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Susceptibility of pike-perch *Sander lucioperca* to a panel of ranavirus isolates

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ABSTRACT

The host range of ranaviruses was investigated by challenging pike-perch (*Sander lucioperca*) with the following ranavirus isolates: epizootic haematopoietic necrosis virus (EHNV), European sheatfish virus (ESV), European catfish virus (ECV), pike-perch iridovirus (PPIV), short-finned eel virus (SERV) and frog virus 3 (FV3). Pike-perch fry were bath-challenged at 12 °C and 22 °C at 5 weeks post hatching, and the challenge was repeated with EHNV and PPIV in older fish (15 weeks post hatching) at higher densities. A third batch of fish was subjected to intraperitoneal (i.p.) and cohabitation challenge with EHNV, ESV, ECV and PPIV at 16 °C. Statistically significant mortality was observed in EHNV-challenged fish at both temperatures in the five week old fish. No mortalities were seen in older fish challenged with EHNV and PPIV. High mortalities were registered in i.p.-challenged fish, but not in cohabitated fish. Virus re-isolation was possible from the youngest bath-challenged fish in all challenge trials and the i.p.-challenged fish, but not from older fish or cohabitated fish. Susceptibility of pike-perch to ranaviruses appears to be dependent on age of fish and challenge route. The study shows that pike-perch are susceptible to infection with ranavirus under certain conditions, and it is suggested that this be considered when reviewing the legislation on notifiable diseases.

1. Introduction

In 2006, epizootic haematopoietic necrosis disease (EHN) was included on the list of exotic fish diseases in the European Union (EU) legislation. The criteria for listing a disease as exotic are that the disease i) is not established in the Community and the pathogen is not known to be present and ii) it has potential for either significant economic impact if introduced or detrimental environmental impact on wild aquatic animal species (Anonymous, 2006). EHN is caused by the epizootic haematopoietic necrosis virus (EHNV), which has previously induced high mortalities in redfin perch (*Perca fluviatilis*) and morbidity in rainbow trout (*Oncorhynchus mykiss*) in Australia (Langdon et al., 1986; Langdon et al., 1988). These two species are listed as susceptible to EHN in the current legislation (Anonymous, 2006), and surveillance programmes are in place, targeting EHNV infection in these species.

In European-based challenge studies, EHNV did not appear to be as virulent for European stocks of red-fin perch and rainbow trout as reported for Australian fish stock (Ariel and Bang Jensen, 2009; Ariel et al., 2010). However, other important European fish species can be susceptible to EHNV, as has been demonstrated for black bullhead (*Ameiurus melas*) and pike (*Esox lucius*) (Bang Jensen et al., 2009; Gobbo et al., 2010).

EHNV is a member of the genus *Ranavirus* (family *Iridoviridae*), which contains viral isolates obtained from fish, amphibians and reptiles from all over the world. Most viruses in the genus are genetically very closely related in terms of the major capsid protein gene, and can only be differentiated after amplification by polymerase chain reaction (PCR) and subsequent restriction enzyme analysis (REA) or sequence analysis (Hyatt et al., 2000; Marsh et al., 2002; OIE, 2006; Holopainen et al., 2009).

EHNV has not been isolated in the EU. However, ranaviruses closely related to EHNV have been isolated in outbreaks with high mortalities in farmed sheatfish (*Siluris glanis*; European sheatfish virus, ESV) and farmed or wild black bullhead (European catfish virus, ECV) (Ahne et al., 1989; Bigarré et al., 2008; Pozet et al., 1992). A ranavirus isolate (pike-perch iridovirus, PPIV) has also been isolated from apparently healthy farmed pike-perch (*Sander lucioperca*) (Tapiovaara et al., 1998), another (Rmax) from clinically healthy turbot fry (*Psetta maxima*) and another (CodV) from free-living cod (*Gadus morhua*) with an ulcus-syndrome (Jensen et al., 1979; Ariel et al., 2010). Short-finned eel virus (SERV) was found in non-symptomatic short-finned eels (*Anguilla australis*) imported to Italy from New Zealand (Bang Jensen et al., 2009).

Previous challenge studies have shown that ECV, ESV, PPIV and SERV can be pathogenic to European stocks of pike, and that ECV and frog virus 3 (FV3), the type species of the genus, can infect pike

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without causing significant mortality (Bang Jensen et al., 2009). In addition to EHNV, black bullhead has been shown to be susceptible to infection with ECV, but seems resistant to infection with SERV or amphibian ranaviruses (Gobbo et al., 2010).

