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Susceptibility of European sheatfish *Silurus glanis* to a panel of ranaviruses

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Summary

The current study was undertaken in order to assess the risk that different ranaviruses might impose on European sheatfish aquaculture. As the *European sheatfish virus* (ESV) is a known pathogen causing losses in European sheatfish aquaculture, it was assumed that closely related exotic ranaviruses might also be able to infect European sheatfish and probably cause disease and mortality in this species. The differential susceptibility of European sheatfish (*Silurus glanis*) to various ranavirus isolates was assessed at two different temperatures (15°C and 25°C) in a recirculation system. Fish were infected experimentally with a panel of ranavirus isolates including ESV, *European catfish virus* (ECV), European catfish virus isolate 24 (ECV-24), *Epizootic haematopoietic necrosis virus* (EHNV), *Rana esculenta* virus isolate Italy 282/ I02 (REV), short-finned eel virus (SERV), *Bohle iridovirus* (BIV), guppy virus 6 (GV6), doctor fish virus (DFV) and *Frog virus 3* (FV3). Significant mortalities were observed, as expected, in fish infected with ESV at 15°C (100%) as well as at 25°C (86/83%). Fish infected with ECV at 15°C showed no clinical signs of disease (8% mortality), whereas those fish infected at 25°C exhibited a cumulative mortality of 54%. Fatal disease was also induced by Italian isolate ECV-24 at 25°C (81%). Virus isolates ESV, ECV and ECV-24, generally the most genetically closely related viruses, were successfully isolated from dead fish by cell culture with subsequent identification by polymerase chain reaction (PCR) and sequence analysis. However, no mortality or clinical signs of disease were observed in the groups of sheatfish infected with the other ranaviruses investigated in the study, and none of those viruses were re-isolated in cell culture or identified by PCR. It was concluded that European sheatfish are susceptible to infection with ESV, ECV and ECV-24 under laboratory conditions, but not to infection with EHNV, REV, SERV, BIV, GV6, DFV or FV3. For ESV, the incubation period was shorter at 25°C compared to 15°C water temperature, but whereas all fish died after ESV infection at 15°C, some fish survived the infection at 25°C. Futhermore, the very young sheatfish were susceptible to ECV and ECV-24 at 25°C, whereas there was no significant mortality in the group of older sheatfish challenged with ECV at 15°C. Therefore, the clinical characteristics of the disease seem to depend on the age of the fish as well as on the water temperature.

Introduction

Ranaviruses are agents belonging to the genus *Ranavirus* within the family *Iridoviridae* (Jancovich et al., 2011). The enveloped icosahedral virions show a diameter of approximately 160–200 nm (Williams et al., 2005). The genome consists of a single linear double stranded DNA molecule of approximately 105–140 kbp, which is highly methylated, circularly permuted and terminally redundant. The genome G+C content amounts to approximately 49–54% (Willis and Granoff, 1980; Goorha and Murti, 1982; Willis et al., 1984; Murti et al., 1985; He et al., 2002; Jancovich et al., 2003; Song et al., 2004; Tsai et al., 2005).

While the other genera of the family *Iridoviridae* (*Iridovirus, Chloriridovirus, Lymphocystivirus* and *Megalocytivirus*) are known to be adapted to a rather small and defined host range of mainly insects, crustaceans, marine fish and molluscs, respectively, ranaviruses are able to infect several fish, amphibian and reptilian species thereby causing systemic, partially fatal diseases (Chinchar et al., 2009; Jancovich et al., 2010, 2011; Whittington et al., 2010). Systemic infections, necrosis of kidney and spleen as well as diffuse subcutaneous and internal haemorrhages have been observed in infected species (Chinchar, 2002; Williams et al., 2005). Ranaviruses have been isolated as virulent agents from fish, amphibian and reptilian species in many countries worldwide (Hyatt et al., 2002; Duffus et al., 2008; Johnson et al., 2008; Chinchar et al., 2009; Gray et al., 2009; Mazzoni et al., 2009; Hoverman et al., 2010; Torrence et al., 2010; Whittington et al., 2010; Xu et al., 2010).

The first isolated and well-characterized ranavirus was *Frog virus 3* (FV3), and is the type species of the genus *Ranavirus*. It was originally isolated from tumor tissues of leopard frogs (*Rana pipiens*) in North America (Rafferty, 1965; Granoff et al., 1966). Another amphibian host isolate is *Bohle iridovirus* (BIV), which was isolated from burrowing frogs (*Limnodynastes ornatus*) in Australia (Speare and Smith, 1992) during the investigation of occurring unexplained mortalities. *Rana esculenta* virus (REV) is the third amphibian host isolate that was included in the infection trial virus panel. It was isolated from diseased wild green frog tadpoles (*Rana esculenta*, G. Bovo, pers. comm.) in Italy. Other than the viruses with amphibian hosts, EHNV is of particular importance, and was added to the list of notifiable exotic diseases by the World Organisation for Animal Health (Office International des Épizooties, OIE) after causing

severe infections in redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) in Australia (Langdon et al., 1986, 1988; Langdon and Humphrey, 1987).

