

Experimental evidence in support of single host maintenance of a multihost pathogen

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Abstract. The rate at which pathogens are emerging appears to be increasing. Therefore, it is important to determine the factors, such as virulence and host specificity, and how they have been affected by the emergence. In the United Kingdom, ranaviruses, which are double stranded DNA viruses (Family *Iridoviridae*) began to emerge in populations of common frogs (*Rana temporaria*) in the mid-to-late 1980s, followed closely by emergence in common toad (*Bufo bufo*) populations. Here we present experimental evidence that a single host may be able to maintain a multihost pathogen. We exposed common frog tadpoles and common toad tadpoles to ranavirus isolates from the mid-1990s at two different doses. Tadpole survival differed significantly between treatments and this was primarily driven by the dose of the exposure. However, at the low dose, common frog tadpoles exposed to isolates from common frogs experienced higher mortality ($n = 35/\text{treatment}$, log-rank: $P = 0.0320$, Wilcoxon: $P = 0.0835$, $df = 1$) than those exposed to common toad isolates. The high dose caused 75% mortality in common frog tadpoles, but common toads never experienced more than 40% mortality. This, and other evidence provided by this study, show that common frogs are likely to be the primary host of the ranavirus in the UK, and that single host maintenance of ranaviruses can occur in anurans.

Key words: *Bufo bufo*; common frogs; common toads; experimental evidence infection; *Iridoviridae*; multihost pathogen; *Rana temporaria*; ranavirus; single host maintenance; United Kingdom.

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INTRODUCTION

Infectious diseases are emerging at an increased rate (Woolhouse and Gowtage-Sequeria 2005, Jones et al. 2008) providing opportunities for pathogens to infect and cause disease in additional host species (Benmayor et al. 2009). Host range and virulence are dictated both by evolution and by ecology; being shaped by historical interactions and by the more contemporary process of emergence (Woolhouse et al. 2005). Ascertaining if host range and virulence

have increased during emergence, or arisen as a result of previous host/pathogen dynamics, can be challenging, particularly in systems where pathogens are re-emerging or in those where the history of the pathogen is uncertain.

On the other hand, introduced pathogens offer opportunities to investigate the determinants of host range and virulence. The prospects for such studies have increased as the globalization of human activities has led to an increase in such novel introductions (Daszak et al. 2000, Hajek and Tobin 2011, Andreou et al. 2012, Fisher et al.

2012). These introductions can be valuable examples, because the ecological and evolutionary dynamics between the hosts and introduced pathogens are newly established, rather than having been shaped by historical interactions. The pathogens face immune responses that are typically different from those of their historical hosts; similarly the new hosts may be ill equipped by their previous evolution, since few, if any, pathogen strategies for overcoming host barriers to infection are universal (Parrish et al. 2008, but see Fisher et al. 2012).

Introduced pathogens can be capable of infecting multiple host species *de novo*, but the ability to infect multiple hosts is not equivalent to the ability to establish persistent infections and cause disease. Most novel communities of host species present a heterogeneous distribution of susceptibilities due to variation of immune responses in different host species. A novel pathogen is most likely to exploit the most susceptible host species initially, and infections of other species often arise through infrequent spill-over events from this primary host (Woolhouse and Gowtage-Sequeria 2005, Woolhouse et al. 2005). Spill-over or interactions with other, co-occurring pathogens can lead to evolutionary dynamics that result in pathogen divergence, where diverged lineages become adapted to, but not necessarily restricted to, secondary host species (Kawecki 1998, Crill et al. 2000, Nemirov et al. 2002, Parrish et al. 2008, Rouchet and Vorburger 2012). The result of these evolutionary dynamics can manifest as decreased parasitism and virulence in the initial host species and increased virulence in new hosts (Ebert 1998). Introduction need not lead to increased pathogen specialization, though, since coevolution can also favour generalist strategies (Hall et al. 2011) and host-switching does not always require adaptation by the pathogen (Nam et al. 2011).

Host range expansion by viruses has repeatedly caused novel disease emergence in wildlife, and some of these cases can be attributed to pathogen introduction (Roelke-Parker et al. 1996, Woolhouse et al. 2005, Härkönen et al. 2006, Bruemmer et al. 2010, Lawson et al. 2012). This appears to be the case for members of the genus *Ranavirus*, iridoviruses that exploit an exceptionally diverse range of vertebrate hosts (Hedrick et al. 1992, Mao et al. 1999, Hyatt et al. 2002, De Voe

et al. 2004, Gray et al. 2009*a, b*, Jensen et al. 2009). Ranaviruses often affects multiple hosts simultaneously at a single location (Gray et al. 2009*a*), but it is uncertain if multihost infection and disease dynamics are possible during initial emergence events or require more prolonged history.

