NOTE

Herpesvirus skin disease in free-living common frogs *Rana temporaria* in Great Britain

L. H. V. Franklinos[1,4], J. Rodriguez-Ramos Fernandez[1,5], H. B. Hydeskov[1,2], K. P. Hopkins[1], D. J. Everest[3], A. A. Cunningham[1], B. Lawson[1,*]

[1]Institute of Zoology, Zoological Society of London, Regent's Park, London, NW1 4RY, UK

[2]Veterinary Epidemiology, Economics and Public Health, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, AL9 7TA, UK

[3]Animal Plant and Health Agency Weybridge, Addlestone, Surrey, KT15 3NB, UK

[4]*Present address*: University College London, Gower Street, London, WC1E 6BT, UK

[5]*Present address*: IDEXX Laboratories Limited, Grange House, Sandbeck Way, Wetherby, West Yorkshire, LS22 7DN, UK

ABSTRACT: Infectious disease is a significant driver of global amphibian declines, yet despite this, relatively little is known about the range of pathogens that affect free-living amphibians. Recent detection of the tentatively named *Ranid herpesvirus 3* (RHV3), associated with skin disease in free-living common frogs *Rana temporaria* in Switzerland, helps to address this paucity in knowledge, but the geographic distribution and epidemiology of the pathogen remains unclear. Syndromic surveillance for ranid herpesvirus skin disease was undertaken throughout Great Britain (GB), January 2014 to December 2016. Reports of common frogs with macroscopic skin lesions with a characteristic grey appearance were solicited from members of the public. Post-mortem examination was conducted on one affected frog found dead in 2015 at a site in England. In addition, archived samples from an incident involving common frogs in England in 1997 with similar macroscopic lesions were further investigated. Transmission electron microscopy identified herpes-like virions in skin lesions from both the 1997 and 2015 incidents. RHV3, or RHV3-like virus, was detected in skin lesions from the 2015 case by PCR and sequencing. Our findings indicate that herpesvirus skin disease is endemic in common frogs in GB, with widespread distribution at apparently low prevalence. Further research into the role of host immunity, virus latency and the significance of infection to host survival is required to better understand the epidemiology and impact of cutaneous herpesvirus infections in amphibian populations.

KEY WORDS: *Ranid herpesvirus 3* · Skin disease · Great Britain · Europe · *Rana temporaria* · Amphibian · Herpesvirus

INTRODUCTION

Over 70% of amphibian species are in decline worldwide, and multiple drivers are implicated, including infectious diseases, habitat loss, over-exploitation, pollution, pesticide use and introduced species (Hayes et al. 2010). In Great Britain (GB), long-term population declines of common frogs *Rana temporaria* have been reported in association with *Ranavirus* disease (Teacher et al. 2010). In addition,

the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been detected in British amphibians (Cunningham et al. 2005), but the conservation significance of *Bd* to amphibians in GB remains unknown. There is a paucity of knowledge about other infectious disease threats to British anurans and their possible impacts at both the individual and population level.

Ranid herpesviruses belong to the genus *Batrachovirus*, family *Alloherpesviridae* (Van Beurden & Engelsma 2012). There are currently 3 ranid herpesviruses reported (Essbauer & Ahne 2001). *Ranid herpesvirus 1* (RHV1) is the causative agent of Lucké renal carcinoma in North American leopard frogs *Rana pipiens* (Lunger 1964). *Ranid herpesvirus 2* (RHV2) was isolated incidentally during Lucké tumour investigations (Rafferty 1965). Experimental infection with RHV2 in leopard frog embryos and larvae led to mortality (Tweedell 1989) but did not induce neoplasia (Granoff 1999). Proliferative skin disease associated with herpesvirus infection in free-living frogs was first reported to affect the agile frog *Rana dalmatina* in Italy (Bennati et al. 1994) and has since been found to affect this species in Switzerland (A. A. Cunningham unpubl. data). Recently, Origgi et al. (2017) characterised herpesvirus infecting common frogs with similar skin lesions in Switzerland. Both Bennati et al. (1994) and Origgi et al. (2017) described intranuclear inclusion bodies consistent with herpesviral infection on histopathological examination of affected skin and herpesvirus-like virions on transmission electron microscopy (TEM). Origgi et al. (2017) used molecular characterisation to identify the virus as a novel herpesvirus, which they tentatively named *Ranid herpesvirus 3* (RHV3). No molecular characterisation was conducted by Bennati et al. (1994), so it is not known if RHV3 was the causative agent in *R. dalmatina*.