To date, EHNV has remained exotic to Europe, and knowledge about possible impacts on European aquaculture species is scarce (Jensen et al., 2009; Gobbo et al., 2010). ESV, ECV, and its variant ECV-24 represent European piscine ranavirus isolates. ESV was the cause of mass mortalities in European sheatfish (*Silurus glanis*) in warm water facilities in Germany (Ahne et al., 1989), whereas ECV was isolated from American catfish (black bullhead, *Ameiurus melas*) in outbreaks associated with high mortalities in France and Italy (Pozet et al., 1992; Bovo et al., 1993). Short-finned eel virus (SERV) is a ranavirus that was isolated incidentally from short-finned eels (*Anguilla australis*) imported into Italy (Bovo et al., 1999).

The two Southeast Asian ranavirus isolates, doctor fish virus (DFV) and guppy virus 6 (GV6), also referred to as ornamental ranaviruses, infect the marine doctor fish (*Labroides dimidatus)*, and the freshwater guppy (*Poecilia reticulate*), respectively (Hedrick and McDowell, 1995).

For identification and characterization of ranaviruses, the major capsid protein (MCP) gene is of major importance. Polymerase chain reaction (PCR) amplifying the MCP gene has been accepted as a reliable diagnostic tool, and is also a suitable approach for the differentiation between various ranaviruses (Hyatt et al., 2000; Marsh et al., 2002; Holopainen et al., 2009; Ohlemeyer et al., 2011).

The current study was undertaken in order to assess the risk that different ranaviruses might impose on European sheatfish aquaculture. The experiments were carried out within the framework of the RANA project (Risk Assessment of New and Emerging Systemic Iridoviral Diseases for European Fish and Aquatic Ecosystems) funded by the European Commission within the 6th Framework Programme 2002–2006 (SSPE-CT-2005-006459). European sheatfish were investigated along with other species such as rainbow trout, pikeperch, perch, and black bullhead (Ariel and Jensen, 2009; Jensen et al., 2009, 2011; Gobbo et al., 2010) because of their economical and ecological significance not only to European aquaculture but also to wild fish populations. It was hypothesized that because it is well established that sheatfish are susceptible to ranaviruses (ESV), it might be possible that the closely-related as well as the more distantly-related ranavirus isolates such as those infecting amphibian hosts could also infect sheatfish. Furthermore, clarification as to whether sheatfish can act as carriers of different ranavirus isolates is also needed.

Materials and methods

European sheatfish (*Silurus glanis*)

European sheatfish (*Silurus glanis*) obtained from different sheatfish or poly-culture fish farms were tested for presence or absence of ESV and other ranaviruses by PCR and cell culture (CCLV Rie88; CCLV Rie173). Experiments were performed using virus-free indoor farm sheatfish, routinely kept at 23–24°C, obtained from a commercial German breeder. After adaptation to the aquarium facility conditions, the fish were tested for virus (ranavirus, VHSV, IHNV, KHV) and bacteria (*Aeromonas salomonicida, Yersinia ruckeri*) absence, again by PCR or reverse transcriptase PCR (RT-PCR), respectively.

Infection experiments

Fish were adapted to the respective water temperature (15°C/25°C ± 1°C) and fed commercial sheatfish food obtained from the breeder (45% protein and 12% total lipid content, energy content 19.9 MJ kg^{-1}, feeding rate 2%/ day). For the virus infection, sheatfish were divided into groups and challenged by immersion with a virus concentration of 10^4 $TCID_{50}$ per ml for 1 h at water temperatures of 15°C or 25°C, respectively. Due to limited aquaria facilities, experiments were carried out in three periods. For each experiment, negative control groups were kept at the same temperatures and handled according to the infected groups. Aquaria (400-L tank size, up to 70 fish of 5–25 g body weight per tank; 300 L h^{-1} pump rate, 6–8 mg L^{-1} oxygen concentration, pH between 7.8 and 8.2) were inspected twice a day over the duration of the experiment. Dead fish were documented, sampled and frozen at -20°C for further processing. All remaining fish were euthanized by a benzocaine overdose at the end of the experiment and frozen at −20°C for further investigation by PCR and virus re-isolation.

Experiment (1). European sheatfish with weights of ca. 10 g in duplicate groups of ca. 35 (n = 70) individuals were infected with EHNV, ESV, ECV, SERV, REV, DFV and GV6, respectively, at a water temperature of 15°C. After immersion with a virus titre of 10^4 $TCID_{50}$ per ml for 1 h, fish were kept in tanks with a re-circulation water system at 15°C. The experiment was completed at 28 days post-infection (dpi).

Experiment (2a). European sheatfish of the same batch as in experiment 1 were challenged at 25°C water temperature. Groups of 35 sheatfish per tank were immersed with ESV, SERV, REV, DFV and GV6 as described above and kept at 25°C for another 28 days.

Experiment (2b). A new batch of very young European sheatfish was ordered. After an adaptation period, the circa 0.5 g fish were divided into groups of 40 individuals per tank for infection with ESV, ECV, ECV-24, BIV, EHNV and FV3 at 25°C water temperature. The experiment was terminated at 12 dpi because of severe cannibalism.

