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Susceptibility of the European common frog *Rana temporaria* to a panel of ranavirus isolates from fish and amphibian hosts

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ABSTRACT: Ranaviruses are an emerging group of viruses and have been implicated in an increase of epidemics in susceptible species. They have a wide host range, infecting fish, amphibians and reptiles, with some isolates able to infect multiple species from different animal classes. Whilst some information exists on the pathogenicity of ranaviruses to novel hosts, there is none on the pathogenicity of fish ranaviruses to amphibians; this information is needed to develop measures to prevent the further spread of ranaviral disease in the aquatic environment. We undertook bath infection trials to assess the susceptibility of the European common frog *Rana temporaria* to 9 ranavirus isolates comprising doctor fish virus (DFV), European sheatfish virus (ESV), epizootic haematopoietic necrosis virus (EHNV), guppy virus 6 (GV6), pike-perch iridovirus (PPIV) and short-finned eel ranavirus (SERV) from fish hosts, and Bohle iridovirus (BIV), frog virus 3 (FV3) and *Rana esculenta* virus 282/I02 (REV) from amphibians. Animals were challenged as tadpoles at 15 and 20°C and as recent metamorphs at room temperature (20 ± 1°C) to investigate the effect of temperature and amphibian developmental stage on virus pathogenicity. Tadpoles were susceptible to FV3, PPIV and REV, but refractory to the other ranaviruses. Post-metamorphs were susceptible to FV3 and REV but refractory to BIV (the other ranaviruses were not tested). Significant mortality occurred in post-metamorphs and in tadpoles challenged at 20°C but was low in tadpoles challenged at 15°C. This study presents the first evidence of mortality in an amphibian species after challenge with ranavirus originally isolated from fish.

KEY WORDS: Ranavirus · Amphibian disease · Common frog · Frog virus 3 · Pike-perch iridovirus · Disease transmission · Metamorphosis · Temperature

INTRODUCTION

Ranaviruses are an emerging group of viral pathogens (Chinchar 2002) belonging to the *Ranavirus* genus in the family *Iridoviridae*, a group of large, icosahedral, double-stranded DNA viruses (Chinchar et al. 2005). They have a wide susceptible host range, infecting fish, amphibians and reptiles, and have been implicated in an increase of epidemics in these animals (Chinchar 2002).

There are currently 6 ranavirus species recognised by the International Committee on Taxonomy of Viruses (ICTV 2009), 3 of which infect fish (*Epizootic haematopoietic necrosis virus* [EHNV], *European catfish virus* [ECV] and *Santee-Cooper ranavirus* [SCRV]). EHNV has caused epidemics in wild redfin perch *Perca fluviatilis* in south-east Australia (Langdon et al. 1986), resulting in temporary population declines (Whittington et al. 2010), and has also been isolated from farmed rainbow trout *Oncorhynchus*

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mykiss (Whittington et al. 1994). ECV has caused disease outbreaks in Europe in black bullhead *Ameiurus melas* (Pozet et al. 1992, Bovo et al. 1993) and sheatfish *Silurus glanis* (Ahne et al. 1989), reducing wild stocks of black bullhead (Whittington et al. 2010). SCRV has caused epidemics in North America, affecting wild largemouth bass *Micropterus salmoides* (Plumb et al. 1996). This ranavirus also appears to be impacting on wild populations, with losses of larger and/or older fish in particular (Whittington et al. 2010). Two strains of SCRV, doctor fish virus (DFV) and guppy virus 6 (GV6) have also been isolated from ornamental fish imported into America from South East Asia (Hedrick & McDowell 1995). Ranavirus species which infect amphibians include *Ambystoma tigrinum virus* (ATV), *Bohle iridovirus* (BIV) and *Frog virus 3* (FV3). ATV has caused epidemic disease in tiger salamanders *Ambystoma tigrinum* in the USA (Jancovich et al. 1997) and Canada (Bollinger et al. 1999). BIV was isolated in Australia from a single outbreak in ornate burrowing frogs *Limnodynastes ornatus* (Speare & Smith 1992). FV3 was originally isolated from an asymptomatic leopard frog *Rana pipiens* in North America (Granoff et al. 1966). However, other strains of FV3 recognised by the ICTV have been implicated in epidemic disease incidents in amphibians in Europe, the Americas and Asia (Wolf et al. 1968, Cunningham et al. 1996, Zupanovic et al. 1998, Weng et al. 2002). In the UK, disease epidemics appear to be causing localised long-term declines in populations of the common frog *Rana temporaria* (Teacher et al. 2010).

The perceived threat of ranaviral disease to aquatic animals resulted in the listing of EHNV in fish and, later, infection with ranavirus in amphibians by the World Organisation for Animal Health (OIE) in the Aquatic Animal Code, requiring member countries to report any occurrences to the OIE and implement OIE standards to limit their spread.

Infection with ranavirus is generally characterised by systemic disease involving multiple internal organs such as liver, spleen, kidneys and gut, and typically results in mortality (Chinchar 2002). However, ranaviruses have also been isolated from apparently asymptomatic animals (Tapiovaara et al. 1998, Bovo et al. 1999) and can also cause low-level mortality (Whittington et al. 1994). It has been suggested that these animals could act as reservoirs of the virus (Gray et al. 2009).

In laboratory trials, ranavirus isolates from natural infections have been demonstrated to be able to infect novel host species (Langdon 1989, Schock et al. 2008, Haislip et al. 2011), including species from different animal classes (Moody & Owens 1994). Additionally, in the wild the same ranavirus was isolated from naturally infected sympatric fish and amphibians (Mao et al. 1999). This ability to cross species barriers increases the potential for ranavirus isolates to infect naïve and potentially highly susceptible species (Hyatt et al. 2002). The susceptibility of a number of fish species has been assessed against novel ranavirus isolates from fish and amphibian hosts (Moody & Owens 1994, Ariel & Bang Jensen 2009, Bang Jensen et al. 2009, Gobbo et al. 2010), as has the susceptibility of amphibians against isolates from amphibians (Cullen et al. 1995, Cullen & Owens 2002, Cunningham et al. 2007, Schock et al. 2008, 2009). Results from these studies have identified additional fish and amphibian species (other than the original host) to be susceptible to ranavirus infection. There have been no published studies to date on the susceptibility of amphibians to ranaviruses isolated from fish hosts. Compiling such data is important in order to develop control strategies to prevent potential further spread of ranaviral diseases (Whittington et al. 2010). Therefore, the aim of this study was to assess the susceptibility of a common amphibian species to a panel of ranaviruses isolated from fish and amphibian hosts. The European common frog *Rana temporaria* was chosen as the test amphibian species. Common frogs are native to the UK and much of Europe and are the most abundant and easily accessible frog species in the UK. The secondary aims of this study were to investigate the effect of temperature and amphibian developmental stage on susceptibility.

