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RESEARCH ARTICLE

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Experimental infection trials with European North Atlantic ranavirus (*Iridoviridae*) isolated from lumpfish (*Cyclopterus lumpus*, L.)

Felix Scholz[1,2] | Niccolò Vendramin[3] | Niels Jørgen Olesen[3] | Argelia Cuenca[3] | Tine Moesgaard Iburg[3] | Luca Mirimin[2] | Ian O'Connor[2] | Neil M. Ruane[4] | Hamish D. Rodger[5] | Eugene MacCarthy[2]

[1]PHARMAQ Analytiq, Oranmore, Ireland

[2]Marine and Freshwater Research Centre, Galway-Mayo Institute of Technology, Galway, Ireland

[3]DTU Aqua, National Institute of Aquatic Resources, Copenhagen, Denmark

[4]Marine Institute, Oranmore, Ireland

[5]VAI Consulting, Kinvara, Ireland

Correspondence
Felix Scholz, PHARMAQ Analytiq, Unit 7b Oranmore Business Park, Oranmore, Co. Galway H91XP3F, Ireland.
Email: felixlscholz@hotmail.com

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Abstract

European North Atlantic ranavirus (ENARV, *Iridoviridae*), is a ranavirus species recently isolated from lumpfish (*Cyclopterus lumpus*, L.), which are used as cleaner fish in Atlantic salmon (*Salmo salar*) farming in Northern Europe. This study aimed to investigate (1) the virulence of ENARV isolates from Ireland, Iceland and the Faroe Islands to lumpfish; (2) horizontal transmission between lumpfish; and (3) virulence to Atlantic salmon parr. Lumpfish were challenged in a cohabitation model using intraperitoneally (IP) injected shedders, and naïve cohabitants. IP challenge with isolates from Iceland (1.9×10^7 $TCID_{50}$ ml^{-1}) and the Faroe Islands (5.9×10^7 $TCID_{50}$ ml^{-1}) reduced survival in lumpfish, associated with consistent pathological changes. IP challenge with the Irish strain (8.6×10^5 $TCID_{50}$ ml^{-1}) did not significantly reduce survival in lumpfish, but the lower challenge titre complicated interpretation. Horizontal transmission occurred in all strains tested, but no clinical impact was demonstrated in cohabitants. Salmon parr were challenged by IP injection with the Irish isolate, no virulence or virus replication were demonstrated. A ranavirus qPCR assay, previously validated for fish ranaviruses, was first used to detect ENARV in tissues of both in lumpfish and Atlantic salmon. This study provides the first data on the assessment of virulence of ENARV isolates to lumpfish and salmon, guidelines for the diagnosis of ENARV infection, and poses a basis for further investigations into virulence markers.

KEYWORDS
Atlantic salmon (*Salmo salar*), cleaner fish, cohabitation challenge trial, European North Atlantic ranavirus (ENARV), lumpfish (*Cyclopterus lumpus*), virulence

1 | INTRODUCTION

Salmon lice, *Lepeophtheirus salmonis* Krøyer, are ectoparasitic copepods that pose a major challenge to the Atlantic salmon (*Salmo salar*) farming industry in Northern Europe (Iversen et al., 2015). One approach to salmon lice control is the use of cleaner fish. Wrasse (*Labridae* spp.) have been used for this purpose since the early 1990s (Bjordal, 1990; Deady et al., 1995), and lumpfish (*Cyclopterus lumpus*) are an effective alternative, with the advantage of being easier to farm and more suited to cold water temperatures than wrasse

Scholz and Vendramin contributed equally to this work.

species (Bolton-Warberg, 2017; Imsland et al., 2014). Demand for cleaner fish has grown steadily and it is estimated that a total of 50 million individuals will be required in Northern Europe in 2020, most of which is expected to be farm-reared lumpfish (Powell et al., 2018). The lumpfish farming industry currently remains dependent on wild-caught broodstock (Brooker et al., 2018), posing a potential biosecurity risk through unknown health status of wild fish (EURL, 2016).

Multiple new infections have been documented in cleaner fish in recent years, including species of uncertain clinical significance and pathogens that may pose a potential risk to cohabited salmon (Guðmundsdóttir et al., 2019; Marcos-López et al., 2017; Ruane et al., 2018; Scholz et al., 2017; Skoge et al., 2018; Toffan et al., 2019). The transmission of *Neoparamoeba perurans* between lumpfish and salmon has been demonstrated experimentally (Haugland et al., 2016), but very few pathogens known to affect cleaner fish have been investigated in this regard, and the transmission of viral disease from lumpfish to salmon has not been demonstrated to date. One putative pathogen in need of further investigation is a ranavirus that has been detected in lumpfish in Ireland, Iceland, Scotland and the Faroe Islands (Rimstad et al., 2017; Stagg et al., 2020). Full genome sequencing of three isolates and phylogenetic analysis based on concatenated iridovirus core genes indicated these are a putative new species of ranavirus with the proposed name of European North Atlantic ranavirus (ENARV) (Stagg et al., 2020). ENARV also encompasses isolates from Atlantic cod (*Gadus morhua*), provisionally termed cod iridovirus, and turbot (*Scophthalmus maximus*), provisionally termed ranavirus maxima (Ariel et al., 2010; Stagg et al., 2020). These findings indicate the persistence of ENARV in the northern European marine environment, and therefore the likelihood of this virus posing a recurring challenge to lumpfish.