Pike-perch is widespread in European inland waters, where it is an ecologically important species due to its status at the top of the food chain, and according to the Food and Agriculture Organization of the United Nations (FAO, 2010), the production of pike-perch is increasing rapidly in Europe, and considerable efforts are made in order to increase the stocks and develop more efficient ways of rearing fry and young fish (Jokelainen et al., 2009). Even though PPIV was isolated from pike-perch, we have no knowledge of the pathogenicity of ranaviruses to this species. The aim of the study presented here was to supplement the knowledge on the host range of ranavirus by determining the susceptibility of pike-perch to different ranavirus isolates, and to confirm its susceptibility to PPIV under experimental settings. Another aim was to investigate whether those isolates that proved to be pathogenic to pike-perch could be transmitted via natural transmission pathways such as co-habitation with infected fish.

2. Materials and methods

2.1. Virus

The panel of viral isolates used in the study and their references are listed in Table 1. In addition to ranaviruses isolated from fish (EHNV, ESV, ECV, PPIV and SERV) one amphibian virus isolate (FV3) was included in the challenge experiments, as it has previously been demonstrated that fish can be susceptible to or be carriers of ranaviruses isolated from amphibians (Moody and Owens, 1994; Mao et al., 1999; Bang Jensen et al., 2009). Furthermore, FV3 has been shown to be genetically closely related to PPIV (Holopainen et al., 2009).

The ECV-24 isolate was obtained from an outbreak of ECV in black bullhead in Italy (Gobbo et al., 2010). Before use, the isolates were propagated in epithelioma papulosum cyprini cells (EPC, Fijan et al., 1983) at 20 °C in Eagle's minimal essential medium (EMEM) according to recommendations by Ariel et al. (2009). When a complete cytopathic effect (CPE) of the monolayer was observed, the cell culture medium with virus was harvested and titrated. The titre was calculated according to the method of Reed and Muench (1938).

2.2. Challenges

Three different challenges were performed as summarised in Table 2. The aim of the first challenge was to investigate the susceptibility of pike-perch to a panel of ranaviral isolates at 12 and 22 °C via a bath challenge. The second challenge expanded the first challenge to older fish at higher densities. The third challenge tested the possibility of transmission via natural routes during co-habitation with infected fish.

2.3. Fish

All pike-perch originated from the same supplier. This producer obtained eggs from wild-caught brood stock, and hatched the eggs at the production site. Fry were then allocated to two different rearing systems. In one, the fish were intensively reared in an indoor facility using a re-circulated water system with a temperature of 28 °C. The other system was an extensive facility, where the fry were reared in an outdoor concrete pond, with no heating, and thus mimicking natural conditions. For the challenges described in this paper, we obtained fish from both rearing facilities. The batch of fish used in challenge I was obtained from the intensive rearing system, whereas the batch used for challenge II was obtained from the extensive rearing system.

The batch used for challenge III was from the same batch as challenge II, but 6 weeks older (see Table 2). Upon arrival at the research facility, a sample of fish from each batch was submitted for bacteriological examination on blood agar and Ordals agar (Lorenzen, 1993) and virological examination according to Commission Decision 2001/183/EC (Anonymous, 2001). No pathogens were detected. Upon arrival at the research facility, the fish for challenge I were distributed in 28 individual 10 L tanks, with approx. 30 fish in each. In half the tanks, the water temperature was 12 °C and in the other half 22 °C, corresponding to the temperatures at which the challenge would be carried out (Table 2). The fish were allowed 2 weeks of acclimatisation before commencement of the challenges. The same procedure was followed for the subsequent batches of fish, with the following exemptions: for challenge II, the number of fish in each tank was 50, and the total number of tanks was 12. For challenge III, the number of fish in each tank was 10, and the total number of tanks was 10. The water temperature in challenge III was 16 °C. Throughout the challenges, the water pH was kept at 7, and the General Hardness was kept at 3 dH via ion exchange. All fish were fed commercial dry food according to their age and size.

2.4. Bath challenges

For the bath challenges (challenges I and II) the following procedures were followed: the water volume in the tank was reduced to 2 L and water flow stopped. Virus (diluted with cell culture medium) was added, reaching a final concentration of 1×10^4 $TCID_{50}$/mL. After 1 h the

Table 1
Virus isolates used in the study.