MATERIALS AND METHODS

Challenge isolates and culture

The ranavirus isolates used in the study and details of their original isolation are listed in Table 1. They include 6 species recognised by the ICTV, plus 3 less extensively characterised isolates recovered from fish and amphibians in Europe. Their identity was confirmed prior to use by sequencing of the complete viral major capsid protein (MCP) gene. All sequencing was carried out by H. Tapiovaara and R. Holopainen (Finnish Food Safety Authority Evira, Finland) in accordance with to the methods described by Hyatt et al. (2000).

Viruses were propagated and titrated using day-old epithelioma papulosum cyprini (EPC) cell monolayers (Fijan et al. 1983) and minimal essential

Table 1. Ranavirus isolates used in this study and details of their original isolation

Virus	Acronym	Host	Country of isolation	Source
Bohle iridovirus	BIV	Ornate burrowing frog *Limnodynastes ornatus*	Australia	Speare & Smith (1992)
Doctor fish virus	DFV	Doctor fish *Labroides dimidatus*	North America (fish imported from Asia)	Hedrick & McDowell (1995)
European catfish virus	ECV	Black bullhead *Ameiurus melas*	Italy	Bovo et al. (1993)
Epizootic haematopoietic necrosis virus	EHNV	Redfin perch *Perca fluviatilis*	Australia	Langdon et al. (1986)
Frog virus 3	FV3	Leopard frog *Rana pipiens*	North America	Granoff et al. (1966)
Guppy virus 6	GV6	Guppy *Poecilia reticulata*	North America (fish imported from Asia)	Hedrick & McDowell (1995)
Pike-perch iridovirus	PPIV	Pike-perch *Stizostedion lucioperca*	Finland	Tapiovaara et al. (1998)
Rana esculenta virus 282/I02	REV	Green frog *Pelophylax esculentus*	Italy	G. Bovo (pers. comm.)
Short-finned eel ranavirus	SERV	Short-finned eel *Anguilla australis*	Italy (fish imported from New Zealand)	Bovo et al. (1999)

medium (GMEM, Glasgow modification of Eagle's medium, Sigma) supplemented with 2% foetal calf serum, penicillin (100 IU ml^{-1}) and streptomycin (100 µg ml^{-1}). This cell line was demonstrated to consistently produce high titres of ranavirus *in vitro* (Ariel et al. 2009). Incubation temperature was 25°C for all isolates except for short-finned eel ranavirus (SERV), which was incubated at 20°C.

Virus for use in challenge experiments was recovered from stock frozen at −70°C and propagated in 75 cm^2 flasks of EPC cells. Once complete cytopathic effect (CPE) was observed, virus and culture media were stored at +4°C (for up to 1 mo) prior to use. Virus was titrated by inoculating 10-fold serial dilutions onto cells in 96-well microtitre trays in 8-well replicates. Virus titre was calculated after 7 d incubation, using the method of Reed & Muench (1938), as 50% tissue culture infectious dose ($TCID_{50}$). On the day of challenge, virus titre was re-measured to determine whether there was any loss of infectivity during storage and to ensure, in retrospect, that viable virus had been used for the challenge.

Animal and husbandry conditions

All common frogs used in the study were taken, as spawn, from a pond in England that had no known prior history of ranaviral disease. Spawn was added to stock tanks containing approximately 30 l fresh water (supplied at 1 l min^{-1}) at a temperature of 10–15°C. When stock tadpoles reached metamorphic climax (Stage 42–46; Gosner 1960), they were transferred to larger tanks (300 l) containing a central area of shallow fresh water (2 l, supplied at approximately 1 l min^{-1}) surrounded by moist compost and bark. Plastic tubing was added for environmental enrichment. Tadpoles were fed ad libitum with cooked lettuce, spinach that had been frozen and thawed and a commercial non-salmonid fish feed. After metamorphosis, animals were fed daily with live flies *Drosophila* sp. and banded crickets *Gryllodes sigillatus* (Livefoods Direct). A real-time day length was provided with 30 min dawn/dusk and day illumination set to provide approximately 200 lux light at the water surface.

Experimental challenge

Experiments were conducted under the authority of a UK Home Office Licence and with the approval of the Centre for Environment Fisheries and Aquaculture Science Weymouth Ethical Review Committee. Prior to the start of each challenge experiment, a sub-sample of animals was tested for the possible presence of viable ranavirus in cell culture, using the virus re-isolation methods described below. All tests were negative.

Animals were challenged as tadpoles and recent post-metamorphs in single groups of 30. Challenge was by bath immersion for 1 h, to mimic a natural infection route. Virus suspension in GMEM was added to fresh water (1/100 dilution) to provide a

challenge dose of approximately 10^4 $TCID_{50}$ virus ml^{-1}.

Tadpoles were challenged 2–3 wk post hatch (approximate Stage 26) with the complete panel of viruses at temperatures of 15 and 20°C (±1°C). A temperature of 20°C was chosen because ranaviruses have been shown to replicate best at or above this temperature (Ariel et al. 2009). A temperature of 15°C was also chosen to represent temperatures experienced in the wild, within in the UK, at the time of hatching. Where necessary, water temperature was raised to the desired temperature prior to challenge, with a maximum increase of 2°C per day. Challenge was carried out in 1 l plastic bowls, with aeration provided, floated individually in experimental tanks to maintain the desired challenge temperature.