Ranaviruses may be transmitted by vectors carrying them outside their native ranges, including introduced, commercially traded herpetofauna from which ranaviruses have frequently been isolated (e.g., Une et al. 2009). Once arrived at a new location the evidence suggests that ranaviruses are capable of producing infection, since they are experimentally transmittable to novel hosts (Jankovich et al. 2001, Pearman et al. 2004, Whittington et al. 2010). The introduction of virus to populations of a novel host species can result in local adaptation by the virus (Storfer et al. 2007, Ridenhour and Storfer 2008).

In the UK, unusual common frog (*Rana temporaria*) mortality events that were subsequently shown to be caused by ranaviruses were first detected in 1985 (Cunningham et al. 1996). Infections in common toads (*Bufo bufo*) were detected soon after the initial detection of ranaviruses in common frogs (Hyatt et al. 2000, Cunningham et al. 2007*a*). Since then, common frog mortality events in the UK caused by ranaviruses have increased substantially in geographic range. There is a serious impact on the common frog populations: approximately half of ranaviruses emergence events in frog populations result in substantial and persistent population declines (Teacher et al. 2010). Diseased or dead toads have rarely been detected and, apart from common frogs, no mass mortality events associated with ranaviruses emergence have been reported in the UK involving other amphibians. This evidence suggests that ranaviruses in the UK are incapable of eliciting sustained disease in multiple hosts, in contrast to the reports of ranaviruses affecting amphibian communities in North America (Duffus et al. 2008). It is conceivable that toad mass mortality events are occurring but go undetected. Toads have experienced inexplicable and significant declines in areas where ranavirus emergence was first detected in British frogs (Cunningham et al. 1996, Carrier and Beebe 2003, Teacher et al. 2010). Ranavirus isolates from toads and frogs

are genetically similar (but not identical; Hyatt et al. 2000), and toad isolates have been experimentally transmitted to frog hosts, resulting in fatal disease (Cunningham et al. 2007a). These latter findings suggest that amphibian ranaviruses in the UK are effective at exploiting both frogs and toads as hosts.

Here we report the results of experiments where we tested the potential for British ranaviruses to infect and cause disease in both *R. temporaria* and *B. bufo*. We used isolates derived several years after the initial emergence of ranaviruses in the UK from both species. Our goal was to determine if isolates from each host species had equivalent capacity to elicit disease in both hosts, or if there was any evidence of host specialization.

METHODS

Ethics statement

All experiments were fully licensed by the Home Office (License No. 07928346219) and all procedures and designs were subject to full ethical review before implementation.

Virus culture and titration

We used four viruses that were isolated from visibly diseased, wild amphibians collected in the UK in the early 1990s (Cunningham et al. 2007a, b). Two (BUK 2, BUK 3) were isolated from common toads, and two (RUK 11 and RUK 13) from common frogs (Hyatt et al. 2000, Cunningham et al. 2007a, b). Viruses isolated from common frogs were associated with different disease syndromes: RUK 11 was isolated from a *R. temporaria* adult presenting substantial internal haemorrhages (haemorrhagic syndrome) and RUK 13 was isolated from a frog presenting superficial skin ulcers (ulcerative syndrome; Cunningham et al. 2007b), unlike those obtained from *B. bufo* where only one disease syndrome has been observed (Hyatt et al. 2000).

Viruses were cultured in confluent lawns of fathead minnow (*Pimephales promelas*) cells (FHM) purchased from the European Collection of Cell Cultures (No. 88102401, ECACC, Oxford, UK). FHM cells were first propagated at 25°C in Eagle's minimum essential media (EMEM: Sigma-Aldrich, Andover, UK), supplemented with 1% L-glutamine (Sigma-Aldrich), 0.005% penicil-

