Review article

Major disease threats to European amphibians

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Disease threats to amphibians in Europe are generally poorly understood. The effects that disease can have on amphibian populations can range from minimal to local extirpation. Currently, two infectious agents are emerging as disease threats to European amphibian populations: *Batrachochytrium dendrobatidis* (Bd), which is the causative agent of amphibian chytridiomycosis, and ranavirus(es). Both pathogens are listed by the World Organization for Animal Health (OIE). The incidence of other infectious diseases, such as amphibiocystidium, might also be increasing. In this review, we discuss known and potential disease threats to European amphibians, including their current and potential impact on amphibian populations, and factors driving their emergence and spread. We provide recommendations on how to proceed with investigations into cases where disease is thought to be involved in mortality or decline. We also stress that a multidisciplinary approach to these investigations is required.

Key words: amphibiocystidium, *Batrachochytrium dendrobatidis*, chytridiomycosis, ranaviral disease, ranavirus

INTRODUCTION

Infectious diseases can cause catastrophic population declines, local extirpations, or even global species extinctions of wildlife (de Castro & Bolker, 2005), but even in the absence of such obvious effects, parasites are important determinants of host population dynamics (Anderson & May, 1979). Key factors that contribute to the impact of infectious disease include host population size, the transmission dynamics of the pathogen (density and/or frequency dependence) and the ability of the pathogen to utilize alternative, or reservoir, hosts or to remain viable in the environment (de Castro & Bolker, 2005; Ryder et al., 2007). Even large, robust populations, however, can be negatively impacted by disease, especially if the pathogen is new to the host in evolutionary terms (Cunningham et al., 2003), and this is of growing concern to conservation biologists (see Scott, 1988, for a discussion of the importance of the impacts of disease on populations).

Added to this, in Europe there appears to be a lack of knowledge regarding many aspects of amphibian biology (Pasmans et al., 2006). This is cause for concern, since it has been estimated that by 2050, depending only on predicted climate alterations, 31% of amphibians (n=42) in Europe will have experienced range contractions (Araújo et al., 2006). Compounded with other factors, such as reduced water availability (Araújo et al., 2006), land use change, environmental pollution and infectious disease, this could lead to marked declines and range restrictions even for amphibian species that are currently considered to be widespread and common in Europe (see Acevedo-Whitehouse & Duffus, 2009, for a discussion of the impacts of environmental change and wildlife health).

In this article, we discuss three major categories of infectious disease threat to amphibians in Europe: 1) amphibian chytridiomycosis, 2) ranavirus disease and 3) other infections for which the impact is currently unknown. We review the biology of the causative agent, clinical signs of disease, mode of transmission and spread, and known impacts on the distributions of amphibians in Europe. We provide starting points for investigations into cases where disease is thought to be responsible for, or to have played a role in, mortality and/or population declines, and we stress the importance of a multidisciplinary approach.

BATRACHOCHYTRIUM DENDROBATIDIS

Batrachochytrium dendrobatidis (*Bd*) is a non-hyphal, zoosporic chytridiomycete fungus that is the causative agent of the amphibian disease chytridomycosis (Berger et al., 1998). At first linked to amphibian mortality events and declines in both Australia and Central America (Berger et al., 1998), it has since been associated with mass mortality events and, in some cases, population declines and species extinctions affecting amphibians in six continents, including several European species (Bosch et al., 2001; Bosch & Martínez-Solano, 2006; Skerrat et al., 2007; Garner et al., 2009; Bielby et al., 2009). *Bd* is classified as an emerging infectious agent in amphibians (Daszak et al., 1999), is considered to be a pandemic pathogen (Pasmans et al., 2006) and, in 2008, was listed as a notifiable pathogen by the World Organization for Animal Health (OIE, 2008). Although the origin of *Bd* remains unknown, a series of genetic studies has demonstrated remarkably low genetic diversity amongst isolates from disparate parts of the world (Morehouse et al., 2003; Morgan et al., 2007; James et al., 2009) pointing to a rapid, clonal pandemic spread of the organism.

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Table 1. Summary of countries and species from which *Batrachochytrium dendrobatidis* (*Bd*) infection has been reported in Europe.