Post-metamorphs were challenged approximately 1 mo after metamorphosis with FV3, *Rana esculenta* virus 828/IO2 (REV) and BIV only. Challenge was carried out at room temperature (20 ± 1°C) in small plastic covered boxes containing sufficient virus suspension to bathe their ventral surface without the animals being out of their depth. Negative control animals (tadpoles and post-metamorphs) were mock challenged in groups of 30 in the same way, replacing the virus suspension with culture medium only.

After challenge (Day 0), animals were removed from challenge containers and directly transferred (in the same groups of 30) to experimental tanks. During experiments tadpoles were housed in 30 l tanks half-filled with fresh water supplied at 0.2–0.4 l min^{-1}. Post-metamorphs were housed in covered plastic boxes (height 20 cm, width 30 cm, length 40 cm) divided into an area of moist compost and bark and approximately 1 l of fresh water, with a drainage hole for water to be changed.

Post-challenge observations and histopathology sampling

Animals were observed for clinical signs and mortality at least 2 times each day for a period of 30 d. During this time, any moribund animals were euthanized on ethical grounds. Cannibalism on dead tadpoles occurred in five 15°C tanks and four 20°C tanks, even though tanks were checked at least twice daily and any mortalities removed. Additionally, during Week 1 of the first post-metamorph challenge experiment a number of frogs escaped from the negative control tank, resulting in only 23 animals being accounted for although 30 animals had been challenged (Table 2). Missing animals were not included in any calculations. At the end of the 30 d period, all surviving animals were euthanized. Euthanasia was by overdose of tricane methane sulfonate (MS-222) buffered using sodium bicarbonate to a pH of 7.

For particular ranaviruses (FV3, pike-perch iridovirus [PPIV] and REV), additional single groups of 30 animals were challenged as indicated above and sampled for histopathological investigations. An additional negative control group of 30 animals was also mock-challenged. Animals were challenged as tadpoles at 20°C and as post-metamorphs (FV3 and REV only). Beginning on Day 4 post challenge, up to 3 animals were euthanized per group daily until no animals remained, selecting any individuals showing lethargy or appearing moribund. If no animals displayed these signs, they were selected randomly.

Virus re-isolation

Mortalities, animals sacrified on ethical grounds and animals sacrified at the end of the experiment were frozen whole at −70°C for future testing for virus presence. Mortalities and animals sacrified on ethical grounds were processed individually. The only exceptions were from the 15°C tank challenged with DFV, where 4 mortalities from the same day were pooled, the 20°C tank challenged with BIV where 2 mortalities from the same day were pooled on 2 occasions and the 20°C negative control tank where 3 or 4 mortalities from the same day were pooled on 3 occasions. All mortalities were tested except in tanks where cumulative mortality was over 85%, when only 9 mortalities were tested, taken from the beginning, middle and end of the post-challenge period. Where numbers were sufficient, 5 of the animals sacrified at the end of the experiment from the same tank were pooled for testing. A group size of 5 animals was chosen because it was considered, from previous results, that one positive animal in a group would still be detected. Where fewer than 5 animals survived in a particular group, all survivors were pooled.

Because of their small size, all animals were processed whole. Samples were diluted 1/10 in GMEM and homogenised using a pestle, mortar and sterile sand, and then centrifuged at 3000 rpm (2000 × *g*) for 20 min. Homogenate was removed and filtered using a 0.45 µm filter (Sartorius) and inoculated in duplicate onto cells in 24-well trays in 3 serial 10-fold dilutions (1/100, 1/1000 and 1/10000 of the original tissue). The inoculated cultures were incubated for 7 d

Table 2. *Rana temporaria*. Cumulative mortality and infection rate in common frog tadpoles and post-metamorphs after bath challenge with 9 different ranavirus isolates at 15 and 20°C. Virus abbreviations as in Table 1. See 'Materials and methods' for information regarding the variation in the number of animals and the number of mortalities tested

Virus	Challenge			Negative control		
	No. animals	No. mortalities	No. mortalities with virus isolated/no. tested	No. animals	No. mortalities	No. mortalities with virus isolated/no. tested
15°C/tadpole						
BIV	30	1	0/1	30	0	0/0
DFV	30	5	0/5	30	0	0/0
ECV	28	2	0/2	30	1	0/1
EHNV	30	0	0/0	30	0	0/0
FV3	28	9	7/9	30	0	0/0
GV6	30	0	0/0	30	0	0/0
PPIV	28	6	5/6	30	0	0/0
REV	25	7	5/7	30	0	0/0
SERV	29	1	0/1	30	0	0/0
20°C/tadpole						
BIV	30	8	0/8	30	11	0/11
DFV	30	2	0/2	29	2	0/2
ECV	29	2	0/2	29	2	0/2
EHNV	30	2	0/2	29	2	0/2
FV3	29	29	9/9	30	6	0/6
GV6	30	0	0/0	29	2	0/2
PPIV	29	27	9/9	30	11	0/11
REV	30	29	9/9	29	2	0/2
SERV	30	1	0/1	29	2	0/2
20°C/post-metamorph						
BIV	30	0	0/0	30	0	0/0
FV3	30	29	9/9	23	5	0/5
REV	30	26	9/9	23	5	0/5

and checked regularly for CPE. After 7 d, cells showing no CPE were passaged following a single freeze–thaw cycle. Cells were first frozen at −70°C for approximately 30 min and then thawed at room temperature (19–22°C) to release any intracellular virus. For each sample, the 1/100 and 1/1000 dilutions were then pooled and inoculated onto a fresh 24-well tray in 3 serial dilutions (1/10, 1/100 and 1/1000). Inoculated cells were incubated for 7 d and samples with no CPE after this time were considered cell culture negative.

So that viral load could be determined for each animal, homogenate from individual mortalities from FV3, PPIV and REV tanks was also inoculated directly onto EPC cells in 96-well microtitre trays in 10-fold serial dilutions with 8-well replicates. Virus titre for each animal was calculated as previously described. Homogenate was not filtered prior to inoculation so that virus could be accurately quantified.