Fish viruses	Abbreviation	Country of origin	Original host	Reference	Isolate obtained from
Epizootic haematopoietic necrosis virus	EHNV	Australia	Red-fin perch (*Perca fluviatilis*)	Langdon et al. (1986)	R. Whittington, University of Sydney, Australia
European sheatfish virus	ESV	Germany	European sheatfish (*Siluris glanis*)	Ahne et al. (1989)	W. Ahne, University of Münich
European catfish virus	ECV	France	Black bullhead (*Ameiurus melas*)	Pozet et al. (1992)	G. Bovo, Instituto Zooprofilattico delle Venezie, Italy
European catfish virus	ECV-24	Italy	Black bullhead (*Ameiurus melas*)	Gobbo et al. (2010)	G. Bovo, Instituto Zooprofilattico delle Venezie, Italy
Pike-perch iridovirus	PPIV	Finland	Pike-perch (*Sander lucioperca*)	Tapiovaara et al. (1998)	H. Tapiovaara, National Food and Veterinary Research Institute of Finland
Short finned eel virus	SERV	Italy	Short-finned eel (*Anguilla australis*)	Bang Jensen et al. (2009)	G. Bovo, Instituto Zooprofilattico delle Venezie, Italy
Frog virus					
Frog virus 3	FV3	North America	Leopard frog (*Rana pipiens*)	Granoff et al. (1966)	W. Ahne, University of Münich, Germany

Table 2
Challenges performed in the study. Virus abbreviations are explained in Table 1.

Challenge	Average weight of fish	Age of fish	Rearing conditions	Temperature (°C)	Transmission route	Viruses
I	1–2 g	5 weeks	Intensive	12 and 22	Bath	EHNV, ESV, ECV, PPIV, SERV and FV3
II	1–2 g	15 weeks	Extensive	12 and 22	Bath	EHNV and PPIV
III	5–7 g	21 weeks	Extensive	16	Cohabitation	EHNV, ESV, ECV-24 and PPIV

water flow was restarted. The negative control fish were exposed to the same treatment, but using an equal volume of cell culture medium instead of the virus dilution. Treatments were carried out in duplicate for each virus/temperature combination. The temperatures used in the challenges are shown in Table 2. Due to limited capacity in the experimental facility, challenge with SERV at 12 °C was carried out at the same time as challenge III, when the fish were approximately 21 weeks old.

Dead fish from each aquarium were recorded, sampled, pooled and frozen at −20 °C daily for subsequent virological examination. The period of observation was 28 days, after which all surviving fish were euthanized. 1–2 fish from each tank were then fixed in 10% neutral buffered formalin for histology and the remaining fish were pooled and frozen for subsequent virological examination.

2.5. Statistical analysis

Statistical analysis was performed on bath challenges where mortalities were seen using the Statistical Analysis System software (SAS version 9.1, SAS Institute, Cary, NC, USA). A 5% significance level was used. Differences in the cumulative mortalities between challenged and control fish in the duplicate aquaria were tested by an analysis of variance (PROC MIXED in SAS). In the analysis the proportion of dead fish out of total fish in the tank was the continuous outcome. The temperature in the aquarium (12 °C and 22 °C), the viral isolate (EHNV, ESV, ECV, PPIV, SERV and FV3), and the interaction between temperature and viral isolate were investigated as fixed effects, in order to take into account the duplicate tanks for each temperature/virus combination. The variation between the two aquaria within temperature and viral isolate constitutes the residual variation.

2.6. Transmission challenge

In challenge III, five fish were injected intraperitoneally (i.p.) with 100 μL of viral suspension, and cohabitated with five naïve fish from the same batch for each of four viral isolates at 16 °C (±2 °C). The number of fish in the challenge was limited to five because of limited resources and justified by the precedence of Langdon (1989) who used small numbers of fish to successfully demonstrate transmission of EHNV between infected and cohabitated fish. To distinguish between exposed and naïve fish, the latter had the caudal dorsal fin clipped. The four viral isolates were (final titre in brackets): EHNV (4×10^6 $TCID_{50}$/mL), ESV (5.9×10^6 $TCID_{50}$/mL), PPIV (4×10^7 $TCID_{50}$/mL) and ECV-24 (4.8×10^6 $TCID_{50}$/mL). In the negative control treatment, the five fish were injected with phosphate buffered saline (PBS) and cohabitated with five naïve fish for control. The fish were observed and sampled daily as described for the bath challenges. After 36 days any surviving fish were euthanized, and fish from each tank were pooled and frozen for subsequent virus re-isolation.

2.7. Virus re-isolation

In order to confirm that mortalities were caused by infection with ranavirus, the sampled pools of fish were processed for virus re-isolation, according to Commission Decision 2001/183/EC (Anonymous, 2001) as described below.