Location	Common name	Species	Reference
Spain	Common midwife toad	*Alytes obstetricans*	Bosch et al., 2001
	Common toad	*Bufo bufo*	Bosch & Martínez-Solano, 2006
	Fire salamander	*Salamandra salamandra*	Bosch & Martínez-Solano, 2006
	Mallorcan midwife toad	*Alytes muletensis*	www.spatialepidemiology.net/bd_maps
	Pyrenean brook salamander	*Euproctus asper*	www.spatialepidemiology.net/bd_maps
	Palmate newt	*Lissotriton* (formerly *Triturus*) *helviticus*	www.spatialepidemiology.net/bd_maps
	Alpine newt	*Ichthyosaura* (formerly *Triturus*) *alpestris*	www.spatialepidemiology.net/bd_maps
	Pygmy marbled newt	*Triturus pygmaeus*	www.spatialepidemiology.net/bd_maps
	Marbled newt	*Triturus marmoratus*	www.spatialepidemiology.net/bd_maps
	Mediterranean tree frog	*Hyla meridionalis*	www.spatialepidemiology.net/bd_maps
	Iberian green frog	*Rana perezi*	www.spatialepidemiology.net/bd_maps
	Natterjack toad	*Bufo calamita*	www.spatialepidemiology.net/bd_maps
	Western spadefoot toad	*Pelobates cultripes*	www.spatialepidemiology.net/bd_maps
	Iberian painted frog	*Discoglossus jeannae*	www.spatialepidemiology.net/bd_maps
	Iberian frog	*Rana iberica*	www.spatialepidemiology.net/bd_maps
	Spanish (or ribbed) newt	*Pleurodeles waltl*	www.spatialepidemiology.net/bd_maps
UK	North American bullfrog	*Lithobates catesbeianus* (formerly *Rana catesbeiana*)	Garner et al., 2005
	Common toad	*Bufo bufo*	www.spatialepidemiology.net/bd_maps
	Natterjack toad	*Bufo calamita*	www.spatialepidemiology.net/bd_maps
Germany	Edible frog	*Rana esculenta*	www.spatialepidemiology.net/bd_maps
Switzer-land	Species not reported		Garner et al., 2005
	Edible frog	*Pelophylax esculentus* (formerly *Rana esculenta*)	www.spatialepidemiology.net/bd_maps
	Water (or pool) frog	*Rana lessonae*	www.spatialepidemiology.net/bd_maps
	Alpine newt	*Ichthyosaura* (formerly *Triturus*) *alpestris*	www.spatialepidemiology.net/bd_maps
	Common midwife toad	*Alytes obstetricans*	www.spatialepidemiology.net/bd_maps
Denmark	Common frog	*Rana temporaria*	www.spatialepidemiology.net/bd_maps
	Edible frog	*Pelophylax esculentus* (formerly *Rana esculenta*)	www.spatialepidemiology.net/bd_maps
France	North American bullfrog	*Lithobates catesbeianus* (formerly *Rana catesbeiana*)	www.spatialepidemiology.net/bd_maps
	Common midwife toad	*Alytes obstetricans*	www.spatialepidemiology.net/bd_maps
Italy	Sardinian newt	*Euproctus platycephalus*	Bovera et al., 2008
	Water (or pool) frog	*Rana lessonae*	Simoncelli et al., 2006
	Apennine yellow-bellied toad	*Bombina pachypus*	Stagni et al., 2002 (in Bovero et al., 2008)
	Italian agile (or Lataste's) frog	*Rana latastei*	Garner et al., 2004 (in Bovero et al., 2008)
	North American bullfrog	*Lithobates catesbeianus* (formerly *Rana catesbeiana*)	www.spatialepidemiology.net/bd_maps
	Edible frog	*Pelophylax esculentus* (formerly *Rana esculenta*)	www.spatialepidemiology.net/bd_maps
	Sardinian tree frog	*Hyla sarda*	www.spatialepidemiology.net/bd_maps

Clinical signs, pathology and diagnosis

The clinical signs of *Bd* infection are relatively non-specific and vary among species, life history stages and environmental conditions. *Bd* is an intracellular pathogen that infects only the keratinized tissues of the body (i.e. skin of metamorphosed amphibians, mouthparts of anuran larvae) (Daszak et al., 1999). Post-metamorphic amphibians suffering from chytridiomycosis can be lethargic, appear dehydrated, exhibit excessive or patchy skin shedding, loss of appetite, reddening of the skin (especially of the underside of the upper thighs and pelvic patch) and even the ulceration or necrosis of digits (Densmore & Green, 2007; Bovero et al., 2008; Bielby et al., 2009). Additionally, there may be apparent neurological

signs, such as the loss of the righting reflex or the absence of the flight response (Densmore & Green, 2007). In adult caudates, the signs of disease might also include the ulceration or necrosis of part, or all, of the tail (Densmore & Green, 2007). These clinical signs, however, are non-specific and can be caused by a range of other diseases, hence the signs of disease are not reliable indicators of the presence of chytridiyomycosis or *Bd* infection and more detailed investigations are required.

Infected anuran larvae often exhibit depigmentation of the mouthparts due to the loss of part, or all, of the keratinized oral disc (e.g. Lips, 1999). The use of mouth part depigmentation is not a reliable diagnostic method for the presence of *Bd*, however, as other conditions, including overwintering, can result in similar lesions (Rachowicz, 2002; Rachowicz & Vredenburg, 2004; Felger et al., 2007).

There is a variety of techniques available to detect *Bd* infections. Amphibian chytridiomycosis was first reported through the use of histological examination of infected tissues, with the detection of intracellular zoosporangia (Berger et al., 1998). Whilst this method provides a "gold standard" for confirming infection as the pathogen can be visualized, histological examination has a low sensitivity of detection relative to molecular methods, especially in animals with subclinical infection (Hyatt et al., 2007). Also, histology is an invasive diagnostic method, conducted on clipped toes in live adult amphibians or on skin or larval mouthparts from dead amphibians. Boyle et al. (2004) and Hyatt et al. (2007) provide detailed protocols for sampling amphibians for the detection of *Bd* using molecular methods.