In order to ensure that the correct challenge virus had been used and there was no cross-contamination between tanks, the identity of virus recovered from mortalities was confirmed by sequencing the complete viral MCP gene. Three mortalities were tested from each tank. All sequencing was carried out by H. Tapiovaara and R. Holopainen (Finnish Food Safety Authority Evira, Finland) using the method described by Hyatt et al. (2000). Virus used for the sequencing was extracted directly from tissue homogenates.

Histopathology and immunohistochemistry

Euthanized animals, with the visceral cavity opened to allow prompt fixation of the internal organs, were placed whole into 10% neutral-buffered formalin (NBF) for a minimum of 48 h. Animals were processed to paraffin wax using a vacuum infiltration processor using standard protocols. Embedded blocks were sectioned at 3–4 µm thickness using a rotary microtome and sections were stained with haematoxylin and eosin (H&E). Sections were examined using a Nikon E800 light microscope with images captured using Lucia™ software.

Immunohistochemistry (IHC) was used to confirm that pathological signs were attributable to ranavirus infection. The primary antibody was a polyclonal antibody raised in rabbit against ECV (Bovo et al. 1993), kindly provided by G. Bovo, Instituto Zooprofilattico Sperimentale delle Venezie, Italy. The antibody cross-reacts with other ranavirus isolates and is specific to ranaviruses (Gobbo et al. 2010, G. Bovo pers. comm.). Briefly, sections were de-waxed and de-hydrated and incubated in 3% H_2O_2 to block endogenous peroxidases. Sections were then re-hydrated in a decreasing alcohol series and incubated for 1 h in Tris-buffered saline (TBS; Dako) with 0.1% trypsin and 0.1% $CaCl_2$ at 37°C. After rinsing in phosphate-buffered saline (PBS; Dako), sections were incubated for 20 min in PBS supplemented with 5% bovine serum albumin (BSA). Sections were then incubated with the primary antibody diluted 1:1500

in TBS supplemented with 2.5% BSA for 60 min. After rinsing in TBS, sections were incubated with biotinylated goat secondary antibody (Dako) diluted 1:400 in TBS with 2.5% BSA for 20 min. After rinsing again in TBS, sections were incubated with strept ABComplex/HRP (Dako) for 30 min. After a final rinse in TBS, sections were incubated in the dark with chromogen substrate AEC (Sigma) for 20 min. Incubations were at room temperature unless stated otherwise. Sections were washed in distilled water and counterstained with Mayer's haematoxylin for 30 s and mounted in glycerol gelatine for microscopic examination as described above.

RESULTS

Common frog tadpoles were susceptible to FV3, REV and PPIV, but were refractory to the other ranaviruses tested. Post-metamorphs were also susceptible to FV3 and REV but refractory to BIV (the other viruses were not tested). Mortality data are given in Table 2.

The percentage cumulative mortalities of tadpoles after challenge with FV3, PPIV and REV at 20°C are shown in Fig. 1. There was significant mortality at this temperature, with cumulative mortality reaching 93% for PPIV, 97% for REV and 100% for FV3 at the end of the trial. Mortality began 4 d after challenge with REV, and 6 and 7 d after challenge with PPIV and FV3, respectively, and continued consistently until 13 d post challenge for REV and 22 d post challenge for PPIV and FV3. After this time, either mortality was reduced or all animals were dead. Fig. 2 shows the percentage cumulative mortality of tadpoles after challenge at 15°C with the same viruses. Cumulative mortality was lower, reaching 21% for PPIV, 28% for REV and 32% for FV3 at the end of the trial. Mortalities began Day 4 post challenge in REV and FV3 challenged tadpoles, and Day 13 in tadpoles challenged with PPIV. After onset, mortalities at 15°C continued at a low rate until the trial was terminated at Day 30. Percentage cumulative mortality following challenge of post-metamorphs is shown in Fig. 3. Mortality at the end of trial reached 87% for REV and 97% for FV3. Mortality began 7 d post challenge with REV and 9 d post challenge with FV3. Subsequently, post-metamorphs died consistently until 20 d post challenge for REV and 17 d post challenge for FV3, after which mortality was reduced.

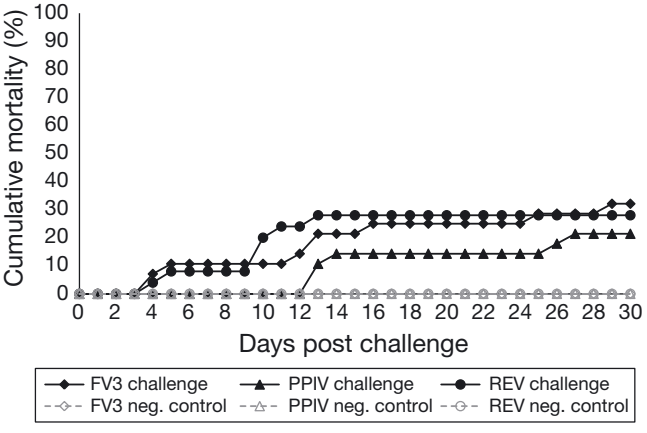

Fig. 2. *Rana temporaria*. Percentage cumulative mortality of common frog tadpoles after bath challenge with frog virus 3 (FV3), *Rana esculenta* virus 282/I02 (REV) and pike-perch iridovirus (PPIV) at 15°C

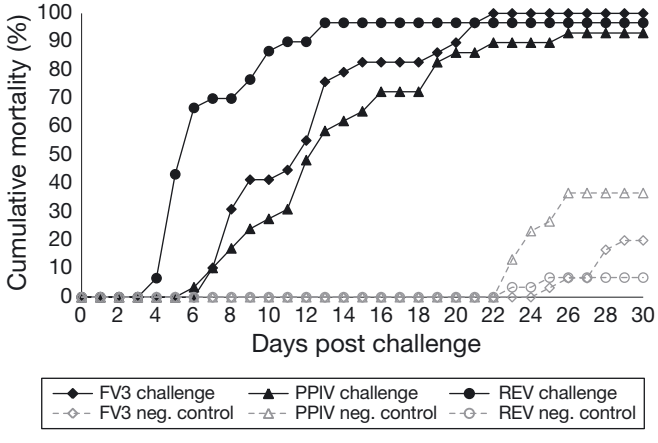

Fig. 1. *Rana temporaria*. Percentage cumulative mortality of common frog tadpoles after bath challenge with frog virus 3 (FV3), *Rana esculenta* virus 282/I02 (REV) and pike-perch iridovirus (PPIV) at 20°C