Cells and virus isolates

Viruses included in the experiments were EHVN, ESV, ECV, FV3, BIV, REV, SERV, GV6 and DFV. Origin and references are described in Holopainen et al., 2009 (Table 1). Identification of all ranavirus isolates was confirmed by PCR and sequence analysis prior to their use in infection experiments. ECV-24 was isolated from American catfish (*Ameiurus*

Table 1
Oligonucleotides used for PCR amplification and sequencing of ranavirus major capsid protein (MCP) gene fragments

PCR product	Oligonucleotide designation	Nucleotide sequence (5′–3′)	Oligonucleotide position	Product size (bp)
MCP1A	MCP1[a]	CAGCGTGTATCTTATAATAAAAAGAAATG	97318–97347[c]	591
	MCP2Arev	GAAGAAGAATGGGAGGGGAAGAAC	97908–97885[c]	
MCP2A	MCP3Afor	CCTCTCATTCAACGACATCAGC	97698–97719[c]	750
	MCP4Arev	ACCAGCGAGTAGTACTCGACTC	98447–98426[c]	
MCP3	MCP5[a]	CGCAGTCAAGGCCTTGATGT	98244–98263[c]	585
	MCP6R[a]	AAAGACCCGTTTTGCAGCAAAC	98828–98807[c]	
Rana MCP	RanaMCP-F[b]	CCAGTCCACATGGTCAACCC	98182–98201[c]	516
	RanaMCP-R[b]	GATAATGTTGTGGTTGATGGCC	98697–98676[c]	

[a]Oligonucleotides published by Hyatt et al. (2000).
[b]Oligonucleotides published by Ohlemeyer et al. (2011).
[c]Oligonucleotide position relative to FV3 genome, AY548484.

melas) in Italy (Bovo et al., 1993) and kindly provided by G. Bovo (Instituto Zooprofilattico delle Venezie, Italy).

All viruses were propagated onto bluegill fry-2 cell line CCLV Rie88 or on epithelioma papulosum cyprini cell line CCLV Rie173 in 75 cm^2 cell culture flasks (Corning, NY, USA). Cultivation was carried out at 20°C in 2.5% CO_2 atmosphere, using MEM Glasgow BHK 21 serum based on Earle's balanced salt solution, containing 10% foetal calf serum. Cell culture supernatant from infected cells was harvested when a complete cytopathic effect (CPE) was observed. Cells were frozen at −20°C and thawed twice. Cell debris was removed by centrifugation at 1100 ***g*** for 15 min and 200 μl of the supernatant were used for titration and DNA extraction, respectively. Titration was carried out on cell line CCLV Rie88 in 96 well plates, incubated at 20°C and evaluated at 10 dpi.

Virus re-isolation

For re-isolation of the viruses from sheatfish organ material, the procedure was: Dissected pieces of internal organ tissue or of the complete fish were used for examination, depending on the weight of the individual fish. European sheatfish in experiments 1 and 2a weighed ca. 10 g. Portions of the heart, liver, spleen and kidney were dissected and homogenized. Fish in experiment 2b weighed ca. 0.5 g; thus, the complete animal was homogenized for further examination. In trials where no clinical signs of disease or mortality were observed, samples of five fish each were pooled. Dead fish were processed individually.

Approximately 300 mg of homogenate was diluted in 1 ml cell culture medium containing 0.01% enrofloxacin (Baytril, Bayer) and mixed thoroughly. After incubation at 4°C overnight, samples were centrifuged at 800 ***g*** for 5 min. Clarified supernatant was delivered directly or at a final dilution of 1:10 onto the cell line Rie88. Incubation was performed in a culture medium containing penicillin/streptomycin (PAA Laboratories Pasching) at a final concentration of 100 U ml^{-1} (penicillin) and 0.1 mg ml^{-1} (streptomycin) at 20°C. Four hours after adsorption, the inoculum was replaced by a fresh cell culture medium containing PAA. Cell culture was observed daily and occurrence of CPE was recorded. After incubation at 20°C for 10 days, cell culture supernatant was used for a further passage in BF2 cells under conditions as described above.

DNA extraction

DNA extraction from 200 μl of purified cell culture supernatant was performed with the QIAamp DNA Mini Kit according to the instruction manual (protocol A for viral DNA; Qiagen, Hilden, Germany). For DNA extraction from tissue homogenates, 25 mg of the homogenate was processed with QIAamp DNA Mini Kit according to the instruction manual (tissue protocol; Qiagen). DNA was stored at −20°C. Each DNA extraction procedure was conducted by one negative extraction control (PBS) and the positive extraction was secured by application of a specific sheatfish ß-actin gene PCR.

