Vol. 478: 223–230, 2013
doi: 10.3354/meps10208

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published March 25

High prevalence of viral haemorrhagic septicaemia virus (VHSV) in Norwegian spring-spawning herring

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ABSTRACT: Viral haemorrhagic septicaemia (VHS) is a viral disease known to cause high mortality in many teleost species, both in wild and farmed populations. The effects of this virus infection on the population dynamics of Atlantic herring *Clupea harengus* are still unknown; however, high mortalities have been reported in infected Pacific herring *C. pallasii*. We investigated Atlantic herring from the Norwegian spring-spawning (NSS) stock during the spawning season and found a much higher prevalence of VHS virus (VHSV) than had been identified in previous surveillance studies. Positive results were found in 19% of the samples analysed using viral cell culture on pooled brain, spleen and kidney tissue samples from 5 fish. Real-time RT-PCR performed on the same pooled samples revealed 33% positive samples, while analysis of individual organs resulted in a prevalence of 12–13%. Gills, which were analysed only by real-time RT-PCR, displayed a prevalence of 69%. It is not clear whether the virus detected in the gills reflects an infection or a passive carrier status. Sequencing of positive samples from gills and internal organs revealed high identity levels with VHSV of genotype Ib detected previously in Norwegian herring. The high prevalence identified in our study indicates a need for further research into the significance of VHSV infection on the herring population.

KEY WORDS: VHSV genotype Ib · Real-time RT-PCR · Gills · *Clupea harengus*

INTRODUCTION

Viral haemorrhagic septicaemia viruses (VHSVs) have been detected in over 70 different fish species, mainly in the northern hemisphere (Skall et al. 2005, Thompson et al. 2011). A low prevalence of VHSV has previously been reported in Atlantic herring *Clupea harengus* populations in the North Sea, Skagerrak, Kattegat, Baltic Sea and in UK waters (Mortensen et al. 1999, King et al. 2001, Brudeseth & Evensen 2002, Dixon et al. 2003), even when using sensitive PCR assays (Matejusova et al. 2010). VHSV has not been linked to disease or mortality in Atlantic herring; however, disease outbreaks caused by VHSV in Pacific herring *Clupea pallasii* and several other wild fish species have been reported in the USA and Canada (Meyers et al. 1994, Hedrick et al. 2003, Elsayed et al. 2006, Groocock et al. 2007, Elston & Meyers 2009, VHSV Expert Panel and Working Group 2010). Susceptibility and high mortality rates have also been demonstrated experimentally in Pacific herring using North American VHSV isolates (Kocan et al. 1997, Hershberger et al. 2007). Indexes for ulceration, presence of VHSV and *Ichthyophonus hoferi* infections have been used in age-structured assessment models to estimate population biomass of Pacific herring with promising results (Marty et al. 2010). Earlier collapses of North Sea herring populations in the 1960s suggest the need for elucidation of all factors affecting stock dynamics (Dickey-Collas et al. 2010).

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VHSVes are grouped into 4 main genotypes (I–IV); some of which are further divided into sub groups (a–f). High mortality due to VHS infections is well known in aquaculture, and the disease is a particular problem in rainbow trout *Oncorhynchus mykiss*, which are highly susceptible to genotype Ia (Skall et al. 2005). Until recently, documentation of high mortality in wild fish was scarce. However, in 2003, a novel variant of the VHSV (genotype IVb) was detected in the Great Lakes region of North America and has since caused major mortalities in several wild species (Elsayed et al. 2006). Studies have confirmed the broad host range (Kim & Faisal 2010) and low viral genetic diversity (Thompson et al. 2011) of this variant. The low species specificity identified in the Great Lakes and the rapid spread to new areas indicate that VHSV may be a much higher risk factor to wild fish populations than previously assumed (VHSV Expert Panel and Working Group 2010).