Current understanding of the geographical distribution and epidemiology of ranid herpesvirus skin disease in free-living common frogs in Europe remains limited. Here we describe a study in GB to address this knowledge gap through syndromic surveillance and disease investigation.

MATERIALS AND METHODS

Syndromic surveillance

Reports of morbidity and mortality in wild amphibians were received from members of the public as part of a scanning surveillance scheme across GB over the period January 2014 to December 2016 inclusive. Frogs with ranid herpesvirus skin disease are known to exhibit light-grey candle wax-like lesions with a widespread distribution over the dorsal and ventral body, with lesions apparently becoming flattened and dark grey over a period of weeks until they resolve completely (Fig. 1). A syndromic incident definition for ranid herpesvirus skin disease comprised a report of one or more common frogs with clinical signs of skin thickening with grey discolouration affecting the dorsal body surface. For inclusion, the report had to have photographic evidence of skin lesions verified by a veterinarian or the reporter had to confirm that the lesions closely resembled an example photograph of an affected frog (Fig. 1a). For each site where lesions consistent with herpesviral skin disease was confirmed, the number of affected common frogs observed, the habitat type (urban, suburban, rural; determined by subjective assessment relating to density of housing at location) and age of pond were summarised. Wild common frogs found dead with suspected herpesviral skin disease were submitted for post-mortem examination if in a reasonable state of preservation. The spatial and temporal distribution of ranid herpesvirus skin disease incidents was summarised over the study period. The home owners of incident sites in 2014 and 2015 were contacted by email 1 yr after their original report to obtain an indication of repeated occurrence.

Pathological investigations

Post-mortem examinations comprised systematic external and internal inspections of all body systems, combined with parasitological, microbiological and histopathological examinations. A suite of tissue samples, including skin, was taken for histopathological examination when the state of tissue preservation permitted. These samples were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) using routine methods. Additionally, a subset of sections were stained with Gram's, Ziehl-Neelsen (ZN), Masson's trichrome and periodic acid-Schiff (PAS) stains to screen for other/concurrent infectious agents that might be associated with the lesions.

If skin lesions were present, they were sampled and archived at −80°C. Unfixed samples were thawed and examined using negative contrast stain TEM as described by Everest et al. (2010). In addition, the aetiology of skin lesions in common frogs from an inci-
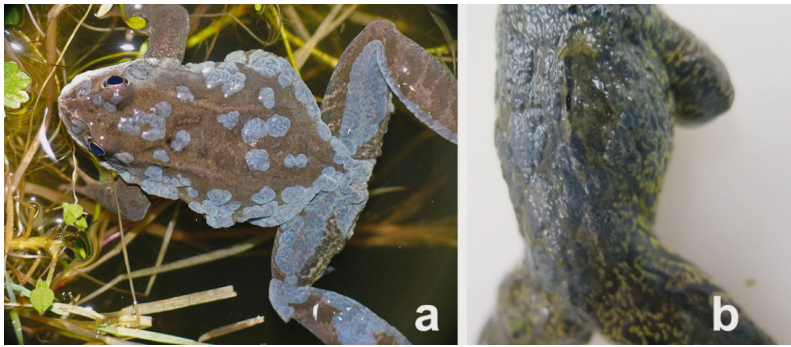
Fig. 1. Macroscopic lesions of herpesvirus-associated skin disease in (a) a live common frog showing skin thickening with grey-blue discolouration over the dorsal body surface and legs, and (b) a common frog examined post-mortem in 2015, with regressing skin lesions showing coalescing dark-grey-to-blue, well-demarcated areas of raised, thickened skin with a mixed irregular and smooth surface

dent that occurred in England in 1997 was further investigated. For these cases, a formalin-fixed skin lesion was retrieved from a paraffin-embedded block and processed for ultra-thin section TEM examination as described by Everest et al. (2018).

