	0.0013	KF987380–KF987393	
IA242	Puget Sound, Washington, USA	2010	7	0.003	0.0016	KF987394–KF987400	
IA243	Puget Sound, Washington, USA	2010	8	0.002	0.0014	KF987401–KF987408	
IA244	Puget Sound, Washington, USA	2010	8	0.003	0.0015	KF987409–KF987416	
IA290	St. Lawrence River, Quebec, Canada	2011	16	0.000	0.0003	KF987575–KF987590	
IA291	St. Lawrence River, Quebec, Canada	2011	16	0.000	0.0003	KF987591–KF587606	
IA292	St. Lawrence River, Quebec, Canada	2011	8	0.001	0.0007	KF987607–KF987614	
IA293	St. Lawrence River, Quebec, Canada	2011	8	0.002	0.0012	KF987615–KF987622	
IA294	St. Lawrence River, Quebec, Canada	2011	16	0.003	0.0008	KF987623–KF987638	
Atlantic herring							
IA303	Atlantic Ocean, Iceland[b]	2011	8	0.003	0.0014	KF987692–KF987699	
IA304	Atlantic Ocean, Iceland[b]	2011	7	0.004	0.0017	Kf987700–KF987706	
IA307	Atlantic Ocean, Iceland[b]	2011	7	0.005	0.0019	KF987707–KF987713	
IA308	Atlantic Ocean, Iceland[b]	2011	8	0.004	0.0014	KF987714–KI987721	
Atlantic mackerel							
IA288	Atlantic Ocean, Algarve, Portugal[c]	2011	8[h]	0.036	0.0047	KF987567–KF987574	
Atlantic salmon							
gb 9-27	Connecticut River, Massachusetts, USA	2008	7	0.003	0.0015	GU059874–GU059880	
IA106	Connecticut River, Massachusetts, USA[d]	2009	8	0.003	0.0016	KF987164–KF987171	
IA108	Connecticut River, Massachusetts, USA[d]	2009	9	0.003	0.0013	KF987172–KF987180	
gb 9-80	Merrimack River, Massachusetts, USA	2008	9	0.007	0.0019	GU059881–GU059889	
IA246	Merrimack River, New Hampshire, USA[d]	2009	8	0.003	0.0013	KF987417–KF987424	
IA247	Merrimack River, New Hampshire, USA[d]	2009	16	0.002	0.0010	KF987425–KF987440	
IA248	Merrimack River, New Hampshire, USA[d]	2009	8	0.003	0.0016	KF987441–KF987448	
Chinook salmon							
IA171	Chena River, Alaska, USA[e]	2004	9	0.002	0.0011	KF987232–KF987240	
IA189	Chena River, Alaska, USA[e]	2006	9	0.002	0.0010	KF987267–KF987275	
IA169	Salcha River, Alaska, USA[e]	2004	9	0.003	0.0015	KF987214–KF987222	
IA170	Salcha River, Alaska, USA[e]	2004	9	0.003	0.0015	KF987223–KF987231	
IA190	Salcha River, Alaska, USA[e]	2006	9	0.002	0.0014	KF987276–KF987284	
IA192	Salcha River, Alaska, USA[e]	2006	9	0.002	0.0014	KF987285–KF987293	
IA177	Yukon River, Alaska, USA[e]	2004	8	0.004	0.0017	KF987241–KF987248	
IA185	Yukon River, Alaska, USA[e]	2006	9	0.003	0.0016	KF987249–KF987257	
IA187	Yukon River, Alaska, USA[e]	2006	9	0.003	0.0014	KF987258–KF987266	
Copper rockfish							
gb IA011	Puget Sound, Washington, USA	2006	5	0.011	0.0027	GQ402855–GQ402859	GQ370769, GQ370790
Dolly Varden							
IA264	Aquaculture, Yamanashi Pref., Japan[f]	2002	8	0.002	0.0010	KF987486–KF987493	
Great sculpin							
IA298	Bering Sea, Alaska, USA	2011	8	0.000	0.0000	KF987660–KF987667	
IA300	Bering Sea, Alaska, USA	2011	8	0.002	0.0015	KF987676–KF987683	

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Table 2 (continued)

Host species Isolate ID	Collection location	Year	No. of clones	*p*-distance Mean	SE	GenBank acc. no(s). ITS	18S
Greenland halibut							
IA260	Atlantic Ocean, Canada, NAFO area 3K	2010	8	0.002	0.0010	KF987467–KF987474	
IA262	Atlantic Ocean, Canada, NAFO area 3K	2010	11	0.003	0.0012	KF987475–KF987485	
IA339	Bering Sea, Alaska, USA	2011	8	0.004	0.0014	KF987759–KF987766	
IA340	Bering Sea, Alaska, USA	2011	11	0.003	0.0014	KF987767–KF987777	
IA377	Davis Strait, Canada, NAFO area 0B	2011	8	0.003	0.0016	KF987800–KF987807	
Pacific cod							
IA338	Bering Sea, Alaska, USA	2011	7	0.002	0.0010	KF987752–KF987758	
Pacific halibut							
IA251	Cook Inlet, Alaska, USA	2010	10	0.002	0.0012	KF987449–KF987458	
IA253	Cook Inlet, Alaska, USA	2010	8	0.003	0.0014	KF987459–KF987466	
IA363	Pacific Ocean, Oregon, USA	2011	7	0.002	0.0012	KF987786–KF987792	
IA364	Pacific Ocean, Oregon, USA	2011	7	0.005	0.0019	KF987793–KF987799	
IA316	Prince William Sound, Alaska, USA	2011	8	0.003	0.0016	KF987722–KF987729	
Pacific herring							
IA147	Lynn Canal, Alaska, USA[e]	2007	8	0.004	0.0018	KF987181–KF987188	
IA148	Lynn Canal, Alaska, USA[e]	2007	7	0.003	0.0012	KF987189–KF987195	
IA084	Prince William Sound, Alaska, USA[a]	2009	8	0.004	0.0017	KF987114–KF987121	
IA085	Prince William Sound, Alaska, USA[a]	2009	8	0.004	0.0014	KF987122–KF987129	
IA086	Prince William Sound, Alaska, USA[a]	2009	9	0.002	0.0009	KF987130–KF987138	
IA150	Prince William Sound, Alaska, USA[e]	2009	9	0.004	0.0016	KF987196–KF987204	
gb IA002	Puget Sound, Washington, USA	2005	12	0.005	0.0012	GQ402831–GQ402842	GQ370767, GQ370788
IA233	Puget Sound, Washington, USA	2010	9	0.003	0.0016	KF987308–KF987316	
IA234	Puget Sound, Washington, USA	2010	9	0.002	0.0013	KF987317–KF987325	
IA235	Puget Sound, Washington, USA	2010	9	0.003	0.0015	KF987326–KF987334	
IA236	Puget Sound, Washington, USA	2010	6	0.001	0.0009	KF987335–KF987340	
IA237	Puget Sound, Washington, USA	2010	15	0.002	0.0014	KF987341–KF987355	
IA238	Puget Sound, Washington, USA	2010	8	0.003	0.0016	KF987356–KF987363	
gb IA051	Sitka Sound, Alaska, USA	2007	15	0.007	0.0016	GQ402872–GQ402886	GQ370773, GQ370794
IA151	Sitka Sound, Alaska, USA[e]	2008	9	0.002	0.0011	KF987205–KF987213	
IA099	Sitka Sound, Alaska, USA[a]	2009	7	0.003	0.0016	KF987139–KF987145	
IA100	Sitka Sound, Alaska, USA[a]	2009	9	0.002	0.0012	KF987146–KF987154	
IA101	Sitka Sound, Alaska, USA[a]	2009	9	0.002	0.0014	KF987155–KF987163	
Pacific staghorn sculpin							
IA220	Puget Sound, Washington, USA	2010	14	0.002	0.0014	KF987294–KF987307	
Plain sculpin							
IA347	Bering Sea, Alaska, USA	2011	8	0.002	0.0011	KF987778–KF987785	
Puget Sound rockfish							
IA023	San Juan Channel, Washington, USA[a]	2005	13[h]	0.017	0.0035	KF987101–KF987113	
Rainbow trout							
IA273	Aquaculture, Epirus Periphery, Greece[g]	2011	8	0.001	0.0009	KF987494–KF987501	
IA276	Aquaculture, Epirus Periphery, Greece[g]	2011	7	0.002	0.0012	KF987502–KF987508	
IA278	Aquaculture, Epirus Periphery, Greece[g]	2011	11	0.002	0.0012	KF987509–KF987519	
IA281	Aquaculture, Epirus Periphery, Greece[g]	2011	7	0.003	0.0013	KF987520–KF987526	
IA282	Aquaculture, Epirus Periphery, Greece[g]	2011	10	0.002	0.0013	KF987527–KF987536	
IA283	Aquaculture, Epirus Periphery, Greece[g]	2011	7	0.003	0.0015	KF987537–KF987543	
IA284	Aquaculture, Epirus Periphery, Greece[g]	2011	8	0.001	0.0010	KF987544–KF987551	
gb RBT11	Aquaculture, Idaho, USA	2008	10	0.005	0.0015	GQ402928–GQ402937	GQ370776, GQ370801
gb RBT12	Aquaculture, Idaho, USA	2008	10	0.003	0.0012	GQ402938–GQ402947	GQ370777, GQ370797
gb RBT13	Aquaculture, Idaho, USA	2008	16	0.003	0.0012	GQ402948–GQ402963	GQ370778, GQ370798
Walleye pollock							
IA299	Bering Sea, Alaska, USA	2011	8	0.002	0.0015	KF987668–KF987675	
IA301	Bering Sea, Alaska, USA	2011	8	0.002	0.0015	KF987684–KF987691	
IA337	Bering Sea, Alaska, USA	2011	7	0.002	0.0013	KF987745–KF987751	
Yellowtail rockfish							
IA297	Pacific Ocean, Washington, USA	2011	7[h]	0.029	0.0036	KF987653–KF987659	

[a]Archived material from the US Geological Survey, Marrowstone Marine Field Station, Nordland WA, USA
[b]Fresh isolates from epizootic occurring in Iceland herring stocks
[c]Fresh isolates from mackerel fishery collected at Oceanario de Lisboa, Lisbon, Portugal
[d]Archived material form the US Fish and Wildlife Service, Northeast Fish Health Center, Lamar PA, USA
[e]Archived material from the Alaska Department of Fish and Game, Fish Health Laboratory
[f]Archived material from the Nippon Veterinary and Life Sciences University, Yamanashi Prefecture, Japan
[g]Fresh isolated from epizootic occurring in Greek trout farms
[h]Isolate contained divergent haplotypes that were considered separately in phylogeny

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and Bayes factors indicated there was very strong support (2lnBayesFactor > 10) for selection of the 6-partition model over all other partition schemes (see Table S3 in the Supplement at www.int-res.com/articles/suppl/d120p125_supp.pdf).

The *Ichthyophonus* phylogeny based on rDNA ITS data under a 6-partition model contained 6 major divisions with limited lower-order structure (Fig. 2). The majority of the isolates (71 of 98 isolates) were nearly identical, producing a large polytomy (Fig. 2: Clade D) that included parasite material from 13 NE Pacific and 4 Atlantic hosts sampled across a wide geographic range. A well-supported (posterior probability = 0.987) daughter clade (Fig. 2: Clade E) within this polytomy included 8 isolates exclusively from American shad in the Merrimack and St. Lawrence Rivers. A single clade (Fig. 2: Clade C) included all the isolates derived from freshwater aquaculture hosts, rainbow trout and Dolly Varden, and included haplotypes from Puget Sound rockfish and Atlantic mackerel. Clades D and C formed an unresolved tricotomy with isolates from Greenland halibut captured in the NW Atlantic (Fig. 2: Clade B). The 2 remaining clades contained more divergent ITS isolates: Clade A included haplotypes from yellowtail rockfish and Atlantic mackerel and Clade F contained a single isolate from an American shad collected in the St. Lawrence Waterway. Mean pairwise percent nucleotide differences between the major clades varied from 0.4 to 10.6 for ITS1 and from 0.6 to 11.7 for ITS2 (Table 3).

The *Ichthyophonus* phylogeny based on 18S sequences (Fig. 3) had limited structure, but effectively related some of our isolates to those of other researchers. The 18S sequence from the unique American shad isolate, IA293, was identical to that of *I. irregularis* described by Rand et al. (2000). The partial 18S sequence of isolate IA297 from yellowtail rockfish was identical to sequences previously isolated from yellowtail rockfish and Pacific ocean perch *Sebastes alutus* (Criscione et al. 2002). Further, these 3 isolates grouped with an IA288 haplotype isolated from Atlantic mackerel (Clade A). Freshwater isolates in Clade C formed a separate clade in the 18S phylogeny. This is contrary to the results presented by Rasmussen et al. (2010), where 18S sequences were identical between freshwater and marine types. Here we amplified and sequenced a larger section of 18S rDNA than that used by Criscione et al. (2002) and Rasmussen et al. (2010), and discovered 4 transitions that occur in a span of 14 nt near the 3′ end of the 18S sequence.

DISCUSSION

The most prominent feature of the ITS rDNA phylogeny of *Ichthyophonus* is the large polytomy containing a majority of isolates from both the Atlantic and Pacific Oceans. (Fig. 2: Clade D). This ubiquitous marine form was widely distributed in pelagic (herring, mackerel), semi-pelagic (walleye pollock, Pacific cod), benthic (copper rockfish, Greenland halibut, Pacific halibut, cottid sp.) and anadromous (Atlantic salmon, Chinook salmon, American shad) hosts, thus confirming, for at least 1 species in the genus, the long held supposition of low host specificity that was attached to *I. hoferi* (McVicar 1999). This parasite type is also the only one we can associate with severe epizootics in wild hosts. Isolates obtained during epizootics in Chinook salmon from the Yukon River (Kocan et al. 2004), American shad from the Columbia River (Hershberger et al. 2010) and Atlantic herring from Iceland (Óskarsson & Pálsson 2011) are all contained in Clade D (Fig. 2).

The predominance of a single *Ichthyophonus* type with nearly identical ITS sequences across a large host and geographic range suggests broad scale transmission processes with little allopatry imposed by different host species, or conversely, that the ITS locus is not variable enough to differentiate phylogenetic structure resulting from host or geographic separation of *Ichthyophonus* stains. Waterborne transmission has been demonstrated among rainbow trout in freshwater (Yokota et al. 2008), but to date we have been unable to replicate this phenomenon in a common marine host, Pacific herring (Gregg et al. 2012). At higher trophic levels, it is clear that *Ichthyophonus* spp. can be acquired through consumption of infected prey fishes (Gustafson & Rucker 1956, McVicar & McLay 1985, Jones & Dawe 2002). These infections may be dead ends in many predatory species, and selection on a parasite life history that is closed in 1, or a few, forage species could result in the fairly uniform parasite distribution we encountered. In the Bering Sea, White et al. (2014) proposed that walleye pollock could be a source of *Ichthyophonus* infection for many other fish species. At many life history stages, walleye pollock are forage for other species, and may even transmit *Ichthyophonus* into the Bering Sea food chain as infected fisheries offal. In this scenario, a selection process that homogenized the *Ichthyophonus* population in pollock, could result in the uniform genetic distribution in other fishes in the Bering Sea. We isolated *Ichthyophonus* from 5 new hosts in the Bering Sea, from walleye pollock, and from Chinook salmon

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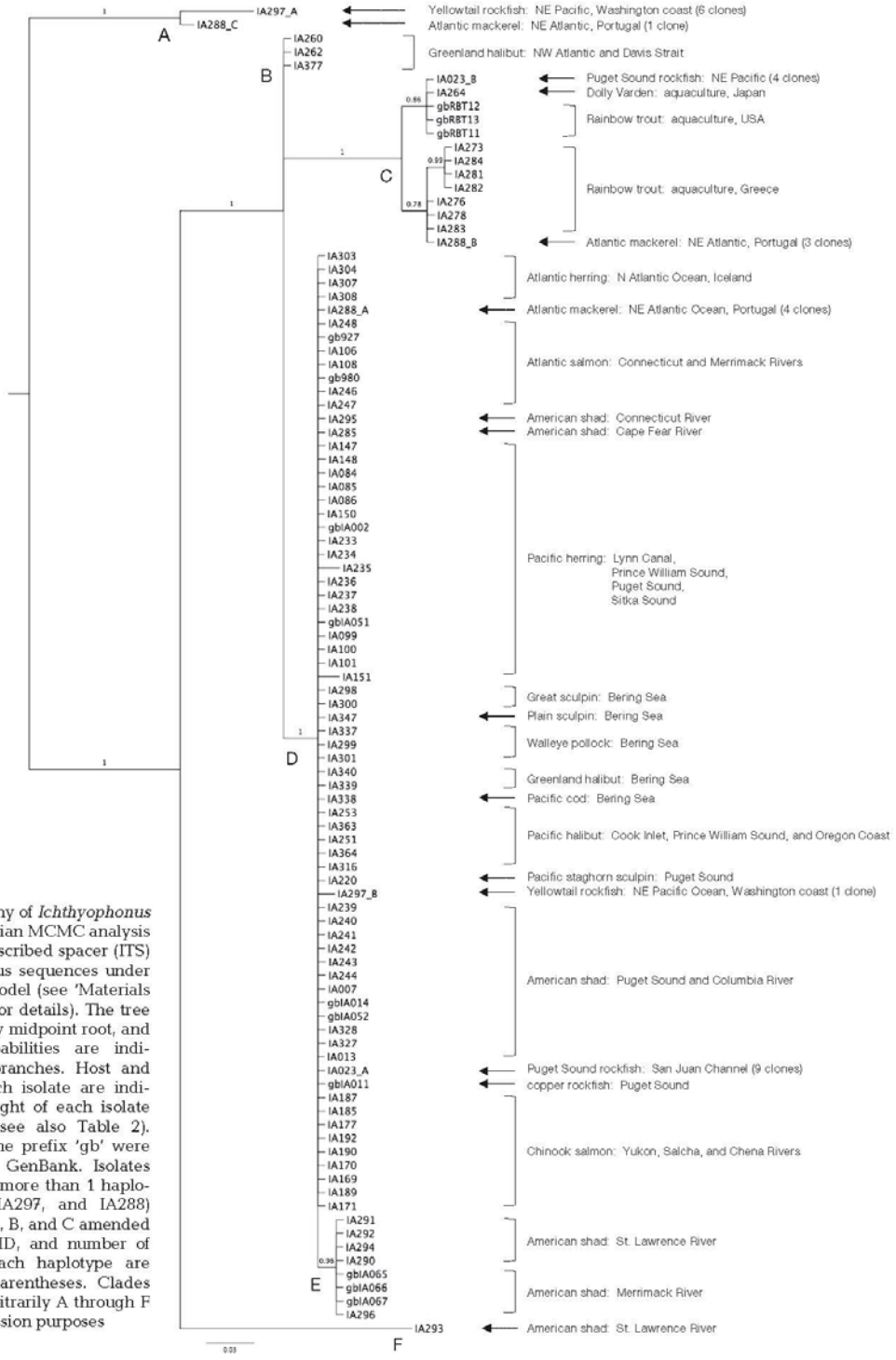

Fig. 2. Phylogeny of *Ichthyophonus* based on Bayesian MCMC analysis of internal transcribed spacer (ITS) rDNA consensus sequences under a 6-partition model (see 'Materials and methods' for details). The tree has an arbitrary midpoint root, and posterior probabilities are indicated above branches. Host and location of each isolate are indicated to the right of each isolate identification (see also Table 2). Isolates with the prefix 'gb' were obtained from GenBank. Isolates that contained more than 1 haplotype (IA023, IA297, and IA288) have suffixes A, B, and C amended to the isolate ID, and number of clones with each haplotype are indicated in parentheses. Clades are labeled arbitrarily A through F for discussion purposes

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Table 3. Mean pairwise nucleotide differences (%) between major clades of the *Ichthyophonus* phylogeny (Fig. 2). Percent difference calculated separately for internal transcribed spacer 1 (ITS1) and ITS2 regions. Length of ITS1 alignment = 245 nt. Length of the ITS2 alignment = 311 nt

	ITS1 % diff./ITS2 % diff.				
	Clade F	Clade E	Clade D	Clade C	Clade B
Clade A	8.0/11.7	6.5/9.1	6.5/9.6	7.3/10	6.5/8.8
Clade B	9.4/2.6	0.8/1.0	0.4/0.9	0.8/4.3	
Clade C	10.2/6.1	1.6/5.2	1.2/4.9		
Clade D	9.8/3.0	0.4/0.6			
Clade E	10.6/3.5				

in the Yukon River watershed (presumably infected in the Bering Sea). The consensus ITS sequences of all these isolates were nearly identical.

In contrast to the ubiquitous marine form, the 4 other *Ichthyophonus* types detected in marine and anadromous fishes had narrow host distributions. Two were found in American shad (Clades E and F), a single species was encountered in Greenland turbot in the NW Atlantic (Clade B), and 2 sister-isolates (Clade A) occurred in Atlantic mackerel (IA288_C) and yellowtail rockfish (IA297_A). Due to the relatively small sample size within hosts, it was not possible to determine whether these forms are specialists in the hosts encountered or if they are multi-host parasites that occur less frequently than the ubiquitous marine type. Regions of identical 18S DNA sequence, though not conclusive, suggest that isolate IA293, from yellowtail rockfish, may also be capable of infecting a sympatric congener, Pacific Ocean perch (Criscione et al. 2002), and that IA293 isolated from American shad may be a species previously described in yellowtail flounder (Rand et al. 2000).

Despite great geographic separation of the isolates (i.e. Greece, North America, and Japan), parasites

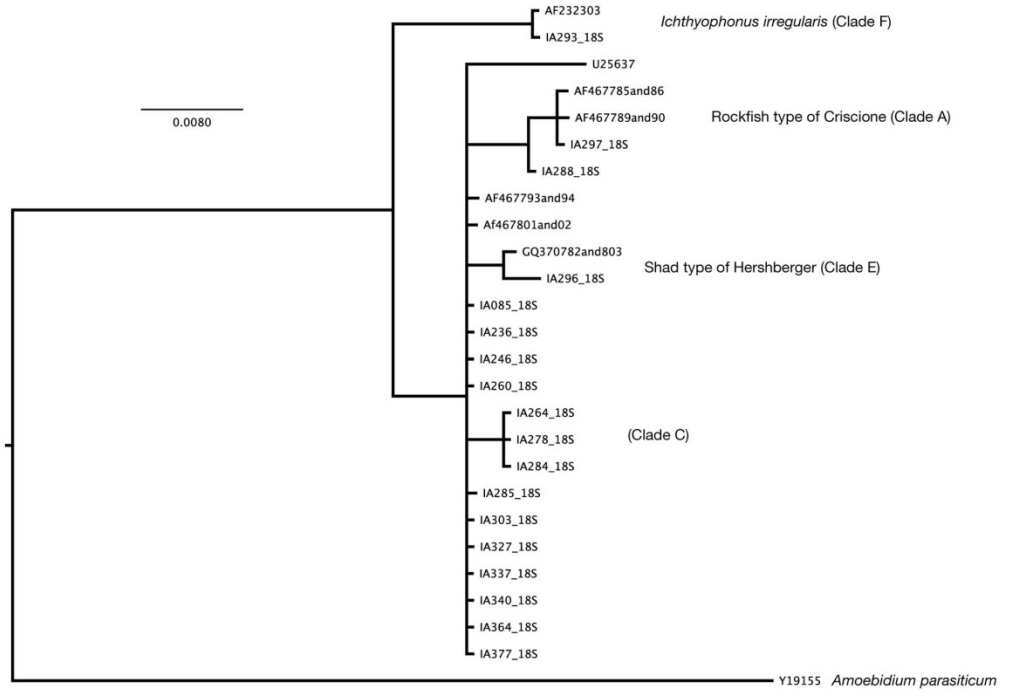

Fig. 3. Phylogeny of *Ichthyophonus* based on Bayesian MCMC analysis of 18S rDNA sequences (see 'Materials and methods' for details). Sequences were generated for a subset of samples used in internal transcribed spacer (ITS) phylogeny and selected from GenBank based on the publications of Ragan et al. (1996), Rand et al. (2000), Criscione et al. (2002), and Hershberger et al. (2010). Clade designations reference the ITS phylogeny presented in Fig. 2. *Amoebidium parasiticum* is used as the outgroup

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from freshwater aquaculture hosts fell into a single clade (Fig. 2: Clade C). This ITS genotype was first identified in rainbow trout from North America by Rasmussen et al. (2010). Clade C haplotypes also occurred in the minority of clones from 2 marine isolates that contained variable ITS sequences, a Puget Sound rockfish from the inland marine waters of Washington State and an Atlantic mackerel captured near Portugal. We do not know if freshwater aquaculture infections originate in wild sympatric hosts, are introduced from a marine source, or are the legacy of an historic introduction and have been perpetuated by the global movement of animals and animal products. Some evidence for local introduction existed in the structure of Clade C, where a well-supported dichotomy mimicked the Pacific versus Atlantic distribution of the isolates. A daughter clade containing rainbow trout from western North America, Dolly Varden from Japan, and the haplotype from Puget Sound rockfish formed a sister group to that containing rainbow trout from Greece and the haplotype isolated from Atlantic mackerel. Anecdotal reports attribute *Ichthyophonus* spp. epizootics in aquaculture to the feeding of unpasteurized tissues of wild freshwater (Erickson 1965) or marine (Rucker & Gustafson 1953, Slocombe 1980, Egusa 1983, Athanassopoulou 1992) hosts. There were no isolates from wild freshwater hosts in this study; however, if *Ichthyophonus* spp. were introduced to aquaculture from marine sources, the absence of the ubiquitous marine species in the fish farms is notable, and may indicate rapid adaptation from standing genetic diversity. Improved facultative survival of the Clade C type has been demonstrated in freshwater (Hershberger et al. 2008) and may explain the absence of other forms in our aquaculture samples. A more focused study with extensive sampling in and around aquaculture facilities is necessary to determine the processes that establish and maintain *Ichthyophonus* spp. in fish farms.

The 6 main clades identified in the ITS rDNA phylogeny of *Ichthyophonus* may represent separate species. Mean pairwise nuleotide differences for ITS1 and/or ITS2 (Table 3) among Clades A, C, and F, and between these clades and all others, were greater than the intraspecific variation that occurs within 75 to 77% of fungal species (Nilsson et al. 2008). However, there are no consensus methods for delimiting species within *Ichthyophonus*, primarily because only 1 modern species description has been published.

Rand (1994) provided a thorough morphological and histochemical description of an irregular form of *Ichthyophonus* that was detected during a large scale survey of yellowtail flounder in the North Atlantic. Sequences of 18S rDNA from this irregular form were different from the 2 *Ichthyophonus* 18S sequences available at the time (Ragan et al. 1996, Spanggaard et al. 1996), and *I. irregularis* was established (Rand et al. 2000). While Rand et al. (2000) provided a good model for the description of novel species within the group, it is likely that the 18S locus is not variable enough to delimit species (Rasmussen et al. 2010), and as more genotypes are described, a new set of lineage-relevant phenotypic characteristics may replace the histochemical and morphological characteristics they used. We feel that species delimitation should be confirmed by multiple genetic markers to insure that the process occurs in a robust phylogenetic framework (Boenigk et al. 2012). As a practical matter, the recondite life history of *Ichthyophonus*, coupled with the varied morphology that can occur in culture and in host tissues, may result in a species identification process that relies heavily on molecular markers and less so on morphological characteristics.

We cannot determine if the present taxonomic units include the species originally described by Plehn & Mulsow (1911). However, their material was isolated from farmed rainbow trout in freshwater, suggesting that the specific name *I. hoferi*, if retained, be used solely for the ITS genotypes of Clade C. Sequences of the 18S rDNA gene indicated that our Clade F likely corresponds to *I. irregularis* (Rand et al. 2000) and this relationship can be confirmed by sequencing ITS rDNA from the archived holotype. Neither formal description nor specific epithet exists for the remaining taxonomic units presented here (i.e. Clades A, B, D, and E).

A limitation of this study is the inability to determine if the multi-haplotype isolates detected in yellowtail rockfish (IA297), Puget Sound rockfish (IA023), and Atlantic mackerel (IA288) resulted from heterozygous parasites or co-infections. Hundreds of copies of the ribosome coding sequence can occur at more than 1 location in the genome, opening the possibility of polyzygous individuals. However, the arrangement of these genes in tandem repeats and concerted evolution at rDNA multi-locus genes has a strong homogenizing effect (Ganley & Kobayashi 2007) that can maintain homogeneity through biased gene conversion during clonal reproduction (Hillis et al. 1991). Variation was low among clones of the other 91 isolates we sequenced; therefore, we hypothesize that these multiple sequences were the result of co-infections rather than heterozygosity.

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This hypothesis will need to be confirmed by sequencing hundreds of clones from single spore isolations of these mixed haplotype isolates.

The prevalence and genetic distribution of *Ichthyophonus* spp. isolated from American shad in their native range were markedly different from those in the NE Pacific, where shad are an introduced species. Infections were detected in only 8 of 1077 adult shad returning to rivers from the NW Atlantic, whereas 73.7% (28/38) of juveniles captured in the Puget Sound were infected and 28.3% (17/60) of adults returning to the Columbia River were infected. Three *Ichthyophonus* types were detected in the 11 isolates sequenced from the Atlantic region, while only the ubiquitous marine type was detected in the 12 isolates sequenced from the Pacific region. Based on a smaller sample, Hershberger et al. (2010) posited that American shad on the east and west coasts of North America carried different *Ichthyophonus* types, but our data show that the form present in the Columbia River also occurs in the NW Atlantic. Infections in the NE Pacific, while more numerous, seem to represent a subset of the ITS genotypes present in the NW Atlantic. We detected the highest *Ichthyophonus* genetic diversity in the area where the first large *Ichthyophonus* epizootics were reported (i.e. Gulf of St. Lawrence and Gulf Maine) (Cox 1916, Fish 1934, Sindermann 1958).

CONCLUSION

We have identified 6 taxonomic units within *Ichthyophonus* that may represent different species. However, it appears that the majority of global impacts in wild fish populations result from a single taxon. This ubiquitous marine form, which lacks formal description, is the only one that we can associate with epizootics in wild fishes, as all isolates from the Atlantic herring epizootic near Iceland (Óskarsson & Pálsson 2011), the American shad epizootic in the Columbia River (Hershberger et al. 2010), and the Chinook salmon epizootic in the Yukon River (Kocan et al. 2004) were of this type. Similarly, a single form that is adapted to low salinity environments appears to be widespread in salmonid culture.

The broad host and geographic distribution of these 2 ITS types will confound efforts to use this marker to infer inter-host transmission patterns. More variable molecular markers should be developed to elucidate the transmission dynamics of *Ichthyophonus* spp. in systems were they significantly affect fishery production and aquaculture. While this study represents the first broad examination of species richness within *Ichthyophonus*, only about 10% of nearly 150 reported host species were sampled, and these samples were geographically focused around North America. Further sampling in marine and freshwater environments, especially those of the Eastern Hemisphere, would significantly improve our understanding of *Ichthyophonus* global diversity.

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Appendix 6

The following supplement accompanies the article

Ichthyophonus parasite phylogeny based on ITS rDNA structure prediction and alignment identifies six clades, with a single dominant marine type

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Table S1. Fish species reported as hosts of parasites in the genus *Ichthyophonus*. List includes infections reported under pseudonyms. DIA = diadromous, SW = salt water (marine), FW = freshwater. Dash indicates provenance of infected host not available from publication.

Family Species (common name)	Region	Habitat	Citation
Anguillidae			
Anguilla japonica (Japanese eel)	Taiwan	DIA	1
Clupeidae			
Alosa pseudoharengus (alewife)	NW Atlantic	DIA	2,3,4
A. sapidissima (American shad)	NE Pacific, NW Atlantic	DIA	5, 6, 7
Clupea harengus (Atlantic herring)	N Atlantic	SW	2, 3, 4, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23
C. pallasii (Pacific herring)	NE Pacific	SW	6, 7, 24, 25, 26, 27, 28, 29, 30, 31, 32
Sprattus sprattus (sprat)	NE Atlantic	SW	8, 19, 21
Tenualosa ilisha (hilsa shad)	Iraq	FW	33
Cyprinidae			
Acanthobrama centisquama	Iraq	FW	33
A. marmid (kalashpa)	Iraq	FW	33
Alburnus caeruleus	Iraq	FW	33
Aspius vorax (shelej)	Iraq	FW	33
Barbus barbulus (abu-barattum)	Iraq	FW	33
B. grypus (shabbout)	Iraq	FW	33
Capoeta damascina (gel khorok)	Iraq	FW	33
C. trutta (barg bidy)	Iraq	FW	33
Carasobarbus luteus (himri)	Iraq	FW	33
Carassius auratus (goldfish)	Africa, France, Iraq	FW	33, 34, 35, 36
C. carassius (crucian carp)	India, Iraq	FW	33, 35, 37
Cyprinion macrostomum	Iraq	FW	33
Cyprinus carpio (common carp)	Iraq,Utah	FW	33, 35, 38
Danio rerio (zebra danio)	–	FW	35
Hypophthalmichthys nobilis (bighead carp)	Africa	FW	36
Luciobarbus esocinus (mangar)	Iraq	FW	33
L. xanthopterus (gattan)	Iraq	FW	33
Mesopotamichthys sharpeyi (bunni)	Iraq	FW	33
Pethia conchonius (rosy barb)	–	FW	35
P. stoliczkana (sonnenfleckbarbe)	–	FW	35

Puntius johorensis (striped barb)	–	FW	35
P. semifasciolatus (Chinese barb)	–	FW	35
Rhodeus amarus (bitterfisch)	–	FW	35
Systomus tetrazona (sumatra barb)	–	FW	35
Tanichthys albonubes (white cloud mountain minnow)	–	FW	35
Tinca tinca (tench)	–	FW	35
Trigonostigma heteromorpha (harlequin rasbora)	–	FW	35
Lebiasinidae			
Nannostomus trifasciatus (threestripe pencilfish)	–	FW	35
Characidae			
Aphyocharax anisitsi (bloodfin tetra)	–	FW	35
Gymnocorymbus ternetzi (black tetra)	–	FW	35
Hemigrammus ocellifer (head-and-tailight tetra)	–	FW	35
H. unilineatus (feather fin tetra)	–	FW	35
Hyphessobrycon anisitsi (Buenos Aires tetra)	–	FW	35
H. flammeus (flame tetra)	–	FW	35
H. rosaceus (rosy tetra)	–	FW	35
Paracheirodon innesi (neon tetra)	–	FW	35
Loricariidae			
Ancistrus brevipinnis (cascudo)	–	FW	35
Bagridae			
Mystus pelusius (abu-zummair)	Iraq	FW	33
Siluridae			
Silurus triostegus (esbele)	Iraq	FW	33
Salmonidae			
Oncorhynchus kisutch (coho salmon)	NW Pacific	DIA	25, 39
O. mykiss (rainbow trout)	France, Germany, Japan, Greece, United States	FW	1, 6, 15, 23, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48
O. nerka (sockeye salmon)	NE Pacific	DIA	39, 49
O. tshawytscha (Chinook salmon)	NE Pacific	DIA	5, 24, 27, 29, 39, 50, 51
Salmo salar (Atlantic salmon)	NE Atlantic, Barents Sea	DIA	14, 15, 52, 53
S. trutta (brown trout/sea trout)	NE Atlantic, Germany, Switzerland, Tasmania	DIA/FW	35, 54, 55, 56, 57
Salvelinus fontinalis (brook trout)	Germany, Canada	DIA	35, 57, 58
S. malma (Dolly Varden)	Japan	FW	42
Osmeridae			
Hypomesus pretiosus (surf smelt)	NE Pacific	SW	28
Plecoglossidae			
Plecoglossus altivelis (ayu)	Japan	FW	59
Lotidae			
Lota lota (burbot)	North America	FW	50

Gadidae			
Gadus macrocephalus (Pacific cod)	NE Pacific	SW	7, 60
G. morhua (Atlantic cod)	NE Atlantic, NW Atlantic	SW	14, 15, 35, 61
Melanogrammus aeglefinus (haddock)	NE Atlantic, NW Atlantic	SW	14, 15, 16, 22, 35, 54, 62
Merlangius merlangus (whiting)	NE Atlantic	SW	15
Micromesistius poutassou (blue whiting)	NE Atlantic	SW	14, 15, 22
Gadus chalcogrammus (walleye pollock)	NE Pacific	SW	7, 63, 64
Melamphaidae			
Scopelogadus beanii (Bean's bigscale)	Atlantic Ocean	SW	22, 65
Gobiidae			
Brachygobius xanthozona (bumblebee fish)	–	FW	35
Mullidae			
Mullus barbatus barbatus (red mullet)	–	SW	35
Trichiuridae			
Aphanopus carbo (black scabbard fish)	NE Atlantic	SW	66
Scombridae			
Scomber colias (Atlantic chub mackerel)	Mediterranean	SW	40
S. scombrus (Atlantic mackerel)	NE Atlantic, NW Atlantic	SW	3, 14, 67, 68, 69
Mastacembelidae			
Mastacembelus mastacembelus (mar mahi)	Iraq	FW	33
Osphronemidae			
Betta splendens (Siamese fighting fish)	–	FW	35
Macropodus opercularis (paradise fish)	–	FW	35
Trichogaster fasciata (banded gourami)	–	FW	35
Trichopodus trichopterus (three spot gourami)	–	FW	35
Anabantidae			
Anabas testudineus (climbing perch)	–	FW	35
Carangidae			
Seriola dumerili (greater amberjack)	–	SW	35
S. quinqueradiata (yellowtail)	Japan, NW Pacific	SW	1, 70, 71
Trachinotus ovatus (pompano)	NE Atlantic	SW	68
Trachurus trachurus (Atlantic horse mackerel)	Mediterranean, NE Atlantic	SW	40, 68
Citharidae			
Citharus linguatula (spotted flounder)	Mediterranean	SW	72

Scophthalmidae			
Scophthalmus maximus (turbot)	NE Atlantic	SW	73, 74
Paralichthyidae			
Citharichthys stigmaeus (speckled sanddab)	NE Pacific	SW	28
Pleuronectidae			
Hippoglossus stenolepis (Pacific halibut)	NE Pacific	SW	7, 60
Limanda ferruginea (yellowtail flounder)	NW Atlantic	SW	22, 61, 62, 75, 76, 77
Platichthys flesus (European flounder)	NE Atlantic	SW	19, 54
P. stellatus (starry flounder)	NE Pacific	SW	32
Pleuronectes platessa (European plaice)	NE Atlantic	SW	14, 15, 16, 22, 74, 78
Pseudopleuronectes americanus (winter flounder)	NW Atlantic	SW	2, 79
Reinhardtius hippoglossoides (Greenland halibut)	NE Pacific, NW Atlantic	SW	7
Cichlidae			
Hemichromis bimaculatus (jewelfish)	France	FW	34
Oreochromis mossambicus (Mozambique tilapia)	–	FW	35
O. niloticus (Nile tilapia)	Africa	FW	36, 80
Pterophyllum scalare (freshwater angelfish)	France	FW	34, 35
Rocio octofasciata (Jack Dempsey)	France	FW	34, 35
Melanotaeniidae			
Melanotaenia nigrans (black-baded rainbowfish)	–	FW	35
Telmatherinidae			
Marosatherina ladigesi (Celebes rainbowfish)	–	FW	35
Aplocheilidae			
Aplocheilus panchax (blue panchax)	–	FW	35
Poeciliidae			
Heterandria formosa (least killifish)	–	FW	35, 81
Limia nigrofasciata (blackbarred limia)	–	FW	35
Poecilia reticulate (guppy)	–	FW	35, 82
P. sphenops (molly)	–	FW	35
Xiphophorus hellerii (green swordtail)	–	FW	35, 83
X. maculatus (southern platyfish)	–	FW	35, 82
Fundulidae			
Fundulus heteroclitus (mummichog)	NW Atlantic	SW	4
Ambassidae			
Parambassis lala (highfin glassy perchlet)	–	FW	35

Mugilidae			
Chelon subviridis (greenback mullet)	Iraq	FW	33
Liza abu (abu mullet)	Iraq	FW	33, 84
L. aurata (golden grey mullet)	Mediterranean	SW	73
L. saliens (leaping mullet)	Mediterranean	SW	73, 85
Mugil capito (thinlip grey mullet)	Mediterranean	SW	73, 85
M. cephalus (flathead grey mullet)	SW Indian Ocean	SW	35, 86
Pomacentridae			
Dascyllus trimaculatus (threespot dascyllus)	–	SW	35
Premnas biaculeatus (spinecheek anemonefish)	–	SW	35
Moronidae			
Dicentrarchus labrax (European seabass)	Mediterranean	SW	35, 73, 87
Sparidae			
Acanthopagrus schlegelii (blackhead seabream)	Japan	SW	1
Dentex dentex (common dentex)	Mediterranean	SW	35, 88
Diplodus annularis (annular seabream)	–	SW	35
Pagrus pagrus (red porgy)	–	SW	35
Sarpa salpa (salema)	NE Atlantic	SW	68
Sparus aurata (gilthead seabream)	Mediterranean, NE Atlantic	SW	35, 40, 73
Spondyliosoma cantharus (black seabream)	–	SW	35
Scatophagidae			
Scatophagus argus (spotted scat)	–	SW	35
S. tetracanthus (scatty)	–	SW	35
Sciaenidae			
Leiostomus xanthurus (spot)	NW Atlantic	SW	89, 90
Lutjanidae			
Pristipomoides filamentosus (opakapaka)	Hawaii	SW	91
Oplegnathidae			
Oplegnathus fasciatus (barred knifejaw)	Japan	SW	92
Serranidae			
Serranus scriba (painted comber)	–	SW	35
Percidae			
Perca fluviatilis (European perch)	–	FW	35
Trachinidae			
Echiichthys vipera (lesser weever)	–	SW	35

Sebastidae			
Sebastes alutus (Pacific Ocean perch)	NE Pacific	SW	24, 93
S. auriculatus (brown rockfish)	NE Pacific	SW	26
S. caurinus (copper rockfish)	NE Pacific	SW	6, 26
S. emphaeus (Puget Sound rockfish)	NE Pacific	SW	27, 28, 32
S. flavidus (yellowtail rockfish)	NE Pacific	SW	7, 24, 93
S. maliger (quillback rockfish)	NE Pacific	SW	60
S. melanops (black rockfish)	NE Pacific	SW	60
S. pinniger (canary rockfish)	NE Pacific	SW	93
S. reedi (yellowmouth rockfish)	NE Pacific	SW	93
S. ruberrimus (yellow eye rockfish)	NE Pacific	SW	60
Zoarcidae			
Zoarces americanus (eelpout)	NW Atlantic	SW	94
Hexagrammidae			
Ophiodon elongatus (lingcod)	NE Pacific	SW	26, 60
Cottidae			
Cottus aleuticus (coastrange sculpin)	NE Pacific	SW	30
C. asper (prickly sculpin)	NE Pacific	FW	39
Leptocottus armatus (Pacific staghorn sculpin)	NE Pacific	SW	7, 95
Myoxocephalus joak (plain sculpin)	NE Pacific	SW	7
M. octodecemspinosus (longhorn sculpin)	NW Atlantic	SW	61
M. polyacanthocephalus (great sculpin)	NE Pacific	SW	7

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Table S2. Partitions, alignment length, and substitution models used in phylogenetic analysis of *Ichthyophonus* rDNA sequences. Complete sequence included internal transcribed spacer (ITS) 1 and 2, and the 5.8s portion of the large sub-unit. Loop partition consists of unpaired regions of the molecule, and stems are paired bases. Akaike Information Criteria (AIC) was used to select the best fit model for each partition from 24 candidate models[a].