The prevalence of VHSV in wild fish identified during summer surveys appears to be higher than that identified in the winter months. It is not known if this is related to spawning cycles or other biological factors (Mortensen et al. 1999). Few previous investigations of VHSV in Atlantic herring have focused on spawning season or particular herring stocks. The aim of this study was to investigate the prevalence of VHSV in Norwegian spring-spawning (NSS) herring during the spawning season. As different stocks of herring feed and spawn in the same areas, genotyping of the fish was necessary to confirm the identity of the sampled fish. The majority of previous VHSV screenings have utilized cell cultures inoculated with pooled organ samples from 5 fish. (Skall et al. 2005). The most frequently tested organs include the heart, kidney and spleen. Brain, liver or gonads have been included in some studies, while gills have only been tested occasionally. Two additional aims of our study were, therefore, to test individual organs to examine viral distribution in carrier fish and to compare the sensitivity of virus isolation in cell culture with real-time RT-PCR (rRT-PCR) detection.

MATERIALS AND METHODS

Sampling

Herring were caught by commercial fishing boats along the west coast of Norway during the main spring-spawning season, from February to April 2010 (Fig. 1). The fish were kept on ice for a maximum of 24 h, and delivered to the Institute of Marine Research (IMR) in Bergen. On each sampling date, 15–40 fish were examined and length and weight were measured. Brain, spleen and kidney from 5 fish were pooled in viral transport medium (Eagle's minimum essential medium, pH 7.6, supplemented with 10% newborn bovine serum and 100 µg ml^{-1} gentamicin) for virus isolation. Gill, brain, spleen and kidney from the same fish were sampled in this order and stored individually in RNAlater® (Ambion). The brain was longitudinally divided; one half was stored in transport medium and one half in RNAlater. Mature gonads were only present at the first 2 samplings and were sampled into RNAlater. All equipment used to collect samples was cleaned with alcohol, flamed between use on each organ, and changed between individual fish. RNAlater samples were stored at 4°C for 24 h before being frozen at –20°C, while samples in transport medium were immediately stored at –80°C.

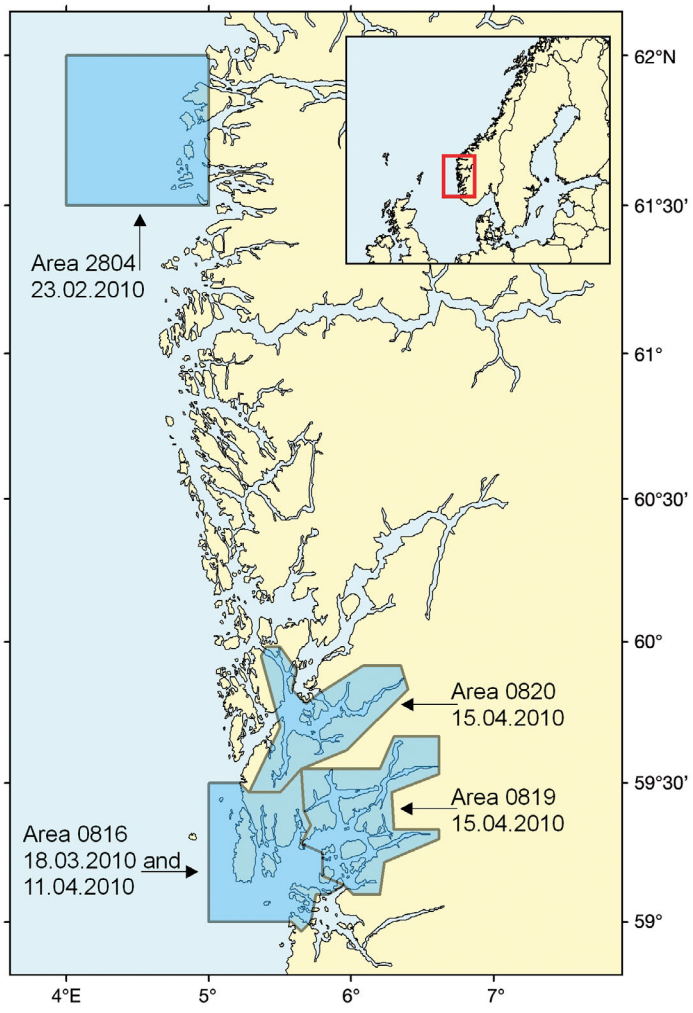

Fig. 1. Atlantic herring *Clupea harengus* capture areas on the west coast of Norway. Dates are d.mo.yr