PCR amplification

The major capsid protein (MCP) gene was used for identification of ranavirus DNA by PCR amplification. Primers MCP 3Afor and MCP 4Arev (Table 1) were used for viral genome detection in tissues and cell cultures after infection with ESV, ECV, ECV-24, FV3, REV, SERV, BIV and EHNV. Additionally, the primer pairs MCP 1/ MCP 2A rev and MCP 5/ MCP 6R (Table 1) were used in terms of positive results in the first PCR. Thus, the combination of the three overlapping major capsid protein gene PCR products (MCP1A, 2A and 3, Table 1) enabled the analysis of the complete MCP nucleotide sequence. In addition, the Rana MCP PCR (Table 1) was used for detection of GV6 and DFV (Ohlemeyer et al., 2011). Oligonucleotides were manufactured by BioTez (Berlin-Buch). DNA from at least five European sheatfish, pooled in one sample per virus isolate and experiment, were examined by PCR after DNA extraction. In cases of occurring mortality, fish were examined individually.

PCR was carried out using the HotStarTaq Master Mix kit according to the instruction manual (Qiagen). Each 25 μl PCR reaction mix contained 12.5 μl HotStar Taq Master Mix, 0.5 μl of each oligonucleotide (10 pmol μl^{-1}), 9.5 μl water and 2 μl DNA template.

Thermal cycler conditions were: after an initial activation step of 95°C for 15 min, the denaturation, annealing, and

extension conditions were 93°C (1 min), 55°C (2 min), 72°C (0.5 min), respectively (40 cycles), followed by a final incubation at 72°C (3 min). Each PCR reaction was complemented with a negative control (water) and a positive control (ESV). As a positive control for successful DNA extraction from organ tissue, sheatfish beta actin gene was determined (data not shown) and used for amplification in a specific PCR with primers ß-actin2 for (5′ACCTCACAGACTACCTCATG) and ß-actin3rev (5′TAGAAGCATTTGCGGTGGAC). Only PCR reactions with positive sheatfish ß-actin PCR outcome (765 bp) were evaluated.

Gel electrophoresis, extraction and cloning of PCR products

Gel electrophoresis was conducted with 1% agarose gels stained with ethidium bromide (2 μl per 70 ml). The 10 μl PCR product mixed with 2 μl DNA loading buffer was applied to the gel. Electrophoresis was carried out at 80 V for 40 min. PCR products were visualised under UV irradiation and respective products were cut out and eluted with QIAquick Gel Extraction kit (Qiagen) according to the manual instructions. 1.5 μl of the respective eluate were ligated into 0.5 μl pGEM T easy vector system (Promega Corporation**,** Madison, WI, USA), adding 2.5 μl according ligation buffer and 0.5 μl T4 DNA ligase (Fermentas). After overnight incubation at 8°C, the plasmid was transformed into bacteria (*E. coli* DH10B) and the transformation mix was plated on LB agar plates. After overnight incubation at 37°C, at least 12 single bacteria colonies were processed for DNA mini preparation. DNA was stored at −20°C.

Sequence analysis

In the case of a positive PCR result, at least three samples per experiment and virus were sequenced and analysed. Strong PCR products were sequenced directly after elution. Weak PCR products were sequenced after cloning in vector system pGEM T easy, transformation of *E. coli* and DNA mini preparation. At least three independently derived clones per PCR product were sequenced for confirmation. Sequencing reaction was carried out as a cycle sequencing using the Big Dye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems) according to the instruction manual. In total, the sequence reaction mix contained 10 μl, consisting of 2 μl Big Dye Ready Reaction Mix, 1 μl 5x sequencing buffer, 1 μl oligonucleotide (3 pmol μl^{-1}), 4 μl sterile water and 2 μl of the eluate. Thermal cycler conditions (Eppendorf Mastercycler gradient) were: An initial activation step at 96°C (1 min) was followed by 30 cycles of denaturation at 96°C (0.5 min), annealing at 65°C (0.25 min) and elongation at 72°C (1.5 min for direct sequencing of the PCR product, 3 min for sequencing of mini DNA). Subsequently, PCR products were purified for sequence analysis (Sigma Spin Post-Reaction Purification Columns, Sigma) according to the instruction manual. After denaturation with Hi-Di™ Formamide (Applied Biosystems), sequencing was carried out with the 3130 Genetic Analyzer (ABI, Applied Biosystems). The complete MCP gene sequence was reassembled from sequence fragments MCP1A, MCP2A and MCP3. In terms of DFV and GV6, the Rana MCP PCR fragment (Ohlemeyer et al., 2011) was analysed. Sequence analysis was performed with Sequence Scanner Software v1.0 (ABI, Applied Biosystems), GCG-X-Win32, version 11.1.3. UNIX (Accelrys). 'NCBI blast 2 sequences' was used for analysis of nucleotide sequence pair percent identity; 'NCBI blastn' was used for comparison with published sequence data.

Results

Cumulative mortality

Experiment (1) 15°C: EHNV, ESV, ECV, SERV, REV, DFV and GV6. While no mortality occurred in the control group and in the group of European sheatfish infected with SERV at 15°C water temperature, mortality of varying degrees was observed in the groups of sheatfish challenged with ESV, EHNV, ECV, REV, DFV and GV6. Mortality started at 4 dpi and stopped at 20 dpi, depending on the virus (Fig. 1). Total cumulative mortality culminated at 16% for EHNV, 8% for ECV, 10% for REV, 9% for DFV, and 11% for GV6. However, no obvious clinical signs of disease were observed in these groups, and no change in the constitution or behaviour of the sheatfish was detected. In contrast, ESV induced severe mortality starting at 16 dpi. All fish died within 10 days after the mortality began (Fig. 1). Dead sheatfish showed diffuse subcutaneous haemorrhages in the region of the lower jaw as well as in the fins.