Partition	Aligned length (nt)	Substitution Model
Complete sequence	719	GTR+I[b]
ITS1	245	HKY+Γ[c]
5.8S	162	K80[d]
ITS2	312	HKY+Γ
Loops	375	HKY+Γ
Stems	341	HKY+I[e]

[a]24 candidate models reviewed in Posada & Crandall (2001).
[b]General time-reversible model with a proportion of invariable sites (I).
[c]Model from Hasegawa et al. (1985, with rate heterogeneity (Γ)
[d]Model from Kimura (1980).
[e]Model from Hasegawa et al (1985) with a proportion of invariable sites (I).

Table S3. 2ln Bayes Factor comparisons and ln likelihood (ln*L*) of ITS gene trees resulting from 4 partitioning strategies. 2ln Bayes Factor > 10 indicates very strong evidence against H_0 (Kass & Raftery 1995). Nearly identical values of duplicate MCMC's were omitted for brevity.

H_0	2ln Bayes Factor				ln*L*
	6 Partitions[a]	3 Partitions[b]	2 Partitions[c]	No Part.	
No partitioning	788.6	84.8	476.8	—	–1779.763
2 Partitions	311.8	–392.0	—		–1541.351
3 Partitions	703.8	—			–1737.362
6 Partitions	—				–1385.442

[a]Partitioned by stem and loop within each region (ITS1, 5.8S, ITS2)
[b]Partitioned by region (ITS1, 5.8S, ITS2)
[c]Partitioned by stem and loop

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Appendix 7

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Journal of Fish Diseases

Persistence of external signs in Pacific herring *Clupea pallasii* Valenciennes with ichthyophoniasis

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Abstract

The progression of external signs of *Ichthyophonus* infection in Pacific herring *Clupea pallasii* Valenciennes was highly variable and asynchronous after intraperitoneal injection with pure parasite preparations; however, external signs generally persisted through the end of the study (429 days post-exposure). Observed signs included papules, erosions and ulcers. The prevalence of external signs plateaued 35 days post-exposure and persisted in 73–79% of exposed individuals through the end of the first experiment (147 days post-exposure). Among a second group of infected herring, external signs completely resolved in only 10% of the fish after 429 days. The onset of mortality preceded the appearance of external signs. Histological examination of infected skin and skeletal muscle tissues indicated an apparent affinity of the parasite for host red muscle. Host responses consisted primarily of granulomatous inflammation, fibrosis and necrosis in the skeletal muscle and other tissues. The persistence and asynchrony of external signs and host response indicated that they were neither a precursor to host mortality nor did they provide reliable metrics for hindcasting on the date of exposure. However, the long-term persistence of clinical signs in Pacific herring may be useful in ascertaining the population-level impacts of ichthyophoniasis in regularly observed populations.

Keywords: Clupea pallasii, external signs, histopathology, *Ichthyophonus*, Pacific herring.

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Introduction

Ichthyophonus hoferi (hereafter referred to generically) is a mesomycetozoean parasite (Herr *et al.* 1999), primarily of marine fishes, that has caused recurring disease (ichthyophoniasis) epizootics and fish kills in herring *Clupea* spp. and other fishes throughout the Northern Hemisphere (reviewed in Burge *et al.* 2014). The mechanism(s) precipitating these epizootics in clupeid populations remain unclear. It is possible that epizootics occur after exacerbating factors stimulate the progression of disease in individuals that were previously harbouring chronic, low-intensity infections. Alternatively, it is possible that epizootics are initiated after exposure (or re-exposure) of herring populations to high concentrations of the parasite in the food or environment. Unfortunately, natural routes of *Ichthyophonus* exposure to clupeids and other planktivorous fishes have not been determined.

Herring with overt ichthyophoniasis demonstrate visible internal and external signs of disease. Internally, the disease manifests as white, nodular lesions that typically appear first on the heart and then on most other internal organs including the liver, kidney, spleen and gastrointestinal tract

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(Sindermann & Rosenfield 1954; Sindermann & Scattergood 1954). Although lesions can be observed on the surface of tissues, the parasite and lesions permeate throughout the internal architecture of the affected organs and into the skeletal muscle (Kocan, Gregg & Hershberger 2010). Externally, signs of ichthyophoniasis are classically defined as 'sandpaper skin' in Atlantic herring, *Clupea harengus* L., and Pacific herring, *C. pallasii* Valenciennes. The flank of affected fish often presents with roughened skin surface, raised bumps under the skin and/or open-pigmented ulcers on the skin that resemble flakes of pepper (Sindermann & Scattergood 1954; Kocan *et al.* 2010). In Pacific herring, these external signs are most frequently observed in juvenile (age 0 year) cohorts (personal observation).

Although *Ichthyophonus* infections can progress to disease and population-level epizootics (Burge *et al.* 2014), the parasite can also lead to chronic infections in affected hosts (McVicar 1999). *Ichthyophonus* surveillance efforts in Pacific herring throughout the north-east Pacific Ocean indicate that infection prevalence in adult herring populations typically ranges between 15% and 50%; however, most are low-intensity infections with no visible internal or external signs of the disease (Hershberger *et al.* 2002). In such cases, the parasite is detectable in the tissues only after laboratory diagnostic evaluations using tissue explant culture, polymerase chain reaction or histology with selective staining.

Stock assessment models are important fishery management tools to help guide sustainable harvesting of fish populations, and disease information has been used to improve the capabilities of assessment models for predicting natural mortality in Pacific herring stocks in Prince William Sound, Alaska, USA (Marty *et al.* 2003, 2010). Marty *et al.* (2010) reported that semi-quantitative analysis of prevalence and severity of two grossly visible lesions detected in herring during annual sampling – skin ulcers and white foci in the heart – was useful for explaining and predicting herring population changes that had confounded traditional fisheries assessments. This model suggested that *Ichthyophonus* and viral haemorrhagic septicaemia (VHS) have been significant contributors to mortality of Prince William Sound herring.

A better understanding of the pathogenesis of ichthyophoniasis in Pacific herring could improve estimates of the contribution of *Ichthyophonus* to natural mortality in affected stocks. For example, although *Ichthyophonus*-infected herring present a variety of external signs, it is unknown whether and how the presence and type(s) of external lesions relate to the duration and prognosis of infection. This study investigated the relation between external *Ichthyophonus* lesions, mortality, morphological stages of the parasite and host responses in Pacific herring up to 429 days after the beginning of experimental infection. Specific objectives of the research were to (i) describe the appearance and persistence of ichthyophoniasis external signs in Pacific herring, (ii) determine whether the occurrence of external signs represented a precursor to host mortality, with potential for use as a forecasting tool for informing the natural mortality component of herring population models, (iii) document the morphological stages of *Ichthyophonus* present in superficial (integumental) tissues and underlying skeletal muscle during the course of experimental infection and (iv) describe the progression of histopathological responses in host skin and skeletal muscle tissues associated with the *Ichthyophonus* infections and external lesions.

Materials and methods

Phase one

To examine the appearance and persistence of external signs at early time points post-exposure, 9-month-old specific-pathogen-free (SPF) Pacific herring (Hershberger *et al.* 2010) were distributed into triplicate tanks (97–120 fish per tank) for each of two groups (treatment and control). Herring in the treatment groups were exposed by intraperitoneal injection with *Ichthyophonus* life stages (120 schizonts per 50 μL dose) suspended in phosphate-buffered saline (PBS; pH 7.4; 0.01 M); the injection inoculum was produced using isolated *Ichthyophonus* originating from cultured Pacific herring tissues. Negative control fish were injected with equivalent aliquots of PBS. Sand-filtered seawater temperatures averaged 7.9 °C throughout the post-exposure period.

At 14 days post-exposure (PE), one tank per group (treatment and control) was designated for *Ichthyophonus* external sign examination. Each fish was gently removed, inspected and identified as *Ichthyophonus* positive when raised papules, ulcers or erosions were present. Examinations were

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repeated every 7 days through 105 days PE and every 14 days thereafter, through the end of the study (147 days PE) [$n = 115$ (treatment); $n = 112$ (control)]. The prevalence of positive fish was recorded on each examination day to determine the progression of visible infection indicators. Proportions of fish with external lesions were calculated on each examination day as:

$$\%\ \text{fish with external signs} = \frac{\text{no. examined fish with external signs} + \text{cumulative no. mortalities with external signs}}{\text{no. fish in examination tank at start of study}}$$

The remaining two tanks in each treatment group were designated as subsample and mortality tracking tanks, respectively. Two herring were killed from each subsample tank on each examination day to photographically document the progression of external signs. In addition, a transverse caudal section was collected above the anal fin and fixed in 10% neutral buffered formalin for histological examination of host response and parasite infection intensity and morphology. Mortality was observed throughout the study, and reported values were corrected for live herring subsample removal where appropriate. The infection status of all sampled herring (mortalities and subsamples) was determined by explant culture of heart tissue in Tris-buffered Eagle's minimum essential medium, supplemented with 5% (v/v) foetal bovine serum (MEM-5), 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 100 μg mL^{-1} gentamycin.

Phase two

To examine the persistence of external signs at extended time periods, 10-month-old SPF Pacific herring were divided into treatment (*Ichthyophonus*-exposed) and negative control (PBS-exposed) groups. Herring were exposed to *Ichthyophonus* (90 schizonts per 50 μL dose; treatment) or PBS (50 μL dose; control) by intraperitoneal injection, and mortality was tracked through 182 days PE (8.7 °C). Next, 84 *Ichthyophonus* survivors presenting with external signs were sorted evenly between two tanks supplied with flow through sea water (8.6 °C). Fish from one tank were examined for external signs at intervals between 28 and 60 days through 429 days PE while the second tank served as a source of subsamples, as described for phase one. Infection status of all mortalities and subsamples was confirmed by heart explant culture in MEM-5. Negative controls were maintained in an individual tank until experiment termination and were subsampled in parallel with the treatment group.

Histopathology. Subsets of formalin-fixed caudal sections were examined for parasite morphology and host response. Phase one negative controls and *Ichthyophonus*-exposed subsamples from 14, 21, 42, 63 and 133 days PE were examined ($n = 2$ fish per day). In phase two, 11 herring sampled 429 days PE representing a range of external signs were also examined. Fixed tissue samples were processed and embedded in paraffin wax according to standard protocols and sectioned at 5 μm. Paired slides were stained with Gill's haematoxylin and eosin (H&E) for host response evaluation or periodic acid-Schiff (PAS) (Carson 1997) for assessment of parasite load. Slides were examined with a Zeiss Axiophot light microscope (Carl Zeiss, Inc.).

Ichthyophonus density was determined by recording the number of parasites in at least 10 microscope fields at 100× magnification within the skin (epidermis, dermis and hypodermis), skeletal musculature (red and white skeletal muscle) and the connective tissue septa associated with the skeletal musculature (dorsal, ventral and lateral septa, and myosepta between myomeres). Parasites were identified by morphological stage according to the criteria of Kocan (2013). The abundance of each morphological stage within a tissue type was reported as the number of parasites per 10 microscope fields.

To quantify host response, at least 10 microscope fields for each tissue type were examined at 200× magnification. The distribution of inflammation, fibrosis and necrosis within each tissue was scored as 0 (no host response), 1 (present in <25% of fields examined), 2 (present in 25–50% of fields) or 3 (present in >50% of fields examined). The degree of the host response within the affected tissue was scored as 0 (no response), 1 (mild response), 2 (moderate response) or 3 (severe response). Scores for distribution and degree were added together to obtain an overall host response severity measure for each tissue type (maximum total host response score = 6).

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Results

Mortality

Phase one. Herring mortality began between 4 days (examination and mortality tracking tanks) and 7 days PE (subsample tank). Cumulative percentage mortality among *Ichthyophonus*-exposed fish began to subside or plateaued between 70 and 90 days PE. Cumulative mortality reached 43.3% (examination, Fig. 1), 48.2% (subsample) and 70.8% (mortality tracking) while mortality in control tanks reached 8.8% (examination), 26.8% (subsample) and 4.8% (mortality tracking). Prevalence of *Ichthyophonus* by culture in groups exposed to the parasite was 97.3% (181/186) among all mortalities. *Ichthyophonus* was not isolated from any control fish.

Phase two. Cumulative percentage mortality reached 24.3% (treatment) and 6.5% (control) prior to preselecting fish for external sign examinations. Among preselected herring, cumulative percentage mortality reached 14.2% (subsample tank) and 19.1% (examination tank). Controls were maintained in an individual tank through 429 days PE where subsample-adjusted mortality reached 10.5%. All tissue explant cultures collected from *Ichthyophonus*-challenged fish were positive (83/83) for the parasite. All control subsamples cultured negative (12/12).

External sign prevalence

Phase one. In the examination tank, external lesions were first identified 14 days PE in 0.9% (1/108) of live herring, 10 days after the onset of mortality (Fig. 1).

Among early mortalities (those occurring <14 days PE) in all *Ichthyophonus* treatment groups combined (examination, subsample and mortality tanks), only 2.2% (1/46; 9 days PE) showed external lesions, but 95.7% cultured positive. Among *Ichthyophonus*-exposed fish in the examination tank, the prevalence of external signs increased steadily to 78% at 35 days PE; prevalence then plateaued at 73.2–78.7% through 147 days PE (Fig. 1). None of the Pacific herring in the control group demonstrated external signs during any examination.

Initial external lesions manifested as sparse small black specks. More unequivocal lesions were noted by 21 days PE and appeared as small black papules. By 28 days PE, individual lesions appeared larger and abundant small black papules and/or non-pigmented papules (sandpaper skin) were present, which generally persisted through 119 days PE. Additional signs were also noted between 28 and 119 days PE and included ulcers (some of which appeared to be bleeding) and suspected healing lesions (Fig. 2). In some fish, white tufts emanated from black papules or ulcers. Disparate external sign characteristics persisted through 147 days PE. No consistent progression of external lesions was observed during this experiment.

Phase two. Between 182 days PE and 429 days PE, external signs appeared similar to those documented in phase one. External sign prevalence

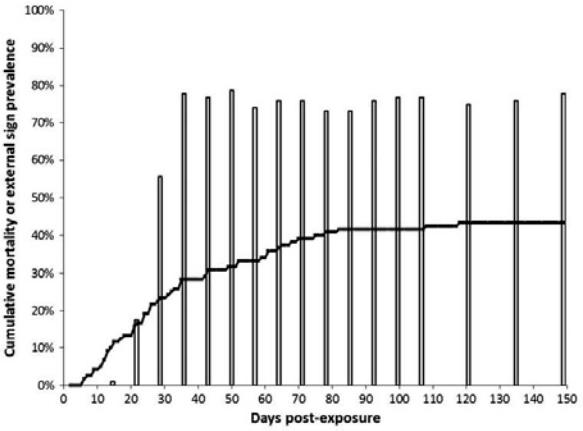

Figure 1 Cumulative mortality (line) and prevalence of external signs (bars) in Pacific herring visually examined at 1- to 2-week intervals following intraperitoneal injection with *Ichthyophonus* (phase one experiment). Positive culture prevalence among all mortalities was 94% (49/52).

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Figure 2 Representative external signs observed in Pacific herring after challenge with *Ichthyophonus* by intraperitoneal injection. (a) No external signs. (b) Large bleeding ulcers (arrow) and blood-infused white tuft (arrowhead). (c) Suspected scarring (arrow) from healing lesions. (d) Skin erosions (arrow). (e) Sandpaper skin (arrow) with pigmented papules along the complete flank.

remained at 100% (42/42) between 181 and 244 days PE. On 244 days PE, prevalence of external signs decreased to 95.2% (40/42) and fluctuated between 92.9% (39/42) and 97.6% (41/42) through 401 days PE. As examinations continued, external lesions became increasingly difficult to identify on a subset of fish, which resulted in minor count fluctuations. By 429 days PE, 90.5% (38/42) of Pacific herring presented with external signs that ranged widely in appearance and included small black papules, scars, white tufts and bleeding ulcers (Fig. 2).

Histopathology

Phase one. All Pacific herring subsampled from the treatment group between 14 and 133 days PE cultured positive for the parasite ($N = 10$); however, external lesions were not observed until 21 days PE (one of two fish positive). Both fish in each subsequent histopathology sample from this tank (42–133 days PE) showed grossly visible external ichthyophoniasis lesions (data not shown). By histopathology, *Ichthyophonus* infections were detected in one of two herring sampled on 21 and 42 days PE and in both fish sampled on 63 and 133 days PE (Fig. 4). Predominant parasite stages included plasmodia and schizonts (Figs 3 & 4), which were observed in all histologically positive herring, with plasmodia present at the highest densities (Fig. 4). The multinucleate spherical schizonts were characterized by a surrounding non-cellular multilaminate membrane; the multinucleate spherical plasmodia lacked this membrane. Degenerating schizonts, which retained a multilaminate membrane and stained PAS positive but lacked definitive internal structure (Fig. 3), were infrequently observed at 42 and 63 days PE (Fig. 4). Parasites were not observed by culture or histopathology in any negative samples.

Among the tissue types examined, only red skeletal muscle was consistently infected on 21 through 133 days PE, with highest parasite densities occurring at 21 and 133 days (Fig. 4). Infection of the connective tissue septa associated with the musculature was observed at 63 and 133 days PE, with highest parasite densities recorded for day 63 (Fig. 4). Parasites were also observed at low densities in the white skeletal muscle of one fish each on 21 and 133 days PE and in the hypodermis of the skin of two fish sampled at 133 days PE (data not shown); all parasite stages observed in these tissues consisted of plasmodia (≤2 parasites per 10 microscope fields) or schizonts (≤1 parasite per 10 microscope fields).

Host response was first evident in most tissues at 21 days PE, when parasites were first observed (Fig. 5). Granulomatous inflammation (Fig. 3) was the most prevalent host response with the highest recorded severity scores (Fig. 5). Fibrosis (Fig. 3) was often observed along with inflammation, especially at 42 days PE onwards (Fig. 5), while necrosis (Fig. 3) was only detected at 63 and 133 days PE (Fig. 5).

Host responses appeared more extensive than parasite distribution and were sometimes observed in samples and tissues from *Ichthyophonus* treated fish in which no parasites were detected. For example, at 21 and 42 days PE, inflammation was identified in red skeletal muscle and connective tissue septa of both sampled fish despite the detection of parasites in only one herring (Fig. 5). Host

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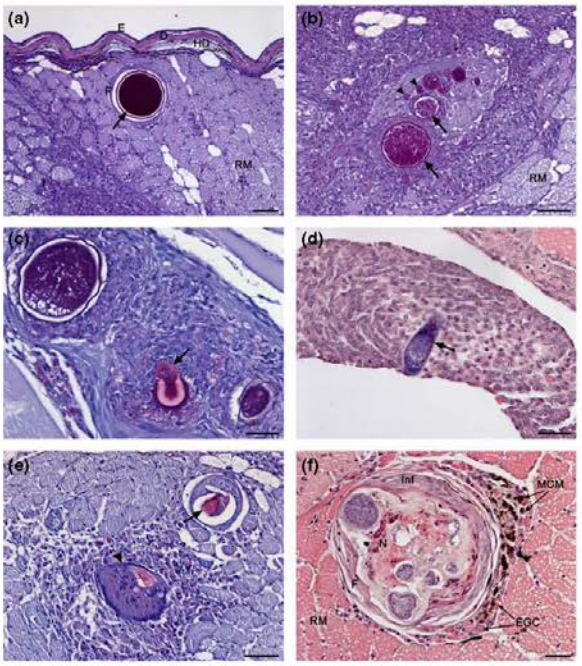

Figure 3 Representative parasite morphological stages and host responses in histologically processed caudal sections from Pacific herring with *Ichthyophonus*. Photomicrographs of (a) and (b) were taken from fish sampled 63 days post-exposure (phase 1). Photomicrographs (c) through (f) were taken from fish sampled 429 days post-exposure (phase 2). (a) Schizont in red skeletal muscle (RM) surrounded by fibrosis. Also shown: epidermis (E), dermis (D), hypodermis (HD); PAS stain, scale bar = 50 μm. (b) Plasmodia (arrowheads) and schizonts (arrows) surrounded by granulomatous inflammation in red skeletal muscle (RM); PAS stain, scale bar = 50 μm. (c) Plasmodium (arrow) exiting a degenerated schizont in white skeletal muscle. Two schizonts are also present; PAS stain, scale bar = 25 μm. (d) Plasmodium with amoeboid morphology (arrow) surrounded by epithelioid macrophages in red skeletal muscle; H&E stain, scale bar = 25 μm. (e) Degenerating schizont (arrow) and multinucleate giant cell (arrowhead) surrounding an apparent degenerating schizont in red skeletal muscle; PAS stain, scale bar = 50 μm. (f) Cluster of schizonts associated with granulomatous inflammation (Inf), necrosis (N) and fibrosis (F). Melanin-containing macrophages (MCM) and eosinophilic granular cells (EGC) are also present; H&E stain, scale bar = 25 μm.

responses also tended to progress to integumental tissues (i.e. hypodermis, dermis and epidermis) in advance of parasite observations. For instance, inflammation and fibrosis were seen in the hypodermis of the skin 42 days PE while *Ichthyophonus* was only observed in muscle tissues at that time point and was not detected in the hypodermis until 133 days PE. Furthermore, parasites were never observed in the dermis or epidermis, whereas inflammation and fibrosis occurred in these tissues of one fish at low severity (≤2) at 63 days PE (data not shown).

Granulomatous inflammation was characterized by the presence of typical epithelioid (activated) macrophages with pale eosinophilic cytoplasm. Eosinophilic granular cells were observed in 89% of inflammatory foci among fish examined from 21 to 133 days PE. Host responses were absent in all negative controls.

Phase two. Ichthyophonus stages (plasmodia, schizonts and degenerating schizonts) were observed at low infection intensity in histological sections of two fish that recovered from external signs by 429 days PE (Fig. 6). Parasites were observed in both red and white skeletal muscle and associated connective tissue septa, but were only detected in the hypodermis of the skin. In

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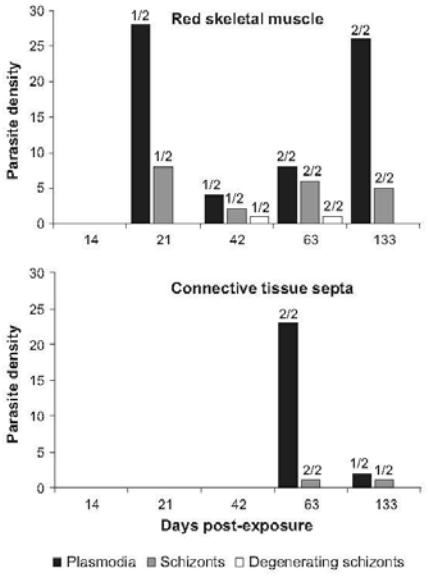

Figure 4 *Ichthyophonus* infection intensity in red skeletal muscle and the connective tissue septa associated with the skeletal musculature (dorsal, ventral and lateral septa, and myosepta between myomeres) of Pacific herring sampled at various times following an intraperitoneal injection challenge (phase one experiment). Density of a given parasite morphological stage was calculated as the mean number of parasites per 10 microscope fields in PAS-stained histological sections examined at 100× magnification. Prevalence of a parasite morphological stage at each time point (number of positive fish divided by the number of fish examined) is shown above each bar. *Ichthyophonus* prevalence and intensity in white skeletal muscle and skin hypodermis were low and are not shown (see text for results), and no parasites were observed in the dermis and epidermis.

cohorts that continued to demonstrate external signs, plasmodia, schizonts and degenerating schizonts occurred at the highest prevalence and density (Fig. 6). Plasmodia were observed exiting degenerating schizonts, and non-spherical amoeboid plasmodia were also seen in the absence of degenerating schizonts (Fig. 3); both were observed at low prevalence and intensity (Fig. 6). *Ichthyophonus* was consistently detected at the highest prevalence and intensity within red skeletal muscle. Other highly affected tissues included white skeletal muscle, connective tissue septa and the skin hypodermis. Although plasmodia and schizonts were also noted in the dermis of the skin, they occurred at low prevalence and density, and no parasites were observed in the epidermis.

Host responses of the two fish without external signs showed inflammation, fibrosis and necrosis in red and white skeletal muscle and associated connective tissue septa, but only in the hypodermis of the skin (Fig. 7). The host responses in affected tissues were generally mild, with a slightly higher mean inflammation severity score observed in the connective tissue septa than in the other affected tissues. Among the fish showing external signs of ichthyophoniasis, inflammation was observed in all tissue types at high prevalence and varying severity (Fig. 7). Fibrosis and necrosis were detected in all tissues except the epidermis, but generally occurred at lower severity than inflammation. Red skeletal muscle of all nine herring with grossly visible signs of infection showed inflammation, fibrosis and necrosis as well as the highest severity of fibrosis and necrosis, while the highest inflammation severity was observed in the connective tissue septa. Inflammation was the only host response to occur in epidermal tissue (Fig. 7).

In addition to typical epithelioid macrophages present in all inflammatory lesions, eosinophilic granular cells were present in 96% of granulomatous inflammatory foci. Melanin-containing macrophages (Fig. 3) were observed in inflammatory foci in red skeletal muscle (nine of 11 fish), connective tissue septa (six of 11 fish) and hypodermis (five of 11 fish). Multinucleate giant cells (Fig. 3) were seen in the red skeletal muscle (three of 11 fish) as well as the white skeletal muscle, connective tissue septa, dermis and hypodermis (one of 11 fish). In the red skeletal muscle, about 50% of multinucleate giant cells surrounded PAS positive, apparently degenerating *Ichthyophonus* parasites (Fig. 3). However, no parasites were associated with giant cells in other tissue types. Regenerating red skeletal muscle, characterized by small-diameter, basophilic-staining fibres and enlarged nuclei, was observed in one infected fish (photograph not shown).

Discussion

Overt signs of ichthyophoniasis were not a precursor to disease-related mortality in experimentally infected herring in this study. Mortality began at least 7 days prior to the onset of external

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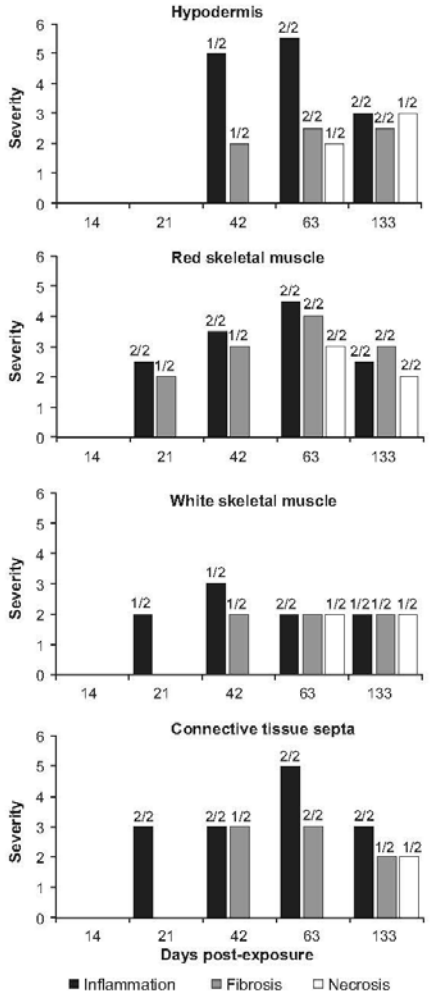

Figure 5 Prevalence and severity of host responses (inflammation, fibrosis and necrosis) in skin hypodermis, skeletal muscle and connective tissue septa associated with the skeletal musculature (dorsal, ventral and lateral septa, and myosepta between myomeres) of Pacific herring sampled for histopathological analysis at various times following an intraperitoneal injection challenge with *Ichthyophonus* (phase one experiment). Host responses in H&E-stained histological sections were scored using an additive scale that included distribution and degree of each response (maximum severity score = 6). Prevalence of a host response at each time point (number of positive fish divided by the number of fish examined) is shown above each bar. Inflammation and fibrosis were observed at low severity in the epidermis and dermis of a single fish at 63 days post-exposure and are not shown (see text for results).

indicators in examined herring. Furthermore, among all mortalities of *Ichthyophonus*-positive fish occurring within 14 days PE, only one individual was identified as having external lesions. A lack of correlation between the two metrics was further indicated by mortality that continued for 5 weeks beyond the plateau of lesion prevalence. Whether external sign progression and mortality correspond in Pacific herring under natural conditions will remain subject to debate until natural exposure routes are identified. Current hypotheses of *Ichthyophonus* pathogenesis are predicated on the assumption that *Ichthyophonus* is consumed orally leading to an intestinal-dependent route of infection (Spanggaard *et al.* 1994; Spanggaard, Huss & Bresciani 1995). If these hypotheses are accurate, rapid detection of the parasite in vital internal organs of orally exposed rainbow trout *Oncorhynchus mykiss* (Walbaum) (McVicar & McLay 1985) suggests that the onset of death would still precede external clinical indicators of disease under natural exposure scenarios.

Although asynchronous among exposed individuals, external signs generally progressed to papules (sandpaper skin), black nodules and ulcerative lesions, similar to those previously described in naturally or experimentally infected herring (Sindermann & Scattergood 1954; Kocan *et al.* 2010), within 3–4 weeks PE. Visual appearance of external signs remained varied throughout the examination period. It is conceivable that the asynchronous occurrence of external signs was associated with the infection method used in the present study. It is unknown whether intraperitoneal injection might have resulted in different route(s) of parasite dissemination than would occur in natural infections, although the majority of experimentally exposed fish in this study had cardiac infections, similar to findings in naturally infected herring (Rahimian & Thulin 1996; Rahimian 1998; Hershberger *et al.* 2002). Accurate enumeration of *Ichthyophonus* life stages and identification of infective stages within a parasite preparation are also notable challenges (Kocan *et al.* 2010) that could contribute to non-uniform distribution of infectious stages among test fish.

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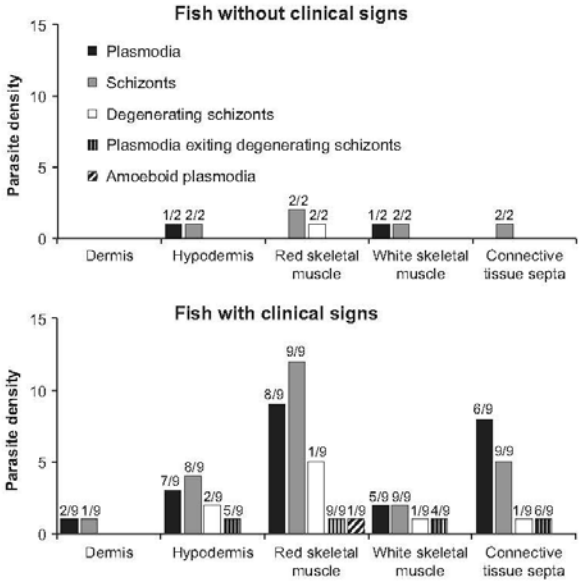

Figure 6 *Ichthyophonus* prevalence and infection intensity in skin and muscle tissues of Pacific herring sampled 429 days after an intraperitoneal injection challenge (phase two experiment). Separate graphs are included for fish without and with grossly visible external signs of ichthyophoniasis. Density of a given parasite morphological stage was calculated as the mean number of parasites per 10 microscope fields in PAS-stained histological sections examined at 100× magnification. Prevalence of a parasite morphological stage in a given tissue (number of positive fish divided by the number of fish examined) is shown above each bar. No parasites were detected in the epidermis of any fish (data not shown).

Among the variety of external indicators observed, white tufts appeared to protrude from lesions along the flanks of a small proportion of infected fish. Histopathological examination of one fish with this lesion type revealed a large aggregation of inflammatory cells protruding from an ulceration that extended into the red skeletal muscle. No *Ichthyophonus* was observed within the protruding inflammatory cell aggregate. The possible contribution to the 'tufted' appearance by mucus, secreted by cells in intact epidermis adjacent to the lesion, would have required preservation of the tissues in a fixative that preserves external mucus as well as tissue morphology (Powell, Speare & Burka 1992).

External lesions persisted in slightly more than 90% of Pacific herring for 14 months. The long-term persistence of clinical signs may be useful at ascertaining ichthyophoniasis-related mortality in regularly observed populations. For example, a large and rapid decrease in lesion prevalence within a population would likely indicate loss of affected individuals rather than recovery from external signs. In such models, age structure may need to be incorporated along with potential recovery rates. Although healing occurred in about 10% of test fish, it is possible that the age of examined herring (~2 years at study termination) marked a transition point for further recovery. Circumstantial observations suggest that external signs are rarely observed in older cohorts of wild Pacific herring, but higher *Ichthyophonus* prevalence is well documented in older fish (Kocan *et al.* 1999; Hershberger *et al.* 2002; Marty *et al.* 2003, 2010). Tracking of external signs in Pacific herring beyond 2 years of age is necessary to better inform models on the relationship between age structure and recovery from lesions. The use of external indicators to hindcast exposure history (i.e. time of initial parasite exposure), however, is not recommended because complete recovery in any proportion of individuals would bias back-projections. Further, asynchrony in the kinetics of lesion progression makes it impossible to back-calculate the date of exposure using this metric.

Histological analysis of skin and muscle tissues suggested a long-term affinity of *Ichthyophonus* for red skeletal muscle. High densities of the parasite have been noted previously in red skeletal muscle of several species (Sindermann & Scattergood 1954; Sitja-Bobadilla & Alvarez-Pellitero 1990; Marty *et al.* 1998; Rahimian 1998; Kocan *et al.* 2010). This is likely associated with high blood supply, which is hypothesized to be a major

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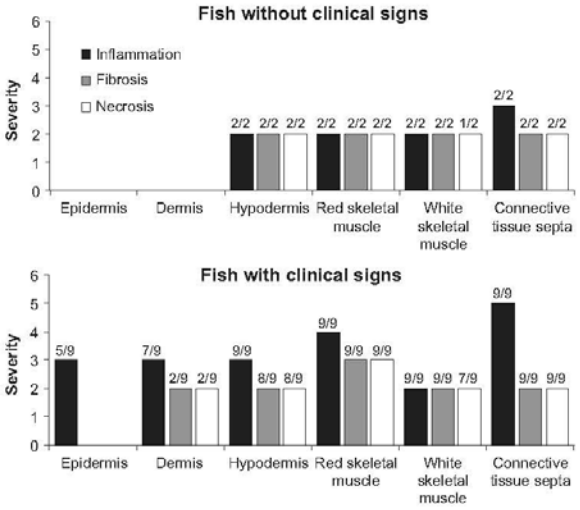

Figure 7 Prevalence and severity of host responses (inflammation, fibrosis and necrosis) in skin, skeletal muscle and muscle-associated connective tissue of Pacific herring sampled 429 days after an intraperitoneal injection challenge with *Ichthyophonus* (phase two experiment). Separate graphs are included for fish without and with grossly visible external signs of ichthyophoniasis. Host responses observed in H&E-stained histological sections were scored using an additive scale that included distribution and degree of each response (maximum severity score = 6). Prevalence of a host response in each tissue (number of positive fish divided by the number of fish examined) is shown above each bar.

mechanism of parasite transport within the fish (Sindermann & Scattergood 1954; McVicar & McLay 1985; Sitja-Bobadilla & Alvarez-Pellitero 1990; Spanggaard *et al.* 1995; Kocan *et al.* 2010; Kocan, LaPatra & Hershberger 2013). Once present within muscle tissue, *Ichthyophonus* can migrate to and through the skin where it is presumed to be released into water (Kocan *et al.* 2010). Interestingly, the parasite was only detected in skin tissue (dermis) in a small number of fish at 429 days PE in the present study. However, the epidermis and dermis were often missing from lesioned areas of the skin, and parasites may have been sloughed along with these tissues. Serial sections of skin and muscle tissue from fish sampled at early and late time points PE might be useful to better understand when *Ichthyophonus* begins to migrate through dermal and epidermal tissue and when or whether it ceases to be released into the surrounding environment.

