CHAPTER 1

Viral and Bacterial Diseases of Amphibians

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I. INTRODUCTION

At least six groups of viruses have been reported to affect amphibians, including caliciviruses, herpesviruses, and iridoviruses (Johnson and Wellehan 2005). Only ranaviruses are known to cause widespread mass mortality and have been studied in detail; hence a review of this group of viruses forms the majority of this chapter. Various strains of ranavirus are found worldwide and some appear to have spread recently (Hyatt *et al.* 2000). Indicative of the broad host range of ranaviruses and their potentially devastating effects, ranaviral disease was listed by the World Organization for Animal Health (OIE) as an internationally notifiable disease in 2008. Although their impacts on populations of declining species are a concern, there is currently no evidence that they have caused permanent declines or extinctions (Daszak *et al.* 2003). Nevertheless, because of their potential impacts on naïve populations, as well as on species that are facing multiple threats, it is important that the risk of spreading these pathogens is minimized (Cunningham *et al.* 2007a).

There have been few investigations into other amphibian viruses. Apart from Frog Erythrocytic Virus (FEV), their impact on wild populations has not been studied. Lucké tumor herpesvirus has been well described, but the other viruses found associated with disease in amphibians have been reported in single papers with little or no experimental work. Their significance as pathogens of amphibians is therefore largely unknown. In addition other viruses, not reviewed here, such as arboviruses (including West Nile Virus), retroviruses and an adenovirus, can infect frogs but their pathogenicity to amphibians is low or unknown (Densmore and Green 2007).

There are no substantiated reports of bacteria causing outbreaks in wild frogs, and cases of bacterial disease were rare during large surveys for disease in the United States and Australia (Berger 2001; Green *et al.* 2002). Bacterial diseases, including septicaemia, are associated with significant mortality in captivity and are associated with poor husbandry. For details on prevention, management and treatment of bacterial diseases in captivity see Wright and Whitaker (2001). Zoonotic bacteria carried by wild and captive amphibians with minimal effect on themselves, but which are potential risk to humans (e.g., *Salmonella* and *Leptospira*), are not included in the present review.

II. VIRAL DISEASES

A. Ranaviruses

A recent spate of research on ranaviruses was stimulated by the interest in global amphibian declines (Chinchar 2002). It built on earlier work that initially arose out of the discovery of ranaviruses in Lucké tumor research (Granoff *et al.* 1969), then fish mortalities (Langdon 1989), and subsequently their possible use as a biological control agent for the cane toad (*Bufo marinus*) in Australia (Speare 1990; Pallister *et al.* 2007). Ranaviruses are significant infectious agents that can cause great mortality in free-living amphibian populations (Cunningham *et al.* 1996) although they are not currently believed to be responsible for amphibian declines (Daszak *et al.* 2003). Amphibian ranaviruses are enveloped icosahedral DNA viruses in the family Iridoviridae (Hengstberger *et al.* 1993). Isolates causing disease have been found in wild and cultured amphibians in Australia (Speare and Smith 1992), the Americas (Wolf *et al.* 1968; Zupanovic *et al.* 1998b; Majji *et al.* 2006), Asia (He *et al.* 2001; Zhang *et al.* 2001; Weng *et al.* 2002), and Europe (Cunningham *et al.* 1996). These include Tadpole edema virus, Frog virus 3, *Rana catesbeiana* virus Z, *Ambystoma tigrinum* virus, Bohle iridovirus, and UK ranavirus.

In North America, ranaviruses are responsible for massive mortality in amphibian larvae and recent metamorphs, while die-offs rarely occur in adults (Green *et al.* 2002). These ranavirus-induced mortality events often occur during summer and involve hundreds to thousands of moribund and dead larvae within a few days (Green *et al.* 2002). Additionally, adult amphibians can be chronically infected carriers, maintaining infection in a population (Wolf *et al.* 1969; Brunner *et al.* 2004; Robert *et al.* 2007). In contrast, ranaviral disease in the United Kingdom typically causes massive, synchronous annual mortality of adult frogs (Drury *et al.* 1995). Molecular work suggests there has been recent spread of ranaviruses both locally and globally. For instance, the strains that occur in the United Kingdom and in captive-breeding facilities worldwide may have originated from North America (Hyatt *et al.* 2000)

1. Taxonomy and Molecular Epidemiology

Currently there are six commonly recognized species of ranavirus (ICTVdB Management 2006), three of which were isolated from amphibians and the others from fish. Species of ranavirus are differentiated on the basis of genetic sequences, particularly the sequencing regions of the major capsid protein (Mao *et al.* 1997, Hyatt *et al.* 2000), but also by restriction fragment length polymorphism (RFLP) profiles and by DNA hybridization (Hyatt *et al.* 2000), virus protein profiles, and the range and specificity of the host (Hyatt *et al.* 2000, ICTVdB Management 2006). However, while isolates of the same species are largely homologous, or even identical in MCP sequence (≥ 95% sequence identity), they can be quite distinct in RFLP profiles (Hyatt *et al.* 2000; Majji *et al.* 2006; Schock *et al.* 2008), host range, and virulence (Brunner, unpublished data), as well as in tissue tropisms (Cunningham *et al.* 2007).

Frog Virus 3 (FV3), the type ranavirus, was isolated from aclinically infected leopard frogs (*Rana pipiens*) collected in the United States in 1962 (Granoff *et al.* 1965). Since then, FV3 or FV3-like viruses, such as Tadpole edema virus (TEV), *Rana catesbeiana* virus Z (RCV-Z), and UK Ranaviruses (RUK, BUK), have been associated with amphibian mortality in North America (Wolf *et al.* 1968, Wolf *et al.* 1969, Petranka *et al.* 2003, Greer *et al.* 2005, Bank *et al.* 2007, Majji *et al.* 2006, Schock *et al.* 2008), South America (Zupanovic *et al.* 1998a, 1998b), the United Kingdom (Drury *et al.* 1995), and Southeast Asia (Kanachanakhan 1998, Zhang *et al.* 2001).

The second distinct amphibian ranavirus species to be discovered was Bohle Iridovirus (BIV), isolated from newly metamorphosed ornate burrowing frogs (*Limnodynastes ornatus*) in Queensland, Australia (Speare and Smith 1992; Hengstberger *et al.* 1993). It remains the only isolate of this species although subsequently ill wild *Litoria caerulea* had positive PCR for BIV, but the virus could not be isolated (Cullen and Owens 2002). Antibodies against ranaviruses were detected in cane toads throughout most of their range in Australia at an overall prevalence of 2.7% (range 0-18%). The identity of the ranavirus that induced the antibodies is unknown since the test is not species specific and no viruses were isolated from any cane toads (Zupanovic *et al.* 1998b).

Lastly, the *Ambystoma tigrinum* virus (ATV) was isolated from a threatened sub-species of tiger salamander (*Ambystoma tigrinum stebbinsi*) in southern Arizona, USA in 1995 (Jancovich *et al.* 1997). Similar ATV-like viruses have been isolated from many locations in western North America (Bollinger *et al.* 1999; Jancovich *et al.* 2005; Ridenhour and Storfer 2008), but not elsewhere.

Ranaviruses can cause aclinical infections in resistant animals, which may facilitate the spread of disease via the inadvertent movement of infected animals (Robert *et al.* 2007). The trade of amphibians for food, research, and as pets has likely played a role in the movement of pathogens such as ranaviruses both within and among continents (Cunningham and Langton 1997; Jancovich *et al.* 2005; Galli *et al.* 2006; Picco and Collins 2008).

Phylogenetic analyses of ATV isolates based on sequence from the MCP and DNA methyltransferase genes, as well as two non-coding regions, suggest a single introduction and radiation of these salamander ranaviruses, with little genetic divergence among isolates (<1.1%), but a rather complex phylogeography (Jancovich *et al.* 2005). Nested-clades analyses suggest long-distance dispersal. One clade encompassed isolates from southern Arizona to Saskatchewan, as well as isolates from the bait trade and one from the axolotl (*Ambystoma mexicanum*) colony at Indiana University (Jancovich *et al.* 2005). ATV has been frequently isolated from tiger salamander larvae used as fishing bait (Picco and Collins 2008) and so at least some of these dispersals are probably due to moving infected bait. An analysis of concordance between the phylogenies of ATV and the tiger salamander host showed an overall lack of congruence, but when three isolates of presumably anthropogenic origins were excluded, the trees of the host and virus were identical, indicating co-evolution between host and parasite (Storfer *et al.* 2007).