From challenge I, up to seven samples of pooled fish collected on different days, from each tank was tested, always including the samples from the end of the trial. From challenges II and III, all samples of pooled, dead fish were tested, including samples from the end of the trial. In tanks where no mortality was seen, only the fish sampled at the end of the trial were available for virus re-isolation. For each sample, the fish were homogenised whole using a mortar, pestle and sterile sand. For samples with >5 fish, 5 fish were pooled, and the rest discarded. The samples were diluted in 2 mL transport medium consisting of sterile EMEM with 10% new-born calf serum, TRIS-buffer and penicillin (100 U/mL)/streptomycin (100 μg/mL) and centrifuged at 4000 *g* at 5 °C for 20 min. After addition of hexamycin (25 μL/mL) to the clarified supernatant, the samples were incubated at 5 °C overnight or at 15 °C for 4 h. The samples were then inoculated onto cultures of 24 hour-old EPC-cells in 24-well trays, in final dilutions of 1:100, 1:1000 and 1:10,000. The inoculated cell cultures were incubated at 20 °C (temperature adapted according to Ariel et al., 2009) for one week, with regular examination for CPE. After one week, samples with no CPE were re-inoculated onto a new EPC cell culture. After one more week, if no CPE was observed, the samples were considered to be virus negative. Cell cultures with CPE were stored at −80 °C.

2.8. Histopathology

From each tank in challenges I and II, 1–2 fish sampled after euthanasia at the end of the trial were examined histologically. From challenge I, 1–2 fish from each virus × temperature combination were examined, totalling 21 fish. After fixation in 10% neutral buffered formalin for at least 24 h, whole fish were embedded in paraffin. All the samples were processed according to standard histological procedures, sectioned and stained with haematoxylin and eosin (H&E) and then prepared for immunohistochemistry using the protocol described by Evensen and Lorenzen (1996). Briefly, a polyclonal rabbit anti-ECV antibody (kindly provided by Dr. Bovo, Instituto Zooprofilattico Sperimentale delle Venezie, Italy) was used as the primary antibody. This antibody cross-reacts with all ranavirus isolates used in this study and is specific for ranaviruses (Gobbo et al. 2010). A section from EHNV-infected pike proven to be positive by this IHC-protocol was used as positive control (Bang Jensen et al. 2009). On each glass-slide were two tissue sections from the same fish. The tissue sections were dewaxed and hydrated through gradients of alcohol, and incubated with 5% BSA for 20 min at room temperature. One tissue section on each slide was then incubated with polyclonal rabbit anti-ECV antibody diluted 1:1500 in 2.5% BSA for 1 h at room temperature, and the other was incubated with the same, only in a 1:750 dilution. After washing with TBS, a biotinylated swine anti-rabbit antibody (E0353, Dako) diluted 1:300 in 2.5% BSA was added and the slides were incubated for 1 h at room temperature. The slides were then washed and incubated with Streptavidin-AP (D0396, Dako) diluted 1:200 in 2.5% BSA for 40 min at room temperature, washed and chromogen substrate was added (Fast-Red, Kem-En-Tec) for 20 min. Finally, the slides were counterstained with haematoxylin and mounted with Aquamount (BDH 36226 Merck) and examined using a light microscope. This procedure was repeated 2 times.

2.9. Polymerase chain reaction (PCR)

PCR targeting the complete ranavirus major capsid protein (MCP) gene was performed to confirm the identity of isolates after virus re-isolation. DNA from the following samples were extracted and analysed: from 1st passage in EPC cells: 3 EHNV-infected fish, 2 ESV-, ECV-, PPIV and FV3-infected fish (respectively) and 1 SERV-infected fish. From organ material: 1 ESV-, ECV-, PPIV- and FV3-infected fish (respectively) and 2 SERV-infected fish. In total, for each ranavirus isolate used in the experiments, the respective PCR and sequencing analyses were performed on at least three independent samples. Material from non-challenged control fish and cells were used as negative controls. DNA was extracted with QIAamp DNA mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The complete viral MCP gene was amplified and sequenced in three overlapping fragments, using the primers described by Hyatt et al. (2000): MCP-1 (CAC CGT GTA TCT TAT AAT AAA AAG GAA ATG), MCP-2R (GGC TCC GTC CTG GCC TGT G), MCP-3 (GAG GCC AAG CGC ACA GGC TAC), MCP-4R (TTG GAG CCG ACG GAA GGG TG), MCP-5 (CGC AGT CAA GGC CTT GAT GT) and MCP-6R (AAA GAC CCG TTT TGC AGC AAA C). The amplification was performed using *Taq* polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA) and conditions described by Hyatt et al. (2000). PCR amplification of the partial viral DNA polymerase gene was performed as described by Holopainen et al. (2009), using the primers DNApol-F (GTG TAY CAG TGG TTT TGC GAC) and DNApol-R (TCG TCT CCG GGY CTG TCT TT). PCR products were separated by agarose gel electrophoresis and single products were eluted applying the QIAquick gel extraction kit protocol (Qiagen, Valencia, CA, USA). PCR products were sequenced directly using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction products were purified using DyeEx 2.0 Spin kit (Qiagen) and analysed with ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The resulting sequence data were analysed with the Sequencing Analysis Software 5.1 (Applied Biosystems). Multiple nucleotide sequence alignments were done with ClustalX 1.81 (Thompson et al. 1997).