Spherical schizont and plasmodium morphologies were the most common parasite forms identified in histological samples regardless of time PE. Schizonts (often called 'resting spores' or 'spores') are the morphological stage most frequently reported from infected herring (Sindermann & Scattergood 1954; Marty *et al.* 1998; Rahimian 1998). Consistent presence from 21 to 429 days PE lends further evidence for the persistence of schizonts and supports their importance for long-term parasite survival in a living host. The importance of the apparently motile forms of *Ichthyophonus* observed (e.g. spherical and amoeboid plasmodia, and plasmodia exiting from degenerating schizonts) is not well understood. One hypothesis suggests that these morphologies are important for parasite dispersal within the host (Spanggaard *et al.* 1995), and the presence of spherical plasmodia from 21 to 429 days PE suggests that dispersal of parasites within the host tissues examined was an ongoing process throughout the study.

Overall, a complex host response consisting of granulomatous inflammation, fibrosis and late-onset necrosis was present in *Ichthyophonus*-infected fish. Inflammation was the first notable host response to occur and was initially documented 21 days PE at mild to moderate levels in three tissues. By 429 days PE, inflammation was recorded in all six examined tissues in a high proportion of herring. Similar ichthyophoniasis-related chronic inflammation and fibrosis, with variable reports of necrosis, have been previously documented in several fish species (Sitja-Bobadilla & Alvarez-Pellitero 1990; Rahimian 1998). Eosinophilic granular cells, which were present in inflammatory lesions from 21 days PE onwards, show close functional similarity to mammalian mast cells, and recruitment of these cells is a common feature of persistent inflammatory reactions in teleosts (Reite & Evensen 2006). Two other inflammatory cell types, melanin-containing

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macrophages and multinucleate giant cells, which also have been reported in naturally infected fish (McVicar & McLay 1985; Rahimian 1998), were only observed at 429 days PE and may be indicators of long-standing *Ichthyophonus* infections in herring. Aggregates of melanin-containing macrophages can develop in association with chronic inflammatory lesions in various parts of a fish's body and may be involved in antigen trapping and presentation to lymphocytes, as well as other functions such as sequestration of products of cellular degradation or potentially toxic materials (Agius & Roberts 2003). Multinucleate giant cells are common in fungal and parasitic infections and foreign-body-type reactions in fish (Ferguson 1989) and likely arise from the fusion of individual macrophages (Secombes 1985). Fibrosis was present 21 days through 429 days PE, and encapsulation of spores in fibrotic tissue is hypothesized to be a primary mechanism of slowing or stopping the spread of infection (McVicar & McLay 1985). Schizont encapsulation in somatic tissues in late phases of infection may also inhibit viable organisms from advancing through the skin and into surrounding environments.

In summary, this study demonstrated the persistence of external clinical signs for more than 1 year in the majority of Pacific herring experimentally infected with *Ichthyophonus*, although the occurrence of external signs was not predictive of mortality, and the asynchrony of the signs did not allow their use for estimating the duration of an infection. The persistence of external signs was accompanied by the continued presence of parasites (principally schizonts and plasmodia with highest densities usually in red skeletal muscle) and associated host response (granulomatous inflammation, fibrosis and necrosis). Many features of the experimental infections were similar to those described from naturally infected fish, although it is unknown how differences in fish stocks or environmental variables such as temperature might influence results. Finally, our understanding of the pathogenesis of ichthyophoniasis in herring could be significantly improved by more complete knowledge of the natural route(s) of infection for planktivorous fishes.

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Appendix 8

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ARTICLE

Optimization of a Plaque Neutralization Test (PNT) to Identify the Exposure History of Pacific Herring to Viral Hemorrhagic Septicemia Virus (VHSV)

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Abstract

Methods for a plaque neutralization test (PNT) were optimized for the detection and quantification of viral hemorrhagic septicemia virus (VHSV) neutralizing activity in the plasma of Pacific Herring *Clupea pallasii*. The PNT was complement dependent, as neutralizing activity was attenuated by heat inactivation; further, neutralizing activity was mostly restored by the addition of exogenous complement from specific-pathogen-free Pacific Herring. Optimal methods included the overnight incubation of VHSV aliquots in serial dilutions (starting at 1:16) of whole test plasma containing endogenous complement. The resulting viral titers were then enumerated using a viral plaque assay in 96-well microplates. Serum neutralizing activity was virus-specific as plasma from viral hemorrhagic septicemia (VHS) survivors demonstrated only negligible reactivity to infectious hematopoietic necrosis virus, a closely related rhabdovirus. Among Pacific Herring that survived VHSV exposure, neutralizing activity was detected in the plasma as early as 37 d postexposure and peaked at approximately 64 d postexposure. The onset of neutralizing activity was slightly delayed in fish reared at 7.4°C relative to those in warmer temperatures (9.9°C and 13.1°C); however, neutralizing activity persisted for at least 345 d postexposure in all temperature treatments. It is anticipated that this novel ability to assess VHSV neutralizing activity in Pacific Herring will enable retrospective comparisons between prior VHS infections and year-class recruitment failures. Additionally, the optimized PNT could be employed as a forecasting tool capable of identifying the potential for future VHS epizootics in wild Pacific Herring populations.

Viral hemorrhagic septicemia virus (VHSV) was once thought to be exclusively associated with the European Rainbow Trout *Oncorhynchus mykiss* industry; however, the virus is now known to impact fishes throughout temperate regions of the northern hemisphere, where different genogroups and subtypes occur in distinct ecoregions (reviewed in Hershberger et al. 2016). Genogroup IVa (hereafter referred to as VHSV, unless specified) occurs in wild and cultured marine fishes throughout coastal areas of the North Pacific Ocean, where it periodically causes disease (viral hemorrhagic septicemia; VHS) and associated fish kills (reviewed in Garver et al. 2013). Throughout their range, wild Pacific Herring

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Clupea pallasii are highly susceptible to the disease and associated epizootics (Hershberger et al. 2016). The population-level impacts of these recurring VHS epizootics are difficult to assess; nevertheless, a leading hypothesis accounting for the 1989–1993 decline and failed recovery of Pacific Herring populations in Prince William Sound, Alaska (Hulson et al. 2008), involves mass mortalities from VHS epizootics (Marty et al. 1998, 2003, 2010).

Forecasts for the annual spawning Pacific Herring biomass in Prince William Sound are based on outputs from an age-structured assessment model (Quinn and Deriso 1999). These model forecasts are used by resource managers to assess whether the spawning population can support annual harvest from several fisheries. Since its inception in 1988, the age-structured assessment model has undergone numerous adjustments (Muradian 2015), including incorporation of a disease-specific mortality index (Marty et al. 2010), that have more effectively aligned model output estimates with observed prespawn biomass estimates (Thomas and Thorne 2003). Total annual mortality in the age-structured assessment model is calculated as the summation of "natural mortality" and disease-specific mortality. The latter is based on a disease index intended to capture annual mortality from both VHS and ichthyophoniasis, another disease of Pacific Herring (Marty et al. 2003). The disease index is calculated as the relative frequency of VHSV-positive fish multiplied by the relative frequency of fish demonstrating ichthyophoniasis-related cutaneous and subcutaneous ulcers. Data used to populate the disease index and forecast abundance are based on fish health examinations of prespawn aggregations of Pacific Herring from March to April of the previous year.

The inclusion of infection and disease data as annual mortality indicators represents a major advancement in our ability to forecast the recruitment and abundance of marine fish populations; however, the quantification of disease impacts must be compatible with ecological principles that govern each host–pathogen system. Unfortunately in the case of VHSV and Pacific Herring, there is no indication that infection prevalence data provide a reliable forecaster of annual host mortality, especially when the prevalence data are represented by geographically limited surveillances from a temporally narrow window. For example, during enzootic periods, VHSV is perpetuated in populations of Pacific Herring and other marine fishes at an extremely low infection prevalence that often falls below the reasonable detection threshold of typical virus surveillance techniques (Hershberger et al. 2016). However, epizootics occur very rapidly in these populations as a result of increased susceptibility and/or elevated infection pressures from changing host and environmental conditions. Therefore, annual fish health assessments that occur during typical enzootic periods are not capable of forecasting the punctuated VHS epizootics that can rapidly occur weeks or months after the assessments are finished.

Rather than relying on VHSV prevalence data, a more biologically relevant approach to forecasting the potential for future VHS epizootics might involve the quantitative assessment of host exposure history. For example, naive Pacific Herring are highly susceptible to VHS (Kocan et al. 1997); however, individuals that survive exposure to the virus develop a long-lived adaptive immunity that confers future resistance to the disease (Hershberger et al. 2010). Therefore, development of a diagnostic technique that is capable of assessing the prior exposure history of Pacific Herring to VHSV could provide a quantitative assessment of herd immunity, thereby indicating the potential for future VHS epizootics within a population. This study was intended to develop and optimize a serological tool capable of assessing the prior exposure history of Pacific Herring to VHSV. Specifically, we attempted to optimize a plaque neutralization assay capable of detecting VHSV neutralizing activity in the plasma of Pacific Herring.

METHODS

Groups of Pacific Herring with different viral exposure histories (exposed and unexposed) and rearing temperature regimes were developed as sources of plasma for the optimization of a plaque neutralization test (PNT). All groups consisted of age-1 and age-2 specific-pathogen-free (SPF; Hershberger et al. 2010) Pacific Herring (N = 204–264 fish/group) that were maintained in flow-through 760-L tanks (one tank per group). Exposed groups survived prior laboratory-induced VHS epizootics that resulted from static immersion in 1.0–1.2 × 10^3 PFU/mL of a VHSV isolate (#99–292 from Atlantic Salmon *Salmo salar*). The VHSV exposures and subsequent fish rearing occurred at cool (mean = 7.4°C; range = 6.6–10.1°C), ambient (mean = 9.9°C; range = 7.4–12.9°C), and warm (mean = 13.1°C; range = 9.4–14.7°C) temperatures. Typical temperature-influenced VHS mortality ensued (Hershberger et al. 2013), with cumulative mortalities reaching 72.3% (7.4°C), 79.3% (9.9°C), and 40.0% (13.1°C) by 345 d postexposure. High viral titers (mean > 1×10^7 PFU/g) were recovered from kidney–spleen–brain pools of subsampled mortalities through 47 d at 7.4°C (43/71 positive), 39 d at 9.9°C (51/70 positive) and 39 d at 13.1°C (5/14 positive). A negative control group consisted of analogous SPF Pacific Herring, maintained in ambient seawater temperatures, that were exposed to phosphate buffered saline in lieu of VHSV; cumulative mortality was 26.5% and the virus was not isolated from any subsampled mortalities (n = 12). Survivors were subsampled from the treatment and negative control groups at 0, 37, 64, 113, 182 and 345 d postexposure (n = 30/time point), and the remaining survivors in the warm treatment group were again sampled 385 d postexposure. During subsampling, fish were euthanized in excess (>250 mg/L) buffered tricaine methanesulfonate (MS-222), and blood was collected by caudal sever and exsanguination through the caudal artery into heparinized capillary tubes. Whole blood

was centrifuged at 12,700 relative centrifugal force (Thermo Micro MB); plasma was stored at –80°C until processed.

Optimization of the PNT.—A modified PNT (LaPatra et al. 1993; Wilson et al. 2014) was optimized to quantify VHSV neutralizing activity in the plasma of Pacific Herring. Optimized methods were intended to maximize assay sensitivity and minimize false positives. The minimum plasma dilution was determined by comparing neutralizing titers in a subset of samples using twofold dilutions of whole plasma from 1:2 to 1:256 (n = 91 treatment fish and 56 negative controls). For determination of neutralizing activity, aliquots of test plasma were diluted using tris buffered Eagle's Minimum Essential Medium (MEM) supplemented with 5% fetal bovine serum; 14 µL of VHSV in MEM (approximately 2×10^3 PFU/mL) was added to an equivalent volume of each serial plasma dilution. Virus plus plasma dilutions were then incubated at 15°C overnight and plated in duplicate onto 96-well plates (10 µL/well) containing confluent monolayers of cells from the Epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983; Winton et al. 2010) that were pretreated with 3.5% polyethylene glycol (20 µl/well; Batts and Winton 1989). After a 30 min virus adsorption period, wells were overlaid with 100 µl of methylcellulose solution and incubated at 15°C for 7 d. Cells were then fixed and stained with a crystal violet and formalin solution, and plaques were enumerated. The average plaque count in each duplicate well was divided by the average plaque count for the respective wells containing dilutions of SPF plasma. Neutralizing titers were reported as the reciprocal of the highest dilution where ≥ 50% reduction in plaque counts occurred, with all lesser dilutions also demonstrating ≥ 50% reductions.

Complement dependency of the PNT was evaluated by comparing neutralizing activities between three plasma preparations: whole plasma, heat-inactivated plasma, and heat-inactivated plasma plus exogenous complement. Test samples consisted of plasma from VHS survivors 64 d postexposure (n = 20), and negative controls (n = 10) consisted of plasma from SPF Pacific Herring. Whole plasma contained all soluble constituents of whole blood minus the separated blood cells. Endogenous complement was removed from heat-inactivated plasma by warming to 45°C for 30 min. Exogenous complement consisted of whole plasma from SPF Pacific Herring that was diluted 1:10 in MEM supplemented with 5% fetal bovine serum; this complement solution was then added to equal volumes of the plasma–virus dilutions in the appropriate aliquots. Samples were then processed by PNT, and neutralizing activity was compared between the three preparations.

The specificity of the neutralizing activity was assessed by comparing the relative ability of Pacific Herring plasma to neutralize VHSV and infectious hematopoietic necrosis virus, a closely related rhabdovirus. The neutralization titer was compared in paired plasma aliquots from survivors of VHSV exposure (n = 6) and negative controls (n = 6) using the PNT and slightly modified methods, whereby plasma was incubated with infectious hematopoietic necrosis virus (4.0×10^5 PFU/mL) in lieu of VHSV in the appropriate treatment.

The reproducibility of the PNT was assessed by comparing the neutralizing titer in plasma aliquots on repeated sampling days. Plasma samples from VHSV survivors (n = 14) and negative controls (n = 11) were aliquoted into triplicate tubes; each tube was processed using the PNT on three different days.

The prevalence of seropositives was compared between treatment groups in the various experiments using the chi-square statistic, with statistical significance assigned to comparisons with $P < 0.05$.

Influence of rearing temperature on the kinetics of neutralizing titers.—The PNT was employed to assess the effects of rearing temperature on the onset and magnitude of virus neutralizing activity in the plasma of VHSV survivors (n = 91) and negative controls (n = 56); n = 5–13 fish per temperature treatment on each subsampling day. Virus neutralization titers were log transformed, and the effects of time postexposure and temperature were examined by two-way ANOVA with Tukey's honestly significant difference multiple comparisons (R Core Team 2015). Results were considered significant when $P \leq 0.05$.

RESULTS

Optimization of the PNT

Optimal PNT performance was achieved by overnight incubation of two-fold dilutions, starting at 1:16, of whole plasma with known quantities of VHSV at 15°C. When plating plasma–virus incubations from negative control treatments, the optimal viral titer was determined to be from 5–20 PFU/well, with a target of 10 PFU/well. Assay results were rejected and rerun whenever viral titers in negative control plasma fell below this range, as the inherent variability involved with plating very low virus titers resulted in a higher proportion of false positives. Similarly, negative control titers much above this range were rerun, as plaques were too numerous to count in the micro wells. The initial plasma dilution threshold for assigning seropositives was established at 1:16, as lower dilutions (1:2 to 1:8) resulted in 12.5% (7/56) false positives among negative controls (Table 1). At or above this dilution threshold of 1:16, false positives occurred in only 2% (1/49) of the negative controls. However, follow-up studies using the optimized methods indicated that false-positive rates as high as 12% (6/50) can periodically occur in negative controls at dilutions from 1:16 to 1:64 (data not shown). Nevertheless, it was decided not to raise the established seropositive dilution threshold to 1:128, as this higher threshold would have negated the detection of 23% (18/77) of probable seropositive samples from known VHSV survivors (Table 1).

Virus neutralization using the optimized PNT methods was both reproducible and specific to VHSV. Among plasma samples from VHS survivors, 64% (9/14) were positive for neutralizing titer in all three independent runs and an additional

TABLE 1. Plaque neutralization test results from subsampled Pacific Herring in each temperature treatment. Values indicate the number of plasma samples demonstrating neutralization at each plasma dilution.

Temperature treatment (°C)	Days postexposure	Degree days	Neutralization titer 0	2	4	8	16	32	64	128	256	Positives[a]/total
Negative control (unexposed)												
7.4	0[b]		8	1	1							0/10
9.9	0[b]		5									0/5
13.1	0[b]		5									0/5
9.9	37		5									0/5
9.9	64		3	1		1	1					1/6
9.9	113		7									0/7
9.9	182		10	1	1	1						0/13
9.9	345		5									0/5
Treatment (VHSV survivors)												
7.4	37	222	4					1	1			2/6
9.9	37	315	1					3			1	4/5
13.1	37	444	1					1	1		2	4/5
7.4	64	384								3	2	5/5
9.9	64	544							1	3	1	5/5
13.1	64	768								1	5	6/6
7.4	113	678	1						2	2	1	5/6
9.9	113	961									5	5/5
13.1	113	1,356	1								4	4/5
7.4	182	1,092	2					1	1		3	5/7
9.9	182	1,547								1	4	5/5
13.1	182	2,184	1					1		2	1	4/5
7.4	345	2,070				1		1	2		3	6/7
9.9	345	2,933								3	2	5/5
13.1	345	4,140	2					1		3	2	6/8
13.1	385	4,620							1	2	3	6/6

[a] Samples were considered seropositive at plasma dilutions ≥ 1:16; all lesser dilutions (1:2–1:8) were considered negative because of the potential for false positives.
[b] Negative controls from day 0 consisted of SPF Pacific Herring from each temperature treatment that were subsampled immediately prior to VHSV exposure.

19% (3/14) were positive in 2/3 independent runs. Similarly, negative results consistently occurred in all three independent runs from all negative controls (n = 11) and 11% (2/14) of VHS survivors (Table 2). The neutralizing activity detected in the plasma of VHSV survivors was virus-specific, as VHSV neutralization was detected in 5/6 plasma aliquots (median titer = 96), but infectious hematopoietic necrosis virus neutralization was detected in only 1/6 aliquots at the lowest serum dilution (Table 3).

The optimized PNT was complement dependent. Among VHSV survivors, heat inactivation of the plasma resulted in the complete loss of all virus-neutralizing activity, with neutralizing titers detected in 90% (18/20) of aliquots containing whole plasma but 0% (0/20) of samples containing heat-inactivated plasma ($P < 0.00001$; chi-square test). Virus neutralizing activity in the heat-inactivated samples was mostly restored by the addition of exogenous SPF complement, with 80% (16/20) of samples demonstrating neutralizing activity (Table 4). Interestingly, although the addition of exogenous SPF complement to heat-inactivated samples failed to fully restore assay sensitivity to the levels detected in whole plasma (80% versus 90%, respectively), a general increase in neutralizing titers did occur relative to whole plasma (median titers = 256 versus 96, respectively). Among negative controls, neutralizing activity was not detected in any samples of whole or heat-inactivated plasma (n = 10); however, neutralizing activity was detected at a low titer (32) in one sample after exogenous SPF complement was added to the heat-inactivated plasma.

Influence of Rearing Temperature on the Kinetics of Neutralizing Titers

The optimized PNT methods successfully detected VHSV neutralizing activity in the plasma from previously exposed Pacific Herring reared at all temperature treatments, starting

TABLE 2. Reproducibility of plaque neutralization test results in VHS survivors using the optimized methods. Aliquots of each sample were processed independently on each of 3 d. Viral hemorrhagic septicemia virus neutralization was not detected in any plasma samples ($n = 11$) from negative-control Pacific Herring (13.1°C and 9.9°C) on any of the three processing days.

Temperature treatment (°C)	Days postexposure	Degree days	Daily neutralization titer		
			1	2	3
7.4	37	222	0	0	0
9.9	37	315	128	64	32
13.1	37	444	256	32	0
9.9	64	544	256	256	256
13.1	64	768	256	128	128
7.4	113	678	64	64	0
9.9	113	961	128	32	32
13.1	113	1,356	128	64	64
7.4	182	1,092	256	256	64
9.9	182	1,547	32	16	0
7.4	345	2,070	0	0	0
7.4	345	2,070	256	128	64
9.9	345	2,933	256	256	128
13.1	385	4,620	256	256	128

on the first subsampling day (37 d postexposure) and continuing through the end of the experiment (345–385 d postexposure). The kinetics of virus neutralization were tempered at the cooler temperature (7.4°C), at which the onset of seropositives was slightly delayed (Figure 1A) and the median neutralization titers were lower (Figure 1B) relative to fish reared at the warmer temperatures. Median neutralizing titers peaked at 64–113 d postexposure in all temperature groups, and significant differences from negative controls (Tukey's honestly significant difference; $P < 1 \times 10^{-8} - 1.4 \times 10^{-3}$) occurred in all temperature treatments and time points from 37 d postexposure onwards, with the exception of the cool treatment for which significant differences from controls occurred from 64 d postexposure onward (Figure 1B; Table 1).

DISCUSSION

Previous studies indicated that Pacific Herring develop long-term resistance after surviving VHS (Hershberger et al. 2010) and that this resistance can be transferred to naive individuals through passive immunization (Hershberger et al. 2011); however, early efforts to quantify neutralizing titers in the plasma of these immune individuals using a PNT produced negative or inconsistent results (Hershberger et al. 2011; P. K. Hershberger, unpublished data). These early failures led to the attempted development of an indirect enzyme-linked immunosorbent assay (ELISA) to detect Pacific Herring antibodies to VHSV. The basic architecture of this ELISA consisted of a viral antigen basal layer, a Pacific Herring test plasma, a mouse-anti-Pacific

TABLE 3. Specificity of the optimized plaque neutralization test based on the relative ability of plasma to neutralize VHSV and the closely related infectious hematopoietic necrosis virus (IHNV). Resluts are presented for the treatment group (VHSV survivors) as neutralization of VHSV and IHNV was not detected in any plasma samples ($n = 6$) from the unexposed negative controls (7.4°C and 9.9°C).

Temperature treatment (°C)	Days postexposure	Degree days	Neutralization titer	
			VHSV	IHNV
7.4	37	222	64	0
9.9	113	961	256	0
7.4	182	1,092	256	0
7.4	345	2,070	64	16
13.1	345	4,140	0	0
13.1	345	4,140	128	0

TABLE 4. Complement dependency of the optimized plaque neutralization test. Each row provides a comparison of neutralizing titers between treatment aliquots (whole plasma, heat-inactivated plasma, and heat-inactivated plasma + exogenous complement) from a single fish. All fish were subsampled 64 d after exposure to either saline (negative control) or VHSV (treatment). Samples failing to neutralize VHSV at any dilution ≥ 1:16 were assigned a neutralization titer of 0.

	Plasma treatment		
Fish treatment	Whole	Heat inactivated	Heat inactivated + complement
Negative control	0	0	0
Negative control	0	0	0
Negative control	0	0	0
Negative control	0	0	0
Negative control	0	0	0
Negative control	0	0	32
Negative control	0	0	0
Negative control	0	0	0
Negative control	0	0	0
Negative control	0	0	0
Treatment	64	0	256
Treatment	128	0	2,048
Treatment	16	0	16
Treatment	64	0	0
Treatment	256	0	2,048
Treatment	256	0	2,048
Treatment	256	0	1,024
Treatment	64	0	0
Treatment	128	0	16
Treatment	0	0	256
Treatment	32	0	256
Treatment	0	0	0
Treatment	32	0	64
Treatment	256	0	128
Treatment	512	0	2,048
Treatment	256	0	1,024
Treatment	256	0	2,048
Treatment	128	0	1,024
Treatment	32	0	2,048
Treatment	16	0	0

Herring monoclonal antibody (α-herring-IgM MAb IA1.2; Purcell et al. 2012), and a horseradish peroxidase-labeled goat-anti-mouse immunoglobulin (goat α-mouse IgG HRP) top layer. An analogous indirect ELISA for Rainbow Trout antibodies against VHSV (Genogroup I) was shown to identify a higher proportion of antibody-positive individuals than did a PNT, especially at later weeks postexposure (Jørgensen et al. 1991; Oleson et al. 1991). However, after extensive optimization efforts, the Pacific Herring ELISA was abandoned, as it failed to detect antibodies among survivors of a single VHSV exposure; further, antibodies were detected only intermittently among hyperimmunized individuals (L. M. Hart and M. K. Purcell, unpublished data). These discouraging results from the indirect ELISA launched a reevaluation and optimization of the PNT methods described here.

The optimized PNT methods were highly sensitive, specific, and reproducible for identifying and quantifying VHSV neutralizing activity in the plasma of Pacific Herring. An important assay adjustment involved increasing the reactive incubation time of test plasma with VHSV from 30–60 min (Dorson and Torchy 1979; Oleson and Jorgensen 1986; LaPatra et al. 1993; Hershberger et al. 2011; Millard and Faisal 2012) to overnight (Ahne and Jorgensen 1993; Wilson et al. 2014). It is possible that neutralizing antibodies or other molecules in the plasma of some fishes require longer contact times with intact virions to ensure complete neutralization. Another important assay modification involved the use of

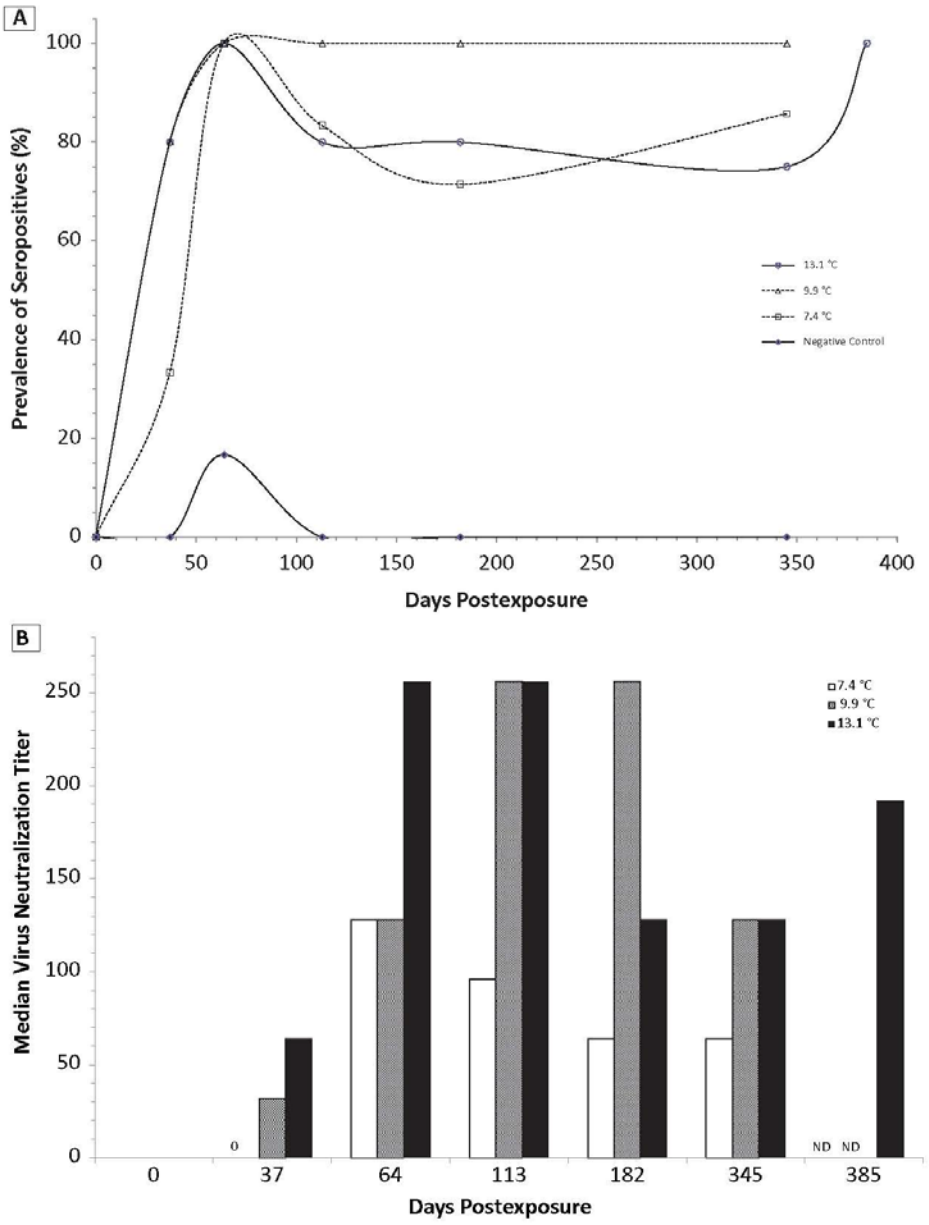

FIGURE 1. Kinetics of virus neutralizing activity in the plasma of VHSV survivors. Fish were considered **(A)** seropositive when neutralizing activity was detected at plasma dilutions ≥ 1:16, and **(B)** neutralizing titers were reported as the reciprocal of the highest dilution where ≥ 50% reduction in plaque counts occurred, with all lesser dilutions also demonstrating ≥ 50% reductions. The median virus neutralization titer in unexposed negative controls was 0 on all subsampling days. The abbreviation ND indicates that there was no data for those points as subsamples were not collected from 7.4°C and 9.9°C groups 385 d postexposure. Daily *n* for each datum is indicated in Table 1.

whole, unmodified Pacific Herring plasma in the optimized PNT. Similar to an analogous PNT used to detect Rainbow Trout antibodies against VHSV–Genogroup I (Dorson and Torchy 1979), the neutralization of VHSV–Genogroup IVa using Pacific Herring plasma was complement dependent (Table 1). In a successful effort to satisfy this complement dependency, the optimized PNT relied on the use of raw, unmodified plasma containing endogenous complement. The traditional approach of utilizing heat-inactivated plasma supplemented with exogenous complement was dismissed as an unviable option for two reasons. First, it is anticipated that this optimized PNT will be applied on a population scale and used to assess the exposure history and future susceptibility of wild Pacific Herring to VHS epizootics. As such, all reagents for the optimized PNT must be readily available to all diagnostic laboratories. Unfortunately, a commercial supply of complement from SPF Pacific Herring is not available, and there is some evidence indicating that exogenous complement from other fish (e.g. Rainbow Trout) is not a suitable replacement for that of Pacific Herring (Hershberger et al. 2011). Second, sensitivity of the PNT, or the ability of the assay to detect neutralizing activity in the plasma of known VHS survivors, was higher (90% versus 80%) and the prevalence of false positives was lower (0% versus 10%) using whole plasma rather than heat-inactivated plasma plus exogenous complement. However, neutralizing titers were typically higher with heat-inactivated plasma plus exogenous complement. Causes for these differences remain undetermined, but it is possible that some thermal degradation of endogenous complement may have occurred during the sample collection or processing procedures.

The optimized PNT methods provided a promising tool capable of identifying the exposure history of Pacific Herring to VHSV; however, an understanding of the precise immunological mechanism(s) responsible for the virus neutralization requires further investigation. The adaptive immune response detected by the PNT is certainly antibody mediated, as virus neutralization persisted in the plasma of VHS survivors even after heat-labile proteins were removed and replaced with those from naive Pacific Herring. However, because the optimized PNT methods utilized whole plasma, it is important to consider that a portion of the reported virus neutralization capacity may be attributable to nonantibody proteins, including endogenous complement and/or interferon (deKinkelin and Dorson 1973; Dorson and de Kinkelin 1974; de Kinkelin et al. 1982; Rogel-Gaillard et al. 1993).

The ability for long-term detection of VHSV neutralizing activity in the plasma of Pacific Herring suggests that the optimized PNT may be developed into a population surveillance tool that is capable of assessing the potential for future VHS epizootics. Unlike a previously described PNT for Rainbow Trout, in which serum neutralizing titers declined after 24 weeks (Olesen et al. 1991), the optimized PNT for the Pacific Herring continued to return high neutralizing titers for at least 345 d postexposure, regardless of temperature (Figure 1). This long-term persistence of plasma neutralization corresponds with in vivo observations of Pacific Herring, which develop long-term protection against the disease and do not undergo subsequent bouts of the disease, even when reexposed to VHSV under environmental conditions that are conducive for disease outbreaks (Hershberger et al. 2016). Therefore, a tool capable of deducing the exposure history of Pacific Herring to VHSV, such as the optimized PNT, may be capable of forecasting the potential for future VHS epizootics within defined populations. For example, populations predominated by individuals containing little plasma neutralizing activity would be assigned low herd immunity and a corresponding high potential for future epizootics if future exposures occur concomitantly with appropriate environmental conditions. Conversely, populations predominated by individuals demonstrating high plasma neutralizing activity would be assigned high herd immunity and a corresponding low potential for future VHS epizootics, as individuals would be refractory to the disease. Additionally, it may be possible to use ongoing Pacific Herring assessment surveys to track the annual VHSV exposure history of Pacific Herring age-classes to determine if or when prior VHS epizootics may have occurred. When combined with population recruitment data from annual Pacific Herring surveys, this type of a priori approach may prove beneficial for assessing the involvement of VHSV in year-class failures from particular regions, such as Prince William Sound.

Although the optimized PNT methods offer potential for identifying Pacific Herring exposure histories to VHSV, additional validation studies are required before their wide application to wild populations. Most importantly, unlike the studies described here that employed laboratory-reared SPF Pacific Herring, the optimized PNT methods must be validated using groups of wild Pacific Herring that demonstrate various degrees of VHS susceptibility. Additionally, although not a requisite for field validation, future laboratory studies should also provide more detailed profiles of plasma neutralizing titers, particularly during the early days after VHSV exposure and during the extended periods beyond 1 year postexposure. A more complete understanding of the Pacific Herring immune response to VHSV infection could be obtained by evaluating these plasma neutralization profiles concomitantly with the expression of innate and adaptive immune response genes.

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Appendix 9

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COMMUNICATION

Influence of Temperature on the Efficacy of Homologous and Heterologous DNA Vaccines against Viral Hemorrhagic Septicemia in Pacific Herring

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Abstract

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

Pacific Herring *Clupea pallasii* are highly susceptible to viral hemorrhagic septicemia (VHS; Meyers et al. 1994; Kent et al. 1998; Kocan et al. 2001; Hedrick et al. 2003), with laboratory exposures resulting in mortalities as high as 100% (Kocan et al. 1997; Hershberger et al. 2007) and periodic epizootics occurring throughout the coastal areas of the north Pacific (Hedrick et al. 2003; Garver et al. 2013). Phylogenetically, VHS virus (VHSV) is classified into different genogroups that are broadly associated with Europe (I–III) and North America (IV; Benmansour et al. 1997; Stone et al. 1997; Einer-Jensen et al. 2005). Genogroups are further divided into sublineages; examples include North American/Japanese (e.g., genogroup IVa), North American Great Lakes (e.g., genogroup IVb; Elsayed et al. 2006), and continental European (e.g., genogroup Ia; Einer-Jensen et al. 2004).

Once herring are exposed to VHSV, the survivors develop resistance to the resulting disease (Kocan et al. 2001;

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Hershberger et al. 2007, 2010), likely through a combination of early innate and later adaptive immune responses. Early protection is associated with the upregulation of antiviral genes such as Mx that are involved with the interferon response (Hansen et al. 2012; Hershberger et al. 2013). Warm water temperatures are known to improve survival of VHSV infection in several fish species (Jørgensen 1982; Sano et al. 2009; Goodwin and Merry 2011), possibly by expediting the protective effects of host innate responses (Lorenzen et al. 2009; Hershberger et al. 2013). Following nonspecific immune reactions, an adaptive response may develop at later time points (Hershberger et al. 1999, 2011; Kocan et al. 2001), but this remains to be analyzed functionally in herring. In Rainbow Trout *Oncorhynchus mykiss*, the transition from innate to adaptive responses following DNA vaccination is temperature dependent, but at 12–15°C generally occurs 3–5 weeks postvaccination (PV; Lorenzen et al. 2002b, 2009; McLauchlan et al. 2003). While antibody production in VHSV-exposed Pacific Herring is poorly understood, strong evidence exists for adaptive immunity in salmonids as a result of VHSV infection and DNA vaccination (Olesen et al. 1991; Lorenzen et al. 1998).

Protective immune responses to VHSV depend mainly on the viral glycoprotein (Lecocq-Xhonneux et al. 1994), which triggers an innate interferon response (Acosta et al. 2006) and neutralizing antibodies (Lorenzen et al. 1990; Jørgensen et al. 1995). DNA vaccines containing glycoprotein genes from VHSV have been used successfully as protection against VHS in different fish species, including Rainbow Trout (Lorenzen et al. 1998), Olive Flounder *Paralichthys olivaceus* (Yong Byon et al. 2005), Turbot *Scophthalmus maximus* (Pereiro et al. 2012), and Pacific Herring (Hart et al. 2012). The most extensive functional studies of time and temperature effects on level and specificity of protection have so far been conducted in Rainbow Trout (Lorenzen et al. 2009). The innate immunity phase is characterized by cross-protection between heterologous viral species. Such cross-protection is absent in the adaptive phase but is still quite efficacious between VHSV genogroups, although survival is typically higher among groups receiving the homologous vaccine (Lorenzen et al. 1999, 2000).

This study examined the kinetics of the functional immune response in Pacific Herring by comparing the ability of homologous and heterologous glycoprotein DNA vaccines to confer protection against VHSV (genogroup IVa) at different water temperatures and at different times postvaccination (PV). Vaccine efficacy was based on such metrics as survival and neutralizing antibody production.