Genotyping of herring

DNA was isolated from muscle tissues from 3 fish per sample point. For baseline data, 93 NSS-herring and 65 herring from a local herring stock from the west of Norway were analysed (Lindaas-herring). The herring were genotyped using 9 microsatellite markers: Cha1017, Cha1020, Cha1027, Cha1202 (McPherson et al. 2001) and Cpa101, Cpa111, Cpa112, Cpa113, Cpa114 (Olsen et al. 2002). Pairwise genetic distances (F_{ST} values) were estimated using Arlequin version 3.5 (Excoffier et al. 2005), and a phylogenetic tree (UPMGA) (Tamura et al. 2012) was produced using MEGA version 5. LOSITAN (Beaumont & Nichols 1996, Antao et al. 2008) was utilized to identify markers under selection.

Virus isolation

Tissue samples pooled in transport medium were homogenized, centrifuged at low speed, and supernatants inoculated in dilutions of 1:10 and 1:100 onto subconfluent monolayers of BF-2-cells (ECACC, Salisbury, UK) in 24-well tissue culture plates (OIE 2009). Inoculated cultures were incubated at 15°C and inspected at 40–150× magnification after 1 wk for cytopathic effect (CPE). Culture medium was collected from all wells and passaged to new cell cultures. After a further week of incubation, the cultures were again inspected. Medium from wells with evident CPE in the second passage was collected, RNA was extracted and tested for VHSV by rRT-PCR.

Real-time RT-PCR (rRT-PCR)

RNA was isolated from the frozen homogenized tissue pools and individual RNAlater organs with an easyMAG robot (Biomérieux). Extracted RNA was measured using a NanoDrop ND-1000 (NanoDrop Technologies). The diagnostic RT-PCR assay was performed using 100–900 ng template RNA (600–900 ng when possible) utilising a QIAGEN OneStep RT-PCR kit (QIAGEN Nordic) and primers and probe targeting the nucleoprotein (N) gene as described by Duesund et al. (2010). The assay was performed with 0.5 µm of each primer and 0.3 µm probe in a 20 µl reaction, with cDNA synthesis at 52°C for 30 min followed by 15 min at 95°C, 45 cycles of 95°C for 15 s and 60°C for 1 min using the real-time Stratagene PCR system (Stratagene). Samples resulting in a specific signal and cycle threshold (Ct) value <40 were considered positive. Gills, brain, spleen, kidney and gonads from all 105 fish were tested individually. rRT-PCR testing was also done on pooled samples (from 5 fish) containing brain, spleen and kidney tissues. In addition, a selection of samples were tested by PCR for *Francisella noatunensis* subsp. *piscicida* and *Mycobacterium* spp. (Ottem et al. 2008, Zerihun et al. 2011).

Sequencing of virus

A selection of VHSV positive pools and organs were selected for sequence analysis (Table 1). Partial and full length glycoprotein (G) gene sequences were generated from overlapping sequences derived using primer sets GB and Gseq (Einer-Jensen et al. 2004). Three primers were used to obtain a 1217 bp nucleoprotein (N) gene sequence: N-G1F 5′-GCT CAC AGA CAT GGG CTT CA-3′, N-G2R 5′-TGG ATT GGG CTT CTT CTT-3′, N-G3F 5′-GGC TCA ACG GGA CAG GAA-3′. Briefly, the RT-PCR was performed using 5 µl extracted RNA from pooled homogenized tissues or individual organs, 0.5 µm primer concentration in a 50 µl QIAGEN OneStep reaction, with cDNA syn-