Interestingly, the UK ranavirus from common frogs (*Rana temporaria*), RUK, is phylogenetically similar to the FV3-like viruses from North America which, in addition to the fact that animals with signs of ranavirus infection were not observed before the mid-1980s, suggests a recent introduction of the virus into the UK (Hyatt *et al.* 2000; Cunningham *et al.* 2007a). Recently an FV3-like virus was identified during mortality events in natural populations of the endemic *Atelognathus patagonicus* in Argentina (Fox *et al.* 2006). It showed 100% identity with the original FV3 isolate across 500bp of the MCP gene, which is consistent with a recent introduction from North America.

2. Biology

Ranaviruses are composed of linear, double-stranded DNA genome encoding ~ 100 proteins (Williams *et al.* 2000) encapsulated in an icosahedral particle ~130 nm in diameter

with an internal lipid membrane. The capsid may or may not be enveloped (Braunwald *et al.* 1979) but both forms are infective (Chinchar 2002). Ranaviruses cause disease involving multiple organs and tissues in fish, amphibians, and reptiles (Chinchar 2002). The virus attaches to an as yet unidentified, but apparently widely distributed, receptor on host cells. Enveloped virions enter via receptor-mediated endocytosis, losing their envelope and releasing their nucleoprotein core. If the virus is naked, it enters the cell via fusion between the internal lipid membrane and the plasma membrane (Chinchar 2002). Early events in virus replication occur in the cell's nucleus, including transcription of early viral mRNAs and replication of copies of one-unit to two-unit lengths of the viral genome. Late viral mRNAs seem to be transcribed in the cytoplasm (Chinchar 2002). Viral assembly occurs at distinct assembly sites in the cell and the newly formed virions either bud off from the cell or, more commonly, accumulate in the cell until the cell lyses (Goorha and Granoff 1978). The machinery and metabolism of infected cells are rapidly diverted into viral replication (Murti *et al.* 1985a,b; Willis *et al.* 1985; Goorha and Granoff 1999; Chinchar 2002).

Ranavirus particles are environmentally resistant and can remain infectious for long periods in certain environments. Studies of a fish ranavirus (EHNV) showed that it remained infective after drying for over 100 days at 15°C (Langdon 1989). Heating to 60°C for 15 minutes or 40°C for 24 hours inactivated the virus. Brunner *et al.* (2007), however, found that ATV was rendered non-infectious when dried in pond substrate and viral titres declined dramatically in ATV-spiked pond water. Ultraviolet radiation from aquaculture UV water-sterilizers killed BIV rapidly at high flow rates (Miocevic *et al.* 1993).

FV3 replicates optimally in culture between 12°C and 32°C, although it can replicate to some extent both below and above this range (Goorha and Granoff 1974). BIV had a thermal limit of 33°C, above which it would not grow. It was capable of infecting fish and mammalian cell lines (if maintained at <34°C) but not insect cell lines in vitro (Speare and Smith 1992). The Venezuelan Ranavirus replicated readily at temperatures ranging from 18°C to 30°C (Zupanovic *et al.* 1998b).

3. Clinical Signs and Pathology

The gross pathology of ranaviruses appears similar to that associated with bacterial septicaemia. Since opportunistic or resident bacteria can often be cultured from dead or sick frogs, it appears that many ranaviral outbreaks have been misdiagnosed as septicaemia (Green 2001; see also "Bacterial septicaemia 'red leg'" in the "Bacterial Diseases" section below). For a more thorough treatment of ranavirus pathology see Wright and Whitaker (2001).

Frog Virus-3: Frog virus-3 (FV3) was found while searching for a viral cause of the Lucké tumour in *Rana pipiens* in the United States (Granoff *et al.* 1969). Subsequently, much work was carried out on the morphology and life cycle of FV3 in the laboratory, and experimentally it was shown to cause oedema, necrosis, haemorrhage, and death in embryos, tadpoles, and recent metamorphs (Granoff 1989). During experimental infections, metamorphic toads developed haemorrhages, accompanied by massive oedema, in the ventral skeletal musculature, stomach, and intestines (Came *et al.* 1968). In addition, tadpoles of the Italian agile frog (*Rana latastei*) exposed to FV3 sometimes became emaciated (Pearman *et al.* 2004). Mortality in embryos can occur 3-12 days post-exposure with 40-70% survivorship of tadpoles (Tweedell and Granoff 1968). Clinical signs include depigmentation, skin sloughing, and spinal curvature. Generally, the lesions caused by FV3 appear to be milder than those caused by Tadpole oedema virus (Green 2001).

Tadpole Edema Virus: Tadpole Edema Virus (TEV) was first isolated from oedemic *Rana catesbeiana* tadpoles from West Virginia, USA in August 1965 (Wolf *et al.* 1968, 1969). Molecular evidence suggests that TEV is a strain of FV3 (Hyatt *et al.* 2000). Acute fatal disease was reproduced with experimental transmission to tadpoles and adults, although pathogenicity varied among host species (Wolf *et al.* 1968). Terminal changes in tadpoles are obvious oedema, particularly in the ventral regions, and haemorrhaging along the body

and hind limbs (Fig. 1) (Green 2001). Internally, haemorrhages and necrosis typically extend into the stomach, intestines, and skeletal muscles, and may include the mesonephros, bladder, liver, lungs, and spleen (Green 2001). The highest titres of the virus occur in the stomach (Clark *et al.* 1969; Green 2001).

UK Ranavirus: While molecular studies suggest UK ranaviruses are strains of FV3 and may have originated in North America (Hyatt *et al.* 2000), theses viruses are not highly pathogenic to embryos and tadpoles (Cunningham *et al.* 1996, Cunningham *et al.* 2007a). There are several distinct "syndromes" associated with the UK ranaviruses including an "ulcerative syndrome," "haemorrhagic syndrome," and "ulcerative and haemorrhagic

Fig. 1. Ventral view of a moribund larval California red-legged frog (*Rana draytonii*) with oedema and petechial haemorrhages in the hind legs and inguinal region typical of TEV infection. Photograph by Valentine Hemingway.

syndrome" (Cunningham *et al.* 1996). While these syndromes are associated with infection by the bacterium *Aeromonas hydrophila*, Cunningham *et al.* (2007a) demonstrated that bacteria were not the cause. Frogs with the "ulcerative syndrome" have skin ulcerations and, to a lesser extent, necrosis of the legs, while systemic haemorrhages, particularly in the myoskeletal, digestive, renal, and reproductive organs, lungs, and pancreas characterize the "haemorrhagic syndrome" (Cunningham *et al.* 1996, Green 2001). Frogs with either or both syndromes were thin and some experienced lethargy (Cunningham *et al.* 1996). Histological lesions typical of "ulcerative syndrome" include epidermal thickening, and epidermal necrosis, as well as necrosis, granulocytic inflammation, congestion, and haemorrhage in internal organs (Cunningham *et al.* 1996). Experimentally infected *Rana temporaria* died six to eight days post-infection (Cunningham *et al.* 2007b).

Bohle Iridovirus: The only virus to be isolated from Australian frogs, Bohle iridovirus (BIV), was first found in *Limnodynastes ornatus* that died during metamorphosis in captivity (Speare and Smith 1992). The frogs had been collected as tadpoles from a temporary pond at Bohle, a suburb of Townsville, Queensland. BIV and FV3 appear to be closely related (Hengstberger *et al.* 1993). Clinical signs of the Bohle iridovirus were oedema of subcutaneous tissue, especially around the jaw and head, and a swollen abdomen due to ascites (Fig. 2). Subcutaneous haemorrhages occurred on the ventral abdomen, inguinal areas. and lower jaw (R. Speare, unpublished data). Typical pathology in natural and experimental infections included severe renal, pulmonary, hepatic, splenic, and haemopoietic

necroses and haemorrhages. Ranavirus immunoperoxidase stained many cell types in liver, lung, spleen and, in particular, fibrocytes in extensive areas of swollen, necrotic dermis and glomeruli (Cullen *et al.* 1995, L. Berger and R. Speare, unpublished data). In experimental infections, high mortality rates in juvenile frogs typically occurred within 5 to 25 days, depending on dose and type of exposure, but adults were less susceptible (Cullen and Owens 2002). Chronic cases were detected by PCR (Cullen and Owens 2002).