Experiment (2a) 25°C: ESV, SERV, REV, DFV and GV6. At 25°C water temperature, no sheatfish died in the control group or in the group infected with GV6. Mortality occurred in the groups infected with SERV (3%), REV (11%) and DFV (14%). No clinical signs of disease were detected.

However, 86% of the European sheatfish infected with ESV died (Fig. 2). The highest mortality occurred between 4 and 6 dpi. In fact, 67% of the sheatfish died at 5 dpi (Table 2). Similar subcutaneous bleedings as described in experiment 1 were observed.

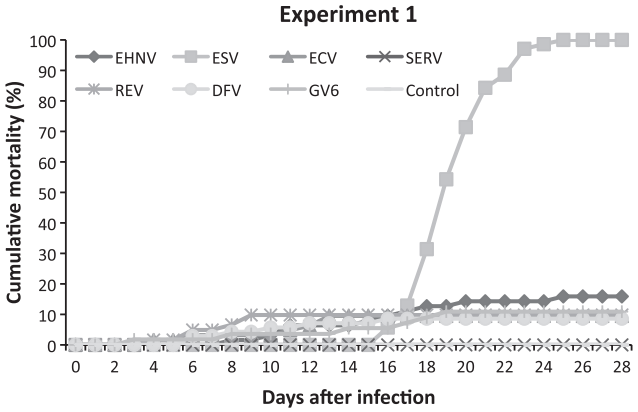

Fig. 1. Cumulative mortality in experiment 1, European sheatfish (*Silurus glanis*), from 0 to 28 dpi with EHNV, ESV, ECV, SERV, REV, DFV, and GV6 at 15°C water temperature, including a control group treated with culture medium

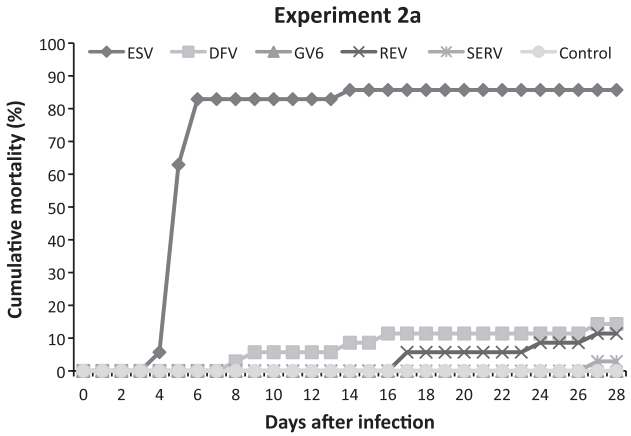

Fig. 2. Cumulative mortality in exp. 2a, European sheatfish from 0 to 28 dpi with ESV, DFV, GV6, REV, and SERV at 25°C water temperature, including a control group treated with culture medium

Experiment (2b) 25°C: ESV, ECV, ECV-24, BIV, EHNV and FV3. The numbers of European sheatfish had already decreased in some of the tanks due to cannibalism prior to infection with ESV, ECV, ECV-24, BIV, EHNV or FV3 at 25°C. Therefore, the numbers of individuals per tank were not consistent. During the experiment no dead fish were observed in the control group or in the group infected with EHNV. There was only one dead fish in the group infected with BIV (3%) and two dead fish in the FV3 group (7%). No clinical signs of disease were observed in these groups.

High mortality was observed in all groups infected with ESV, ECV and ECV-24 (Fig. 3). The cumulative mortality of 81% in the ECV-24 infected group occurred between 5 and 10 dpi, with a peak at 5 and 6 dpi; in fact, 80% of mortality after infection with ECV-24 occurred within these 2 days. The group infected with ECV showed a mortality of 55% between 3 and 6 dpi. In this group, the main mortality (89%) was observed between 5 and 6 dpi, similar to the group infected with ECV-24. The cumulative mortality in the group infected with ESV culminated in 83% between 7 and 9 dpi. European sheatfish suffering from infections with ESV, ECV and ECV-24 showed coordination problems such as tumbling and apathy as well as subcutaneous haemorrhages.

Unfortunately, the experiment had to be terminated at 12 dpi because of severe losses due to cannibalism (Fig. 4). Although no obvious mortality was observed in some groups, the actual number of surviving fish decreased rapidly, and single sheatfish showed an intensive weight gain. Regarding the total mortality rates including losses due to cannibalism, no significant mortality could be determined, since a reduction of between 60% and 95% occurred in all groups, including the negative controls (Fig. 4, Table 2).