lin-streptomycin (Sigma-Aldrich), 0.005% nystatin (Gibco, Invitrogen, Paisley, UK), and 10% research grade foetal bovine serum (Hyclone, Perbio Science, Northumberland, UK). Confluent flasks of FHM cells were then inoculated with 1000 µL of virus isolate. Twenty-five milliliters of maintenance media (EMEM supplemented with 1% L-glutamine, 0.005% penicillin-streptomycin, 0.005% nystatin, and 1% research grade fetal bovine serum, all suppliers as above) was added to each flask and all flasks were then incubated at 25°C. Flasks were monitored daily for the formation of viral plaques and once plaques were observed and FHM cells ceased to adhere to the flask, virus was harvested and stored at -80°C. Each isolate was standardized by passaging three times before final harvest and titration. To generate experimental negative controls we mock-harvested FHM cell cultures that had not been exposed to virus. To do this, we substituted maintenance media for culture media in confluent flasks of FHM cells and left flasks to incubate for 3 days, which we observed to be the average number of days required to bring virus cultures to harvest stage after the addition of maintenance media. After 3 days we scraped cells into the media and stored the cell solution at -80°C.

We determined the number of plaque forming units per milliliter (PFU/mL) for each virus using serial dilutions of harvested virus titrated in duplicate into six well flasks with confluent FHM cells (10^{-3} to 10^{-8} , 1 mL per well per dilution, one isolate per plate, two plates per isolate; Duffus et al. 2008). Plates were incubated at 25°C for 24 hours, following which 2 mL of maintenance media was added to each well. Plates were returned to 25°C and monitored daily for plaque formation. As soon as plaques were first detected in the well with the highest concentration virus, we removed the media from all wells of the plate and fixed cells in 100% methanol. After 10 minutes we removed the methanol, stained cells using a 0.05% crystal violet-20% methanol solution for 20 minutes and rinsed the wells with sterile water to removed excess stain. Plaques were counted by eye and we averaged plaque counts to generate PFU/mL for each isolate harvest.

Experimental design and procedure

Rana temporaria eggs (10 clutches) were collect-

ed from a pond located in Faversham, Kent, England, and *B. bufo* eggs (10 clutches) from a pond located in Cowden, Sussex, England, in March 2009. Infection with ranavirus and ranaviriosis have not been detected at either site (Teacher et al. 2010, Duffus et al. 2013). Eggs were brought to the Institute of Zoology, Zoological Society of London and transferred into 84-L plastic tubs (Really Useful Box Company, Normanton, UK) containing approximately 45 L of tap water aged for 48 hrs. Clutches were split among tubs by species and no more than three clutches were kept in any one box. We partially changed water every few days and removed any components of clutches that did not exhibit development. Hatched tadpoles were combined in single species tubs, where water was changed every two days and Tetra Tabimin pellets (Tetra Fish, Southampton, UK) were provided ad libitum.

Tadpoles were allocated to the experiments, one per amphibian species, when the tadpoles had reached Gosner stage 25 (Gosner 1960). Due to time differences in egg availability, the two experiments were not run simultaneously. Experimental treatments were the same in each experiment and included 35 individuals per treatment, for a total of 315 animals per experiment/amphibian species. Tadpoles were bath exposed in groups of five in Petri dishes (Nunc, Roskilde, Denmark) to one of nine different treatments: negative control, 10^6 PFU (high dose) of one of the four isolates, or 10^4 PFU of one of the four isolates. All exposures were standardized to 30 mL total volume and the volume of harvested virus was kept constant across all treatments. Tadpole groups were exposed for 18 hrs, after which individual tadpoles were transferred either into Petri dishes containing 30 mL aged tap water (*R. temporaria*) or 75 cm² tissue culture flasks (Nunc) containing 140 mL aged tap water (*Bufo bufo*). Tadpoles were maintained on a three- (*R. temporaria*) or four-day (*Bufo bufo*) water change and frog tadpoles were fed 125 µL of a dilution of Tabimin pellets (6 finely ground pellets suspended in 50 mL aged tap water) every second day until the end of the experiment. Toad tadpoles were fed as per *R. temporaria* until the 10th day, when we reduced food concentration to 3 pellets in 50mL and pipetted 100µL of food suspension into each flask

once every 2 days. To limit the possibility of contamination amongst experimental treatments, we kept animals separated by treatment and species, but rotated the position in the rearing room of each treatment and each tadpole within a treatment daily. Initially we checked for evidence of disease and death daily, but once mortality commenced checks were done twice daily until mortality rate had declined significantly. We recorded date of death (or survival to the end of the experiment) and visible signs of disease commonly reported for tadpoles suffering from ranaviriosis (e.g., Greer et al. 2005) for each animal. Because our goal was to measure impacts before metamorphosis, animals reaching Gosner Stage 43–44 were euthanized and counted as survivors. Both experiments were completed after 30 days and survivors were euthanized using an overdose of MS-2,2,2 [1g/L Tricane methylsulphonate (Thompson and Joseph, Norwich, UK) buffered to pH 7 with sodium bicarbonate]. All carcasses were stored in 2 mL microcentrifuge tubes in 100% ethanol for molecular diagnosis of infection.