Batrachochytrium dendrobatidis in Europe

The first report of *Bd* in Europe was from Germany in 2000 from imported animals from South America and from captive-bred individuals from both Germany and Belgium (Mutschmann et al., 2000). Infection with *Bd* in wild amphibians in Europe was first reported in 2001 from Peñalara Natural Park in central Spain, where infection with the pathogen was associated with mass mortalities of recently metamorphosed common midwife toads (*Alytes obstetricans*) and the extirpation of this species from 86% of its known distribution in the park (Bosch et al., 2001). Subsequently, mortalities of fire salamanders (*Salamandra salamandra*) and common toads (*Bufo bufo*) due to chytridiomycosis have occurred in the park (Bosch & Martínez-Solano, 2006). Interestingly, whilst this has led to fire salamander declines, the common toad has expanded its range and population density within Peñalara Natural Park (Bosch & Rincón, 2008). This response, apparently due to the decline of the common midwife toad, is the first evidence in Europe of an indirect ecological effect associated with the emergence of *Bd* (Bosch & Rincón, 2008).

Further studies of *A. obstetricans* in Spain have shown that most study sites are *Bd* free and, even in sites known to be *Bd*-positive, most toads test negative for *Bd* (Walker et al., 2010). This might be because, for example, *Bd*-infected toads die shortly after infection, the diagnostic test used was not sensitive enough to detect all infected animals, or sampling was biased towards uninfected animals (e.g. perhaps due to the timing of sampling or the behaviour of infected toads). On first inspection, these findings seem to be in direct contradiction to the large-scale extirpation of *A. obstetricans* in Peñalara Natural Park due to chytridiomycosis (Bosch et al., 2001), and indicate some of the difficulties involved in elucidating the epidemiology of *Bd* infection and its impact on wild animals at the population level. Walker et al. (2010) found that whilst *A. obstetricans* mortality and declines due to chytridiomycosis were evident in high altitude regions (e.g. Peñalara, the Pyrenees, the Cordillera Cantabrica), *Bd* can infect the same species at lower altitudes in the absence of declines. Thus environmental context is an important factor to consider when investigating *Bd* infection and its possible outcomes.

In addition to mainland Spain, *Bd* has been found on the Spanish island of Mallorca, where it infects the single-island endemic Mallorcan midwife toad (*Alytes muletensis*). This vulnerable species has been the subject of a captive breeding and restocking project since the mid 1980s and genotyping has identified this conservation effort as the most likely source of *Bd* introduction to the island (Walker et al., 2008). Interestingly, there is no evidence of chytridiomycosis impacting the Mallorcan midwife toad populations on Mallorca and experimental infection studies using *Bufo bufo* have shown the Mallorcan strain of *Bd* to have a lower virulence than strains from elsewhere (Fisher et al., 2009). Cases such as these illustrate the need for long-term surveillance data, including pathological samples, to link disease and decline.

An opportunistic survey for *Bd* infection conducted using tissue samples collected from across Europe for other research purposes from 1994 to 2004 detected infection in 20 of 28 species and in five (Italy, Portugal, Spain, Switzerland, UK) of 13 countries, with apparently high infection prevalence in Spain and Switzerland (Garner et al., 2005). The date of first detection of *Bd* in Spain was 1997, whilst in Switzerland it was 1998, in Italy and Portugal, 2003 (T. Garner, personal communication) and in the UK, 2004 (Cunningham et al., 2005). An apparently high prevalence of *Bd* infection in Switzerland was noted to be of particular interest because the positive animals appeared to be healthy at the time of sample collection and because there had been no reports of amphibian mortalities in Switzerland (Garner et al., 2005). This is now being followed up to ascertain if the high prevalence of *Bd* infection is impacting amphibian populations in Switzerland. Detailed investigations are required in order to differentiate between infection, disease and population impacts.

In three European countries (France, Italy and the UK), *Bd* was initially found in introduced North American bullfrogs (*Lithobates catesbeianus*, formerly *Rana catesbeiana*) (Cunningham et al., 2005, Garner et al., 2006). This species, along with the cane toad (*Bufo marinus*) and the African clawed frog (*Xenopus laevis*), is considered to be a possible vector of the global introduction and spread of *Bd* (Weldon et al., 2004; Garner et al., 2006, Fisher & Garner, 2007). It is possible that the North American bullfrog (Ficetola et al., 2007, 2008) and the African clawed

frog (e.g. South Wales, UK, Measey, 2001; Sicily, Italy, Faraone et al., 2008) – two invasive species that are widespread in Europe – are vectors of *Bd* introduction to other European countries.