PCR analyses

Each described tissue sample was tested by PCR MCP 2A first (Table 1). In the case of a positive MCP2A result, complementary PCR using MCP 1A and MCP 3 were undertaken, which gave positive results (Table 1). DFV and GV6 challenge samples were tested by PCR Rana MCP (Ohlemeyer et al., 2011). In the case of a positive MCP PCR, at least three samples/clones per experiment and virus were sequenced for virus identification and sequence analysis, respectively. Therefore,

Table 2
Mortality and virus re-isolation results

				Mortality		Positive PCR/ total no. fish tested		Virus re-isolation/ total no. fish tested	
Trial	Temperature	Weight of fish (g)	Challenge	No. dead fish/ total in tanks	Percent mortality	Dead fish	Surviving fish	Dead fish	Surviving fish
1	15°C	10	EHNV	10/63	16	0/6	0/10	0/6	0/10
			ESV	70/70	100	20/20	0/0	**20/20**	0/0
			ECV	5/63	8	0/4	0/10	0/4	0/10
			SERV	0/70	0	0/0	0/10	0/0	0/10
			REV	6/61	10	0/5	0/10	0/5	0/10
			DFV	6/70	9	0/5	0/10	0/5	0/10
			GV6	6/55	11	0/5	0/10	0/5	0/10
			Control	0/70	0	0/0	0/10	0/0	0/10
2a	25°C	10	ESV	30/35	86	10/10	0/4	**10/10**	0/4
			SERV	1/35	3	0/1	0/5	0/1	0/5
			REV	4/35	11	0/4	0/5	0/4	0/5
			DFV	5/35	14	0/5	0/5	0/5	0/5
			GV6	0/35	0	0/0	0/5	0/0	0/5
			Control	0/35	0	0/0	0/5	0/0	0/5
2b	25°C	0.5	EHNV	0/32 (27/32*)	0 (84*)	0/0	0/5	0/0	0/5
			ESV	25/30 (3/30*)	83 (10*)	23/23	2/2	**22/23**	**1/2**
			ECV	18/33 (2/33*)	55 (6*)	16/16	10/13	**15/16**	**5/13**
			ECV-24	30/37 (5/37*)	81 (14*)	28/28	2/2	**28/28**	0/2
			BIV	1/30 (26/ 30*)	3 (87*)	0/1	0/3	0/1	0/3
			FV3	2/30 (16/30*)	7 (53*)	0/2	0/12	0/2	0/12
			Control	0/35 (25/35*)	0 (71*)	0/0	0/10	0/0	0/10

*Reduction of fish number due to cannibalism.

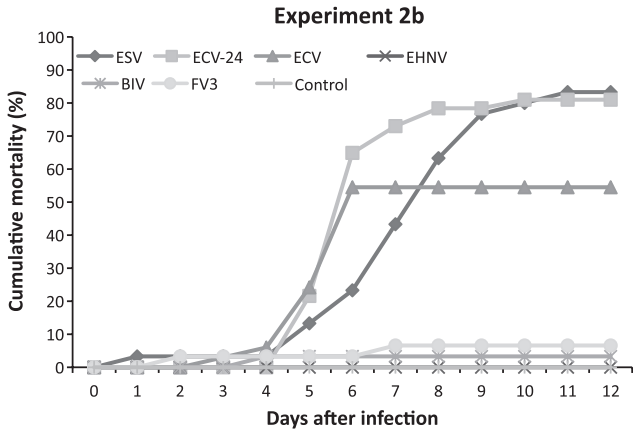

Fig. 3. Cumulative mortality in exp. 2b, European sheatfish from 0 to 12 dpi with ESV, ECV-24, ECV, EHNV, BIV, and FV3 at 25°C water temperature, including a control group treated with culture medium

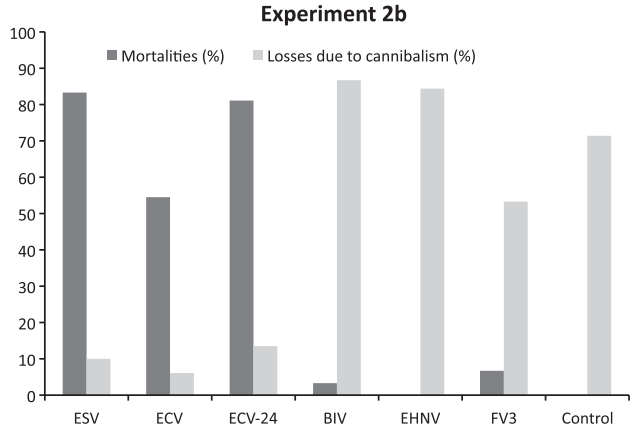

Fig. 4. Cumulative mortality and losses due to cannibalism in exp. 2b in European sheatfish 12 dpi with ESV, ECV-24, ECV, EHNV, BIV, and FV3 at 25°C water temperature, including a control group treated with culture medium compared to counted number of losses (in %) due to cannibalism in respective tanks

the complete MCP gene sequence (1392 bp) was analyzed. No nucleotide exchanges in the MCP gene sequence were detected for ESV, ECV and ECV-24. All PCR results are summarized in Table 2.

