Ranavirus ecology in common frogs (Rana Temporaria) from United Kingdom: transmission dynamics, alternate hosts and host-strain interactions.

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RANAVIRUS ECOLOGY IN COMMON FROGS (*RANA TEMPORARIA*) FROM THE UNITED KINGDOM: TRANSMISSION DYNAMICS, ALTERNATE HOSTS AND HOST-STRAIN INTERACTIONS

A Thesis Submitted for the Degree of *Philosophiæ Doctor*

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STATEMENT OF ORIGINALITY

I certify that that the data presented in this thesis are original and are the product of my own toils. All ideas and quotes from other sources are referenced in accordance with the standard practice associated with publications in the biological sciences. I also acknowledge the supervision of Dr T.W.J. Garner and Prof R. A. Nichols.
This thesis combines the styles of UK, European and North American dissertations, in what I hope brings together their best aspects.

Signed:

Amanda L. J. Duffus

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ABSTRACT OF THESIS

The ranaviruses (Family: *Iridoviridae*) are a group of emerging pathogens in amphibians. Ranavirus(es) were introduced to the UK in the late 1980s and have been associated with mass mortality events in common frogs (*Rana temporaria*) in south east England. While the signs associated with the disease are well known in common frogs, little is known about the ecology of the disease in any amphibians in the UK. This thesis begins the process of the eludication of the ecology of the ranavirus in common frogs. To test the two different hypotheses for the transmission/maintenance of ranavirus(es) in North American amphibians, investigations into the life history stages of common frogs (*Rana temporaria*) affected by the ranavirus were undertaken. Eggs and tadpoles were screened using standard molecular methodologies for the presence of the virus. No infections were found in eggs (n = 720), one infection was found in a tadpole (n = 288), but adults were commonly infected with the virus. A mathematical model was developed to investigate if the ranavirus could be maintained in populations of common frogs when only adult-to-adult horizontal transmission of the ranavirus occurred. Under certain circumstances, the virus can persist for long periods of time when this occurs. This is the first attempt to mathematically quantify the dynamics of a ranavirus.

The potential of alternate or reservoir hosts of the ranavirus(es) in the UK were also examined. This permitted for the identification of new amphibian host and for the isolation and characterization of ranaviruses from different hosts. Phylogenetic analyses revealed that all of the viral isolates were genetically similar at both loci examined.

Experimental work examining the association between ranavirus isolates from different hosts in common frog and common toad (*Bufo bufo*) tadpoles was also performed. At low doses, isolates from common frogs caused higher mortality in common frog tadpoles than isolates from common toads. However, in common toad tadpoles, no such relationship was observed.

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Publications

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CHAPTER 1: GENERAL INTRODUCTION

Portions of this Introduction have been submitted for publication as:

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Amphibian Declines

It is now an established fact that amphibians are declining on a global scale and are the most threatened group of vertebrates on the planet (Houlahan et al. 2000; Stuart et al. 2004). The rates of decline and extinction for amphibians are the highest in the Neotropics, but the two families most affected, the Ranidae and Bufonidae (Stuart et al. 2004), are well represented temperate regions as well. These declines have been linked to many different factors, including anthropogenic environmental change and emerging infectious diseases (Alford and Richards 1999; Daszak et al. 1999; Houlahan et al. 2000). The disease which has been most extensively associated with amphibian declines is chytridiomycosis, caused by infection with *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998). The emergence of *Bd* has been cited as the causative agent in the decline of many amphibian species around the globe (e.g. Central American and Australia: Berger et al. 1998; Europe: Bosch et al. 2001; North America: Fellers et al. 2001). However, another group of emerging infectious agents, the iridoviruses, have also been linked to amphibian population declines (e.g. leopard frogs in Canada; Schock and Bollinger 2005; common frogs in the UK; Teacher 2009), but they have often been overlooked, despite multiple reports of their involvement in mass mortalities (e.g. Green et al. 2002; Greer et al. 2005).

The Influence of Disease

Disease can be an important factor in determining host population dynamics (Anderson and May 1979). In some cases, disease can cause population declines or result in local extirpations of species if certain conditions arise (de Castro and Bolker 2005; Ryder et al. 2007). Key conditions that contribute to population declines due to disease are: small host population sizes; the transmission dynamics of the pathogen;

and the ability of the pathogen to use alternate or reservoir hosts (de Castro and Bolker 2005; Ryder et al. 2007).

The number of diseases caused by emerging pathogens is increasing and in the last 20 years 30 new diseases in humans alone, have been described (Bossart 2007). In amphibians there are three main emerging infectious agents, *Bd* (e.g. Berger et al. 1998), iridoviruses (e.g. Cunningham et al. 1996) and other infections (e.g. trematode infections; Johnson et al. 2002). The focus on *Bd* as the preeminent pathogen responsible for amphibian declines has left us with large gaps in knowledge about other infectious diseases and their roles in amphibian population dynamics. In Europe, if this situation is combined with the lack of knowledge about the natural history and population status of the majority of amphibian species, we are not going to be able to adequately assess the impacts of disease (Pasmans et al. 2006). This should be a great cause for concern because by 2050, if only the predicted changes in climate are accounted for, the majority of suitable habitat for amphibians will be lost (Araújo et al. 2006). If this is considered alongside other forms of habitat loss, anthropogenic environmental pollution, and the effects of emerging infectious diseases, this will spell disaster, for even what are currently considered to be the most common European amphibian species.

One difficulty in examining the role of infectious disease in population declines is the delineation between an infection and a disease. Infection is the presence of a pathogen or a potentially pathogenic agent in a host or in the host population (Scott 1988). Disease, however, requires the presence of infection combined with a measurable effect (e.g. clinical signs), the severity of which are determined by physical examination (Scott 1988). The line between infection and disease has been blurred with the advent of sensitive molecular tests for pathogens. The assumption that the presence of the genetic material (either DNA or RNA) of the pathogen, detected using modern methods, is the same as observing signs of disease is unequivocally false. This is especially true with investigations that examine the presence/absence of *Bd* in amphibians since only swabs are usually taken for molecular analysis and the animals are only inspected briefly, if at all, for the presence of clinical signs of disease.

To over come these shortfalls, a set of guidelines for determining the role of disease in amphibian mortality and declines have been established (Daszak et al.

2003), however they are seldom adhered to. These guidelines are as follows: The first step in examining a pathogen that is thought to contribute to mortality or decline is fulfilling Koch's postulates (Daszak et al. 2003). In the most basic form these postulates are as follows: 1 - The pathogen occurs in all cases of the disease; 2 – The pathogen is responsible for the clinical signs of the disease as well as the pathological changes associated with the disease; 3 – The pathogen is not associated with another disease; 4 – The pathogen, when isolated and introduced into a naïve individual causes the same disease (Modified from Evans 1976). Once Koch's postulates have been supported, then the pathogen must be identified as the cause of the majority of the mortality in a mortality/decline event (Daszak et al. 2003). The establishment of disease-induced mortality, based on pathological investigations, as the actual cause of the population decline is essential if the link between disease and decline is to be made (Daszak et al. 2003). This is needed since in some cases, although disease has been responsible for mortality or even mass mortality events, other causes can be responsible for decline. A good example of this is the presence of *Bd* in the Savannah River Site in South Carolina, where infections, but not disease, were found in historical samples and Daszak et al. (2005) argue that the declines of amphibians in the area was associated with climatic change and reproductive failure.

This illustrates the care needed to adequately and accurately determine if the pathogen present is in fact the agent responsible for the decline or even local extirpation of an amphibian species. It also shows that while advancements in diagnostic techniques are useful, caution must be used in interpreting their meaning, making accurate assessments of the effects of disease difficult.

The Iridoviruses

The iridoviruses (Family: *Iridoviridae*) are a group of viruses which affect invertebrates and ectothermic/poikilothermic vertebrates with four different genera: Ranavirus, Lymphocystivirus, Iridovirus, and Chloiridovirus (Mao et al. 1997; Chinchar 2002; Chinchar et al. 2009). The viruses which are part of the Ranavirus and Lymphocystivirus genera are those which are known to infect vertebrates, whereas, the viruses in the Iridovirus and Chloridiovirus genera are those which affect invertebrates (Mao et al. 1997; Chinchar 2002; Chinchar et al. 2009). The lymphocystiviruses are currently only known to infect fish (Chinchar 2002). The

ranaviruses affect a wide range of animals, including fish, reptiles (including turtles and tortoises), and amphibians (Ahne et al. 1997; Chinchar 2002).

The ranaviruses are defined by their morphology and the signs of disease that result from infection (Chinchar 2002). The viral particles may be enveloped or non-enveloped, ranging in size from 130nm to 200nm (Chinchar 2002). The genomes are circular, double-stranded DNA ranging from 100kb to 210kb in length, they are also highly methylated and have cytosine-guanine concentrations of over 50% (Schetter et al. 1993; Ahne et al. 1997; Tidona et al. 1998; Chinchar 2002).

Iridovirus Infections in Reptiles:

The investigation into iridovirus infections in reptiles, turtles and tortoises is a relatively new area of research, consisting mostly of opportunistic descriptions of disease. The majority of the iridoviruses found in these animals have been similar to frog virus 3 (FV3, the type-virus of the ranaviruses; Chinchar 2002) or have been designated as new types of viruses. (Please see Table 1.1 for a summary of iridoviruses currently described in reptiles, turtles and tortoises.)

The signs associated with iridovirus infections range from asymptomatic to severe/fatal and include: upper respiratory tract lesions; lethargy; dehydration (Westhouse et al. 1996; Allender et al. 2006; Hyatt et al. 2002), necrosis of internal organs (Hyatt et al. 2002) and intracellular inclusions (Johnsrude et al. 1997). These signs may also be accompanied by the development of secondary bacterial infections (Marshang et al. 1999).

Interestingly, captive reptiles have been described with iridoviral disease caused by infection with an invertebrate iridovirus (Just et al. 2001). Disease signs associated with iridovirus infections were seen in bearded dragons (*Pogona vitticeps*), a chameleon (*Chamaeleo quadricornis*) and a frilled lizard (*Chamydosaurus kingii*) (Just et al. 2001). When the identity of the virus responsible was determined, it was found to have 97% homology with the major capsid protein (MCP) 97% homology with the *Chilo iridescent virus* MCP and 100% homology with *Gryllus bimaculatus* iridescent virus MCP, both invertebrate iridoviruses which had not previously been known to infect vertebrates (Just et al. 2001). This is an important discovery since it demonstrates the ability of the iridoviruses to make large host jumps, which can have implications for animal husbandry and indicates the need for complete investigations into the range of potential viral hosts.

Table 1.1. Summary of iridoviruses currently known to occur in reptiles. The species affected, virus type/name and citation are provided.

Common Name	Species Name	Virus	Location	Reference
Gopher Tortoise	*Gopherus polyphenes*	Unclassified	USA	Westhouse et al. 1996
Soft-shelled turtle	*Trionyx sinnesis*	Soft-Shelled Turtle Virus	China	Chen et al. 1999; Huang et al. 2009
Hermann's Tortoise	*Testudo hermanii*	FV3-Like	Captive	Marschang et al. 1999
Fer de Lance	*Bothrops moojeni*	Snake Erythrocyte Virus	Captive	Johnsrude et al. 2001
Bearded Dragon	*Ponga vitticeps*	Iridescent Virus-Like	Captive	Just et al. 2001
Chameleon	*Chamaeleo quadricornis*			
Frilled Lizard	*Chamydosaurus kignii*			
Eastern Box Turtle	*Terrapene carolina carolina*	FV3, FV3-Like	USA	Allender et al. 2006
Leopard Tortoise	*Geochelone pardalis pardalis*	FV3-Like	Captive	Benetka et al. 2007
Green python	*Chondropython viridis*	Unclassified – New	Imported to Australia	Hyatt et al. 2002
Burmese Star Tortoise	*Geochelone platynota*	Burmese Star Tortoise Virus	Captive	Johnson et al. 2007

Iridoviral Infections in Fish:

The study of iridoviral infections and disease in fish has been extensive due to the economic importance of the species involved. Many different iridoviruses have been isolated and characterized from ornamental and economically important species. (Please see Table 1.2 for a brief summary of fish iridoviruses.) One of the first documented iridovirus outbreaks in fish was originally recorded as viral erythrocytic necrosis (VEN; Smail 1982). The virus responsible for VEN had, what would now be considered to be, the typical iridovirus morphology and classified as an icosahedral cytoplasmic deoxyribovirus (ICDV; Smail 1982). Subsequently, ICVDs were renamed Iridoviruses and placed into the newly created viral family *Iridoviridae* (Smail 1982).

Iridoviruses thrive in aquaculture facilities because of the high density of animals, as well as the high pathogenicity and virulence of the pathogen. Outbreaks iridoviruses in aquaculture facilities can have mortality rates of over 95% (Langdon and Humphries 1987; Bloch and Larsen 1990; Hedrick et al. 1990) and therefore can incur substantial financial losses. The severity of iridovirus infections in fish range from asymptomatic (Berry et al. 1983) to severe/fatal (Schuh and Shirley 1992). Clinical signs associated with iridoviral infections in fish include: lethargy; emaciation; oedema; systemic lesions or haemorrhages; tissue necrosis and viral inclusions especially in gills, liver, spleen and pancreas (Langdon and Humphries 1987; Hedrick et al. 1990; Bloch and Larsen 1990; Fraser et al. 1993; Tamai et al. 1997; Gibson-Kueh et al. 2003).

Recent investigations into fish iridoviruses have implicated a relationship between the trade in ornamental fish and the emergence of iridoviruses in economically important species (Go et al. 2006). This study suggests that the iridoviruses originated in Asia and the trade in ornamental fish has been responsible for the geographical spread of the iridoviruses, which in turn facilitated the increase in the number of host species (Go et al. 2006). Furthermore, since many of the iridoviruses found in fish are similar to FV3 (e.g. Tamai et al. 1997l; Mao et al. 1999), iridoviruses may have spread to amphibians from fish through this route. This hypothesis is supported by the discovery of an identical ranavirus isolated from sympatric three-spined sticklebacks (*Gasterostelus aculeatus*) and red-legged frog tadpoles (*Rana aurora*; Mao et al. 1999), which suggests that fish could be alternate/reservoir host of amphibian ranaviruses.

Table 1.2. A brief summary of fish species affected by iridovirus infections. They are classified as to whether the species is ornamental or economically important and the type of iridovirus isolated or characterised from the species is also included.

Common Name	Species Name	Class of Fish	Virus	Location	Reference
Turbot	*Scophthalmus maximus*	Economic	Unclassified	Denmark (Aquaculture)	Bloch and Larsen 1990
White Sturgeon	*Acipenser transtotanus*	Economic	Unclassified	USA	Hedrick et al. 1990
Sheatfish	*Silurus glanis*	Economic	Icosahedral Cytoplasmic Deoxyribovirus	Germany (Aquaculture)	Ahne et al. 1990
Gold Fish	*Crarassius auratus*	Ornamental	FV3-Like	USA	Berry et al. 1983
Angel Fish	*Pterophyllum scalare*	Ornamental	Unclassified	Canada; Not Disclosed	Shuch and Sherly 1990; Paperna et al. 2001
Dwarf Gouramis	*Colisa lalia*	Ornamental	Unclassified	Australia	Anderson et al. 1993;
Gouramis	*Trichogaster trichopterus*	Ornamental	Similar to Haemapoetic Necrosis Virus (HNV)	USA; Not Disclosed	Fraser et al. 1993; Paperna et al. 2001
Guppies	*Poecillia reticulate*	Ornamental	Unclassified	Australia	Hedrick and McDowell 1995
Doctorfish	*Labroides dimitatus*	Ornamental			
Red Seabream	*Pageus major*	Economic	FV3-Like	Japan (Aquaculture)	Tamai et al. 1997
Stripped Jack	*Caranx delicatissimus*	Economic			
Red-Finned Perch	*Perce fluviatilis*	Economic	Epizootic haematopoetic virus (EHNV)	Australia	Langdon et al. 1986; Reddacliff and Whittington 1996
Rainbow trout	*Oncorhynchus mykiss*	Economic	EHNV	Australia	Reddacliff and Whittington 1996
Swordtail	*Xiphophorus hellerii*	Ornamental	Lymphocystis – Like	Not Disclosed	Paperna et al. 2001
Flounder	*Paralichthyes olicaceus*	Economic	Lymphocystis	China	Zhang et al. 2004

Iridoviruses Infections in Amphibians

Salamanders and Newts:

The first ranavirus infections described in wild urodeles were from a mass mortality event in endangered Sonoran tiger salamanders (*Ambystoma tigrinum stebbinsi*) that occurred in 1995/96 in Arizona, USA (Jancovich et al. 1997). This mortality event was the first ranavirus-associated mass mortality event in North America and the first to be documented in any wild urodele species. Subsequently, in 1997, mass mortalities in tiger salamanders (*A. tigrinum*) in Saskatchewan, Canada were described (Bollinger et al. 1999). The viruses involved in all of these events are similar and considered to be the *Ambystoma tigrinum* virus (ATV) or ATV-like (see Shock et al 2008). Ranavirus-associated mortality events and ranavirus infections in North American urodele species continue to be reported (e.g. Green et al. 2002; Docherty et al. 2003; Duffus et al. 2008), however, this is likely to be only a small portion of those that actually occur. (Please see Table 1.3 for a summary of ranavirus infections in Australian and North American amphibians, European species will be dealt with in greater detail later on).