We selected from each treatment, where possible, the first 10 animals to die and the first 5 animals that survived to the end of the experiment; these individuals were euthanized for screening for the presence of the ranavirus. In treatments where 10 animals did not die, all animals that died were included and the rest of the sample was made up of euthanized individuals. We aseptically sampled a small triangular section from the left side of every tadpole (each sample contained viscera and skin) and extracted DNA from these tissues using the Wizard SV96 Genomic DNA Purification System (Promega, Southampton, UK). We used extractions to amplify a 500-bp region of the major capsid protein (MCP) of frog virus 3 (FV3) using the primer set originally developed by Mao et al. (1996). All polymerase chain reactions (PCR) were completed using the Multiplex PCR kit (QIAGEN, Crawley, UK) and with the following thermocycler settings: initial five minute denaturing step at 95°C, followed by 35 cycles of 95°C for 45 sec, 52°C for 45 sec, 72°C for 45 sec. Positive controls and negative extraction controls were included in all PCR plates. We screened every extraction at least twice and any ambiguous amplifications or runs where positive PCR

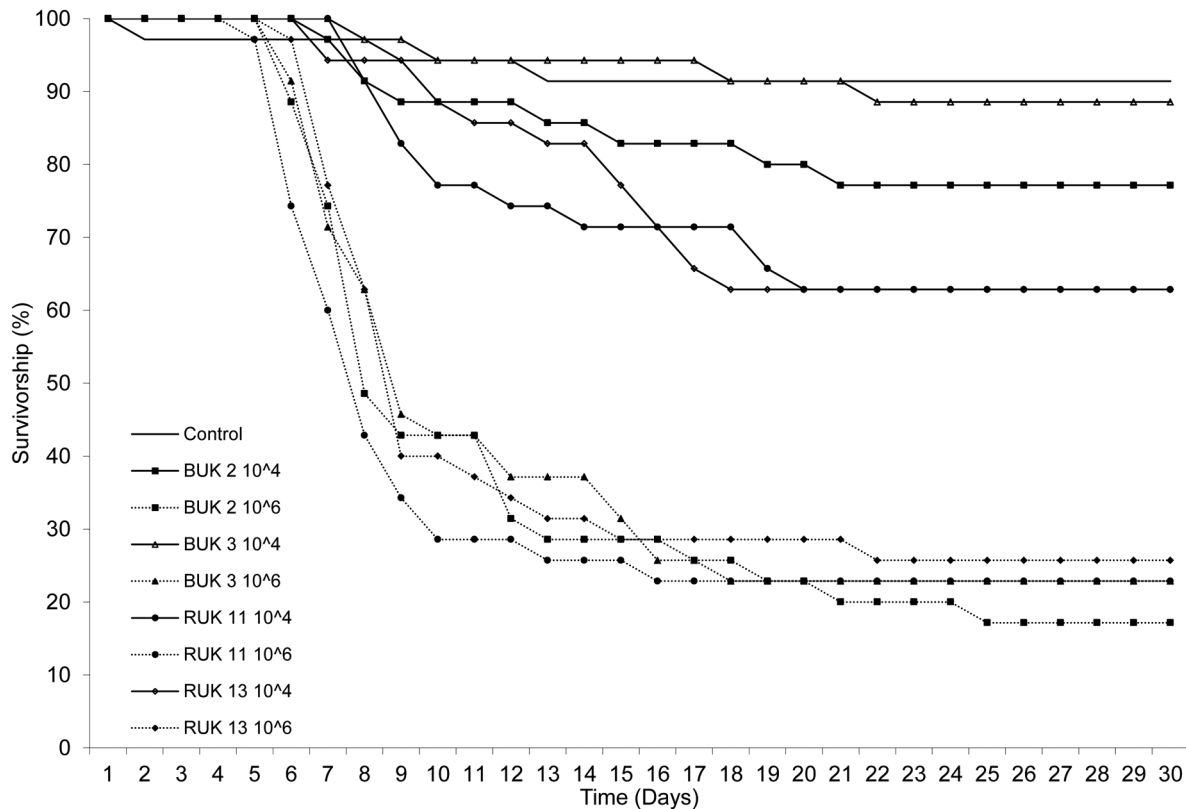


Fig. 1. Survivorship of common frog (*Rana temporaria*) tadpoles exposed to different doses and isolates of ranaviruses from the UK ($n = 35/\text{treatment}$).

controls failed were re-amplified: ambiguous amplifications or failed runs were exceptionally rare. PCR products were visualized on 1% agarose gels stained with ethidium bromide. Amplifications generating a 500-bp fragment were scored positive for infection with ranavirus.

