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Investigations into the Life History Stages of the Common Frog (*Rana temporaria*) Affected by an Amphibian Ranavirus in the United Kingdom

Ranaviruses are emerging infectious disease agents that affect a wide range of ectothermic and poikilothermic vertebrates: fish, reptiles (including turtles and tortoises) and amphibians (Ahne et al. 1997; Chinchar et al. 2009; Miller et al. 2011). In the United Kingdom (UK), amphibian ranaviruses began to emerge in the late 1980s and early 1990s in southeast England (Cunningham et al. 1996) and manifested as adult mass morbidity and mortality events (Cunningham et al. 1993; Cunningham et al. 1996; Drury et al. 1995).

Evidence for local ranavirus outbreaks in the UK have, to date, relied exclusively upon reports of moribund or dead adult common frogs (e.g. Cunningham et al. 1993; Cunningham et al. 1996; Drury et al. 1995; Teacher 2009; Teacher et al. 2009; Teacher et al. 2010). In the majority of the cases, few frogs were collected from each site and these were usually in poor condition and thus uninformative for post mortem examinations. The sole focus on adults leaves open the question of whether earlier life history stages are susceptible to and involved in ranavirus outbreaks in the UK, as they are in North America. For example, an outbreak of the Regina Ranavirus (RRV) affected both larval and adult Tiger Salamanders (*Ambystoma tigrinum*) in Saskatchewan, Canada (Bollinger et al. 1999). Susceptibility to ranavirus infection also changes throughout development (e.g., Haislip et al. 2011; Warne et al. 2011). Therefore, it is not appropriate to assume that all life history stages are equivalent.

In Common Frogs (*Rana temporaria*), it is unknown if ranavirus infections occur naturally in eggs or tadpoles, even in locations where there is a known history of outbreaks in adults. We attempted to establish if ranavirus infections are present in the eggs and tadpoles of Common Frogs and to confirm the presence of ranavirus infections in adult Common Frogs using molecular methods.

From April to June 2007, Common Frog tadpoles were collected from 15 ponds (N = 20/pond) distributed over a 40 km^2 area around central London, UK. Eight of the ponds were known to have a history of repeated ranavirus infections in adult Common Frogs and six of the ponds were historically free of ranavirus (see Teacher 2009, Teacher et al. 2009, and Teacher et al. 2010 for pond selection). One additional site had an unknown ranavirus infection history. No die-offs were apparent in any of these ponds during our collections. All of the sites are on privately owned land, so in order to maintain confidentiality we are unable to provide more detailed location information than is provided in Tables 1 and 2.

Live tadpoles were transported in a common container in pond water to the Institute of Zoology, Zoological Society of London, London, UK. Upon arrival, tadpoles were euthanized using an overdose of MS-222 (1g/L tricaine methanesulphonate, Thompson & Joseph Ltd., Norwich, UK) buffered to pH 7.0 with sodium bicarbonate. Tissue samples were then dissected out and frozen at -80°C for ranavirus screening. In the case of larger tadpoles, tissues included the right anterior quarter of the body, and in smaller individuals, the central half was used. This sampling method ensured that commonly used tissues (e.g., liver and kidneys) for ranavirus screening were contained in the sample. Instruments were disinfected with a Virkon solution (40%; Antec International Ltd, Suffolk, UK) then rinsed thoroughly in fresh water to avoid cross contamination.

In February and March 2008, portions of four freshly laid broods of eggs were collected from each of six locations (three historically ranavirus infected sites, including Ealing and Deal, and three historically ranavirus infection free sites, including Eltham 1 and Farham 1, as per Teacher 2009, Teacher et al. 2009, and Teacher et al. 2010). A sample of ≈ 120 eggs per clutch was taken and immediately preserved in 100% ethanol. From this, a subsample of 30 eggs per clutch was taken for screening for the presence of the major capsid protein (MCP) gene of frog virus 3. Egg jelly was removed from each egg prior to DNA extraction to remove potential surface contamination as per Duffus et al (2008).

Beginning in 2006 adult frogs from suspected ranavirus mortality events were collected with the cooperation of pond owners, FrogLife (Registered Charity No. 1093372 in England and Wales), and the South Essex Wildlife Hospital. All animals received by A.L.J.D. underwent complete post mortem examinations, with hepatic tissue taken for molecular testing for the presence of the major capsid protein (MCP) of frog virus 3. The carcasses are archived at the Institute of Zoology.