The genus *Ranavirus* is one of five recognized genera in the *Iridoviridae*, which comprises large, double-stranded DNA-viruses (Chinchar et al., 2017). Six species are currently recognized by the International Committee on the Taxonomy of Viruses (ICTV), but further putative species are described. Ranaviruses typically show a low host specificity (Whittington et al., 2010), some species even being pathogenic to both fish and amphibian species (Ariel & Owens, 1997; Deng et al., 2020; Mao et al., 1999; Moody & Owens, 1994; Waltzek et al., 2014). This indicates a possible threat of transmission between lumpfish and cohabited species in sea pens, that is, Atlantic salmon and wrasse species. Species in the genus *Ranavirus* have caused high mortality in wild fish stocks and in aquaculture, both in freshwater and marine environments, though reports of repetitive ranavirus outbreaks are rare in finfish. Epizootic haematopoietic necrosis virus (EHNV) is the causative agent of Epizootic haematopoietic Necrosis (EHN). EHN has caused high mortalities in wild redfin perch, *Perca fluviatilis* L. (Langdon et al., 1986; Langdon & Humphrey, 1987) and disease in cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Australia (Langdon et al., 1988). Epizootic Haematopoietic Necrosis (EHN), is not present in the EU, and is currently listed as Category A disease under Commission Implementing Regulation (EU) 2018/1882 (https://eur-lex.europa.eu/eli/reg_impl/2018/1882/oj), entailing mandatory culling of infected stocks if the virus is detected. Singapore grouper iridovirus (SGIV) has caused high mortalities in brown spotted grouper, *Epinephelus tauvina* (Fauskal), in Singapore (Chua et al., 1994; Qin et al., 2003). In Europe, high mortality in catfish, *Ictalurus melas* (Rafinesque), was attributed to European catfish virus (Pozet et al., 1992) and in sheatfish, *Silurus glanis* L. to European sheatfish virus (Ahne et al., 1990).

The clinical significance of ENARV is currently unknown. Ranavirus maxima was isolated from clinically healthy turbot fry in a facility in which an iridovirus was previously detected in fish tissues during a disease outbreak with 70% mortality (Ariel et al., 2010; Bloch & Larsen, 1993). The cod iridovirus was isolated when investigating the cause of ulcus syndrome in wild cod, though the virus could not be linked to the condition (Ariel et al., 2010; Jensen et al., 1979). The lumpfish ENARV isolate from Ireland was isolated from fry with elevated mortality, but there are no reports of disease or mortality associated with isolates from Iceland, Scotland and the Faroe Islands. Different strains of ranavirus species can show differences in virulence (Goldberg et al., 2003). The differences in clinical condition of ENARV infected lumpfish may consequently be due to variations in virulence between ENARV isolates. This is potentially supported by a study which found that the Irish ENARV isolate was closely related to, but slightly divergent from other lumpfish isolates examined based on nucleotide differences in the whole genome of three isolates and a non-synonymous substitution in the sequence of the partial Major Capsid Protein (MCP) gene in the Irish isolate (Stagg et al., 2020). Pilot challenge trials with the Irish isolate resulted in productive infection of lumpfish juveniles (0.2 g average weight) following immersion challenge without consistent pathology that could be attributed to the virus. Fish appeared to clear the infection over the course of 6 weeks. Infection could not be demonstrated in lumpfish (15.1 g average weight) following immersion challenge or in Atlantic salmon smolts (110 g average weight) following immersion challenge or IP injection. A major limitation to the evaluation of these trials was the lack of a quantitative molecular assay and dependence on cell culture with unknown sensitivity (Scholz, 2018). In this study, cohabitation challenge trials were set up using lumpfish and ENARV isolates from Ireland, Iceland and the Faroe Islands. Atlantic salmon parr were challenged with the Irish isolate by IP injection. The aims of the study were to (1) study the virulence of ENARV isolates from Ireland, Iceland and the Faroe Islands to lumpfish, including potential variations in virulence; (2) demonstrate horizontal transmission between lumpfish; and (3) investigate virulence to Atlantic salmon parr using the Irish ENARV isolate. A TaqMan real-time quantitative PCR for the detection of ranaviruses (Stilwell et al., 2018) was first used to detect ENARV in fish tissues.

2 | MATERIALS AND METHODS

2.1 | Fish

The experiments were carried out at DTU-Aqua (Kgs Lyngby, Denmark) in accordance with current animal welfare regulations

under licence 2013-15-2934-00976. The protocols were approved by the Danish animal research authority. Atlantic salmon juveniles (mean weight 5 g) were imported from a Danish commercial farm free of listed diseases according to current EU Council implementing regulation 2018/1882 (https://eur-lex.europa.eu/eli/reg_impl/2018/1882/oj). Lumpfish juveniles (mean weight 0.2 g) were imported from a commercial hatchery in Iceland, parental stock was tested negative by qPCR for viral haemorrhagic septicaemia virus (Jonstrup et al., 2013), as well as by cell culture (epithelioma papulosum cyprinid; EPC and bluegill fry-2; BF-2 cell lines), and bacteriology. Fish used in experiments were also tested for the presence of potential pathogens during their 2 weeks acclimatization and quarantine period. Salmon ($n = 5$) and lumpfish ($n = 5$) were screened by bacteriology (kidney material streaked onto marine agar and blood agar, incubated for 2 weeks at 15°C), cell culture ((EPC) and (BF-2) cell lines, protocol as outlined under virus re-isolation below) and parasitology (skin and gill scrapes examined by light microscope). Tissue samples from a further five salmon (homogenated cranial section including viscera) were screened by qPCR for IPNV - infectious pancreatic necrosis virus (Lockhart et al., 2007), PRV-1 piscine orthoreovirus 1 (Palacios et al., 2010) and ranavirus (Stilwell et al., 2018); a further 5 lumpfish were screened for ranavirus and nodavirus following protocols from Stilwell and Baud respectively (Baud et al., 2015; Stilwell et al., 2018). All results were negative.