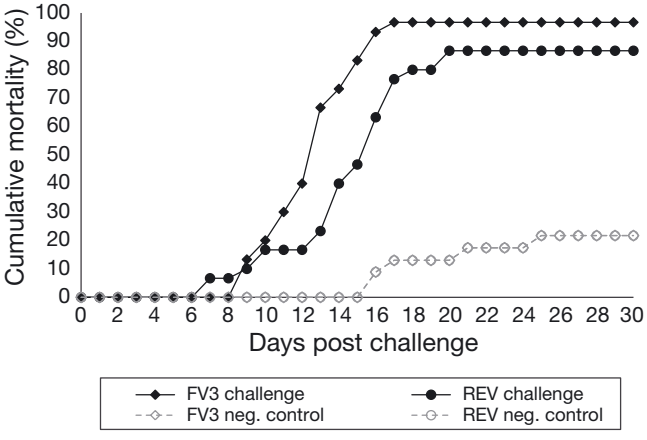

Fig. 3. *Rana temporaria*. Percentage cumulative mortality of common frog post-metamorphs after bath challenge with frog virus 3 (FV3) and *Rana esculenta* virus 282/I02 (REV) at room temperature (20 ± 1°C)

Tadpoles infected with FV3, PPIV and REV either showed no visual signs of infection or, more commonly, became lethargic and tended to swim at the surface of the water or lie at the bottom of the tank. Infected tadpoles sometimes appeared bloated with a distended abdomen often filled with clear liquid (Fig. 4). Infected post-metamorphs often showed no visual signs of disease. Some animals became lethargic and tended to crouch in the tank corners. However, this was not always indicative of being infected; not all animals that displayed this behaviour died and their behaviour may have been due to disturbance caused by the lifting of the tank lid. Haemorrhaging was observed on the limbs of some post-metamorph mortalities; additionally, digits were occasionally missing or ulcerated (Fig. 5).

At a temperature of 20°C mortality occurred on a number of occasions between Days 21 and 30 post challenge in challenged and control animals. Mortality tended to occur in animals that did not develop correctly during metamorphosis, with the majority of mortalities having poorly developed legs. The possibility that these animals drowned could not be excluded. At a temperature of 15°C a number of mortalities occurred in challenged and control animals throughout the trial, including most notably 5 tadpoles challenged with DFV that died on Day 9 (n = 4) and on Day 14 (n = 1) post challenge.

Amphibian development

Metamorphosis was markedly slower in tadpoles held at 15°C than at 20°C. At the end of the 30 d trial, tadpoles held at 15°C did not appear to have undergone significant development, with hind limb buds only observed on close examination (Stage 26–27), whereas the majority of tadpoles held at 20°C had completed metamorphosis by the end of the trial. As metamorphosis progressed in negative control tadpoles held at 20°C, they became lethargic and tended to rest on the bottom of the tank or float at the surface. Tail resorption (Stage 40–41) began approximately 15–17 d into the post-challenge period. Resorption of the gills occurs at the same time as or slightly earlier than tail resorption and, at this time, the lungs become the main respiratory organs (Shi 1999). Pieces of floating plastic with a small ramp into the water were added to the tanks as tail resorption began so that animals could emerge from the water. Metamorphosis was complete in some individuals at approximately 21 d post challenge.

Fig. 4. *Rana temporaria*. Infected common frog tadpole

Fig. 5. *Rana temporaria*. Infected common frog post-metamorph

Virus re-isolation

Virus re-isolation results are given in Table 2. Virus was recovered from all tadpole mortalities tested that were challenged with FV3, PPIV and REV at a temperature of 20°C. Virus was also consistently re-isolated from tadpoles challenged with the same viruses at 15°C, although not all dead animals tested positive for virus. Virus was recovered from all FV3 and REV challenged post-metamorphs tested. Virus was not recovered from any other mortalities. CPE was characterised by initial condensation and rounding of cells within the cell monolayer, rounded cells detached forming plaques around which more rounded cells formed with eventual destruction of the complete cell layer. Where CPE occurred, it was always evident prior to passage and was observed in each of the duplicate wells at all 3 tissue homogenate dilutions. The titre of infectious virus recovered

from mortalities was consistently at least 1×10^7 $TCID_{50}$ g^{-1} whole animal tissue (Fig. 6), at least a 1000-fold increase from the initial challenge dose of 1×10^4 $TCID_{50}$ ml^{-1}. In all but one case, virus was not recovered from animals sacrified at the end of the post challenge period. The exception was that REV was recovered from the group of 4 REV-challenged post-metamorph survivors.

Histopathology and immunohistochemistry

Histopathology consistent with ranavirus infection was observed in common frog tadpoles exposed to REV at Day 5 post exposure, particularly in those tadpoles that appeared lethargic or were consistently observed at the water surface. Hepatocellular necrosis associated with the loss of normal trabecular pattern of the liver was observed (Fig. 7A). Specimens from Day 6 post exposure displayed similar hepatic pathology and focal necrosis of the gastric glands. Tadpoles sampled at Days 7 and 8 did not show significant pathology. A single animal sampled at Day 9 exhibited renal haematopoietic cell necrosis. Post-metamorphs challenged with REV displayed fewer degenerative lesions, restricted to necrosis of connective tissue seen in only one animal.