METHODS

Cloning of the heterologous DNA vaccine pcDNA3-vhsG (DK-3592b, genotype Ia) has previously been described elsewhere (Heppell et al. 1998). For cloning of the homologous DNA vaccine, purified RNA from the isolate BC-99-292 (genotype IVa) originally isolated from Atlantic Salmon *Salmo salar* in British Columbia, Canada, was used as a starting point. Primers with the unique restriction sites (underlined) BamHI in the sense primer 5′AATTGGATCC ACC**ATG**GAATGGAATACTTTTTTCTT3′ and EcoRI in the antisense primer 5′AATTGAATTC**TCA**GACCATCTGGC TTCTGGA3′, respectively, were used in the reverse transcriptase (RT)-PCR amplification, which allowed insertion of the gene downstream of the CMV promoter in the pcDNA3 vector. The initial Met and final stop codons of the encoded G gene are shown in bold. Significant G protein expression was ensured by including the optimized Shine–Dalgarno sequences upstream of the ATG initiation codon as described previously (Einer-Jensen et al. 2009). Functionality of the plasmids was evaluated in vitro by transfection of *Epithelioma papulosum cyprini* (EPC) cell cultures and subsequent immunological detection of the viral proteins using the in-house-produced VHSV G-protein-specific monoclonal antibodies (MAbs) IP1H3 and IP1D11 (Lorenzen et al. 1988, 1998). Finally, the plasmid constructs were confirmed by DNA sequencing of the inserted G genes.

Specific pathogen-free (SPF) Pacific Herring ages 1.5 year (Hershberger et al. 2010) were randomly separated into four treatment groups, including saline control (20 µL of phosphate-buffered saline [PBS] per fish), plasmid control (pcDNA3, 4.0 µg/fish), heterologous vaccine (pcDNA3-vhsG [isolate DK-3592b, genotype Ia], 4.0 µg/fish; Heppell et al. 1998), and homologous vaccine (pcDNA3-vhsG [isolate BC-99-292, genotype IVa], 4.0 µg/fish). All treatments were administered by injection into the left epaxial muscle. Immediately after vaccine administration; fish from each treatment were separated into designated ambient (9.0°C) or warm (12.6°C) holding tanks, yielding a total of eight groups (320–353 fish/group; ambient and warm, saline, plasmid, Ia, and IVa). For the 12.6°C group, water temperature was slowly raised over the course of one day. All temperature groups were maintained in 760-L tanks, supplied with single-pass seawater.

Vaccine efficacy was assessed twice, at early (2 weeks) and late (10 weeks) times PV, by controlled exposures to VHSV corresponding to the homologous genotype IVa isolate BC-99-292. Exposures to VHSV, made at the same temperatures as applied during immunization, consisted of herring from each group that were transferred to triplicate tanks (29–32 herring/tank) and challenged with VHSV by static immersion ($6.0–8.0 \times 10^3$ pfu/mL) for 1 h. The early exposure (2 weeks PV) corresponded to 135 and 189 degree days (DD), respectively, and those during the late exposure (10 weeks PV) corresponded to 639 and 895 DD, respectively. Mortality was tracked for 15 d postexposure, after which each experiment was terminated by killing all survivors in an overdose of buffered tricaine methanesulfonate (MS-222). Kidneys were subsampled

from between one and three mortalities each day at 3, 6, 9, and 12 d postexposure to confirm viral presence.

Plasma virus neutralization (VN) titers were assessed from subsamples (n = 4–5) of Pacific Herring in each treatment group at 2 or 10 weeks PV just prior to viral exposure. Fish were killed by immersion in buffered MS-222, tails were transected, and blood was collected from the caudal artery into heparin-containing capillary tubes (Fisher). Blood was centrifuged at 12,700 × g (Thermo Micro MB) and plasma was decanted and stored at –80°C until processed using a modified 50% plaque neutralization test (PNT; adapted from Wilson et al. 2014). First, EPC cells (Fijan et al. 1983; Winton et al. 2010) were seeded at a calculated density of 100,000 cells per well on 96-well flat-bottom microtiter plates (Costar) and grown to confluency at 25°C. Next, individual plasma samples were serially diluted (1:8–1:1024) in Tris-Cl–buffered Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum. An equivalent volume of VHSV (genotype IVa) diluted in Tris-Cl–buffered Auto-Pow (TAP; 0.94% MEM, 0.30% tryptose phosphate broth, 1.3% Tris), yielding 5.3×10^3–1.6×10^4 pfu/mL, was added to each dilution, resulting in final plasma dilutions of 1:16–1:2048. A pool of plasma collected from four SPF Pacific Herring (negative control) was diluted similarly. All plasma–virus preparations were incubated overnight at 15°C and viral titers were confirmed by plaque assay (Hershberger et al. 2010). On the following day, each well was pretreated with 20 µL of 35 mL/L polyethylene glycol (PEG) for 15 min; 10 µL of each sample dilution was plated in duplicate and incubated for 30 min. Afterwards, each well was overlayed with 100 µL of methylcellulose and plates were incubated for 7 d at 15°C. Cells in each well were then fixed and stained with crystal violet–formalin solution and plaques were enumerated. Duplicate averages were calculated for each plasma dilution, and test sample averages were divided by the plaque count for analogous dilutions of negative control plasma. Virus neutralization titers reflected the highest reciprocal dilution at which a minimum of 50% reduction in plaques occurred. For virus neutralization titers to be reported, all lesser dilutions for said sample were also required to have a minimum of 50% reduction in plaques. Each plasma sample was tested by the PNT on two separate days and was recorded as positive if neutralization occurred on both days. Positive sample titers are reported as the 2-d mean reciprocal dilution.

Percent survival at 15 d postexposure was arcsin-transformed, and differences among means were examined by two-way ANOVA (R Core Team 2015). Multiple comparisons were performed using Tukey's honestly significant difference (HSD; R Core Team 2015). Results were considered significant at $P \leq 0.05$. Survival was reported as the back-transformed mean ± SD of arcsin-transformed values. Relative percent survival (RPS) was calculated using 15-d cumulative mortality:

$$\text{RPS} = 1 - \left(\frac{\text{Cumulative \% mortality of treatment group}}{\text{Cumulative \% mortality of ambient saline-injected group}}\right) \times 100.$$

Viral titers in tissues were evaluated by plaque assay on 24-well plates, using four serial dilutions on each sample (Hershberger et al. 2010). Any plaque counts above 99 in the highest dilution (>4.0×10^7 pfu/g) were deemed too numerous to count and were assigned a value of 1.0×10^8 pfu/g tissue or pfu/mL water. All viral titers are reported as geometric means of positive samples.

To check for coinfections with erythrocytic necrosis virus (ENV), spleen tissue was collected from all groups just prior to viral exposure 10 weeks PV and preserved in RNAlater (Qiagen). Total RNA was extracted from spleen tissue using the RNeasy Tissue Kit (Qiagen) following standard methods. Reverse transcription of RNA to DNA was accomplished using the High-Capacity cDNA Synthesis Kit (Thermofisher, Inc.), and cDNA was diluted 1:5 prior to PCR. Reverse transcriptase quantitative PCR (RT-qPCR) for Mx-1 and the normalizing gene ARP-P0 was performed as previously described (Hansen et al. 2012). Quantification of the ENV ATPase-like gene copy number was evaluated as previously described (Purcell et al. 2016), except that spleen cDNA was used as the starting template.

RESULTS

Vaccination of Pacific Herring using homologous and heterologous DNA vaccines tended to result in the highest levels of protection against VHS at 2 weeks PV, but only at the elevated temperature (12.6°C). When evaluated by RPS alone, there was an indication of temperature-dependent early vaccine–induced protection in the 12.6°C groups given the IVa and Ia constructs, as RPS was similar between the two groups but greater than RPS of the other 12.6°C treatments. No similar pattern was evident in the 9.0°C groups (Table 1). Cumulative survival indicated a similar pattern; IVa and Ia constructs had elevated survival outcomes compared with all other groups; however, the results were not significant (two-way ANOVA, F = 2.213, df = 3, P = 0.126) between any of the individual groups (Figure 1A). VHSV was detected in mortalities from all treatments (n = 1–3/d; 2.1×10^3–1.0×10^8 pfu/g).

Vaccination of Pacific Herring using homologous and heterologous DNA vaccines resulted in protection against VHS at 10 weeks PV. Percent survival between treatment groups was compared without inclusion of the 12.6°C saline control group, which experienced unanticipated high survival after exposure to VHSV. The unanticipated survival presumably occurred due to interference from an unintended infection with ENV. Spleens tested for expression of the ENV ATPase (n = 5 /group) by RT-qPCR (Purcell et al. 2016) showed ENV RNA in subsampled fish from the 12.6°C saline control group (5/5) with a mean of 5.9 log copies of ENV ATPase per reaction. In contrast, ENV RNA was not detected in any samples from other treatment groups except in one fish from the IVa vaccine group (9.0°C), which had a much lower ENV copy number (1.4 log copies per reaction; data not

TABLE 1. Relative percent survival (RPS) 15 d after exposure of Pacific Herring to VHSV (genogroup IVa). All values are relative to a negative control group that was injected with saline (9.0°C) in lieu of vaccine. The relative percent survival of the 12.6°C saline group at 10 weeks postvaccination (PV) is elevated due to coinfection with erythrocytic necrosis virus.

		Relative percent survival (%)	
Vaccine treatment	Temperature (°C)	2 weeks PV	10 weeks PV
Homologous IVa	9.0	0.0	40.4
	12.6	21.9	51.1
Heterologous Ia	9.0	0.9	11.1
	12.6	22.7	27.3
Plasmid	9.0	0.0	2.2
	12.6	5.7	4.2
Saline	12.6	7.9	48.3

presented). Spleens were also evaluated for the antiviral gene Mx (Hershberger et al. 2013); expression was not significantly different among the eight treatment groups (Kruskal–Wallis, $P = 0.2811$, $n = 5$/group; data not presented). Mean survival of the two homologous treatment groups (51.8% [12.6°C IVa], 41.2% [9.0°C IVa]) and the 12.6°C (26.9%) nonhomologous treatment group was significantly higher than for all negative controls (Figure 1B; Tukey's HSD, $P = 5.0 \times 10^{-5}$–4.6×10^{-2}). Relative percent survival showed trends similar to that of mean survival: both vaccines elicited superior survival at 12.6°C than at 9.0°C. Further, RPS of homologous vaccinated groups (IVa) at both temperatures was higher than that of heterologous (Ia) vaccinated groups (Table 1).

The detection of VN was primarily a function of time PV. Virus neutralization was not detected in plasma samples collected from any groups at 2 weeks PV. However, neutralizing titers were observed in 40–60% of plasma samples collected from vaccinated herring and one 12.6°C saline sample at 10 weeks PV. Neither VN prevalence nor titer was influenced by water temperature (Table 2).

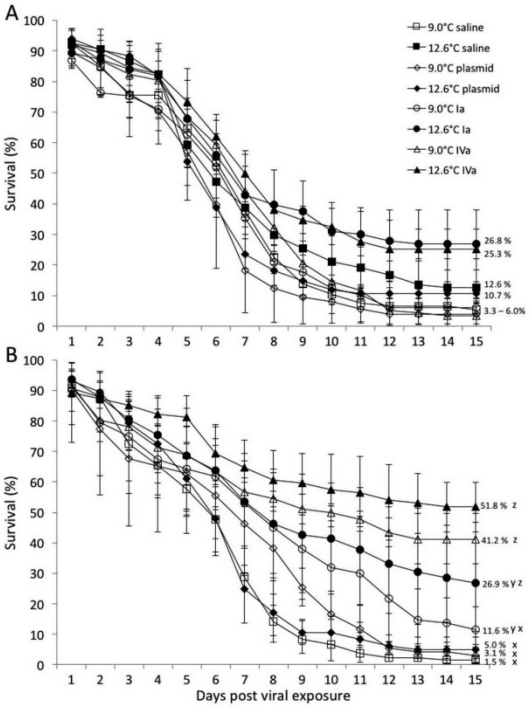

FIGURE 1. Survival of Pacific Herring exposed to VHSV at 2 weeks **(A)** and 10 weeks **(B)** after injection with homologous vaccine (IVa), heterologous vaccine (Ia) or plasmid/saline (negative controls). Because of coinfection with erythrocytic necrosis virus, the results for saline samples are removed from 10 weeks comparisons at 12.6°C. Bars indicate standard deviation. All data points represent back-transformed percentages corresponding to the means of arcsine-transformed proportions from triplicate tanks. Different letters indicate statistically significant differences between day-15 means (Tukey's HSD, $P < 0.05$).

DISCUSSION

The results presented here suggest that Pacific Herring mount both innate and adaptive antiviral immune defense mechanisms upon vaccination with a DNA vaccine encoding the VHSV G protein. The study complements our earlier report on partial protection against VHS in Pacific Herring following DNA vaccination (Hart et al. 2012). The earlier reported protection, with RPS values of 34–48%, was less prominent than that found for Rainbow Trout and Olive Flounder (Lorenzen et al. 2002b; Yong Byon et al. 2005), and the characteristic early antiviral protection seen following DNA vaccination of Rainbow Trout with a heterologous DNA vaccine (Lorenzen et al. 2002a, 2002b), was not observed (Hart et al. 2012). As in the earlier study, we found no early protection in vaccinated fish kept at ambient temperature (9°C) and challenged 2 weeks PV, while both the homologous and the heterologous vaccines mediated elevated and similar survival at 12.6°C at 2 weeks PV. Whether the higher number of DD reached at 12.6°C than at 9°C, or a general less efficient activation of the immune response by the DNA vaccines at 9°C, can explain this difference remains to be determined. The results stress the importance of inclusion of temperature in host–pathogen interactions in fish. As in salmonids, the early protection most likely resulted from innate nonspecific immunity, reflecting that the G gene-based DNA vaccine tends to stimulate an immune response like that seen during a virus infection (Lorenzen et al. 2002b; Kurath 2008). Accordingly,

TABLE 2. Mean virus neutralization titers in the plasma of Pacific Herring from each treatment group. Titer values represent the reciprocal dilution at which a minimum of 50% reduction in plaques occurred (mean value of two independent tests). Dashes indicate a titer value of zero.

Treatment	Sample number					Positive prevalence	Mean titer
	1	2	3	4	5		
2 weeks postvaccination							
9.0°C saline	–	–	–	–	–	0/5 (0%)	0
9.0°C plasmid	–	–	–	–	–	0/5 (0%)	0
9.0°C Ia	–	–	–	–	–	0/5 (0%)	0
9.0°C IVa	–	–	–	–	–	0/5 (0%)	0
12.6°C saline	–	–	–	–	–	0/5 (0%)	0
12.6°C plasmid	–	–	–	–	–	0/5 (0%)	0
12.6°C Ia	–	–	–	–	–	0/5 (0%)	0
12.6°C IVa	–	–	–	–	–	0/5 (0%)	0
10 weeks postvaccination							
9.0°C saline	–	–	–	–	–	0/5 (0%)	0
9.0°C plasmid	–	–	–	–	–	0/5 (0%)	0
9.0°C Ia	384	272	–	–	–	2/5 (40%)	328
9.0°C IVa	256	96	768	–	–	3/5 (60%)	373.3
12.6°C saline	32	–	–	–	–	1/5 (20%)	32
12.6°C plasmid	–	–	–	–	–	0/4 (0%)	0
12.6°C Ia	192	1,152	1,024	–	–	3/5 (60%)	789.3
12.6°C IVa	384	384	48	–	–	3/5 (60%)	272

nonspecific antiviral genes are upregulated at early time points post-VHSV exposure (Avunje et al. 2012; Hansen et al. 2012; Hershberger et al. 2013) and tend to be expedited by warm temperature in Pacific Herring (Hershberger et al. 2013). The vaccine-mediated expression of the G protein presumably represents a key element in the triggering of the early interferon upregulation (Acosta et al. 2006; Martinez-Lopez et al. 2014). Analogously, equal levels of early cross-protection at 7 d PV have also been reported in Rainbow Trout given DNA vaccines encoding G genes of different variants of infectious hematopoietic necrosis virus (IHNV), a closely related rhabdovirus (Peñaranda et al. 2011).

The unexpected high survival of unvaccinated 12.6°C saline controls at 10 weeks PV (Figure 1B) was likely due to nonspecific cross-protection resulting from a coinfection with ENV, which necessitated elimination of this group from the statistical analysis. It remains to be solved whether the occurrence of elevated ENV replication, particularly in the 12.6°C saline group, could have been due to accidental presence of a few ENV carriers in this group or to tank-related conditions. Although the immune mechanisms associated with ENV infection are largely unknown, and Mx expression was not elevated, preliminary data from separate trials have demonstrated cross-protection against VHS in Pacific Herring that are coinfected with ENV (Hart and Hershberger unpublished). Furthermore, intracrythrocytic viruses such as piscine orthoreovirus have been shown to stimulate production of antiviral proteins in Atlantic Salmon erythrocytes (Wessel et al. 2015). Similarly, protective effects of subclinical infections with heterologous viruses have been reported earlier for both VHSV and IHNV in Rainbow Trout (de Kinkelin et al. 1992; LaPatra et al. 1995).

Protection at 10 weeks PV was likely the result of acquired immunity. Exposure to the homologous vaccine tended to confer the strongest protection and survival levels closely aligned to those of herring passively immunized prior to IVa VHSV exposure (Hershberger et al. 2011). Similar trends have been noted in salmonids where homologous DNA vaccines against VHSV or infectious hematopoietic necrosis virus (IHNV) tend to provide slightly better protection (Lorenzen et al. 1999, 2000; Corbeil et al. 2000; Garver et al. 2005; Peñaranda et al. 2011) in the late (adaptive) phase of protection. Similar levels of VN against the IVa isolate in both vaccinated groups did not suggest variation in glycoprotein neutralization epitopes as an explanatory variable in survival outcomes in the present study. Adaptive responses have been proposed previously in Pacific Herring (Hershberger et al. 2011) but direct species-specific evidence has been lacking. Although VN was not detected in a portion of the plasma samples collected from vaccinated herring 10 weeks PV, the absence of VN activity in sera from vaccinated fish does not correlate with lack of protection (Lorenzen et al. 1998). Low antibody titers have been shown to be protective against IHNV (LaPatra et al. 1994; Traxler et al. 1999), and neutralizing antibodies may circulate at levels below the detection threshold of the neutralization assay. Nonneutralizing antibodies and

cellular immune responses are also likely to be involved in protection (Kim et al. 2000; Lorenzen et al. 2000; Adelmann et al. 2008; Takami et al. 2010).

Apart from ornamental specimens, vaccination of Pacific Herring does not have an applied perspective. However, the results presented here demonstrate the usefulness of VHSV DNA vaccines as tools for analyzing herring immune reactions and as an effective surrogate for live virus in studies aimed at understanding viral kinetics and immune responses to subsequent viral exposure. Despite the use of a homologous DNA vaccine, the protective effect against VHS found here in Pacific Herring was considerably lower than that consistently reported for Rainbow Trout at both low (ambient) and higher temperatures at early and late time points PV. This suggests some overall quantitative or qualitative differences in host–virus interactions. Perhaps by adapting to different life strategies, Pacific Herring as a pelagic marine species and Rainbow Trout being an anadromous species, the two fishes have developed diverse immune response patterns. The fact that VHSV originates from the marine environment (Einer-Jensen et al. 2004) possibly further implies that Pacific Herring has evolved with the virus much longer than Rainbow Trout has. These aspects should be approached by future studies.

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Appendix 10

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COMMUNICATION

Infecting Pacific Herring with *Ichthyophonus* sp. in the Laboratory

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Abstract

The protistan parasite *Ichthyophonus* sp. occurs in coastal populations of Pacific Herring *Clupea pallasii* throughout the northeast Pacific region, but the route(s) by which these planktivorous fish become infected is unknown. Several methods for establishing *Ichthyophonus* infections in laboratory challenges were examined. Infections were most effectively established after intraperitoneal (IP) injections with suspended parasite isolates from culture or after repeated feedings with infected fish tissues. Among groups that were offered the infected tissues, infection prevalence was greater after multiple feedings (65%) than after a single feeding (5%). Additionally, among groups that were exposed to parasite suspensions prepared from culture isolates, infection prevalence was greater after exposure by IP injection (74%) than after exposure via gastric intubation (12%); the flushing of parasite suspensions over the gills did not lead to infections in any of the experimental fish. Although the consumption of infected fish tissues is unlikely to be the primary route of *Ichthyophonus* sp. transmission in wild populations of Pacific Herring, this route may contribute to abnormally high infection prevalence in areas where juveniles have access to infected offal.

The mesomycetozoean parasite (Herr et al. 1999) *Ichthyophonus* sp. causes epizootics that can have population-level impacts on planktivorous marine fishes, particularly clupeids (reviewed by McVicar 2011 and Burge et al. 2014). Investigations into these events are typically restricted to descriptive observations of the epizootics, such as reports of parasite prevalence and infection intensity. A basic understanding of the host and environmental co-factors involved with these epizootics remains elusive, with hypotheses based principally on factor analysis of associated environmental data (Kramer-Schadt et al. 2010). Unfortunately, this epizootiological approach is incapable of establishing cause-and-effect relationships, and essential details of *Ichthyophonus* sp. life history in the marine environment remain largely undefined.

One of the most obvious information gaps concerning *Ichthyophonus* sp. life history involves the natural route(s) of exposure for wild clupeids and other planktivores. Controlled laboratory studies have repeatedly demonstrated that *Ichthyophonus* sp. transmission to piscivorous and scavenging fishes occurs via the consumption of infected tissues (Rucker and Gustafson 1953; Gustafson and Rucker 1956; Jones and Dawe 2002; Gregg et al. 2012; Kocan et al. 2013). However, empirical demonstration of an effective exposure route for planktivorous fishes has not been achieved (P. K. Hershberger, unpublished data). For example, although the horizontal transfer of *Ichthyophonus* sp. occurs easily between Rainbow Trout *Oncorhynchus mykiss* cohabitating in freshwater (Gustafson and Rucker 1956; Yokota et al. 2008), attempts to demonstrate an analogous mechanism of transfer for Pacific Herring *Clupea pallasii* have been unsuccessful (Gregg et al. 2012). Reasons for this disparity require further investigation but may involve genotypic (Rasmussen et al. 2010) or phenotypic (Hershberger et al. 2008) differences between freshwater and seawater forms of the parasite. Because the natural mechanisms of *Ichthyophonus* sp. transmission to planktivores remain unknown, laboratory exposure experiments involving Pacific Herring have been mostly limited to injection of parasite isolates into the host's coelom (e.g., Kocan et al. 1999). Although coelomic injection is effective for establishing infections in laboratory test animals, this unnatural exposure

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route is inappropriate for addressing many of the basic questions about the natural disease process.

The goal of this study was to further investigate the possible routes of *Ichthyophonus* sp. transmission to Pacific Herring. Specifically, we investigated whether *Ichthyophonus* sp. could be transmitted to Pacific Herring by (1) a single feeding or multiple feedings of infected fish tissues or (2) various exposure mechanisms using parasite isolates from cultures.

METHODS

A controlled feeding trial was performed to investigate the possible transmission of *Ichthyophonus* sp. to Pacific Herring through the consumption of infected tissues. Experimental fish consisted of specific-pathogen-free (SPF; Hershberger et al. 2010) age-0 Pacific Herring (139 d old; FL [mean ± SD] = 35 ± 5.4 mm; weight = 0.32 ± 0.18 g) in each of three experimental treatments: single exposure ($N = 65$), multiple exposure ($N = 46$), and negative control ($N = 29$). Each treatment occurred in a separate 760-L tank that was supplied with single-pass, sand-filtered seawater (mean temperature = 8.6°C). Prior to initiation of the experiment, fish in all treatment groups were weaned onto minced tissues from SPF Pacific Herring for 20 d. Tissues used as feed during the experiment were obtained from colonies of laboratory-reared, age-2 Pacific Herring that were either SPF (negative control group) or infected with *Ichthyophonus* by prior intraperitoneal (IP) injection with parasite suspensions isolated from wild Pacific Herring. Feed tissues were prepared by euthanizing the donor fish in sodium-bicarbonate-buffered tricaine methanesulfonate (MS-222), discarding the head and caudal fin, confirming *Ichthyophonus* infection status by microscopically examining liver squash preparations for the presence of parasite schizonts, and mincing all remaining tissues in a blender. Upon initiation of the experiment, minced tissues (mean ± SD = 65 ± 10 g/d) from the appropriate donor fish were poured into the respective treatment tanks on day 0 of the experiment (single-exposure group) or on 16 occasions (approximately every other day) throughout the duration of the experiment (multiple-exposure group and negative control group). The consumption of Pacific Herring tissues was confirmed on day 1 (22 h after exposure to tissues) by subsampling fish ($n = 10$) from each treatment group, fixing the stomach in a 10% solution of neutral buffered formalin, and examining histological sections of the stomach bolus for the presence of consumed tissues. The experiment was terminated 30 d after the initial feeding, and all survivors were euthanized via an overdose of buffered MS-222.

A second experiment was performed to examine the infectivity of *Ichthyophonus* sp. culture isolates to Pacific Herring by various exposure routes. Experimental fish consisted of SPF age-0 Pacific Herring (219 d old; FL [mean ± SD] = 63 ± 8.0 mm; weight = 2.0 ± 0.90 g) that were distributed among four treatments: gastric intubation, gill exposure, positive control, and negative control. Each treatment occurred in a separate 260-L tank that was supplied with single-pass, sand-filtered seawater (mean temperature = 8.6°C). The exposure inoculum was prepared by pooling *Ichthyophonus* sp. isolates (originally obtained from wild Pacific Herring) and suspending the parasite stages in phosphate-buffered saline. Individual fish within each treatment group received an average of 710 parasite schizonts (and an unquantifiable number of other parasite life stages) per 50-μL exposure dose. All experimental fish were anesthetized in buffered MS-222 prior to parasite exposure. Pacific Herring in the intubation treatment ($N = 40$) were exposed by gastric gavage of parasite suspensions into the stomach; those in the gill exposure group ($N = 42$) were exposed by lifting the left operculum and flushing parasite suspensions over the gill lamellae; those in the positive control group ($N = 40$) were exposed via IP injection of parasite suspensions; and those in the negative control group ($N = 33$) were intubated and injected with 50-μL aliquots of phosphate-buffered saline in lieu of *Ichthyophonus* sp. suspensions. To investigate the efficacy of the gastric intubation procedure, Pacific Herring were subsampled ($n = 3$ fish/sample) at 1 and 24 h postexposure; the gastrointestinal tract from each subsampled fish was fixed in 10% neutral buffered formalin and was processed by using standard histological sectioning methods. Histological sections were mounted on glass slides, stained using chromogenic in situ hybridization (CISH) procedures specific for *Ichthyophonus* sp. (Conway et al. 2015), and examined microscopically (100× magnification) for the presence of *Ichthyophonus* sp. The experiment was terminated at 29 d postexposure, when all surviving fish in each treatment were euthanized with an overdose of buffered MS-222.

The infection status of all sampled Pacific Herring (survivors and mortalities) from both experiments was assessed by microscopically examining tissue explant cultures for *Ichthyophonus* sp. life stages (Hershberger 2014). Hearts and livers were aseptically removed and cultured in tris-buffered Eagle's minimum essential medium supplemented with 5% fetal bovine serum, penicillin at 100 IU/mL, streptomycin at 100 μg/mL, and gentamycin at 100 μg/mL. Cultures were incubated at 15°C for 14 d, after which the prevalence of infection was determined by microscopic examination (40× magnification) for the presence of *Ichthyophonus* sp. stages.

The chi-square test was used to compare infection prevalence among treatment groups; statistical significance was assigned to comparisons with P-values less than 0.05.

RESULTS

Feeding the infected tissues to Pacific Herring resulted in successful transmission of *Ichthyophonus* sp.; however, transmission efficacy increased considerably after repeated feedings. The total prevalence of infection (mortalities plus survivors) was significantly greater among Pacific Herring that received multiple feedings with infected tissues (65%) than among those that received only a single feeding (5%; $P < 0.0001$; Table 1).

TABLE 1. Prevalence (%) of *Ichthyophonus* sp. infection as determined by explant culture of heart and liver tissues from Pacific Herring after they were fed infected fish tissues in a single exposure (on day 0) or in multiple exposures (16 feedings, occurring every other day). Tissues from survivors were cultured 30 d after the initiation of feeding.

	Infection prevalence (number positive/total)		
Treatment group	Mortalities	Survivors	All fish
Single exposure	5 (1/20)	4 (2/45)	5 (3/65)
Multiple exposures	38 (9/24)	96 (21/22)	65 (30/46)
Negative control	0 (0/13)	0 (0/16)	0 (0/29)

Ichthyophonus sp. was not detected by culture in any fish from the negative control group.

Single exposure of Pacific Herring to *Ichthyophonus* sp. isolates produced infections in more than 50% of challenged fish when the parasite was administered by IP injection but not when administered by gill exposure or gastric gavage. The total prevalence of infection (mortalities plus survivors) was significantly greater among Pacific Herring that were injected with isolate suspensions (74%) than among those that were exposed by gastric intubation (12%; $P < 0.0001$; Table 2). *Ichthyophonus* sp. stages were observed in CISH-stained histological sections of stomach bolus from two of the three individuals sampled from the intubated group at 24 h postexposure (Figure 1) but not in any of the three fish that were sampled at 1 h postexposure. *Ichthyophonus* sp. was not detected via culture in any fish from either the gill exposure group or the negative control group (Table 2).

DISCUSSION

The natural mechanism(s) responsible for *Ichthyophonus* sp. transmission to Pacific Herring must be highly efficient and ubiquitous throughout the northeast Pacific Ocean, as infection prevalence in the region is consistently high (Hershberger et al. 2002, and in press; Jones and Dawe 2002). Results from this study indicated that the primary routes of *Ichthyophonus* sp. transmission to Pacific Herring are unlikely to include either gill exposure to suspended parasite stages or the periodic ingestion of infected fish tissues. Although a single feeding with infected tissues did result in some transfer of *Ichthyophonus* sp., the transmission efficacy was low and resulted in an infection prevalence of only 5% (Table 1). The observed infection prevalence after multiple feedings (65%) was similar to the prevalence that would be expected after 16 independent feedings (56%), assuming a transmission efficacy of 5% for each independent exposure,

$$P_e = \frac{100\left[N_0 - N_0\,(1 - E_t)^{N_e}\right]}{N_0},$$

where P_e = the expected prevalence of infection resulting from multiple exposures; N_0 = the total number of uninfected individuals (i.e., 46 fish) at the beginning of the experiment; E_t = the efficacy of transmission for each independent exposure (0.05); and N_e = the number of exposures (i.e., 16).

Our results differed from those anecdotally reported for Atlantic Herring *Clupea harengus*; where force-feeding or multiple volitional feedings with "fungus material" resulted in only a low infection prevalence (4–12%), and single feedings—no matter how massive—failed to initiate any infections (Sindermann and Scattergood 1954). However, attempts to compare the infection prevalence values reported here and those reported by Sindermann and Scattergood (1954) are confounded by differences in host species, parasite genotype, exposure level, and sensitivity of the respective diagnostic techniques. Both Atlantic Herring and Pacific Herring are primarily planktivores, and there is no evidence substantiating the premise that repeated feedings of infected fish tissues occur under typical conditions. On rare occasions, clupeids can become cannibalistic on age-0 conspecifics (Holst 1992); however, the lack of support for cannibalistic transmission of *Ichthyophonus* sp. in Pacific

TABLE 2. Prevalence (%) of *Ichthyophonus* sp. infection as determined by explant culture of heart and liver tissues from Pacific Herring after exposure to pathogen culture isolates by various routes. Tissues from survivors were cultured at 29 d postexposure.

	Infection prevalence (number positive/total)		
Treatment group	Mortalities	Survivors	All fish
Gill exposure	0 (0/2)	0 (0/39)	0 (0/41)
Gastric intubation	40 (2/5)	7 (2/29)	12 (4/34)
Positive control (intraperitoneal injection)	100 (4/4)	71 (25/35)	74 (29/39)
Negative control	0 (0/6)	0 (0/26)	0 (0/32)

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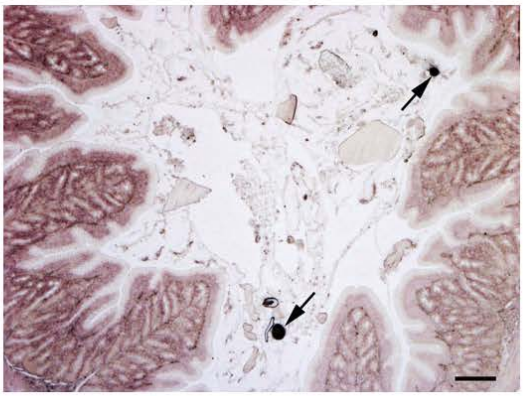

FIGURE 1. Histological section of a Pacific Herring stomach collected 24 h after the fish was subjected to gastric intubation with *Ichthyophonus* sp. isolates from culture (scale bar = 100 μm). Staining by chromogenic in situ hybridization for *Ichthyophonus*-specific DNA indicates the presence of *Ichthyophonus* life stages in the stomach lumen (arrows). *Ichthyophonus* was not detected via explant culture of heart and liver tissue from this fish. [Figure available online in color.]

Herring is indicated by the typically low infection prevalence in juvenile (prey) cohorts (Hershberger et al., in press) combined with the low transmission efficacy we observed for fish that received a single feeding of infected tissues. Furthermore, although adult Pacific Herring can consume copious numbers of conspecific eggs during spawning periods, there is no evidence that *Ichthyophonus* sp. is vertically transferred to or associated with eggs.

Although the consumption of infected fish tissues is unlikely to be the primary route of *Ichthyophonus* sp. transmission for Pacific Herring in the northeast Pacific Ocean, it may represent an important mechanism in localized schools of Pacific Herring. For example, although *Ichthyophonus* sp. infection prevalence is usually very low in juvenile Pacific Herring, elevated infection prevalence can occur among groups of juveniles that residualize in areas adjacent to offal discharges from fish processing plants (Hershberger et al., in press).

Infectious *Ichthyophonus* sp. cells are released from the skin surface of highly infected Pacific Herring (Kocan et al. 2010); however, the results of our experiments with a variety of direct transmission routes and the results of previous laboratory experiments involving cohabitation of *Ichthyophonus*-infected Pacific Herring and SPF conspecifics (Gregg et al. 2012) suggest that direct transmission is not the principal means of infection for this host. Therefore, we hypothesize that an intermediate invertebrate host may be involved in the transmission process for Pacific Herring and other planktivorous fishes. Similar to many other fish parasites, *Ichthyophonus* sp. infectivity to Pacific Herring may be enhanced after the pathogen passes through an intermediate host. Identification of an intermediate host would provide an important link in understanding the marine life cycle of this enigmatic pathogen.

Our experiments were not intended to assess host mortality or parasite pathogenicity; however, significant host mortalities occurred during both experiments, presumably as a result of anticipated constraints in the experimental design. For example, very small (mean FL = 35 mm), newly metamorphosed juvenile Pacific Herring were used during the first experiment, where cumulative mortality in the treatment groups ranged from 31% to 52% (Table 1). Although some mortality in the treatment groups may have resulted from of ichthyophoniasis disease, the majority of these mortalities likely resulted from emaciation and failure of the experimental Pacific Herring to feed on the blended fish tissues. At the end of the feeding experiment, most of the *Ichthyophonus*-positive Pacific Herring in the multiple-exposure group demonstrated heavy external signs of ichthyophoniasis. Similarly, mortalities in the second experiment ranged from 5% to 19% and were probably attributable to handling trauma associated with the challenge procedures. This hypothesis is supported by the observation that the negative control group experienced the most handling trauma (mock intubation plus mock IP injection) and the highest rate of mortality.

Laboratory experimentation using an in vivo model for *Ichthyophonus* sp. and Pacific Herring has been limited by an inability to successfully establish infections by any means other than IP injection using parasite isolates from cultures. This unnatural exposure route severely limits the types of question that can be addressed with this model system. However, the ability to reliably establish *Ichthyophonus* sp. infections in Pacific Herring after multiple feedings with infected tissues opens new avenues of research that were previously unavailable, including questions of disease kinetics, pathogenicity, and infective dose.

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Appendix 11

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The parasite *Ichthyophonus* sp. in Pacific herring from the coastal NE Pacific

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Abstract

The protistan parasite *Ichthyophonus* occurred in populations of Pacific herring *Clupea pallasii* Valenciennes throughout coastal areas of the NE Pacific, ranging from Puget Sound, WA north to the Gulf of Alaska, AK. Infection prevalence in local Pacific herring stocks varied seasonally and annually, and a general pattern of increasing prevalence with host size and/or age persisted throughout the NE Pacific. An exception to this zoographic pattern occurred among a group of juvenile, age 1+ year Pacific herring from Cordova Harbor, AK in June 2010, which demonstrated an unusually high infection prevalence of 35%. Reasons for this anomaly were hypothesized to involve anthropogenic influences that resulted in locally elevated infection pressures. Interannual declines in infection prevalence from some populations (e.g. Lower Cook Inlet, AK; from 20–32% in 2007 to 0–3% during 2009–13) or from the largest size cohorts of other populations (e.g. Sitka Sound, AK; from 62.5% in 2007 to 19.6% in 2013) were likely a reflection of selective mortality among the infected cohorts. All available information for *Ichthyophonus* in the NE Pacific, including broad geographic range, low host specificity and presence in archived Pacific herring tissue samples dating to the 1980s, indicate a long-standing host–pathogen relationship.

Keywords: Ichthyophonus, Pacific herring.

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Introduction

Common throughout the North Pacific Ocean, Pacific herring *Clupea pallasii* Valenciennes are members of a species assemblage that is collectively referred to as forage fish. By occupying an ecological niche in the middle of the food web, the forage fish assemblage is often characterized in the 'Wasp Waist Hypothesis' as the critical determinant of energy flow and productivity in some coastal marine systems (Rice 1995; Cury *et al.* 2000). Contrary to systems controlled by top-down or bottom-up forces, where limits on

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Journal of Fish Diseases

ecosystem productivity are exerted at the level of primary production or at the level of highest order predators, respectively; the Wasp Waist Hypothesis asserts that forage fish exert top-down control on all lower trophic levels and bottom-up control on all upper trophic levels (Fauchald *et al.* 2011). As such, sudden changes in forage fish abundances, demographics and assemblages cascade throughout multiple trophic levels in the ecosystem. For example, a sudden mass mortality of pilchards in south Australia (Griffin *et al.* 1997; Jones *et al.* 1997; Ward *et al.* 2001), presumably caused by disease from an introduced herpesvirus (Hyatt *et al.* 1997; Gaughan, Mitchell & Blight 2000; Whittington *et al.* 2008), was followed by diet shift, breeding failure and starvation of closely associated seabirds (Bunce & Norman 2000; Dann *et al.* 2000). Large oscillations in abundance are common in forage fish populations (Schwartzlose & Alheit 1999), yet ecological drivers of these population changes remain poorly understood. Determining the causes of population oscillations in highly migratory marine fishes is particularly difficult, owing largely to observational difficulties; however, cyclic declines in red grouse populations from Britain can be initiated by host/parasite interactions (Hudson, Dobson & Newborn 1998; Redpath *et al.* 2006). We hypothesize that analogous ecological mechanisms can influence populations of wild marine fishes.