2.2 | Virus

ENARV isolates originated from Ireland (F140-16), Iceland (F24-15) and the Faroe Islands (F214-1011-1), referred to as ENARV IE, ENARV IS and ENARV FO hereafter. For challenge trials, the ENARV isolates were propagated on EPC cells at 15 degrees (Ariel et al., 2009). Upon appearance of full cytopathic effect with disruption of cell monolayer, the cell culture supernatant was collected and sterile filtered (0.22 μm). Viral isolates were titrated according to routine procedures on EPC cells (Reed & Muench, 1938). Titre obtained from infected cell culture supernatant was diluted to obtain a final concentration of 1.9×10^7 $TCID_{50}$ ml^{-1} (ENARV IS), 5.9×10^7 $TCID_{50}$ ml^{-1} (ENARV FO) and 8.6×10^5 $TCID_{50}$ ml^{-1} (ENARV IE), respectively.

2.3 | Challenge protocol

Table 1 provides an overview of the experimental design and survival. Lumpfish were challenged via IP injection (shedders) and by cohabiting naïve fish with shedders. For IP challenge, the fish were anaesthetized with benzocaine (80 mg/L, Sigma), injected with 0.1 ml challenge material and tagged with visible implant elastomer tags (Northwest Marine Technology, Inc.) according to the manufacturers' instructions. Tags were injected subcutaneously beside

TABLE 1 Overview of challenges, routine sampling protocol and mortality

						Routine samples for virology and qPCR (S/C)					
Tank	Fish	Isolate	IP challenge titre	No. of fish (S/C)	Time	1 W	2 W	3 W	4 W	HE	*p* value (S/C)
1	LF	n/a	Negative control	15/15	4 W	2/2	2/2	2/2	2/2	0/5	n/a
2				15/15		–	–	–	2/2	2/3	.1501/.108
3	LF	ENARV IS	0.1 ml 1.9×10^7 $TCID_{50}$ ml^{-1}	21/21	4 W	3/3	3/3	3/3	1/3	9/0	n/a
4				18/18		–	–	–	0/5	16/1	**<.0001**/.2504
5				18/18		–	–	–	0/5	10/8	**.0025**/.1534
6	LF	ENARV FO	0.1 ml 5.9×10^7 $TCID_{50}$ ml^{-1}	21/21	4 W	3/3	3/3	3/3	1/3	10/4	n/a
7				18/18		–	–	–	0/5	17/0	**<.0001**/.0578
8				18/18		–	–	–	0/5	15/1	**<.0001**/.2637
9	LF	ENARV IE	0.1 ml 8.6×10^5 $TCID_{50}$ ml^{-1}	21/21	4 W	3/3	3/3	3/3	3/3	3/4	n/a
10				18/18		–	–	–	0/5	3/6	.8360/.4735
11				18/18		–	–	–	0/5	0/0	.1173/.5501
12	AS	n/a	Negative control	35/0	4 W	3	3	3	5	2	n/a
13	AS	ENARV IE	0.1 ml 8.6×10^5 $TCID_{50}$ ml^{-1}	35/0		3	3	3	5	1	.1493
14				35/0		3	3	3	5	6	.5505

Note: See Section 2.5 for histology samples. Fewer shedders sampled for ENARV IS and FO after week 4 are due to reduced survival. *p* is mortality compared to corresponding control replicate in tank 2. *p* of control is compared to a theoretical scenario of 100% survival in an equal number of fish.

Abbreviations: S/C, shedders/cohabitants; W, week; LF, lumpfish; AS, Atlantic salmon; n/a, not applicable; HE, fish that reached the humane endpoint.

p values below the significance threshold (< .05) are in bold.

the dorsal fin. Negative controls for IP challenges were injected with 0.1 ml EMEM (Eagle's minimum essential medium with tris-buffer and 10% newborn calf serum, pH 7.6) and tagged as above. Lumpfish were challenged with all isolates in triplicate and with duplicate negative controls. Salmon were challenged with ENARV IE by IP injection only, protocol as above. Salmon were challenged in duplicate with one negative control. The duration of experiments was 4 weeks.

2.4 | Husbandry

Fish were held in eight-litre tanks on a flow-through system. Municipal dechlorinated water was used for salmon, and mixed with salt (Koral Salt, Aquaconstruct, Copenhagen) to a final concentration of 20 practical salinity units (PSU) for lumpfish. Temperature was maintained at 12°C. Fish were fed a commercial diet (1.5% body weight/day) and tanks were cleaned twice daily for the first week. Husbandry procedures were adjusted after week 1 following incidences of aggression in lumpfish. Feeding was increased to every 2 h (to satiation), lights were dimmed and lumpfish were provided with artificial sea weed. Fish were monitored for abnormal behaviour and signs of clinical disease at least twice daily.

2.5 | Sampling protocol

Table 1 provides an overview of challenges, including the routine sampling protocol.

In lumpfish trials, three shedders and three cohabitants from one replicate/isolate and two IP injected and two cohabitants from one control tank/isolate were sampled for cell culture and qPCR analysis after every week. These replicates are not considered in the survival analysis. In the further challenge replicates, survival was analysed, and cohabited fish were sampled after week 4 to confirm horizontal transmission. In the second control replicate, shedders and cohabitants were sampled after week 4. Fish that reached the humane endpoint (defined as lack of response to repeated stimuli or observation of visible pathology) were sampled for cell culture and qPCR, except for two shedders per isolate, which were sampled for histology. Further samples taken for histology were one cohabited fish (ENARV IS) that reached the humane endpoint and three fish per isolate and challenge model randomly sampled after week 4.

In salmon trials, three fish per tank were sampled every week for cell culture and qPCR analysis. Three fish per tank after week 4, and two challenged fish that reached the humane endpoint were sampled for histology.

Samples for bacteriological analysis and microscopic analysis of wet mounts (skin and gill scrapes) were taken from a total of five fish with clinical disease, if present, in every tank.

Fish were euthanized with an overdose of anaesthetic for sampling or when the experimental humane endpoint was reached in accordance with Danish national regulation implementing EU animal welfare regulations under licence 2013-15-2934-00976.