In Europe, the first reported mass-mortality in wild urodeles occurred in alpine newt (*Mesotrition alpestirs cyreni*) larvae from the Iberian Peninsula (Balsiero et al. in press). However, previously, in the Netherlands and Belgium, mortality associated with ranavirus infection was reported in red-tailed knobby newts (*Tylototriton kweichowensis*) imported from Asia (Pasmans et al. 2008). Initially in good breeding condition, the animals, kept by different hobbyists, started to experience mortality (Pasmans et al. 2008). Heavy nematode infections were discovered in the lungs and were treated with fenbendazole, however, deaths continued and virological investigations led to the isolation of a ranavirus with 99.8% homology to the major capsid protein (MCP) of FV3 (Pasmans et al. 2008).

The signs associated with ranavirus infections in urodeles are conserved and include: the development of polyps on the skin, external and internal haemorrhages, emaciation, lethargy, oedema of body cavities, discoloration of the liver and viral inclusions (Jancovich et al. 1997, Bollinger et al. 1999, Docherty et al. 2003; Pasmans et al. 2008). The presence of these signs are important indicators of ranavirus infections in urodeles.

Table 1.3. Brief summary modern ranaviruses known to infect American and Australian amphibians, taking into account the new species names for hosts.

Common Name	Species Name	New Species Name*	Virus	Location	Reference
Cane Toad	*Bufo marinus*		Iridovirus-like	Australia	Spear et al. 1991
Ornate Burrowing Frog	*Limnodynates ornatus*		Bohle Iridovirus	Australia	Spear and Smith 1992; Hengstberger et al. 1993
Sonoran Tiger Salamander	*Ambystoma tigrinuim stebbinsi*		*Ambystoma tigrinum* virus	USA	Jancovich et al. 1997
Tiger Salamanders	*Ambystoma tigrinum diaboli*		Regina Ranavirus	Canada	Bollinger et al. 1999
Red-legged Frog	*Rana aurora*	*Aurorana aurora*		USA	Mao et al. 1999
Pig frog	*Rana grylio*		FV3-like	Asia	Zhang et al. 2001
North American Bullfrog	*Rana catesbeiana*	*Lithobates catesbeianus*	Not Classified	USA	Green et al. 2002
Mink frog	*Rana septentrionalis*		Not Classified		
Pickerel Frog	*Rana palustris*	*Lithobates palustris*	Not Classified		
Green Frog	*Rana clamitans*	*Lithobates clamitans*	Not Classified		
Spring Peeper	*Pseudacris crucifer*		Not Classified		
Spotted Salamander	*Ambystoma maculatum*		FV3-like	USA	Docherty et al. 2003
Tiger Salamanders	*Ambystoma tigrinum daiboli*		Regina Ranavirus-like		
Tiger Salamanders	*Ambystoma tigrinum melanostictum*				
Wood Frogs	*Rana sylvatica*		FV3-like	Canada	Greer et al. 2005
Leopard frogs	*Rana pipiens*	*Lithobates pipiens*	FV3-like		
North American Bullfrogs	*Rana catesbeiana*	*Lithobates catesbeianus*	*Rana catesbeiana* virus Z	South America	Majji et al. 2006
Grey Treefrog	*Hyla versicolor*		FV3-like	Canada	Duffus et al. 2008
Eastern Spotted Newts	*Notophthalmus viridescens*		FV3-like		

* As per Frost et al. 2006 and Che et al. 2007

Frogs and Toads:

Ranavirus infections in anurans are well documented in both North America and Europe. The ranid frogs appear to be the group of anurans which are most dramatically affected by ranavirus emergence. Wood frog (*Rana sylvatica*) tadpoles can have mortality rates reaching nearly 100% in the wild when a ranavirus first emerges (Greer et al. 2005). Adult common frogs (*Rana temporaria*) that are infected with a ranavirus usually develop severe disease with a high chance mortality (Cunningham et al. 1996; Please see Table 1.4 for a summary of European amphibians known to be affected by ranaviruses).

The first documented ranavirus-associated mortalities in amphibians were described in common frogs from the southeast of England (Cunningham et al. 1993; Drury et al. 1995; Cunningham et al. 1996). Reports of unusual mortalities in common frogs began in the late 1980s and by 1992 the 'Frog Mortality Project' was set up to investigate them (Cunningham et al. 1995). This project identified an FV3-like virus as the causative agent of the disease responsible for the observed mass mortalities in common frogs (Cunningham et al. 1993; Drury et al. 1995; Cunningham et al. 1996; Hyatt et al. 2000). Ranavirus-associated disease was also identified in common toads (*Bufo bufo*) in the southeast of England during this time (Hyatt et al. 2000; Cunningham et al. 2007b).

In common frogs there are two different disease syndromes associated with ranavirus infections, the ulcerative form and the haemorrhagic form (Cunningham et al. 1996). The ulcerative form is characterized by the presence of ulcerations of the skin (which may include the skeletal muscle) and necrosis of the digits (Cunningham et al. 1996). Whereas, the haemorrhagic form is characterized by the presence of haemorrhages in the internal organs, most commonly the gastrointestinal and reproductive tracts (Cunningham et al. 1996). Additionally, frogs that show the ulcerative form of ranaviral disease are usually thin or emaciated, whereas, those that suffer from the haemorrhagic form can be in good condition with large fat bodies and well developed reproductive tracts, i.e. in females eggs are present in the oviducts and males have large testes (personal observation). The presence of two different disease syndromes as a result of ranavirus infection is very unusual. In North America, the signs of ranaviral disease in anurans is conserved and similar to those described for urodeles.

There is evidence that the emergence of the ranavirus in UK common frogs has had measurable negative effect. There are data that suggest common frog populations have declined in southeast England where the ranavirus has emerged (Teacher 2009). Also, the emergence of the ranavirus has been found to be a significant selection pressure on common frog populations (Teacher et al. 2009a&b). In populations that have been affected by the ranavirus for a prolonged period of time (~10 years, which is about 5 frog generations), selection of certain MHC haplotypes has been documented (Teacher et al. 2009a).

The effects of ranavirus emergence, in a naïve group of amphibians, has been assessed experimentally using the Italian agile frog, *Rana latastei* (Pearman et al. 2004; Pearman and Garner 2005). When tadpoles were exposed to different doses of FV3, mortality was dose dependant, with animals that were exposed to higher doses experiencing mortality at a shorter time interval from exposure than lower doses (Pearman et al. 2004). The potential for transmission of FV3 through scavenging interactions was also tested, and tadpoles that contracted the virus by scavenging dead, infected tadpole corpses exhibited higher mortality than those exposed to an infected carcass but were not able to consume it (Pearman et al. 2004).

Further experiments using the *R. latastei*-FV3 system were performed, this time factoring in the genetic variation of the host (Pearman and Garner 2005). Tadpoles from populations with low genetic diversity experienced high rates of mortality when exposed to low concentrations (10^4 plaque forming units/mL) of FV3 (Pearman and Garner 2005). While there was more variation in the survival of tadpoles exposed to the same low concentration of FV3 from populations with higher genetic diversity, on average they experienced lower mortality rates (Pearman and Garner 2005). Time to death also differed significantly between high and low genetic diversity source populations, further supporting the hypothesis that greater genetic diversity buffers against the negative effects of ranavirus emergence in naïve populations (Pearman and Garner 2005).

Reports of ranavirus infections from mainland Europe are becoming more common. In 2008, a large scale mortality event in common midwife toad (*Alytes obstetricans*) tadpoles was discovered on the Iberian Peninsula (Balseiro et al. 2009). The tadpoles were suffering from systemic haemorrhages (involving eyes, gills, skin and/or internal organs) and the agent responsible was determined to be a ranavirus,

tentatively called the common midwife toad virus (Balseiro et al. 2009). In 2008 an extremely large mass mortality event (approximately 1200 frogs) occurred in Denmark (Ariel et al. 2009). *Pelophylax esculentus* (formerly *Rana esculenta*) seems to be the only amphibian species involved in the event and investigations determined a ranavirus was the causative agent (Ariel et al. 2009). The mortality event occurred when there was an extremely high density of frogs in the pond, as well as a period of hot weather (Ariel et al. 2009). The association of the mortality with warm weather is not unexpected as mortality events in the UK peak during the warmest part of the summer (personal observation).

There are many more species of amphibians in Europe that have not been examined for the presence of the ranavirus. Many of these species are currently in decline and it is possible that the ranavirus is a contributing factor. There is a need to examine multiple species of amphibians that can, are thought to and/or are known to carry ranavirus infections. Therefore, detailed examinations into the transmission dynamics of known amphibian-ranavirus systems, as well as investigations into host-species interactions and virulence of the ranavirus(es) present in Europe are necessary.

Table 1.4 Summary of locations and species known to be affected by a ranavirus in Europe.

Location	Common Name	Species	Reference
UK	Common Frog	*Rana temporaria*	Cunningham et al. 1996
	Common Toad	*Bufo Bufo*	Hyatt et al. 2000
Croatia	Edible Frogs	*Pelophylax esculentus* (formerly *Rana esculenta*)	Kunst and Valpotić 1968; Fijan et at. 1991
Eastern Europe	Edible Frogs	*Pelophylax esculentus* (formerly *Rana esculenta*)	Mişcalencsu et al. 1981
Spain	Common Midwife Toad	*Alytes obstetricans*	Balseiro et al. 2009
	Alpine Newts	*Mesotrition alpestirs cyreni*	Balseiro et al. in press
Demark	Edible Frogs	*Pelophylax esculentus* (formerly *Rana esculenta*)	Ariel et al. 2009
Belgium and the Netherlands (Imported)	Red Tailed Knobby Newts	*Tylototriton kweichowensis*	Pasmans et al. 2008

The Host: Specificity, Alternatives and Implications

Amphibian ranaviruses are throught to be multi-host pathogens (Schock et al. 2008) or infections of amphibian communities (Duffus et al. 2008). Since many pathogens are capable of infecting multiple host species, many aspects of pathogen biology including ecology, transmission dynamics and pathogenesis are unknown, the true scale and impacts of infection are not understood (Woolhouse et al. 2001). In the case of amphibian ranaviruses, which are known to have broad host species jumps (Mao et al. 1999), they are often mistreated as single host pathogens, leading to potentially incorrect assumptions about pathogen dynamics.

In the event of a ranavirus-associated mass mortality, sympatric amphibian species are seldom screened for ranavirus infections. In 1997, a mass mortality event of tiger salamanders (*Ambystoma tigrinum diaboli*), occurred in Saskatchewan, Canada, however, despite the presence of other amphibian species, none were screened for the presence of the virus (Bollinger et al. 1999). When, in 1999, ranavirus associated mass mortality events began in south eastern Ontario, no other potential host species were examined for the presence of viral infections, other than those involved in the mortality (*Rana sylvatica* or *Lithobates pipiens*; Greer et al. 2005; Charbonneau 2006).

The first attempts to examine ranavirus infections in the context of the amphibian community were in 2005 (Duffus 2006; Duffus et al. 2008). The study examined sites that were previously known to have ranavirus infections in wood frog tadpoles (Charbonneau 2006; Duffus et al. 2008). The aquatic amphibian community in the main study pond was diverse consisting of three urodele and four anuran species, all of which had ranavirus infections (Duffus et al. 2008). This study provided the first evidence of community-wide ranavirus infections and demonstrates the importance of considering all amphibian species present, regardless of whether they are involved in ranavirus-associated mortality or showed signs of ranaviral disease.

Experimentation with amphibian ranaviruses has a much longer history and has considered the potential for alternative hosts, but not necessarily their role in maintaining the virus within a species or community. The first attempts to examine host-specificity dates back to the late 1960s. Clark et al (1968) successfully produced ranaviral disease in red efts (also called eastern spotted newts, *Notophthalmus*

viridescens) and were able to re-isolate the virus from the animals which showed signs of disease. However, when North American bullfrogs (*Lithobates catesbieanus*) were submitted to the same treatment, no disease was seen, nor were attempts to re-isolate the virus successful (Clark et al. 1968). These experiments were the first to demonstrate that amphibian ranaviruses showed host-specificity with respect to infection and the subsequent development of disease.

Further experiments examining host-specificity were not performed until the 1990s. Bohle Iridovirus (BIV), was originally isolated from *Limnodynates ornatus* (Spear and Smith 1992; Hengstberger et al. 1993), and used in infection trials of three different Australian anuran species. Experimental infections in *Litoria latopalmata* and *Limnodyates terraeginae* revealed that not only was the route of exposure important in the development of disease, but also, the life history stage at which animals were exposed (Cullen et al. 1995). When *Limnodyates terraeginae* tadpoles were infected with BIV using a bath exposures, mortality was dependant on the concentration of BIV that was used (Cullen et al. 1995). However, when *Limnodyates terraeginae* metamorphs were infected by bath exposure, they showed fewer signs of disease (2 of 8), than those that were in contact with an infected individuals (4 of 8), or those that were injected with BIV (6 of 8; Cullen et al. 1995). Further experiments with *Litoria caerulea* demonstrated that metamorphs, but not adults, developed disease when exposed to BIV (Owens and Cullen 2002). Therefore, both the route of exposure and the life history stage that exposure occurs, are both important factors in the development of ranaviral disease.

Interest began to develop in alternate hosts of amphibian ranaviruses in the wild. Experiments to determine potential alternate hosts of ATV in the area where the original outbreak occurred were performed (Jancovich et al. 1997; Jancovich et al. 2001). Three sympatric amphibian and three sympatric fish species were examined for susceptibility to ATV infections to determine if they could act as alternate hosts (Jancovich et al. 2001). Infection trials were only successful in two amphibian species, *Ambystoma gracile* and *Notophthalmus viridescens* (Jancovich et al. 2001). In the absence of field data to support their conclusions, the authors suggest that ATV was not maintained within the population of Sonoran tiger salamander, but was brought in by other means (Jancovich et al. 2001).

Attempts to examine broader host-strain associations of amphibian ranaviruses have also been undertaken. ATV and FV3-like viruses were used in exposure experiments in *A. tigrinum* and two anuran species (Schock et al. 2008). ATV exposure resulted in higher mortality in *A. tigrinum* metamorphs than FV3-like viruses (Schock et al. 2008). Whereas, exposure to FV3-like viruses resulted in higher mortality in the anuran metamorphs than ATV exposure (Schock et al. 2008). Here, the isolates were more virulent in the group of amphibian that they were originally derived from (Schock et al. 2008). While these results appear to show compelling evidence of host-strain associations, low sample sizes and poor experimental design require caution when interpreting the results and further, statistically robust investigations are required to make concrete conclusions.

With the emergence of ranaviral disease in the UK, an interesting experimental system has also emerged. Ranaviral disease presenting in two different syndromes (as previously described) is unique to common frogs. This system provides a venue for examining host-strain interactions, the evolution of an emerging pathogen and viral persistence in host populations. However, despite a long term data set of nearly twenty years, the disease dynamics in common frogs is poorly understood and an important experimental system is left underutilized.