Statistical analysis

We tested for differences in survival amongst treatments in each experiment using both log-rank analysis and Wilcoxon tests, as in both experiments data were right-censored (Kleinbaum and Klein 2005, Machin et al. 2006) and the ratio of hazards was greater earlier in the experiments (Kleinbaum and Klein 2005). When we detected a significant difference using either approach, we used Proportional Hazard Models to determine if dose or isolate type had the greater impact on survival (Kleinbaum and Klein 2005, Machin et al. 2006). We used a proportional hazard model because the hazard was continu-

ous and the model allows the inclusion of categorical variables (Machin et al. 2006). Relationships between infection prevalence and signs of disease were examined with the controls removed and the direction of the differences was established using a Fisher's exact test. All statistics were performed with JMP 8.0 (SAS Institute, North Carolina, USA).

RESULTS

Frog survival differed significantly amongst treatments ($n = 35/\text{treatment}$, log-rank: $P < 0.0001$, Wilcoxon: $P < 0.0001$, $df = 8$; Fig. 1). This was primarily driven by the dose (proportional hazards, $n = 315$, $df = 2$, $P < 0.0001$). Further analysis on low dose treatments revealed a significant difference, with RUK isolates causing significantly more mortality than BUK isolates in exposed tadpoles ($n = 35/\text{treatment}$, log-rank: $P = 0.0320$, Wilcoxon: $P = 0.0835$, $df = 1$;

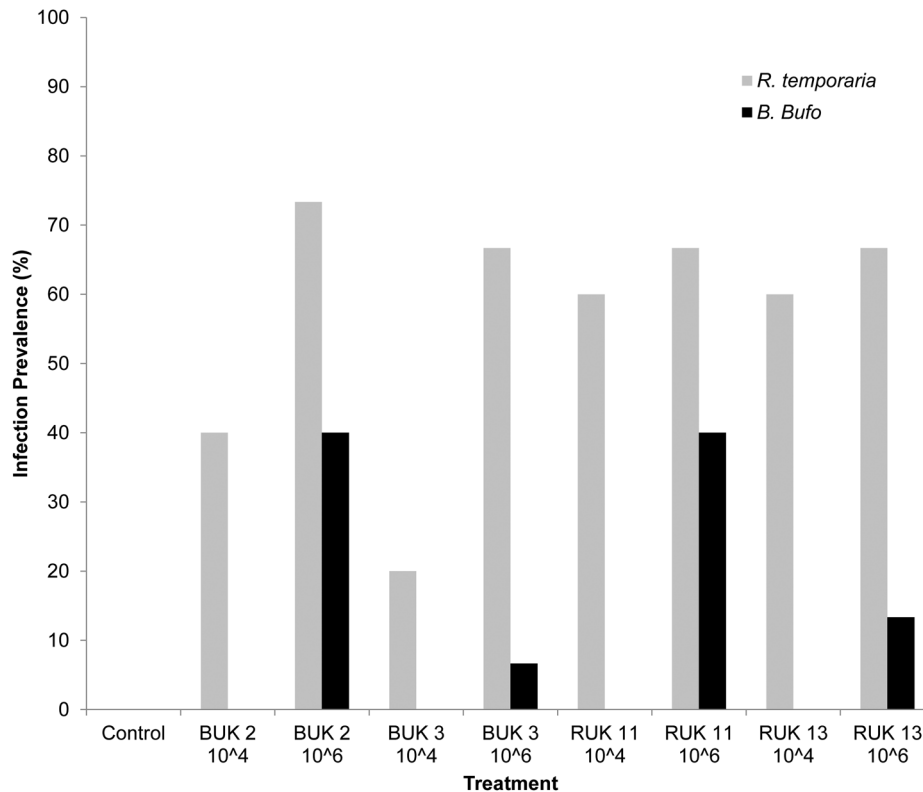


Fig. 2. Infection prevalence in tadpoles (*Rana temporaria* and *Bufo bufo*) exposed to different doses and isolates of ranaviruses from the UK (note that the y-axis stops at 50%; $n = 15/\text{treatment}$).