Ichthyophonus hoferi is a parasite that causes systemic infections primarily in marine fishes. Taxonomic and phylogenetic uncertainties have accompanied *I. hoferi* since its original description (reviewed McVicar 2011); however, the organism is currently considered a member of the Mesomycetozoea, a monophyletic class of organisms that arose near the time when animals diverged from fungi (Mendoza, Taylor & Ajello 2002). A lack of distinguishing characteristics provided in the original species description (von Hofer 1893), combined with a high degree of morphological plasticity in life history stages (Okamoto *et al.* 1985), likely resulted in the grouping of several closely related organisms into a species assemblage that is collectively referred to as *I. hoferi* (reviewed in McVicar 2011). Phenotypic (Rand 1994; Hershberger *et al.* 2008) and genotypic (Rand *et al.* 2000; Criscione *et al.* 2002; Halos *et al.* 2005; Hershberger *et al.* 2010; Rasmussen *et al.* 2010) differences in *I. hoferi* isolates from fishes provide preliminary evidence for apparent speciation within the genus. Consequently, several attempts at species designations have been made, including *Ichthyophonus hoferi*, *Ichthyophonus irregularis*, *Ichthyophonus gasterophilum*, *Ichthyophonus lotae*, *Ichthyophonus intestinalis* (reviewed in Rand 1990; Rand *et al.* 2000); however, the species descriptions are often incomplete, duplicative and based on subjective criteria. To avoid further perpetuation of confusion surrounding the *Ichthyophonus* spp. complex, the organism(s) will hereafter be referred to generically as *Ichthyophonus*.

Ichthyophonus is perhaps the most ecologically and economically significant pathogen of wild marine fishes throughout the world, based on its low host specificity, broad geographic range and recurring association with epizootics that result in massive fish kills and population-level impacts (reviewed in Burge *et al.* 2014). The parasite has been reported in more than 35 species of marine fishes, and more than 80 species are reported as susceptible to infection (reviewed in McVicar 2011). Additionally, recurring epizootics have been reported in Atlantic herring *Clupea harengus* populations throughout the coastal regions of the Atlantic Ocean, Chinook salmon in the Yukon River (Alaska and Canada), yellowtail flounder in the western North Atlantic and American shad in the Columbia River (Washington and Oregon) (reviewed in Burge *et al.* 2014). *Ichthyophonus* is also endemic in some populations of Pacific herring (Hershberger *et al.* 2002; Jones & Dawe 2002), where mortality from the resulting disease remains a leading hypothesis accounting for the population decline and failed recovery in Prince William Sound (Marty *et al.* 2003, 2010). Details regarding the epizootiology of *Ichthyophonus* in Pacific herring remain largely uninvestigated, including its geographic range, seasonal and interannual persistence, and differences in infection prevalence within and between genetically distinct host metapopulations. The objective of this study was to examine these information gaps using the results from *Ichthyophonus* prevalence surveys in populations of Pacific herring that were performed throughout the northeast Pacific Ocean over a recent 11-year period.

Methods

The prevalence of *Ichthyophonus* was assessed in populations of Pacific herring from California to Alaska during 2003–13 (Table 1). Throughout this range, Pacific herring were collected by trawl,

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Table 1 Prevalence of *Ichthyophonus* in Pacific herring populations

Year	Stock	Site	Collection date	Gear type	Adult/ Juvenile (A/J)	Mean fork length mm (SD)	*Ichthyophonus* prevalence
2003	Puget Sound	Pt Orchard/Madison	February 5	Trawl	A	176 (19)	31% (28/120)
		Skagit Bay	February 11	Trawl	A	161 (10)	42% (25/60)
		Port Susan	February 12	Trawl	A	171 (14)	47% (28/60)
		Port Gamble	February 25	Trawl	J	122 (24)	25% (15/60)
2004	Puget Sound	Cherry Point	May 4	Trawl	A	218 (17)	53% (37/70)
		Cherry Point	May 23	Gill Net	A	202 (21)	40% (24/60)
2005	Puget Sound, WA	Port Gamble Bay	February 2	Trawl	A	181 (15)	53% (32/60)
		Port Townsend Bay	February 2	Trawl	J	115 (15)	13% (8/60)
		Port Gamble Bay	March 3	Gill Net	A	214 (9)	68% (41/60)
		Port Gamble Bay	March 9	Gill Net	A	191 (17)	48% (57/120)
		Kilisut Harbor	March 17	Gill Net	A	226 (11)	50% (26/52)
		Hood Canal	April 28	Trawl	A	191 (17)	69% (35/51)
		Cherry Point	May 2	Trawl	A	178 (11)	27% (16/60)
		Cherry Point	May 3	Gill Net	A	213 (22)	47% (28/60)
		Cherry Point	May 11	Gill Net	A	216 (23)	53% (32/60)
		Cherry Point	May 26	Gill Net	A	228 (28)	52% (32/62)
		Hood Canal	June 7	Trawl	A	179 (11)	73% (44/60)
	Cook Inlet, AK	Kamishak Bay	May 16	Purse Seine	A	ND	38% (20/53)
		Kamishak Bay	May 16	Purse Seine	A	ND	55% (24/44)
2006	Puget Sound, WA	Case Inlet	January 17	Trawl	A	170 (13)	5% (3/59)
		Quartermaster Harbor	January 17	Trawl	A	168 (11)	18% (11/60)
		Pt Orchard/Madison	February 2	Trawl	A	177 (20)	14% (8/59)
		Skagit Bay	February 2	Trawl	A	174 (10)	34% (20/59)
		Holmes Harbor	March 30	Gill Net	A	193 (13)	50% (30/60)
	Cook Inlet (AK)	Kamishak Bay	May 18	Purse Seine	A	ND[a]	18% (11/60)
		Kamishak Bay	May 21	Purse Seine	A	ND[a]	17% (10/60)
		Kamishak Bay	May 22	Purse Seine	A	ND[a]	10% (10/60)
2007	Puget Sound, WA[a]	Johnson Point	January 18	Trawl	A	181 (8)	7% (4/59)
		Pt Orchard/Madison	February 1	Trawl	A	181 (11)	17% (10/60)
		Skagit Bay	February 8	Trawl	A	184 (11)	37% (22/60)
		Cherry Point	April 30	Trawl	A	184 (13)	25% (15/60)
	Cook Inlet, AK	Kamishak Bay	May 16	Purse Seine	A	ND[a]	32% (19/60)
		Kamishak Bay	May 27	Purse Seine	A	ND[a]	20% (12/59)
		Kamishak Bay	May 27	Purse Seine	A	ND[a]	28% (17/60)
	Prince William Sound, AK	St. Matthews Bay	April 5	Cast Net	A	224 (17)	42% (25/60)
		Simpson Bay	April 19	Purse Seine	J	86 (6)	15% (9/60)
		Sawmill Bay	November 30	Purse Seine	A	215 (21)	25% (15/60)
		Simpson Bay	December 2	Purse Seine	A	187 (13)	37% (22/60)
	Sitka Sound, AK	S. Cannon Island	April 9	Cast Net	A	215 (18)	28.3% (17/60)
	Lynn Canal, AK	Benjamin Island	November 10	Trawl	A	199	11% (7/61)
2008	Puget Sound, WA	Drayton Pass	January 15	Trawl	A	144 (7)	2% (1/60)
		Pt Orchard/Madison	February 5	Trawl	A	154 (16)	7% (4/60)
		Skagit Bay	February 2	Trawl	A	176 (17)	23% (14/60)
		Holmes Harbor	March 13	Trawl	A	193 (8)	48% (29/60)
	Prince William Sound, AK	Fish Bay	March 19	Purse Seine	A	236 (27)	33% (19/58)
		Unknown	March 17	Purse Seine	J	141 (11)	20% (12/59)
		Whale Bay	March 24	Purse Seine	J	149 (22)	15% (9/60)
		Port Gravina	November 8–12	Purse Seine	A	197 (23)	24% (19/80)
		Simpson Bay	November 8–12	Purse Seine	J	65 (7)	0% (0/78)
	Sitka Sound, AK	Beli Rock	March 5	Purse Seine	A	262 (14)	30% (18/60)
		N. Middle Island	March 26	Purse Seine	A	249 (14)	28% (17/60)
	Lynn Canal, AK	Breadline	February 23	Trawl	A	ND	5% (3/61)
		Cohen Island	April 12	Trawl	A	ND	5% (3/61)
		Aaron Island	May 10	Trawl	A	ND	19% (11/59)
2009	Puget Sound, WA	Pt Orchard/Madison	February 2	Trawl	A	170 (9)	3% (2/60)
		Skagit Bay	February 2	Trawl	A	166 (23)	18% (11/60)
		Port Gamble	February 12	Trawl	A	169 (12)	27% (16/60)
		Holmes Harbor	March 18	Trawl	A	193 (20)	22% (13/60)

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Table 1 *Continued*

Year	Stock	Site	Collection date	Gear type	Adult/ Juvenile (A/J)	Mean fork length mm (SD)	*Ichthyophonus* prevalence
	Cook Inlet, WA	Kamishak Bay	May 8	Purse Seine	A	ND[a]	3% (2/60)
		Kamishak Bay	May 21	Purse Seine	A	ND[a]	2% (1/60)
	Prince William Sound, AK	Port Gravina	March 20	Purse Seine	A	199 (15)	43% (26/60)
		Port Gravina	March 20	Purse Seine	J	168 (11)	25% (15/60)
		Simpson Bay	March 22	Purse Seine	J	94 (8)	13% (8/60)
		Snug Corner Cove	April 13	Cast Net	A	217 (27)	26% (16/62)
		Port Gravina	April 4–9	Gill Net	A	222 (24)	45% (27/60)
		Port Gravina	November 15	Purse Seine	A	179 (17)	12% (7/60)
		Elrington Pass	November 17	Purse Seine	A	216 (19)	17% (10/60)
		Simpson Bay	November 19	Purse Seine	J	87 (14)	5% (3/60)
		Eaglek Bay	November 14	Purse Seine	J	98 (4)	3% (1/29)
		Lower Herring Bay	November 16	Purse Seine	J	99 (4)	0% (0/14)
		Simpson Bay	November 19	Purse Seine	J	70 (12)	5% (1/20)
	Sitka Sound, AK	Guide Island	February 15–16	Trawl	A	256 (15)	40% (32/80)
		Unknown	March 24	Purse Seine	A	270 (19)	46% (20/44)
		St. John Baptist Bay	March 26	Purse Seine	A	248 (23)	31% (21/67)
		Unknown	March 27	Purse Seine	J	175 (7)	4% (3/69)
	Lynn Canal, AK	Amalga Trench	February 11–12	Trawl	A	203 (15)	7% (3/44)
		Fritz Cove	March 18–19	Trawl	A	ND	13% (8/60)
		Benj. Isl. Trench	November 24	Gill Net	A	210 (14)	18% (11/60)
		Benj. Isl. Trench	December 7	Gill Net	A	198 (23)	8% (5/60)
	San Francisco Bay, CA	Pt. Chauncey	February 11	Gill Net	A	155 (15)	0% (0/81)
		Pt Chauncey	February 25	Gill Net	A	149 (18)	0% (0/60)
2010	Puget Sound, WA	Squaxin Pass	January 28	Trawl	A	140 (12)	3% (2/60)
		Holmes Harbor[b]	March 23	Trawl	A	171 (15)	28% (17/60)
	Cook Inlet, AK	Kamishak Bay	May 4	Purse Seine	A	ND[a]	2% (1/60)
		Kamishak Bay	May 18	Purse Seine	A	ND[a]	3% (2/60)
	Prince William Sound, AK	Port Gravina	March 16	Purse Seine	A	213 (14)	18% (11/60)
		Port Fidalgo	March 19	Purse Seine	A	200 (15)	23% (14/60)
		Simpson Bay	March 20	Purse Seine	J	109 (23)	13% (8/60)
		Cordova Harbor	June 2–13	Cast Net	J	85 (12)	35% (17/49)
		Cordova Harbor	August 18	Cast Net	J	44 (2.8)	0% (0/18)
		Cordova Harbor	September 28 – October 7	Cast Net	J	50 (5.9)	0% (0/22)
		Simpson Bay	November 2	Purse Seine	J	73 (6.8)	0% (0/38)
		Port Fidalgo	November 4	Purse Seine	J	77 (3.7)	0% (0/22)
		Eaglik	November 5	Purse Seine	J	90 (8.7)	0% (0/34)
		Whale Bay	November 10–11	Purse Seine	J	95 (33)	3% (2/58)
	Sitka Sound, AK	Indian River	March 22	Purse Seine	A	242 (22)	27% (16/60)
		Boarder/Sitka Rocks	March 23	Purse Seine	A	209 (28)	15% (9/60)
		Kruzof Island	March 24	Purse Seine	A	241 (25)	37% (22/60)
	Lynn Canal, AK	Shelter Island	March 15–16	Gill Net	A	202 (20)	5% (3/56)
		Bridget Cove	April 26	Cast Net	A	212 (11)	13% (5/40)
2011	Cook Inlet, AK	Kamishak Bay	May 4	Purse Seine	A	ND[a]	0% (0/60)
		Kamishak Bay	May 13	Purse Seine	A	ND[a]	2% (1/60)
	Prince William Sound, AK	Lower Herring Bay	March 11	Cast Net	J	95 (3.9)	2% (1/60)
		Eaglik Bay	March 15	Cast Net	J	113 (22)	5% (3/60)
		Port Fidalgo	March 16	Cast Net	J	76 (5.8)	10% (6/60)
		Port Gravina	April 4	Cast Net	A	219 (20)	27% (16/60)
		Port Gravina	April 6	Purse Seine	A	253 (13)	47% (28/60)
		Port Gravina	November 21	Purse Seine	A	205 (20)	63% (19/30)
		Port Gravina	November 22	Purse Seine	J	157 (12)	13% (4/30)
		Simpson Bay	November 15	Cast Net	J	60 (6.1)	0% (0/57)
		Whale Bay	November 20	Cast Net	J	83 (8.2)	0% (0/60)
		Simpson Bay	December 13	Cast Net	J	60 (5.0)	0% (0/60)

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Table 1 *Continued*

Year	Stock	Site	Collection date	Gear type	Adult/ Juvenile (A/J)	Mean fork length mm (SD)	*Ichthyophonus* prevalence
	Sitka Sound, AK	Bear Cove	March 24	Cast Net	J	108 (11)	2% (1/60)
		Long Island	March 22	Purse Seine	A	232 (16)	18% (11/60)
		Salisbury Island	April 6	Cast Net	A	228 (20)	20% (12/60)
	Lynn Canal, AK	Halibut Cove	January 12	Gill Net	A	ND	2% (1/60)
		Amalga Trench	January 28	Trawl	A	ND	10% (6/60)
		Amalga Trench	April 9	Gill Net	A	ND	18% (11/60)
		Auke Bay[c]	April 18, June 4	Beach Seine	A	202 (15)	18% (11/60)
	British Columbia, Canada	Little Qualicum	March 1	Purse Seine	A	180 (16)	23% (14/60)
		Sydney Inlet	March 17	Purse Seine	A	189 (14)	8% (5/60)
		Prince Rupert,	March 23	Purse Seine	A	183 (16)	20% (12/60)
		Kwakshua Inlet	March 23	Purse Seine	A	167 (18)	22% (13/60)
		Prince Rupert	March 24	Purse Seine	A	194 (16)	27% (16/60)
		Haida Gwaii	March 26	Purse Seine	A	191 (12)	8% (5/60)
		Haida Gwaii	March 30	Purse Seine	A	192 (13)	5% (3/60)
2012	Cook Inlet, AK	Kamishak Bay	May 7	Purse Seine	A	ND[a]	2% (1/60)
	Prince William Sound, AK	Simpson Bay	January 11	Cast Net	J	57 (2.8)	0% (0/60)
		Simpson Bay	April	Cast Net	J	ND	3% (1/30)
		Port Gravina	March 28	Purse Seine	A	218 (16)	42% (25/60)
		Port Gravina	March 31	Purse Seine	A	215 (21)	40% (24/60)
		Fidalgo Bay	April 2	Purse Seine	A	231 (19)	35% (21/60)
		Port Gravina	November 15	Purse Seine	A	159 (14)	3% (2/60)
	Sitka Sound, AK	N. Khasiana Isl.	April 3	Cast Net	A	232 (23)	20% (12/60)
		St. John Bay	April 4	Purse Seine	A	214 (24)	32% (19/60)
		Sitka breakwall	April 4	Cast Net	A	225 (22)	10% (6/60)
	Lynn Canal, AK	Tee Harbor	June 8	Hook-and-Line	A	176 (13)	0% (0/60)
2013	Puget Sound, WA	Hood Canal[d]	May 19	Trawl	A	171 (18)	57% (25/44)
	Cook Inlet, AK	Kamishak Bay	May 20	Purse Seine	A	ND[a]	2% (1/60)
	Prince William Sound, AK	Port Gravina	March 27	Purse Seine	J	147 (16)	3% (2/60)
		Port Gravina	March 31	Purse Seine	A	232 (20)	34% (20/59)
		Port Gravina	April 1	Purse Seine	A	225 (23)	32% (19/60)
	Sitka Sound, AK	Apple Islands	March 29	Cast Net	A	246 (28)	18% (11/60)
		Silver Bay	March 30	Purse Seine	A	251 (16)	18% (11/60)
		Unknown	March 30	Purse Seine	A	226 (26)	18% (11/60)
	Craig, AK	Diamond Point	February 20	Cast Net	A	214 (23)	22% (13/60)

[a]ND: Herring lengths in Cook Inlet from 2006 to 2013 were recorded as standard length, not fork length.
[b]Biased population sample: largest fish were removed from this sample for other purposes prior to determination of *Ichthyophonus* prevalence.
[c]Herring from Lynn Canal on 18 April and 4 June 2011 were transported to a tank at the NOAA Ted Stevens Marine Science Laboratories prior to sampling for *Ichthyophonus*. Tank confinement likely had little impact on the reported prevalence, as *Ichthyophonus* is not easily transferred between Pacific herring through cohabitation (Gregg *et al.* 2012).
[d]Sample consisted of post-spawn herring.

gill net, purse seine, cast net, beach seine or hook-and-line. *Ichthyophonus* prevalence at each location was determined by tissue explant culture. The heart from each fish was aseptically removed and immersed in *Ichthyophonus* growth medium (>1:5 W:V), consisting of tris-buffered Eagle's Minimum Essential Medium supplemented with 5% foetal bovine serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 100 μg ml^{-1} gentamycin (MEM). Explant cultures were examined microscopically (40× magnification) for the presence of *Ichthyophonus* after 7 and 14 days; any cultures without detectable *Ichthyophonus* schizonts or hyphae after 14 days were considered negative. Fork length or standard length, sex and age (from scales) were recorded from a portion of the sampled fish. *Ichthyophonus* prevalences were compared by chi-square (χ^2) test, with statistical significance assigned to comparisons with $P < 0.05$.

The historical presence of *Ichthyophonus* in Puget Sound was evaluated from formalin-fixed

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Pacific herring specimens archived at the University of Washington Fish Collection. Hearts were removed from archived specimens and embedded in paraffin blocks, following standard histological procedures. Thin sections (2–3 μm) of the hearts were stained with periodic acid–Schiff (PAS) and examined microscopically for the presence of PAS-positive *Ichthyophonus* schizonts and hyphae.

Results

Ichthyophonus occurred in Pacific herring populations throughout the eastern North Pacific Ocean, ranging from Puget Sound, WA north to the Gulf of Alaska (Table 1); the parasite was not detected in any Pacific herring samples as far south as San Francisco Bay, CA ($n = 141$). On an annual basis, the prevalence of *Ichthyophonus* infection was not consistent in Pacific herring stocks throughout this range; for example, infection prevalence in adults during 2008 ranged from 5% in Lynn Canal, AK to 48% in Holmes Harbor (Puget Sound), WA. This disparate prevalence pattern also occurred on smaller geographic scales, with the infection prevalence in adult Pacific herring throughout Puget Sound, WA ranging from 5% in Case Inlet to 50% in Holmes Harbor during 2006. Similarly, infection prevalence differed among Pacific herring collected throughout British Columbia during 2011 (Table 1), with higher prevalence occurring in prespawn populations that overwintered in nearshore locations than those that overwintered in offshore locations (Fig. 1). Interannual changes also occurred in the infection prevalence from specific sampling locations; for example, the infection prevalence in adult Pacific herring from Cook Inlet, AK declined from 38–55% in 2005 to 2–3% in 2009.

Among samples of prespawn Pacific herring collected in the spring, the prevalence of *Ichthyophonus* generally increased with host size and age (Figs 2–5). For example, the annual spring infection prevalence in Prince William Sound increased with size class from 15 to 60% in 2008, 16–80% in 2009, 13 to 33% in 2010, 13–47% in 2011 and 4–50% in 2013; the trend was less apparent in 2012, where an apparent high prevalence in a small size cohort (161–180 mm) was likely skewed by small sample size ($n = 4$; Fig. 3a). Among Pacific herring collected in the spring, *Ichthyophonus* prevalence in each age class followed an analogous pattern as size; for example, the infection prevalence in Lower Cook Inlet increased from 0% in age 2- to 3-year cohorts to 50% in age 8+ year cohorts during 2006, and from 7% among age 2- to 3-year cohorts to 67% in age 8+ year cohorts during 2007 (Fig. 4b). The infection prevalence in Lower Cook Inlet dropped dramatically after 2007, after which the parasite was detected only in low percentages among the largest size classes/oldest age cohorts (Fig. 4a,b). This increased prevalence with size/age was also generally reflected in lower infection prevalence among collections of juveniles than of adults (Table 1). However, a single exception to this pattern occurred with the detection of an unusually high *Ichthyophonus* prevalence (35%) in juvenile cohorts from Cordova Harbor (Prince William Sound, AK) during June, 2010. The infection prevalence of Pacific herring collected in

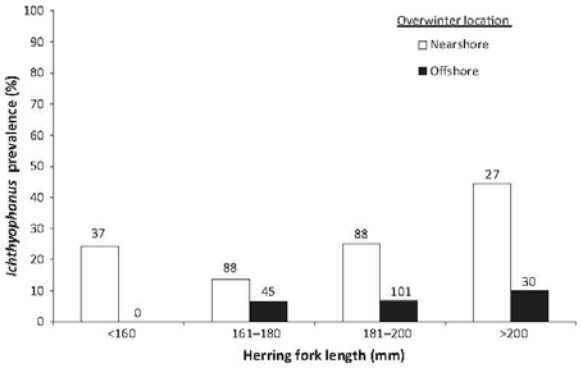

Figure 1 *Ichthyophonus* infection prevalence in each size class of Pacific herring collected during the spring of 2011 from British Columbia. Nearshore samples included herring from Little Qualicum, Prince Rupert and Kwakshua Inlet; offshore samples included those from Sydney Inlet and Haida Gwaii (Table 1). Numerals above the bars indicate sample size (n).

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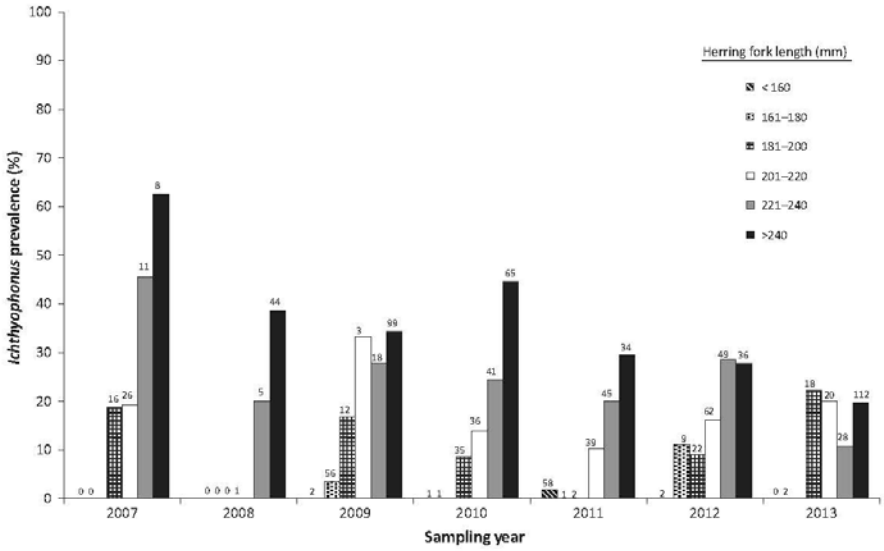

Figure 2 *Ichthyophonus* infection prevalence in each size class in spring samples of Pacific herring from Sitka Sound, AK during the spring. Numerals above the bars indicate sample size (*n*).

the spring (Fig. 3a) did not necessarily correspond with that of samples collected in the fall of the same year (Fig. 3b).

A consistent pattern between *Ichthyophonus* prevalence and Pacific herring sex did not occur (Table 2), as the infection prevalence was not significantly ($P = 0.97$) different between males and females that were combined from all locations and years (25.2% and 25.1%, respectively). Similarly, interannual infection prevalence was not significantly different ($P \geq 0.09$) between males and females from general geographic locations including Puget Sound (33.2% and 30.0%, respectively), Cook Inlet (11.2 and 8.8%, respectively), Prince William Sound (30.8% and 31.0%) and Lynn Canal (25.2% and 25.1%). Statistical significance ($P \leq 0.03$) appeared to occur in some intra-annual comparisons between males and females from some locations, including Puget Sound in 2003 (64.0% and 29.7%, respectively) and 2010 (19.7% and 2.7%, respectively), Prince William Sound in 2010 (27.0% and 6.8%, respectively), Sitka Sound in 2011 (11.8% and 28.8%, respectively) and Lynn Canal (8.1% and 34.8%, respectively). However, these apparent patterns were likely reflective of a type 1 error due to multiple testing, as the *Ichthyophonus*-dominant sex was inconsistent among these groups, with males having a higher prevalence in three groups and females having a higher prevalence in two groups.

Analysis of archived samples indicated that *Ichthyophonus* occurred in Pacific herring populations since at least 1986 (Table 3). Histological sections of Pacific herring hearts with PAS-positive *Ichthyophonus* schizonts were identified in archived samples that were collected from Puget Sound on 21 October 1986 (6/9 samples), 18 August 1987 (3/8 samples), 13 October 1987 (3/11 samples) and 14 October 1987 (1/9 samples).

Discussion

The widespread distribution of *Ichthyophonus* in Pacific herring throughout the west coast of North America provides some indication of the mechanisms involved in the perpetuation and transmission of the parasite. The natural route(s) of *Ichthyophonus* transmission in Pacific herring remain unresolved, and laboratory studies have

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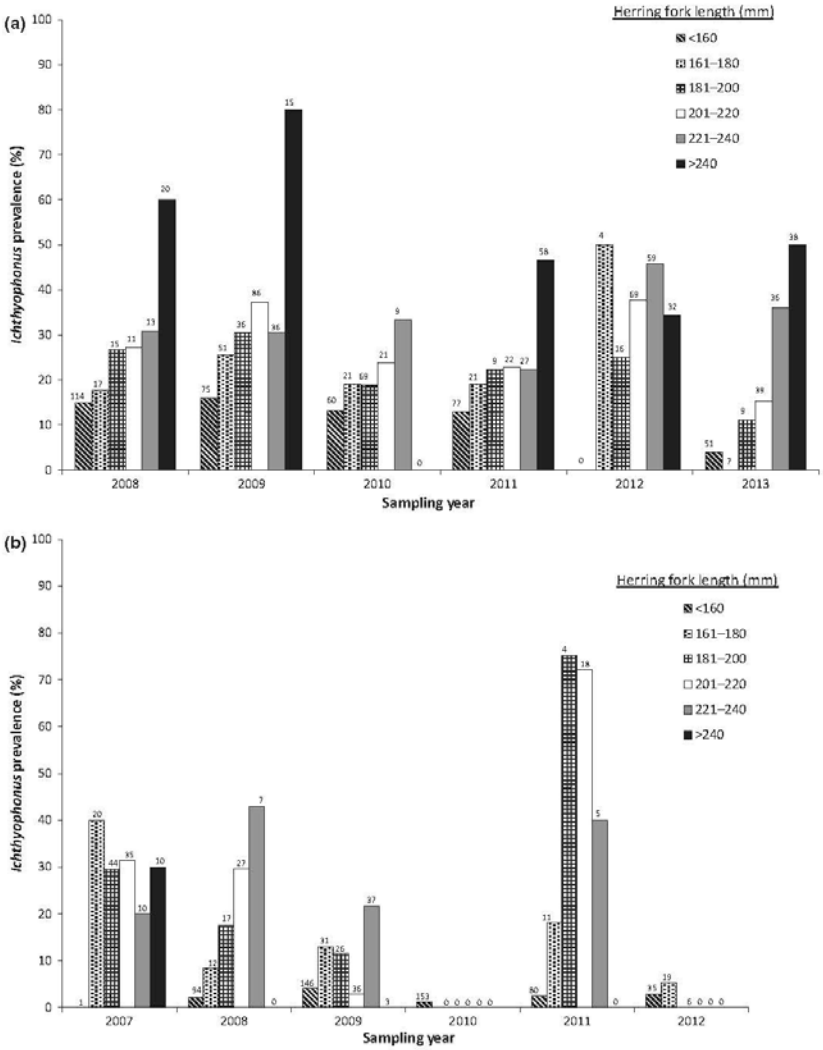

Figure 3 *Ichthyophonus* infection prevalence in each size class of Pacific herring collected from Prince William Sound, AK during the spring (a) and fall (b). Numerals above the bars indicate sample size (*n*). Note that the spring/fall sampling years are offset on the respective horizontal axes.

generally been unsuccessful at demonstrating transmission by host cohabitation, immersion in parasite isolates or feeding with infected tissues or isolates (Gregg *et al.* 2012). A leading hypothesis accounting for the transmission of *Ichthyophonus* to Pacific herring includes the possible involvement of an intermediate host, where the parasite likely develops into a stage that becomes more infectious to Pacific herring. This hypothesis is further supported by extreme plasticity in

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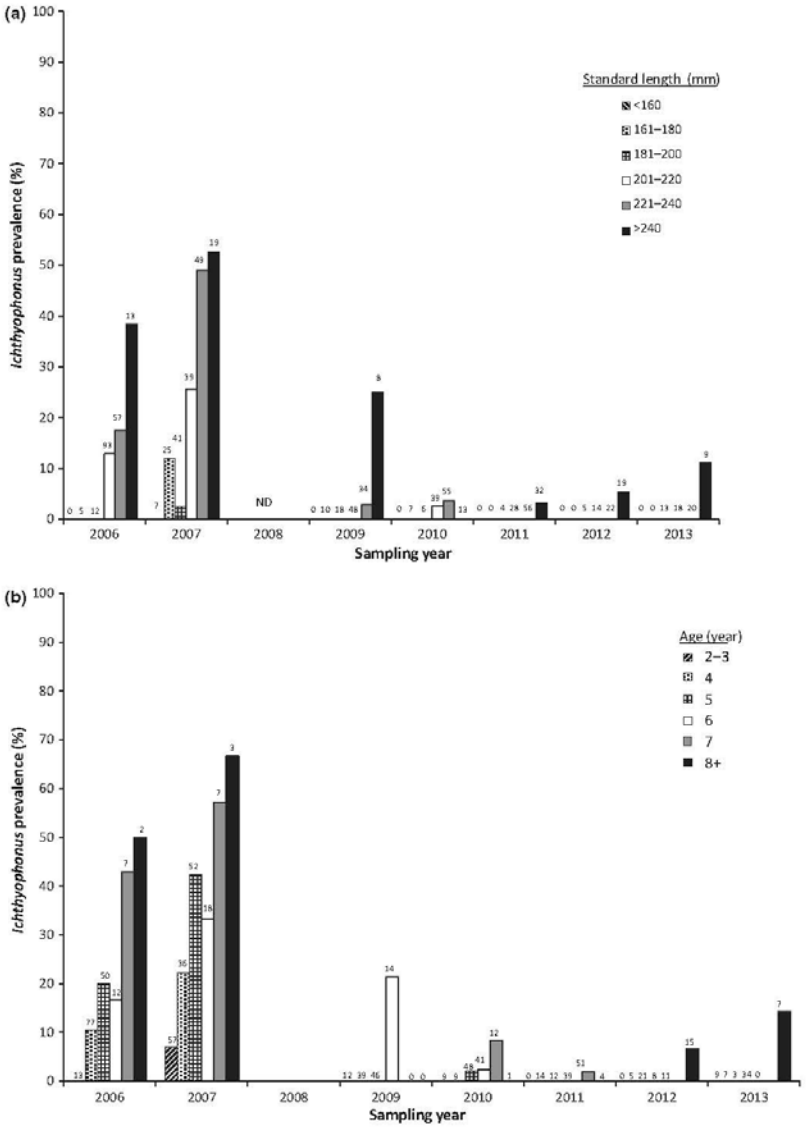

Figure 4 *Ichthyophonus* infection prevalence in each size (a) and age (b) class of Pacific herring collected from Cook Inlet, AK during the spring. Numerals above the bars indicate sample size (*n*).

Ichthyophonus morphology that can be induced by manipulating various host and culture conditions. If an intermediate host (or hosts) exists, then the results from this study indicate that its geographic range must be very expansive throughout the NE Pacific. Further, patchiness or seasonality of this

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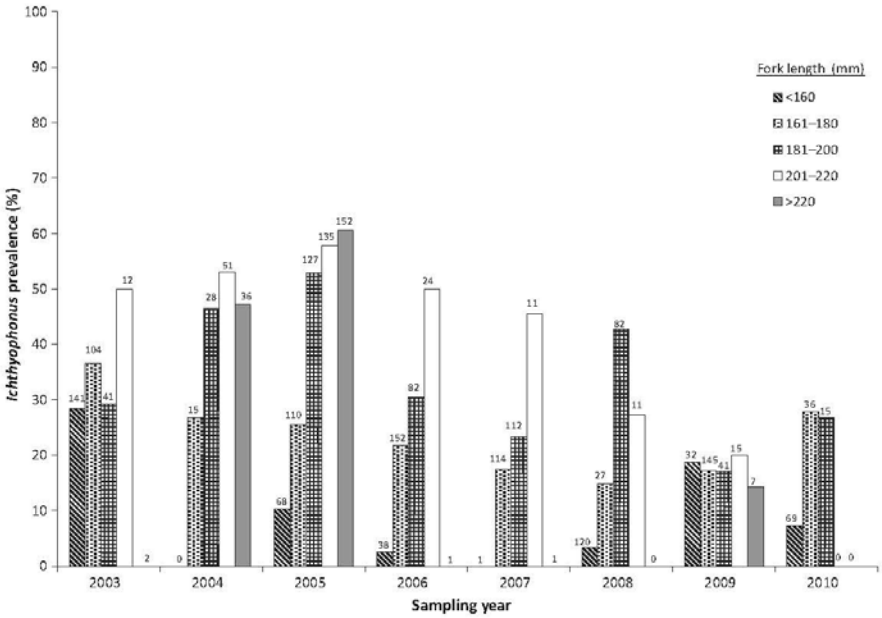

Figure 5 *Ichthyophonus* infection prevalence in each class of Pacific herring collected from Puget Sound, WA during the spring. Numerals above the bars indicate sample size (*n*).

proposed intermediate host may partially explain the differences in infection prevalence that we detected over relatively small geographic distances within Puget Sound (Table 1) and between Pacific herring populations in British Columbia that overwinter in nearshore vs. offshore locations (Fig. 1). Similar spatial differences in *Ichthyophonus* prevalence also occur in populations of Atlantic herring, which have been hypothesized to result from exposure differences resulting from host seasonal migration patterns (Kramer-Schadt, Holst & Skagen 2010).

For Pacific herring, a lack of detections from San Francisco Bay, CA suggests that the southern extreme of *Ichthyophonus* range may end north of this latitude (Table 1) and the northern extreme of its range ends south of the Bering Sea (Kocan, Hershberger & Winton 2004). An expansive area exists between the two southernmost sampling locations in this study (Puget Sound and San Francisco Bay), and Pacific herring populations throughout this gap tend to be relatively small; however, it is likely that *Ichthyophonus* occurs in some of these stocks, as the parasite can occur in high prevalence among another clupeid, American shad (*Alosa pseudoharengus*), from the Oregon/Washington boarder (Hershberger *et al.* 2010). Although these results indicate that Pacific herring represent a dominant host species for *Ichthyophonus* in the NE Pacific, other species appear to maintain the parasite northward into the Bering Sea, where a paucity of infections in Pacific herring (Kocan *et al.* 2004) are replaced by often-high prevalences in Chinook salmon and walleye pollock (White, Morado & Friedman 2014). The geographic extremes of the *Ichthyophonus* range in Pacific herring likely shift on an interannual basis in response to changing climatic conditions, ocean circulation patterns, water temperatures and invertebrate assemblages. For example, the prevalence of *Ichthyophonus* in Pacific herring from southern Cook Inlet, AK, near the current northern boundary of the *Ichthyophonus* range in Pacific herring, declined from 20–32% in 2007 to 2–3% in 2009. We hypothesize that this recent decrease in infection prevalence at the northern extreme of

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Table 2 Comparison of *Ichthyophonus* prevalence between male and female Pacific herring

Puget sound	% infected males (*n*)	% infected females (*n*)	*P* value (χ^2)
2003	64.0% (86)	29.7% (138)	1.0×10^{-7}
2004	48.2% (56)	45.1% (71)	0.86
2005	52.9% (121)	55.8% (120)	0.89
2006	24.3% (152)	22.9% (140)	0.87
2007	20.3% (143)	22.6% (93)	0.94
2008	29.2% (96)	27.1% (59)	0.93
2009	20.8% (101)	16.5% (91)	0.56
2010	19.7% (76)	2.7% (37)	0.03
Total	33.2% (831)	30.0% (749)	0.19
Cook Inlet			
2006	15.7% (89)	14.4% (90)	0.97
2007	27.4% (113)	25.4% (67)	0.90
2009	2.9% (69)	0% (51)	0.61
2010	5.3% (57)	1.6% (63)	0.54
2011	1.3% (76)	2.3% (43)	0.74
2012	2.8% (36)	0% (24)	0.84
2013	0% (23)	2.7% (37)	0.81
Total	11.2% (463)	8.8% (375)	0.30
Prince William Sound			
2007	28.9% (45)	27.9% (68)	0.92
2008	32.6% (43)	21.4% (42)	0.36
2009	28.3% (152)	28.9% (142)	0.99
2010	27.0% (37)	6.8% (44)	0.03
2011	39.1% (87)	42.3% (78)	0.79
2012	29.9% (107)	40.4% (89)	0.16
2013	29.7% (64)	35.1% (57)	0.66
Total	30.8% (535)	31.0% (520)	0.98
Sitka Sound			
2007	27.8% (36)	30.4% (23)	0.94
2008	31.0% (29)	29.0% (31)	0.91
2009	30.2% (63)	45.8% (48)	0.13
2010	24.1% (83)	28.1% (96)	0.66
2011	11.8% (68)	28.8% (96)	0.03
2012	21.1% (123)	20.4% (49)	0.92
2013	18.7% (91)	18.0% (89)	0.94
Total	22.1% (493)	27.3% (338)	0.09
Lynn Canal			
2009	9.55% (42)	19.0% (58)	0.31
2010	15.2% (33)	4.8% (63)	0.17
2011	8.1% (37)	34.8% (23)	0.02
Total	10.7% (112)	15.2% (144)	0.38
Total (all locations, all years)	25.2% (2434)	25.1% (2176)	0.97

the parasite range in Pacific herring reflects a wandering northern boundary that is influenced by ocean and climatic conditions.