Preliminary investigations into the relationships between the two disease syndromes in UK common frogs have shown that the source of the virus used and the route of exposure are important factors in the development of disease (Cunningham et al. 2007a). When adult common frogs were exposed to a tissue homogenate (derived from a frog with the haemorrhagic ranaviral disease) via bath exposure, no disease developed (Cunningham et al. 2007a). However, when the same experiment was repeated with a tissue homogenate derived from an animal that had the ulcerative form of ranaviral disease, some animals did develop the ulcerative form of ranaviral disease (Cunningham et al. 2007a). When frogs were exposed via immersion to a culture of virus isolated from a diseased animal with the haemorrhagic or ulcerative form of the disease, signs of the haemorrhagic and/or ulcerative ranaviral disease developed (Cunningham et al. 2007a). This suggests that there are different ranaviruses present in common frogs in the UK, which may be akin to quasi-species seen in RNA viruses (Cunningham et al. 2007a). When common frogs were exposed to a ranavirus isolate from a common toad, found

diseased in the wild, haemorrhagic syndrome developed (Cunningham et al. 2007b). The implications of this are toads may be acting as reservoirs/alternate hosts for the ranavirus, however, the sample sizes here are again small and it is difficult to ascertain the true relationship between diseased common frogs and common toads because the reciprocal experiment was not performed. Understanding if or how the ranavirus is spread between species is important for predicting viral persistence and the effects of infection at a population level.

Modelling of Disease Dynamics: Ranavirus-Amphibian Systems

Micro- and macro-parasites can affect the population dynamics of their hosts (Anderson and May 1979; May and Anderson 1979), this is true for the amphibian ranavirus system (e.g. UK common frogs; Teacher 2009). Models are important tools for understanding the impacts of infectious diseases on host populations and also to predict future dynamics of both the host and pathogen (Anderson and May 1979). In some cases the pathogen may in fact be the key factor which modulates the population dynamics of the host (Anderson and May 1979), this is why understanding a pathogen and its effects are extremely important, especially in populations which are affected by other stressors (Acevedo-Whitehouse and Duffus 2009).

The amphibian-ranavirus system has not received much attention with respect to understanding how the virus persists in affected populations or other aspects of pathogen dynamics. Currently, there are only two models which examine the maintenance and transmission of ranaviruses in amphibians, neither of which are based on mathematical models. However, both are based on experimental and field data, and because of this, provide good starting points for understanding host-pathogen dynamics in this exciting system.

The first model was developed for the *Ambystoma tigrinum-Ambystoma tigrinum* virus (ATV) system. This system is characterized by the use of an intraspecific reservoir (Brunner et al. 2004). When young-of-the-year *Ambystoma tigrinum* individuals were screened for the presence of ATV, an average prevalence of 78% infection was seen, with only 25% of these individuals showing signs of disease (Brunner et al. 2004). When the same pond was examined two years later during the spring migration, infected individuals were found returning to the pond

(Brunner et al. 2004). While at first the two year gap in examining animals retuning to the pond may seem odd, the biology of the tiger salamanders suggests that they do not migrate to the pond the year after they metamorphose, but in the second, so this maintains continuity in the cohort of salamanders being studied (Brunner et al. 2004 and references therein). In laboratory experiments, 50% of *A. tigrinum* larvae infected with ATV recovered, while a further 40% of infected individuals retained the infection at a chronic and sub-lethal level (Brunner et al. 2004). Therefore, in *A. tigrinum*, ATV infection is maintained in the host population and re-introduced to the larvae in the pond by adults retuning to breed (Brunner et al. 2004; Figure 1.1).

The importance of the Brunner et al. (2004) model is that the adults act as reservoirs of ATV for the larvae and only a single species is involved in pathogen dynamics. Amplification of ATV infections occurs in the larvae, where the majority of disease induced mortality incurred (Brunner et al. 2004). However, a sufficient number of infected larvae survived, metamorphosed and then returned to the pond as adults, re-introducing the infection and closing the transmission circle (Brunner et al. 2004). This model was the first to demonstrate how a ranavirus could be maintained in an amphibian community.

The second model of ranavirus transmission in amphibians is based on a community of multiple amphibian species. The model assumes that vertical/pseudovertical transmission, parent-to-offspring transmission either in the gamete (vertical) or in the reproductive fluids/tract of either parent (pseudovertical), can occur in at least one of the species present in the community and that intraspecific as well as interspecific horizontal transmission occurs (Duffus et al. 2008). Groups of susceptible, infected or dead individuals are not assumed to be made up of a single species, but instead composed of multiple species (Duffus et al. 2008). This model allows for the development of genetically based resistance to ranavirus infection (Duffus et al. 2008). It also has some key assumptions: the same strain of the ranavirus is present in all species that are infected and can be transmitted between all species; horizontal transmission is the predominant mode of transmission; transmission can occur through scavenging of infected corpses; amphibian species present vary in their susceptibility to ranavirus infection; and asymptomatic carriers of the ranavirus are present in multiple species (Duffus 2006; Duffus et al. 2008; Figure 1.2).

Although both models demonstrate routes of transmission and the method of maintenance of ranavirus infections, they provide little predictive value. The key value of mathematical models in the case of ranavirus is the ability to examine difference scenarios, including under which conditions the virus will be maintained. Therefore, a mathematical approach to understanding the dynamics of ranaviruses would be of great value, especially in UK common frogs, since they have been shown to be declining at sites where the pathogen has emerged (Teacher 2009). Models would provide a valuable tool for uncover the circumstances which permit the long term persistence of ranaviruses where mass mortalities have routinely been documented and also indicate directions for future/further research.

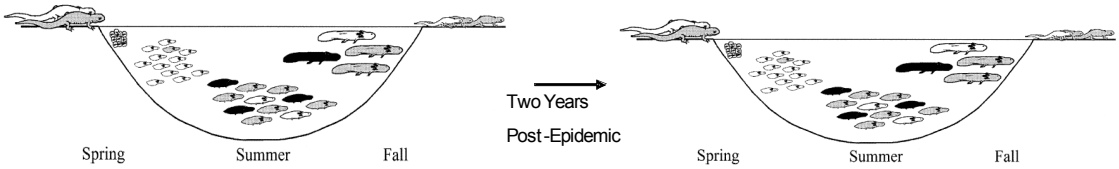

Figure 1.1. Diagram of the intraspecific reservoir transmission of ATV in tiger salamanders. In the spring, breeding adults return to the pond in a mixture of disease free (white) and infected (grey) individuals. The density of the larvae is high and disease transmits between the infected (grey) and susceptible (white) individuals. As the season progresses, the number of infected individuals increases, disease induced mortality occurs (back) and the population density decreases. When the metamorphs leave the pond, some are disease free and others are infected and in two years when they return the cycle starts again. (Adapted from Brunner et al. 2004)

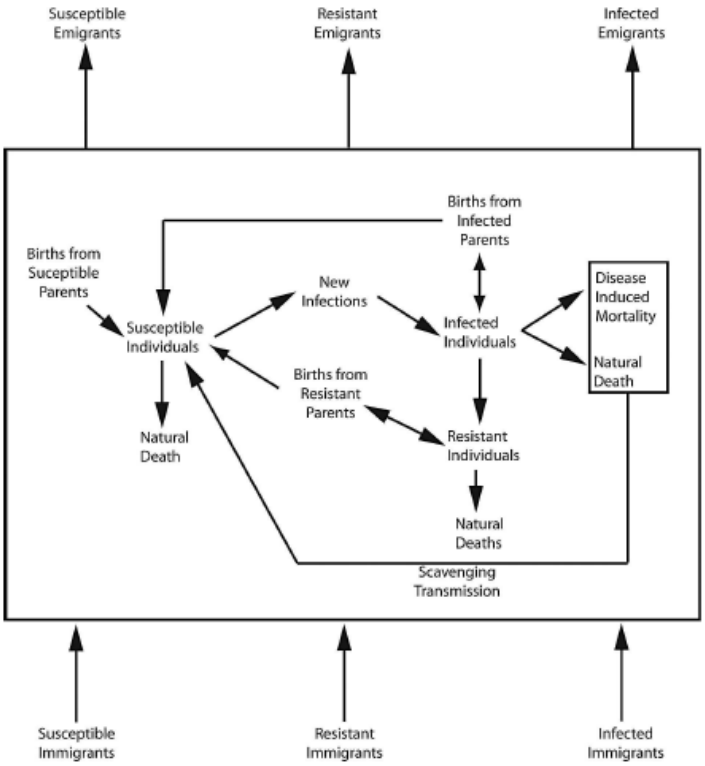

Figure 1.2. The community based transmission model of an FV3-like virus in aquatic amphibians. The outer box shows the limit of the amphibian population. The arrows show direction of infection or movement of individuals. The two types of mortality seen in infected individuals are combined in one box because scavenging off of either type will result in transmission of the virus. (As per Duffus et al. 2008)

Outline of the Thesis

The main question that this thesis addresses is the ecology of the ranavirus in the UK, focussing on the common frog (*Rana temporaria*) and what factors influence the long term persistence of the virus. Each chapter examines the ranavirus(es) present in the UK in a manner which contributes to understanding the central question. The second and third chapters focus specifically on the ranavirus in common frogs. The fourth and fifth chapters take a more applied approach in examining host-strain associations in UK ranavirus isolates. The final chapter serves as a brief summary of the main findings of the thesis and indicates future direction of research which I feel are necessary to gain a better understanding of ranaviruses in the UK.

Chapter 2: Intraspecies Dynamics in Rana temporaria

Here, I take a systematic approach to investigating the ranavirus in common frogs. By examining the different life history stages of common frogs for ranavirus

infections, I test the validity of both North American models of ranavirus dynamics. I test eggs and tadpoles from infected and disease free common frog populations for the presence of ranaviral DNA. Also, I test adult frogs from the archives at the Institute of Zoology and those obtained from mortality events, suspected to be the result of ranavirus outbreaks for the presence of the virus. This chapter provides detailed evidence of the life history stages of common frogs that are affected by the ranavirus in the UK and provides the basis for the next chapter.

Chapter 3: Modelling Transmission Dynamics in Adults

In this chapter, I take a mathematical approach to understanding the dynamics and persistence of ranavirus infections in populations of adult UK common frogs. I explore the conditions under which long term viral persistence is predicted to occur in terms of a simple susceptible-infected (SI) model and parameters derived from the relevant scientific literature. I also explore the viability of viral persistence in the face of disease induced population declines which have been reported by Teacher (2009). Furthermore, I consider the infection dynamics and conditions under which both disease syndromes seen in UK common frogs can persist. I highlight areas where further behavioural and experimental research is needed to gain biologically relevant estimates of different parameters to ensure that the models are applicable to what is seen in common frog populations.

Chapter 4: Alternate Hosts for UK Ranaviruses – A Phylogenetic Approach

Here, I investigate other UK amphibian species for the presence of ranavirus infections. Using viral isolation and phylogenetic methods, I examine the relationships between different UK ranavirus isolates and assess the potential host-strain associations and alternate hosts of the virus. I use a two loci method to examine the phylogeny, which will provide a more accurate picture of the relationships between the different viral isolates and therefore permit for a better understanding of how and if the virus can be maintained in amphibian species other than common frogs.

Chapter 5: Experimental of Virulence and Host-Specificity of UK Ranavirus Isolates

In this chapter, I present the first statistically robust experiments which examine host-specificity, virulence and rates infection between different ranavirus isolates. I expose common frog (*R. temporaria*) and common toad (*Bufo bufo*) tadpoles to four different viral isolates (two derived from naturally infected adult

common frogs and two from naturally infected adult common toads) at two different concentrations. The survivorship data, infection prevalence data, and presence of signs at death will all be used to make inferences about host-strain relationships and virulence of UK ranavirus isolates. These experiments will also be invaluable for determining the primary host of the ranavirus in the UK.

Chapter 6: Conclusions and Summary

Here, I summarize briefly the main conclusions of the chapters and indicate future directions of research which would be helpful for understanding the ecology and evolution of the ranaviruses.

CHAPTER 2: INTRASPECIES RANAVIRUS DYNAMICS IN *RANA TEMPORARIA*: AN EXAMINATION OF THE LIFE HISTORY STAGES AFFECTED BY THE RANAVIRUS IN THE UNITED KINGDOM

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Abstract

Ranaviruses are considered to be an emerging infectious agent in amphibian species. In the UK the infection began to emerge almost 20 years ago, yet, little effort has been made to determine the ecology of the virus or how it is transmitted in what appears to be its primary host, the common frog (*Rana temporaria*). In this chapter I evaluate two ecological models of transmission that have been proposed to explain the epidemiology of ranaviruses in North America. The first, the intraspecific transmission model, proposes that transmission only occurs between individuals of the same species, with the infection being amplified in the larval stages. The second, the community dynamics transmission model, includes vertical/pseudovertical transmission within the same species, but also includes transmission of the ranavirus between other sympatric amphibian species. I found no evidence of infection in eggs (n = 740), and only one infection in tadpoles (n = 288). These findings suggest a rejection of both transmission models that were developed to explain disease transmission in North America. The only consistently infected life history stage were the adults; a finding which demonstrates the need for further investigation into how the disease can be maintained within *R. temporaria* populations.

Introduction

Ranaviruses are classified as emerging infectious agents of amphibian populations. They are large double-stranded DNA viruses from the viral family *Iridoviridae* (Chinchar 2002). Ranavirus infects both ectothermic and poilkilothermic vertebrates: fish, reptiles (including turtles and tortoises), and amphibians (Ahne et al. 1997; Chinchar 2002). In the United Kingdom a ranavirus emerged in the late 1980s and early 1990s and was first detected in adult common frogs (*Rana temporaria*) that had died in mass mortality events (Drury et al. 1993; Cunningham et al. 1995). In the majority of animals examined, pox-virus like particles were detected through histology and electron microscopy (Drury et al. 1993; Cunningham et al. 1995). These particles were isolated and cultured, then determined to be an iridovirus, which upon further characterization were found to be a ranavirus (Drury et al. 1993; Cunningham et al. 1995). The final finding of post mortem examinations was that the causative agent of the disease and mortality events in *R. temporaria* was indeed the virus (Cunningham et al. 1996).

In the UK, when a *R. temporaria* mortally event is reported, which is thought to have been caused by the ranavirus, current practice is to examine only adults for the presence of the pathogen. This concentration on adults maybe inappropriate since, evidence from other anuran species suggests that multiple life history stages (eggs, larvae metamorphs) can be infected by the ranavirus. For example, multiple mass mortality events involving anuran and caudate larvae have been described in North America – one such mass mortality event associated with an outbreak of a ranavirus infections occurred in Saskatchewan, Canada in 1997 and affected both adult and larval tiger salamanders (*Ambystoma tigrinum*) in four different populations (Bollinger et al. 1999). These mortality events showed the typical clinical signs of ranaviral disease, such as dermal lesions or ulcerations, gastrointestinal ulcers and liver necrosis (Bollinger et al. 1999). The virus from these outbreaks was isolated and characterized using molecular methods, resulting in the discovery of a new strain of the ranavirus, called the Regina Ranavirus (Bollinger et al. 1999).

A subsequent descriptive study of amphibian mortality and morbidity events in the United States between 1996 and 2000 reported that iridoviruses contributed wholly or in part to the majority of these events (Green et al. 2002). (See Table 2.1

for a summary). The presence of iridoviruses in such a large proportion of amphibian morbidity and/or mortality events illustrates its potential importance in amphibian population declines.

Table 2.1. Summary of mortality and disease events associated with iridovirus infections in the United States of America between 1996 and 2000 (n = 64). (Adapted from Green et al. 2002.)