2.6 | Virus re-isolation

For virus re-isolation lumpfish (whole fish) or salmon (cranial section including head and viscera) were sampled individually into 4 ml of cooled sterile EMEM. Samples were homogenized using a mortar, pestle and sterile sand and cleared by low-speed centrifugation (4000 g) according to procedures given in EURL diagnostic manuals for VHS/IHN (https://www.eurl-fish-crustacean.eu/fish/diagnostic-manuals/vhs). The supernatant was used for virology and qPCR analysis. For virology, gentamycin was added to reach a final concentration of 1 mg/ml, the sample was incubated for 12 h at 5°C and inoculated at final dilutions of 1:100 and 1:1000 onto sub-confluent monolayer cell cultures (EPC) in 24-well tissue culture plates as described EURL diagnostic manual (link as above). Inoculated cultures were incubated at 15°C and inspected regularly with a microscope for the occurrence of cytopathic effect (CPE) at 40× magnification. Samples were considered negative if no CPE was visible after two blind passages of 1 week each.

2.7 | Virus detection by qPCR

Supernatant for qPCR analysis was frozen at −80°C until processed, and 200 µl of the cell supernatant was used to purify DNA, using a QIAcube and the QIAamp DNA Mini Kit (QIAGEN, Germany) following manufacturer's instructions. Real-time PCR was conducted using the protocol of Stilwell et al. (2018) with some modifications. In brief, qPCR was carried out in 20 µl volumes containing 5 µl of purified DNA, 12.5 µl Brilliant 2 qPCR Master Mix (Agilent technologies), 2.25 µl primers (0.9 µM final concentration), and 0.625 µl probe (0.25 µM final concentration). RNAse-free water was used as a negative control. In each purification procedure a 10-fold dilution of virus stock solution (F214-1011-1) was included as a positive control. DNA from this isolate was included in each run of qPCR as a positive PCR control. Thermocycling was performed on Mx3005P and Mx3000P qPCR-systems (Stratagene) with the following settings: 95°C for 15 min, followed by 50 cycles of 94°C for 10 s and 60°C for 1 min. MxPro (v. 4.10) software was used for qPCR analysis with default options. The cut-off value for positive samples was set at 35 Cts. Samples yielding a Ct value between 35 and 40 were considered weak positive/suspect, and assessed on a case-by-case basis depending on shape of amplification plot, samples with a Ct above 40 were considered negative, in accordance with DTU Aquas' internal Standard Operating Procedures in infectious trials. The efficiency of the qPCR assay was evaluated by testing cell culture supernatant and 10-fold dilution series up to 10^{-6} for all three isolates in duplicate.

2.8 | Histology

Fish were sampled whole except for tail sections (removed behind the vent), the ventral abdomens were slit to facilitate formalin penetration. Samples were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax blocks, sectioned (4 µm)

longitudinally, and stained with haematoxylin and eosin (H&E). Histopathology findings were considered consistent if present in over 50% of challenged fish sampled but not in controls.

2.9 | Statistical analysis

Kaplan–Meier survival analysis was performed using GraphPad PRISM 7 software. Significance of variations in survival was assessed through a log-rank (Mantel-Cox) test within individual challenge models, that is, IP injected or cohabited fish and respective negative controls and significance threshold (p) was set at .05.

3 | RESULTS

3.1 | Survival and clinical disease

Survival of lumpfish shedders injected with ENARV IS and ENARV FO was significantly reduced compared to controls (Figure 1, Table 1). The survival of salmon and lumpfish IP challenged with ENARV IE was not significantly reduced. The survival of lumpfish groups challenged by cohabitation (all isolates), was not significantly reduced (Figure 1, Table 1). Reduced survival in the first 2 weeks showed no correlation to challenge model or isolate. Pathology of the tail fin and peduncle, attributed to observed aggressive behaviour, was associated with reduced survival in week 1. Changes in husbandry were implemented and the incidence of reduced survival attributed to aggression was 70%, 12% and 0%, respectively, in the following weeks.

Statistically significant reduced survival in weeks 3 and 4 correlated to IP challenge with ENARV IS and ENARV FO. Reduced survival in ENARV IE shedders was not statistically significant (Table 1, Figure 1a, c, e). Clinical signs associated with reduced survival in weeks 3 and 4 were bloating, ascites, haemorrhaging around the brain and spinal cord (Figure 2) and haemorrhaging in the body wall of fish in good body condition (all isolates). Reduced survival in cohabited fish and negative controls was associated either with pathology attributed to aggression or the humane endpoint triggered by poor body condition and lethargic behaviour. No presentations associated with = infectious disease were seen on external examination or necropsies in salmon, and no indications of other infections were seen on wet mounts or bacteriology samples.

3.2 | Virus isolation and qPCR results

Ct (cycle threshold) values for stock virus samples and sequential dilutions were consistent between replicates and the amplification curves were parallel over the 7 orders of magnitude tested (diluted to 10^{-6}, data not shown). qPCR efficiency ranged from 93% to 96% and R^2 from 0.998 to 0.999, undiluted samples were not considered in this calculation.

All Ct values and corresponding cell culture results from challenge trials are listed in Table 2. Ct values (mean and range) from weekly routine samples are presented in Figure 1.

3.2.1 | ENARV IS and ENARV FO

Results were consistent between these isolates. All shedder samples were tested positive by both qPCR and cell culture. Mean Ct values of routine shedder samples decreased from week 1 to 3 and were increased slightly in one fish sampled for each isolate after week 4 (Figure 1b, d). Ct values of shedders that reached the humane endpoint decreased and were consistently low in weeks 3 and 4, averaging 14.5 ($n = 6$) and 15.5 ($n = 9$) for ENARV IS and 15.7 ($n = 14$) and 19 ($n = 1$) for ENARV FO replicates, respectively. All cohabitant samples tested negative in week 1, but routine cohabitant samples became consistently positive by qPCR and cell culture from week 2 on. The mean Ct values of routine cohabitant samples decrease between week 2 and week 3, but samples at week 4 showed no continued decrease (Figures 1b, d). Results from cohabitants that reached the humane endpoint in week 2 ($n = 8$) were inconsistent, five fish tested positive on qPCR but only one fish tested positive by cell culture (ENARV IS).