Molecular characterisation

DNA extraction was conducted using the DNeasy Blood & Tissue Kit (QIAGEN) for frozen skin lesions and using the GeneRead DNA FFPE Kit (QIAGEN) for the formalin-fixed paraffin-embedded skin lesion, following the manufacturer's instructions. DNA extractions were stored at −20°C prior to testing. Initially, a pan-herpesvirus PCR protocol using 10 degenerate and deoxyinosine-substituted primers was implemented as described by Ehlers et al. (1999) targeting a region of the DNA polymerase gene. DNA from *Bovine alphaherpesvirus 1* was used as a positive PCR control. Additionally, a PCR protocol targeting a partial sequence of the DNA polymerase gene of RHV3 was performed, as described by Origgi et al. (2017). The cycling reaction was carried out on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). PCR products were visualised under UV light on a 2% agarose gel: 2 µl loading buffer containing GelRed (Cambridge Bioscience) was added to 2 µl of each PCR product. A negative extraction control (reagents only with no tissue to confirm absence of contamination during the DNA extraction protocol) and a negative PCR control (molecular grade water to confirm absence of contamination during the PCR protocol) were also included. Bidirectional Sanger sequencing was conducted on PCR

amplicons of the anticipated size (circa 250 base pairs) (GENEWIZ) to confirm product identity.

RESULTS

Syndromic surveillance

During the period January 2014 to December 2016 inclusive, 484 opportunistic reports of common frog mortality or morbidity were received. Of these, 18 incidents from 16 sites complied with our syndromic definition of ranid herpesvirus skin disease. These incidents originated from 14 sites across GB, from a mix of urban, suburban and rural garden ponds. All ponds associated with these reports had been established for over 6 yr. All the incidents occurred from January to April inclusive and the majority of reports (10/18) were received in 2015. A total of 33 adult or subadult common frogs with skin lesions were observed across the sites. In most of the reports, only one individual was affected at each site (mode 1; range 1–6). Mortality was reported in 6 affected common frogs from 3 sites, including the frog submitted for post-mortem examination. Follow up data were available from 3 sites, 2 of which reported observations of similarly affected frogs in the consecutive year.

Pathological investigations

Post-mortem examinations were performed on a total of 177 common frogs during the study period. Of these, only 1 common frog had skin lesions consistent with herpesviral infection. The adult male common frog was found dead in March 2015 within an allotment in Greater London, England; the location of the nearest pond was unknown. Another dead common frog with similar skin lesions was seen at the same site within 2 wk of the first sighting but was decomposed and was not submitted for examination. At post-mortem examination, the submitted frog was subjectively assessed to be in good body condition on the basis of muscle mass and the presence of fat bodies. Approximately 40% of the body surface had several multifocal-to-coalescing, dark-grey-to-blue, shiny, well-demarcated areas of raised, thickened skin with a mixed irregular and smooth surface (Fig. 1b). The lesions extended over the dorsum and

ventrum with the dorsal and inguinal skin regions most severely affected. No other significant lesions were detected on gross examination. No significant findings were detected on parasitological or microbiological examination.

Histopathological examination of the skin lesions revealed multifocal, well-demarcated areas of epidermal, and sometimes dermal, hyperplasia up to 4 times thicker than unaffected skin, with sloughing of superficial keratinized cells (Fig. 2). Affected areas of skin contained mild infiltrates of granulocytes and lymphocytes and multifocal intra- and inter-cellular oedema. Some of the skin glands appeared to be mildly to moderately dilated with larger lumina and flattening of the lining epithelium compared to most skin glands examined; infrequently they contained sloughed cells and granulocytes. Almost all skin glands contained acellular amphophilic mucous-like material. In some areas, keratinocytes were moderately disorganised, but the maturation pattern was overall orderly. Stains for infectious agents (ZN, PAS, Gram's) revealed mixed bacteria and, rarely, fungal fragments admixed with sloughed cells on the skin surface. These organisms could be ante or post-mortem secondary invaders. In addition, there was multifocal and moderate granulocytic nephritis with small areas of necrosis. Some tissues were autolysed, which hindered interpretation.

Negative contrast stain TEM of skin lesions revealed low numbers of circular viral particles of approximately 100 nm diameter, which were morphologically consistent with *Herpesviridae.* Small numbers of pleomorphic membranous particulate structures were detected in the skin lesion sample homogenate analysed by negative contrast stain TEM. These particles had dark-centred cores, were all of a size (circa 100 nm diameter) and had a shape and surface morphology consistent with a herpes virus.