Common Name	Latin Name	Number of Iridovirus-Associated Mortality Events
Wood Frogs	*Rana sylvatica*	4
Tiger Salamanders	*Ambystoma tigrinum*	7
Mink Frogs	*Rana septentrionalis*	1
Blue-Spotted Salamanders	*Ambystoma maculatum*	4
Pickerel Frogs	*Rana palustris*	3
Bullfrogs	*Rana catesbieana*	5
Green Frogs	*Rana calmitans*	1
Spring Peeper	*Pseudacris crucifer*	1

In 1999, a mass mortality of wood frog (*Rana sylvatica*) tadpoles occurred near Peterborough, Ontario, Canada (Greer et al. 2005). Mortality events re-occurred at this site in 2001 and 2002 and from 2003 to 2005 ranavirus infections were detected in *R. sylvatica* tadpoles (Greer et al 2005; Charbonneau 2006; Duffus et al. 2008). It was estimated that when the disease emerged in this population, mortality of the tadpoles was nearly 100%, as no metamophs were observed to emerge from the pond in 1999 (Greer et al. 2005). In subsequent years, mortality associated with ranavirus infections appear to have declined, however, ranavirus infections in *R. sylvatica* tadpoles were still detected (Duffus 2006). Despite the presence of multiple amphibian species in this pond, ranavirus-associated mortality was not observed in any other species (Duffus et al. 2008). A similar situation had previously been found in the same area earlier by Greer et al. (2005), who examined 4 other mortality events and found that despite the presence of other amphibian species in the pond that was infected only a single species was affected by mortality. These observations led to the question of how the ranavirus is maintained in populations and communities of amphibians.

There are currently two models of transmission of the ranavirus in amphibians that address the question of virus transmission and maintenance in populations and communities. The first, the intraspecific model (IM) examines the potential for alternate life history stages of a single species to act as reservoir. The model is based on both laboratory and field data for Ambystoma tigrinum virus (ATV) infections in various life history stages of *Ambystoma tigrinum* (Brunner et al. 2004). In this system, there are no alternative amphibian hosts for ATV present in the ponds, nor any evidence of vertical transmission (Brunner et al. 2004). Although there can be a high mortality rate associated with ATV infections, especially in metamorphs, some animals do survive and appear to recover from the disease and carry sub-lethal, transmissible infections (Brunner et al. 2004). These sub-lethal infections can persist for long periods of time and can be returned by terrestrial survivors (Brunner et al. 2004). When the infection is brought back to the pond it is amplified in the larvae, completing the cycle (Brunner et al. 2004). In this model, persistent sub-lethal ATV infections in metamorphs which leave the pond and return as sub-lethally infected adults are the source of the recurring infections leading to a chronically infected population (Brunner et al. 2004).

The second model includes the potential for intraspecific ranavirus transmission, but examines the transmission of a ranavirus at the community level and is based on an amphibian community of more than 5 different species (Duffus et al. 2008). In wood frogs, *Rana sylvatica*, laboratory and field studies showed it was unlikely that ranavirus infections persisted in the species through intraspecific transmission alone, even though both vertical/pseudovertical transmission and horizontal transmission of the ranavirus have been described (Duffus et al. 2008). With vertical/pseudovertical transmission of the ranavirus in wood frogs, the infection can be re-introduced to the pond the next year, potentially infecting the larvae of other amphibian species present, leading to amplification of the infection (Duffus et al. 2008).

The ambystomatid larvae present show persistent levels of ranavirus infection and are assumed to be the source of the ranavirus for other species in the pond (Duffus et al. 2008). Therefore, the community transmission dynamics include both intra- and interspecific aspects and permits the ranavirus to remain in a community,

even when high (~100%) mortality has been seen in one of the species (Duffus et al. 2008).

The current study tests the models developed from the work on ranaviruses from North American species in order to investigate the dynamics of the ranavirus in *R. temporaria* populations in the UK, specifically the IM model, since the majority of amphibian mortality reports are from common frog adults. In determining the life history stages which are affected by the ranavirus, I assess the routes of transmission within the population and the potential for the ranavirus to persist in the population via intraspecific transmission. In this study I assess all life history stages (except for metamorphs) of *R. temporaria* for the presence of the ranavirus and examine how the ranavirus is maintained in the population through intraspecific transmission.

Methods

Tadpole Collections

In the spring of 2007, tadpoles (n = 20) were collected from 14 ponds with the permission of the pond owners. Ponds varied with respect to their disease history. A pond was classified as Ranavirus positive if there were records of mortality events at least every second year, for approximately ten years and considered ranavirus negative if no mortality events had been recorded over the same period. (See Teacher (2009) for more details on site selection.) Using these criteria, I classified seven of the ponds as ranavirus positive sites, six as ranavirus negative sites and one as having unknown status. Tadpoles collected were transported back live to the Institute of Zoology, Zoological Society of London, in water from their own pond. Upon arrival, tadpoles were euthanized by an overdose of MS-2,2,2 (1g/L of tricane methane sulphonate, Thompson and Joseph Ltd., Norwich, UK) buffered with sodium bicarbonate to a pH of 7.

Tissue samples were taken from the tadpole for testing for the presence of the ranavirus. In the case of larger individuals the right anterior quarter of the body was used, in the case of smaller animals it was the central half. These samples were frozen at -80°C for screening for the ranavirus. The remainder of the tadpole was also frozen at -80°C for future use, should the tadpole test positive for the ranavirus and be needed for viral isolation.

Egg Collections

In February and March 2008, four freshly laid broods of eggs were collected from each of six locations (three ranavirus positive and three ranavirus negative). In order to allow spatial analysis of infections within each egg mass, two subsamples were taken from each. One comprised eggs from the centre of the brood ($n \approx 60$), the other combined eggs from four points on the outer edge ($n \approx 60$). Half of the eggs collected were put into ethanol, for ranavirus detection. The other half was permitted to develop until Gosner Stage 25 after which the tadpoles were euthanized and stored for DNA extraction.

An initial sample of 15 eggs from the inner sample and an additional 15 eggs from the outer sample were tested for the presence of the ranavirus. The jelly was removed from the egg before DNA extraction (as per Duffus et al. 2008). The remainder of the eggs and tadpoles were stored in ethanol for further analysis, if required. The total sample size collected was based on calculations of minimum sample sizes needed for prevalence studies in Naing et al. (2006) and Dell et al. (2002).

Adult Collections

Adult common frogs that were archived at the Institute of Zoology, Zoological Society of London had liver tissue sampled for ranavirus screening. Other samples of adult frogs were obtained from garden pond owners in cooperation with Froglife and also from the South Essex Wildlife Hospital. The animals underwent a post mortem examination, from which various samples were take, as well as liver tissue for ranavirus screens. The carcasses were then placed into the frozen archives at the Institute of Zoology.

Extraction and Screening Methodology

DNA Extractions and Screening for the Ranavirus

DNA was extracted from amphibian tissues and eggs using the Wizard SV96 Genomic DNA Purification System (Promega, Southampton, UK). The DNA extracted from both animal tissues and cell culture, were screened for the presence of the ranavirus using the following methods. The primers used for the screen are for the major capsid protein (MCP) of frog virus 3 (FV3) originally developed by Mao et al. (1996). The polymerase chain reaction (PCR) reagents used were from the

QIAGEN Multiplex kits (QIAGEN, Crawley, West Sussex, UK). The thermocycle settings were as follows: 95°C for 45 seconds, 52°C for 45 seconds, 72°C for 45 seconds, for 35 cycles, then 4°C for infinity as per Pearman et al. (2004).

The PCR products were visualized on a 1% agarose gel stained with ethidium bromide. A band at the 500 base pair mark indicates the presence of ranaviral DNA in the sample.

Notes on Ensuring PCR Reliability

The DNA extracted from each sample was screened twice for the presence of the MCP. The positive PCR controls were originally taken from animals that had previously tested positive using PCR and had been extracted using the same method as described above. Alternatively, I used a UK viral isolate, which had been extracted using the QIAGEN DNeasy blood and tissue kit (QIAGEN, Crawley, West Sussex, UK).

Negative extraction controls were used (usually 6 per plate, placed in every second or third row) to screen for contamination. Only one plate showed any contamination, and in this case it was detected in four of the six controls. The samples from this plate were re-extracted and analyzed again.

Positive PCR controls were used on each plate. If the positive PCR controls failed to amplify, the run was determined to be a failure and that plate was re-run. This only occurred on three occasions.

In cases where an ambiguous PCR result was found, the sample was also re-run, PCR results were classified ambiguous if one replicate was positive and the other negative, or if there were shadows or a faint band at the 500 base pair line. This type of result occurred 17 times in the study.

Results

Tadpoles:

Only one tadpole of the 288 screened was positive for the presence of the ranavirus (see Table 2.2).

Eggs:

No eggs tested positively for the presence of the ranavirus (n = 720).

Adults:

There were ranavirus infections present in every year except 2005 (in which only two frogs were available for testing). The number of adults tested in each year and the number of infections identified are described in Table 2.3. The apparent large increase in the number of ranavirus positive specimens in 2008 can be explained by an increase in the collection effort. During June a joint publicity campaign between the Zoological Society of London and Froglife was launched. Note that the proportion of ranavirus infected adults in this study is not necessarily representative of the whole population or of the distribution of common frogs in the UK. The majority of the common frogs that were examined were from sites that were known to be ranavirus positive or from unusual mortality events that were thought to be associated with a ranavirus outbreak.

Table 2.2. Prevalence and infection rates of the ranavirus in common frog (*Rana temporaria*) tadpoles in the spring of 2007 in various locations in the south east of England.

Location	Gosner Stages	Site Ranavirus Status	Number Ranavirus Positive	Number Tested	Prevalence
London, NW10	25-26	Negative	0	19	0%
Fareham 1	30-32	Negative	0	20	0%
Fareham 2	32	Positive	0	20	0%
Fareham 3	33-36	Negative	0	20	0%
London, NW1	27-34	Unknown	0	20	0%
Eltham 1	26-27	Negative	0	20	0%
Eltham 2	30-40	Negative	0	20	0%
London, N12	27-40	Negative	0	20	0%
Worthing	36-38	Positive	0	20	0%
Dagenham	36-40	Positive	0	20	0%
Deal	28-33	Positive	1	20	0.05%
Ealing	35-39	Positive	0	20	0%
Ladywell	30-40	Positive	0	20	0%
Isleworth*	38-40	Positive	0	8	0%
Tooting	37-41	Negative	0	20	0%
Total			***1***	***288***	***3.5×10^{-3} %***

* This population had very few tadpoles present and 8 was the maximum that could be found.

Table 2.3. The number of ranavirus infections of adult common frogs (*Rana temporaria*) by year which were sent to the Institute of Zoology or were collected from unusual mortality events. Results from PCR based screens. The number of known sites represents the number of sites with known ranavirus histories, where as, the number of unknown sites refer to animals tested that had no location information to accompany them.

Year	Number of Infections	Number of Frogs Tested	Number of New Sites	Number of Known Sites	Number of Unknown Sites
2004	1	5	2		1
2005	0	2	1		1
2006	5	15	2	2	5
2007	4	65	5	6	3
2008	27	48	12		3

Discussion

The negligible prevalence of the ranavirus in *R. temporaria* tadpoles was extremely unexpected. Other anuran amphibians, such *R. sylvatica* in Central Ontario, Canada, have chronic ranavirus infections as tadpoles. When infection was detected in a pond which had experienced multiple mortality events in *R. sylvatica* tadpoles, at least 32% of individuals were infected (average over all developmental stages), with higher prevalence seen at earlier developmental stages (Duffus et al. 2008). The failure to detect infected tadpoles in the case of *R. temporaria*, suggests that both the IM and CDTM transmission models are inappropriate, since they both require the infection to persist in tadpoles.

No infections were found were found in the *R. temporaria* eggs. This was again unexpected for an anuran species, since in laboratory reared *R. sylvatica* eggs, the highest prevalence (~20%) was found in the egg; infection of later life history stages only occurred in one individual (Duffus et al. 2008). This result reinforces the conclusion that the CDTM should be rejected for *R. temporaria* in the UK. There is no evidence for vertical (or pseudovertical) transmission of the ranavirus, even in ponds where a large proportion of the adult *R. temporaria* are diseased.

With the rejection of both the IM and CDTM models, our attention must now be re-focused on the infection rates of the adults. It would appear that, if *R.*

temporaria are the only amphibian present at the site, the pathogen must either be maintained is through adult to adult transmission or continual re-introduction of the virus by external sources. As yet, some key information is not available, particularly the length of the infective period and the recovery rate. Nevertheless, since the observed mortality in adult *R. temporaria* is extremely high when the ranavirus emerges in a population, and these mortality events tend to re-occur for several years (Cunningham et al. 1996), it is likely that an alternative host of the ranavirus is required to maintain the pathogen in the population.

Further investigation is needed to establish the potential for adult-to-adult transmission of the ranavirus and the feasibility of this route maintaining the infection. The existence of a reservoir, of alternative hosts for the ranavirus and the length of time the virus remains viable in the environment all need to be investigated in order to explain how and why ranavirus infection can persist in adult *R. temporaria* given that there appears to be no transmission to future generations through their eggs or tadpoles.

CHAPTER 3: MODELLING THE TRANSMISSION OF THE RANAVIRUS IN POPULATIONS OF COMMON FROGS (*RANA TEMPORARIA*) IN THE UNITED KINGDOM: A GUIDE FOR FURTHER RESEARCH

Abstract

In this chapter, I explore the transmission dynamics of the ranavirus present in common frog (*Rana temporaria*) populations in the context of a simple susceptible-infected (SI) model, using parameters derived from the literature. I then explore the effects of disease induced population decline on the dynamics of the ranavirus. I then extend the model to consider the infection dynamics in populations where both ranaviral disease syndromes, the ulcerative and haemorrhagic forms, are present. The preliminary investigation into this system indicates that under certain circumstances both disease syndromes compete for hosts. When the ulcerative form is present in a population to which the haemorrhagic form is then introduced, the haemorrhagic form of the disease needs to be highly contagious to persist. I highlight areas where further research and experimental evidence is needed and hope that this will act as a guide for further research into the system.

Mathematical models are helpful tools for understanding how pathogens behave in host populations (Anderson and May 1979; May and Anderson 1979). Aspects such as pathogen persistence and pathogen effect on host demography can all be explored mathematically. Models can also be used to predict future trends of both host and pathogen. In the amphibian-ranavirus system, to date, no attempt has been made to examine the transmission dynamics of the virus mathematically. Only two attempts have been made to formalize the hypothesised transmission dynamics of a ranavirus using data from the lab and field to create vector diagrams. The first examines the route of ranavirus transmission and amplification when intraspecies transmission is thought to occur (Brunner et al. 2004). The second examines the possible routes of transmission at the level of an aquatic amphibian community (Duffus et al. 2008). While these are both important starting points for the development of mathematical models, the situation described in these two studies do not apply to the situation observed in the ranavirus and common frog (*Rana temporaria*) dynamic in the UK. As I have shown in Chapter 2, unlike other anuran species (e.g. wood frogs, *Rana sylvatica*; Duffus et al. 2008), there is no evidence of infections in the eggs or tadpoles of common frogs and the infections appear to be limited to adults. Given this, the route of transmission is between the adults (Figure 3.1; Note: the infection status of juveniles is currently unknown).

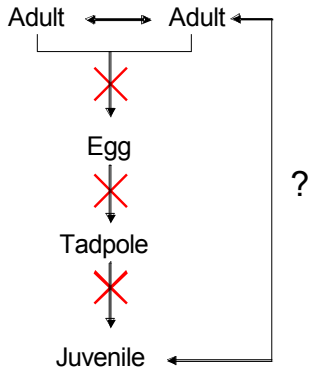

Figure 3.1. Hypothesized transmission dynamics for the ranavirus present in common frogs (*Rana temporaria*) in a typical UK population. Transmission occurs between adults and the infection status of juvenile frogs remains unknown.

With the benefit of long term data on persistence of ranavirus infections in common frogs (e.g. Teacher 2009 and Cunningham 2001) we know that the ranavirus can persist in adult frog populations for many years. The pressing question is: "Can the ranavirus persist in these populations of common frogs if only adult to adult horizontal transmission occur?" In the simplest case of ranavirus infection, I assume a susceptible-infected (SI) model with no recovery, because of the high mortality rate associated with ranavirus infection. I followed the method for developing mathematical models described by Otto and Day (2007) and distinguished between two groups of frogs within the population: susceptible (S) and infected (I) individuals. Additionally, I assumed that population size remains constant, all recruits to the population are susceptible to the ranavirus and all individuals are equally susceptible to infection. This situation is illustrated below in Figure 3.2.