3. Results

3.1. Mortality

In challenge I, high mortalities were registered in most treatments including the negative controls. No mortality was observed in the fish infected with SERV at 12 °C. Mortality started at day 3–5 and levelled out at around day 19 for fish kept at 22 °C, whereas mortality started later, at day 8 at the earliest, for fish kept at 12 °C (Fig. 1). The mortality curves were noticeably steeper for EHNV, ESV and ECV exposed fish than for the negative control group at 22 °C, whereas for PPIV, SERV and FV3 exposed fish they looked very similar to the negative control. For fish challenged with EHNV at 12 °C the mortality curves were steeper and the end point mortality was higher than for the negative control fish. For the group of fish challenged with ESV at 12 °C the mortality started earlier (days 8 and 11) than the negative control group (days 10 and 15), and the end point mortality was higher (average 58%) compared with negative controls (average 41%) (Table 3). For fish challenged with ECV, PPIV, SERV and FV3 at 12 °C the mortality curves were very similar to the negative controls.

A statistically significant difference was found between mortality in the EHNV exposed group and the negative control group at both 12 °C and 22 °C ($p<0.001$) (Table 3). For ESV, ECV, PPIV, SERV and FV3 no statistically significant difference was recorded between challenged fish and negative controls at either temperature ($p>0.05$). The only symptoms seen in the fish in challenge I were a darkening of the skin and lethargic behaviour. These symptoms were seen in some of the fish in all tanks, irrespective of infection status or temperature.

In challenge II, one fish died in a tank infected with EHNV at 22 °C, and one dead fish was found at both temperatures in the negative control treatments (Table 3). These were the only mortalities observed, and none of the fish showed any signs of disease.

The results from the transmission experiment (challenge III) are presented in Table 4. No mortalities were seen in fish cohabitated with virus inoculated fish, but one fish cohabitated with negative control fish died. In the virus inoculated fish, high mortalities were observed in the fish i.p. infected with EHNV, ESV and ECV-24, whereas no mortalities were seen in fish challenged with PPIV or in the negative control group.

3.2. Virus re-isolation

Virus was re-isolated from samples from all virus/temperature combinations in challenge I, except for fish infected with SERV at 12 °C (Table 3). The percentage of positive virus re-isolation was high for fish infected with EHNV, ESV, ECV and PPIV, whereas it was lower for fish infected with SERV and FV3 (Table 3).

Virus could not be isolated from the pike-perch euthanized at the end of the trials for fish infected with SERV at 12 °C or any other virus at 22 °C (Table 3).

In challenge II, virus was re-isolated from the fish that died during challenge with EHNV at 22 °C and from fish infected with EHNV at 12 °C and euthanized at the end of the challenge (Table 3). Virus re-isolation was unsuccessful from fish challenged with PPIV at either temperature in this challenge.

In challenge III, the inoculated virus was re-isolated from all i.p. infected fish, but no virus could be found in any of the co-habitated fish (Table 4), and neither in the one negative control fish that died during the challenge.

3.3. Histopathology

Pathological lesions were observed in H&E stained pike-perch from challenge II infected with EHNV, ECV and PPIV at 12 °C, but not in any fish infected at 22 °C or in fish infected with ESV or FV3 at 12 °C. The lesions were present in the kidneys of affected fish, and were characterised by foci of infected cells showing hypertrophy, vacuolization and pyknotic nuclei or karyorrhexis. Melanocytes were present in large numbers in the kidneys, and the renal interstitium was destroyed, with extensive cell necrosis and haemorrhaging. No positive staining was obtained by IHC using the anti-ECV antibody in any samples, except for the positive control, no matter which dilution of primary antibody.

3.4. PCR

Amplification of the ranavirus MCP and DNA polymerase gene could be achieved from those samples that were positive in virus re-isolation. The complete MCP gene (1392 bp) and partial DNA polymerase gene (560 bp) sequences were successfully obtained from all PCR positive samples. The sequence analysis of these positive samples confirmed that the ranavirus isolates obtained from the infected fish were identical to the isolates used for infection. No nucleotide changes were detected within the coding region of the MCP or DNA polymerase gene after passages of viruses in cell culture or fish. The ranavirus MCP or DNA polymerase gene could not be amplified from organ tissue or cell culture material obtained from negative controls or from exposed fish where virus re-isolation had not been possible.

4. Discussion

The present study showed that pike-perch can readily be infected with ranavirus under conditions that mimic their natural environment.