Ambystoma tigrinum Virus: Experimental infections of *Ambystoma tigrinum* virus (ATV) in tiger salamanders caused 40 to 100% mortality within two to three weeks (Brunner *et al.* 2005). Infected animals may show haemorrhaging in internal organs as well as white skin polyps, skin sloughing with mucus and ulcers, bloody mucus from the cloaca, and lethargy, and may refuse food, while others may die without clinical signs (Fig. 3) (Jancovich *et al.*

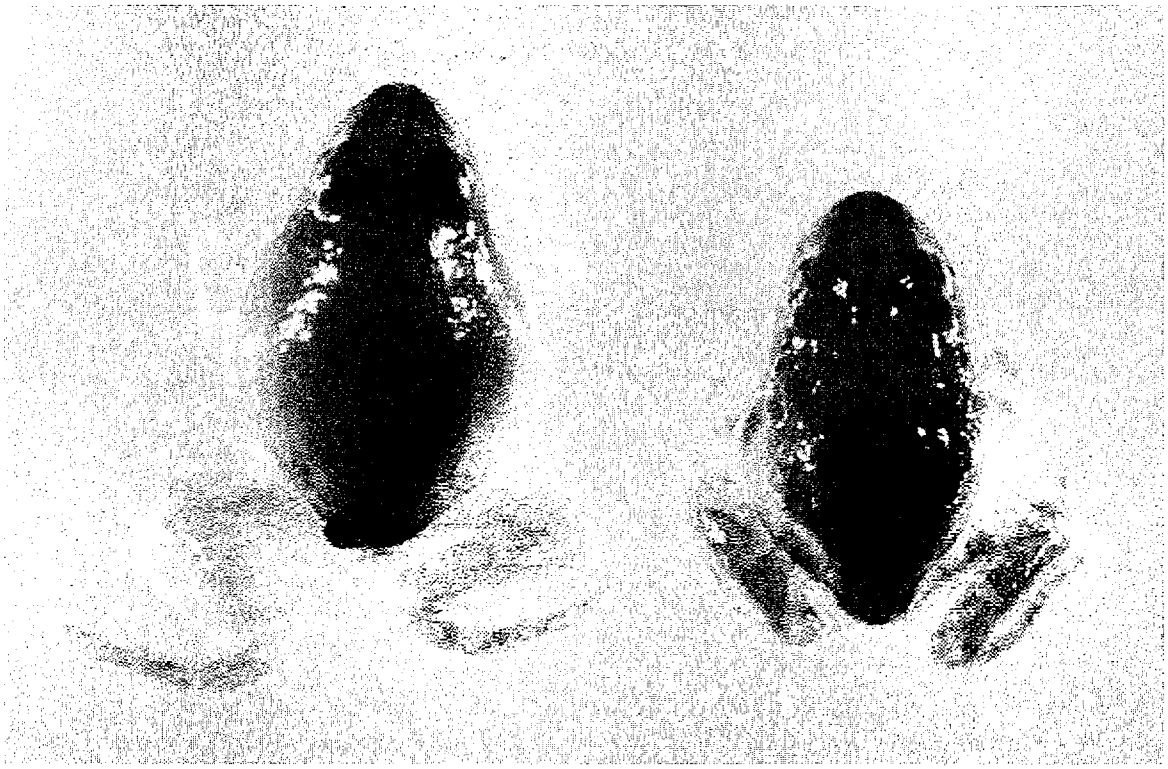

Fig. 2. Metamorphs of *Limnodynastes ornatus* with ascites. This frog died from Bohle Iridovirus during the original outbreak from which the virus was first isolated. Photograph by Rick Speare.

1997; Chinchar 2002). Further, infected lung, skin, and liver cells are larger than are uninfected cells (Jancovich *et al.* 1997). Infected animals may be able to maintain chronic, sublethal or aclinical infections, likely contributing to maintenance of infection in the population (Brunner *et al.* 2004). Mortality in wild populations occurs primarily in larvae in the northern part of their range and in larvae and neotonic adults in the southern part of the salamander's range (J. Brunner, unpublished data).

Croatian Ranavirus: A ranavirus-like agent called "viral haemorrhagic septicaemia of frogs" was found in dying captive *Rana esculenta* from Croatia that had lethargy, oedema, haemorrhages, and skin necrosis (Fijan *et al.* 1991).

Rana catesbeiana Virus Z: A ranavirus similar to other ranaviruses, such as FV3, was isolated from cultured *R. catesbeiana* tadpoles in the USA (Majji *et al.* 2006). *Rana catesbeiana* virus Z (RCV-Z) appears to be much more pathogenic than FV3, causing mortality in 50-100% of exposed tadpoles (Majji *et al.* 2006). Similar to other ranaviruses, symptoms

included oedema in the abdomen, haemorrhaging in ventral regions, and lethargy (Majji *et al.* 2006).

Venezuelan Ranavirus: Seven ranaviruses with strong similarities to BIV and FV3 were isolated from wild-caught *Bufo marinus* and *Lepodactylus* spp. in Venezuela (Zupanovic *et al.* 1998b). Infected animals had no external lesions or internal symptoms (Zupanovic *et al.* 1998b).

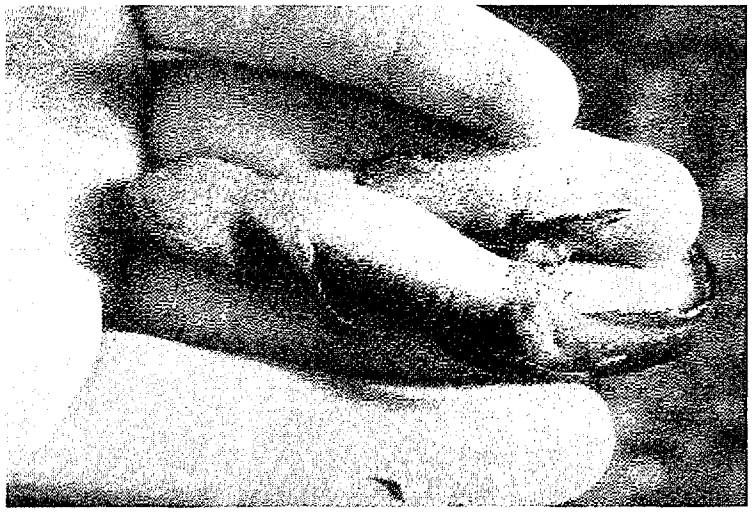

Fig. 3. Moribund ATV-infected larval tiger salamander (*Ambystoma tigrinum*) with oedema of the hind legs and with petechial haemorrhages on the jaw, inguinal region, legs, and tail. Photograph by Jesse Brunner.

4. Epidemiology

Impacts on Populations: While the first ranaviruses were isolated in the 1960s (Granoff *et al.* 1965; Wolf *et al.* 1968), it was not until the 1990s that ranaviruses were recognized as causing mass mortality in wild populations of amphibians (ATV: Jancovich *et al.* 1997; RUK: Drury *et al.* 1995). Ranaviruses have since been identified as the agents of mass mortality events around the world. Most outbreaks of amphibian ranaviruses have been reported from North America and the United Kingdom, although this may represent a bias in reporting rather than the actual distributions.

Ranavirus-associated die-offs are characterized by a rapid onset with massive mortality involving nearly all of the individuals in a pond (Green *et al.* 2002); entire cohorts can be killed (Petranka *et al.* 2003; Brunner *et al.* 2004). Die-offs also tend to recur in the same ponds or wetlands for several years in a row (Green *et al.* 2002; Cunningham *et al.* 2007a; Petranka *et al.* 2007; Greer and Collins 2008). Whereas ranaviruses tend to cause recurrent mortality, they are not associated with catastrophic amphibian declines (Green *et al.* 2002; Daszak *et al.* 2003).