Table 1. *Clupea harengus*. Pooled samples and individual organs from Atlantic herring found viral haemorhagic septicaemia virus (VHSV)-positive by cell culture and real-time RT-PCR (rRT-PCR). Parentheses: prevalence of positive samples; NS: not sampled; *: specific sequence data (n = 16) obtained from the indicated VHSV-positive sample types for verification

Sampling date 2010	No. of fish sampled	No. of pools[a]	No. of positive pools[a]		No. of positive organs from individual fish by rRT-PCR				
			Cell culture	rRT-PCR	Brain	Spleen	Kidney	Gills	Gonads
25 Feb	40	8	0	2	1	1	0	12 (30%)	0 / 40
19 Mar	20	4	1	2*	4	3*	5	19* (95%)	2 / 20
12 Apr	15	3	0	0	1	0	2	14* (93%)	NS
16 Apr	15	3	0	0	1	2	1*	13* (87%)	NS
22 Apr	15	3	3	3*	6	7*	6	14* (93%)	NS
Total	105	21	4 (19%)	7 (33%)	13 (12%)	13 (12%)	14 (13%)	72 (69%)	2 (3%)

[a]Brain, spleen and kidney tissues pooled from 5 fish

Table 2. *Clupea harengus*. Length and weight of herring (n = 105) caught in the 5 respective catches, including standard deviation (SD) and condition factor (K). Fulton's formula was used to calculate the condition factor: $K = W \times 100\ L^{-3}$, where W = weight and L = length. Average condition factor is based on the average weight and length measures

Sampling date 2012	Length (cm)				Weight (g)				Condition factor			
	Max.	Min.	Avg.	SD	Max.	Min.	Avg.	SD	Based on max. values	Based on min. values	Average condition factor	SD
25 Feb	33.0	29	31.73	1.10	385	310	337.07	26.19	1.07	1.27	1.06	0.14
19 Mar	31.5	28	29.65	1.13	247	170	211.27	24.89	0.79	0.77	0.81	0.01
12 Apr	31.5	26	28.57	1.36	249	164	200.40	24.84	0.80	0.93	0.86	0.10
16 Apr	32.0	27	29.30	1.31	283	177	229.93	31.08	0.86	0.90	0.91	0.03
22 Apr	29.5	20	27.72	2.39	267	152	219.40	31.11	1.04	1.90	1.03	0.61

thesis at 50°C for 30 min followed by 15 min at 95°C, 40 cycles of 95°C for 1 min, 55°C 1 min and 72°C for 90 s. The RT-PCR products were visualized on an agarose gel and purified using the ExoSAP-IT protocol (Usb) prior to sequencing with BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Obtained sequences (Acc. nos. JQ755255–JQ755265) were aligned and compared to related VHSV sequences using Vector NTI Advance 11 (Invitrogen).

RESULTS

Sampling

All 105 fish examined appeared to be in good condition (Table 2) and showed no signs of disease or ulcers, with the exception of 2 individuals sampled on 19 March that displayed swollen spleens. These 2 individuals tested positive for VHSV in the gills only, and none of them tested positive by PCR for *Francisella noatunensis* subsp. *piscicida* or *Mycobacterium* spp.

Genotyping of fish

The herring were genotyped successfully with the 9 microsatellite markers. No marker showed signs of selection, and the pairwise F_{ST} tests showed that the 15 herring sampled for VHSV detection clustered together with the NSS herring and were significantly different from the local herring stock (Lindaas-herring) (Fig. 2).

Virus detection

VHSV was isolated in cell culture from 4 of 21 pooled samples, while rRT-PCR on the same pooled material added 3 more positive pools. The detection rate with rRT-PCR using individual organs was even higher, with a total of 72 VHSV RNA-positive gill samples, indicating a prevalence of 69%. Of these 72 individuals, 22 were also positive in at least one internal organ. No fish showed positive results on internal organs and negative results on gills. Only 2 of the 60 gonad samples tested VHSV positive, and one of these fish also displayed high amounts of virus in all other organs tested. The RNA concentrations extracted from 7 of the rRT-PCR-negative gill samples and most of the brain samples were low (<50 ng μl^{-1}) and false-negative results for these samples cannot be ruled out.