DNA from eggs, tadpole tissues and adult hepatic tissue were extracted using the Wizard SV96 Genomic DNA Purification System (Promega, Southampton, UK) then screened for the presence of *Ranavirus* DNA using the polymerase chain reaction (PCR) primers and procedure of Pearman et al. (2004) and reagents from Multiplex PCR kits (QIAGEN, Crawley, UK). All samples were screened twice to ensure the repeatability and accuracy of the results. Any ambiguous results were re-screened. Positive PCR and negative extraction controls were used throughout. Originally, positive controls consisted of DNA extracts from known positive animals (Teacher 2009), but later were DNA from pure ranavirus cultures from UK frogs (Duffus 2010). The PCR products were visualized on a 1% agarose gel stained with ethidium bromide and the presence of a 500bp band was considered diagnostic for the presence of ranaviral DNA. Confidence intervals on the prevalence of infection were calculated using the calculator at http://vassarstats.net.

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TABLE 1. Prevalence and infection rates of the ranavirus in Common Frog (*Rana temporaria*) tadpoles in the spring of 2007 in various locations in the south east of the United Kingdom.

Location	Gosner stages	Site ranavirus history	Number ranavirus positive	Number tested	Prevalence	CI
London, NW10	25–26	Negative	0	19	0	0–0.209
Fareham 1	30–32	Negative	0	20	0	0–0.201
Fareham 2	32	Positive	0	20	0	0–0.201
Fareham 3	33–36	Negative	0	20	0	0–0.201
Camden	27–34	Unknown	0	20	0	0–0.201
Eltham 1	26–27	Negative	0	20	0	0–0.201
Eltham 2	30–40	Positive	0	20	0	0–0.201
London, N12	27–40	Negative	0	20	0	0–0.201
Worthing	36–38	Positive	0	20	0	0–0.201
Dagenham	36–40	Positive	0	20	0	0–0.201
Deal	28–33	Positive	1	20	0.05	0.003–0.269
Ealing	35–39	Positive	0	20	0	0–0.201
Ladywell	30–40	Positive	0	20	0	0–0.201
Isleworth*	38–40	Positive	0	8	0	0–0.402
Tooting	37–41	Negative	0	20	0	0–0.201

CI = Confidence Interval. * Only 8 tadpoles were found at this site.

TABLE 2. Number of infections in adult *Rana temporaria* screened for the presence of the ranavirus from various location in the United Kingdom from 2006 to 2008.

Year	Location	Site ranavirus history	Number ranavirus positive	Number tested	Prevalence	CI
2006	Unknown Site A	Unknown	5	9	56%	0.227–0.847
	Unknown Origin	Unknown	0	5	0%	0–0.537
2007	Uxbridge	Unknown	0	20	0%	0–0.201
	Camden	Unknown	0	11	0%	0–0.321
	Cowden	Unknown	1	2	50%	0.027–0.973
	Barnet	Unknown	0	1	0%	0–0.945
	Arylesy	Unknown	0	13	0%	0–0.283
	Brighton	Unknown	2	4	50%	0.092–0.908
	Charton	Unknown	0	1	0%	0–0.945
	Unknown Origin	Unknown	0	2	0%	0–0.802
	Ealing	Positive	0	1	0%	0–0.945
	Bexhill-on-Sea	Negative	0	2	0%	0–0.802
	Dagenham	Positive	0	4	0%	0–0.604
	Eltham 2	Positive	0	2	0%	0–0.802
	Teddington	Positive	0	2	0%	0–0.802
2008	Bristol	Unknown	0	2	0%	0–0.802
	Lewes	Unknown	0	1	0%	0–0.945
	Peterborough	Unknown	0	1	0%	0–0.945
	Bournemouth	Unknown	0	1	0%	0–0.945
	Plymouth*	Unknown	2	2	100%	0.198–1
	Wokingham*	Unknown	2	2	100%	0.198–1
	Carshalton	Unknown	1	2	50%	0.027–0.973
	Preston†	Unknown	7	11	64%	0.261–0.796
	Whitstable†	Unknown	8	15	53%	0.274–0.777
	Unknown Origin	Unknown	3	3	100%	0.310–1
	Southampton	Unknown	1	1	100%	0.055–1

* Denotes that the positive result was done through isolation methods and confirmed with PCR.
† Denotes that the animals come from an unusual or mass mortality event.
CI = Confidence Interval.