An archived report of amphibian disease obtained from a member of the public in March 1997 described a single site in Devon, England, with approximately 70 common frogs of which more than 50% were affected with abnormal grey discolouration and thickening of the skin. Four frogs (3 males, 1 female) from the site were examined post-mortem after being found dead or being euthanased. All frogs displayed multifocal-to-coalescing, dark grey, well-demarcated areas of thickened skin with an irregular and smooth surface affecting the head, limbs, dorsum and ventrum. One affected frog also had cloacal prolapse. Histopathological examination was performed on the skin lesions of 3 of these frogs. All had areas of epidermal hyperplasia, which was up to 7 times the thickness of the unaffected epidermis. This thickening was partially due to the presence of large and abundant intercellular spaces that often contained rounded keratinocytes, necrotic keratinocytes and amphophilic granular material. These intercellular spaces were mostly in the outer third of the epidermis. A few nuclei with an amorphous glassy appearance which might have been intranuclear inclusions were observed within the skin lesions of 2 of the frogs. TEM ultra-thin section examination revealed multiple aggregates of virions, with a size, shape and surface morphology consistent with herpesvirus, which were located both intra- and extra-cellularly (Fig. 3). The clustering of the virions on TEM was consistent with the glassy appearance of nuclei observed on histology being due to the presence of inclusion bodies.
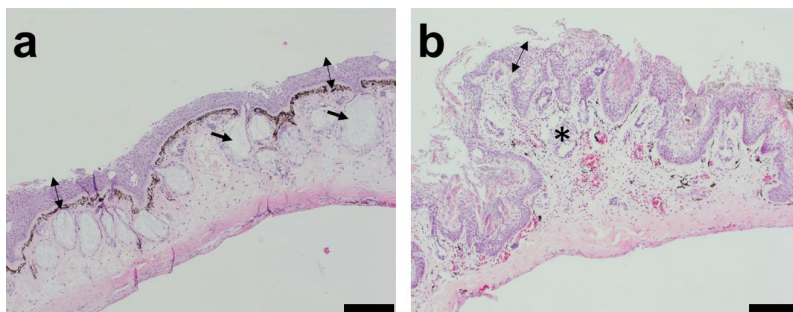

Fig. 2. Photomicrographs of (a) lesions of herpesvirus skin disease in the 2015 common frog case showing epidermal hyperplasia (double-headed arrows) and some glands that appear to be distended (arrows) (scale bar = 200 nm), and (b) microscopic lesions of herpesvirus skin disease in the same frog showing epidermal hyperplasia (double-headed arrow) and dermal hyperplasia and inflammation with oedema (asterisk) (scale bar = 200 nm). (H&E stain)

Molecular characterisation

All skin lesions tested from the 3 frogs sampled in 1997 and the frog sampled in 2015 were PCR-negative using the pan-herpesvirus protocol. Using the PCR protocol targeting RHV3, however, a 214 bp consensus sequence (Genbank MG800826) was derived from the skin lesion of the 2015 common frog with 100% sequence identity to that obtained by Origgi et al. (2017) (Genbank accession number KX832224). DNA extracted from formalin-fixed paraffin-em-
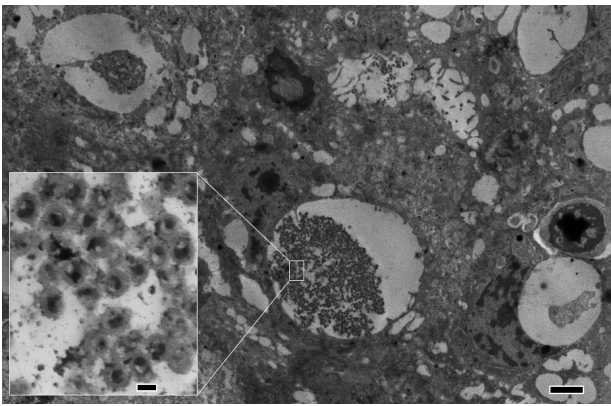
Fig. 3. Common frog from the 1997 incident. Ultra-thin section (90 nm) of a skin lesion displaying, within a cell, large numbers of virus particles with dark dense cores of approximately 100 nm diameter, typical of herpesvirus and surrounded by a membranous structure (scale bar = 500 nm). Inset: image of the same lesion at higher magnification (scale bar = 100 nm) showing virus particles within a cell

bedded skin lesions from the 3 frogs from 1997 did not amplify a PCR product. The extractions from these samples contained low concentrations (0.258–0.880 ng μl^{-1}) of DNA compared to the extracts that did amplify (77.6 ng μl^{-1}). An internal positive control amplified successfully, excluding PCR inhibition as the reason for the lack of amplification.