Green *et al.* (2002) found that the majority of amphibian die-offs (25 of 44 investigated) in the United States were caused by iridoviruses, presumably ranaviruses. ATV has a wide distribution in North America, having been isolated from tiger salamanders during die-offs throughout the western cordillera and central plains (Bollinger *et al.* 1999; Green *et al.* 2002; Jancovich *et al.* 2005). Similarly, FV3-like viruses have been associated with amphibian die-offs — generally involving anurans but also spotted salamander larvae (*Ambystoma maculatum*) and adult eastern spotted newts (*Notophthalmus viridescens*) (Petranka *et al.* 2003; Duffus *et al.* 2008) — across North America (Mao *et al.* 1999; Green *et al.* 2002; Petranka *et al.* 2003; Greer *et al.* 2005; Gray *et al.* 2007; Duffus *et al.* 2008; St. Amour *et al.* 2008; Schock *et al.* 2008).

FV3-like ranavirus caused recurrent, catastrophic die-offs of larval *Ambystoma maculatum* and *Rana sylvatica* in a network of natural and constructed ponds and wetlands in North Carolina from 1997, when they were first detected (although monitoring had begun in 1994), until at least 2006 (Petranka *et al.* 2003, 2007). In most years well over half of the

ponds surveyed experienced die-offs. Juvenile recruitment was dramatically affected by these die-offs, as well as by droughts, which led to a reduction in breeding activity in later years (Petranka *et al.* 2007). Populations of both species, however, remained extant in this complex. It is worth noting that die-offs and amphibians with signs consistent with ranavirus infection have not been observed in other ponds and wetlands in the region (Harp and Petranka 2006), suggesting that outbreaks of FV3 can be highly localized.

Populations of *Ambystoma tigrinum nebulosum* in northern Arizona and the endangered *A. tigrinum stebbinsi* in southern Arizona have experienced recurrent, catastrophic die-offs since at least the mid-1980s (Collins *et al.* 1988); these events were later attributed to the ranavirus, ATV (Jancovich *et al.* 1997), yet these population persist (Greer and Collins 2008). At the level of a pond, however, ranavirus epidemics may cause local extinctions; tiger salamanders were apparently extirpated in some ponds in Saskatchewan after 3 to 4 years of ATV-related die-offs (Carey *et al.* 2003).

Unlike ATV, which has only been found in central and western North America (Jancovich *et al.* 2005), and BIV, which has been detected in the wild only once, in Queensland, Australia (Speare and Smith 1992; Hengstberger *et al.* 1993), FV3 is globally distributed. FV3-like viruses have also been reported from dead and moribund amphibians in captive populations in North America (Robert *et al.* 2007; Miller *et al.* 2008) and in frog farms in Brazil and Uruguay (Galli *et al.* 2006), Thailand (Kanachanakhan 1998), and China (Zhang *et al.* 1999).

The most extensive amphibian ranavirus epidemics have occurred in the United Kingdom since the mid 1980s, where FV3-like ranaviruses have caused annual mass die-offs of common frogs (*Rana temporaria*) and common toads (*Bufo bufo*) in garden-ponds across much of the country, but focused in the south and east (Drury *et al.* 1995; Cunningham *et al.* 1996; Cunningham *et al.* 2007a,b). FV3-like ranaviruses were the putative cause of these die-offs (Drury *et al.* 1995; Cunningham *et al.* 2007a) affecting tens of thousands of frogs across the United Kingdom but, again, there is little evidence of population declines (Daszak *et al.* 2003). Still, as Daszak *et al.* (2003) argued, the potential for ranaviruses to impact amphibian populations should not be discounted.

There are at least two reasons why populations may remain extant, or even abundant, in the face of high ranaviral mortality. First, natural mortality of tadpoles is normally very high due to predation and to drying of ponds but only a small fraction of the individuals need to survive in order for adequate recruitment to occur. Ranaviruses may, therefore, cause only compensatory mortality, with little added effect on long-term population dynamics. Secondly, many affected species are rather common and widespread. Consequently, local populations may be rescued by immigration from other ponds and the metapopulation would persist. Of course habitat loss and alteration of habitat would undermine this effect.

Life stages: Most die-offs caused by ranaviruses involve larvae or recent metamorphs inhabiting permanent water (Speare and Smith 1992; Jancovich *et al.* 1997; Green *et al.* 2002; Petranka *et al.* 2003; Fox *et al.* 2006). Several studies involving experimental exposures have shown that larvae are much more susceptible to BIV and FV3-like viral infections than are adults (Wolf *et al.* 1968; Clark *et al.* 1969; Cullen and Owens 2002; Gantress *et al.* 2003; but also see Cullen *et al.* 1995), and the patterns of mortality in the wild bear this out. The ranaviruses in the United Kingdom are the exception: larvae are affected, but most of the mortality is observed in adult frogs and toads (Cunningham *et al.* 1996; Cunningham *et al.* 2007a).

Range in Host Taxa: The three species of amphibian ranaviruses have a broad range in host taxa. Overall, the array of hosts of ranaviruses under ecologically realistic conditions remains poorly resolved but, potentially, it is important to the epidemiology of ranaviruses in amphibian communities. Several of the amphibian ranavirus are capable of infecting hosts from three vertebrate classes: Amphibia, Pisces and "Reptilia". BIV was highly pathogenic

for mammalian cells in vitro, but presumably in vivo protection is provided by the host's body temperature being above the maximum for replication of BIV (Speare and Smith 1992).

BIV was first isolated from the ornate burrowing frog (*Limnodynastes ornatus*), but has since been found to infect other captive or experimental hosts, including *Bufo marinus*, *Litoria terraereginae*, *L. caerulea*, *L. albogutatta*, *Cyclorana brevipes*, and *L. latopalmata*, as well as the native barramundi fish (*Lates calcarifer*) and reptiles (Owens 1994; Cullen *et al.* 1995; Ariel 1997; Cullen and Owens 2002; Moody and Chinchar 2002; Williams *et al.* 2005). Juvenile *L. terraereginae*, *L. caerulea*, and *L. latopalmata* were highly susceptible to BIV, while larval *L. terraereginae* and adults of *L. caerulea* and other species were less susceptible. Mortality varied with dose and route (Cullen *et al.* 1995).

Frog Virus 3-like viruses are also infectious to frogs, salamanders, fishes, and reptiles. Mao *et al.* (1999) found apparently identical FV3-like viruses in sympatric three-spine sticklebacks (*Gasterosteus aculeatus*) and a tadpole of the northern red-legged frog (*Rana aurora*), and Johnson and Jacobson (2004) isolated a ranavirus identical in MCP sequence from a captive Burmese star tortoise (*Geochelon latynota*) and an ill southern leopard frog (*Rana utricularia*) in the tortoise's enclosure. FV3-like viruses commonly infect both frogs and salamanders under natural conditions. In the 1960s TEV, an FV3-like virus isolated from *Rana catesbeiana*, was found in tadpole of *Bufo americanus*, *B. woodhousii fowleri*, and *Spea intermontana* (Wolf *et al.* 1968, 1969). More recently, Petranka *et al.* (2003) reported FV3-like viruses from sympatric *Ambystoma maculatum* and *Rana sylvatica* in North Carolina, and Duffus *et al.* (2008) found apparently identical FV3-like viruses in larvae of *Pseudacris* spp., *Hyla versicolor* and ambystomatid salamanders in south-central Ontario. Schock *et al.* (2008), however, found that while *Ambystoma tigrinum*, *Rana sylvatica*, *R. pipiens*, and *Hyla regilla* could be experimentally infected with both FV3 and ATV, in at least one pond, strains of ATV and FV3 were co-circulating in distinct host species. *Rana temporaria* United Kingdom virus (RUK) has been reported in the common frog (*Rana temporaria*) (Cunningham *et al.* 2006) and a similar virus, *Bufo bufo* United Kingdom virus (BUK), has been found to infect both the common toad (*Bufo bufo*) and (experimentally) the common frog (Cunningham *et al.* 2007b).