Tadpoles challenged with FV3 first displayed histopathological changes at Day 6 post exposure, with one tadpole exhibiting mild renal haematopoietic cell necrosis, a feature present in tadpoles sampled on Days 7 and 9; these tadpoles also exhibited hepatocellular necrosis (Fig. 7C). A bacterial infection was observed in one tadpole sampled 8 d after challenge. Post-metamorphs challenged with FV3 also displayed hepatocellular necrosis with necrotic changes seen in blood cells within the sinusoids of the liver. In addition, necrosis of the connective tissue associated with the gut wall, Hardarian gland and spleen was seen in some animals.

Tadpoles challenged with PPIV displayed mild to severe necrosis of the renal haematopoietic tissue and glomeruli. In the most severely affected tadpole, hepatocellular necrosis and focal inflammation in the subcutaneous loose connective tissue was also seen. IHC confirmed the presence of ranavirus in tissues with pathological changes (Fig. 7B,D). No pathology or immunolabelling for ranavirus was observed in the negative controls.

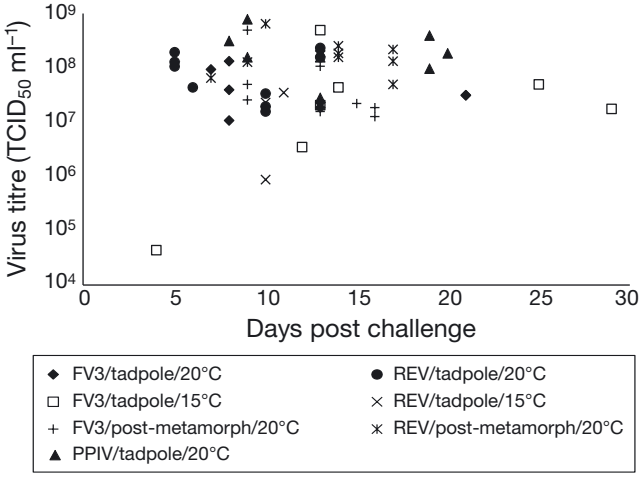

Fig. 6. *Rana temporaria*. Viral load from individual common frog tadpole and post-metamorph mortalities after challenge with 3 different ranavirus isolates at 15 and 20°C. Virus abbreviations as in Table 1

DISCUSSION

Results from this study show that common frog tadpoles are susceptible to the ranaviruses FV3, PPIV and REV after experimental challenge by bath immersion. Cumulative mortality was high after challenge at 20°C, and was similar for each of the 3 viruses, ranging from 93 to 100%. In 2008, the European Food Safety Authority published criteria for assessing host species susceptibility in a report detailing scientific opinion of the panel on animal health and welfare (EFSA 2008). The criteria were evidence of replication or growth of the organism, presence of a viable organism, presence of specific clinicopathological changes, and specific location of the pathogen. In our study, although only 9 mortalities were tested for each virus (taken from the beginning, middle and end of the mortality period), high titres of virus were re-isolated from each one, indicating that virus was able to replicate within the host and that mortality was due to the viral infection. Additionally, histopathological changes consistent with ranavirus infection were observed in infected animals, particularly in the liver and kidney, and the presence of ranavirus associated with these changes was supported by IHC results. Mortality was significantly lower after challenge at 15°C, reaching between 21 and 32%, and virus was not recovered from all mortalities; however, when virus was recovered, it was in high titres, providing evidence of viral replication within the host at this temperature.

Most ranaviruses can grow at temperatures between 10 and 28°C; however, the highest titres obtained in cell cultures are observed towards the top of this range, with optimal growth temperatures of