In general, Ct values identified for individual organs indicate a large variation in viral load between organs: gills Ct 24–40, brain Ct 31–39, spleen Ct 19–39, kidney Ct 23–40. The highest levels of VHSV RNA were detected in 7 fish in which all analysed organs were positive, with inter-organ Ct values ranging between 19 and 39. No organ clearly stands out as consistently containing the highest viral load. For example, some brain samples were positive, while spleen and kidney samples from the same fish

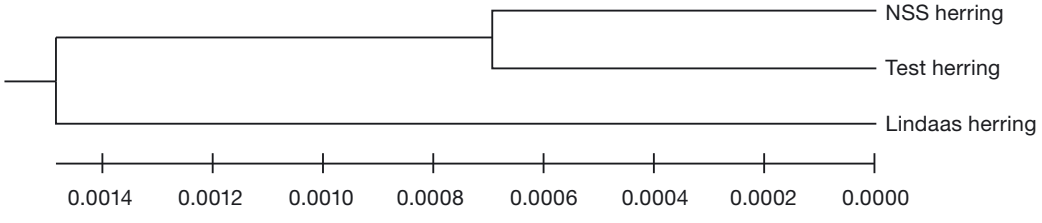

Fig. 2. Phylogenetic tree (UPGMA) of Norwegian spring spawning herring (NSS herring), a local herring stock (Lindaas herring) and samples from 15 herring from this study (test herring)

were negative and vice versa. In 3 fish, spleen samples were positive while kidney samples were negative. In 4 other fish the opposite was found. An overview of all results and Ct values are available in the electronic supplement (www.int-res.com/articles/suppl/m478p223_supp.pdf).

Sequencing

In total, sequence data was obtained from individual organ samples (7 gills, 2 spleens, 1 kidney) from 8 herring and 6 pooled tissue homogenates (Table 1). Herring that were VHSV positive only in gill samples, and pooled samples found positive by rRT-PCR but negative by virus isolation, were included in the selection. Sequencing was performed on a range of samples but was not successful for samples with low viral load. The G gene and N gene sequences confirmed that the herring isolates belong to genotype Ib and are closely related (99–100% identity). Identical sequences were obtained from gill and spleen samples from the same herring. The sequences were also closely related (99–100% identity) to VHSV sequences previously detected in herring in a Norwegian fjord (Duesund et al. 2010).

DISCUSSION

Our results show a much higher prevalence of VHSV in Atlantic herring than has been found in previous studies (Skall et al. 2005). We tested a total of 105 Atlantic herring from the NSS herring stock for the presence of VHSV during the spawning season. The herring were caught at 5 different locations by a commercial fishing fleet over a 2 mo period. rRT-PCR analysis of gills resulted in positive detection of VHSV in 72 individuals. Of these, 22 individuals tested positive for VHSV in at least one additional internal organ. A total of 7 individuals tested positive in all organs analysed. Viral prevalence can be influenced by many parameters including temperature, climate change, density of fish, condition factor and health status of fish, spawning season, disease outbreaks, virulence of the virus in relation to the fish species, testing methods and sampling regimes. One or several of these factors may be responsible for the unique high prevalence detected in our study.

VHSV is pathogenic for many different species of fish, including herring. It has been suspected as a cause of the decline in the Pacific herring population in Alaska, USA, and Canada, as high amounts of VHSV have been detected during disease outbreaks (Elston & Meyers 2009). Challenge trials on Pacific herring, with VHSV isolates from the US (genotype IVa) resulted in high mortality rates (Hershberger et al. 2007). Challenge trials with European virus isolates should be performed on Atlantic herring since the effect of this virus infection on these stocks is unknown. The high prevalence identified in our study is the first indication that VHSV might influence Atlantic herring stocks. Challenge trials with 3 stocks of Pacific herring showed that survivors from a primary challenge have low susceptibility to a secondary challenge with VHSV (Hershberger et al. 2010), indicating that fish might develop immunity. This should, therefore, be taken into account when prevalences in wild fish stocks are interpreted. A low prevalence could indicate either a low exposure rate or immunity due to previous exposure.