3.2.2 | ENARV IE

Mean Ct values in routine lumpfish shedder samples were positive and stable at weeks 1, 2 and 3, cell culture was positive in these samples. At week 4, Ct values were higher (Figure 1f), and 2/3 cell culture samples were negative. Shedders that reached the humane endpoint consistently tested positive on qPCR and cell culture. Routine samples from cohabitants inconsistently show weak positive signal in qPCR that could not be corroborated by cell culture. Cohabitants that reached the humane endpoint in week 2 ($n = 2$) were weakly positive by qPCR, but this was not corroborated by cell culture. In week 3, samples ($n = 4$) were positive by qPCR and cell culture.

Salmon routine samples were positive by qPCR at weeks 1, 2 and 3. Ct values were higher in week 4, with largely only weak positive results and only 2 below a Ct of 35. (Figure 1h). Cell culture results of salmon were positive in week 1, inconsistent at weeks 2 and 3, and negative at week 4. Atlantic salmon humane endpoint samples ($n = 3$) were found positive by cell culture and qPCR at week 3.

None of the samples from the lumpfish and salmon control groups showed CPE in culture or were detected positive for the presence of ENARV by qPCR.

3.3 | Histology

3.3.1 | IP challenged lumpfish

Lumpfish that reached the humane endpoint (all isolates) had necrosis in the kidney (interstitial haematopoietic and lymphoid

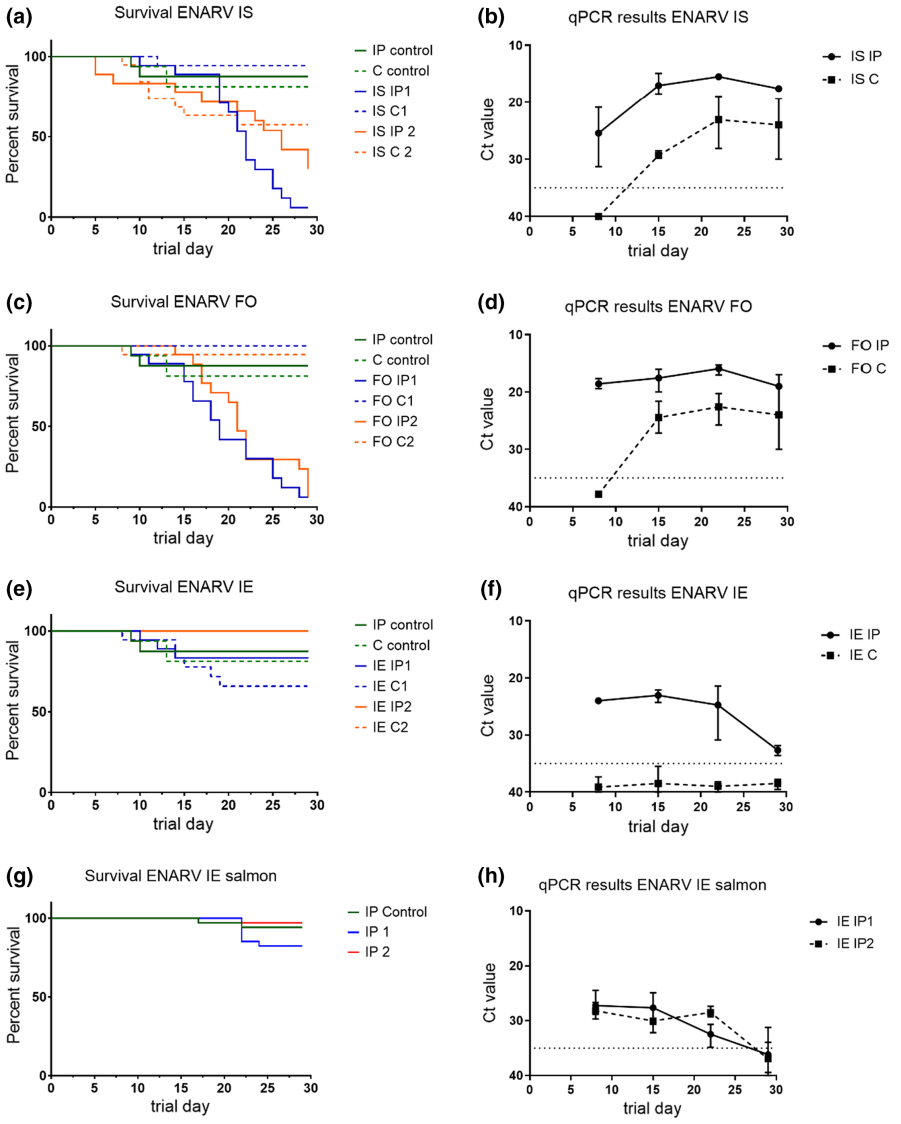

FIGURE 1 (a, c, e, g) Survival curves of ENARV challenged fish for 2 replicates and negative controls. (b, d, f, h) qPCR results (mean, error bars indicate range) of routine samples as described in Tables 1 and 2. The cut-off value is indicated by the horizontal dotted line. The last value for fish IP injected with ENARV IS and FO is based on a single fish. *IS, Iceland; FO, Faroe Islands; IE, Ireland; IP, intra peritoneal challenge; C, cohabitation challenge.