ATV may have the most restricted array of hosts. Based on experimental challenges of the northwestern salamander (*Ambystoma gracile*) and the eastern newt (*Notophthalmus viridescens*), as well as *Rana pipiens* and *R. catesbeiana* and three species of fish (*Gambusia affinis*, *Lepomis affinis*, and *Oncorhynchus mykiss*) with ATV-SRV (an isolate from *A. tigrinum stebbinsi* in the San Rafael Valley in southern Arizona), Jancovich *et al.* (2001) concluded that ATV was restricted to salamanders. Recent experiments with ATV-RRV (an ATV isolate from Roussell Pond, Regina, Saskatchewan, Canada), however, demonstrated that anurans can be infected with ATV (Schock *et al.* 2008). ATV has yet to be detected infecting any species of fish.

Seasonality: One hallmark of ranavirus epidemics is their seasonality—mortalities primarily occur in late spring and in summer (Green *et al.* 2002; Petranka *et al.* 2003; Brunner *et al.* 2004; but see Gray *et al.* 2007). One potential explanation for this seasonality is that amphibians are particularly vulnerable to ranaviral infection in the late spring and in summer when the larvae of many species begin to metamorphose. Many components of the amphibian immune system are down-regulated just prior to metamorphosis (Rollins-Smith 1998; Carey *et al.* 1999). This does not appear to be the case, however, for ATV in tiger salamanders—Brunner *et al.* (2004, 2005) found that ATV-infected larvae that metamorphose have higher survival rates. Gray *et al.* (2007) also found that the prevalence of infections by FV3-like virus in bullfrog larvae (*Rana catesbeiana*) in eight wetlands in Tennessee, United States, decreased with Gosner stage, up through stage 41, which may reflect increasing immunocompetence during development or, alternatively, increasing disease-induced mortality rates (McCallum and Dobson 1995). Adding to the ambiguity, there was no trend in prevalence with developmental stage in the larvae of sympatric green

frogs (*Rana clamitans*) (Gray *et al.* 2007). Moreover, developmental changes in susceptibility would not seem to explain the seasonality of ranavirus-associated mortality of adult *Rana temporaria* and *Bufo bufo* in the United Kingdom, which tends to peak in July, August, and September, although mortality was observed for at least 40 weeks at one location (Cunningham *et al.* 1996). Instead seasonal changes in prevalence and mortality may reflect changes in temperature or other environmental factors (Rojas *et al.* 2005; Raffel *et al.* 2006; Gray *et al.* 2007), or simply the dynamics of recurrent, growing epidemics. For instance, at high elevations in northern Arizona, U.S.A. mass die-offs involving hundreds to thousands of primarily larval *Ambystoma tigrinum nebulosum* occur in natural ponds and in earthen stock-watering tanks in the summer or early autumn (Berna 1990; Brunner *et al.* 2004; Greer and Collins 2008). A similar seasonal pattern has been observed in Saskatchewan and Manitoba, Canada (Bollinger *et al.* 1999; Schock 1999). Brunner *et al.* (2004) argued that summer or autumn die-offs were the more conspicuous peaks of epidemics that began in early spring, when the occasional aclinically infected adult introduced the virus into ponds when they returned to breed. These authors showed that while many, or even most, larvae die during the epidemic, some young of the year leave the pond infected. A few of these apparently survive with sublethal infections because a small fraction of adults returning to ponds to breed are infected with ATV (Brunner *et al.* 2004). The persistence of sublethal or even aclinical infections may explain both the recurrence and seasonality of ATV epidemics, and may be important to the epidemiology of other ranaviruses as well. Interestingly, in southern Arizona, where ponds remain ice-free year-round and the salamanders remain in ponds in both metamorphic and neotenic forms, epidemics have been observed throughout the year (Collins *et al.*, unpublished data).

Effect of Habitat: Several studies of ranaviruses in amphibian populations have found that artificial or human-impacted bodies of water are more prone to epidemics than are natural ones. Greer and Collins (2008) found that ponds modified to retain water for livestock were over four-times more likely to experience epidemics than were natural sinkholes. With little emergent vegetation in which to hide, *Ambystoma tigrinum* larvae aggregated at the edges of modified ponds in a "halo," which may have led to increased contact rates and therefore to viral transmission among larvae (Greer and Collins 2008). Similarly, there was a slight increase in the proportion of FV3 die-offs occurring in constructed ponds of the Tulula wetlands complex in North Carolina, compared to those occurring in the natural reference ponds in the complex (Petranka *et al.* 2003). Gray *et al.* (2007) found that the prevalence of an FV3-like virus was higher among *Rana clamitans* in wetlands with access by cattle compared to those without cattle access, although this trend was not significant among sympatric *Rana catesbeiana*. The authors suggested that this difference was due to these two species having different tolerances to poor water quality (Gray *et al.* 2007). Degree of human influence and proximity to industrial activity and to human habitation were all significantly associated with higher FV3 prevalence in *R. clamitans* (presumably adults) in central and northeastern Ontario, Canada (St. Amour *et al.* 2008).

A higher position within a catchment basin in Acadia National Park in Maine, United States, seemed to predispose wetlands for ranaviral outbreaks (Gahl and Calhoun 2008), although the reasons for this are not clear.

5. Resistance to Infection

Amphibians may fight ranaviral infections via both acquired and innate immunity. A first line of defence includes antimicrobial peptides of the skin (Erspamer 1994) which have been shown in some species to be effective against ranavirus *in vitro* (Chinchar *et al.* 2001; Rollins-Smith *et al.* 2002). Antibodies specific to ranavirus are also produced, although their efficacy appears to be species specific (Chinchar *et al.* 1984). There seems to be a distinction between anuran and caudate immune responses to ranaviral infections. *Rana catesbeiana* tadpoles that recovered after exposure to FV3 or RCV-Z appeared to be protected from future lethal infection with RCV-Z (Majji *et al.* 2006). Brunner and Schock (unpublished data) found that surviving an experimental exposure to ATV does not provide protection

against later exposures (i.e., no immune memory). Cotter *et al.* (2008) found that although experimentally infected *Ambystoma mexicanum* mounted an impressive immune response, they lacked the production of lymphocytes by the spleen that are associated with the ability of adult *Xenopus* to clear ranaviral infections (Robert *et al.* 2005; Maniero *et al.* 2006). This may be an important function in species that are able to clear ranaviral infections.

The outcome of infection varies greatly with genetic identity of individuals as well as with life history stage (Brunner *et al.* 2005; Pearman and Garner 2005).

Survivorship when infected with ranavirus also appears to be related to dosage. When groups of tadpoles of the Italian agile frog (*Rana latastei*) were exposed to different dosages of FV3, the animals that received the lowest dosage survived the longest (Pearman *et al.* 2004). Duffus *et al.* (2008) found that when tadpoles of the wood frog (*Rana sylvatica*) were exposed to increasing dosages of FV3, there was a corresponding increase in the proportion of infected tadpoles. Brunner *et al.* (2005) demonstrated that the proportion of tiger salamanders (*Ambystoma tigrinum*) that became infected with ATV increased with dose of inoculum, as did mortality, whereas time to death was reduced. Dose varies with route of exposure and this may play an important role in the outcome of infection (Cullen and Owens 2002; Brunner *et al.* 2005).

6. Transmission and Spread

Local transmission of ranavirus can occur via several direct and indirect routes. While a variety of laboratory studies have demonstrated that ranaviruses can be transmitted via indirect routes, i.e. fomites, soil, contaminated water (Pearman *et al.* 2004; Duffus *et al.* 2005; Harp and Petranka 2006; Brunner *et al.* 2007), it appears that more direct contact, i.e. touching, biting, cannibalism, necrophagy, may be required for transmission in a natural setting (Jancovich *et al.* 1997, Brunner *et al.* 2004, 2007; Pearman *et al.* 2004; Parris *et al.* 2005; Harp and Petranka 2006).

The rate and outcome of infection seem to vary with the route of exposure. For example, when infected tadpoles of the wood frog (*Rana sylvatica*) were introduced into a tank with uninfected tadpoles, mortality was upwards of 98%, while tadpoles exposed only to water and sediment from a site with an active ranavirus die-off did not experience a catastrophic die-off and individuals primarily developed aclinical ranaviral infections (Harp and Petranka 2006). Movement of water or sediment via fomites or aclinically infected animals between sites may facilitate the spread of ranaviruses (Harp and Petranka 2006).