More recently, *Bd* has been found on the Mediterranean island of Sardinia, a place with a unique assemblage of endemic amphibians and reptiles (Bovero et al., 2008). Mortality due to chytridiomycosis has been detected in the endangered Sardinian newt (*Euproctus platycephalus*), which has been in decline since the early 1980s (Bovero et al., 2008). Although it is tempting to attribute these declines to the emergence of *Bd*, causation has not yet been demonstrated. Also on Sardinia, mass mortalities attributed to chytridiomycosis have been found in the Tyrrhenian painted frog (*Discoglossus sardus*) (Bielby et al., 2009). Unlike many recent studies of decline and mortality associated with *Bd*, Bielby et al. (2009) confirmed the diagnosis of chytridiomycosis using histopathological investigations. As in most studies that cite chytridiomycosis as the cause of death, however, systematic postmortem examinations were not performed, therefore other possible causes, or contributory factors, were not ruled out.

The number of species and regions in Europe that are known to be affected by *Bd* continues to increase. A website (www.spatial epidemiology.net/bd_maps) has been set up to display spatial *Bd* detection data globally, but although this is probably the best resource currently available for obtaining such information, even this is only partially populated with relevant data. Also, in Europe, as elsewhere, most information on *Bd* has been obtained through opportunistic "swab-and-go" tactics (see Duffus, 2009a, for further discussion). Systematic *Bd* surveillance programmes combined with longitudinal population monitoring and detailed pathological investigations of disease or mortality events are required in order to obtain reliable information on *Bd* prevalence, the incidence of chytridiomycosis and the impacts, if any, of *Bd* infection on amphibian populations. Currently, many researchers fail to distinguish between infection and disease, which limits the usefulness of their results (Duffus, 2009a).

There is a growing body of evidence (e.g. Berger et al., 2005; Fisher et al., 2009) demonstrating variation in virulence or pathogenicity amongst *Bd* isolates, despite their clonal reproduction and spread. In Europe, for example, experiments using the common toad as a model have demonstrated differences in infection outcomes among three different European isolates, with a strain from Mallorca exhibiting lower virulence (as determined by death due to chytridiomycosis) than strains isolated from either the UK or from mainland Spain (Fisher et al., 2009). These authors infected common toad tadpoles with *Bd* and followed the development of the animals through to metamorphosis. Animals that survived to metamorphosis weighed significantly less than unexposed, control animals if they had been exposed to either the Mallorcan or the mainland Spanish strains of *Bd*, but not if they had been exposed to the more virulent UK strain (Fisher et al., 2009). This might be because susceptible, or infected, animals exposed to the UK strain did not survive until metamorphosis. Strain variation is likely to be an important factor in determining the impact of *Bd* emergence in naive populations (Fisher et al., 2009) and this is a compelling reason why countries in which *Bd* already occurs should not be complacent about taking measures to prevent the incursion of additional *Bd* strains. This is further supported by the findings of Goka et al. (2009) who showed that the North American bullfrog (*Lithobates catesbianus*) carries *Bd* strains with the highest amount of genetic diversity found so far and that new strains of *Bd* have been introduced into Japan by the pet trade.

Experiments using the common toad as a model were performed by Garner et al. (2009) to examine the relationship between different levels of *Bd* exposure and selected life history traits. Exposure of tadpoles to *Bd* decreased the time to metamorphosis and also decreased body mass at metamorphosis. This was most pronounced for animals exposed to lower levels of *Bd*, with higher mortality rates amongst tadpoles exposed to higher doses of *Bd* (Garner et al., 2009). Reduced body mass was associated with a lower chance of surviving metamorphosis (Garner et al., 2009). When animals were exposed to *Bd* as metamorphs, higher doses were again associated with higher rates of mortality, with the effect being more pronounced in toadlets with lower body mass. This series of experiments indicates that, in addition to causing overt mortality, infection with *Bd* can have more subtle effects on life history traits. The impacts of *Bd* on European amphibians are poorly understood and more research into the ecology, transmission dynamics and fitness effects of infection on individuals and populations are required.

RANAVIRUSES

Ranaviruses are icosahedral double-stranded DNA viruses that comprise the genus *Ranavirus* in the family Iridoviridae; frog virus 3 (FV3) is the type virus of this group (Chinchar, 2002; Chinchar et al., 2009). This genus appears to have a global distribution (although ranaviruses have not yet been reported from Africa) and it contains pathogens of fish, amphibians and reptiles (Chinchar et al., 2009). In amphibians, ranaviruses can cause mass mortality events, usually of the larval stages (Jancovich et al., 1997; Bollinger et al., 1999; Green et al., 2002; Greer et al., 2005; Duffus et al., 2008). Although ranaviruses have been known since 1965 (Granoff, 1989), both the number of ranavirus-associated amphibian mortality events and the number of new ranaviruses isolated from amphibians have greatly increased since the 1980s and ranaviruses are considered as emerging infections in amphibians (Daszak et al., 1999). Recently, ranavirus infection of amphibians has been listed as notifiable by the World Organization for Animal Health (OIE, 2008), an indication of the severity of the potential impact of ranaviruses on amphibians.