Fig. 1). Infection prevalence was not significantly different among treatments ($n = 120$, $df = 7$, $\chi^2 = 12.540$, $P = 0.0841$), nor was it different between isolates (likelihood ratio: $n = 120$, $df = 1$, $\chi^2 = 3.249$, $P = 0.3548$). Dose had a significant effect on probability of infection; individuals exposed to 10^6 PFUs were significantly more likely to be infected than those exposed to 10^4 PFUs, although this effect was driven by variation of infection amongst treatments involving toad-derived isolates (Fisher's exact test, $n = 120$, $P = 0.0211$; Fig. 2). The most common sign of ranaviral disease that we observed in frog tadpoles was abdominal haemorrhages, with or without abdominal bloating. The prevalence of visible disease was significantly different among treatments (likelihood ratio: $n = 276$, $df = 14$, $\chi^2 = 91.0349$, $P < 0.0001$; Fig. 3). Stronger doses were more likely to generate visibly diseased tadpoles (likelihood ratio: $n = 276$, $df = 1$, $\chi^2 = 68.559$, $P < 0.0001$), but the type of isolate did not have a significant effect on how frequently disease was

presented (likelihood ratio: $n = 276$, $df = 1$, $\chi^2 = 5.047$, $P = 0.5378$).

Survival again differed significantly amongst treatments in the toad experiment ($n = 32\text{--}35/\text{treatment}$, log-rank $P < 0.0001$, Wilcoxon $P = 0.0001$, $df = 8$; Fig. 4), and again dose was the most important predictor of survival (log-rank $P < 0.0001$, $df = 3$). However, whereas high doses caused on average 75% mortality in frogs (Fig. 1), toads exposed to high doses never experienced more than 40% mortality, and only came close to this level of mortality when exposed to high doses of RUK 11 (Fig. 3). High doses of the other 3 isolates caused, on average, slightly more than 20% mortality. Infection at time of death differed significantly among treatments (likelihood ratio: $n = 120$, $df = 7$, $\chi^2 = 30.381$, $P < 0.0001$; Fig. 4) and again isolate did not affect prevalence of infection (likelihood ratio: $n = 120$, $df = 3$, $\chi^2 = 6.912$, $P = 0.748$; Fig. 2). As with frogs, toads exposed to 10^6 PFUs had significantly more infections than those exposed to 10^4 PFUs (Fisher's exact test, n

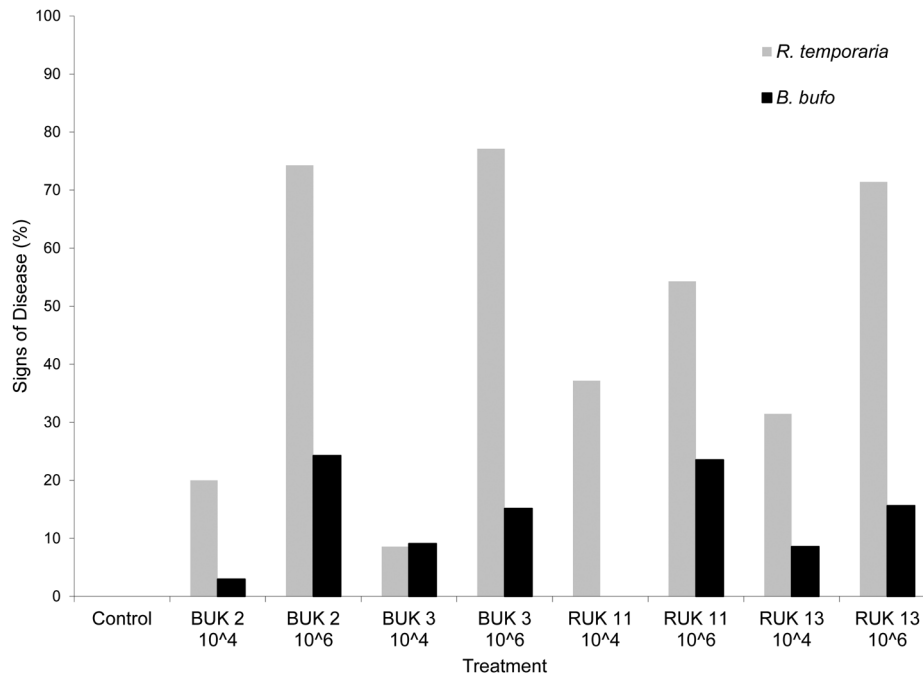


Fig. 3. Frequencies of signs of ranavirosis in tadpoles (*Rana temporaria* and *Bufo bufo*) exposed to different doses and isolates of ranaviruses from the UK ($n = 35/\text{treatment}$).