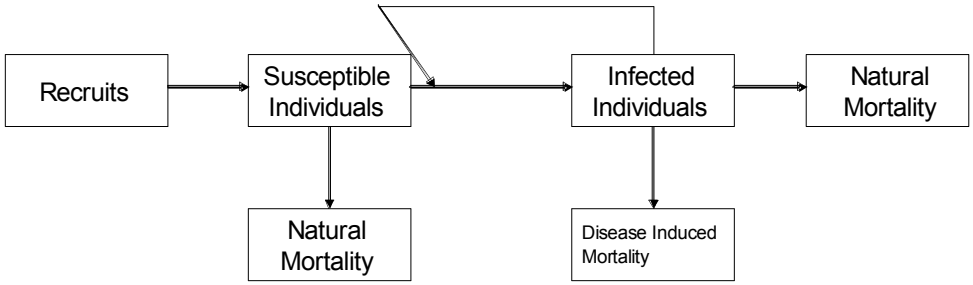

Figure 3.2. A diagrammatic representation of the transmission of the ranavirus in a population of adult common frogs with the population broken into component parts. Lines which touch the corner of a box denote the contact occurs with that group, but that the result moves the animal into a different groups. All other arrows represent the direction of movement of individuals between the different groups within the population.

I then assigned variables to the different categories, the contact rate (Ψ) and the likelihood of transmission (σ) for a contact. The contact rate here is defined as the number of different individuals that one animals comes into contact with. Whereas, the likelihood of transmission is the probability that the infection will be transmitted at any given contact. In the model the following variables replace the standard names of the groups: A_R – recruits, A_S – susceptible, A_I – infected, M_N – natural mortality rate, and M_D – mortality due to disease (Figure 3.3).

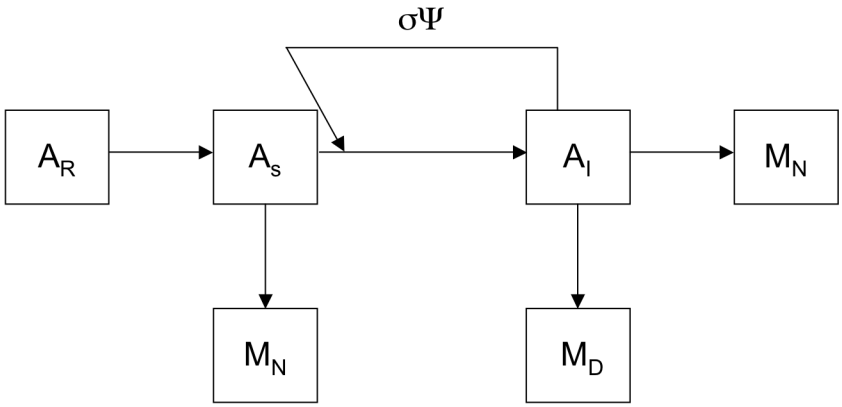

Figure 3.3. Diagrammatic representation of the relationship of different groups of adults within the population as algebraic variables without a time factor.

I then added both time constraints and the interaction between A_S and A_I defined as the transmission likelihood and contact rates (as per Otto and Day 2007; Figure 3.4). Here I chose to use a discrete time model since the life history of amphibians is characterized by discrete events that reoccur on a yearly basis. Since common frogs are at the highest population density in the pond when they are breeding, I assumed that contact rate is overwhelmingly determined through contacts during breeding. I also assume M_D occurs primarily during the summer (ranavirus-associated mortality peaks between mid-July and mid-August, Chapter 2) and is therefore temporally distinct from transmission. (See Figure 3.5 for a schematic of important life history events and timing.) I illustrate the interactions between different groups using a table of events (Table 3.1; Otto and Day 2007).

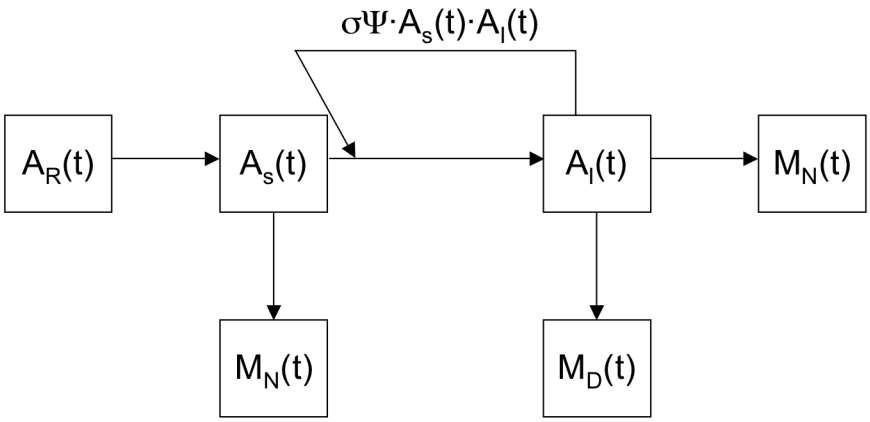

Figure 3.4. Diagrammatic representation of the different groups of the population with time and the interaction between A_S and A_I accounted for.

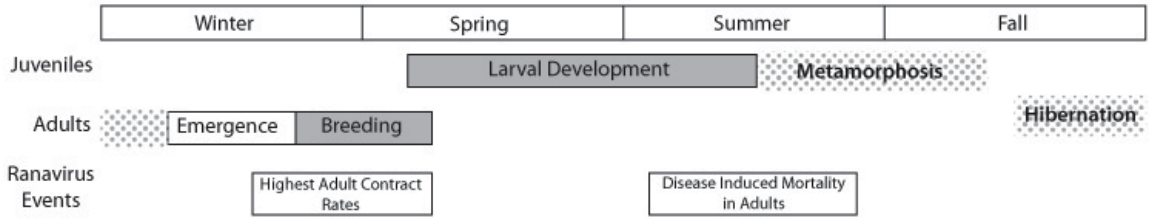

Figure 3.5. Annual cycle of important life history events for common frogs (*R. temporaria*) and important events for ranavirus. Boxes shaded in grey are events that occur in the aquatic environment, stippled boxes are those that straddle land and water.

Table 3.1. A table of events for the interaction of individuals within the ranavirus infected population (as per Otto and Day 2007). (Δ is the change with respect to the number of individuals entering or leaving a given portion of the population.)

		Result of Contact	
Interaction	**Number of Contacts**	A_S	A_I
A_S x A_S	$\sigma\Psi \cdot A_S(t) \cdot A_S(t)$	No Δ	No Δ
A_S x A_I	$\sigma\Psi \cdot A_S(t) \cdot A_I(t)$	- Δ	+ Δ
A_I x A_I	$\sigma\Psi \cdot A_I(t) \cdot A_I(t)$	No Δ	No Δ

I used the information from Figure 3.4 and Table 3.1 to derive a set of equations to examine the dynamics of the ranavirus in adult common frogs. In the initial equations for the system (Equations 1 and 2) i represents the initial value of the variable in the system. Equation 3 is the dynamics of susceptible individuals after the initial exposure, i.e. at t + 1, and Equation 4 represents the dynamics of the infected individuals at t + 1. Equation 5 is the basic reproductive rate of the pathogen in the population.

$$A_s(t) = A_S(i) - \sigma\Psi \cdot A_s(i) \cdot A_I(i) + A_R(i) - M_N(i) \qquad \textbf{Eq}^{\underline{n}}\ \textbf{1}$$

$$A_I(t) = A_I(i) + \sigma \cdot \Psi A_s(i) \cdot A_I(i) - [M_N(i) + M_D(i)] \qquad \textbf{Eq}^{\underline{n}}\ \textbf{2}$$

$$A_s(t+1) = A_s(t) - \sigma\Psi \cdot A_s(t) \cdot A_I(t) - M_N(t) + A_R(t) \qquad \textbf{Eq}^{\underline{n}}\ \textbf{3}$$

$$A_I(t+1) = A_I(t) + \sigma\Psi \cdot A_s(t) \cdot A_I(t) - [M_N(t) + M_D(t)] \qquad \textbf{Eq}^{\underline{n}}\ \textbf{4}$$

$$R_o = \sigma\Psi \cdot A_s(t)/[M_N(t)+M_D(t)] \qquad \textbf{Eq}^{\underline{n}}\ \textbf{5}$$

The basic reproductive rate of a parasite is important in determining how and if a pathogen will spread in a host population and also is important for pathogen evolution within the host population (Frank 1996; Day 2002). For the ranavirus to persist in a population $R_o \geq 1$ because the infection must be transmitted to at least one other individual. To determine under what conditions the ranavirus would remain or spread in a population, I modified Equation 5 by removing $M_D(t)$ because I assume a successful introduction:

$$R_o = \sigma\Psi \cdot A_s / M_N(t) \qquad \textbf{Eq}^{\underline{n}}\ \textbf{6}$$

To ensure that the responses in the model are caused by disease, it is necessary to demonstrate that there are no effects on population sizes in the absence of disease (Figure 3.6, All calculations and graphs were done in Microsoft Excel).

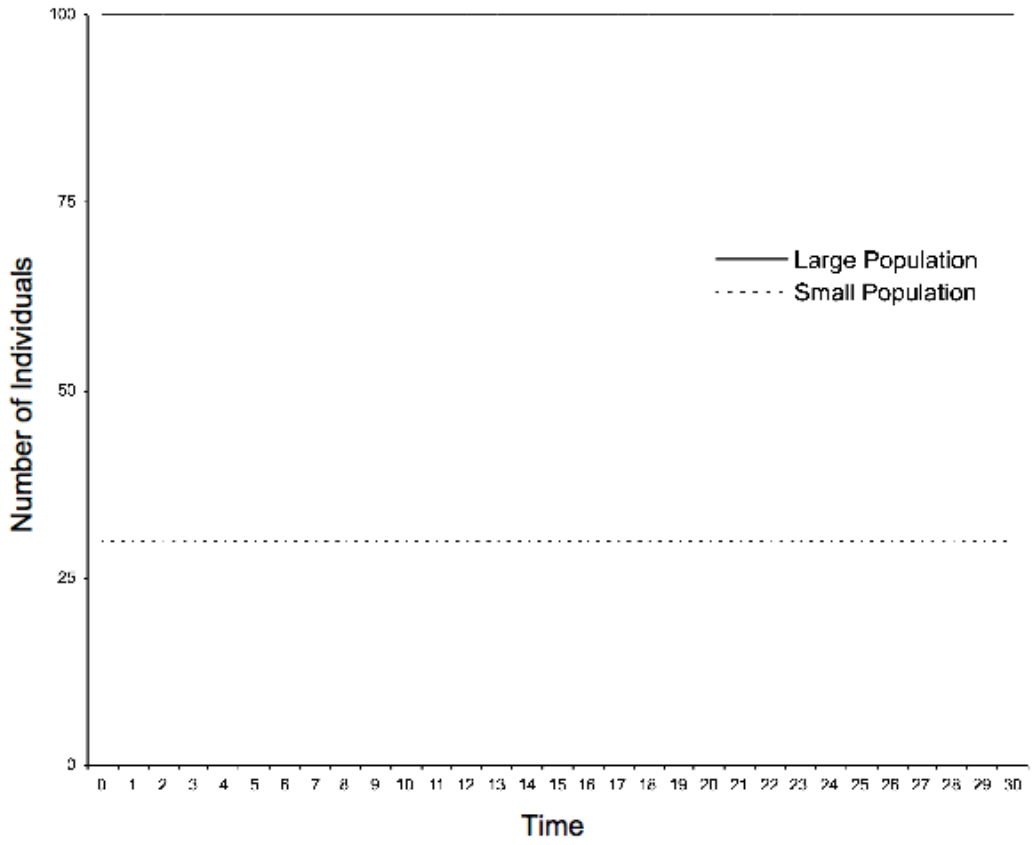

Figure 3.6. The model presented in Eq$^{\underline{n}}$ 3 without disease present. The large population is 100 individuals at the start and the small population is 30 ($M_D = 0.2$).

A_s represents the number of susceptible adults in the population, the important interactions in the equation are between σ and Ψ and this relationship needs to be explored graphically to determine when the conditions for $R_o \geq 1$ exist. If we assume a population size of 99 (A_S) with an initial introduction of 1 infected individual (A_I) and an M_N of 20% (i.e. 20 individuals/annum), values of σ and Ψ under which $R_o \geq 1$ are illustrated in Figure 3.7. To test if similar assumptions are valid at a smaller population size, I repeated the process using alternative values for A_S = 49 and M_N = 10% (i.e. 5 individuals/annum) and introduced one infected individual (total population size 50; Figure 3.7).

There are several different estimates of natural mortality rates for common frogs. The most extreme is a mortality rate of a minimum of 75% for individuals over the age of three years and about 50% for younger frogs (Gibbons and McCarthy 1984). A better estimate of adult mortality in common frogs is presented by Miaud et al. (1999). They consider a wider range of populations across a greater geographical area, which averages to 20%, the estimate I used in the following models. If the population size is altered (and the same mortality rate used), only the position of the curves on the graph is altered in the up or down the y-axis (Figure 3.7).

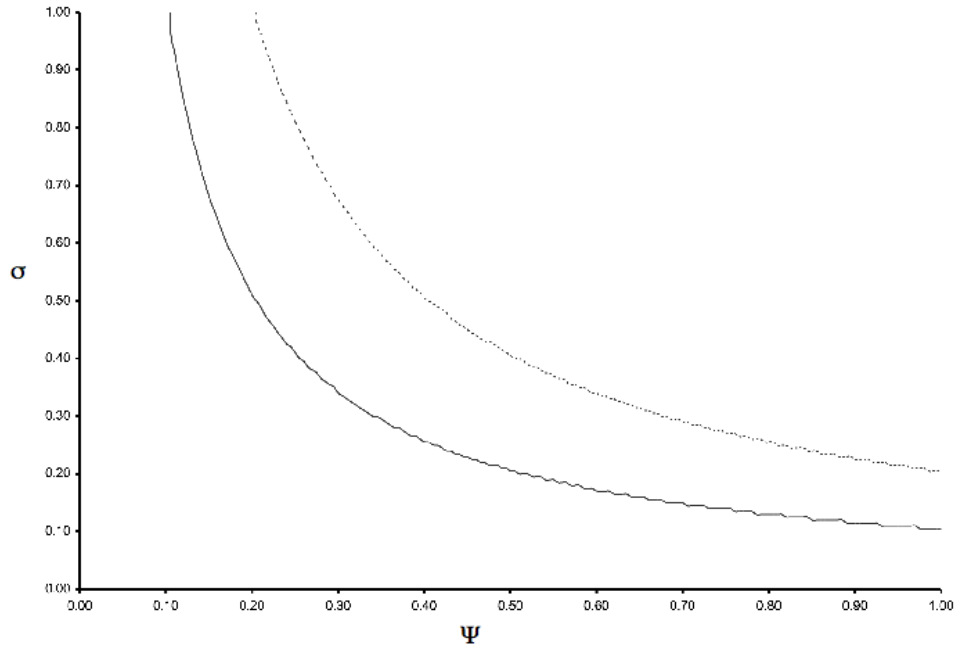

Figure 3.7. The interaction between σ and Ψ under which $R_o \geq 1$, when A_S = 99 and an M_N = 20% (upper curve - dashed) and when the initial conditions of A_S = 49 and an M_N = 10% (lower curve). When the value of R_o is below 1 (below the line) the ranavirus infection will not persist in the population. However when the value $R_o \geq 1$ the introduction of the ranavirus will result in ranavirus establishment in a host population. (Eq^{n} 6)

With the establishment of the conditions which permit ranavirus persistence in the population after an initial introduction, I next examine the behaviour of A_S and A_I under what I believe to be biologically meaningful conditions. I used experimental data for ranavirus exposures in the literature to generate relevant estimates of σ (Table 3.2). Estimates of Ψ are more difficult to ascertain, however, since the model assumes that the highest contact rate occurs during breeding, I use logic based on host biology to make an estimate of a range of contact rates. I assume a contact rate of 30-60% during the breeding season (Ψ = 0.3→0.6). I ignore the potential for sex specific contact rates because mating behaviour in the host species suggests that male-male contact rates are extremely high. To examine the predicted behaviour of A_S with contact rates (Ψ) between 0.3 and 0.6 I first assume: σ = 0.3; M_N = 0.2; M_D = 0.75 with a starting population comprised of A_I = 1 and A_s = 99 (Figure 3.8).