One of the most important issues in epidemiology and conservation is whether transmission scales with host density (McCallum *et al.* 2001; de Castro and Bolker 2005). When transmission is density-dependent, pathogens can (at least theoretically) drive the population only so low because at some threshold transmission becomes so rare that the pathogen fades from the population. If, however, transmission continues unabated even as the host population declines, then extinction is possible. The evidence from ranaviruses is mixed. In a controlled environment, when infected *Rana sylvatica* tadpoles were placed in a pool with healthy tadpoles, more than 98% of the tadpoles died, regardless of initial tadpole density in the pool (Harp and Petranka 2006). In contrast, Greer *et al.* (2008) found that infection rates in naïve tiger salamander larvae increased with the density of ATV-infected hosts to which they were exposed, although not in a strictly density-dependent manner. Interestingly, greater densities of infected larvae led to an earlier death for the newly exposed larvae.

Necrophagy and cannibalism of infected animals are also potentially important routes of direct transmission. Necrophagy, in particular, seems to be a density-independent form of transmission; infected carcasses are a steady source of infection regardless of host density. Transmission by necrophagy and cannibalism is common in host species such as *Ambystoma tigrinum*, *Rana sylvatica*, and *R. latastei* (Pearman *et al.* 2004; Harp and Petranka 2006; Brunner *et al.* 2007) and infections acquired by these routes seem to be more lethal.

Tadpoles of *R. latastei* that fed on carcasses of FV3 infected tadpoles became infected and were more likely to die than were tadpoles in close proximity to infected carcasses but without direct contact (Pearman *et al.* 2004). *Rana sylvatica* tadpoles allowed to consume infected tadpole carcasses died sooner than did those exposed to infected carcasses but not allowed to eat them (Harp and Petranka 2006).

Larvae of *Ambystoma tigrinum nebulosum* were also easily infected with ATV when allowed to eat tissue from dead, infected larvae, but they also were easily infected via contact with mucous from infected larvae or with a short "bump" with infected larvae (Brunner *et al.* 2007). All of these modes of direct contact are common in amphibian larvae and it is likely that all of these behaviours contribute to ranaviral transmission in the wild.

Transmission through indirect routes has also been demonstrated in the laboratory. Langdon (1989) showed that ranaviruses could survive in a laboratory for over 90 days. In addition, several studies have found that larvae could become infected with ranavirus when exposed to water that previously housed infected larvae and/or sediment from a ranavirus die-off site or when inoculated with ranavirus (Brunner *et al.* 2004; Harp and Petranka 2006; Duffus *et al.* 2008; Brunner *et al.* 2007), although ranaviruses seem to decay in pond water (Brunner *et al.* 2007). Harp and Petranka (2006) found that the tadpoles exposed to water and sediment from a die-off site experienced only a low mortality rate, and were instead aclinically infected. They hypothesized that this may be a mode of maintaining infection in the population, as well as potentially moving it to other sites with migrating, infected amphibians.

Spread of ranaviruses between sites may be due to movement of water, sediment via fomites, or sublethally or aclinically infected animals (Harp and Petranka 2006). Most understanding of transmission of ranavirus originates, however, from experiments in the laboratory, leaving one to extrapolate how the dynamics of transmission play out in a natural and more complex setting.

For a discussion of long-distance, anthropogenic spread of ranaviruses, see Section 2 "Taxonomy and Molecular Epidemiology".

7. Diagnosis

The current routine techniques for diagnosing ranaviruses in amphibians include immunohistochemistry, viral isolation from tissues and cell culture, and ELISA and PCR. Sublethal or aclinical infections may only be detectable by cell culture or PCR.

Immunohistochemistry: Wax-embedded tissue sections can be labelled with an anti-ranaviral immunohistochemical marker so as to visualize infection within tissues (see Cunningham *et al.* [2008] for a detailed description). Variations on this technique include immunofluorescence and immunoelectron microscopy (Zupanovic *et al.* 1998b).

Cell Culture: Ranaviruses can be grown from fresh or frozen tissues in various fish (e.g. EPC and FHM), mammalian, or amphibian cell lines (Hengstberger *et al.* 1993) as long as those cells grow at ectothermic temperatures (<25°C). Often samples from amphibian tissues only show cytopathic effects after two or three passages (Brunner, unpublished data). The species of the cultured ranavirus can be identified by sequencing a portion of the major capsid protein gene using a technique described by Marsh *et al.* (2002).

ELISA: Enzyme-Linked ImmunoSorbent Assay (ELISA) is used for detection of anti-ranaviral antibodies in amphibian sera. Both Whittington *et al.* (1997) and Zupanovic *et al.* (1998b) provided detailed descriptions of this technique.

PCR: Polymerase chain reactions (PCR) using primers designed to amplify portions of the major capsid gene have become the most common method of detecting ranaviruses. For instance, the MCP4 and MCP5 primers described by Mao *et al.* (1996) amplify an approximately 500bp region of the MCP gene in all known ranaviruses. Quantitative real-time PCR reactions have also been developed (Mao *et al.* 1996; Brunner 2004). Galli *et al.*

(2006) provided a useful description of sample extraction and PCR procedures. Various tissues have been used to detect ranaviral infections in epidemiological studies and laboratory experiments, although the sensitivity of PCR tests seems to vary among tissues. St. Amour *et al.* (2007) found that toe clips, which can be taken non-lethally, were less sensitive for ranaviral detection in green frogs than were liver samples. Similarly, Greer *et al.* (2007) found non-lethal tail clips to be less sensitive than were whole-body homogenates of experimentally infected tiger salamander larvae. They also found that the ability to detect ATV infections increased with time from infection. Thus, these non-lethal sampling methods should be viewed as providing conservative estimates of infection.

8. Management

Given recent declines in amphibian populations worldwide and the impact novel pathogens have had on naïve populations, it is essential to avoid spreading pathogens or artificially increasing rates of infection. Disinfection of equipment used in handling captive and wild amphibians and effective quarantine guidelines are key to effectively managing these risks. Since ranaviral disease has been listed by the OIE as a globally notifiable disease, the international legislative requirements will result in a greater focus on surveillance and quarantine for ranaviruses.

Control Options: Control of ranaviruses within a site that is already infected may be particularly difficult given the possibility that aclinically infected animals may maintain infection in a population (Brunner *et al.* 2004; Robert *et al.* 2007). There are no vaccines available for ranaviruses and, given the logistical difficulties and expenses associated with developing and distributing a vaccine, it seems unlikely that one will be forthcoming. Culling amphibian populations would also be difficult given the secretive nature of many amphibian species, and likely ineffective if transmission is not density dependent (Greer *et al.* 2008). Currently there are no known treatments for ranaviruses. The only method for their control, therefore, is to limit human-influenced spread of the pathogen.

Disinfection: Whether working with captive or wild amphibians, it is essential to minimize the risk of amplifying transmission of pathogens or of exposing animals to new strains. Much of the risk can be managed through careful disinfection of potential fomites both between sites and between animals.

At the level of the site, it is important to disinfect waders, boots, boats, float tubes, nets, seines, traps, vehicle tyres and undercarriage, and any other equipment that comes in contact with the water.

Before disinfecting equipment, it must be scrubbed clean to permit all surfaces to contact the decontamination solution. Several common disinfectants (70% ethanol, 70% isopropyl alcohol, 10% household bleach) appear to be effective in inactivating ranaviruses if applied liberally for sufficient time, and when used in conjunction with mechanical scrubbing; for more detail see Brunner and Sesterhen (2001). In particular, a dilute solution of bleach is effective, has broad spectrum, is inexpensive, and oxidizes quickly (Speare *et al.* 2004; D. Green personal communication). Ethanol (70%) is effective against the fish ranavirus, EHNV (Langdon 1989) and can inactivate ranaviruses if given sufficient time or if used to flame equipment (Brunner and Sesterhen 2001; Brunner unpublished data). Because ethanol is relatively expensive, it is generally used only on forceps, scissors, and other instruments. Quaternary compounds are also effective and have the advantage of being less corrosive than bleach, but they require careful rinsing to remove soapy residues. Any disinfectant must be applied for the specified amount of time (Speare *et al.* 2004) to be effective. Finally, if using a chemical disinfectant, the equipment can be rinsed with sterile water, thus lowering the potential for release of the chemical into the habitat and decreasing the wear on equipment.