$= 120$, $P < 0.0001$), an effect driven by the lack of detectable infection in any toads exposed to low doses (Fig. 2). We did not observe abdominal or indeed any hemorrhages affecting toads and bloating was rare. We did commonly observe skin sloughing, and taken together, signs did differ significantly amongst treatments (likelihood ratio: $n = 269$, $df = 7$, $\chi^2 = 23.523$, $P = 0.0014$; Fig. 4), an effect driven by increasing dose strength (Fisher's exact test, $n = 269$, $P < 0.0001$). As with frogs, isolate did not have an effect on the frequency of observable signs of disease (likelihood ratio: $n = 269$, $df = 3$, $\chi^2 = 0.337$, $P = 0.9529$).

DISCUSSION

Highly virulent ranavirosis had been circulating in UK common frog populations for close to ten years when the ranaviruses used in this study were cryobanked, after isolation from wild UK amphibians. This time period is more than adequate for ranavirus evolution in response to host immunity (Ridenhour and Storfer 2008) and *R. temporaria* in populations experiencing sustained ranavirosis exhibit the molecular signal of

selection at immunogenetic loci important for combating infection with ranaviruses (Teacher et al. 2009a). Nevertheless, the responses of *R. temporaria* tadpoles we exposed to ranaviruses were broadly congruent with those commonly observed when novel pathogens emerge in highly susceptible hosts (Fig. 1; Pearman and Garner 2005, Warnecke et al. 2013): infection, disease and mortality were predominantly dose-dependent. We did detect divergence in virulence among isolates derived from different host species when frog tadpoles were exposed to low doses (Fig. 1), but any evidence of specialization of viruses was swamped by force of infection, as all four UK ranavirus isolates caused approximately 80% mortality in common frog tadpoles at high doses (Fig. 1).

The fact that frog-derived isolates induced stronger mortality at low doses in *R. temporaria* could be explained as decreased virulence of toad isolates in the primary host (*R. temporaria*). Theory predicts lower virulence in the primary host in cases where novel pathogens diverge and a new pathogen lineage exploits a novel, secondary host (Ebert 1998). This same theory predicts increased virulence of divergent pathogen line-