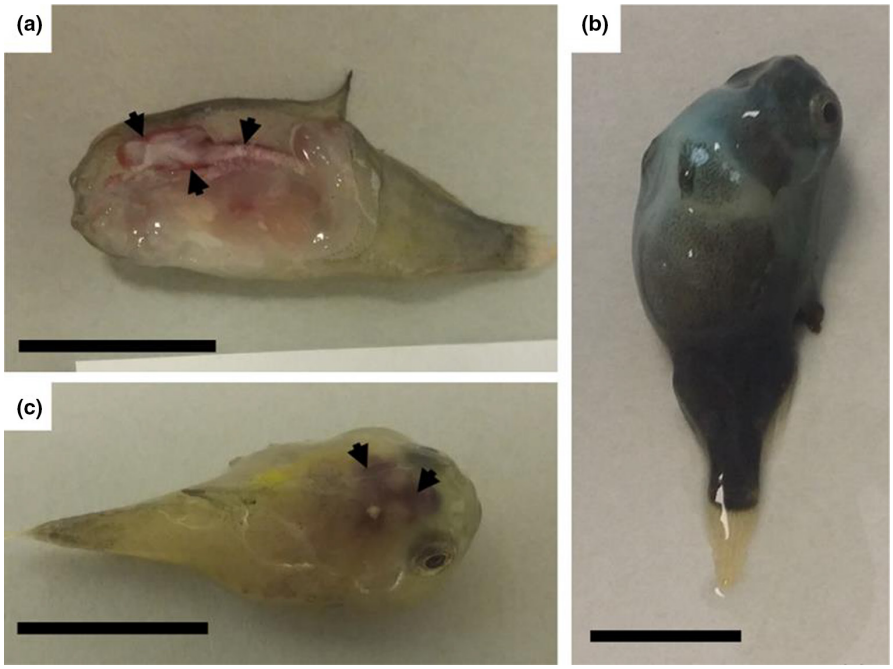

FIGURE 2 Pathology attributed to IP injection with ENARV. (a) Haemorrhaging around brain and spinal cord in sagittal section (arrows) and (c) The same fish viewed externally. (b) Severe abdominal distension in an otherwise good condition fish. Scale bars represent 1 cm.

tissue) (Figure 3b), stomach and oesophagus (mucosa and lamina muscularis) (Figure 3a), liver (peripheral hepatocytes) and peritonitis. Spleens were not visible in sections consistently, but necrosis was present in 2 ENARV FO challenged fish. Peritonitis was consistent in random ENARV IS and FO samples, other pathologies seen in fish that reached the humane endpoint were not present consistently in random samples. Pathology in ENARV IE random samples was mild compared to other isolates. One fish had peritonitis and necrosis in liver and kidney, two had mild focal gastrointestinal necrosis as described above.

Further pathological changes were present inconsistently (all isolates), including multifocal necrosis throughout the liver in addition to peripheral hepatocytes, inflammatory response associated with necrotic foci in kidney, liver and the gastrointestinal tract, necrosis in skeletal muscle, thymus, subdermal tissue, peripancreatic fat and spongiform myocardium, congestion around brain and inflammation in visceral organs adjacent to peritonitis. No comparable pathologies were seen in negative controls.

3.3.2 | Cohabitant lumpfish

One ENARV IS challenged cohabitant showed mild focal necrosis in the oesophageal mucosa and lamina muscularis. No consistent pathological changes were present in other cohabitant samples.

3.3.3 | Salmon

Chronic focal granulomatous inflammation was present in the livers of two salmon (random samples, IP challenged), no pathologies were seen in negative controls.

4 | DISCUSSION

In recent years, the salmon farming industry has increasingly moved towards non-medicinal approaches to salmon lice control (Overton et al., 2019). One approach currently implemented is the biological control of salmon lice through cleaner fish. This trend is likely to continue, and in order to supply cleaner fish an increase in transport of lumpfish and lumpfish ova between European countries is expected. The impact of a new pathogen, or a new pathogen strain introduced into new hydrographic areas could be potentially high (EURL, 2016) and understanding the impact of infectious agents is a basic requirement to informed health management decisions. This study, together with pilot trials (Scholz, 2018), provides the first experimental data on ENARV infections in lumpfish.

The ENARV IE titre used in this study was approximately 2 log lower than the ENARV IS and FO titres. This was not intentional but was discovered on back titration of the challenge material. Results from ENARV IE challenges differed from ENARV IS and FO challenges, but virulence of viruses, including ranaviruses, can be dose dependent (Brunner et al., 2005; van Beek et al., 1988, 2000). The variation in challenge titre therefore invalidates a direct comparison of results between ENARV IE and the other isolates, a direct comparison is possible only between ENARV IS and FO challenges and between lumpfish and salmon challenged with ENARV IE. The following discussion is structured accordingly.

Infection trials with ENARV IS and FO were developed according to the following chain of events. IP challenge was followed by viral replication in the host, horizontal transmission of the virus to naïve cohabited fish and statistically significant reduction in survival in IP-challenged fish which was associated with consistent clinical signs. Viral replication was demonstrated by decreasing Ct values, and the virus was re-isolated consistently from shedders (Tables 1, 2, Figure 1). The lowest Ct values measured were in fish that reached the humane endpoint, and in random specimens collected during the phase of reduced survival. However, IP injection is not a natural infection route and consequently the results in IP-challenged fish do not necessarily reflect the virulence of ENARV isolates under field conditions. It has previously been demonstrated that IP challenge with ranaviruses can reduce survival while immersion challenge under comparable conditions did not (Ariel & Jensen, 2009; Jensen et al., 2011; Whittington & Reddacliff, 1995).

IP challenge was associated with consistent clinical signs. Severe abdominal distension and ascites, haemorrhaging around the brain, spinal cord and in the muscle of the abdominal wall were associated with reduced survival in lumpfish shedders. Abdominal distension and ascites are common signs of disease in lumpfish and can be caused by various infectious aetiologies (Scholz et al., 2018). Degeneration and necrosis of vascular endothelial cells and haemorrhaging in internal organs can occur with ranavirus infections (Whittington et al., 2010). Necrosis in kidney and gastrointestinal tract were attributed to ENARV IP challenge on histology. Necrosis in the spleen was observed, but spleens were not consistently sectioned due to small size and position. This is consistent with descriptions of systemic necrotizing infections, including necrosis of haematopoietic tissue and in the gastrointestinal tract, described for other ranavirus infections in fish (Whittington et al., 2010). Peritonitis and necrosis of the peripheral liver were consistent in ENARV IS and FO IP-challenged fish. It seems likely that this presentation is associated with ENARV exposure through IP challenge and is therefore not necessarily applicable to natural infections. Further lesions (see Section 3.3) were present inconsistently, but absence of pathological findings in negative control samples indicate that these could also be attributed to the infection.