Reason(s) for the increased *Ichthyophonus* prevalence with Pacific herring size and age (Figs 2–5) remain unknown; however, this pattern is consistent over broad spatial–temporal scales in populations of Pacific (Hershberger *et al.* 2002; Marty *et al.* 2003) and Atlantic herring (Kramer-Schadt *et al.* 2010). This zoographic pattern is consistent with that of a chronic infection that accumulates in a population via recurring exposures throughout the lifetime of the host. The timing, location and route of *Ichthyophonus* exposures to Pacific herring remain unknown; however, this accumulation hypothesis suggests that the clearance or elimination of the parasite from infected host tissues must be a rare event. It is important to consider this *Ichthyophonus* pattern with Pacific herring age/size when surveying wild populations. For example, a standard 60 fish sample from a population may contain a biased age/size distribution due to sampling gear/techniques; this size bias is subsequently reflected in the reported *Ichthyophonus* infection prevalence for the population (Holst 1996). Therefore, it is recommended that *Ichthyophonus* prevalence in Pacific herring populations be reported by age/size class (i.e. Figs 1–3) rather than as a single prevalence from a geographic stock/location (i.e. Table 1).

A clear exception to this direct relationship between Pacific herring age/size and *Ichthyophonus* prevalence occurred in a sample from Cordova Harbor (June 2010), where juveniles (mean length 85 mm, age 1 year) had an unusually high prevalence of 35% (Table 1). Interestingly, these juvenile Pacific herring were also experiencing an epizootic of viral erythrocytic necrosis (71% infection with 92% of the infections scored as moderate-to-high intensity; data not shown) and heavy infestations with sea lice (predominantly *Caligus clemensi*). The aggregation of juvenile Pacific herring around boat harbours is not unusual; however, juvenile cohorts sampled from boat harbours in Puget Sound typically demonstrate low prevalence of *Ichthyophonus* infection (P K Hershberger, unpublished data). Although additional studies are needed to determine the source of the infection, it is possible that these abnormal fish health conditions were a reflection of activities associated with a fish processing plant located adjacent to Cordova Harbor. Raw offal from this processing plant is discharged directly into the water, and it is possible that exposure to infected offal increased the infection pressures to nearby juvenile Pacific herring. Follow-up efforts later in the summer were unsuccessful, as the age 1+ year cohorts disappeared from the harbour, and were replaced by newly metamorphosed, age 0-year cohorts; none of which tested positive for *Ichthyophonus* (Table 1).

Although *Ichthyophonus* appears to typically persist in Pacific herring at chronic levels that accumulate in populations over time, several lines of

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Table 3 *Ichthyophonus* in historical specimens of Pacific herring from Puget Sound archived at the University of Washington Fish Collection. *Ichthyophonus* status was determined by microscopic evaluation of periodic acid–Schiff-stained histological sections of heart tissues

UW archive number	Collection location	Collection date	Fork length (mm)	*Ichthyophonus* status
UW 428	Elliot Bay	10 November 1888	215	Not Detected
			224	Not Detected
UW 426	Puget Sound	Pre-1929	206	Not Detected
			193	Not Detected
No Data	Dabob Bay	16 January 1979	214	Not Detected
			206	Not Detected
UW 42796	Nisqually River delta	17 January 1980	219	Not Detected
			240	Not Detected
			239	Not Detected
UW 45151	Dabob Bay	26 July 1980	174	Not Detected
			198	Not Detected
			182	Not Detected
			184	Not Detected
			197	Not Detected
			203	Not Detected
			194	Not Detected
UW 045319	Dabob Bay	21 October 1986	210	Positive
			183	Positive
			201	Positive
			183	Positive
			185	Positive
			191	Not Detected
			206	Not Detected
			202	Positive
			179	Not Detected
UW 045266	Dabob Bay	5 May 1987	163	Not Detected
			185	Not Detected
			181	Not Detected
			198	Not Detected
UW 045192	Dabob Bay	18 August 1987	181	Not Detected
			174	Not Detected
			165	Positive
			168	Positive
			179	Not Detected
			151	Not Detected
			168	Not Detected
			159	Positive
UW 45227	Dabob Bay	14 October 1987	198	Not Detected
			178	Not Detected
			176	Positive
			162	Not Detected
			157	Not Detected
			169	Not Detected
			187	Not Detected
			176	Not Detected
			192	Not Detected
UW 045248	Dabob Bay	14 October 1987	221	Not Detected
			204	Not Detected
			179	Not Detected
			178	Not Detected
			182	Not Detected
			157	Not Detected
			161	Not Detected
			183	Not Detected
UW 045099	Dabob Bay	13 October 1987	216	Positive
			192	Not Detected
			213	Not Detected
			191	Positive
			174	Not Detected

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Table 3 *Continued*

UW archive number	Collection location	Collection date	Fork length (mm)	*Ichthyophonus* status
			198	Positive
			179	Not Detected
			176	Not Detected
			176	Not Detected
			194	Not Detected
			166	Not Detected
UW 045192	Mouth of Nisqually River	17 January 1989	210	Not Detected
			211	Not Detected
			205	Not Detected

evidence indicate that the parasite may periodically contribute to negative impacts on Pacific herring population dynamics. Laboratory exposures indicate that *Ichthyophonus* can be highly pathogenic to Pacific herring, with intraperitoneal injections resulting in host mortality with a mean day-to-death of 36 days (Kocan *et al.* 1999). Subsequent studies have indicated that this post-exposure mortality can be reproduced most commonly in younger (age 0 year) age cohorts (P K Hershberger, personal observation), likely as a result of factors involving dose per fish size (Okamoto, Nakase & Sano 1987). Regardless, massive epizootics and associated fish kills periodically occur in populations of adult Atlantic herring, often culminating in population-level impacts (reviewed in Burge *et al.* 2014). Causes of these periodic epizootics remain undetermined, but a leading hypothesis asserts that they result from episodic and punctuated exposures to high levels of the parasite. This hypothesis infers that increases in infection prevalence/intensity and epizootics may result from situations where Pacific herring feeding aggregations become sympatric with zooplankton aggregations that serve as intermediate hosts for the parasite (Kramer-Schadt *et al.* 2010); however, additional research efforts are needed to determine whether an intermediate host exists. Additionally, *Ichthyophonus* may impact Pacific herring demographic patterns in Puget Sound, WA, where increased infection prevalence with host size and age (Hershberger *et al.* 2002) occurred concomitantly with a loss of older age cohorts and a resulting decreased median age of the populations (Landis & Bryant 2010). Similarly, the prevalence of *Ichthyophonus* decreased from 62.5% (5/8) to 19.6% (22/112) in the largest size class (>240 mm) from Sitka Sound during 2007–13 (Fig. 2). It is likely that this size-specific decrease in *Ichthyophonus* prevalence resulted from selective mortality among the infected cohorts, as the heaviest infection intensities observed throughout this 7-year survey (2007–13) occurred in Sitka Sound during 2012 (Fig. 6). Analogous demographic changes occurred in populations of Atlantic herring after ichthyophoniasis epizootics (Tibbo & Graham 1963).

Seasonal, geographic and demographic patterns in *Ichthyophonus* prevalence are difficult to interpret, largely because of the highly migratory nature of Pacific herring throughout the region. For example, novel acoustic tagging results indicate that many Pacific herring in north-east PWS migrate to the southern PWS and presumably into the Gulf of Alaska, shortly after spawning (M A Bishop personal communication). Therefore, it is difficult to directly compare the spring and fall infection prevalences from a particular location (Fig. 3a,b), as samples may have consisted of different groups of Pacific herring with inconsistent exposure histories. However, interannual differences in spring infection prevalence are easier to compare, as Pacific herring typically demonstrate general fidelity to broad geographic spawning regions. Decreases in infection prevalence, such as those observed in Lower Cook Inlet (Fig. 4a/b) and among the largest size cohorts in Sitka Sound (Fig. 2), were likely the result of proximate or ultimate mortality among the infected cohorts. Laboratory exposure studies provide no indication that, once infected, Pacific herring are capable of completely clearing *Ichthyophonus* infections; rather, infected individuals experience either acute mortality (Kocan *et al.* 1999) or survival with persistent infections (Hershberger 2012). Elevated mortality of infected cohorts could occur from selective predation on *Ichthyophonus*-infected cohorts if infected individuals with decreased swimming performance (Kocan *et al.* 2006) are more easily captured by predators. This predator selection hypothesis is supported by field observations during an *Ichthyophonus* epizootic, when 60–80% of

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Figure 6 Gross signs of heavy ichthyophoniasis in the heart of a Pacific herring collected from Sitka Sound, AK during 2012. The heart and pericardium were nearly completely displaced by whitish coloured parasitic material. With the exception of Sitka Sound in 2012, high-intensity *Ichthyophonus* infections such as this were rare.

Atlantic cod stomachs contained heavily infected Atlantic herring (Kramer-Schadt *et al.* 2010).

All available information indicates a long-standing host/pathogen relationship between Pacific herring and *Ichthyophonus* rather than a recent introduction in the NE Pacific. Although few archived samples of Pacific herring were available for *Ichthyophonus* assessment, the parasite was detected in formalin-fixed Pacific herring samples from Puget Sound dating back to 1986 (Table 3). Inability to detect *Ichthyophonus* in any of the earlier samples should not be interpreted as absence of the parasite in the years prior to this year, as only a limited number of samples were processed ($n = 16$ from 1888 to 1980). Further, although histological assessment of PAS-stained slides is effective at identifying high-intensity *Ichthyophonus* infections, the technique demonstrates low sensitivity for low-intensity infections (Kocan, Dolan & Hershberger 2011); therefore, low-intensity cases were likely misdiagnosed as false negatives. A long-standing host–pathogen relationship was further supported by the ubiquity of *Ichthyophonus* in Pacific herring populations throughout coastal waters of the NE Pacific, ranging from Washington State north to the Gulf of Alaska.

Progressive steps in fisheries management are integrating *Ichthyophonus* prevalence data into Pacific herring stock assessment models in Prince William Sound, AK (Marty *et al.* 2010); however, further refinement of these disease parameters is required to accurately reflect annual disease-related mortality. Currently, annual mortality from *Ichthyophonus* is assigned to a percentage of the population demonstrating characteristic lesions that are detected during spring stock assessments. However, laboratory studies indicate that not all symptomatic individuals necessarily die from the resulting disease; further, some asymptomatic individuals likely die from the disease if exposure occurs sometime after the spring stock assessments are performed. Additional epizootiological and controlled laboratory studies are necessary for developing multivariate models which could assess the association of explanatory values and identify confounding variables. However, this approach is currently restricted by a paucity of information regarding the *Ichthyophonus* life cycle and the involvement of Pacific herring in completion of this cycle. It is recommended that future research efforts address this information gap by investigating natural routes of infection for Pacific herring, including the possible involvement of intermediate hosts.

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Appendix 12

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VIABILITY AND INFECTIVITY OF *ICHTHYOPHONUS* SP. IN POST-MORTEM PACIFIC HERRING, *CLUPEA PALLASII*

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ABSTRACT: *Ichthyophonus*-infected Pacific herring, *Clupea pallasii*, were allowed to decompose in ambient seawater then serially sampled for 29 days to evaluate parasite viability and infectivity for Pacific staghorn sculpin, *Leptocottus armatus*. *Ichthyophonus* sp. was viable in decomposing herring tissues for at least 29 days post-mortem and could be transmitted via ingestion to sculpin for up to 5 days. The parasite underwent morphologic changes during the first 48 hr following death of the host that were similar to those previously reported, but as host tissue decomposition progressed, several previously un-described forms of the parasite were observed. The significance of long-term survival and continued morphologic transformation in the post-mortem host is unknown, but it could represent a saprozoic phase of the parasite life cycle that has survival value for *Ichthyophonus* sp.

One of the defining characteristics of *Ichthyophonus* sp. is its ability to survive and undergo dramatic morphologic changes following the death of the fish host (Daniel, 1933; Fish, 1934; Dorier and Degrange, 1960; McVicar and McLay, 1985; Okamoto et al., 1985; Rahimian, 1998). Since *Ichthyophonus* sp. was first described, investigators have hypothesized that the morphologic forms observed in the post-mortem host represented part of the parasite's life cycle, related in some way to transmission (Fish, 1934; Sindermann and Scattergood, 1954; Spanggaard and Huss, 1996). Empirical evidence has demonstrated that the schizont in the living or recently deceased host is the infectious stage for piscivorous fish (McVicar and McLay, 1985; Okamoto et al., 1985; Rand and Cone, 1990) and that direct fish-to-fish water-borne transmission also occurs (Gustafson and Rucker, 1956; Erickson, 1965; Yokota et al., 2008). However, there have been no long-term studies on the viability and infectivity of *Ichthyophonus* sp. in the post-mortem host.

In the living fish host *Ichthyophonus* sp. occurs primarily as a spherical schizont surrounded by a non-cellular capsule that shields the organism from the host's immune defenses; this complex is often encased in a granuloma of host origin. While the host is alive the schizont increases in volume but otherwise remains relatively inactive. Following the death of the host, the schizont segments into 1 or more un-encapsulated plasmodia that migrate out of the capsule into the dead host tissue (Kocan, 2013), or alternatively, the schizont produces germ tubes (i.e., hyphae) through which the plasmodia migrate into an apical swelling where they morph into a new schizont (Spanggaard et al., 1994; McVicar, 2011). The ultimate fate or significance of these stages in the post-mortem host has not been empirically determined, but some investigators believe they may be related to similar stages observed in vitro (Okamoto et al., 1985; Spanggaard et al., 1994, 1995). In order to determine the significance of these post-mortem changes, we used a herring–sculpin model designed to address the following questions: (1) How long does *Ichthyophonus* sp. remain viable in post-mortem host tissue? and (2) For how long can *Ichthyophonus* sp. be naturally transmitted to a new vertebrate host following the death of an infected host?

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MATERIALS AND METHODS

Source of *Ichthyophonus*-infected tissue

There are no recognized criteria for assigning species to this parasite, so the term *Ichthyophonus,* without a species designation, will be used hereafter. *Ichthyophonus* was maintained in laboratory reared specific pathogen-free (SPF) Pacific herring, *Clupea pallasii,* (Hershberger et al., 2007) by periodic i.p. injection of culture-derived schizonts originally isolated from wild herring in tris-buffered Eagles Minimum Essential Medium (Gibco-Life Technologies, Grand Island, New York) supplemented with 5% fetal bovine serum (MEM-5), plus antibiotics (Hershberger et al., 2006). *Ichthyophonus* isolated from these experimentally infected herring was used to infect SPF herring for this study.

Sentinels

Pacific staghorn sculpin, *Leptocottus armatus,* a natural host for *Ichthyophonus,* were wild-caught as young-of-the-year from an *Ichthyophonus*-free population that has been sampled for over 10 yr without evidence of infection (Kocan et al., 2013). Prior to exposure the sculpin were acclimatized to laboratory conditions for ≥14 days in 1 m diameter community tanks supplied with flow-through filtered and ultraviolet-treated natural seawater and fed freeze-dried krill and chopped, previously frozen squid. Once acclimated the sculpin were fed (e.g., offered) *Ichthyophonus*-infected decomposing herring tissue for up to 13 days.

Viability (objective 1)

Ichthyophonus viability in post-mortem herring was determined in 2 separate studies, in which decomposing tissue was evaluated by in vitro explant culture, histology, and direct microscopic observation of fixed post-mortem tissue. Experimentally infected herring were killed with an overdose of buffered tricaine methanesulfonate (MS-222) approximately 12 mo post-exposure and examined for the presence of *Ichthyophonus* schizonts by microscopic evaluation of brain tissue squashes. Herring that were confirmed positive by this method were then placed into individual plastic flow-through troughs (7.6 × 20 × 6.5 cm; ~1.3 L) supplied with sand filtered and ultraviolet-treated seawater with a mean temperature of 7.5 C and average flow rate of 77 ml/min (range 44–130 ml). This design allowed for the natural autolysis and microbial degradation of the post-mortem herring without disturbance by macro-scavengers (Sorg et al., 1997).

In the first study, heart and somatic muscle tissue from 3 infected herring was cultured in MEM-5 on days 0–5 post-mortem, then microscopically evaluated for hyphal growth after 14 days in culture. Based on the results of the first study, a second study was conducted under similar conditions, but sampling was extended from 5 to 29 days. Portions of the heart, liver, gut, and somatic muscle from 2 herring were sequentially placed into tubes containing MEM-5 from days 0 to 29 post-mortem; portions of the same tissues were also fixed in 10% neutral buffered formalin (NBF), processed for histology, and stained with Periodic acid-Schiff reagent (PAS) for histologic–histochemical identification of the parasite. Herring were scored as positive if *Ichthyophonus* was observed in any of the cultured organs. When autolysis and microbial degradation resulted in tissues becoming unrecognizable, usually ≥7 days incubation, the resultant slurry was microscopically examined directly, as well as placed into culture.

Table I. *Ichthyophonus* sp. detected by in vitro culture and histology in post-mortem herring, *Clupea pallasii*.

Days post-mortem	First study +/n (%)	Second study +/n (%)	Histology (study 2)
0	3/3 (100)	6/6 (100)	+
1	3/3 (100)	2/2 (100)	+
2	3/3 (100)	2/2 (100)	+
3	3/3 (100)	2/2 (100)	+
4	3/3 (100)	2/2 (100)	+
5	3/3 (100)	2/2 (100)	+
7		2/2 (100)	+
9		1/2 (50)	+
11		2/2 (100)	+
12		2/2 (100)	+
13		2/2 (100)	−
14		2/2 (100)	+
15		2/2 (100)	+
16		2/2 (100)	−
19		2/2 (100)	−
21		2/2 (100)	−
23		2/2 (100)	+
25		1/2 (50)	−
27		1/2 (50)	+
29		2/2 (100)	−

Owing to the difficulty of visualizing 3 dimensional forms of the parasite by single plane histology, morphological changes to *Ichthyophonus* in the post-mortem herring were further investigated by directly examining autolyzed organs and tissue slurry collected from the carcasses at various time points post-mortem. Heart, liver, viscera, and unidentifiable autolyzed herring tissues were sampled from infected carcasses on days 0, 5, 15, and 22 post-mortem; fixed in 10% NBF to arrest parasite development; and stained with PAS, without being processed for histology. These samples were examined microscopically at ×100–200 magnification for morphological changes to *Ichthyophonus*.

Infectivity (objective 2)

Prior to exposure (defined under "Sentinels" above), sculpin were not fed for ≥48 hr, then placed into individual 10 L tanks with flowing seawater where they were fed infected herring tissue on successive days post-mortem; any tissue remaining after 48 hr was removed from the tank. Individual sculpin were then maintained in separate tanks and fed a diet of freeze-dried krill and chopped squid for up to 60 days prior to necropsy. At the end of the study surviving sculpin were killed with an overdose of buffered MS-222 and necropsied; then their tissues were cultured.

In the first study, individually housed sculpins (mean weight = 6.5 g ± 2.46) were fed pooled viscera and muscle from infected herring on days 0–5 post-mortem. All sculpin were killed 60 days post-exposure, necropsied, and examined for the presence of *Ichthyophonus* by in vitro explant culture of cardiac muscle. Negative controls consisted of 6 sculpin fed tissues from SPF herring at the beginning and end of the study.

Following the initial study, a second infectivity study was conducted to verify the results of the first study and extended the exposure period from 5 to 13 days. Individually housed sculpin (mean weight 22.3 g ± 12.52) were fed pooled viscera (~2.5 g) and muscle (~8 g) from infected herring each day beginning on day-0–day-5, and on days 7, 9, 11, and 13 post-mortem. Negative controls consisted of 9 sculpin sampled prior to commencing the study to confirm their SPF status.

Following exposure to infected herring tissue, sentinels in the second study were housed separately for 60 days at a mean temperature of 8.6 C and fed a maintenance diet of freeze-dried krill, chopped squid, and commercial fish pellets. Sculpin that died during the course of the study were necropsied, and their tissues were cultured to evaluate the presence of *Ichthyophonus*. Surviving sculpins were killed at the end of the study and similarly examined.

RESULTS

Viability (objective 1)

Cultures of heart and somatic muscle tissue from all herring in the first study were *Ichthyophonus*-positive from day-0–day-5 post-mortem. In the second (extended) study *Ichthyophonus* was cultured from 35 of 38 herring carcasses (92.1%) sampled on day-0–day-29, and was detected histologically on day-0–day-12, and days 14, 15, 23, and 27 (Table I).

Herring organs remained intact and were recognizable during the first 5 days post-mortem, but by ≥7 days, the heart, liver, spleen, and kidney became increasingly unrecognizable as a result of autolysis and microbial degradation. Conversely, the intestine and skeletal muscle could be visually identified for up to 29 days. This sequence of events follows the first 2 recognized stages of animal decomposition, autolysis and microbial degradation (Sorg et al., 1997; Vass, 2001). Decomposition stages 3 (decay) and 4 (dry) were precluded by the experimental design, i.e., exclusion of macro-invertebrate scavengers from the flow-through chambers.

During the first 48 hr post-mortem, histologic evaluation of herring tissue revealed *Ichthyophonus* morphing from the typical schizont present in the living host to un-encapsulated amoeboid plasmodia or producing germ tubes. From days 15 to 22 post-mortem, un-encapsulated plasmodia, germ tubes, and several previously unidentified PAS-positive morphologic forms were observed (Fig. 1).

Germinal discs developed on the surface of many schizonts within 8 hr of the host's death, followed by growth of germ tubes. By 24–48 hr post-mortem numerous amoeboid plasmodia were seen vacating the schizont capsule, or alternatively, the content of the schizont migrated through germ tubes to an apical swelling, which expanded and reformed into new schizonts (Fig. 2).

Germinating schizonts were observed throughout the 29-day post-mortem sample period, as were plasmodia observed exiting the schizont capsules (Fig. 3A, C, E, F). Spherical thick walled cells (≥2 μm) resembling merozoites also appeared in apical hyphal swellings as well as in newly formed schizonts (Figs. 1E, 3B, D).

Infectivity (objective 2)

The combined results from both studies showed infectivity to be highest during the first 24–48 hr post-mortem, then decreasing linearly ($r^2 = 0.746$; $P = 0.0057$; df = 6), through day-5, reaching 0% by day-6 post-mortem (Fig. 4). In the first study all sculpin consumed all or most of the infected herring offered within 2–3, hr, and all survived to the end of the study. In this study transmission continued for 3 days, decreasing from 100% to 33% to 0% on days 0–1, 2–3, and 4–5, respectively; the 6 negative controls were not infected. In the second study infectivity decreased from 60–100% on days 0–2, to 50% on day-5, and 0% on days 7–13, closely paralleling the results of the first study, but extending infectivity from 3 to 5 days.

In the second study, 14 of the 24 sculpin fed post-mortem herring tissues on day-0–day-5 died, and 10 survived until the end of the study. Of the 14 dead sculpin, 5 did not consume any herring and were *Ichthyophonus*-negative; these were omitted from the study. Infection prevalence (5/9) in the remaining sculpin that died between 12 and 45 days post-

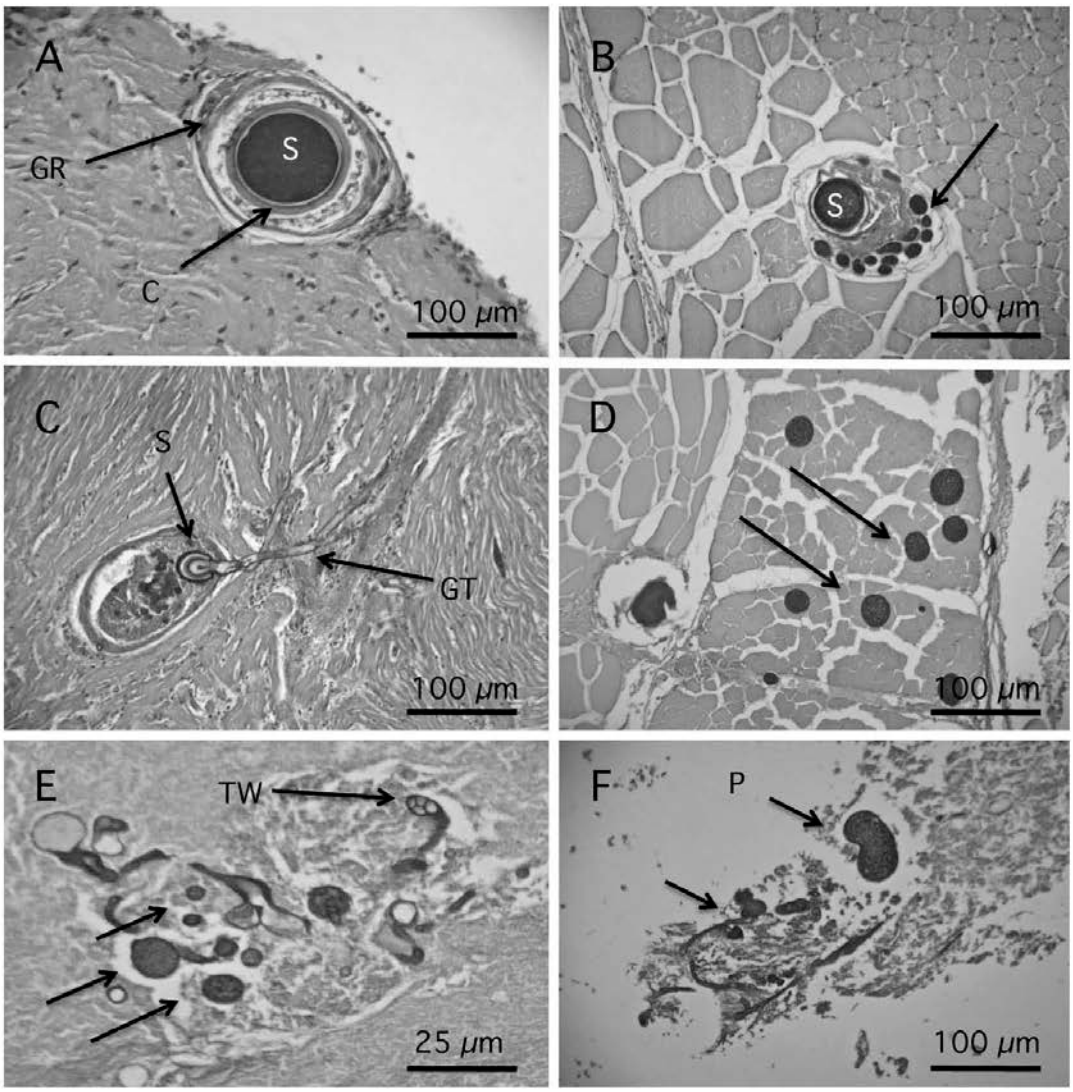

FIGURE 1. Periodic acid-Schiff (PAS) stained histologic sections of post-mortem herring (*Clupea pallasii*) tissue. (**A**) Typical multinuclear schizont (S) with multilaminate capsule (C) and granuloma (GR). (**B**) Shrunken schizont (S) surrounded by multiple plasmodia (arrow). (**C**) Empty schizont capsule (S) in granuloma with germ tubes (GT) penetrating surrounding tissue. (**D**) Multiple un-encapsulated plasmodia (arrows) migrating into dark muscle. (**E**) Thick walled cells in apical tip of germ tube (TW), small plasmodia (arrows), and unidentified structures. (**F**) Un-encapsulated plasmodium (P) and unidentified PAS-positive cells. Time post-mortem: A = 0 hr; B = 24 hr; C = 48 hr; D = 36–48 hr; E = 15 days; F = 22 days.

exposure was not different from that of the 10 sculpin that survived to the end of the study (3/10); Fisher's exact test ($P = 0.68$; $n = 19$).

Owing to progressive autolysis and microbial decomposition, it was not possible to accurately quantify the amount of herring tissue ingested by each sculpin in the second study beyond 5 days post-mortem. Over time the tissues became gelatinous or liquid, precluding an accurate determination of whether they were eaten or simply disintegrated into the surrounding water and washed away. Consequently, it is possible that *Ichthyophonus*-negative sculpins exposed beyond day-5 ingested little or no *Ichthyophonus*-infected tissue.

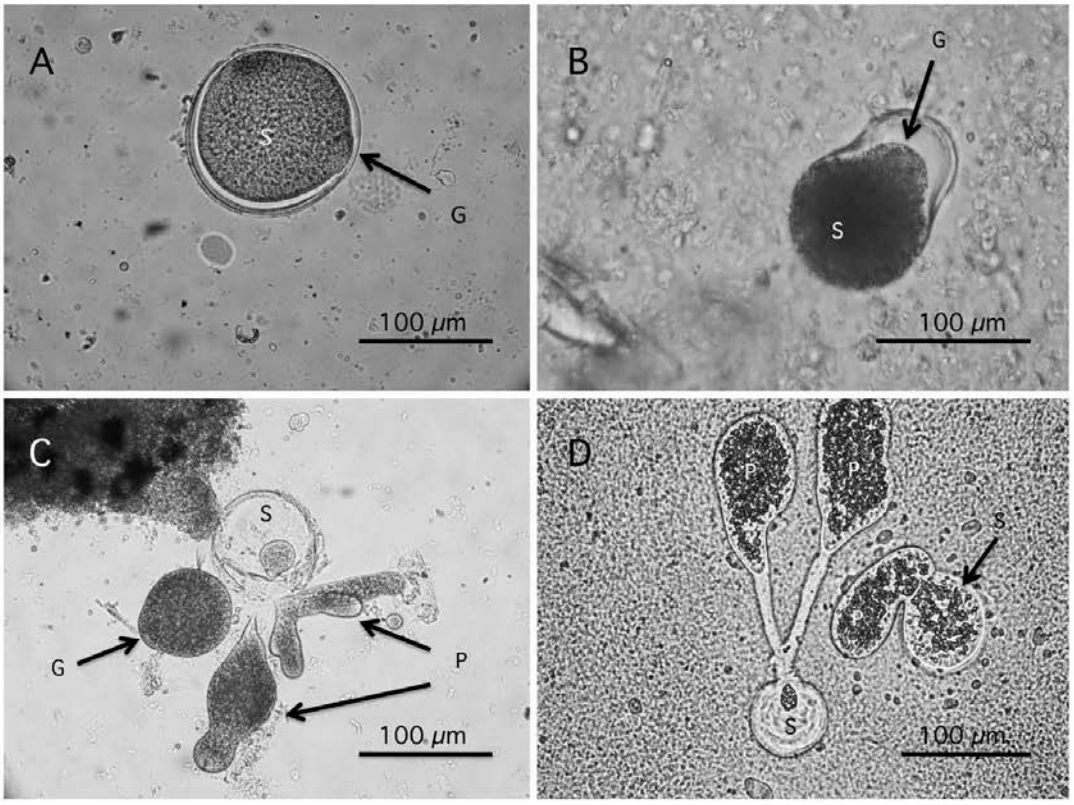

FIGURE 2. Several morphological changes to *Ichthyophonus* observed in liver squashes of Pacific herring (*Clupea pallasii*) 8–48 hr post-mortem. (**A**) Germinal disc (G) on the surface of an *Ichthyophonus* schizont. (**B**) Non-cellular capsule deforming in response to germinal growth (G) of schizont (S). (**C**) Plasmodia (P) migrating away from an empty schizont capsule (S), and a second schizont in early germination (G). (**D**) Empty schizont capsule (S) with bifurcated germ tubes and apical swellings filled with plasmodia (P), and a second schizont with amoeboid plasmodium exiting the capsule. Hours post-mortem: A = 8; B = 24; C = 24–36; D = 48.

DISCUSSION

Viability (objective 1)

Ichthyophonus was identified in post-mortem carcasses of infected Pacific herring for up to 29 days post-mortem when incubated in flowing seawater at ambient temperatures. During the first 48 hr post-mortem the parasite underwent a series of previously described morphologic changes (Okamoto et al., 1985; McVicar, 2011; Kocan et al., 2013). Although *Ichthyophonus* became increasingly more difficult to find in each carcass as decomposition progressed, it continued to undergo morphological transformations, and the cells within apical swellings underwent cytokinesis to produce thick walled merozoite-like cells. This prolonged presence of the parasite in the host carcass, along with morphologic transformation, suggests the possibility of there being a previously unrecognized saprozoic phase of the *Ichthyophonus* life cycle.

While plasmodial cells were common in herring tissue ≤48 hr post-mortem (Figs. 1B, D; 2C, D), several novel cells (Fig. 3B, E) appeared in decomposing tissue 5–22 days post-mortem. Of these novel cells, the most striking were the thick walled merozoite-like cells within the apical tips of germ tubes (Fig. 3B, D). These have not been previously reported to occur in vivo or in vitro, but rather they appear to be derived from a cell similar to that pictured in Figure 2D. This form may represent a step in the development of a previously unrecognized stage of *Ichthyophonus* that develops after the death of the host. Although superficially similar to cells seen in vitro, the cells in the apical tips of germ tubes in the post-mortem herring have not been previously reported.

Previous long-term *Ichthyophonus* survival studies have demonstrated that cultured schizonts (previously identified as "spores") could survive in sterile saline and artificial seawater for up to 20 mo, after which they were capable of germinating in vitro (Spanggaard and Huss, 1996). However, when whole infected Atlantic herring, *Clupea harengus*, and "isolated nodules" excised from the same fish were kept in sterile saline for 7 days,

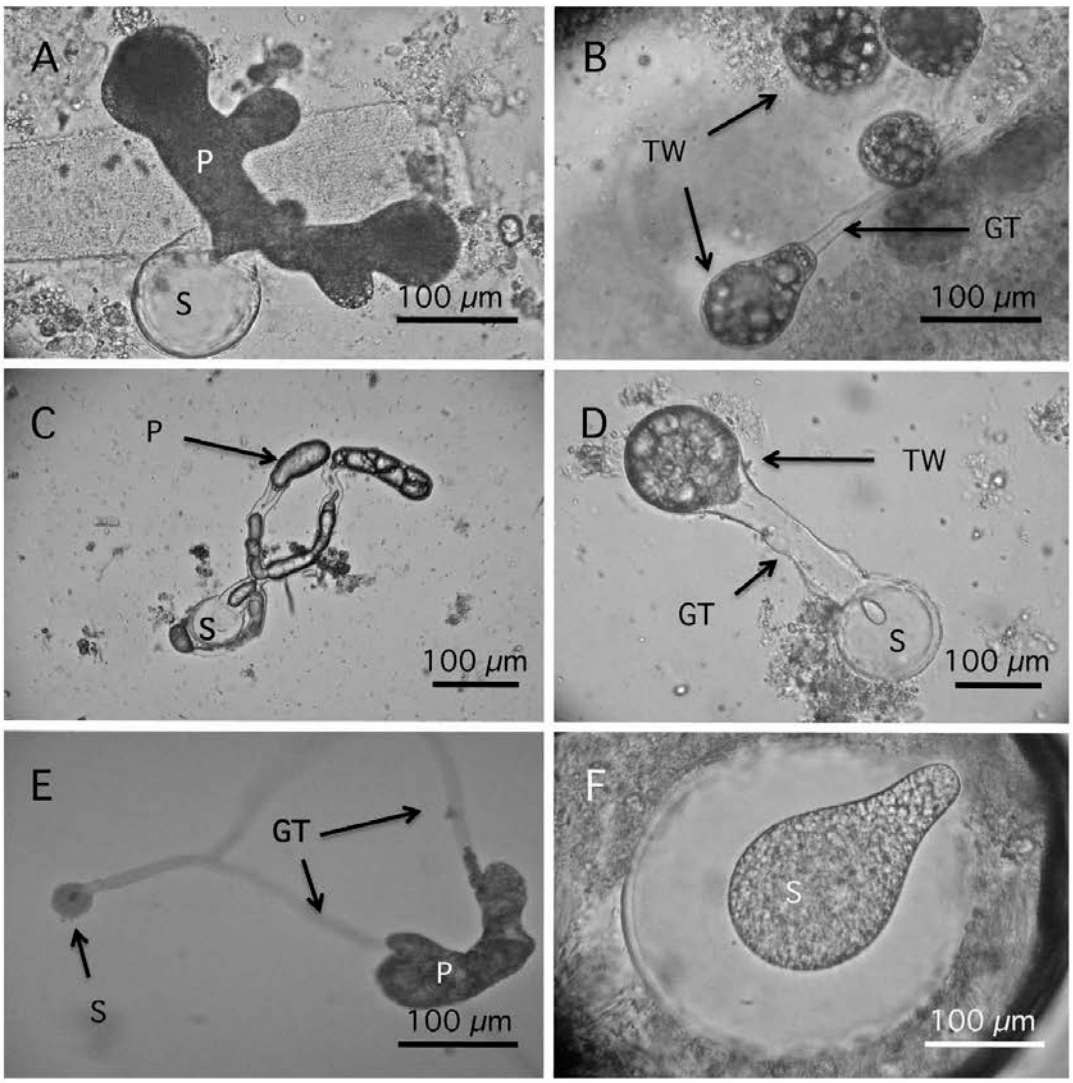

FIGURE 3. Novel morphological forms of *Ichthyophonus* observed in decomposing herring carcasses. (**A**) Plasmodium (P) exiting a schizont capsule (S). (**B**) Thick walled merozoite-like cells (TW) within the apical swelling of germ tubes (GT). (**C**) Empty schizont capsule (S) with plasmodia (P) migrating through germ tubes. (**D**) Empty schizont capsule (S) with thick walled cells (TW) at the end of a single germ tube (GT). (**E**) Empty schizont capsule (S) with a large plasmodium (P) at the end of a bifurcated germ tube (GT). (**F**) A newly germinating schizont (S) in decomposing herring tissue. Days post-mortem: A, B = 5; C, D = 15; E, F = 22.

they showed no signs of development beyond 24 hr (Daniel, 1933). However, when "macroscopic nodules of fungus material" obtained from infected herring muscle were placed into sterile seawater, hyphae that were present when the nodules were excised disappeared after 24 hr, and only "resting spores" (i.e., schizonts) remained after 180 days (Sindermann and Scattergood, 1954). Although these studies were conducted under different conditions, when combined with the present study they show that *Ichthyophonus* can survive and remain viable for extended periods outside the host, but that conditions present in the decomposing carcass are required for development beyond the schizont stage. Based on their in vitro studies, Okamoto et al. (1985) supported

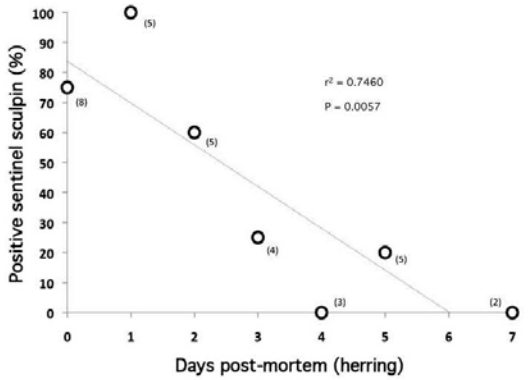

FIGURE 4. Loss of *Ichthyophonus* infectivity in post-mortem herring, *Clupea pallasii*, for Pacific Staghorn sculpin, *Leptocottus armatus*. Pooled data from 2 studies; number sign (#) = n.

this hypothesis when they stated that growth and structure of *Ichthyophonus* "reflect the available nutrients and physical conditions surrounding *I. hoferi*."