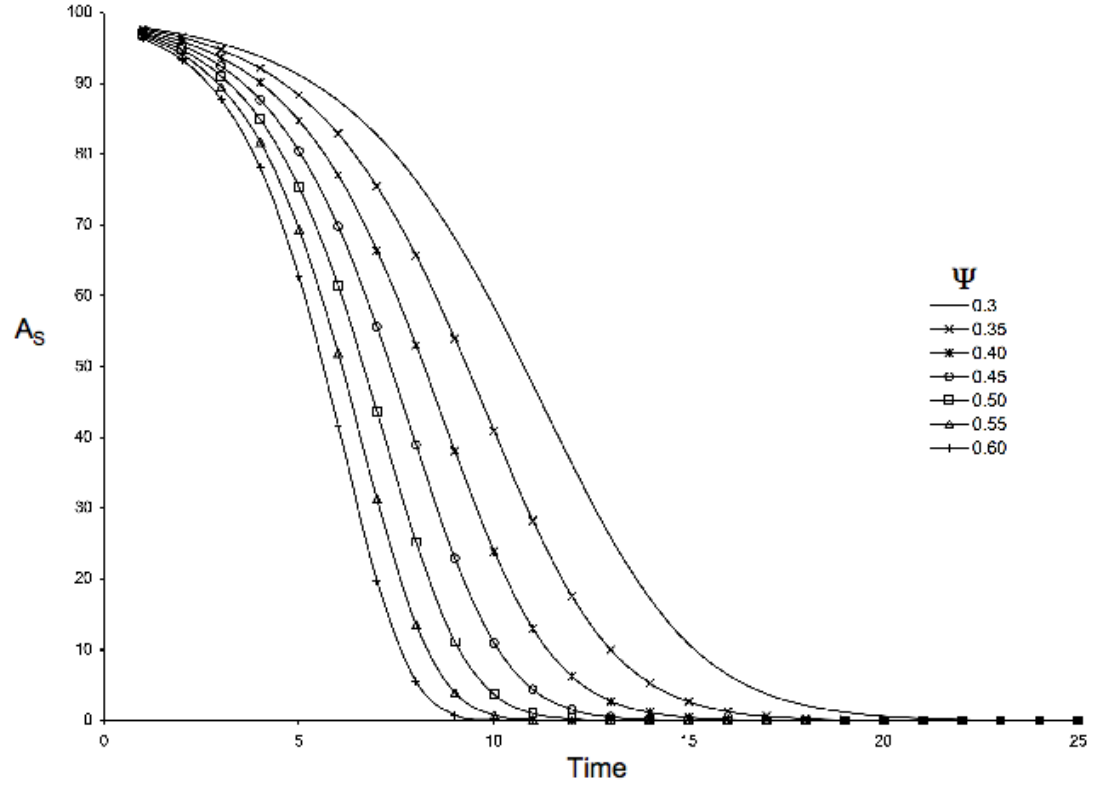

Figure 3.8. Predicted values of A_s with varying values of Ψ while other values remain constant at: σ = 0.3; M_N = 0.2; M_D = 0.75. Starting population composition is A_I = 1 and A_s = 99 and time is in years. (Eq^n 3)

Table 3.2. Estimates for σ derived from the literature. Note: Experiments where the exposure was via inoculation have not been included in these estimates. No distinction has been made between the types of ranavirus associated disease that the virus was derived from. All data from Cunningham et al. 2007a.

Development of Disease		Disease Prevalence	Type of Experiment/ Exposure Type	Estimate of σ
No. with Disease	Total No. Exposed			
3	20	15%	Immersion with virus from naturally disease tissue, with and without bacteria	0.15
9	20	45%	Immersion with virus from naturally disease tissue to animals with skin wounds, with and without bacteria	0.45
9	10	90%	Immersion in virus isolated from naturally diseased animals from virus culture	0.90
5	5	100%	Immersion in virus isolated from naturally diseased animals from virus culture to animals with wounded skin with the same concentration of virus from naturally infected tissues	1
2	5	40%	Immersion in virus from naturally diseased animals to animals with wounded skin with the same concentration of virus as the isolated virus from culture	0.40

The median population size of common frogs in garden ponds in England, where most ranavirus emergence has been reported, is 31 individuals (Teacher 2009). To change the population size requires the re-evaluation of R_o. For simplicity, I now assume A_S is 29 and one infected individual is introduced into the population (Figure 3.9). Even when population size is small and natural mortality rates vary, there are cases where $R_o \geq 1$.

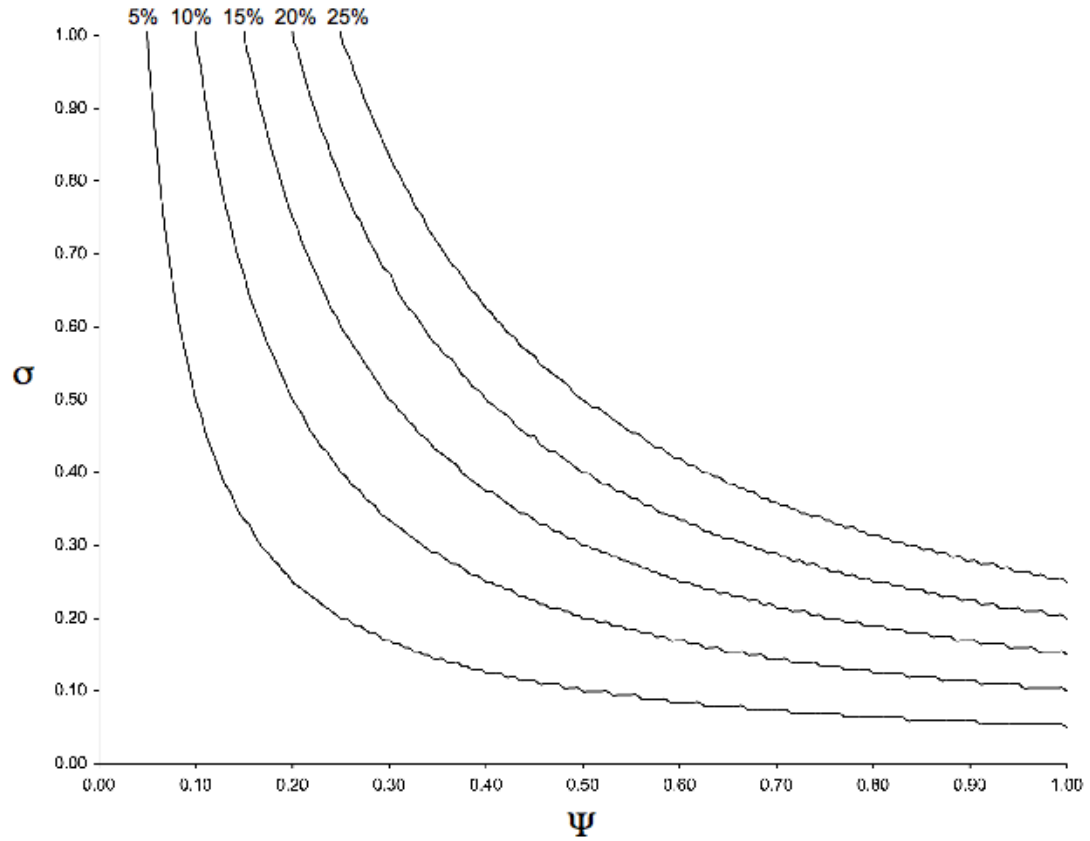

Figure 3.9. The interaction between σ and Ψ under which $R_o \geq 1$, when the initial conditions of $A_S = 29$ and an M_N is varied. The value of M_N can be found at the origin of each line. (Eq^n 3)

The reported mortality rates for ranavirus infections are extremely high (e.g. 90% to 100%, Green et al. 2002; Greer et al. 2005). However, these estimates of mortality are from tadpoles and mortality in common frogs occurs in adults. The median number of frogs killed in ranavirus-associated mortalities for the UK is reported to be 30 individuals (Teacher 2009). Using this information and the median population size of 31 that Teacher (2009) reported, I roughly estimate disease related mortality rate to be 97%, which is biologically possible for ranavirus infections. However, if this were the case in adult common frogs, the ranavirus infection would not persist in the population after one time step (data not shown). A more useful estimate of disease induced mortality is likely between 60% and 80% of common frogs which become infected, while the best estimates for the mortality rate associated with ranavirus infection that allows for ranavirus persistence are between 75% and 80%. Figure 3.10 illustrates a closer view of the dynamics of the ranavirus system and time period of emergence that the ranavirus in the UK is currently in.

Since we are relatively near the beginning of disease emergence, it is possible that if certain mortality rates are true, the disease dynamics of the population has not stabilized (Figure 3.10).

Using an estimated σ of 0.3 (from data from Table 3.2), an average M_D of 0.775 (the average value of 0.75 to 0.8), a Ψ value of 0.45 (average value of 0.3 to 0.6) and an A_{total} of 30 (as per the median population size described by Teacher 2009), with the initial conditions of A_s of 29 and A_I of 1, Figure 3.11 is obtained. Figure 3.11 is what can be considered to be the 'average' expectation of ranavirus emergence under those conditions. The interaction between A_s and A_I takes over 70 years to stabilize to post epidemic dynamics (Figure 3.11; data not shown), illustrating that under the present conditions, the ranavirus infections seen in populations of common frogs appear to be self-sustaining and adult to adult transmission is enough to maintain the infection within the population.

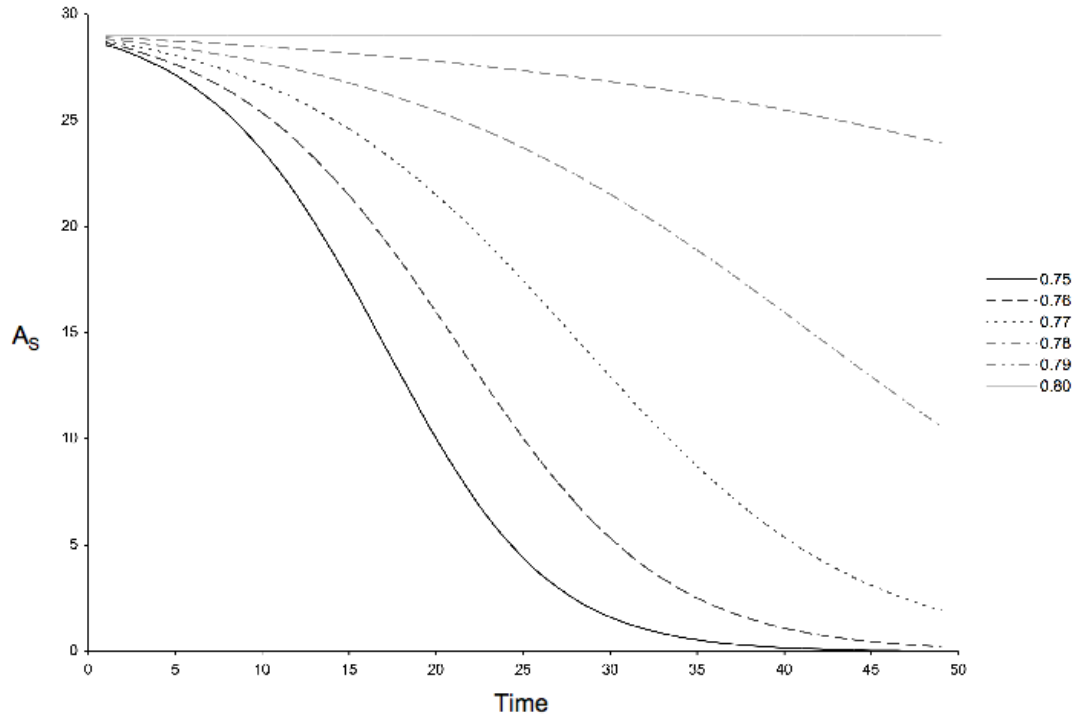

Figure 3.10. Illustration of predicted values for A_S with different disease induced mortality rates while other values remain constant at: Ψ = 0.45; σ = 0.3; M_N = 0.2; the starting population comprised of A_I = 1 and A_s = 29. (Eq^n 3)

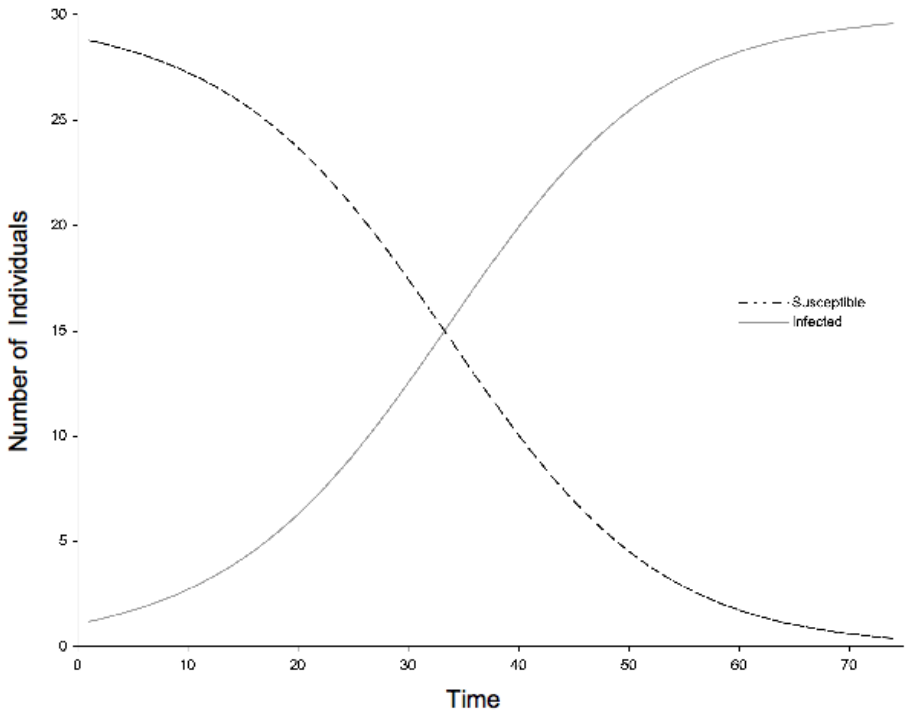

Figure 3.11. The average expectation of the ranavirus epidemic dynamics in a population of adult common frogs (*Rana temporaria*). ($\Psi = 0.45$; $\sigma = 0.3$; $M_N = 0.2$; $M_D = 0.775$; starting population comprised of $A_I = 1$ and $A_s = 29$. Time is in years. A_I: Eq^n 4; A_s: Eq^n 3)

Factoring in Population Decline:

In populations of common frogs where the ranavirus has emerged the median population decline between 1996/7 and 2008 is ~ 83% (Teacher 2009). This is a clear violation of the assumption that the population size remains constant. It has also been demonstrated that the declines experienced by populations were proportional to the size, i.e. larger populations experienced larger declines (Teacher 2009). This is a confirmation that the assumption of all adults being equally susceptible to the ranavirus is valid, at least with respect to the original introduction of the ranavirus.

The mortality due to disease is clearly greater than the ability of common frog populations to recruit individuals and the emergence of the ranavirus in the UK has resulted in the decline of common frog populations because of the disease (Teacher 2009). However estimating the disease-induced decline from the available data is difficult. Intuitively, greater rates of decline should occur at the beginning of the epidemic because that it is when the population of susceptible adults is greatest. However, since year specific data are unavailable, a crude estimate of yearly decline due to ranavirus emergence can be estimated by dividing the median decline by 10

years (this information is based on the manner in which Teacher (2009) selected study sites) and a value of 5.3% is obtained. Factoring in this annual disease induced decline into the population dynamics, the total predicted population decline over years 1 to 10 is 31%, and from year 11 to 20 a total decline of 57% of the initial population size (Figure 3.12).