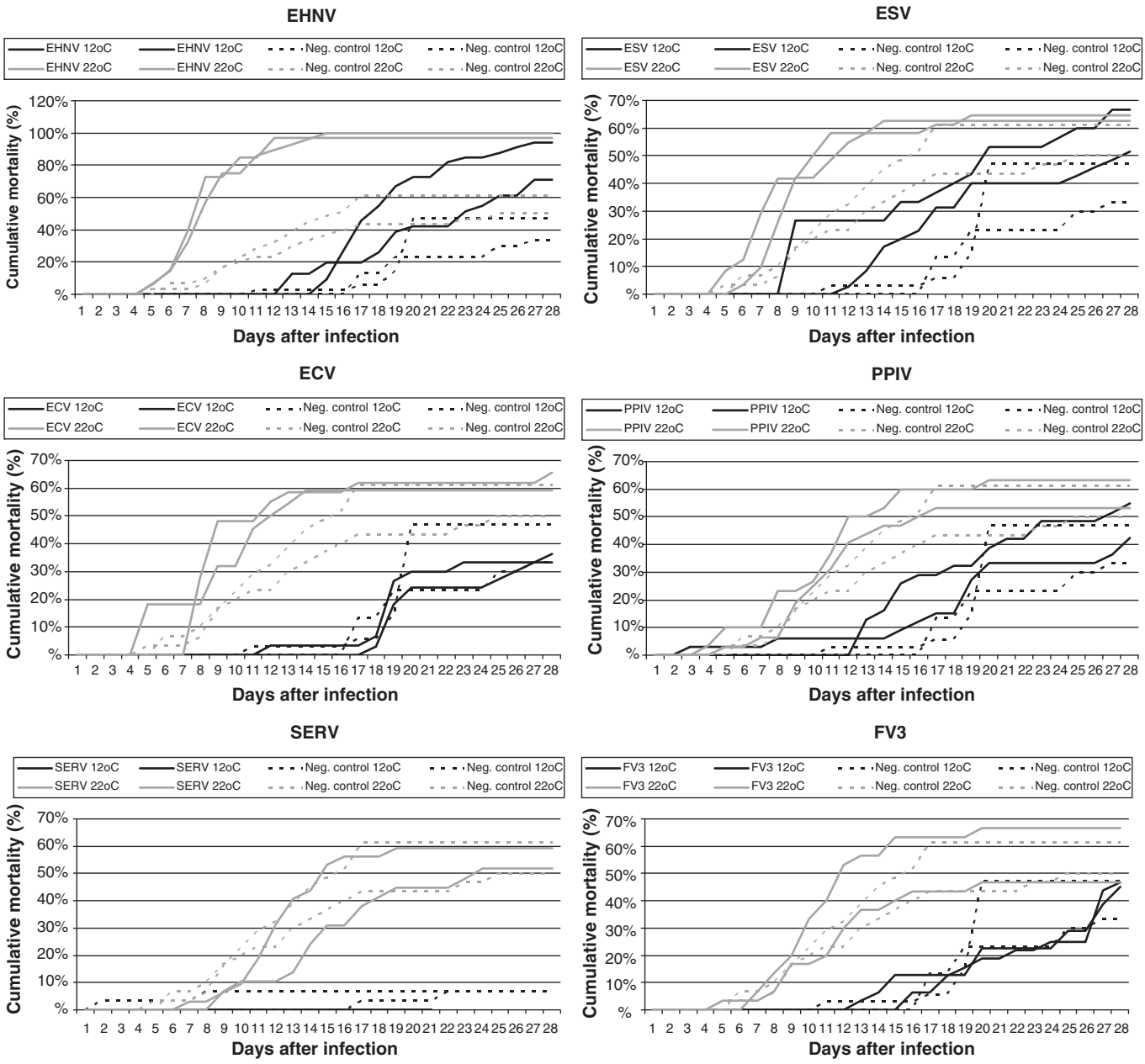

Fig. 1. Challenge I. Cumulative mortality in duplicate tanks for infection of pike-perch with 6 *Ranavirus* isolates at 12 °C and 22 °C. Negative controls are shown in dashed lines for comparison. For virus abbreviations, see Table 2.

Virus was re-isolated from fish challenged with all six virus isolates, and sequencing analysis confirmed the isolates to be identical to those the fish were exposed to. Because of the high background mortality, it was only possible to detect statistically significant mortality in fish infected with EHNV. As virus was re-isolated in fish surviving until the end of the challenge, it is likely that pike-perch can act as carriers for ranaviruses under certain conditions.