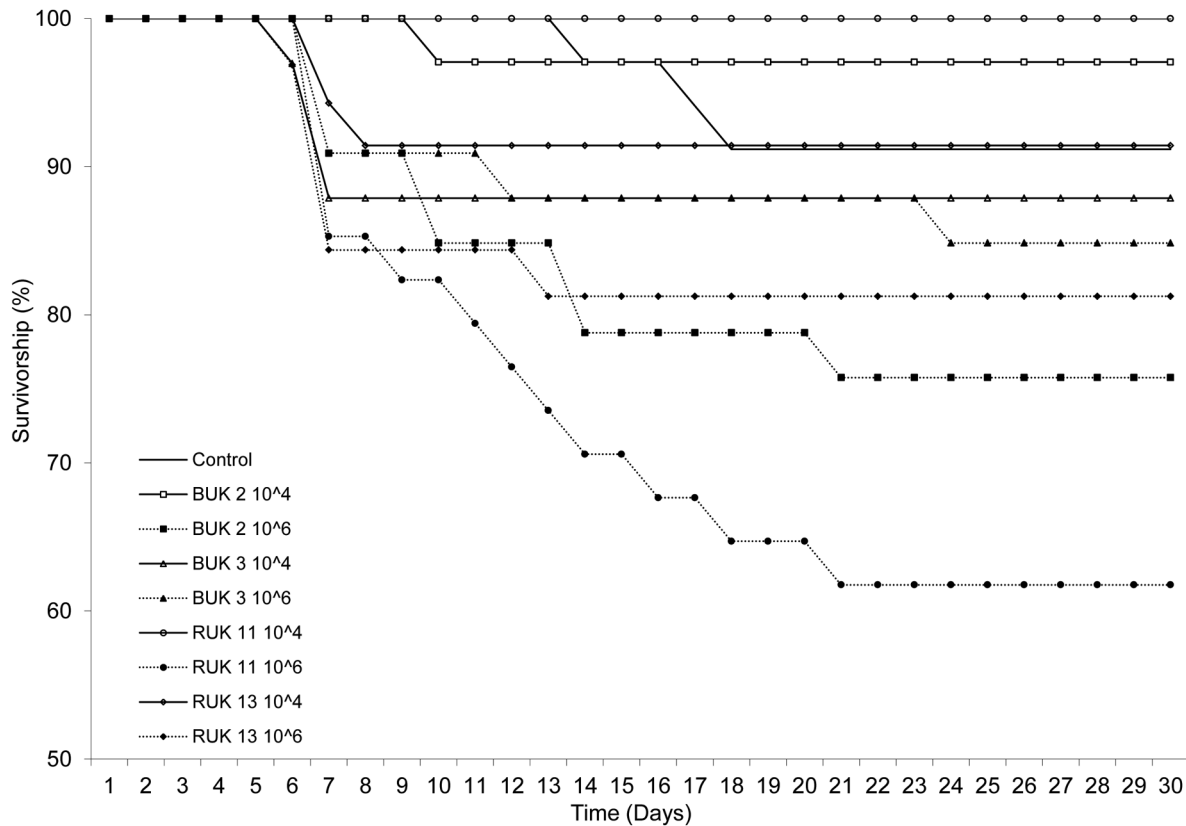


Fig. 4. Survivorship of common toads (*Bufo bufo*) tadpoles exposed to different doses and isolates of ranaviruses from the UK ($n = 35/\text{treatment}$).

ages in the secondary host species, but prevalence of infection and patterns of disease in *B. bufo* did not support this (Figs. 2 and 3). In this host, infection and mortality were exclusively dose-dependent. An alternative explanation for pathogen divergence evident in common frogs is that ranaviruses isolated from frogs have evolved elevated virulence in the primary host, which is consistent with evidence of selection on host immunity (Gandon 2004, Teacher et al. 2009a). Ranavirosis in the UK has been causing mass mortality in common frogs for almost 30 years (Cunningham et al. 1996) and shows no sign of abating. Frog populations are responding to emergent ranavirosis (Teacher et al. 2009a, b) so coevolution is a predicted response. However, our experimental results indicate that if coevolution is occurring between UK ranaviruses and UK frog hosts, this dynamic is not being influenced significantly by the ability of UK ranaviruses to elicit low levels of infection and

mortality in common toads.

Patterns of infection and disease in toads instead indicate that ranaviruses are unlikely to be sustained in toad populations without the presence of the primary host species carrying infections. Infection was only detected in toads exposed to high doses, and even then very few animals became infected as a result of exposure. Signs of disease exhibited by toads did not include the superficial skin lesions that have been reported in larvae of North American amphibians dying from ranavirosis (Greer et al. 2005, Duffus et al. 2008) and the skin and abdominal haemorrhages that were typically presented by infected *R. temporaria* tadpoles. Haemorrhages are a source of infectious ranavirus that can be transmitted both directly and indirectly to susceptible hosts (Pearman et al. 2004, Cunningham et al. 2007b). Because toads do not present these signs of disease, they are less likely to be a significant source of infectious

ranavirus and rare infections in UK common toads appear most likely to have arisen through spill-over from diseased *R. temporaria*.

Given that toads are likely to be dead-end ranavirus hosts, that ranavirosis is relatively unknown in other UK amphibians, and persistent disease is reported in frog populations where other alternative hosts (e.g., fish, reptiles) are lacking, ranavirus may be maintained in the UK in a single, primary host species, *R. temporaria*. Precedence exists for highly virulent ranavirosis to be maintained in a single host species, even with significant mortality in that host (Brunner et al. 2004). Survival was still possible in *R. temporaria* exposed to both dose concentrations of ranavirus and some survivors did exhibit detectable infections. If these survivors could maintain infections while recruiting into later life history stages and then return to their natal site with transmissible infections or transmit infections to returning frogs before dying, infection would be sustained in manner comparable to salamander species affected by persistent ranavirosis (Brunner et al. 2004). Alternatively, infection could be maintained in adult frogs that experience infection but do not always succumb to disease. In support of this proposal, it has been observed that breeding common frogs presenting the systemic haemorrhagic form of ranavirosis often present healed skin ulcers that indicate a bout of disease the preceding year (Cunningham et al. 2007b).

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