Only one of the 288 tadpoles collected and screened for the presence of the MCP of FV3 was positive (Table 1). This tadpole was collected from Deal, a historically positive site where the owners intensively manage the amphibian population (i.e. actively bring in eggs and/or animals from other populations). This infection was confirmed using virus culture techniques (Duffus 2010). Unfortunately, no adult frogs were obtained from this site so we cannot ascertain whether adult frogs were concurrently or subsequently infected. No eggs screened positively for the MCP of FV3 (even those from historically positive sites such as Deal and Ealing) (N = 30 eggs/brood, 4 broods/site, 6 sites; 3 negative and 3 positive). In contrast, adult ranavirus infections were documented during all years of this study: 5 of 14 (36%) in 2006, 3 of 65 (5%) in 2007, and 24 of 41 (59%) in 2008 (Table 2). None of the frogs obtained from historically ranavirus positive sites were positive for FV3, presumably because of small sample sizes ($N \leq 4$), and hence low power to detect infections (Table 2). Animals of all three life history stages that were tested from the site in Ealing were negative for the virus, even though this site has a long history of ranavirus infection (Teacher 2009), although just a single adult animal was screened.

The extremely low prevalence of the ranavirus in Common Frog tadpoles was unexpected, as North American ranid populations seem to have persistent infections after a long history of ranavirus outbreaks (e.g., Duffus et al. 2008). Ranid tadpoles appear to be especially susceptible to infection with a variety of ranaviruses (e.g., several North American isolates; Hoverman et al. 2011; FV3 in Europe, Pearman et al. 2004; Pearman and Garner 2005). Since the susceptibility of ranid tadpoles to infection changes throughout development (e.g., Warne et al. 2011), it is possible that the sampling strategy of our study missed affected tadpoles. The tadpoles that were collected were Gosner stage 30–40 and individuals affected by the virus may have already succumbed to ranaviral disease. Future studies would benefit from sampling across developmental stages. Alternatively, there may have been very few infected tadpoles because of minimal opportunities for viral transmission from adults to tadpoles or between tadpoles. The concentration of ranavirus particles in water may not be sufficient to result in infection under natural conditions. The infection rate for some amphibian species under experimental conditions are known to be dose dependent (Brunner et al. 2005; Pearman et al. 2004).

The absence of infections in eggs is not wholly unexpected because the prevalence of ranaviral infection in ranid eggs is thought to be very low (see Duffus et al. 2008). Therefore, it is plausible that vertical or pseudovertical (where the offspring are infected by the parents but not directly through infected gametes) transmission occurs infrequently, if at all in Common Frogs.

Adult Common Frogs did test positive for infection in all years of this study. The apparent large increase in the number of infections in 2008 can be explained by an increase in sampling effort attributed to a joint campaign between the Zoological Society of London and FrogLife. The samples obtained were from frogs that had died and that pond owners themselves had submitted to the study. It is important to note that the number of ranavirus infected adults in this study does not necessarily represent the prevalence in the population where they had originated or of the true distribution of ranavirus infections in common frog populations in the UK. Although our samples of adults were small and unevenly distributed across the UK (primarily in the Southeast), it is clear that ranavirus infections are relatively common. Moreover, in many cases they were associated with unusual or large scale mortality events (e.g., Preston in the Northwest and Whitstable in the Southeast; unpubl. data).

The majority of the Common Frogs examined in this study were from historically ranavirus positive sites or from unusual mortality events that were thought to be associated with a ranavirus outbreak. However, the true extent of the distribution of ranavirus(es) in the UK needs to be determined with a structured and non-biased sampling regime that looks at multiple species and uses more sensitive molecular methods (e.g., quantitative real time PCR) to ensure that no infections are missed.

Our study is the first to address the possibility of ranavirus infections in Common Frogs affecting life history stages other than adults. While further investigations into the transmission, maintenance, and alternative hosts are required to fully understand viral dynamics, our results are consistent with the conclusion that ranavirus infections in Common Frogs in the UK predominantly affect adults. This ranavirus continues to cause mass mortality events more than 20 years since it began to emerge. A detailed understanding of the ecology and dynamics of this pathogen are essential if any conservation or management plans are to be successful.

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