All instruments and tools that come into contact with animals should be disinfected between each animal. Each animal should be handled with new, disposable gloves, and

gloves should be changed between each animal. A new or disinfected bag or container should be used to hold each animal individually. Collectively, these procedures will help lower contact rates between the animals and avoid a positive bias when testing for prevalence of infection.

In the laboratory, several other options are available for decontaminating equipment. Autoclaving or exposure to UV light are effective in killing viruses, again when the equipment is first scrubbed and the method is applied for the specified amount of time (Speare *et al.* 2004). In addition, any water or other materials contacting captive animals must be disinfected, using bleach or autoclaving, before disposal.

In commercial frog enterprizes, if water is circulated by pumps, potential ranaviruses can be killed by use of commercial UV sterilizers (minimum effective dose of UV is 2.6 x 104 uW.sec/cm2) which are effective at high flow rates and even up to 70% turbidity (Miocevic *et al.* 1992).

Refer to Speare *et al.* (2004) or Kast and Hanna (2008) for more details on disinfecting equipment. Note that ranaviruses are more resistant than is the amphibian chytrid fungus *Batrachochytrium dendrobatidis:* guidelines for killing this fungus may not be adequate for ranavirus.

Quarantine Guidelines: There are several published quarantine guidelines intended to decrease the risk of introducing pathogens into a captive setting or of releasing captive, infected animals. Detection of ranaviruses poses special consideration since they can be difficult or impossible to detect in aclinically infected animals. A brief synopsis follows, but one should also consult the papers by Lynch (2001) and Ferrell (2008) for a more thorough treatment of this topic.

When obtaining animals, it is important to consider their origin and whether captive sources follow strict health guidelines (Lynch 2001). On arrival, animals should be held in a separate quarantine room for a minimum of 60 days and their health status checked regularly; they should be tested for fungal, bacterial, viral, and parasitic infections (Lynch 2001; Ferrell 2008). During this time clinical signs of some pathogens of concern, including ranavirus, may become manifest. Unfortunately, some animals that are infected with ranavirus remain aclinical. Brunner *et al.* (2004) found that aclinical ATV infections recrudesced in salamander larvae when they were co-housed with others under apparently stressful conditions. This may improve the ability to detect infections in newly obtained animals, but one should not rely on aclinical infections becoming patent. Currently there is no definitive test for individual animals for ranavirus that does not involve sacrificing the animal. There are several options for proxies on groups of animals. An ELISA test on sera can test for antibodies, thereby providing information on whether animals were ever exposed to ranavirus (Whittington *et al.* 1997; Zupanovic *et al.* 1998b). Additionally, PCR tests of toe-clips or tail-clips can provide a rather high confidence level about infection within the group, if a large enough number of animals are tested (Greer and Collins 2007; St. Amour and Lesbarreres 2007). Similarly, sacrificing a proportion of animals in the group to test organ homogenate or whole-animal homogenate via PCR will provide definitive testing for those animals and, depending on the proportion of animals tested, give a rather high level of confidence (Mao *et al.* 1996). Any animals to be released into the wild should be certified disease free (Lynch 2001; Ferrell 2008).

During the minimum 60-day holding period, quarantined animals should be held in a separate room from other individuals. It is important to minimize human contact with animals, use dedicated equipment, wear new, disposable gloves at all times, and use a disinfectant foot-bath when entering and exiting the room (Lynch 2001; Ferrell 2008). Additionally, all surfaces should be disinfected regularly; cages and cage materials should be disinfected when removing animals, and all waste water must be treated (Lynch 2001; and Ferrell 2008).

Risk of introduction: Anthropogenic movement of animals infected with ranavirus may be one of the most important sources for spread of this pathogen to naïve populations of

amphibians (Daszak *et al.* 2001). Amphibians are transported worldwide for food, research, and in the pet and bait trade. There is little control over this movement or the husbandry of the animals (Daszak *et al.* 2001; Schlaepfer *et al.* 2005). Animals to be transported for these activities should be subjected to the quarantine methods described above, holding animals in confinement, examining and testing animals for pathogens, treating as necessary, and disinfecting all equipment and water used. For a more through treatment of this topic, refer to the paper by Daszak *et al.* (2001).

9. Discussion

Although ranaviruses have not been linked to catastrophic amphibian declines, they can cause high levels of mortality in affected amphibian populations (Cunningham *et al.* 1996). Ranavirus can survive for long periods in the environment under some conditions (Langdon 1989), they are multi-host pathogens (Schock *et al.* 2008), and there is little if any regulation on the movement of amphibians around the globe, all of which increase the ability of ranaviruses to emerge in, and have an impact upon, amphibian populations. Given the myriad of other threats to amphibians and the fact that amphibians exist in increasingly fragmented populations and so may be less able to rebound after catastrophic mortality, the potential population-level impacts of ranaviruses should not be dismissed (Cunningham *et al.* 2007a). For example, populations may be unable to recover from infection with *Batrachochytrium dendrobatidis* (amphibian chytrid fungus), if mortality rates are further increased. Both regulation and enforcement to limit human-initiated movement of amphibian pathogens, including ranaviruses, is imperative if there is to be constraint upon the impact of emerging infectious diseases on these sensitive populations.

B. Frog Erythrocytic Virus

Frog Erythrocytic Virus (FEV) was discovered in populations of *Rana catesbeiana*, *R. clamitans* and *R. septentrionalis* in Ontario, Canada (Gruia-Gray *et al.* 1989; Gruia-Gray and Desser 1992). FEV is a large (up to 450 nm in diameter), enveloped, double-stranded DNA virus of uncertain classification within the Iridoviridae. Viral inclusions in the cytoplasm of red blood cells are seen by light microscopy of blood smears, and a large proportion of cells may be infected. Infected red blood cells change shape from oval to spheroidal, and heavily infected frogs can become anaemic (Gruia-Gray *et al.* 1989; Gruia-Gray and Desser 1992). A survey showed that infection is more common in juvenile bullfrogs (30% overall) than in adults (9%), and that it peaked in August/September (Gruia-Gray and Desser 1992). Infected juveniles were slightly less likely to be recaptured (4%) than were uninfected juveniles (9%), suggesting that the virus contributed to the mortality of young bullfrogs. FEV is transmitted between frogs by mosquitoes or midges, and is not transmitted by water, orally, or by leeches.

Similar large viruses have been found in red blood cells of amphibians in Costa Rica, Brazil, South Africa, China and the United States (Bernard *et al.* 1968; de Sousa and Weigl 1976; de Matos *et al.* 1995; Speare *et al.* 1991; Alves de Matos and Paperna 1993; Werner 1993).

C. Lucké Tumour Herpesvirus

Lucké tumour herpesvirus (LTHV) has been reported only from the northern leopard frog, *Rana pipiens* in the United States (McKinnell and Carlson 1997). Recently LTHV has been classified as Rana herpesvirus 1 (RaHV-1) (Davison *et al.* 1999) in the family Herpesviridae. Genomic studies indicated that RaHV-1 belongs to the fish virus lineage of the herpesvirus family rather than to the lineage populated by mammalian and avian viruses (Davison *et al.* 1999). This virus induces renal adenocarcinoma in *R. pipiens* in the United States; the tumour was well described by Lucké (1934). Its transmissible nature was recognized in 1938 and a virus was initially suggested as the cause due to the intranuclear inclusions (Lucké 1938), a surmise that was later confirmed.

Vertical transmission occurs with eggs becoming infected, but tumours are slow to develop. Clinical signs in adults are bloating, lethargy and death, which only occur when the tumour is large or has metastasized (Anver and Pond 1984). Single or multiple white nodules occur in the kidneys and grow into large masses. The tumour is an infiltrating and destructive adenocarcinoma or, less often, it is orderly and adenomatous (Lucké 1934). Although the gross appearance of the tumour remains relatively unchanged, there are significant seasonal differences in its microscopic appearance. Winter tumours display cytopathic characteristics associated with the presence of virus (enlarged nuclei with eosinophilic inclusions) whereas those in summer lack virus (McKinnell 1973). Metastasis of the cancer also depends on temperature. Studies have shown that above 22°C virus replication does not occur and viral particles are not present in the tumour (Anver and Pond 1984). Surveys of wild *Rana pipiens* for the Lucké tumour have found prevalences of up to 12.5% (McKinnell 1969). Since the 1960s, however, the prevalence of Lucké renal adenocarcinoma in Minnesota has decreased. This is thought to be due to the population declines and reduced density of *R. pipiens* (McKinnell *et al.* 1980).