In experiment 1, all mortalities were investigated individually by PCR. Regarding tissue samples from dead European sheatfish challenged with EHNV (6), ECV (4), REV (5), DFV (5), and GV6 (5), no ranavirus DNA was detected by PCR. From European sheatfish infected with ESV, 20 of the 70 dead fish were tested by PCR. All fish tested positive for the ranavirus MCP gene. In addition, ten of the surviving European sheatfish from the control group as well as from the groups challenged with ECV, EHNV, SERV, REV, DFV and GV6 were tested by PCR in pools of five fish per sample, all with negative results.

All tested samples of fish dying after being infected with SERV (1), REV (4) or DFV (5) in experiment 2a were negative by PCR. However, ten of the fish that died from ESV also tested positive to PCR. Tissue material of five surviving fish from each group infected with SERV, REV, DFV and GV6 as well as from the control group were pooled, respectively, and tested PCR negative. Four surviving fish that had been exposed to ESV were also negative by PCR.

From experiment 2b, one dead fish from the group exposed to BIV and two from the group exposed to FV3, were tested individually. No ranavirus genome was detected by PCR. Mortality occurring in the groups infected with ESV, ECV and ECV-24, continuously resulted in positive PCR outcomes. All 23 samples of ESV infected fish, all 16 samples of ECV and all 28 samples of ECV-24 infected fish gave positive PCR signals. In this experiment, surviving fish were also tested individually. All surviving fish exposed to EHNV (5), BIV (3), or FV3 (12) resulted in a negative PCR as well as those remaining European sheatfish (10) of the control group. Most samples from surviving European sheatfish exposed to ESV (2/2), ECV-24 (2/2) or ECV (10/13), gave positive signals by PCR.

Virus re-isolation

The cell culture virus re-isolation results are summarized in Table 2. All successfully re-isolated viruses were subsequently identified and reconfirmed by PCR and sequencing of the complete MCP gene (1392 bp). At least three clones per virus were sequenced for virus identification and MCP sequence analysis, respectively. No nucleotide exchanges in the MCP gene sequence were detected for ESV, ECV or ECV-24. Cell culture PCR results were in agreement with virus re-isolation results in all experiments.

According to PCR results obtained from organ tissues, virus re-isolation was successful for the 20 samples obtained from European sheatfish that had died after infection with ESV in experiment 1. CPE occurred in the first passage in cell culture approximately 3–4 days after inoculation. In the groups infected with the other ranaviruses, no pathogen was re-isolated in five cell culture passages.

In accordance with organ tissue PCR results, the virus was re-isolated in the first cell culture passage from all 10 examined fish that died after exposure to ESV in experiment 2a, whereas no virus was re-isolated in five cell culture passages from the four fish that survived the infection with ESV. In all other groups virus re-isolation was neither successful in dead (SERV 1, REV 4, DFV 5 samples) nor in surviving fish (five pooled samples per group).

In experiment 2b, virus re-isolation was negative from fish that died after infection with BIV (1) and FV3 (2). In addition, no virus was re-isolated, either from surviving European sheatfish exposed to EHNV (5), BIV (3), and FV3 (12) or from the control group (10) in two cell culture passages. However, virus re-isolation was successful in all sheatfish (28) that died after infection with ECV-24, although no virus was re-isolated from the two fish that survived. Regarding ESV, the virus was re-isolated from 22 of 23 mortality samples that had tested positive by PCR. The sample that tested negative in virus re-isolation originated from a fish that had died at 1 dpi. Virus was re-isolated from only one of the two surviving fish that had tested positive by organ tissue PCR.

Last but not least, virus re-isolation was positive in 15 of 16 PCR positive fish that died after infection with ECV. One fish that died at 3 dpi was negative by virus re-isolation; however, a faint band could be detected in the gel following PCR. Furthermore, from 10 PCR positive samples of the surviving ECV infected fish, virus re-isolation was positive in five samples (50%). No virus was re-isolated from the three ECV infected surviving fish from which organ tissues also proved to be negative by PCR.

Discussion

Three infection trials with European sheatfish were carried out in order to gain knowledge on their susceptibility to different ranaviruses. It can be stated that the susceptibility of European sheatfish to the ranaviruses EHNV, SERV, BIV, REV, FV3, as well as to GV6 and DFV is negligible, at least under the described conditions at 15 and 25°C water temperatures and for European sheatfish of the age and constitution tested in this experiment. It is possible that under natural conditions with unfavourable water conditions or under stress, as well as in combination with other fish pathogens, there may still be an effect on the health of European sheatfish. However, none of these viruses was re-isolated in cell culture from organ tissues of dead or surviving fish (Table 2). None of the infected fish showed clinical signs of disease. In addition, it was not possible to detect the ranavirus genome directly from organ tissue samples by PCR. Therefore, it can be assumed that the tested viruses do not replicate efficiently in this fish species.

The only ranavirus causing significant mortality at 15°C water temperature was ESV (100% mortality). This was expected, since ESV was originally isolated from European sheatfish where it had been causing severe mortality for years (Ahne et al., 1989). However, at this temperature, ECV did not induce a significant mortality rate (8%) and none of the ECV samples tested positive by virus re-isolation. Unfortunately, ECV-24 was not available until after the initiation of experiment 1 and thus could not be investigated at 15°C.