The mortality curves of fish infected with ESV at 12 °C and 22 °C and ECV at 22 °C appeared to be different from the negative controls, and resembled those normally recorded for ranaviral-infection in fish (Bang Jensen et al., 2009; Gobbo et al., 2010). However, any difference in mortality induced by the viral isolates was not sufficiently different from the controls to be statistically significant, due to the medium–high mortality observed in the negative control fish, and the limited number of fish used in the study. The reason for the relatively high mortality in the negative control fish in challenge I is uncertain. There were no indications of any pathogens present, and bacterial and virological examinations were negative. Likewise, no lesions were observed by histology. All the fish used in the three challenges originated from the same source, but the fish used for challenge I had been reared intensively, prior to arrival at the research facility. Thus, these fish had reached an average weight of 1–2 g in 5 weeks, whereas those that were reared under more natural conditions took 15 weeks to reach the same weight. The stress associated with fast growth might have caused these fish to become less tolerant to the unfamiliar environment in the research facility, and thus caused mortality due to ill-thriving. This theory is supported by the fact that there was minimal mortality in the negative controls of challenges II and III which were carried out using the extensively reared fish. This was the first time we have worked with pike-perch, and it might well be that our experimental facility is not well suited for fry of this species. In a previous challenge with pike fry, we also experienced high mortality in the negative control fish, which we ascribed to sub-optimal conditions (Bang Jensen et al., 2009).

Table 3
Challenges I and II: cumulative mortality and virus isolation results for pike-perch challenged with *Ranavirus* isolates. p-values are based on analysis of variance of mortality between ranavirus isolates and negative controls. Bold values indicate $p<0.05$.

Group	Temperature	No. of challenged fish	Mortality (%)	p-value	Virus re-isolation	
					No. of samples	% Positive
Challenge I						
Neg. control	12 °C[a]	60	7		2	0
	12 °C	64	41		10	0
	22 °C	61	56		14	0
EHNV	12 °C	64	83	<**0.001**	14	100
	22 °C	61	98	<**0.001**	12	83
ESV	12 °C	65	58	0.065	14	100
	22 °C	55	64	0.414	14	57
ECV	12 °C	63	35	0.577	13	85
	22 °C	51	63	0.489	13	38
PPIV	12 °C	64	48	0.383	14	100
	22 °C	62	58	0.701	14	79
SERV	12 °C[a]	62	0	nd	2	0
	22 °C	61	56	0.992	14	14
FV3	12 °C	63	46	0.544	12	67
	22 °C	60	57	0.915	16	31
Challenge II						
Neg. control	12 °C	102	1	nd	1	0
	22 °C	114	1	nd	1	0
EHNV	12 °C	124	0	nd	2	50
	22 °C	112	1	nd	3	33
PPIV	12 °C	114	0	nd	2	0
	22 °C	109	0	nd	2	0

nd = not done (since mortality was ≤1%).
[a] This control group was used for infection with SERV at 12 °C.

Histopathological lesions were seen only in pike-perch challenged with EHNV, ECV and PPIV at 12 °C, but not in any fish challenged at 22 °C or in fish infected with ESV or FV3 at 12 °C or in any of the negative controls. No samples stained positive with the ranavirus-specific antibody. However, all the samples for histology were taken at the end of the trials, from fish that had survived the challenge. This might explain the lack of positive antibody-staining, and the overall lack of lesions. The lack of lesions in fish that were challenged at 22 °C and survived until the end of the trial corresponds with the lack of virus re-isolation in these fish, and indicates that these fish were either refractory to the virus or that they were able to clear themselves of infection.

It was not possible to induce any mortality in 15 week old fish, despite the increased density of fish during the trials, and virus isolation was only possible from EHNV-infected fish and not PPIV-infected. The effect of stocking density of fish on mortality and susceptibility to disease is not clear. Several studies have been made with rainbow trout, and in 10 of 23 studies, high stocking density had an adverse effect on mortality, whereas 2 of 7 studies reported increased levels of plasma cortisol (indicating increased stress) at high stocking density (Ellis et al., 2002). The studies carried out with stocking densities in pike-perch are similarly inconclusive, and no optimum level for stocking density has been found for this species (Molnár et al., 2004). The fish in challenge II were from the same batch as the ones used in the first challenge, but they had been reared extensively and were older. It is well-known that susceptibility of fish to virus-infection changes with the age of the animal. Thus, younger fish have a lesser developed immune response system, and are more susceptible to infection and disease (Press and Jørgensen, 1999). In our study, it seems like the older fish were able to clear themselves of infection with PPIV, since we could not re-isolate it from fish at the end of challenge II. In the third challenge, the fish were even older, but it was possible to infect them by i.p.-injection, and this method of administration induced mortality in 50–100% of fish challenged with EHNV, ESV and ECV-24. This is consistent with previous infection trials with rainbow trout in Australia and redfin perch in Denmark, which showed that it was possible to induce disease by i.p.-injection of EHNV but not by bath at the same temperature (Whittington and Reddacliff, 1995; Ariel and Bang Jensen, 2009). Thus, not only age, but also dose or administration route of virus determines the pathogenicity of ranaviruses.