In North America, ranavirus infection, disease and mortality have been reported mostly in larval urodeles and anurans (e.g. Jancovich et al., 1997; Bollinger et al., 1999; Green et al., 2002; Greer et al., 2005), whereas in Europe, infection is primarily reported as affecting adult anurans (Fijan et al., 1991; Cunningham et al., 1996; Ariel et al., 2009). Ranavirus infection has been reported in amphibians in South America, but isolates were made from tissues pooled from animals caught specifically for

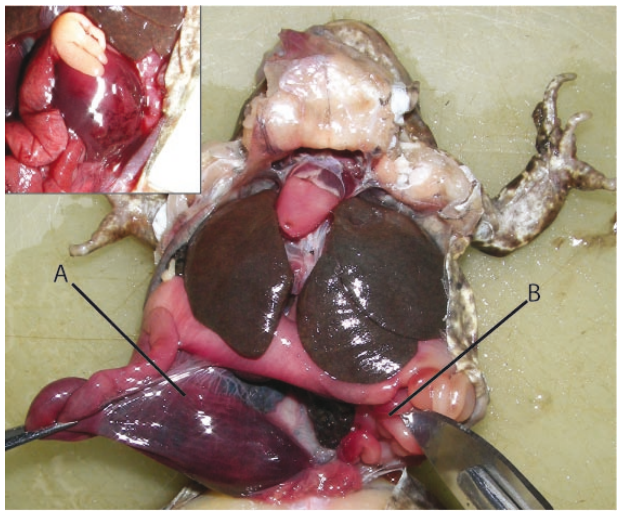

Fig. 1. A common frog (*Rana temporaria*) suffering from ranaviral disease. This frog is an adult female. A) Haemorrhages in the gastrointestinal tract, here most notably affecting the rectum. B) Haemorrhages in the oviducts. In males, the testes can be haemorrhagic (inset; note the apparently healthy fat bodies above the testis).

the purpose and in the absence of clinical examination (Zupanovic et al., 1998b). It seems likely that these isolates were made from apparently healthy animals. Only one ranavirus, named Bohle iridovirus (BIV), has been isolated in Australia. This was cultured from diseased ornate burrowing toad (*Limnodynastes ornatus*) tadpoles (Speare & Smith, 1992). Serological surveys, however, have shown evidence of widespread exposure of introduced cane toads (*Bufo marinus*) to ranavirus in Australia, but no disease has been detected in, and no virus has been isolated from, this species (Zupanovic et al., 1998a).

Clinical signs, pathology and diagnosis

Ranavirus infection does not necessarily result in disease. However, when disease does develop, similar signs are usually seen regardless of the strain of virus or the species of host. Adult amphibians with ranaviral disease tend to be lethargic or just found dead. Affected larvae exhibit behavioural changes, such as abnormal posture and disturbed swimming (including apparent problems with the regulation of buoyancy) (Densmore & Green, 2007; Duffus, personal observation). In most cases, ranaviruses cause an acute, systemic haemorrhagic disease in both larvae and adults. In these cases, haemorrhages can be found in a range of tissues, including skin, organs and muscle; often blood is present within the mouth and within the lumen of the gastrointestinal tract (Cunningham et al., 1996, 2007a,b, 2008) (Fig. 1). In larvae, in addition to systemic haemorrhaging, ranavirus disease often involves an increase in the volume of tissue (oedema) and coelomic (ascites) fluid. In some cases, excessive tissue fluid is present in the subcutaneous lymph sacs (subcutaneous oedema) of affected adult anurans (Fig. 2).

In the common frog in Great Britain, a cutaneous form of ranaviral disease has also been reported (called ulcerative syndrome or US), which is characterized by gross lesions that are limited to the skin with the development of dermal ulceration especially, but not exclusively, affecting the feet, hind limbs and ventral pelvic region (Cunningham et al., 1996, 2007a, 2008; see Figure 2). This form of the disease is much more chronic than the systemic form (called haemorrhagic syndrome or HS) and lesions appear to develop over days or weeks, often resulting in extensive skin ulceration and necrosis of one or more digits or extremities of the limbs (Cunningham et al., 1996). These necrotizing ranavirus lesions can appear similar to the necrotizing form of chytridiomycosis or, in areas where there are harsh winters, to digit loss due to "frost-bite" (Duffus, personal observation).

Although the gross lesions of each type of ranavirus disease (US and HS) appear distinct, virus can be detected in most organs and tissue types regardless of the gross presentation (Cunningham et al., 2008). Often, intracytoplasmic virus inclusions are present within epithelial and parenchymal tissues (i.e. the tissues that make up the bulk of many internal organs). These virus inclusion bodies can take the form of basophilic inclusions comprised primarily of virus particles or of eosinophilic inclusions comprised of endoplasmic reticulum whorls, the formation of which appears to be induced by virus infection (Cunningham et al., 1996). Histological examination reveals multiple necrotic foci widespread within tissues of animals with systemic disease, but microscopic necrotic foci can also be detected in internal tissues of animals with the cutaneous form of ranaviral disease, although, if present, these are less extensive (Cunningham et al., 1996).