D. Herpes-like Virus of Skin

In Italy, up to 80% of a wild population of *Rana dalmatina* had epidermal vesicles associated with a herpes-like virus, but dead frogs were not found (Bennati *et al.* 1994).

E. Calicivirus

A calicivirus was isolated from two captive *Ceratophrys orata* found dead. Both had pneumonia, while one also had oedema and the other had lymphoid hyperplasia (Smith *et al.* 1986).

F. Leucocyte Viruses

Polyhedral cytoplasmic DNA virus was found in the cytoplasm of white blood cells of a Mexican *Rana catesbeiana* that was lethargic and had small exudative ulcers (Briggs and Burton 1973). The large iridovirus found in red blood cells of *Bufo marinus* in Costa Rica also was found in the cytoplasm of reticular cells in the spleen (Speare *et al.* 1991).

III. BACTERIAL DISEASES

A. Bacterial Septicaemia ("Red Leg")

Bacterial septicaemia in amphibians has been termed "red leg" due to the cutaneous reddening that occurs on frogs' ventral thighs (Emerson and Norris 1905). This is an unfortunate name since many non-pathologists and non-veterinary clinicians appear to think that a frog with any erythema (redness) of the legs has a bacterial disease. It is important to realize that this is a very non-specific sign and it cannot be used to diagnose bacterial septicaemia. Bacterial septicaemia must be diagnosed by a combination of histopathology and bacterial culture.

Some massive die-offs in the wild have been attributed to bacterial septicaemia but these diagnoses are dubious due to a lack of histopathological confirmation and testing for other agents, particularly for ranaviruses and *Batrachochytrium dendrobatidis*, the amphibian chytrid (Green *et al.* 2002). Bacteria (including *Aeromonas hydrophila*) were reported in die-offs in *Alytes obstetricans* in the Pyrenees mountains in Spain (Marquez *et al.* 1995), in *Rana muscosa* in California (Bradford 1991) in *Bufo boreas boreas* in Colorado (Carey 1993) and in tadpoles of *Rana sylvatica* in Rhode Island, USA (Nyman 1986). These bacteria can be cultured from frogs with ranaviral disease and chytridiomycosis, particularly when the animals are collected dead (Cunningham *et al.* 1996; Berger *et al.* 1998). They can also be isolated from the intestines of healthy amphibians and from the environment (Carr *et al.* 1976; Hird *et al.* 1983). For example, *Aeromonas hydrophila* was found in the intestines of 46% (102) of 222 healthy leopard frogs (Hird *et al.* 1983). Bacteria may be present in sick frogs as normal

residents, contaminants, or secondary infections. In addition, reddening of the legs can occur in some amphibians showing clinical ranaviral disease and chytridiomycosis (Cunningham *et al.* 1996; Berger *et al.* 1999).

During a die-off of Yosemite toads (*Bufo canorus*) in the 1970s, three of 21 histologically examined toads had evidence of acute septicaemia, one of which also had chytridiomycosis, and all of which had been toe-clipped about two weeks before, which might have led to the infection (Green and Sherman 2001).

Bacterial septicaemia is, however, a common cause of outbreaks in captive amphibians. A range of bacteria may be involved including *Aeromonas hydrophila* and other gram-negative bacteria or combinations of bacteria, such as *Pseudomonas* spp., *Proteus* spp., *Flavobacterium* sp. (Hubbard 1981; Taylor *et al.* 1993; Olson *et al.* 1992). Recently, a new virulent subspecies, *A. hydrophila ranae*, was discovered in *Rana rugulosa* farmed in Thailand and dying from septicaemia (Huys *et al.* 2003). With bacterial septicaemia, gross pathological signs include pale skin, petechiation, ulcers, lethargy, oedema, ascites, pale livers, and haemorrhages in the internal organs. Histological examination may show degenerative myopathy and multiple foci of coagulative necrosis with clumps of bacteria. Variable results were obtained from transmission experiments - the disease usually required inoculation of the bacteria, or bath exposure and stress (Glorioso *et al.* 1974; Dusi 1949; Somsiri *et al.* 1997) although *A. hydrophila ranae* appeared to be more virulent than were other subspecies (Huys *et al.* 2003). In most cases, disease probably occurs secondarily to stress caused by poor husbandry such as overcrowding, dirty conditions, trauma, temperature changes, and also after transport (Hubbard 1981; Glorioso *et al.* 1974).

Recent experimental work using *A. hydrophila* on the host, *Xenopus laevis*, has shown that the amphibian host's genetics is also important in determining susceptibility to pathogenic bacteria (Barribeau *et al.* 2008). The survival and growth of *X. laevis* tadpoles with different major histocompatability complexes (MHC), when exposed to *A. hydrophila*, depended on their MHC haplotypes with heterozygous tadpoles being intermediate between tadpoles with resistant and susceptible MHC haplotypes.

B. Streptococcosis

A non-haemolytic group *B Streptococcus* caused an outbreak killing 80% of about 100,000 farmed bullfrogs (*Rana catesbeiana*) in Brazil (Amborski *et al.* 1983). Septicaemia, necrotizing splenitis, and hepatitis with haemorrhages occurred in frogs. Viral cultures were negative. The outbreak was associated with overcrowding and stress. Mortality due to a similar streptococcus occurred in *R. catesbeiana* being raised for consumption in Uruguay (Mazzoni 2001) and in the United States (Mauel *et al.* 2002). In the latter instance, the agent was identified as *Streptococcus iniae*, a species that is a pathogen of fish and has zoonotic potential (Lehane and Rawlin 2001).

C. Chlamydiosis

Chlamydophila pneumoniae caused chronic pneumonia in a wild immunosuppressed frog, *Mixophyes iteratus*, in Australia (Berger *et al.* 1999) and in a captive colony of *Xenopus tropicalis* in the United States (Reed *et al.* 2000). *C. pneumoniae* is an important human pathogen. A range of chlamydial species infecting healthy amphibians from Switzerland were identified by PCR (Blumer *et al.* 2007). Outbreaks of chlamydiosis in captive amphibians can result in fulminant, multisystemic infections with pyogranulomatus inflammation, causing moderate to high mortality rates in various species (Wilcke *et al.* 1983; Howerth 1984; Honeyman *et al.* 1992).

D. Mycobacteriosis

A number of atypical *Mycobacterium* species infect amphibians; *M. tuberculosis* or *M. bovis* have not been reported. The only report of wild amphibians with mycobacteria was *M. chelonei*

subsp abscessus isolated from four of 66 *Bufo marinus* and two of 86 *B. granulosus* in a survey of Amazonian amphibians (Mok and Carvalho 1984). None of these animals had histopathological lesions, although experimental intraperitoneal inoculation of 29 toads resulted in the death of five animals from mycobacteriosis.

Disease due to mycobacteria has been reported only in captive amphibians and occurs mainly in immunocompromised animals. Several species have been reported. *M. marinum* was experimentally shown to cause a chronic granulomatous non-lethal disease in immunocompetent leopard frogs (*Rana pipiens*) whereas frogs immunocompromised with hydrocortisone developed an acute lethal disease (Ramakrishnan *et al.* 1997). A *M. ulcerans*-like species and *M. leiflandii* both caused outbreaks in captive *Xenopus laevis* (Trott *et al.* 2004; Godfrey *et al.* 2007) while *M. szulgai* caused an outbreak in captive *X. tropicalis* (Chai *et al.* 2006). Infections primarily involve the skin, respiratory tract, or intestines. Frogs have been found with single, large tumour-like masses or with disseminated nodules throughout the internal organs. Organs such as liver, spleen, kidney, or testes may become almost completely destroyed by the infection before the animal dies, usually with cachexia (Reichenbach-Klinke and Elkan 1965). Early granulomas are composed of mostly epithelioid macrophages, which may progress to form encapsulated foci with dry caseous centres. Granulomas typically contain large numbers of acid-fast bacilli.

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