The above data have led us to posit that the ability of *Ichthyophonus* to survive and undergo progressive morphologic transmutation in a post-mortem host may represent a saprozoic stage of an as yet undefined mode of survival. If correct, the novel plasmodia and merozoite-like cells seen in the apical swelling of germ tubes may be stages of the parasite's life cycle that play a role in maintaining the pathogen in the aquatic environment until a suitable host becomes available.

Infectivity (objective 2)

Although *Ichthyophonus* could be isolated from herring tissues for up to 29 days post-mortem, transmission to a new host via ingestion was observed for only 5 days, declining rapidly from 100% on days 0–1 to 0% after day-5. The most plausible explanation for the rapid decline in transmission is that *Ichthyophonus* infectivity decreased over time, ultimately being lost. This is supported by the results of the first infectivity study where the number of positive sculpin decreased from 100% to 0% following ingestion of all infected post-mortem herring for all 5 days. If infectivity did not decrease over time, then the number of infected sculpin should have remained unchanged for the entire exposure period. Alternative hypotheses include: (1) decomposing tissue disintegrated after 5 days, and the infective stage was washed away before it was eaten, or (2) decomposing tissue became progressively unpalatable and was not eaten.

There are 4 stages of decomposition in the post-mortem carcass, beginning with autolysis, followed by microbial degradation (bloat), then active and advanced decay (scavenging), and finally skeletal remains (dry). The timing of these events varies with temperature and mass of the carcass, but they always occur in the same sequence (Sorg et al., 1997). If *Ichthyophonus* remained infectious through the scavenging phase of decomposition, then at any point it would have the potential of being transmitted to some host species willing to consume the infected tissue. Because scavenging in the marine environment is rapid, and experimental transmission to sculpin was ≤5 days, there is a limited window of opportunity for transmission to vertebrate scavengers under natural conditions; however, the surviving *Ichthyophonus* may still be infectious for invertebrate scavengers. Once the integrity of the intact host carcass is lost through scavenging activity, organic debris and any remaining *Ichthyophonus* from the decomposing carcass could mix with sediments, thus providing a mechanism for long-term survival and transmission of the parasite to an invertebrate host. The existence of such an intermediate host has been previously proposed and is highly speculative; its existence remains to be demonstrated.

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Appendix 13

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Full Scientific Report

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Identification of the major capsid protein of erythrocytic necrosis virus (ENV) and development of quantitative real-time PCR assays for quantification of ENV DNA

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Abstract. Viral erythrocytic necrosis (VEN) is a disease of marine and anadromous fish that is caused by the erythrocytic necrosis virus (ENV), which was recently identified as a novel member of family *Iridoviridae* by next-generation sequencing. Phylogenetic analysis of the ENV DNA polymerase grouped ENV with other erythrocytic iridoviruses from snakes and lizards. In the present study, we identified the gene encoding the ENV major capsid protein (MCP) and developed a quantitative real-time PCR (qPCR) assay targeting this gene. Phylogenetic analysis of the MCP gene sequence supported the conclusion that ENV does not group with any of the currently described iridovirus genera. Because there is no information regarding genetic variation of the MCP gene across the reported host and geographic range for ENV, we also developed a second qPCR assay for a more conserved ATPase-like gene region. The MCP and ATPase qPCR assays demonstrated good analytical and diagnostic sensitivity and specificity based on samples from laboratory challenges of Pacific herring *Clupea pallasii*. The qPCR assays had similar diagnostic sensitivity and specificity as light microscopy of stained blood smears for the presence of intraerythrocytic inclusion bodies. However, the qPCR assays may detect viral DNA early in infection prior to the formation of inclusion bodies. Both qPCR assays appear suitable for viral surveillance or as a confirmatory test for ENV in Pacific herring from the Salish Sea.

Key words: Diagnostic validation; inclusion bodies; viral erythrocytic necrosis.

Introduction

Viral erythrocytic necrosis (VEN) is a disease of marine and anadromous fish that is characterized by the presence of cytoplasmic inclusion bodies in erythrocytes, anemia, increased susceptibility to secondary infection, and direct mortality.[3] The disease has been recognized in both the Atlantic and Pacific Oceans in a wide range of fish hosts that include the families *Gadidae*, *Clupeidae*, *Salmonidae*, *Anguillidae*, and *Blenniidae*.[3] The etiological agent, erythrocytic necrosis virus (ENV),[11] was characterized in 2014 as a novel iridovirus using next-generation sequencing technologies.[6] Phylogenetic analysis of a conserved DNA polymerase gene indicated that ENV groups with other erythrocytic iridoviruses from snake (*Thamnophis sauritus* erythrocytic virus) and lizard (*Lacerta monticola* erythrocytic virus) hosts; the authors propose that these viruses may constitute a sixth genus of the family *Iridoviridae*.[6]

A presumptive diagnosis of VEN is based on clinical signs of anemia and the observation of cytoplasmic inclusion bodies in erythrocytes by light microscopy (2014 American Fisheries Society suggested procedures for the detection and identification of certain finfish and shellfish pathogens, http://goo.gl/ybJ3L2). However, there are a number of salmonid-associated intraerythrocytic viruses that form cytoplasmic inclusion bodies (Hedrick RP, et al. Another erythrocytic virus from salmonid fish? Fish Health Section AFS Newsl 1987;15:5; Landolt ML, et al. Detection of an intra-erythrocytic virus in rainbow trout (*Salmo gairdneri*). Fish Health Section AFS Newsl 1977;6:4–6),[7,14] so confirmatory diagnosis typically requires detection of iridovirus-like particles in the cytoplasm of erythrocytes by transmission electron microscopy (TEM; http://goo.gl/ybJ3L2). Conventional PCR primers targeting regions of the genes encoding a DNA polymerase, ATPase-like molecule, RNA polymerase, and ribonucleotide reductase small subunit have been reported.[6] Diagnostic PCR tests for other iridoviruses

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commonly target the gene encoding the major capsid protein (MCP) because this gene tends to be highly virus specific, and sequencing of this gene can provide information about viral genotype.[27] Additionally, this gene has been used to assess phylogenetic relationships within and among iridovirus species.

The objective of our study was to extend the work of Emmenegger et al.[6] by reporting the sequence of the gene encoding the ENV MCP, and developing a quantitative real-time PCR (qPCR) targeting the MCP gene. Only ENV from infected Pacific herring (*Clupea pallasii*) in the Salish Sea has been characterized to date,[6] and there is limited information about the diversity of ENV across its host and geographic range. For that reason, we also developed and validated a second qPCR test that targets a more conserved ATPase-like gene. The analytical and diagnostic properties of the 2 new ENV qPCR assays were compared with the current gold standard diagnostic technique based on examination of stained blood films for the presence of intracytoplasmic inclusion bodies. The molecular assays developed herein will be useful for ENV surveillance within the Salish Sea, confirmatory diagnosis, or for diagnostic cases where the condition of the fish prevents blood film analysis.

Materials and methods

Animals

All live fish experiments were approved by the Western Fisheries Research Center Institutional Animal Care and Use Committee (protocol 2008-31). Pacific herring gametes were collected from various spawning locations in the Salish Sea (Washington State). The rearing of specific pathogen–free (SPF) Pacific herring was performed as previously described[13] in sand-filtered and ultraviolet-irradiated flowing seawater. Throughout their rearing period, SPF fish were fed a diet of progressively larger food items including enriched rotifers *Brachionus plicatilis*, enriched *Artemia franciscana* nauplii (instars 1–2), frozen copepods,[a] and commercially available food pellets.[b] There was no history of VEN disease in the fish stock used for diagnostic validation.

Identification of the ENV major capsid protein

Parallel 454 sequencing of ENV total DNA and the identification of an ENV ATPase-like gene (GenBank accession KJ730210) has been previously described.[6] A search of the 454 dataset revealed a contig with a partial open reading frame (ORF) that encoded a protein with similarity to the iridovirus MCP. To confirm the authenticity of the contig, total DNA was extracted from ENV-infected Pacific herring kidney tissue following the manufacturer's[c] recommendation for tissues. DNA was quantified by spectrophotometer[d] and stored at −20°C until used. Amplification of the putative MCP was achieved following the manufacturer's recommendation[e] for a 25 μL final volume using primers 51F (GCGCTCGTGTCA CACAATAGT) and 1671R (CCAAAATTTGTAGAACCCT GAG; amplicon length 1,620 bp). All conventional PCR and sequencing primers were designed manually and synthesized commercially.[j] Reaction conditions were 1 cycle of 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min.

Sanger sequencing was performed on an automated sequencer[f] with the recommended reagents[g] in a final 10 μL reaction. Sequencing used the first-round PCR primers (51F and 1671R), as well as internal primers 361F (ACG GCAGCGAGTTTATATGTAT) and 1243R (TCCAGA GGATCCGCAGCCAC). Sequence chromatograms were assessed and edited manually.[h] A partial ENV MCP sequence was used in a phylogenetic comparison with other iridoviruses (Table 1). Multiple sequence alignment was performed using ClustalX[25] within the MEGA V6.0 software package.[24] Phylogenetic relationships were inferred using the maximum likelihood method implemented in the MEGA software package, using a Poisson correction model with 1,000 bootstrap iterations.

Primer, probes, and artificial positive controls development

Primers and fluorescent-labeled probes were designed using a commercial program,[i] and sequences are provided in Table 2. Two assays targeting the MCP and ATPase-like genes were designed (hereafter referred to as the MCP and ATPase qPCR assays, respectively). An artificial positive control (APC) plasmid was designed for use as a control and quantitative standard using methods previously reported.[21] Briefly, an artificial construct that encoded target sequences for the ENV primers and probe plus the addition of an artificial probe binding site was synthesized and cloned into the commercial high copy vector.[j] A probe labeled with a VIC reporter dye[k] was designed to bind to the artificial sequence site.[23] The APC plasmid DNA was used as a positive control in separate reaction wells and to generate standard curves. The APC plasmid was used to determine absolute ENV gene copy number based on a plasmid molecular weight of 1.3×10^6 g/mole (both assays), which equates to 4.7×10^{11} copies/μg; copy number calculations were as described.[20] The APC plasmid DNA was diluted to a standard number of copies per microliter, serially diluted in 10-fold increments, and subjected to either the ATPase or MCP qPCR assay. Each reaction includes the ENV-specific primer and probes and the APC probe; the APC probe was included to be consistent with our standard operating procedure for testing unknown samples. However, only fluorescence obtained from the ENV-specific probe was used to generate the APC plasmid DNA standard curve. Linear regression analysis was used to relate quantification cycle (Cq) values to log copy number (r^2 value range 0.994–0.996). All APC plasmid DNA was stored as aliquots at −80°C until used.

Table 1. Comparison of the partial major capsid protein amino acid sequence of erythrocytic necrosis virus (ENV) to other iridoviruses. A total of 357 amino acid positions were present in the final dataset.

Genus/Virus*	Host	GenBank accession	Substitution number relative to ENV	% difference
Unclassified				
IIV-6	Insect	AAK82135	175	46.7
Iridovirus				
IIV-1 (TIV)	Insect	AAA46245	176	46.9
IIV-16 (CzIV)	Insect	AAB82569	177	47.2
IIV-9 (WIV)	Insect	AAB82568	181	48.3
Chloriridovirus				
IIV-3 (MIV014L)	Insect	ABF82044	181	48.3
Lymphocystivirus				
LCDV-1	Fish	AAW48181	192	51.2
LCDV-China	Fish	AAS47819	195	52.0
Megalocytivirus				
ISKNV	Fish	BAL04525	202	53.9
RSIV	Fish	BAC66968	202	53.9
TRBIV	Fish	AAT01301	202	53.9
Ranavirus				
EHNV	Fish	AAO32315	203	54.1
BIV	Amphibian	AAO32316	204	54.4
ECV	Fish	ACO90017	204	54.4
FV-3	Amphibian	AAB01722	204	54.4
TFV	Amphibian	AAK55105	204	54.4

* IIV = *Invertebrate iridescent virus*; TIV= *Tipula* iridescent virus; CzIV = *Costelytra zealandica* iridescent virus; WIV = *Wiseana* iridescent virus; MIV014L = *Aedes taeniorhynchus* iridescent virus; LCDV-1 = *Lymphocystis disease virus 1*; ISKNV = *Infectious spleen and kidney necrosis virus*; RSIV = red seabream iridovirus; TRBIV = turbot reddish body iridovirus; EHNV = *Epizootic haematopoietic necrosis virus*; BIV = *Bohle iridovirus*; ECV = *European catfish virus*; FV-3 = *Frog virus 3*; TFV = tiger frog virus.

Table 2. Primer and probe sequences for quantitative real-time PCR (qPCR) targeting the major capsid protein (MCP) and ATPase-like gene.*

Predicted gene	qPCR primer and probes (5′–3′)	Amplicon length
MCP	MCP F: GCCAATCCACTTCCCAATACTC[k]	66 bp
	MCP R: TGCGCGTTCGATAGAAGGT[k]	
	MCP T: 6FAM-CAATGGTGGAGTTCCT-NFQ/MGB[k]	
ATPase-like	ATPase F: CGTAGGGCCCCAATAGTTTCT[k]	100 bp
	ATPase R: GGAGGAAATGCAGACAAGATTTG[k]	
	ATPase T: 6FAM-TCTTGCCGT/ZEN/TATTTCCAGCACCCG-IBFQ[j]	

* The primers and probes were synthesized commercially.[j,k]

Quantitative PCR standard operating procedures

Real-time PCR was conducted using a commercial system.[k] The cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions contained 12 µL of master mix[l] with 900 nM of each forward and reverse primers, 200 nM of fluorescent probe, and 200 nM of APC probe, with 5 µL of diluted DNA. Negative template controls and positive controls (APC plasmid) were included on each plate run. The test interpretation criteria were as follows: 1) no amplification in 2 replicate wells was considered a negative result, 2) sigmoidal amplification curves present in 2 replicate wells was considered a positive result, and 3) sigmoidal amplification curves present in 1 of 2 replicate wells was considered a suspect result. Suspect results would typically be repeated in quadruplicate with the goal of obtaining 2 replicate wells with positive amplification, but no suspect test results were obtained in this study.

Analytical validation

The analytical sensitivity and PCR efficiency of each of the ENV assays were evaluated using standard curves generated with APC plasmid DNA or kidney total DNA derived from

Table 3. Ruggedness of the major capsid protein (MCP) quantitative real-time PCR (qPCR) and ATPase-like qPCR assays. Evaluations included a comparison of master mix composition, incorporation of an internal positive control (IPC)[n] duplex, or incorporation of an artificial positive control (APC) probe into the reaction.*

	PCR conditions				
	GenEx	GenEx	GenEx	Universal	FastStart
IPC duplex	–	+	–	–	–
APC duplex	–	–	+	+	+
MCP qPCR (Cq ± SD)	29.7 ± 0.7	30.8 ± 0.7	29.4 ± 0.7	31.8 ± 3.7	28.8 ± 0.8
ATPase qPCR (Cq ± SD)	30.5 ± 2.1	31.5 ± 1.6	30.2 ± 2.1	39.2†	29.0 ± 1.9

* + = duplex probe included; – = duplex probe not included. GenEx = TaqMan gene expression master mix, Life Technologies Inc., Carlsbad, CA; Universal = TaqMan universal PCR master mix, Life Technologies Inc., Carlsbad, CA; FastStart = FastStart PCR master, Roche Life Sciences, Indianapolis, Indiana.
† Only 1 of the 3 samples tested with the universal master mix amplified with the ATPase qPCR assay.

an ENV-infected Pacific herring. Standard curves consisted of 10-fold serial dilutions that were subjected to qPCR. The ENV qPCRs were tested for cross-reactivity with other selected fish iridoviruses using DNA extracted from *Lymphocystis disease virus 1* (LCDV-1), red seabream iridovirus (RSIV), and *Infectious spleen and kidney necrosis virus* (ISKNV). The within-run repeatability of the ENV qPCRs was estimated by using 10 replicate wells (in the same run) of 3 samples representing different viral copy number. To measure the between-run repeatability of each assay, the 3 samples representing different copy number concentrations were tested in 4 separate runs on 4 different days. Coefficient of variation (CV) was calculated as standard deviation/mean. Assay ruggedness was evaluated by substituting master mix formulations and incorporation of a commercial exogenous internal positive control (IPC[n]; as described in Table 3).

Diagnostic validation

Diagnostic properties of the MCP and ATPase qPCR assays were evaluated using positive and negative populations of Pacific herring created by laboratory challenge. Briefly, tissues from ENV-infected Pacific herring were homogenized in phosphate buffered saline (PBS), subjected to centrifugation, and then 50 µL of the supernatant was injected into naïve Pacific herring (~2.6 g) to create a "true-positive" group (n = 50). Additional naïve herring were injected with PBS to create a "true-negative" group (n = 50). Fish were held in flowing seawater (mean ± SD temperature 8.5°C ± 0.2°C) and were euthanized by an overdose of buffered tricaine methanesulfonate (MS-222) at 16 days postinfection (dpi). The caudal artery was severed, blood films were produced on glass slides, and kidney tissues were sampled and stored at −20°C until used. Blood films were air dried, fixed in methanol, and stained with 3.5% Giemsa in phosphate buffer[9]; the number of inclusion bodies per 100 erythrocytes was determined by light microscopy at 1,000× magnification (http://goo.gl/ybJ3L2). The technician reading the slides had moderate prior experience with blood smear analysis; the reader was not blinded to the test slides. DNA was extracted from kidney tissues using the methods described above and analyzed with the MCP and ATPase qPCR assays. The technician conducting the qPCR analysis was experienced in the method; the technician was not blinded to the test samples. Diagnostic sensitivity (DSe), diagnostic specificity (DSp), and positive and negative likelihood ratios (LRs) were estimated as previously described[2,5,21] or by using EpiTools (http://goo.gl/BimzdF).

ENV infection progression

To investigate ENV progression, 20 naïve Pacific herring were injected with supernatant derived from ENV-infected tissue homogenates as described above. Five fish were sampled from this group at 5, 10, 15, and 20 dpi. Blood and kidney sampling, blood smear analysis, and qPCR detection of ENV DNA were performed as described above. The difference in log mean ENV DNA copy numbers in samples with and without detectable inclusions bodies was evaluated by t-test.[10]

Results

ENV major capsid protein

A contig with similarity to an iridovirus MCP was identified from previously described next-generation sequence data.[6] Sanger sequencing was used to confirm a 1,211 nucleotide region that included a partial ORF encoding 388 amino acids of the putative MCP. This MCP ORF appeared to lack ~70–90 amino acids of the C-terminus based on comparison to MCP sequences of other iridoviruses. We determined partial MCP sequences from an ENV-infected Pacific herring sampled near Port Townsend Bay (Port Townsend, Washington; GenBank KT211480) and near Malcolm Island (North Vancouver Island, British Columbia, Canada; GenBank KT211481). These sequences differed at 1 synonymous nucleotide substitution (0.09% nucleotide difference). The ENV MCP amino acid sequence differed from other iridovirus MCP sequences

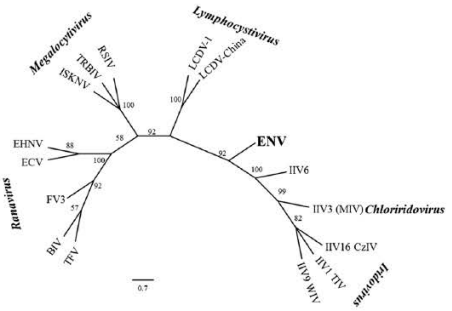

Figure 1. Relationship of erythrocytic necrosis virus (ENV) to other iridoviruses based on partial sequencing of the major capsid protein. Evolutionary relationships were inferred using the maximum likelihood with Poisson correction model. The numbers above the nodes represent bootstrap support after 1,000 replicates. Virus names, iridovirus genus, and GenBank accessions are listed in Table 1.

by 47–55% (Table 1). Although MCP gene sequences of other erythrocytic iridoviruses were not available in GenBank, the ENV MCP sequence determined herein did not cluster with homologs from members of any of the established genera of family *Iridoviridae* (Fig. 1).

Assay development

Nucleotide BLAST[1] searches of the MCP and ATPase qPCR primer and probe sequences revealed no sequences with significant homology. Initial tests of the qPCR assays revealed that infected fish tissues can harbor extremely high ENV copy numbers (<15 Cq); templates with high copy numbers yielded better results when diluted 1:100. We observed no effect on assay sensitivity when an exogenous IPC reagent was included in the reaction (Table 3); the IPC is a control to detect false negatives due to PCR inhibitors. We observed no effect on assay sensitivity when the fluorescent VIC[k]-labeled APC probe was included in the reaction (Table 3); the APC probe is a control designed to detect false-positive reactions due to contamination with the artificial positive control. The IPC and APC probes are both labeled with the same reporter molecule so they were not tested simultaneously. The ATPase qPCR reaction did not perform optimally with a universal commercial master mix[o] as evidenced by higher Cq value, but no Cq differences were observed with the other commercial master mixes[l,p] (Table 3). Our standard operating procedure for the analytical and diagnostic validation used a commercial master mix[l] and included the APC probe in the duplex reaction.

Analytical validation

The slopes of the lines obtained when serial 10-fold dilutions of the APC plasmid were analyzed were −3.5 and −3.6 for the MCP and ATPase qPCR assays, respectively (Fig. 2A, 2C). Similar slopes were obtained for total DNA tested with the MCP (–3.9) and ATPase (–3.7) qPCR assays (Fig. 2B, 2D), indicating similar, and near optimal, PCR reaction efficiencies for both template types. Both the MCP and ATPase qPCR assays reliably detected APC plasmid DNA copies ranging from 5×10^7 to 50 copies per reaction; a final dilution to 5 copies was detected in 3 of 4 technical replicates for both assays (Fig. 2A, 2C). A serially diluted total DNA sample derived from the kidney of an ENV-infected herring, which amplified with a starting Cq value of 22.0 equating to 1.3×10^5 copies per reaction, was reliably detected to a lower limit of an estimated 13 copies per reaction using the ATPase qPCR assay (Fig 2D). The MCP qPCR assay detected 13 copies per reaction in 3 of the 4 technical replicates (Fig. 2B). The MCP and ATPase qPCR assays did not produce detectable amplification when DNA from other iridoviruses (RSIV, ISKNV, or LCDV) was used.

Three ENV-infected Pacific herring DNA samples were tested in 10 replicate wells to evaluate the within-run repeatability. Between-run repeatability was evaluated by testing the same samples in quadruplicate over 4 separate days. For the MCP qPCR assay, the within-run repeatability CV range was 0.6–1.3% and the between-run repeatability CV range was 0.8–1.4% (Table 4). For the ATPase qPCR assay, the within-run repeatability CV range was 0.5–1.7% and the between-run repeatability CV range was 0.5–1.1% (Table 4). There was a general trend toward lower repeatability in sample 3, which had a mean Cq value of 35.9, suggesting repeatability decreased as ENV copy number decreased.

Diagnostic validation of ENV qPCR assays

The MCP and ATPase ENV qPCR assays were tested on a population of 100 Pacific herring for which half the fish were injected with medium ($n = 50$ assumed as true negative) and the other half with ENV-positive tissue homogenates ($n = 50$ assumed as true positives). All 50 true negative fish tested negative by the ATPase qPCR assay, whereas 49 of the 50 fish tested negative by the MCP qPCR assay. All 50 true-positive fish tested positive by both the MCP and ATPase qPCR assays. DSe of both qPCR assays was 1.00 (95% confidence interval [CI]: 0.93–1.00). DSp was calculated for the MCP assay as 0.98 (95% CI: 0.90–1.00) and for the ATPase assay as 1.0 (95% CI: 0.93–1.00; Table 5). The positive and negative LRs for both the MCP and ATPase ENV qPCR assays were LR+ = 99.0 and LR– = 0.01. Technical difficulties made a number of blood smears unreadable and so only 87 total fish were evaluated for viral inclusion bodies. Viral inclusions were noted in 0 of 48 true-negative fish and 38 of 39 true-positive fish. DSe and DSp for blood smear analysis was 0.97 and 1.0, respectively. The diagnostic odds ratio (DOR), which defines how well a test correctly classifies a sample, was highest for the ATPase qPCR assay (9,901), fol-

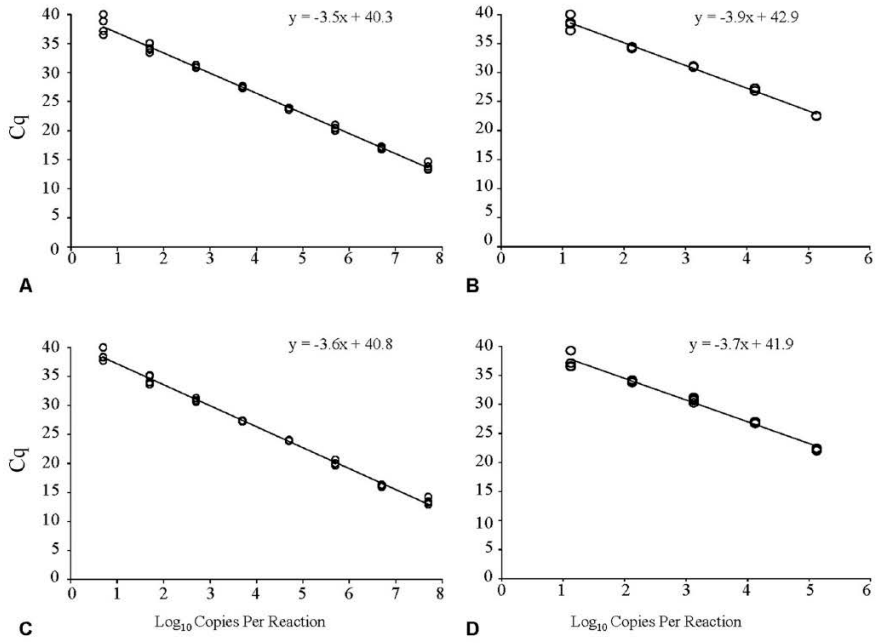

Figure 2. Analytical sensitivity of the erythrocytic necrosis virus (ENV) quantitative real-time PCR assays targeting the major capsid protein (MCP) or ATPase-like gene. Serial dilutions of (**A**) plasmid DNA analyzed with the MCP qPCR, (**B**) ENV-infected Pacific herring (*Clupea pallasii*) total DNA analyzed with the MCP qPCR assay, (**C**) plasmid DNA analyzed with the ATPase qPCR assay, and (**D**) ENV-infected Pacific herring total DNA analyzed with the ATPase qPCR assay. Four replicate wells were analyzed for each dilution.

Table 4. Within-run and between-run repeatability of the major capsid protein (MCP) and ATPase-like quantitative real-time PCR assays.*

Assay/ Sample name	Within-run repeatability		Between-run repeatability	
	Mean Cq ± SD	CV%	Mean Cq ± SD	CV%
MCP				
1	25.5 ± 0.2	0.9	25.8 ± 0.3	1.1
2	30.7 ± 0.2	0.6	31.1 ± 0.4	1.4
3	34.8 ± 0.4	1.3	35.4 ± 0.3	0.8
ATPase				
1	25.3 ± 0.1	0.5	25.6 ± 0.3	1.1
2	30.8 ± 0.2	0.7	30.8 ± 0.2	0.5
3	34.9 ± 0.6	1.7	34.8 ± 0.4	1.1

* Within-run repeatability was evaluated by testing DNA from 3 erythrocytic necrosis virus–infected tissue samples in 10 replicate wells. Between-run repeatability was evaluated by testing the same DNA samples in 4 replicate wells over 4 independent days.

lowed by the MCP assay (9,800), and lowest for blood smear analysis (3,761).

ENV infection progression

Detection of ENV in kidney tissue by qPCR and presence of cytoplasmic inclusion bodies in the erythrocytes were evaluated in laboratory-challenged Pacific herring. Copy numbers determined by the MCP or ATPase ENV qPCR assays were highly correlated ($r^2 = 0.99$; data not shown). ENV DNA was detected at the first sampling time point (5 dpi), continued to increase until20 dpi, and levels remained similar until the final sample at 25 dpi (Fig. 3A, 3B). In contrast, erythrocytic inclusion bodies were not observed until 10 dpi and continued to increase until achieving maximum value at 25 dpi (Fig. 3C). Both ENV qPCR assays detected DNA in 100% of the injected fish, whereas only 72% of the fish had detectable inclusion bodies. The mean log viral load of fish that had detectable intraerythrocytic inclusion bodies was 1.8×10^8 MCP copies per reaction, whereas fish with undetectable intraerythrocytic inclusion bodies had a significantly lower 2.3×10^2 copies per reaction (*t*-test; $p < 0.001$); a similar magnitude of difference between samples was assessed as positive or negative for inclusion bodies and tested using the ATPase qPCR assay ($p < 0.0001$; Fig. 3D).

Table 5. Comparison of 3 diagnostic tests for erythrocytic necrosis virus (ENV), including blood smears to visualize intraerythrocytic inclusion bodies, quantitative real-time PCR (qPCR) for the ENV major capsid protein (MCP) and qPCR for the ENV ATPase-like gene.*

Assay	TP	FN	TN	FP	DSe†	DSp†	LR+	LR–	DOR
Blood smear‡	38	1	48	0	0.97 (0.87–1.04)	1.00 (0.93–1.00)	97.44	0.03	3,761
MCP qPCR	50	0	49	1	1.00 (0.93–1.00)	0.98 (0.90–1.00)	99.01	0.01	9,800
ATPase qPCR	50	0	50	0	1.00 (0.93–1.00)	1.00 (0.93–1.00)	99.01	0.01	9,901

* Diagnostic sensitivity (DSe), diagnostic specificity (DSp), and 95% confidence intervals were calculated using EpiTools (epitools.ausvet.com.au/); other diagnostics parameters were calculated directly.[3,5] Diagnostic tests were evaluated using 50 fish injected with medium and 50 fish injected with ENV. For each assay, the number of fish classified as true positive (TP), false negative (FN), true negative (TN), and false positive (FP) is listed. LR = likelihood ratio; DOR = diagnostic odds ratio.

† Numbers in parentheses are 95% confidence intervals.

‡ Technical difficulties with the blood smears resulted in only 40 samples from the ENV-infected (TP + FN) group and 48 samples from the mock infected (TN + FP) group being evaluated.

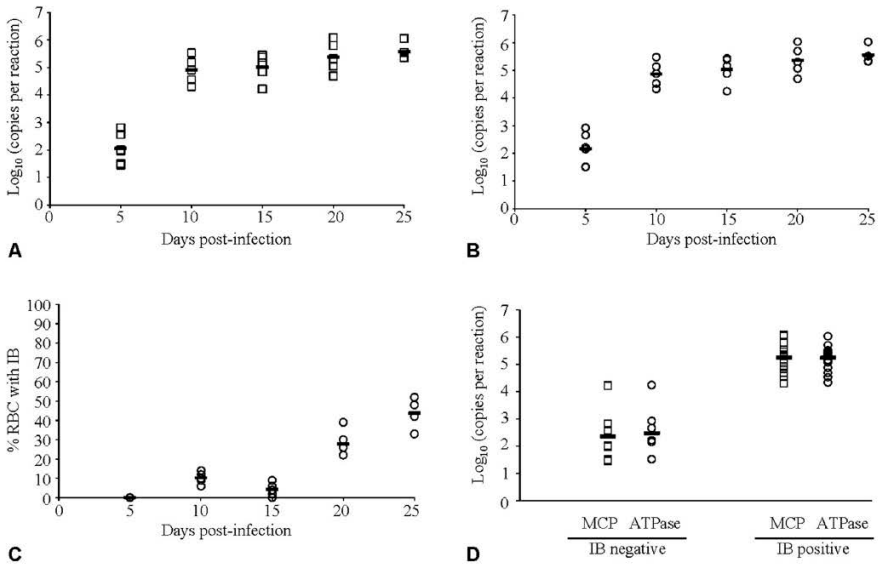

Figure 3. Progression of erythrocytic necrosis virus (ENV) infection in Pacific herring (*Clupea pallasii*) measured with quantitative real-time PCR (qPCR) assays targeting the (**A**) major capsid protein (MCP) or (**B**) ATPase-like gene or (**C**) by determining the percentage of red blood cells (RBC) with visible cytoplasmic inclusion bodies (IBs). Panel (**D**) compares the ENV copy number determined by qPCR in samples that were negative for IBs and samples that tested positive for IBs. In all panels, each point represents an individual fish sample and black bars represent the mean of the group.

Discussion

Phylogenetic analysis of the conserved DNA polymerase gene indicates that ENV groups with several reptilian erythrocytic viruses, which may constitute the sixth genus within the family *Iridoviridae*.[6] In our study, the MCP gene, which is often used for phylogenetic studies of iridoviruses, was evaluated.[27] Viruses from distinct iridovirus genera have ~50% amino acid variation in the MCP gene.[27] Unfortunately, MCP sequences were not available from the reptilian erythrocytic iridoviruses.[4,10,26] However, the ENV MCP differed by 47–54% amino acids when compared to other iridoviruses assigned to 1 of the 5 accepted genera (http://goo.gl/y7kqcg), supporting the conclusion that ENV is a novel member of the family. The disease VEN has been reported from a large number of marine fish species, from both the Atlantic and Pacific Ocean and Northern and Southern hemispheres.[3] It is not clear how ENV may vary across the reported wide host and geographic range or if all previous reports of ENV represent a single virus species. For instance, differences in virion ultrastructure and size were noted in Atlantic herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) that were

presumptively diagnosed with VEN.[16] We made multiple attempts to obtain ENV-infected fish tissues from other geographic locations outside the Salish Sea for use in this study but we were not successful. Thus, these assays should be used cautiously when diagnosing VEN in fish from regions outside the Salish Sea.

Diagnostic validation indicated that the 2 new ENV qPCR assays developed herein have similar DSe and DSp as blood smear analysis. The ATPase qPCR assay yielded the highest DOR value indicating that this test was the most likely to correctly classify the samples; although a very similar DOR was obtained for the MCP qPCR assay. To estimate DSe and DSp, all test samples were classified as either positive or negative by qPCR regardless of viral copy number. Viral copy quantification is not necessarily needed for ENV diagnosis but this information may contribute to a better understanding of the relationship between virus copy number and clinical disease. Our diagnostic validation study in Pacific herring examined only a single time point when intraerythrocytic inclusion bodies were evident. However, sequential sampling revealed detectable viral DNA by 5 dpi, whereas erythrocytic inclusion bodies were not observed until the next time point, 10 dpi. Thus, the qPCR assays may detect ENV DNA during early infection or in light infections. The transient delay in inclusion body formation in our progression study was consistent with previous laboratory challenge studies and confinement studies in Pacific herring, which found detectable erythrocytic inclusion bodies appearing in a low percentage of cells 4–6 days after initiation of the epizootic.[9,12] Although DSe and DSp of blood smear analysis was similar to the qPCR assays, it should be noted that technical difficulties were encountered with the analysis of some blood films, where cell lysis occurred after inadvertent contact with seawater. We were not able to confidently analyze 20% of blood smear slides from the ENV-infected fish. If we had chosen to classify unreadable smears as negative test results and included these results in our DSe and DSp calculations, the molecular assays would have had significantly higher DSe relative to blood smear analysis. However, blood smear analysis has the advantage that it provides additional clinical information regarding the severity of anemia, erythroblast proliferation, and leukocytopenia.

Some naturally infected wild herring with heavy intensities of infection have demonstrated extremely high viral loads with $>5 \times 10^9$ DNA copies per PCR reaction (Cq values <10; data not shown). At times, extracted DNA from ENV-infected herring needed to be diluted 100-fold to obtain amplification within the linear range of the assay. The high virus copy number in samples may increase the likelihood of cross-contamination of samples during DNA extraction and assay preparation, and negative controls should be included throughout the sample processing.[19] It is advisable that samples representing different populations be processed separately to avoid cross-contamination. One approach commonly used to reduce potential false-positive test results is to select a quantification cycle cutoff value (e.g. Cq < 35) with the assumption that low levels of PCR amplification are due to probe degradation, nonspecific amplification, or cross-contamination.[2] In our study, the single false-positive test result obtained with the MCP qPCR had a mean Cq of 38.5 (0.3 viral copies per PCR reaction). Thus, a Cq cutoff of 38 would have eliminated the false-positive test result and maximized the DOR for the MCP qPCR assay. However, it cannot be excluded that the single fish classified as a false positive actually had a low level, covert ENV infection. Unfortunately, we did not have duplicate tissue samples available for retesting because of the small size of the fish used in this study. Further research is needed to determine if ENV can persist in asymptomatic carrier fish and, if so, to determine the accuracy of the existing diagnostic tests to identify these carrier fish. Ultimately, the use of a cutoff value for interpreting qPCR results depends on test purpose and the relative importance of minimizing false-positive or false-negative test results.[2]

Nearly all nonmammalian vertebrates, including fish, have nucleated erythrocytes that are specifically targeted by a variety of pathogens.[15] In fish, a number of viruses are associated with intraerythrocytic cytoplasmic inclusion bodies, including ENV, erythrocytic inclusion body syndrome virus (EIBSV),[14,22] piscine orthoreovirus (PRV),[7] rainbow trout *Oncorhynchus mykiss* erythrocytic virus (Landolt ML, et al., 1977), intraerythrocytic virus–like particle in coho salmon (*O. kisutch*; Hedrick RP, et al., 1987), and viral erythrocytic infection virus (VEIV).[18] Among these, genomic sequence data are available to date for only ENV and PRV,[6,17] and PRV is the only intraerythrocytic virus for which polyclonal antibodies are available.[8] Thus, confirmatory diagnosis of most intraerythrocytic viruses still requires ultrastructural analysis by TEM. The sensitive and specific molecular assays reported herein will facilitate more rapid and accurate confirmatory diagnosis of ENV and will help to distinguish this virus from other intraerythrocytic viruses that form inclusion bodies. Additionally, these molecular assays will be especially useful for diagnostic cases where blood films are not collected from fresh specimens. For example, observations of fish die-offs in remote locations are typically reported by fisheries biologists and/or members of the public who typically freeze samples at −20°C until the appropriate diagnostic authority is identified. Prior to the advent of PCR-based ENV assays, the possible involvement of ENV in these fish die-off events was difficult to assess because of the inability to collect quality blood films from previously frozen carcasses.

The ENV qPCR assays developed in our study can be used as a primary screening surveillance tool for ENV infections or for confirmatory diagnosis of presumptive VEN. However, the diagnostic utility of these ENV qPCR assays should be considered limited because our study only targeted Salish Sea populations of Pacific herring using laboratory challenges with endemic strains of ENV to create populations of known

infection status. More work is needed to validate these ENV assays in natural populations, including their applicability for use in other host species and geographic locations. Sequencing of ENV genes, particularly the MCP gene, from a broad range of fish demonstrating clinical cases of VEN will help to better define the potential genetic diversity present in this viral species.

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Authors' note

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Government of any product or service to the exclusion of others that may be suitable.