There is little information about ranavirus infections in the larval stages of European amphibians. In experimentally infected common frog (*R. temporaria*) tadpoles, signs of disease included oedema and haemorrhages within the coelomic cavity (Duffus, 2009b). In contrast, common toad (*Bufo bufo*) tadpoles experimentally exposed in the

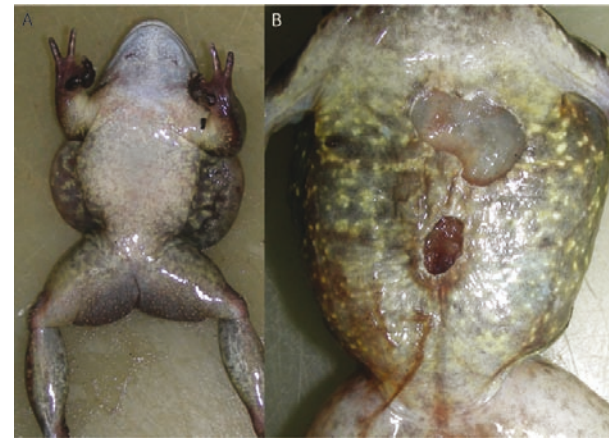

Fig. 2. A) Male common frog (*Rana temporaria*) with a large amount of subcutaneous oedema in the thorax and femoral area of the lower limbs. B) Common frog with two large ulcerations of the skin of the ventral thorax caused by ranavirus infection.

same manner died in the absence of noticeable clinical signs (Duffus, 2009b), possibly because the dark ventral pigmentation of the common toad tadpoles masked any presence of coelomic haemorrhages.

Ranavirus infection can be diagnosed using standard PCR, qPCR, tissue culture or electron microscopy (Cunningham et al., 1996; Pearman et al., 2004, Pallister et al., 2007). Also, viral inclusions in tissues can be detected using light microscopy (e.g. Cunningham et al., 1996; Greer et al., 2005) and infection with ranavirus can be confirmed using ranavirus-specific immunohistochemistry (Cunningham et al., 2008). Ranavirus can be cultured using a variety of cell types of amphibian, fish or mammal origin (e.g. Drury et al., 1995; Jancovich et al., 1997; Bollinger et al., 1999; Hyatt et al., 2000; Duffus, 2009b). Further to the development of cytopathic effect (Cunningham et al., 1996), the presence of ranavirus should be confirmed using electron microscopy or a ranavirus-specific molecular method (e.g. PCR and sequence analysis). The success of virus isolation is dependent upon many factors, with advanced tissue decomposition or repeated freeze–thaw cycles likely to reduce the chances of virus recovery, and therefore all results, including negatives, should be confirmed using at least one other diagnostic method.

As in all disease investigations, systematic postmortem, including histopathological and microbiological, examinations of multiple individuals (if possible, at least five specimens and, ideally, many more of each affected species) should be conducted along with any subsequent diagnostic tests indicated by the postmortem findings.

Amphibian ranaviruses in Europe

Iridovirus infections in European amphibians were first identified by Fijan et al. (1991) when investigating *Rana esculenta* mortality in Croatia. Previous to this, Kunst & Valpotic (1968) had reported a "viral haemorrhagic septicaemia of frogs" affecting *Rana esculenta* (now *Pelophylax esculentus*) in the former Yugoslavia. Whilst it is likely that the causative agent in each case was ranavirus, it is not clear from these reports if the disease outbreaks described occurred in wild, captured or farmed frogs. The first unambiguous reports of ranavirus infection in wild amphibians in Europe were of common frog (*Rana temporaria*) mortalities in the southeast of England (Cunningham et al., 1993, 1996; Drury et al., 1995). These authors reported annually recurring mass mortalities of adult frogs, which were first noticed in the late 1980s and early 1990s and continue to the present (Cunningham et al., 1996; Teacher, 2009; Duffus, 2009b).

The emergence of ranavirus in the UK has had a measurable negative effect on some populations of common frogs, with long-term data sets indicating median population declines of approximately 83% (Teacher, 2009). Emergence of ranavirus has also been associated with the selection of certain MHC genotypes in populations that have been affected by persistent infections for about 10 years (approximately five generations), in comparison to disease-free sites (Teacher et al., 2009).

Investigations of ranavirus in Great Britain using experimental exposures determined that the origin of the virus (infected tissue or cultured virus), the syndrome that the virus was from (US or HS) and the route of exposure (bath exposure or parenteral inoculation) each play an important role in the subsequent development of disease (Cunningham et al., 2007a). For example, when adult common frogs were exposed, via immersion, to tissues from a frog with HS, no lesions developed; frogs exposed, via immersion, to tissues from a frog with US developed US, whilst frogs exposed to virus isolated from either a frog with HS or a frog with US predominantly developed HS, but skin ulceration sometimes also occurred.