The earlier challenge trials on different stocks of Pacific herring did not reveal inter-stock differences in cumulative mortality to either primary or secondary exposure to VHSV (Hershberger et al. 2010). Little is known about variation in VHSV prevalence or susceptibility in Atlantic herring stocks. The Norwegian coast has several local stocks of herring with specific spawning, feeding and wintering areas (Nakken 2008). Most of these stocks spawn in the spring while others spawn in late summer/autumn. Different stocks of herring may mix during some parts of the year. Studies of population dynamics are, therefore, to a large extent performed on stock level to determine information such as fishing quotas. Commercially, the most important stock in Norway, and the world's largest herring stock, is the NSS herring identified and tested in this study. Earlier investigations of VHSV in Atlantic herring have not been aimed at specific stocks, and the populations tested are, therefore, often unknown. We have no data available to indicate whether our results represent a normal prevalence in this stock at the time of spawning. Earlier studies have hypothesized that high prevalence of VHSV could be related to collapses in herring populations (Pearson et al. 1999), and further research is needed to investigate if levels of VHSV can be related to current ecological circumstances in the Norwegian Sea (Huse et al. 2012).

The size and condition factors of the herring tested in our study were considered normal, and variation between fish in the same catch was low (Table 2). Since we have no material for histopathological examination, we have no evidence on whether the VHSV-positive herring had any pathological changes related to VHS or whether they represent healthy car-

rier fish. No skin ulcers or other signs of disease were macroscopically observed in VHSV-positive fish. Few studies have investigated the relationship between skin ulcers and VHSV detection, and any connection is unclear (Marty et al. 2010). External ulcers and subdermal hemorrhages of the skin and fins have been reported from VHSV-infected Pacific herring and Pacific cod *Gadus macrocephalus*. However, ulcers were also seen in fish without VHSV so a clear connection between virus and ulcers has not been proven (Meyers et al. 1994). VHSV has been detected in the skin of a minority of Atlantic cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* displaying skin ulcers (Smail 2000). In the same study, 2330 herring were also examined, and although 47 had hemorrhages, no VHSV was detected.

At the time this paper was written, the Fish-pathogens database (www.fishpathogens.eu) contained 489 VHSV sequences from 26 different fish species, including 99 isolates from Atlantic herring. Of the herring isolates, 78 were reported from the Baltic Sea, 6 from Kattegat, 3 from Skagerrak, 2 from the North Sea and 1 from the English Channel. The genotype was reported for 36 herring isolates. Of the Baltic Sea isolates, 20 were genotyped as Ib, along with all genotyped isolates from the Kattegat, the English Channel and North Sea. Two isolates from Skagerrak and 2 from the Baltic Sea clustered with genotype II. Only one isolate, from Skagerrak, was grouped as genotype III. The finding of VHSV of genotype Ib in Atlantic herring is, therefore, common.

The genotype alone is not sufficient to determine how virulent a VHSV isolate is for different fish species. As long as the mechanisms of virulence remain unclear, challenge trials are the only way to determine the virulence of a VHSV isolate for any particular fish species. Experimental challenge studies with VHSV genotype Ib isolates from herring have shown very low to no mortality in rainbow trout, salmon, halibut *Hippoglossus hippoglossus* and Atlantic cod (King et al. 2001, Skall et al. 2004, Snow et al. 2005, Duesund et al. 2010). However, there have been a few reports of mortalities caused by VHSV of genotype Ib in rainbow trout farmed in the Baltic sea (Nordblom 1998, Nordblom & Norell 2000). A marine VHSV of genotype III has also shown low virulence towards rainbow trout in challenge trails, although outbreaks in farmed fish have occurred (Dale et al. 2009). This shows that the risk of VHSV infection from marine wild fish to farmed salmonids is low, but present. Whether the current genotype Ib isolated from herring is virulent for other fish species needs to be tested further in challenge trials.