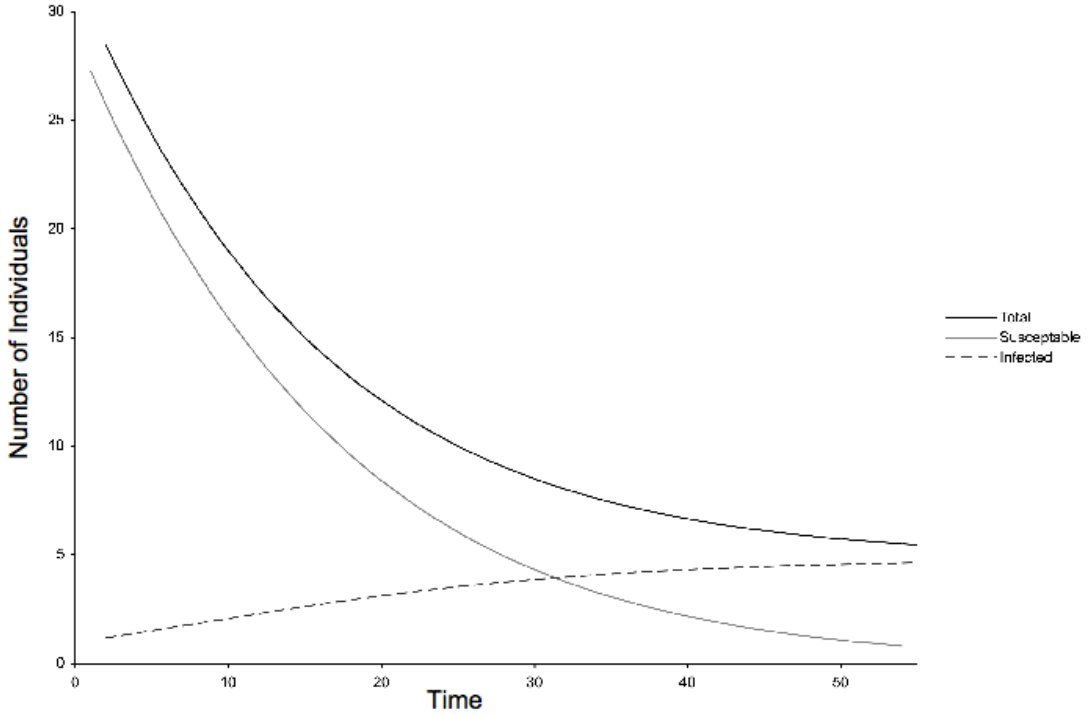

Figure 3.12. Illustration of the predicted dynamics of a common frog population with the ranavirus factoring in an annual population decline of 5.3% for adult common frogs. (Ψ = 0.45; σ = 0.3; M_N = 0.2; M_D = 0.775; starting population comprised of A_I = 1 and A_s = 29. Time is in years. Susceptible: Eq^n 3; Infected: Eq^n 4; Total: Eq^n 3 + Eq^n 4)

Interestingly, the decline predicted by the model is less than the 83% that has been reported by Teacher (2009). Since, populations that were disease free did not suffer from population declines (Teacher 2009), it cannot be argued that additional external factors contributed to the observed decline. This discrepancy could be the result of an underestimate of the disease induced mortality rate or an underestimate of the contact rate in the models or if two different types of ranavirus or two different disease syndromes are circulating in affected populations in my model. Alternatively, because the population size and other calculations are based on medians instead of means, Teacher (2009) may over estimate the true effect that ranavirus emergence has had on populations. In this model, a mix of a median/mean population has been

used and since the median and mean have different mathematical definitions this may be skewing the analysis.

Accounting for Different Disease Syndromes:

The ranaviruses present in UK common frogs exhibit two different disease syndromes, which are not necessarily mutually exclusive. The ulcerative form of the disease is characterized by ulcers of the skin and necrosis of the digits (Cunningham et al. 1996). The haemorrhagic form of the disease is characterized by the presence of internal haemorrhages, most commonly involving the gastrointestinal and reproductive tracts (Cunningham et al. 1996; personal observation). The definitions of the ulcerative and haemorrhagic forms of ranaviral disease used here differ from those used by Cunningham et al. (1996). Here, the ulcerative syndrome also includes ulcers which involved the skeletal muscle. An individual that presents signs of both syndromes is considered to have both.

In experimental exposures, adult common frogs which were exposed to homogenates of infected tissue or virus isolates from cell culture developed different signs of disease (Cunningham et al. 2007a; Table 3.3). Adult frogs exposed to a tissue homogenate derived from skin ulcers only developed the ulcerative form of the disease (prevalence of ~30%) (Cunningham et al. 2007a). Frogs exposed to the tissue homogenate of internal organ tissue from frogs exhibiting the haemorrhagic syndrome did not develop any signs of disease (Cunningham et al. 2007a). When frogs were experimentally exposed to viral isolates from cell culture, those exposed to the isolate derived from the ulcerative tissues developed both ulcerative and haemorrhagic signs of disease (Cunningham et al. 2007a). Frogs exposed to an isolate from the haemorrhagic syndrome only developed the haemorrhagic form of the disease (Cunningham et al. 2007a). This indicates that there is the potential for different strains of the ranavirus to be present in the same individual (Cunningham et al. 2007a) and that both strains can co-exist. These observations are reinforced by the presence of molecular differences between UK isolates (Hyatt et al. 2000; Chapter 4) and the fact that an isolate from common toads (*Bufo bufo*) caused the haemorrhagic form of the disease (Cunningham et al. 2007b).

In natural conditions, frogs are unlikely to be exposed to the same concentration of virus as those in exposure experiments. Estimates of σ for both the ulcerative and haemorrhagic forms can be found in Table 3.3. When the values for

the cultured isolates are removed σ for the ulcerative and haemorrhagic syndromes are 0.33 and 0.20 respectively. This makes sense both intuitively and in the light of higher experimental transmission rates seen with respect to the ulcerative form of the disease.

With the presence of two disease syndromes, caused by different strains of the ranavirus, three different situations with respect to disease dynamics arise that require consideration. The first situation is where only the ulcerative form (A_U) of the disease is present (Figure 3.13). The second occurs when only the haemorrhagic form (A_H) of the disease is present (Figure 3.14). In both cases where only one of the disease syndromes is present, the dynamics of the system are represented by Eq^{ns} 1 – 6. Therefore, the conditions under which $R_o \geq 1$ do not need to be re-evaluated, nor does the fact that each syndrome has a different σ need to be considered.

The third situation, on which I now focus, is the situation where both disease syndromes are present in the population. This requires the development of a new series of equations that include a variable for animals that show signs of both forms of the disease, A_{U+H}. Here, I assume that all animals are equally susceptible to each form of the disease and it remains that way even if one disease syndrome is present (Figure 3.14). I also assume that there is no difference in the disease induced mortality between syndromes and that both forms of the disease are introduced at the same time.

Table 3.3. Estimates for σ derived from the literature taking into account the different disease syndromes and type of syndrome that the virus was obtained from. U indicates the ulcerative form of the disease, H is the haemorrhagic form. The estimate of σ is simply the prevalence of the disease based on the presence of the signs of disease when the experiment terminated. The average estimate of σ is simply the mean of the estimates for each type of virus used for exposure. (All data from Cunningham et al. 2007a)

Form of Disease of the Isolate	Development of Disease				Type of Experiment/ Exposure Type	Estimate of σ	Average Estimate of σ
	No. with U	No. with H	No. with U & H	Total Exp.			
Ulcerative	2	0	0	5	Immersion with virus from naturally disease tissue with bacteria	0.4	0.36
	1	0	0	5	Immersion with virus from naturally disease tissue without bacteria	0.2	
	2	0	0	5	Immersion with virus from naturally disease tissue to animals with skin wounds with bacteria	0.4	
	0	0	0	5	Immersion with virus from naturally disease tissue to animals with skin wounds without bacteria	0	
	2	2	0	5	Immersion in virus isolated from naturally diseased animals from virus culture (RUK 13)	0.8	
Haemorrhagic	0	0	0	5	Immersion with virus from naturally disease tissue without bacteria	0	0.44
	0	0	0	5	Immersion with virus from naturally disease tissue with bacteria	0	
	1	1	1	5	Immersion with virus from naturally disease tissue to animals with skin wounds with bacteria	0.6	
	0	3	1	5	Immersion with virus from naturally disease tissue to animals with skin wounds without bacteria	0.8	
	1	2	1	5	Immersion in virus isolated from naturally diseased animals from virus culture (RUK 11)	0.8	

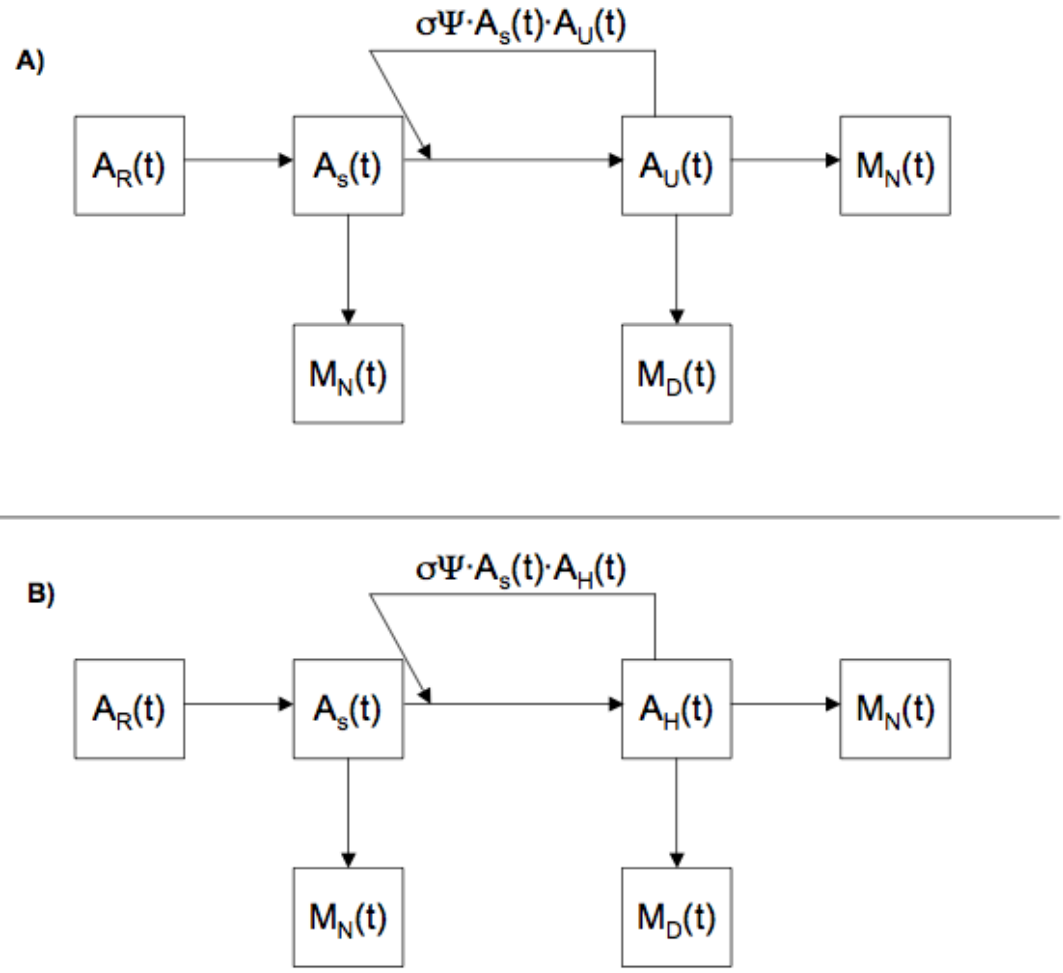

Figure 3.13. Diagrammatic representations of the transmission dynamics of the ranavirus when one 'strain' of the ranavirus is present. A) When only the ulcerative form of the ranavirus is present within the population. B) When only the haemorrhagic form of the disease is present in the population. All of the variables present are the same as described above and all have a time component associated with them.

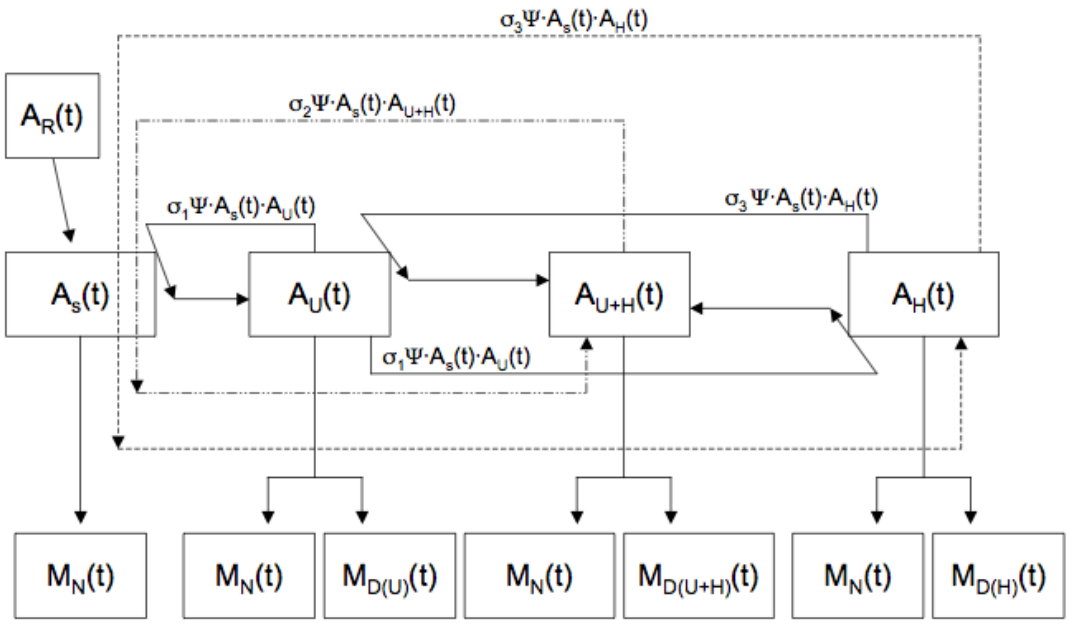

Figure 3.14. Illustration of the complex transmission dynamics of the ranavirus when all three of the observed disease syndromes are present in the population. Dashed lines are used to make the disease syndrome-specific vectors of transmission easier to follow. The box sizes are not representative of the number of individuals in each category. Nor is the order of the boxes representative of when the given disease syndrome was introduced. All factors have time components associated with them.