Ranavirus infection has also been found to cause mortality of the common toad (*Bufo bufo*) in England, with affected animals dying of systemic haemorrhages (Hyatt et al., 2000; Cunningham et al., 2007b). Virus isolates from these animals also cause HS disease in experimentally infected common frogs (Cunningham et al., 2007b). Common toads and common frogs are sympatric in many areas and as it would appear that they share a ranavirus that can infect both species, one species might be acting as a source of infection for the other. This interaction would increase the effective population size, prolonging the presence of ranavirus in the population and increasing the risk of local extirpation or extinction by infection (as per de Castro & Bolker, 2005). The common toad (*Bufo bufo*) has been undergoing recent marked and unexplained declines in southeast England (Carrier et al., 2003), the area where *Ranavirus* infection in amphibians is most prevalent in Great Britain. Cunningham et al., (2007b) speculated that *Ranavirus* might be driving these declines. Although Duffus (2009b) showed that common toad tadpoles are susceptible to *Ranavirus* infection, infection experiments in adult common toads have not been performed. Further work is required to investigate the role, if any, of *Ranavirus* in toad declines in England.

The emergence of frog virus 3 (FV3) in other European amphibians has been explored experimentally. Italian agile frog (*Rana latastei*) tadpoles exposed to different doses of FV3 (Pearman et al., 2004) exhibited dose-dependent effects, with those exposed to higher doses experiencing higher levels of mortality, and with death occurring sooner after exposure, than those exposed to lower doses (Pearman et al., 2004). The FV3–*R. latastei* system was used to investigate the role of host population genetic structure on the impact and dynamics of an introduced pathogen to a naive host species (Pearman & Garner, 2005). Tadpoles from populations with lower genetic diversity experience higher mortality rates than those from populations with higher levels of diversity (Pearman & Garner, 2005). Whilst a greater variation in the mortality rate associated with FV3 exposure was observed in tadpoles from populations with higher genetic diversity, the overall survivorship was higher for these populations (Pearman & Garner, 2005).

Recently, mass mortalities of wild amphibians due to ranavirus infection have occurred in mainland Europe, with reports in 2008 from both Spain and Denmark (Table 2). A mass mortality event of common midwife toad (*Alytes obstetricans*) tadpoles occurred in Spain in 2008 (Balseiro et al., 2009). The tadpoles were suffering from systemic haemorrhages involving the eyes, gills, skin and/or internal organs (Balseiro et al., 2009). The rana-

Table 2. Summary of countries and amphibian species from which ranavirus infection has been reported in Europe.

Location	Common name	Species	Reference
UK	Common frog	*Rana temporaria*	Cunningham et al., 1996
	Common toad	*Bufo bufo*	Hyatt et al., 2000
	Common midwife toad	*Alytes obstetricans*	Duffus, 2009a
	Smooth (or common) newt	*Lissotriton* (formerly *Triturus*) *vulgaris*	Duffus, 2009a
Croatia	Edible frog	*Rana esculenta*	Fijan et al., 1991; OIE, 2007
Spain	Common midwife toad	*Alytes obstetricans*	Balseiro et al., 2009, in press
	Alpine newt	*Ichthyosaura alpestris* (formerly *Mesotriton alpestirs cyreni*)	Balseiro et al., in press
Denmark	Edible frog	*Pelophylax esculentus* (formerly *Rana esculenta*)	Ariel et al., 2009

virus responsible for this mass mortality has tentatively been called the common midwife toad virus (Balseiro et al., 2009). This is the first ranavirus-associated mass mortality in tadpoles reported in Europe. Again in 2008, a ranavirus-associated mass mortality involving both common midwife toad tadpoles and alpine newt (*Mesotrion alpestris cyreni*, now *Ichthyosaura alpestris*) larvae was described in Spain (Balsiero et al., in press). In Denmark, approximately 1200 adult *Pelophylax esculentus* died during a ranavirus disease outbreak (Ariel et al., 2009). As commonly found in North American outbreaks, although multiple amphibian species were present, only one species (in this case, *P. esculentus*) appeared to be affected (Ariel et al., 2009).

It is likely that the low number of reports of ranavirus disease of amphibians in mainland Europe is due, in part at least, to under-surveillance. Garden ponds are the most common habitat for amphibians in Great Britain, from where there are large numbers of reports each year, but in continental Europe amphibians occur mainly in rural areas. When mass mortalities of amphibians do occur in mainland Europe, they are rarely adequately investigated and rarely are follow-up tests for specific pathogens, such as ranaviruses, carried out. Increasing numbers of reports of ranavirus mortality in mainland Europe might reflect increasing interest in, and investigation of, amphibian mortality events. Increased surveillance, pathological investigations and molecular virological studies are required in order to determine the true prevalence of ranavirus mortality of amphibians in Europe, the likely source of the pathogen(s) and the degree of threat posed to European amphibian conservation.

OTHER INFECTIOUS DISEASES AND POTENTIAL PATHOGENS

Whilst chytridiomycosis and ranavirus disease are the two major infectious causes of death of amphibians in Europe, there are undoubtedly many others. This is, however, a profoundly under-studied area of herpetology and veterinary medicine, with few other pathogens recognized as causing mortality in, or negatively affecting fecundity or population survival of, European amphibians. There has been a small number of reports of bacterial infection causing mortality in wild amphibians in Europe, but in all cases the pathological work-up was inadequate to conclude that the bacterium identified was the causative agent. It is probably more likely that bacteria identified as pathogens in past reports were, in fact, either commensal organisms or saprophytic postmortem invaders (Cunningham et al., 1996, 2007a). For example, the bacterium *Aeromonas hydrophila* is a normal commensal of the amphibian skin and intestinal tract, so isolating it from the body of a dead or diseased frog is not surprising and, of itself, is no indication that the organism was implicated in the demise of the animal.