Authors' contributions

MK Purcell, S Pearman-Gillman, and RL Thompson drafted the manuscript. MK Purcell and PK Hershberger contributed to conception and design of the study; contributed to acquisition, analysis, and interpretation of data; critically revised the manuscript; and gave final approval. S Pearman-Gillman contributed to design of the study, and contributed to acquisition, analysis, and interpretation of data. RL Thompson contributed to design of the study; contributed to acquisition and interpretation of data; critically revised the manuscript; and gave final approval. JL Gregg contributed to design of the study; contributed to analysis and interpretation of data; and gave final approval. LM Hart contributed to design of the study; contributed to analysis and interpretation of data; critically revised the manuscript; and gave final approval. JR Winton and EJ Emmenegger contributed to conception of the study; contributed to acquisition of data; critically revised the manuscript; and gave final approval. All authors agreed to be accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Sources and manufacturers

a. Cyclop-eeze, Argent Chemicals, Redmond, WA.
b. Bio-Olympic, Bio-Oregon, Longview, WA.
c. Qiagen DNeasy mini kit, Qiagen Inc., Valencia, CA.
d. NanoDrop 1000, Thermo Scientific, Wilmington, DE.
e. *Taq* PCR core kit, Qiagen Inc., Valencia, CA.
f. ABI 3130 genetic analyzer, Life Technologies, Carlsbad, CA.
g. Big Dye terminator v. 1.1, Life Technologies Inc., Carlsbad, CA.
h. Sequencher V.5.2.4 software, Gene Codes Corp., Ann Arbor, MI.
i. Primer Express software V.3.0 Life Technologies, Carlsbad, CA.
j. pIDT Smart vector, Integrated DNA Technologies, Coralville, IA.
k. ViiA 7 real-time PCR system, Life Technologies Inc., Carlsbad, CA.
l. TaqMan gene expression master mix, Life Technologies Inc., Carlsbad, CA.
m. InStat version 3, GraphPad, La Jolla, CA.
n. TaqMan exogenous internal positive control reagents, Life Technologies Inc., Carlsbad, CA.
o. TaqMan universal PCR master mix, Life Technologies Inc., Carlsbad, CA.
p. FastStart PCR master, Roche Life Sciences, Indianapolis, IN.

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Appendix 14

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Development and Evaluation of a Blocking Enzyme-Linked Immunosorbent Assay and Virus Neutralization Assay To Detect Antibodies to Viral Hemorrhagic Septicemia Virus

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Viral hemorrhagic septicemia virus (VHSV) is a target of surveillance by many state and federal agencies in the United States. Currently, the detection of VHSV relies on virus isolation, which is lethal to fish and indicates only the current infection status. A serological method is required to ascertain prior exposure. Here, we report two serologic tests for VHSV that are nonlethal, rapid, and species independent, a virus neutralization (VN) assay and a blocking enzyme-linked immunosorbent assay (ELISA). The results show that the VN assay had a specificity of 100% and sensitivity of 42.9%; the anti-nucleocapsid-blocking ELISA detected nonneutralizing VHSV antibodies at a specificity of 88.2% and a sensitivity of 96.4%. The VN assay and ELISA are valuable tools for assessing exposure to VHSV.

Viral hemorrhagic septicemia (VHS) is one of the most pathogenic viral diseases of fish worldwide and affects a wide range of host species (1–7). Of the four genotypes, the North American strains of VHS virus are designated types IVa and IVb. Type IVa was originally isolated from asymptomatic marine salmonids in the Pacific Northwest in 1988 (8); it is now known to be endemic throughout the northeast Pacific, where it is highly virulent to populations of Pacific herring (*Clupea pallasii*) and other marine fishes (9). A new freshwater strain, type IVb, was isolated from a muskellunge (*Esox masquinongy*) collected from Lake St. Clair, MI, in 2003 (10). This distinctive sublineage has been isolated from 31 species of fish in the Great Lakes (11) and has been associated with significant die-off events of freshwater drum (*Aplodinotus grunniens*), muskellunge (*Esox masquinongy*), gizzard shad (*Dorosoma cepedianum*), round gobies (*Apollonia melanostomus*), and yellow perch (*Perca flavescens*) in the Great Lakes between 2005 and 2008 (2, 10, 12–16). By 2009, the virus had spread to all of the Great Lakes and several inland lakes. The introduction and spread of this pathogen and the threat it poses to a broad range of hosts resulted in increased surveillance of the virus in Wisconsin and other states within the Great Lakes Basin.

Currently, the surveillance methods for VHS virus (VHSV) detection include virus isolation in cell culture, followed most commonly by confirmation by reverse transcription-PCR (RT-PCR). Fish are tested for VHSV according to the guidelines outlined in the American Fisheries Society Fish Health Section Blue Book (17) and the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals (18). Both approved methods detect the virus but do not detect antibodies indicative of previous virus exposure.

Clinical signs of disease are not consistent among VHSV-susceptible species, and VHSV IVb is not always isolated from clinically affected fish, especially salmonids (19, 20). Differences in susceptibility and mortality rates among different populations of yellow perch have been reported recently (21). The clinical signs and severity of infection also depend on water temperature at the time of infection, stress level, host age, and other environmental factors (15, 22). These variables can affect the narrow window of opportunity to detect VHSV by virus isolation; therefore, diseased or recovered individuals may easily be missed during surveillance efforts.

Methods to detect neutralizing antibodies to VHSV have been developed for surveillance using a complement-dependent neutralization test (50% plaque neutralization test [PNT]) and have been highly sensitive and specific for trout (23–25). However, PNT requires overlay and plaque enumeration steps; further, this method is best suited for small sample sizes. A microneutralization format without the use of overlay might lead to a 50% reduction in the resources and labor required to perform the assay. Another advantage of a virus neutralization assay is that the indicator system is a susceptible cell line for the target virus, which makes the assay inherently species independent.

Competitive and blocking enzyme-linked immunosorbent assays (ELISAs) for the detection of antibodies against mammalian viruses have been in use for decades. Indirect ELISAs have been available for VHSV since 1988 (26). A highly sensitive (92%) indirect ELISA for detecting nonneutralizing antibodies for the surveillance of VHS in farmed rainbow trout (*Oncorhynchus mykiss*) has also been described (25), but it requires a species-specific secondary fish antibody. Thus, these tests are not practical for multispecies VHSV surveillance in the wild, because there are at least 31 species known to be susceptible to VHSV IVb.

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Here, we describe the development and evaluation of a modified virus neutralization (VN) assay and a blocking enzyme-linked immunosorbent assay (ELISA). Both tests were adapted from previously described methods in the World Organisation for Animal Health (OIE) manual (27) and use blood serum samples from uninfected fish and VHS survivors. Although the development of the anti-VHSV nucleocapsid monoclonal antibody used in our ELISA was published in 1988 (26), no competitive ELISA methods have been reported until now. These serological methods broaden the window of detection by demonstrating whether previous exposure to the virus had occurred, which might alleviate the time constraints of surveillance efforts using virus isolation.

MATERIALS AND METHODS

Cell lines. The epithelioma papulosum cyprini (EPC) cell line (American Type Culture Collection, Manassas, VA), originating from fathead minnow (*Pimephales promelas*) epithelial cells (28), was cultured at 25°C according to detailed protocols (18, 29), and the medium was supplemented with tryptose phosphate broth (Teknova; Hollister, CA, USA), 5% fetal bovine serum (FBS) (PAA Laboratories, Inc., Etobicoke, Ontario, Canada), 200 mM L-glutamine (Life Technologies), and buffered with 7.5% sodium bicarbonate solution (Life Technologies). The Chinook salmon embryo (CHSE-214) cell line (American Type Culture Collection, Manassas, VA) was cultured at 20°C, and the medium was supplemented with 10% FBS (PAA Laboratories, Inc.).

Virus isolate. The Great Lakes strain of VHSV (type IVb) was isolated on the EPC cell line. The isolate was obtained from pooled kidney and spleen tissue samples from a freshwater drum in Lake Winnebago during a VHS outbreak in 2007 and confirmed by real-time RT-PCR (30, 31).

Virus propagation and purification. Virus was adsorbed to the EPC cells at a multiplicity of infection (MOI) of 0.01 for 30 min at room temperature and then supplemented with cell culture medium. To propagate virus, flasks were incubated at 15°C for 5 to 7 days or until the first signs of cytopathic effect (CPE) were observed. A plaque-purified stock of VHSV-infected EPC cell supernatant was clarified by the removal of EPC cells, aliquoted for one-time use, and stored at −80°C. Postfreezing, an aliquot was thawed, and the titer of the batch was determined.

CHSE cells were used to propagate virus for ELISA antigen coating. The flasks for propagation were inoculated using the same methods as for the EPC cells but virus was adsorbed for 1 h at room temperature. After two freeze-thaw cycles, cell debris was removed by centrifugation at 4,000 × *g* for 15 min at 4°C in a Sorvall ST40R centrifuge (Thermo) and clarified. The supernatant was purified and concentrated according to the manufacturer's protocols using a Fast-Trap virus purification and concentration kit (Millipore, Billerica, MA). Eluted virus was aliquoted and stored at −80°C. A mock infection was performed in a similar manner to provide cell lysates for determining an optical density baseline in uninfected CHSE cells. Antigen was treated with 10% MEGA-10 detergent (Sigma-Aldrich) for an hour at room temperature prior to diluting in coating buffer for use in the ELISA.

Sera from fish of known infection status. Blood serum samples were obtained from 33 uninfected fish (Table 1), including brown trout (*Salmo trutta*) and yellow perch. A blood serum sample with antibodies to spring viremia of carp virus (SVCV) was obtained from a common carp (*Cyprinus carpio*) (Table 1). The serum samples were collected 4 to 5 months after an SVCV epizootic occurred in May 2002 in Cedar Lake, WI, and tested positive for neutralizing antibodies to SVCV at the Center for Environment, Fisheries, and Aquaculture Science (CEFAS) in Weymouth, United Kingdom, using a competitive ELISA (32). Serum samples were obtained from 28 experimentally infected or wild-caught fish that had survived exposure to VHSV (Table 2), including grass carp (*Ctenopharyngodon idella*), yellow perch, Pacific herring (*C. pallasii*), muskellunge, and freshwater drum. All serum samples were stored frozen at −80°C and then heated to 45°C for 30 min to inactivate complement before use in assays.

TABLE 1 Virus neutralization and blocking ELISA results for VHS-negative group

Serum source	VN titer result	ELISA data	
		% inhibition[a]	Result
Salmo trutta (brown trout) 1[b]	Negative	8.13	Negative
Salmo trutta (brown trout) 2	Negative	31.54	False positive
Salmo trutta (brown trout) 3	Negative	18.88	Negative
Salmo trutta (brown trout) 4	Negative	13.38	Negative
Salmo trutta (brown trout) 5	Negative	19.84	Negative
Salmo trutta (brown trout) 6	Negative	4.65	Negative
Salmo trutta (brown trout) 7	Negative	14.58	Negative
Salmo trutta (brown trout) 8	Negative	20.2	Negative
Salmo trutta (brown trout) 9	Negative	13.50	Negative
Salmo trutta (brown trout) 10	Negative	8.73	Negative
Salmo trutta (brown trout) 11	Negative	13.50	Negative
Salmo trutta (brown trout) 12	Negative	24.73	Negative
Salmo trutta (brown trout) 13	Negative	5.50	Negative
Salmo trutta (brown trout) 14	Negative	8.13	Negative
Salmo trutta (brown trout) 15	Negative	14.34	Negative
Salmo trutta (brown trout) 16	Negative	15.18	Negative
Salmo trutta (brown trout) 17	Negative	9.92	Negative
Salmo trutta (brown trout) 18	Negative	9.90	Negative
Salmo trutta (brown trout) 19	Negative	41.10	False positive
Salmo trutta (brown trout) 20	Negative	11.35	Negative
Salmo trutta (brown trout) 21	Negative	13.03	Negative
Salmo trutta (brown trout) 22	Negative	14.81	Negative
Salmo trutta (brown trout) 23	Negative	16.49	Negative
Salmo trutta (brown trout) 24	Negative	5.14	Negative
Salmo trutta (brown trout) 25	Negative	20.13	Negative
Salmo trutta (brown trout) 26	Negative	14.34	Negative
Salmo trutta (brown trout) 27	Negative	34.29	False positive
Salmo trutta (brown trout) 28	Negative	24.73	Negative
Salmo trutta (brown trout) 29	Negative	2.39	Negative
Salmo trutta (brown trout) 30	Negative	0.24	Negative
Perca flavescens (yellow perch) 1[c]	Negative	12.66	Negative
Perca flavescens (yellow perch) 2	Negative	27.48	False positive
Perca flavescens (yellow perch) 3	Negative	23.90	Negative
Cyprinus carpio (common carp)[d]	Negative	31.45[e]	Negative

[a] Results determined positive at ≥25% inhibition for test sera diluted 1:2 and ≥35% inhibition for undiluted test sera.
[b] Brown trout 1 to 30 were captive broodstock from Westfield, Wisconsin State Fish Hatchery that were never exposed to VHSV.
[c] Yellow perch 1 to 3 were lab-reared at the Great Lakes Water Institute in Milwaukee, WI, and never exposed to VHSV.
[d] Wild-caught from Cedar Lake, WI, following spring viremia of carp virus (SVCV) epizootic in May 2002. The serum sample was positive for neutralizing antibodies to SVCV at the Weymouth Laboratory, Weymouth, United Kingdom, using standard methods (40).
[e] Result from ELISA with undiluted serum, in which the positive threshold is ≥35% inhibition.

All yellow perch used in the study were hatched and reared at the University of Wisconsin, Milwaukee (UWM) School of Freshwater Sciences (SFS) Aquaculture Research Facility, according to previously described methods (33). They were exposed to VHS virus strain IVb (MI03) by intraperitoneal (i.p.) injection at a titer of 1×10^4 PFU/fish (J-Z fish) or 1×10^2 PFU/fish (H-Y fish). At 28 days post-VHSV injection, all yellow perch exhibited mild clinical signs of the disease, such as exophthalmia and hemorrhaging. The fish euthanized on day 64 appeared healthy, exhibiting no clinical signs of VHS, and all plaque assay results were negative.

Hyperimmunized Pacific herring (*C. pallasii*) were produced from laboratory-reared specific-pathogen-free (SPF) colonies (34). Briefly, SPF herring ≥5 years of age were immersed in waterborne VHSV (1.5×10^3

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TABLE 2 Real-time RT-PCR, virus neutralization, and ELISA results for VHS-positive group

Serum source	VHS PCR result	VN titer result	ELISA data	
			% inhibition[a]	Result
Ctenopharyngodon idella (grass carp) 1[b]	Negative	Negative	75.12	Positive
Ctenopharyngodon idella (grass carp) 2[b]	C_T, 39.5	Negative	58.38	Positive
Perca flavescens (yellow perch) HI-14[c]	C_T, 36.5	1:16	72.97	Positive
Perca flavescens (yellow perch) J2-13[d]	Negative	p1:16[j]	78.26	Positive
Perca flavescens (yellow perch) J1-13/J3-11[d]	C_T, 37.8	p1:16[j]	46.96	Positive
Perca flavescens (yellow perch) H4 A[c]	C_T, 35.7	p1:16[j]	36.92	Positive
Perca flavescens (yellow perch) H4 B[c]	C_T, 35.7	p1:16[j]	54.04	Positive
Perca flavescens (yellow perch) H4 C[c]	C_T, 38.6	Negative	55.93	Positive
Perca flavescens (yellow perch) J4 A[d]	C_T, 32.6	Negative	42.30	Positive
Perca flavescens (yellow perch) J4 B[d]	Negative	Negative	27.62	Positive
Perca flavescens (yellow perch) Z1-2[e]	Negative	Negative	81.42	Positive
Perca flavescens (yellow perch) Z2-1[e]	Negative	Negative	95.99	Positive
Perca flavescens (yellow perch) Z2-2[e]	Negative	Negative	57.22	Positive
Perca flavescens (yellow perch) Y1-2[f]	Negative	Negative	49.47	Positive
Perca flavescens (yellow perch) Y3-1[f]	Negative	Negative	47.60	Positive
Perca flavescens (yellow perch) Y3-3[f]	Negative	Negative	56.15	Positive
Clupea pallasii (Pacific herring) 140[g]	Negative	p1:16[j]	42.22	Positive
Clupea pallasii (Pacific herring) 141[g]	Negative	Negative	56.22	Positive
Clupea pallasii (Pacific herring) 142[g]	Negative	p1:16[j]	56.91	Positive
Clupea pallasii (Pacific herring) 143[g]	Negative	Negative	41.23	Positive
Clupea pallasii (Pacific herring) 144[g]	Negative	Negative	30.95	Positive
Clupea pallasii (Pacific herring) 145[g]	Negative	1:32	27.23	Positive
Clupea pallasii (Pacific herring) 146[g]	Negative	p1:16[j]	43.88	Positive
Clupea pallasii (Pacific herring) 147[g]	Negative	Negative	26.15	Positive
Clupea pallasii (Pacific herring) 148[g]	Negative	p1:16[j]	34.67	Positive
Clupea pallasii (Pacific herring) 149[g]	C_T, 38.9	Negative	41.82	Positive
Esox masquinongy (muskellunge)[h]	Negative	1:80	7.94	False negative
Aplodinotus grunniens (freshwater drum)[i]	Negative	1:16	32.32	Positive

[a] Results determined positive at ≥25% inhibition for test serum diluted 1:2.
[b] Injected intraperitoneally (i.p.) with 200 μl of 10^6 PFU/fish VHSV IVb, serum collected 21 days post-i.p. injection.
[c] Injected i.p. with 1×10^2 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.
[d] Injected i.p. with 1×10^4 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.
[e] Injected i.p. with 1×10^4 PFU/ml VHSV IVb, held at 12°C, serum collected 64 days post-i.p. injection.
[f] Injected i.p. with 1×10^5 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.
[g] Hyperimmunized Pacific herring were exposed to 1.5×10^3 PFU/ml VHSV IVa by waterborne immersion for 1 h (day 0). The survivors were reexposed by i.p. injection after 49 days (2.9×10^2 PFU/fish) and 77 days (2.8×10^1 PFU/fish). Serum samples were collected from the hyperimmunized survivors after 112 days.
[h] Survived infection with VHSV IVb. The reference serum was received already diluted at 1:20 and used as the starting dilution for the VN assay. A new aliquot was obtained and used at 1:2 in the ELISA.
[i] Wild-caught on Lake Winnebago in Wisconsin on 9 May 2012. Kidney and spleen tissues tested positive for VHSV by real-time PCR according to previously described methods (31).
[j] p1:16, partial neutralization at this dilution.

PFU/ml) for 1 h. The survivors were reexposed to VHSV by i.p. injection after 49 days (2.9×10^2 PFU/fish) and 77 days (2.8×10^1 PFU/fish). Serum samples were collected from the hyperimmunized survivors 112 days after the initial waterborne exposure.

Virus neutralization. The VN assay to detect VHSV-neutralizing antibodies was modified from the mammalian VN assay protocol based on previously described methods (27). The VHSV VN assay was performed as follows: first, epithelioma papulosum cyprini (EPC) cells were preseeded onto sterile microtiter plates, typically 2 days prior to inoculation to achieve 100% confluence. Next, 50 μl of 100 × the 50% tissue culture infective dose ($TCID_{50}$) of virus (35, 36) was mixed with 2-fold serial dilutions of serum starting at 1:16 in 96-well cell culture microtiter plates (BD Biosciences, San Jose, CA) and incubated at 15°C for 24 h. A back titration plate with 10-fold dilutions of the working dilution of virus ($100 \times TCID_{50}$) was included to confirm the correct virus concentrations. Serum controls (serum without virus) were performed for each sample as well as an antibody positive and negative control on each plate. The cells were treated with 7% polyethylene glycol (PEG) for 10 min (37). Lastly, serum-virus mixtures were inoculated onto the PEG-treated cells, covered, and incubated at 15°C for 5 days. The virus neutralization titers were read as the last serum dilution showing protection of the cell monolayer.

Cross-reactivity of anti-VHSV nucleocapsid monoclonal antibody with SVCV. Mouse anti-VHSV nucleocapsid monoclonal IgG antibody (Aquatic Diagnostics, Stirling, Scotland) (26) was purchased for use in the blocking ELISA. The specificity of the anti-VHSV nucleocapsid monoclonal antibody to VHSV nucleocapsid was assessed by performing a Western blot, as previously described (38, 39). Spring viremia of carp virus (SVCV) is a rhabdovirus that is closely related to VHSV and that also causes disease during the spring season. The lysates were obtained from an isolate circulating during an SVCV epizootic in wild common carp (*C. carpio*) in northwestern Wisconsin (40). VHSV and SVCV lysates were separated on 4 to 20% gradient gels by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad) using a wet transfer Mini Trans-Blot cassette according to the manufacturer's protocols in the Mini-PROTEAN Precast Gels Instruction Manual and Application Guide (Bio-Rad), with the following modifications. The membranes were soaked in Tris-buffered saline containing 0.05% (vol/vol) Tween 20 (TBS-T) with 5% StartingBlock (PBS) blocking buffer (Thermo) overnight at 4°C. The

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membranes were then incubated with anti-VHSV nucleocapsid monoclonal antibody diluted 1:100 in StartingBlock (PBS) blocking buffer at room temperature for 1 h with constant agitation. Three 5-min wash steps with TBS-T were performed after each antibody incubation step. The membranes were then incubated with peroxidase-rabbit anti-mouse IgG (H+L) (Invitrogen) at 1:1,000 in StartingBlock (PBS) blocking buffer for 1 h. A CN/DAB substrate kit (Thermo) was used for chromogenic detection of horseradish peroxidase-bound antibodies and stopped with deionized water.

Blocking enzyme-linked immunosorbent assay. A blocking ELISA was developed using modifications to a previous ELISA method (41). The anti-VHSV nucleocapsid monoclonal antibody utilized in our assay was previously shown to lack neutralizing activity (26). The antibody was purified and conjugated to horseradish peroxidase (HRP) using a commercial laboratory (American Qualex, San Clemente, CA). Alternating rows of purified MEGA-10 detergent-treated VHSV antigen and mock-infected MEGA-10 detergent-treated antigen diluted 1:100 in carbonate-bicarbonate buffer (pH 9.6) (Sigma) were adsorbed to 96-well Immulon 2 HB microtiter plates (Thermo) for 24 h at 21°C in an EchoTherm IN20 incubator (Torrey Pines Scientific) and then blocked with 200 μl StartingBlock (PBS) blocking buffer for 2 h at 20°C. Antigen and blocking buffer were aspirated from the wells using an ELx405 microplate washer (BioTek). Fifty microliters of fish test serum (either straight or diluted 1:2) was added to the wells containing VHSV antigen and mock-infected antigen and incubated for 30 min at 37°C. Directly after incubation (without washing or removal of test sera), 50 μl of the HRP-conjugated monoclonal antibody, diluted 1:5,000 in StartingBlock (PBS) blocking buffer, was added to the wells and incubated with the test sera for an additional 90 min at 37°C. The plates were then washed 3 times with phosphate-buffered saline (PBS) (pH 7.2) containing 0.05% Tween 20 (Sigma) to remove unbound antibodies. PBS was made by diluting 18.46 g of FTA hemagglutination buffer (BD, Chicago, IL) in 2 liters of deionized water. SureBlue 3,3′,5,5′-tetramethylbenzidine (TMB) 1-component microwell peroxidase substrate (KPL, Gaithersburg, MD) was used as an enzyme substrate and chromogen for development of the assay. One hundred microliters of enzyme substrate was added to each well, and the assay was developed for 15 min at 37°C. The reaction was terminated by adding 100 μl of 1% HCl TMB Stop Solution (KPL, Gaithersburg, MD) per well and the optical density (OD) at 450 nm was measured in an ELx808 absorbance microplate reader (BioTek). Multiple modified checkerboard experiments were performed to determine the optimal working dilution for the HRP-conjugated monoclonal antibody and the antigen concentrations for the coating plates.

The serum samples were tested both undiluted and at a 1:2 dilution in PBS wash solution. All OD readings for the samples and controls were adjusted by subtracting the background OD levels in the mock-infected wells. The percent inhibition (%I) was calculated using the formula $\%I = 100 - (100 \times \text{sample}_{OD}/\text{negative control}_{OD})$.

The presence of blue color after incubation with enzyme substrate indicated an absence of anti-nucleocapsid antibodies in a well. A higher concentration of anti-VHSV nucleocapsid serum antibodies in a well resulted in the absence of blue color and therefore higher percent inhibition of the mouse anti-VHSV nucleocapsid monoclonal binding to the VHSV antigen.

ROC analysis. A receiver operating characteristic (ROC) analysis was performed over a range of possible percent inhibition cutoff points for the ELISA (42). The thresholds were based on the percent inhibition values for the infected and uninfected fish.

Viral RNA analysis of serum by real-time RT-PCR. Two published real-time RT-PCR assays were used to detect viral RNA. At the Great Lakes Water Institute, University of Wisconsin (UW)-Milwaukee, RNA was extracted, and real-time PCR (21, 43) was performed on all yellow perch (Table 1). At UW-Madison, RNA was extracted, and real-time RT-PCR was performed (31) on the remaining fish. Any samples crossing the cycle threshold before cycle 40 were considered positive.

RESULTS

Prevalence of neutralizing antibodies in control serum by VN assay. The VN assay was modified from a previously described protocol (27). The results from the pilot studies (not shown) indicated that 24-h incubation of the virus and serum prior to inoculation onto the cells produced more significant neutralization of the virus versus a 30-min or 1-h incubation described in previously developed plaque neutralization test protocols for VHSV I and IVb (24, 44). The pilot studies also showed considerable toxicity at dilutions of 1:2 to 1:8. Thus, an initial dilution of 1:16 was used. Serum samples showing partial or complete protection were considered positive and were designated p1:16 (partial) or 1:16 (complete). If no protection was observed at 1:16, a serum sample was considered to be negative.

Neutralizing antibody titers were not detected in any of the serum samples from fish in the VHS-negative group ($n = 34$). Low VHSV-neutralizing titers were detected in 43% (12/28) of the fish from the VHS-positive group (Table 2), with titers ranging from p1:16 to 1:80. Thus, the VN assay had a specificity of 100% (95% confidence interval, 89.6% to 100%) and a sensitivity of 42.9% (95% confidence interval, 24.5% to 62.8%).

The addition of naive brown trout serum as complement was evaluated in our VN assay and was found to have no effect on neutralization (data not shown). A methylcellulose overlay was also evaluated for the isolation of plaques but was not necessary in reading the last serum dilution showing protection of the monolayer for determining the neutralizing antibody titer in the VN assay (data not shown). Three antibody-positive controls and one antibody-negative control were used to compare results with and without the addition of overlay, and no difference in antibody titer was observed.

Cross-reactivity of anti-VHSV nucleocapsid monoclonal antibody with SVCV. A Western blot under reduced conditions showed staining only with the nucleocapsid protein of VHSV using the anti-VHSV nucleocapsid monoclonal antibody, showing the specificity of the antibody to this protein (results not shown). No staining occurred with the SVCV lysate in a Western blot using the anti-VHSV nucleocapsid monoclonal antibody, indicating no cross-reactivity between our monoclonal detection antibody and SVCV. Specifically, these results show that there is no cross-recognition between the linear epitopes of the N proteins of VHSV and SVCV.

Analysis of anti-VHSV nucleocapsid monoclonal antibody in ELISA. A blocking ELISA is well suited for testing diagnostic samples from wildlife species because a secondary antibody is not required. At the time of assay development, no effective monoclonal antibody against the VHSV glycoprotein was available commercially. The anti-VHSV nucleocapsid monoclonal antibody used in this study was commercially available and effective. The anti-VHSV nucleocapsid monoclonal antibody has an advantage in that it detects persistent antibodies directed against the nucleocapsid. ELISA plates coated with intact viral particles revealed incomplete blocking. Treating the virus with MEGA-10 detergent prior to coating the plates was a critical step to allow for accurate identification of infected and noninfected fish. Presumably, this treatment reveals the target epitope of the nucleocapsid protein and allows the binding of the anti-nucleocapsid monoclonal antibody (45–47).

The efficacy of the anti-VHSV nucleocapsid monoclonal anti-

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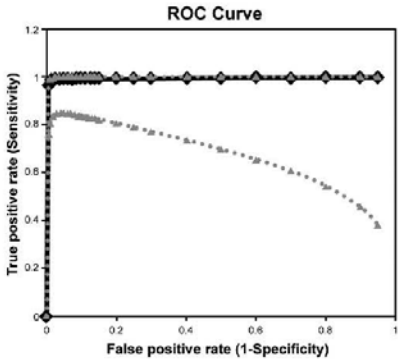

FIG 1 Receiver operating characteristic (ROC) curve of blocking ELISA using 1:2 diluted serum from VHSV-infected fish ($n = 28$) and uninfected fish ($n = 34$). The dashed lines indicate upper and lower 95% confidence intervals for the true-positive fraction, and the solid line indicates the curve for the true-positive rate.

body was evaluated by testing serum samples from the 34 uninfected and 28 previously infected fish. The serum samples were tested both undiluted and at a 1:2 dilution. The serum samples were tested at a 1:2 dilution to eliminate high background issues with hemolyzed serum.

Thirty of 34 serum samples (88.2%) from the VHS-negative group fish diluted 1:2 were negative by ELISA (Table 1). Twenty-seven of the 28 serum samples (96.4%) from the VHS-positive group fish diluted 1:2 were positive by ELISA (Table 2).

ROC analysis for ELISA. A receiver operating characteristic (ROC) curve was performed to derive the optimal percent inhibition threshold for detecting VHSV antibodies in fish serum (Fig. 1). Undiluted samples were considered positive at 35 to 100% inhibition and negative at <35% inhibition. The samples tested at a 1:2 dilution were considered positive at 25 to 100% inhibition and negative <25%, based on the ROC analysis. The area under the ROC curve was 0.994, confirming that the ELISA diagnostic performance characteristics under these thresholds were well correlated with the true status of each serum sample. These cutoff values demonstrated 88.2% specificity (95% confidence interval, 72.5% to 96.6%) and 96.4% sensitivity (95% confidence interval, 81.6% to 99.4%) with serum diluted 1:2. The percent inhibition values from uninfected fish ranged from 0.24% to 41.1% (average, 15.66%) and 7.94% to 95.99% (average, 49.21%) for previously infected fish. The positive predictive value of the ELISA for experimentally infected fish is 87.1% (95% confidence interval, 70.2% to 96.3%) and the negative predictive value is 96.8% (95% confidence interval, 83.2% to 99.5%) (Table 3 and 4).

Viral RNA detection by real-time RT-PCR. Serum samples were tested for VHSV by RT-PCR to determine if there was viral infection at the time blood was collected for fish exposed to VHSV and to determine if the inhibition of antibody binding was occurring in our tests due to antibodies being complexed with virus in the serum.

VHSV RNA was not detected by real-time RT-PCR in sera from uninfected fish. Sera from yellow perch H1-14, J1-13/J3-11, H4 A, H4 B, H4 C, and J4 A were positive, with threshold cycle (C_T) values ranging from 32.6 to 39.5 (Table 2). Serum from grass carp 2 was positive, with a C_T of 39.5, and that from Pacific herring 149 was positive, with a C_T of 38.9. All other sera from fish in the VHS-positive group tested negative for viral RNA.

TABLE 3 Results of blocking ELISA[a]

VHS infection status	No. of known positives ($n = 28$)	No. of known negatives ($n = 34$)
Positive	27	4
Negative	1	30

[a] $n = 62$. The sensitivity is 96.4% and the specificity is 88.2%, both calculated from fish in the VHS-negative and VHS-positive groups.

TABLE 4 Results of VN assay[a]

VHS infection status	No. of known positives ($n = 28$)	No. of known negatives ($n = 34$)
Positive	12	0
Negative	16	34

[a] $n = 62$. The sensitivity is 42.9% and the specificity is 100%, both calculated from fish in the VHS-negative and VHS-positive groups.

DISCUSSION

We successfully developed a virus neutralization assay and a blocking ELISA to detect neutralizing and nonneutralizing antibodies against VHSV, respectively. The VN assay has the advantage of recognizing antibodies that likely confer protective immunity to VHSV and can indicate recent exposure to the virus (25). The blocking ELISA is valuable for identifying nonneutralizing anti-nucleocapsid antibodies, which may persist longer and therefore extend the opportunity to detect VHSV antibodies after initial virus exposure (25, 48). These assays complement viral detection methods by providing a means for determining the exposure histories of wild populations.

Previous studies have described the use of complement-dependent 50% plaque neutralization tests and indirect ELISAs to detect VHSV antibodies in trout (24, 25). These methods are reliable but not practical for screening large populations of fish from multiple species. There are currently no commercially available diagnostic tests in the United States for detecting antibodies to VHSV. Surveillance efforts by virus isolation are labor-intensive, must occur within narrow water temperature windows, and are costly. Although real-time PCR assays are available, these methods generally need to be performed in high-throughput laboratories for large sample sizes. Serological assays, such as our ELISA and VN assay, provide efficient and less costly methods for evaluating the VHSV exposure histories of samples from large wild fish populations or waterbodies.

Our VN assay is different from the traditional 50% plaque neutralization test (24, 44, 49) in that it is performed in a microneutralization format and the antibody titers are read as the last serum dilution showing complete protection of the cell monolayer from VHSV. Additionally, we determined that methylcellulose overlay is not necessary in our VN assay because plaques are not counted to determine the titers. Complement has been shown to enhance neutralization in 50% PNTs when applied to trout serum (23, 48, 50). However, neutralization was not enhanced by the addition of complement in our assay, which may indicate the presence of a different immune mechanism specific to trout. It should be noted that the reduced sensitivity observed in our VN

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assay may be due to VHSV forming a complex with neutralizing antibodies in the serum, which reduces the availability of antibodies for binding to virus neutralization epitopes in the VN assay (45–48). However, this concern is obviated by using the nucleocapsid protein as our target, because antibodies with nucleocapsid affinity presumably do not complex with the intact viral particle, which underscores another value of the anti-nucleocapsid ELISA. It should be noted that four of the eight sera (50%) from our VHS-infected group tested positive for viral RNA but negative in the VN assay. This emphasizes the importance of utilizing parallel assays when testing the virus exposure history of fish.

Although previous experiments have determined that homologous strains of VHSV must be used for neutralization epitopes to be recognized (51, 52), serum samples from five Pacific herring hyperimmunized with VHSV type IVa were able to neutralize type IVb virus in the VN assay at low titers of p1:16 to 1:32. There are 21 amino acid differences between the type IVa and IVb glycoprotein sequences. None of these differences occur in two of the identified glycoprotein-neutralizing epitopes. This suggests there are shared neutralizing epitopes between types IVa and IVb (53, 54). Type IVa glycoprotein epitopes may be similar enough to those of type IVb to react in our VN assay. Indeed, a similar phenomenon was noted when Pacific herring vaccinated with the glycoprotein gene isolated from VHSV type Ia were protected from VHSV type IVb (55). Further investigation is needed to determine whether our VN assay may detect different antibody titers in herring exposed to virus that is homologous to that used in the VN assay. Additionally, although we demonstrate the ability to detect antibodies in hyperimmunized Pacific herring that likely experienced artificially high antibody titers, further investigations are needed to determine the sensitivities of these assays in wild Pacific herring or in those surviving more realistic VHSV exposure histories.

Our new blocking ELISA is a suitable nonlethal method for detecting exposure to VHSV. Considering the broad host range of VHSV type IVb (11), the advantage of a species-independent ELISA is significant for the surveillance of VHSV. The assay can measure the concentrations of antibodies directed against the nucleocapsid in any freshwater species since it does not require a secondary antibody. Furthermore, the ability of the monoclonal antibody to bind to a single viral epitope results in high specificity. We demonstrated by Western blotting that the nucleocapsid monoclonal antibody binding was specific to VHSV versus SVCV, another rhabdovirus that is present in Wisconsin. Previous studies showed a lack of cross-reaction between the antibody used herein and spring viremia of carp virus, infectious hematopoietic necrosis virus, pike fry rhabdovirus, or rhabdovirus anguilla (26, 56). According to the manufacturer of the antibody (Aquatic Diagnostics, Stirling, Scotland), no cross-reaction of the antibody occurs with nodavirus, infectious salmon anemia virus, koi herpesvirus, salmon alphavirus (1, 2, and 3), or *Piscirickettsia salmonis* infected cells. We were not able to test positive sera from transboundary VHSV strains (type I, II, and III); however, previous efforts indicate the anti-nucleocapsid monoclonal antibody detects anti-nucleocapsid antibodies against all strains of VHSV (26).

A feature crucial to the function of our ELISA is the treatment of the viral antigen with MEGA-10 detergent prior to coating the plates (47). Repeated trials showed that treatment of the virus with detergent allowed for better attachment of the nucleocapsid-specific antibody to the virus. This result is probably due to the ability of the detergent to expose the nucleocapsid epitope and make it available for antibody binding.

The large difference in sensitivities between our blocking ELISA (96.4%) and VN assay (42.9%) may be attributed to the immune response kinetics at the time of serum collection. Studies have shown that neutralizing antibodies do not persist as long as nonneutralizing antibodies in trout (25, 48), and neutralizing antibody titers peak at 6 weeks postinfection in rainbow trout infected with VHSV I (24) and at 11 to 16 weeks in muskellunge infected with VHSV IVb (44). It was observed that the majority of VHSV-exposed fish with serum samples collected prior to 6 weeks postinfection had no or low neutralizing antibody titers. Those with low titers may have still been clearing virus while producing protective antibodies, indicated by the presence of viral RNA in the serum sample of a portion of our VHSV-exposed fish. Investigation into the kinetics of viral replication and the related immune response in multiple species are therefore important for further study.

A limitation of this study is the number of serum samples available from VHSV-uninfected and -infected fish. We used serum samples from 27 experimentally infected and one wild-caught fish that had VHSV-positive kidney and spleen tissues as tested by real-time RT-PCR for ELISA development. It is also important to note that our threshold for detecting VHSV antibodies by ELISA may require adjustment when evaluating various wild-caught species due to differing environments and susceptibility. In this light, we note that 4 of 34 serum samples diluted 1:2 from the VHS-negative group were positive on the blocking ELISA. There may be nonspecific reactions occurring that more extensive testing would help reconcile. For the purpose of this assay as a surveillance tool, it is more practical to keep a threshold at a level that maximizes sensitivity.

In summary, the blocking ELISA shows high sensitivity and acceptable specificity, whereas the VN assay has unacceptably low sensitivity but high specificity. When used in parallel, the VN assay and ELISAs correctly identified the VHSV exposure status of all known uninfected and infected fish. Our results highlight that the anti-VHSV nucleocapsid monoclonal antibody used in the blocking ELISA is a good indicator of past exposure to VHSV and may be a reliable time-independent and species-independent diagnostic test suitable for nonlethal surveillance of VHSV. Our nonlethal serological assays will be valuable for assessing VHSV exposure history and might reduce the extensive laboratory effort needed to screen fish for VHSV using virus isolation. Use of the VN assay, blocking ELISA, and virus isolation under actual surveillance conditions is needed to fully demonstrate the interplay between the assays. The collection of additional reference samples is required for continued assay validation to further assess the sensitivity and specificity and determine repeatability, robustness, and ruggedness. Our serological assays might supplement existing VHS surveillance protocols, which might have regulatory implications for fish movement between VHSV-positive and -negative locations in certain jurisdictions or geographic regions.

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