Regarding experiment 2a at 25°C, ESV was again the only virus which led to significant mortality (86%). However, in this experiment a small number of European sheatfish survived the infection with ESV, and interestingly, no virus was re-isolated from these fish but were re-isolated from all mortalities induced by ESV (Table 2). A possible explanation is that these surviving fish were not susceptible to infection and were able to effectively clear the virus.

Losses of individuals due to severe cannibalism in experiment 2b made interpretation of the mortality results problematic. For this reason it could be argued that experiment 2b should not be taken into consideration. However, severe mortalities as a result of virus infection were observed in the groups challenged with ESV, ECV, and ECV-24, with typical clinical symptoms and positive results from virus re-isolation. In the remaining groups mortality was very low with no clinical symptoms and none of the above-mentioned ranaviruses could be re-isolated or identified by PCR (Table 2).

In experiment 2b, younger fish were infected at 25°C water temperature; the visible mortality induced by ESV was comparable (83%) to that observed in experiment 2a. Unfortunately, this experiment had to be terminated on 12 dpi because of severe losses due to cannibalism. Those losses were not calculated as specific virus-induced mortality. However, two fish infected with ESV survived until 12 dpi, and although the virus was re-isolated from only one of these fish in cell culture, both samples were positive by organ tissue PCR. Interestingly, the one fish that died on the first dpi in the group infected with ESV was positive for ranavirus DNA by PCR, but virus re-isolation was not successful (Table 2). This can be explained by the fact that at such an early stage of death, ESV would not have replicated to an amount exceeding the cell culture detection limit of 10^2 to 10^3 $TCID_{50}$ per ml. Even if the mortality results from experiment 2b are regarded as ineligible, the results of the virus re-isolation still lead to the conclusion that ESV, ECV, as well as ECV-24 are able to replicate in the species, since they were re-isolated in cell culture from organ material of nearly all mortalities (ESV 22/23, ECV 15/16, ECV-24 28/28), but none of the other viruses was isolated from the challenged fish in experiment 2b.

The incubation period of ESV was much longer at 15°C than at 25°C water temperature. At 15°C mortality was first observed at 16 dpi and lasted until 26 dpi. Contrary to this, at 25°C mortality was observed much earlier, from 4 to 6 dpi. However, after this short period of severe mortality, only one additional fish died at 14 dpi.

It is likely that ESV replicates less efficiently at 15°C water temperature, but infection is nevertheless lethal for the majority of infected European sheatfish. In experiment 2a and 2b a rapid occurrence of mortality at 25°C was observed, but the cumulative mortality did not reach 100% as was observed at 15°C water temperature. With regard to the temperature-dependence of the fish immune system, it is very likely that the water temperature is responsible for the higher survival rate at 25°C compared to 15°C. The normal rearing temperature for European sheatfish is stated as 23–24°C, so that the decrease of temperature to 15°C induces stress in the fish, resulting in depression of the phagocytic leukocyte activity and immunoglobulin M plasma levels, which may be causative for the 100% mortality in experiment 1 (Chen et al., 2002; Workenhe et al., 2010). However, the temperature did not affect the characteristics of the clinical signs of disease.

Another interesting point is that experiment 2b ECV and its variant ECV-24 also caused severe mortality at 25°C in contrast to experiment 1 at 15°C. The cumulative mortality due to ECV-24 is comparable to the one induced by ESV in this experiment (81%) (Fig. 3). ECV caused a total mortality of 55%, which was confirmed by virus re-isolation. While positive PCR results were detected from 10 of the 13 surviving fish from this group, virus re-isolation was only successful in five of these 10 samples. In accordance with this, the 28 investigated samples from ECV-24 infected dead fish also gave positive PCR signals as well as virus re-isolation results, whereas the two surviving European sheatfish were only positive by PCR. Since experiment 2b only lasted until 12 dpi, it has to be assumed that further virus-induced mortalities might have occurred.

These results highlight the limitations of PCR, which despite being a very sensitive method for detection of viral

genome, does not allow conclusions concerning the presence of replicating ranavirus particles. Virus concentrations in some surviving fish might have been below the detection limit of the cell culture re-isolation method, resulting in negative results.

ESV and ECV are discussed in the context of strains belonging to the same ranavirus because they are closely related genetically. Differentiation between ESV, ECV and ECV-24 by MCP gene PCR is not possible because of the 100% identical MCP gene sequences (Holopainen et al., 2009; Ohlemeyer et al., 2011). The results presented in the current study lead to the assumption that the French/ Italian ranavirus isolates ECV and ECV-24 could be temperature-dependent variants of ESV, since at 15°C the ECV had no significant effect on the fish (Fig. 1), whereas in experiment 2b a high mortality was observed and the virus was re-isolated (Fig. 3, Table 2).

The influence of the age or size of the fish is clearly shown: younger fish at 25°C water temperature are more susceptible to ranavirus ECV than older individuals at 15°C water temperature. Unfortunately, it is not possible to determine the main factor responsible for the higher susceptibility, since only the young sheatfish were challenged with ECV and ECV-24 at 25°C water temperature (Table 2). Therefore, both a high water temperature and young age of sheatfish are possibly influencing factors.

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