DISCUSSION

This study confirms ranid herpesvirus skin disease in free-living British common frogs based on sequence characterisation and electron microscopical examinations. To the best of our knowledge, this is the first molecular identification of RHV3, or RHV3-like, infection in a British amphibian. Whilst the sequence obtained had 100% identity to that derived from common frogs in Switzerland, given the small PCR product, further molecular characterisation is required to determine whether the British and Swiss RHV3 isolates are identical.

Syndromic surveillance of wild amphibians over a 3 yr period demonstrated a widespread spatial distribution of ranid herpesvirus skin disease in common frogs throughout GB and across habitat types. Whilst amphibian mortality incident reports were received throughout the season of amphibian activity (February to October), marked seasonality was noted with reports of ranid herpesvirus skin disease occurring only from January to April. These incidents account for a minority (3%) of all disease incident reports involving common frogs received over the study period.

Although herpes virions were detected on TEM examination of skin lesions in common frogs sampled in 1997, it was not possible to characterise the virus further. Origgi et al. (2017) detected the extracellular aggregation of mature viral particles as a unique feature of RHV3 infection. In this study, we detected virions within the intracellular space, but none were recognisable within the nucleus. In our case, the dissociation of adjacent keratinocytes that is required for particles to move into the extracellular space might not yet have occurred. The failure of the PCR to amplify viral DNA in these frogs could be because of low quantity or poor quality DNA resulting from formalin-fixation and/or long-term storage. However, the possibility that an alternative ranid herpesvirus lineage, not detected by the RHV3 primers, was present, cannot be excluded.

Histopathological examination of the 3 frogs sampled in 1997 identified epidermal hyperplasia in all cases but possible intranuclear viral inclusions were observed in only 2 frogs. Detection of the virus in histological samples might have been aided by immunohistochemical staining but, unfortunately, anti-RHV3 antibodies are currently unavailable. The development of *in situ* hybridization methods would aid localisation of the virus in tissues. On examination using TEM, intranuclear aggregates of virions consistent with viral inclusions were seen in these frogs. Intranuclear viral inclusions were observed by Bennati et al. (1994) with herpesvirus skin lesions in *R. dalmatina* and by Origgi et al. (2017) in *R. temporaria*. The apparent absence of intranuclear viral inclusions in the additional frog from 1997 and the frog examined in 2015 may be due to the state of carcass preservation or the late stage of infection when inclusions may be absent.

The clinical significance of herpesvirus skin disease remains unclear and requires further investigation. The cause of death in the common frogs examined post-mortem that had not been euthanased specifically to study the skin disease remains undetermined. Whilst the skin lesions were extensive, histopathological examination of the skin revealed microscopic abnormalities of mild to moderate severity and invoking only a mild inflammatory response, similar to that described by Origgi et al. (2017). The clinical significance of the disease, therefore, is unclear, but it would appear that the lesions regress over the course of the spring and summer in most affected frogs. Also, the disease occurs perennially within breeding populations (this study, Origgi et al. 2017, A. A. Cunningham unpubl. data), although it is not known if individual animals are affected multi-annually as seen for other alloherpesviruses that can

form long-term latent infections (Hanson et al. 2011). The disease is reminiscent of carp pox (a proliferative skin disease caused by *Cyprinid herpesvirus 1*) which causes raised irregular 'candle wax-like' grey lesions over the skin of carp (*Cyprinus* spp.) in the spring (McAllister et al. 1985).

As with carp pox (McAllister et al. 1985), the seasonal spring occurrence of herpesviral skin disease in the common frog might be a result of immune suppression. This may be associated with overwintering or breeding activity (Guillette, Cree & Rooney 1995, Leary et al. 2008), low ambient water temperature (McKinnell & Carlson 1997), in addition to a possible immunomodulatory effect of the virus (Origgi 2007, Origgi et al. 2017). In carp pox, the skin disease is generally benign to adult fish and this also appears to be the case for herpesvirus skin disease in the common frog, in which the majority of affected animals appear to recover (present study, A. A. Cunningham unpubl. data). The affected 2015 frog was in good body condition, therefore, there is no implication that the skin lesions had led to general debility. The animal had mild nephritis, which might have contributed to its death. Mortality was reported in a further 5 individuals with skin lesions from different sites, but since they were not available for examination it is not known if herpesvirus infection contributed to their death.