The fish sampled in this study were kept on ice for up to 24 h before being delivered to the laboratory, and this may have influenced the results. The titer of live VHSV declines as soon as the fish dies, and this might influence the virus isolation rate in cell culture compared to rRT-PCR which detects viral RNA. Earlier comparisons between virus isolation and PCR assays indicated approximately equal sensitivity (Knüsel et al. 2007). Both cell culture assays and PCR techniques are, however, constantly under improvement, making comparison with previous studies difficult. Our results show that rRT-PCR is equally to more sensitive than cell culture isolation for detection of VHSV of genotype Ib in pooled organ samples. This has also been shown in other studies, especially for detection of VHSV in clinically healthy carrier fish (Cutrin et al. 2009, Hope et al. 2010). The organs that were tested also need to be considered when results are compared. In screening programs in which the aim is detection of several virus types, virus isolation in cell culture may still be the best choice (OIE 2009).

In earlier studies the VHSV prevalence detected in Atlantic herring has ranged between 0 and 16.7% (Skall et al. 2005). The prevalence of 69% identified in gills in the present study is, therefore, unique. It is not clear whether the PCR detection in the gills reflects a true gill infection, or whether the virus is exogenous, possibly bound to the gill surface mucus. However, gill testing will give useful information as to whether the fish is or has been exposed to VHSV present in the water. The low numbers of viral particles indicated in the gills would probably make immunohistochemistry difficult, but this method could be used in future research to determine whether there is in fact a true gill infection.

The gills were sampled first, and one could argue that internal organ samples may have been subsequently contaminated by gill sampling. However, the large number of VHSV-positive gills, and the fact that Ct levels indicated a higher viral load in most internal organ samples than in gill samples, indicate that disinfection of sampling equipment was sufficient. It should also be noted that in the main, scissors were used to sample gills while scalpels were used on other organs, further minimizing the possibility of contamination.

If the aim of any particular study is detection of true VHSV-infected fish, virus isolation and testing of internal organs must be included. In this study, VHSV was detected in one or more internal organs of 21% (22 of 105) of the fish sampled, indicating that VHSV has the ability to infect herring. The infected fish showed a large variation in viral load in different

organs, suggesting that a pool of internal organs would provide the most representative results.

The high prevalence of VHSV found in NSS herring in our study indicates that a marine reservoir exists that might affect the herring population and that could pose a risk to aquaculture. In future VHS studies, age, sex, gonad maturation, size as well as condition factor of the herring in relation to virus load should be investigated. The viral load and pathological changes related to physical condition would provide valuable information on the significance of the infection to the fish population. The number of fish tested in our study was relatively low; larger studies are needed to determine the prevalence and importance of VHSV in different populations of Atlantic herring. Histopathological studies, including immunohistochemistry, could provide valuable answers to how this virus affects herring. Based on the results from this study, the infection seems to have minimal influence on the condition of Atlantic herring. The fish investigated in our study were collected from purse seine catches over a 2 mo period during which specific schools were targeted. The sampling time and fishing method are of great importance when the prevalence in fish is interpreted. In future studies, fish peripheral to the main shoal should be sampled to determine whether the prevalence is higher or lower in weak fish. Such sampling would typically have to be done by trawling larger water masses, since diseased fish often quickly disappear from the water column (Bergh 2007).

Acknowledgements. This study was financially supported by the Norwegian Research Council, Grant No. 190245, 'Viral haermorrhagic septicaemia virus (VHSV) in wild and farmed fish in Norway'. We thank A. C. B. Einen, R. H. Olsen and J. Nordbø for help with the sampling, and K. Gjertsen for producing Fig. 1. We also thank the crew of the fishing vessels and the fish processing facilities for help with logistics. We are also grateful to D. Colquhoun, who corrected our English and gave us many useful comments on the text.

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Editorial responsibility: Jake Rice, Ottawa, Ontario, Canada

Submitted: April 11, 2012; Accepted: November 29, 2012
Proofs received from author(s): March 15, 2013