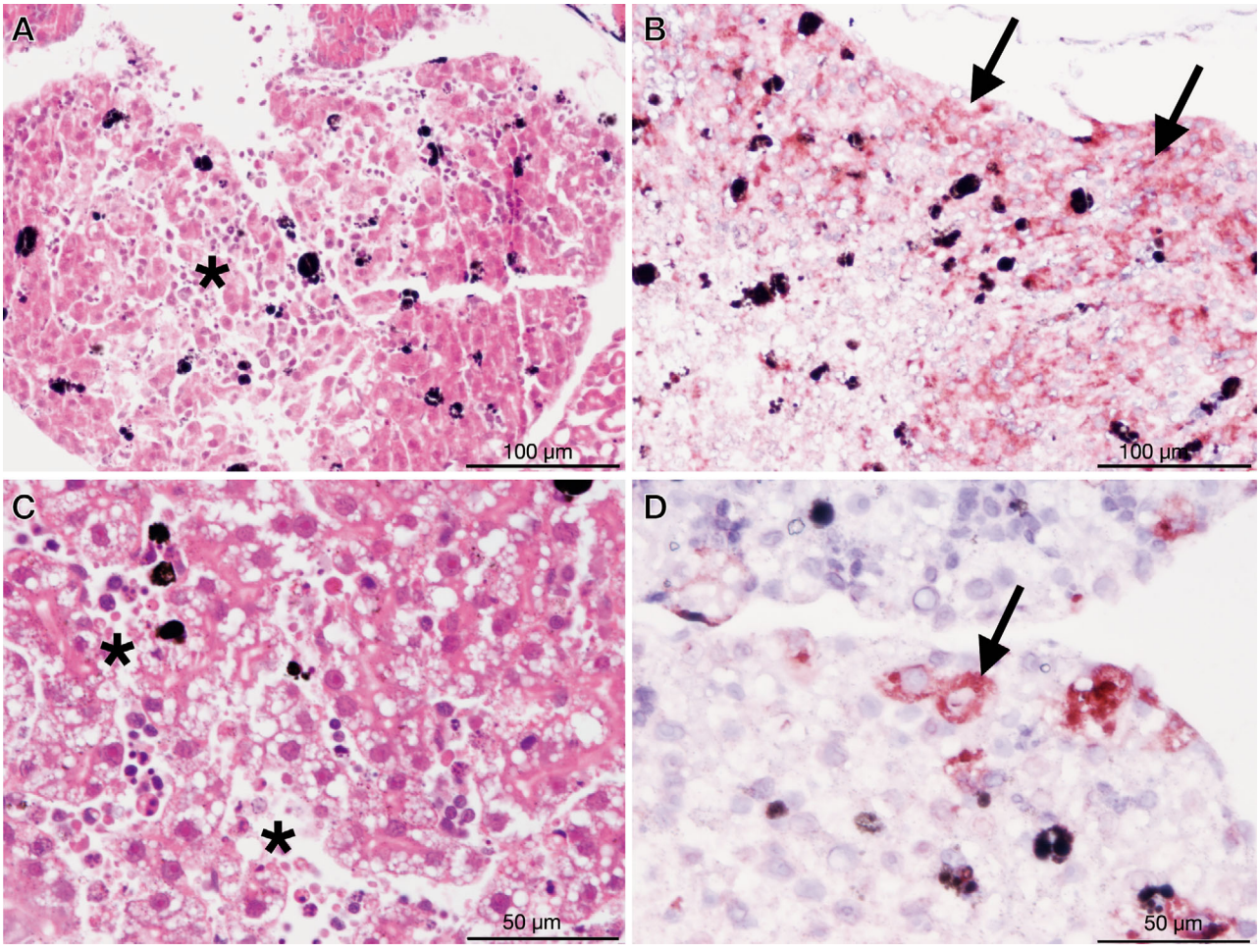

Fig. 7. *Rana temporaria*. (A) Hepatocellular necrosis (*) associated with infection with *Rana esculenta* virus 282/I02 (REV). H&E staining. (B) The same animal as depicted in (A), showing labelling of hepatocytes for the presence of REV. Note the relatively intense staining towards the periphery of the organ (arrows). Immunohistochemistry (IHC). (C) Hepatocellular necrosis (*) associated with infection with frog virus 3 (FV3). Note the disruption to the normal hepatic architecture. H&E staining. (D) The same animal depicted in (C), showing labelling of hepatocytes infected with FV3 (arrow). In this section, hepatocellular necrosis is not as evident as in the H&E section. IHC

24°C for FV3 and PPIV, and 28°C for REV for propagation to high titres (Ariel et al. 2009). The higher water temperature, therefore, likely resulted in more rapid and extensive viral replication within the host, which is a possible explanation for the observed higher virulence. The influence of temperature on ranavirus infection has been studied in larval salamanders *Ambystoma tigrinum* (Rojas et al. 2005). Salamanders were bath challenged with ATV at different temperatures including 10 and 18°C; a temperature of 18°C appeared favourable for the virus to infect and rapidly kill (Rojas et al. 2005), which is consistent with the results of our study. However, high infection and mortality also occurred after challenge at 10°C, although time to death was longer, and mortality did not begin until after 35–40 d. In our study, experiments were terminated at Day 30 so it is possible that further mortality may have occurred in tadpoles challenged at 15°C. This was regarded as unlikely since virus was not re-isolated from surviving animals; however, virus levels may have been below the level of detection of the cell culture test system used, and only 5 surviving animals from each group were tested for virus presence. Delay in onset of mortality in salamanders at the lower temperature in the study of Rojas et al. (2005) may have been linked to slower rate of replication within the host, but the ultimate high mortality indicates that rapid viral replication was not necessary to induce significant mortality.

Another possible reason for the differences in cumulative mortality is that challenge temperature had

an indirect effect on immune function. In our study, amphibians held at 20°C were observed to undergo metamorphosis at a markedly faster rate than those held at 15°C, with tadpole metamorphosis complete at 20°C approximately 21 d post challenge. Amphibians possess distinct immune systems during their larval and adult stages, with dramatic changes occurring during metamorphosis (Chen & Robert 2011). Although key areas of adaptive immune function develop during or after metamorphosis (Chen & Robert 2011), the efficiency of amphibian immune function is reduced during metamorphosis with specific components being downregulated (Rollins-Smith 1998, Carey et al. 1999). It has also been suggested that the energy resources available for mounting an immune response whilst also undergoing metamorphosis may be limited (Warne et al. 2011). Both of these factors are likely to lead to increased susceptibility to infection during metamorphosis. In the wild, mortality in the ornate burrowing frog *Limnodynastes ornatus* due to BIV infection occurred during or soon after metamorphosis rather than at the tadpole stage (Speare & Smith 1992), and after experimental exposure, ranavirus was more virulent to wood frog *Rana sylvatica* tadpoles that were challenged just prior to metamorphosis rather than as earlier stage tadpoles (Warne et al. 2011). Haislip et al. (2011) also demonstrated experimentally that amphibian susceptibility to ranavirus can vary with developmental stage. Susceptibility at 4 developmental stages from embryo (Stage 11) to pro-metamorphosis (Stage 41) was tested for 7 amphibian species and interestingly did not appear to be consistently highest at any one developmental stage. Mortality and infection prevalence were greatest after challenge during metamorphosis in 3 of the 7 species tested, as occurred with common frogs in our study; however, mortality and infection prevalence in 2 of the 7 species tended to be greatest after challenge as tadpoles.

The conditions under which tadpoles undergo metamorphosis have also been shown to affect immune competence (Carey et al. 1999). Tadpoles forced to metamorphose precociously, as likely occurred in the present study, exhibit increased susceptibility to disease during and after metamorphosis compared with controls metamorphosing on a normal schedule (Rollins-Smith 1998); thus, this may also have been a contributing factor.

Common frog post-metamorphs were susceptible to FV3 and REV. Cumulative mortality was similar to that of tadpoles challenged at 20°C, reaching 97 and 87%, respectively, at the end of the challenge experiment, and high titres of virus were recovered from all mortalities tested. Histopathological changes were observed in sampled animals challenged with both viruses, although in REV-challenged animals they were confined to just one individual, so further sampling would be desirable for confirmation. These results are consistent with the hypothesis that susceptibility to ranaviral infection is high in the period during and directly after metamorphosis (Rollins-Smith 1998, Carey et al. 1999, Warne et al. 2011). Similarly, Cullen & Owens (2002) showed that *Litoria caerulea* juveniles were highly susceptible to ranavirus (BIV), whereas adults appeared refractory. In other studies, recent *Limnodynastes terraereginae*, *Litoria alboguttata*, *Cyclorana brevipes* and *Pseudophryne coriacea* metamorphs were also reported to be susceptible (Cullen et al. 1995, Cullen & Owens 2002).