Horizontal transmission of ENARV IS and FO between lumpfish was demonstrated, but survival was not significantly reduced in horizontally infected fish. The detection of viral DNA and viable particles consistently demonstrated infection of cohabitants for ENARV IS and FO from day 14 onwards. Up to week 3, dropping Ct values showed an initial phase of virus replication. At week 4, the mean Ct values were slightly increased, indicating a lowered viral load and suggesting clearance of the virus. QPCR results indicated significant

TABLE 2 Cell culture and qPCR results of routine samples and fish that reached the humane endpoint

	Time points			
Isolate	**W eek 1**	**Week 2**	**Week 3**	**Week 4**
Lumpfish routine samples				
IS – IP	24/+, 31/+, 21/+	18/+, 19/+,15/+	16/+, 15/+, 15/+	17.6/+
IS – C	−/−, −/−, −/−	30/+, 30/+, 29/+	22/+, 19/+, 28/+	29/+, 19/+, 26/+, 20/+, 23/+, 20/+, 25/+, 23/+, 28/+, 22/+, 25/+, 23/+, 30/+
FO – IP	18/+, 18/+, 20/+	16/+, 17/+, 20/+	16/+, 16/+, 15/+, 15/+, 19/+	19/+
FO – C	38/−, 38/−, 38/−	25/+, 22/+, 27/+	20/+, 26/+, 22/+	23/+, 26/+, 24/+, 30/+, 24/+, 27/+, 17/+, 22/+, 29/+, 24/+, 27/+, 23/+, 27/+
IE – IP	24/+, 24/+,24/+	22/+, 23/+, 24/+	22/+, 22/+, 31/+	33/−, 34/−, 32/+
IE – C	37/−, −/−, −/−	36/−, −/−, −/−	38/−, −/−, 39/−	−/−, 38/−, 38/−, 39/−, −/−, 39/−, −/+, 38/−, 39/−, −/−, 39/−, 39/−, 39/−
Lumpfish humane endpoint samples				
IS – IP	20/+, 15/+, 21/+	24/+ (2), 18/+, 16/+, 14/+, 20/+, 14/+	15/+, 15/+, 14/+ (2), 16/+, 13/+	15/+, 15/+, 17/+, 13/+, 16/+, 14/+, 16/+, 17/+ (2)
IS – C	−/−	−/T, −/−, −/−, 34/−, 24/+	36/+	
FO – IP	20/+, 20/+	19/+, 17/+, 18/+, 36/T, 19/+, 18/+, 15/+	18/+, 27/+, 14/+, 14/+ (2), 14/+ (2), 15/+, 15/+, 14/+, 16/+, 15/+, 15/+ (2)	19/+
FO – C	−/− −/−	34/− (2), 32/−		
IE – IP		24/+, 21/+, 20/+, 20/T, 22/+	14/+	
IE – C	−/− (2), −/−	35/T, 39/−	33/+, 36/+, 39/+, 34.6/+	
Atlantic salmon routine samples				
IE – IP	29/+, 28/+, 25/+, 30/+, 26/+, 28/+	31/−, 25/+, 27/−, 30/−, 32/−, 28/+	32/+,31/+, 35/−, 27/+, 29/+, 29/+	36/−, 31/−, −/−, 37/−, 37/−, 36/−, 34/−, 38/−, 37/−, 39/−
Atlantic salmon humane endpoint samples				
IE – IP			33/−, 24.5/+, 26.3/+	

Note: Negative controls are not included. Results are presented as Ct value/cell culture results (positive: + or negative: −). Ct values are rounded. If samples consist of more than one fish the number is indicated in brackets behind the result. Samples with discrepancies between qPCR and cell culture results are underlined.

Abbreviations: IP, challenged by intraperitoneal injection; C, challenged by cohabitation; T, cell culture could not be evaluated due to toxic effect.

viral loads in some cohabitant fish, in part comparable to random samples in IP-injected fish at earlier time points but never as low as values associated with reduced survival. No pathologies were seen in randomly sampled cohabitant fish. Mild oesophageal necrosis consistent with changes seen in IP-injected fish and described in one fish in previous experimental immersion challenges with ENARV IE (Scholz, 2018) was observed in one cohabitant fish that reached the humane endpoint (ENARV IS). This suggests that this necrosis, despite being present in one cohabitant sample only, may be attributed to ENARV infection. The rate at which pathologies potentially develop following horizontal transmission with ENARV is unknown and the trial period would have to be extended to validate results and the observation of decreasing viral loads.