Protozoan and metazoan parasites are also commonly found to infect amphibians in Europe, but only one, *Amphibiocystidium* (formerly *Dermocystidium*; class Mesomycetozoea, order Dermocystida) has been implicated as a significant cause of morbidity or mortality. Disease associated with infection by *Amphibiocystium* or *Amphibiocystidium*-like species has been reported in central Italy (Pascolini et al., 2003), France (Pérez, 1907; González-Hernádez, 2007), Switzerland (Guyénot & Naville, 1922), Czechoslovakia (Broz & Privora, 1952), on the Isle of Rhum, Scotland (Grey, 2008) and in the Scottish Highlands (Duffus, unpublished observations).

Lesions caused by dermocystid protozoa usually are visible as swellings in the skin, although internal organs, particularly the liver, also might be affected (González-Hernádez, 2007). Skin lesions can range from spherical, ovoid or U-shaped (Jay and Pohley, 1981; Pascolini et al., 2003) to carbuncle-like (González-Hernádez, 2007). Also, the localities of the lesions associated with infection may vary: they might be primarily located on the ventral region (Pascolini et al., 2003) or they might not be restricted in their location (González-Hernádez, 2007). According to Densmore & Green (2007), dermocystid infections are usually not fatal and heal on their own, but Pascolini et al. (2003) reported declines in populations of

R. esculenta complex, but not in sympatric *R. lessonae* in central Italy, where there was a high prevalence and infection intensity of *Amphibiocystidium ranae*. Infection with *Bd* was later reported in this population (Simoncelli et al., 2006), however, and it is unclear which, if either, parasite was implicated in the *R. esculenta* complex population declines, further underlining the need for adequate sample collection and detailed pathological investigation, since in the first instance skin samples were not collected.

There is a multitude of other infectious agents and diseases of amphibians, of which relatively little is known, such as herpes (and other) viruses, helminths, protozoa and bacteria. In most cases, due to a lack of information on the parasitic fauna of healthy amphibians, we do not know what a normal parasite burden or composition is and caution is required when investigating and interpreting the results of pathological investigations.

CONCLUDING REMARKS

Recently there has been an increasing awareness of infectious disease as a threat to wildlife conservation (e.g. Smith et al., 2009), but, in general, diseases of wildlife remain poorly studied and understood, with amphibians being no exception. Amphibian chytridiomycosis, and its association with amphibian declines globally, was discovered in 1997 (Berger et al., 1998), and this has since led to a marked and unprecedented interest in amphibian disease. Most attention, however, has been directed only to *Batrachochytrium dendrobatidis*, the causative agent of amphibian chytridiomycosis, and amphibian diseases in general remain profoundly under-studied with few pathogens recognized relative to other host taxa.

When an unusual, or mass, mortality event of amphibians is discovered or reported, it is important to collect as much information about the event and as many affected individuals (sick and freshly dead animals) as possible. The necessity that these animals undergo detailed, systemic postmortem examination by a suitably qualified and knowledgeable expert (ideally a veterinary pathologist) cannot be understated. Using a multidisciplinary approach to understanding the causes and consequences of mortality and decline is important to ensure that the correct conclusions and mitigation measures are made. Further information on amphibian disease investigations, including guidance on conducting postmortem examinations, is provided by Whittaker & Wright (2001) and by Pessier & Pinkerton (2003). Daszak et al. (2003) provide guidelines on establishing causative links between the presence of an infectious agent, the presence of disease and the presence of population declines in amphibians. The purpose of this review is to provide an overview of the most conservation-relevant amphibian diseases currently known to occur in Europe and to give guidance on the investigation of amphibian mortality events. A multidisciplinary team, including the disciplines of herpetology, ecology and veterinary science, is important to ensure that such investigations realize their full potential.

Infectious disease can have important roles in host population dynamics (Anderson & May, 1979) and under certain circumstances can lead to population declines and/or extinctions (de Castro & Bolker, 2005; Ryder et al., 2007). In Europe, the three main disease threats that we have identified are *Bd*, ranaviruses and a general category of others, dominated by protozoa. The impact of infectious diseases on European amphibian populations, however, remains unknown but is likely a serious threat to many species. Increased vigilance, therefore, along with the improved investigation of amphibian disease is required in order to identify the true extent of these threats and to mitigate their impacts.

ACKNOWLEDGEMENTS

Thank you to Frank Pasmans, Trent Garner and Robert Jehle for helpful comments on earlier versions of the manuscript. ALJD is supported by a Queen Mary, University of London Research Studentship and a Natural Science and Engineering Council (NSERC) of Canada Doctoral Award.

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Accepted: 21 April 2010