Naïve fish cohabitated with the infected fish did not contract infection, and virus could not be isolated from them. Two studies on transmission of ranavirus have been published. Langdon (1989) placed 10 redfin perch in a tank which had previously housed EHNV-infected redfin perch, and observed 100% mortality. Furthermore, 4/4 mosquito fish (*Gambusia affinis*) placed in the same tank died; whereas no mortality was observed in 10 smelt (*Retropinna semoni*) subjected to the same treatment. In that study, those species that were susceptible to infection with EHNV by cohabitation were the same as those susceptible to infection by bath-challenge, and the mortalities were all seen within 4–10 days post-challenge. We have previously described that no mortality was seen in redfin perch cohabitated with identical fish that were i.p.-challenged with EHNV, and only 4 of a total of 100 rainbow trout that were subjected to the same treatment died (Ariel and Bang

Table 4
Challenge III: cumulative mortality and virus re-isolation results for pike-perch i.p.-challenged with ranavirus isolates and cohabitant fish.

Virus	Treatment	No. of challenged fish	Mortality (%)	Virus re-isolation	
				No. of samples	% positive
Neg. control	i.p. infected	10	0	2	0
	Cohabitants	10	10	3	0
EHNV	i.p. infected	10	100	7	100
	Cohabitants	10	0	2	0
ESV	i.p. infected	10	90	8	100
	Cohabitants	10	0	2	0
ECV-24	i.p. infected	8	50	5	100
	Cohabitants	10	0	2	0
PPIV	i.p. infected	10	0	2	100
	Cohabitants	10	0	2	0

Jensen, 2009). In that study, virus could be isolated from 2 rainbow trout subjected to cohabitation, but not from any redfin perch.

As pike-perch was shown to be susceptible to at least EHNV by bath-challenge, our transmission-study results do not concur with those described by Langdon (1989), but resembles the results of the redfin perch cohabitation challenge (Ariel and Bang Jensen, 2009). As discussed previously, it is possible that the older pike-perch are not as susceptible as the younger fish to infection by natural routes, supporting the theory that age may be of a great influence for pathogenicity of ranaviruses to pike-perch.

One of the aims of the study was to explore the susceptibility of pike-perch to PPIV. As described, PPIV did not induce mortality in the challenged fish differing from the mortality seen in the negative control group in challenges I and II, but there were 79 and 100% virus re-isolation in challenge I at 12 and 22 °C respectively, indicating that these fish were harbouring an active infection, that did not result in disease. The same was observed in the transmission challenge, where it was possible to re-isolate virus from i.p.-injected fish euthanized at the end of the challenge, even though no mortality was seen. The PPIV isolate was originally isolated from healthy, symptomless pike-perch fingerlings. Subsequent experimental challenges with rainbow trout fry did not cause any mortality, but the virus could be re-isolated up to 24 days post-challenge (Tapiovaara et al., 1998). Previously, we have induced significant mortality in pike-fry infected with PPIV at 12 °C, and re-isolated virus from fish challenged at 22 °C, even though no significant mortality was detected (Bang Jensen et al., 2009). As PPIV has only been isolated once, and not in connection with any mortalities and as it has not proven to be highly pathogenic, PPIV is probably not a serious threat to the European freshwater fish population.

At the time when EHNV was listed by the EU, there was very little knowledge on the pathogenicity of this virus to other species than redfin perch and rainbow trout, and the molecular relationship between EHNV and other ranaviruses had not been thoroughly examined. The study presented here and other recent studies have demonstrated that European stocks of pike, pike-perch and black bullhead are susceptible to EHNV (Bang Jensen et al., 2009; Gobbo et al., 2010), whereas European redfin perch and rainbow trout may not be as susceptible as first assumed (Ariel and Bang Jensen, 2009). Furthermore, it has been demonstrated that even though EHNV is genetically closely related to ECV and ESV (Hyatt et al., 2000; Marsh et al., 2002; Holopainen et al., 2009), there are differences in the pathogenicity of these viral isolates (Ariel and Bang Jensen, 2009; Bang Jensen et al., 2009; Gobbo et al., 2010). EHNV does not occur outside of Australia, whereas all the other virus isolates tested have been found within the European region, suggesting that they might pose a more imminent threat to the European fishes than EHNV.

We suggest that the new knowledge on ranavirus phylogeny and pathogenicity should be taken into account when evaluating which viruses should be listed, and which species should be surveyed.

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