IP injection of lumpfish with ENARV IE did not significantly reduce survival. Negative cell culture results and high Ct values at week 4 indicate that IP-challenged survivors were able to clear the virus (Tables 1, 2, Figure 1). However, the few ENARV IE IP-challenged fish that reached the humane endpoint showed clinical signs and histopathological changes consistent with pathologies seen in the other isolates. Together with cell culture and qPCR results, which include a Ct value of 14 in the one fish sampled in week 3, this indicates that ENARV IE was able to replicate in this host and displayed virulence in single cases (Tables 1, 2, Figure 1). Though not comparable to the doses used for ENARV IS and FO, the IP challenge with ENARV IE at 8.6×10^5 $TCID_{50}$ ml^{-1} is considered significant and similar or substantially lower doses have been used to demonstrate virulence of other viruses in susceptible fish species (Dale et al., 2009; Graham et al., 2011). Horizontal transmission in ENARV IE-challenged lumpfish occurred only inconsistently, with low viral loads detected by qPCR and cell culture in some fish that reached the humane endpoint in week 3. ENARV IE routine cohabitant samples were negative by cell culture, qPCR results were inconsistent and Ct values in positive samples were always high (Table 2, Figure 1). Whether the varying results between ENARV isolates are due to a dose-dependent effect and a critical ENARV load resulting in clinical disease in lumpfish, or whether these are

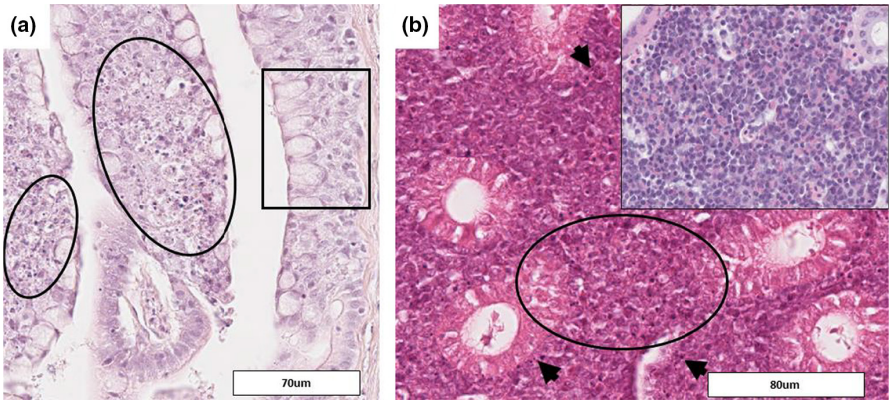

FIGURE 3 Histopathology in a fish sampled randomly after week 4 (ENARV IS IP challenge). (a) Section of oesophagus showing normal appearance (square) and necrosis (circled) in oesophageal mucosa. (b) Necrosis in interstitial kidney haematopoietic and lymphoid tissue (circled, arrows). Insert shows normal kidney tissue.

due to variations in virulence between ENARV isolates cannot be determined.

ENARV has not been reported in farmed Atlantic salmon to date. Nevertheless, virulence of ranaviruses can be dose dependent (Brunner et al., 2005) and an outbreak in cohabited lumpfish could generate a high level of infectious pressure not previously encountered by farmed salmon stocks. Results from this study suggest that the Irish ENARV isolate used was not a high risk to salmon. IP challenge of salmon parr with ENARV IE did not significantly reduce survival, and there was no indication of long-term virus replication in salmon as Ct values increased over time and cell culture results were consistently negative at week 4. The average Ct values in IP-injected salmon were higher after week 1 compared to lumpfish challenged with the same material (Ct 27.7 vs. 24),which potentially indicates a lesser ability of the virus to replicate in salmon and/or a better control efficiency of the immune system. Granulomatous inflammation in the livers of two salmon is not consistent with pathology in lumpfish. This likely presents a reaction to the challenge process rather than a systemic infection, as supported by the chronic appearance of the lesions, lack of further pathology and results from cell culture and qPCR. However, the variations in virulence between isolates observed in lumpfish challenges mean that this result cannot necessarily be applied to other ENARV isolates and further challenges with isolates that demonstrated virulence in lumpfish are advisable.

While this study cannot demonstrate variations in virulence between ENARV isolates, this remains a possible explanation for the results. The potential presence of intrinsic virulence markers in ENARV FO and ENARV IC could be investigated by comparing their full-length genome sequence with those of other isolates showing different mortality phenotypes in vivo (Stagg et al., 2020).

Tail biting was considered the cause of reduced survival in lumpfish in the first 2 weeks, supported by no association with challenge protocol, observed aggressive behaviour, consistent pathology and the absence of consistent infectious aetiology. Tail biting is a common problem in lumpfish hatcheries and there are reports of cannibalism among lumpfish juveniles (Ingólfsson & Kristjánsson, 2002). Reduced survival in the first 2 weeks did not lead to variations between challenged groups and respective control groups that would invalidate the conclusions drawn from the survival analysis. The husbandry measures introduced in week 1 largely mitigated tail biting behaviour by the end of the second week of experiment, thereby making it easier to assess reduced survival ascribed to ENARV IS and FO IP challenge occurred in weeks 3 and 4 (Figure 1).

Some discrepancies were observed between viral isolation on cell culture and qPCR analysis (Table 2). These occurred in samples with high Ct values, indicating a low viral load and a higher sensitivity of the qPCR assay compared to the cell culture protocol used in this study. Most cases of positive qPCR and negative cell culture results in the same sample occurred in ENARV IE challenges, and this was the only isolate that the fish appeared to clear or inactivate. This possibly implied qPCR amplification of DNA from non-viable viral particles. The specificity of the assay was tested by Stilwell et al., 2018 and this study indicates it is suitable for detection of ENARV for surveillance or diagnostic purposes.

In conclusion, this study shows that ENARV can be horizontally transmitted and that some strains can reduce survival of lumpfish juveniles following IP injection associated with consistent pathological changes. Indications of possible lower virulence in the Irish ENARV strain require further investigation, also regarding potential virulence markers. This study found no indication of the Irish ENARV isolate replicating in Atlantic salmon parr. The ranavirus qPCR developed by Stilwell et al. (2018) was demonstrated to be an effective diagnostic tool for ENARV infection. Although not conclusive, this study provides valuable data to inform risk assessments evaluating the risk posed by the use of cleaner fish in aquaculture.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Felix Scholz https://orcid.org/0000-0001-9461-4086
Niccolò Vendramin https://orcid.org/0000-0002-9217-7887
Argelia Cuenca https://orcid.org/0000-0001-8596-1086
Neil M. Ruane https://orcid.org/0000-0002-2001-9652

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