Our findings suggest that ranid herpesvirus skin disease is established and widespread in the common frog in GB, but at apparently low prevalence. Despite herpesviruses being well-studied in mammals, there remains a relative paucity of knowledge of this group of viruses in lower vertebrate hosts (Van Beurden & Engelsma 2012). Further research into the role of host immunity, latency and the possibility of reservoir hosts is required to better understand the epidemiology and impact of herpesvirus infections in wild amphibians. The clinical and population impacts, if any, of herpesvirus infections in non-adult amphibians also need to be ascertained.

LITERATURE CITED

Bennati R, Bonetti M, Lavazza A, Gelmetti D (1994) Skin lesions associated with herpesvirus-like particles in frogs (*Rana dalmatina*). Vet Rec 135:625–626

Cunningham AA, Garner TW, Aguilar-Sanchez V, Banks B and others (2005) Emergence of amphibian chytridiomycosis in Britain. Vet Rec 157:386–387

Ehlers B, Borchers K, Grund C, Frölich K, Ludwig H, Buhk HJ (1999) Detection of new DNA polymerase genes of known and potentially novel herpesviruses by PCR with degenerate and deoxyinosine-substituted primers. Virus Genes 18:211–220

Essbauer S, Ahne W (2001) Viruses of lower vertebrates. J Vet Med B Infect Dis Vet Public Health 48:403–475

Everest DJ, Stidworthy MF, Milne EM, Meredith AL and others (2010) Retrospective detection by negative contrast electron microscopy of faecal viral particles in free-living wild red squirrels (*Sciurus vulgaris*) with suspected enteropathy in Great Britain. Vet Rec 167:1007–1010

Everest DJ, Shuttleworth CM, Grierson SS, Dastjerdi A and others (2018) The implications of significant adenovirus infection in UK captive red squirrel (*Sciurus vulgaris*) collections: how histological screening can aid applied conservation management. Mamm Biol 88:123–129

Granoff A (1999) Amphibian herpesviruses (Herpesviridae) In: Granoff A, Webster R (eds) Encyclopedia of virology. Academic Press, London, p 51–53

Guillette JLJ, Cree A, Rooney AA (1995) Biology of stress: interactions with reproduction, immunology and intermediary metabolism. In: Warwick F, Murphy J (eds) Health and welfare of captive reptiles. Chapman & Hall, London, p 32–81

Hanson L, Dishon A, Kotler M (2011) Herpesviruses that infect fish. Viruses 3:2160–2191

Hayes TB, Falso P, Gallipeau S, Stice M (2010) The cause of global amphibian declines: a developmental endocrinologist's perspective. J Exp Biol 213:921–933

Leary CJ, Garcia AM, Knapp R (2008) Density-dependent mating tactic expression is linked to stress hormone in woodhouse's toad. Behav Ecol 19:1103–1110

Lunger PD (1964) The isolation and morphology of the Lucké frog kidney tumor virus. Virology 24:138–145

McAllister PE, Lidgerding BC, Herman RL, Hoyer LC, Hankins J (1985) Viral diseases of fish: first report of carp pox in golden ide (*Leuciscus idus*) in North America. J Wildl Dis 21:199–204

McKinnell RG, Carlson DL (1997) Lucké renal adenocarcinoma, an anuran neoplasm: studies at the interface of pathology, virology, and differentiation competence. J Cell Physiol 173:115–118

Origgi FC (2007) Reptile immunity. In: Jacobson E (ed) Infectious diseases and pathology of reptiles. CRC Press, Boca Raton, FL, p 131–166

Origgi FC, Schmidt BR, Lohmann P, Otten P and others (2017) *Ranid herpesvirus 3* and proliferative dermatitis in free-ranging wild common frogs (*Rana temporaria*). Vet Pathol 54:686–694

Rafferty KA (1965) The cultivation of inclusion-associated viruses from Lucké tumor frogs. Ann NY Acad Sci 126:3–21

Teacher AGF, Cunningham AA, Garner TWJ (2010) Assessing the long-term impact of ranavirus infection in wild common frog populations. Anim Conserv 13:514–522

Tweedell KS (1989) Herpesviruses: interaction with frog renal cells. In: Ahne W, Kurstak E (eds) Lower vertebrates. Springer, Berlin, p 13–29

Van Beurden S, Engelsma M (2012) Herpesviruses of fish, amphibians and invertebrates. In: Magel G, Tyring S (eds) Herpesviridae — a look into this unique family of viruses. InTech, Vancouver, p 217–242