Common frog tadpoles appeared to be refractory to the other ranaviruses tested. At a temperature of 20°C some mortality occurred in control and challenged tanks towards the end of the experiment. Virus was not recovered from these mortalities, indicating infection was not the cause of death. Mortality occurred predominantly in animals that did not develop well during metamorphosis, particularly animals with poorly developed legs, and was likely a result of this. It is unclear whether the unsuccessful metamorphosis observed in a small proportion of animals was an artefact of the holding conditions used during the experiment or natural wastage. Only a very small proportion of eggs develop successfully into adults in the wild (ca. 0.25%); however, many additional factors, such as predation and adverse environmental conditions, would affect survival in the wild. At a temperature of 15°C some mortality occurred in challenged and control animals throughout the post challenge period, including most notably 5 tadpoles challenged with DFV. Again, virus was not recovered from these mortalities, indicating that they were not due to infection, but their cause is not known. However, this unexplained low-level background mortality may explain why not all mortalities challenged at 15°C with FV3, PPIV and REV tested positive for virus presence, since mortalities that did not test positive for virus may have been part of the background mortality and not due to ranavirosis. It is not possible to conclude whether surviving tadpoles (challenged at 15 or 20°C) were infected with the non-pathogenic ranaviruses. Virus was not recovered from any of the survivors tested at the end of the trial; however, only 5 survivors per treatment were tested.

A number of factors could have affected the results of our study. All common frogs originated from the same pond in England. Since ranavirus has been

reported in the UK on multiple occasions (Cunningham et al. 1996), the possibility of other animals in the pond being infected cannot be ruled out. The chances of this occurring were minimised as much as possible by choosing a pond with no known history of ranaviral disease and by testing a sub-sample of tadpoles for virus presence prior to the experiment. Additionally, it is possible that pathogenicity of the ranavirus isolates was lost during *in vitro* culture. However, other laboratory trials have demonstrated the pathogenicity of many of the isolates originating from fish hosts used in this study to other fish species (Ariel & Bang Jensen 2009, Bang Jensen et al. 2009, Gobbo et al. 2010). Since the isolates were obtained from the same source and virus propagation methods were similar, their apparent lack of pathogenicity to common frogs in this study is, therefore, unlikely to be due to loss of pathogenicity. Our study was designed to be an initial screen of the potential for novel ranavirus isolates, particularly those originally isolated from fish, to cause mortality in amphibians. The main aim was to distinguish isolates with high virulence from those that are apparently non-virulent, rather than to make precise quantification. Animal numbers were minimised as far as possible for ethical reasons, but also for practical considerations of availability of animals and facilities. A sample size of 30 gives good power (>0.66) to detect increases of 20% or more in mortalities. Use of single tanks did not provide replicates to guard against 'tank effects'; however, mortalities were tested for virus presence, and where virus was re-isolated the animal's death as a response to the challenge was shown to be plausibly causal. Additionally, variation in tank parameters such as lighting and flow rate between tanks was minimised as much as possible. Holding the animals individually would have allowed a better estimate of the initial infection rate since animals in our trial were likely exposed to variable challenge doses due to release of virus from infected animals in the same tank. Cannibalism was also observed in tadpoles, and it is likely that tadpoles consumed infected material and that the virus further spread in this way. The challenge scenario used in our study is, however, closer to what would occur during a natural exposure.

Two of the 3 ranaviruses that proved to be pathogenic in our trial were originally isolated from frogs. The susceptibility of common frog to FV3 and REV, whilst novel information, is not unexpected, given that the isolates are pathogenic to other amphibian species. REV was initially isolated from wild-caught edible frog *Pelophylax esculentus* tadpoles in Italy during a disease outbreak in 2002 occurring 2 d after the transfer of tadpoles to aquarium facilities (G. Bovo pers. comm.). FV3 infection has caused disease epidemics in multiple amphibian species (Wolf et al. 1968, 2002, Cunningham et al. 1996, Zupanovic et al. 1998) and a strain of FV3 known as 'Ranavirus UK' has been implicated in disease outbreaks in adult common frogs in the UK (Cunningham et al. 1996).

The third pathogenic ranavirus in this trial was originally isolated from a fish. PPIV was isolated from apparently healthy pike-perch *Stizostedion lucioperca* fingerlings cultivated for restocking purposes in Finland (Tapiovaara et al. 1998). This is the first report of experimental transfer of ranavirus from a fish to amphibian species by bath exposure, with infection resulting in significant mortality. The complete MCP gene sequence for PPIV has recently been published (Holopainen et al. 2009) and compared with the MCP gene sequences of other ranaviruses, including the FV3, BIV, REV, SERV, EHNV and ECV isolates used in this study. Based on the phylogenetic analysis of the sequences, Holopainen et al. (2009) showed that PPIV grouped together with FV3, REV and the other ranaviruses originating from frogs but was less closely related to the ranaviruses originating from fish. The same groupings were observed after analysis of the partial DNA polymerase and neurofilament triplet H1-like protein genes. Also, at the time of the original PPIV isolation, fish were held in freshwater ponds during the summer that were also inhabited by juvenile frogs (H. Tapiovaara pers. comm.) However, there is no information on the health status of the amphibians, and no amphibians were tested for virus presence, so no conclusions can be drawn. There are few data on the pathogenicity of this virus for other species, although pike have been reported to be susceptible (Bang-Jensen et al. 2009).

In summary, this study has shown that common frog tadpoles are susceptible to the ranavirus PPIV, originally isolated from fish, and also to FV3 and REV, originally isolated from amphibians. This is the first time an amphibian species has been shown to be susceptible to a ranavirus from fish. Mortality was high after challenge at 20°C but appears to depend on temperature or stage of tadpole development. These results highlight the need for further studies to investigate the potential differential susceptibility of common frogs to ranavirus isolates at different developmental stages and at different temperatures, and for comparative studies with other amphibian species. Such information is fundamental for understanding transmission dynamics and epidemiology of ranavirus disease in the aquatic environment.

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