Again, using the instructions of Otto and Day (2007), the following steps need to be considered to obtain another set of linear equations to describe the system. In this case since there are two different pathogens circulating in the system, two different equations for R_o are required, one for each pathogen. Using both Figure 3.18 and Table 3.4 the following equations are obtained for the system:

$$A_s(t) = A_S(i) - [\sigma_1\Psi \cdot A_S(i) \cdot A_U(i) + \sigma_2\Psi \cdot A_S(i) \cdot A_{(U+H)}(i) + \sigma_3\Psi \cdot A_S(i) \cdot A_H(i)] - M_N(i) + A_R(i) \quad \textbf{Eq}^{\underline{n}}\ \textbf{7}$$

$$A_U(t) = A_U(i) + [\sigma_1\Psi \cdot A_S(i) \cdot A_U(i)] - [\sigma_1\Psi \cdot A_U(i) \cdot A_H(i) + \sigma_3\Psi \cdot A_H(i) \cdot A_U(i)] - [M_N(i) + M_{N(U)}(i)] \quad \textbf{Eq}^{\underline{n}}\ \textbf{8}$$

$$A_{(U+H)}(t) = A_{(U+H)}(i) + [\sigma_2\Psi \cdot A_S(i) \cdot A_{(U+H)}] + [\sigma_1\Psi \cdot A_U(i) \cdot A_H(i)] + [\sigma_3\Psi \cdot A_H(i) \cdot A_U(i)] - [M_N(i) + M_{N(U+H)}(i)] \quad \textbf{Eq}^{\underline{n}}\ \textbf{9}$$

$$A_H(t) = A_H(i) + [\sigma_3\beta \cdot A_S(i) \cdot A_H(i)] - [\sigma_1\beta \cdot A_U(i) \cdot A_H(i) + \sigma_3\beta \cdot A_H(i) \cdot A_U(i)] - [M_N(i) + M_{N(H)}(i)] \quad \textbf{Eq}^{\underline{n}}\ \textbf{10}$$

$$A_s(t+1) = A_S(t) - [\sigma_1\Psi \cdot A_S(t) \cdot A_U(t) + \sigma_2\Psi \cdot A_S(t) \cdot A_{(U+H)}(t) + \sigma_3\Psi \cdot A_S(t) \cdot A_H(t)] - M_N(t) + A_R(t) \quad \textbf{Eq}^{\underline{n}}\ \textbf{11}$$

$$A_U(t+1) = A_U(t) + [\sigma_1\Psi \cdot A_S(t) \cdot A_U(t)] - [\sigma_1\Psi \cdot A_U(t) \cdot A_H(t) + \sigma_3\Psi \cdot A_H(t) \cdot A_U(t)] - [M_N(t) + M_{N(U)}(t)] \quad \textbf{Eq}^{\underline{n}}\textbf{12}$$

$$A_{(U+H)}(t+1) = A_{(U+H)}(i) + [\sigma_2\Psi \cdot A_S(i) \cdot A_{(U+H)}] + [\sigma_1\Psi \cdot A_U(i) \cdot A_H(i)] + [\sigma_3\Psi \cdot A_H(i) \cdot A_U(i)] - [M_N(i) + M_{N(U+H)}(i)] \quad \textbf{Eq}^{\underline{n}}\ \textbf{13}$$

$$A_H(t+1) = A_H(t) + [\sigma_3\Psi \cdot A_S(t) \cdot A_H(t)] - [\sigma_1\Psi \cdot A_U(t) \cdot A_H(t) + \sigma_3\Psi \cdot A_H(t) \cdot A_U(t)] - [M_N(t) + M_{N(H)}(t)] \quad \textbf{Eq}^{\underline{n}}\ \textbf{14}$$

Ulcerative Syndrome

$$R_{oU} = \sigma_1\Psi\,[A_S(t) + A_H(t)]/M_N(t) + M_{D(U)}(t) + M_{D(H)}(t) \quad \textbf{Eq}^{\underline{n}}\ \textbf{15}$$

Haemorrhagic Syndrome

$$R_{oH} = \sigma_3\Psi\,[A_S(t) + A_U(t)]/M_N(t) + M_{D(H)}(t) + M_{D(U)}(t) \quad \textbf{Eq}^{\underline{n}}\ \textbf{16}$$

Table 3.4. A table of events for the interaction of individuals within the ranavirus infected population (As per Otto and Day 2007). Where Δ is the change in the population dynamic. The order of the interaction is important here.

		Result of Contact			
Interaction	**Number of Contacts**	A_S	A_U	$A_{(U+H)}$	A_H
$A_S \cdot A_S$	$\sigma_{N/A}\Psi \cdot A_S \cdot A_S$	No Δ	No Δ	No Δ	No Δ
$A_S \cdot A_U$	$\sigma_1\Psi \cdot A_S \cdot A_U$	-	+	No Δ	No Δ
$A_S \cdot A_{(U+H)}$	$\sigma_2\Psi \cdot A_S \cdot A_{(U+H)}$	-	No Δ	+	No Δ
$A_S \cdot A_H$	$\sigma_3\Psi \cdot A_S \cdot A_H$	-	No Δ	No Δ	+
$A_U \cdot A_U$	$\sigma_1\Psi \cdot A_U \cdot A_U$	No Δ	No Δ	No Δ	No Δ
$A_U \cdot A_{(U+H)}$	$\sigma_{1\text{ or }2}\Psi \cdot A_U \cdot A_{(U+H)}$	No Δ	-	+	No Δ
$A_U \cdot A_H$	$\sigma_1\Psi \cdot A_U \cdot A_H$	No Δ	-	+	-
$A_{(U+H)} \cdot A_{(U+H)}$	$\sigma_2\Psi \cdot A_{(U+H)} \cdot A_{(U+H)}$	No Δ	No Δ	No Δ	No Δ
$A_{(U+H)} \cdot A_H$	$\sigma_{2\text{ or }3}\Psi \cdot A_{(U+H)} \cdot A_H$	No Δ	No Δ	-	+
$A_H \cdot A_U$	$\sigma_3\Psi \cdot A_H \cdot A_U$	No Δ	-	+	-
$A_H \cdot A_H$	$\sigma_3\Psi \cdot A_H \cdot A_H$	No Δ	No Δ	No Δ	No Δ

To determine all of the conditions that satisfy this model would require an entire thesis on its own. However, preliminary exploration is necessary to ascertain validity of the model. Under the assumptions that $A_S = 28 \rightarrow 14$, $A_H = 1 \rightarrow 14$, with the introduction of $1 \rightarrow 5$ A_U, $M_{D(U)} = M_{D(H)} = 0.775$, $\sigma_1 = 0.3$, $\sigma_3 = 0.25$, $\Psi = 0.45$, $M_N = 0.2$, based on R_o values, A_U and A_H will not coexist. It does not matter if A_U is introduced into a population with A_H or vice versa (data not shown).

Both of these disease syndromes persist together in the wild (personal observation) and in experimental infections (Cunningham et al. 2007a). Also, animals with broken skin are more likely to become infected with the haemorrhagic form of the disease than in the absence of skin damage (Cunningham et al. 2007a). Since virus cultured from an ulcerated individual produced both syndromes (Cunningham et al. 2007a), I assume that the ulcerative syndrome is present and it is the haemorrhagic syndrome that is introduced subsequently, as per the above results.

In the presence of skin wounds σ_3 for A_H can be approximated to 0.7 (from data in Table 3.3). I assume that σ_1, Ψ, M_N, $M_{D(U)}$ and $M_{D(H)}$ remain the same as above. This brings $R_o \geq 1$ and permits for the spread of the haemorrhagic syndrome of the disease in the population. The minimum σ_3 under these conditions for A_H to spread in the population $\sigma_3 \approx 0.46$ (Figure 3.15). However, there is likely to be more than one individual with A_U in the population and this requires consideration (Figure 3.16).

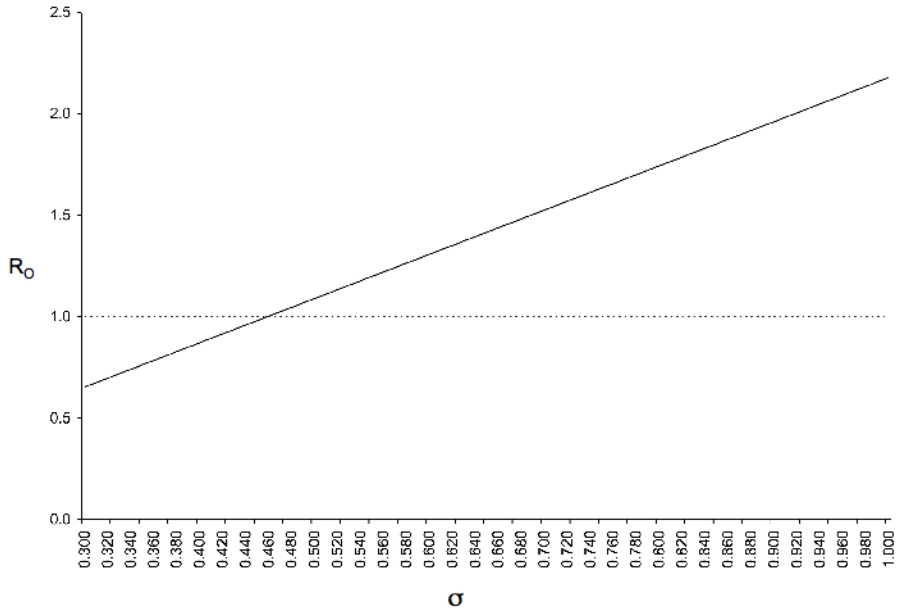

Figure 3.15. R_o values for the introduction of one A_H individual into a population of $A_S = 28$ and $A_U = 1$. ($M_{D(U)} = M_{D(H)} = 0.775$, $\Psi = 0.45$, $M_N = 0.2$; Eq^{n} 15)

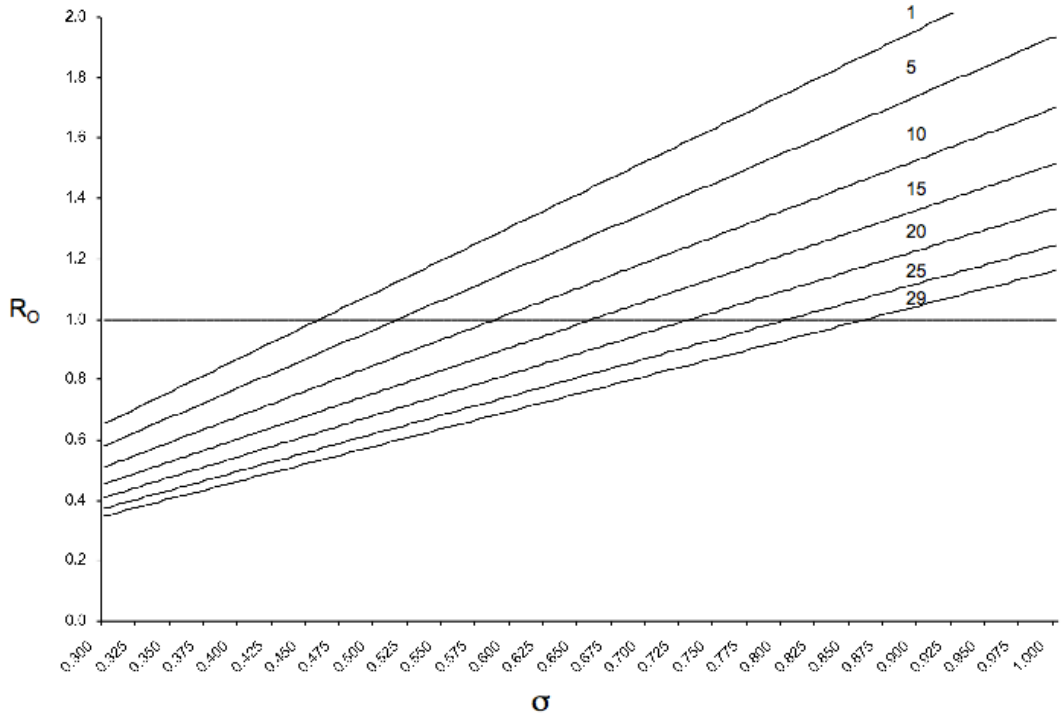

Figure 3.16. R_o values for the introduction of one A_H individual to populations with differing numbers of A_U while the total population size remains constant at 30. The number associated with each line indicates the number of A_U individuals present in the population. ($M_{D(U)} = 0.775$, $\Psi = 0.45$, $M_N = 0.2$; Eq^{n} 15)

Increasing the number of A_U individuals in a population requires a higher transmission rate at each contact for A_H to become established (i.e. $R_o \geq 1$). While at first this may seem counter intuitive, it is not: when there are more A_U individuals in the population there is a higher overall mortality rate because $M_{D(U)} >> M_N$. Hence, there are actually fewer individuals to infect. Even when all of the population (n = 29) is composed of A_U individuals, A_H can become established ($\sigma_3 \approx 0.85$). Although this is a high transmission rate, it is not unlikely in the situation where animals have broken skin, which is characteristic of the ulcerative form of ranaviral disease, demonstrating the plausibility if the model.

The above analysis demonstrates that even under conditions which are estimated from poor and incomplete data, based on R_o values, the two disease syndromes can persist in the same population by adult to adult transmission. It is also extremely interesting that the second model predicts that there will be competition between the two disease syndromes, which could lead to the exclusion of one of the disease syndromes from a population. However, full validation of the model is required as are good estimates of basic parameters.

Importantly, these preliminary models do demonstrate that the persistence of the ranavirus(es) present in the UK by adult to adult transmission is possible. The exact circumstances in a biologically relevant/possible context are still required so that these models can be used as predictive tools. The following are areas which need to be addressed:

- Experimental determination of the transmission rate for both the ulcerative and haemorrhagic forms of ranavirus disease present in the UK.
- Determination of contact rates during the breeding season for adult frogs.
- Experimental assessment of disease induced mortality rates for individuals with the ulcerative form, haemorrhagic form and the combination of the two.
- Full molecular characterization of the viruses responsible for the different syndromes to determine if they are truly different viruses or strains.
- Prevalence data on the different syndromes in wild populations of common frogs to validate the assumptions of the models and to make sure that they are relevant to what is actually occurring.
- Investigations into the progression of an infection to disease in common frogs to determine the length of time that disease takes to develop.
- Determination of viral persistence in the environment.

The ability to address these issues are all experimentally and methodologically possible. The determination of these areas will also be invaluable to conservation strategies which need to address the disease to make valid management decisions.

CHAPTER 4: ALTERNATIVE HOSTS OF THE RANAVIRUS PRESENT IN THE UK: AN EXAMINATION OF INFECTIONS IN OTHER AMPHIBIAN SPECIES USING MOLECULAR PHYLOGENETICS

*The screens of the majority of the common toads (*Bufo bufo*) presented here were part of a preliminary health screen of common toads on Jersey and have been submitted for publication in:*

Fernández-Loras, A., Hidalgo-Vila, J., Hermosilla, C., García, G., López, J., **Duffus, A. L. J.**, Cunningham, A. A., and V. Roca. In Review. Preliminary health screening and possible pathogen determination in a *Bufo bufo* population. Journal of Natural History. Submission No. TNAH-2009-0231

Abstract

Ranaviruses began to emerge in the UK nearly twenty years ago, yet, only preliminary attempts to determine the number of amphibian species that are affect, or to isolate and characterize the virus(es) present have been done. In this chapter, I screened different species of amphibians for ranavirus infections, isolate the virus from infected tissues and use sequence analysis at two loci to make inferences about the phylogenetic relationships between the different isolates. I report the first ranavirus infections in introduced common midwife toads (*Alytes obstetricans*) and smooth newts (*Lissotriton vulgaris*) in the UK. High homology between all ranavirus isolates, suggests that transmission between species is likely to be the result of pathogen spillover.

Introduction

Pathogens can cause population declines, local extirpations or even extinctions, and several factors have been identified that are associated with increased risk of host extinction (de Castro and Bolker 2005). First, if the host population size is small, it will be more susceptible to extirpation due to pathogenic agents (de Castro and Bolker 2005). This aspect of host biology in general renders the host population more susceptible to extirpation, and is not novel with respect to pathogens. Second, if pathogen transmission is not density-dependent, or if transmission is frequency dependent, the pathogen is more likely to cause population decline or local extirpation (de Castro and Bolker 2005, Ryder et al. 2008). This aspect of host-pathogen biology can be more clearly linked to pathogen influences than small host population size. Third, if the pathogen has a reservoir, including alternate or reservoir hosts, it can persist in an area even after the primary host has gone extinct (de Castro and Bolker 2005). Again, this aspect of host-pathogen biology is clearly attributed to the dynamics of the pathogen. A pathogen, which fulfils any of the above criteria, can post a threat to a host population, or indeed, a host species.

The ranaviruses (Family *Iridoviridae*) are a group of emerging pathogens in several taxa, including amphibians (Chinchar 2002; Chinchar et al. 2009). The ranaviruses, which is used as a generalized term for members of this genus, have been associated with morbidity and mortality events in amphibian species in North America (Bollinger et al. 1999; Green et al. 2002; Greer et al. 2005), continental Europe (Balsiero et al. 2009; Ariel et al. 2009) and the UK (Cunningham et al. 1996). Importantly, ranaviruses are known to use reservoir/alternate host species. Since amphibians have complex life histories, it is possible for one life history stage to act as an alternate host for another: this has been described in the *Ambystoma tigrinum* – *Ambystoma tigrinum* virus (ATV) system (Brunner et al. 2004). In this system, infected adults act as the reservoir, bringing the virus to ponds when they return to breed. Adults are presumed to transmit the virus to larval young-of-the-year, where the infection is amplified and the majority of mortality occurs (Brunner et al. 2004). Some infected larvae do not die but are recruited into the sexually mature cohort and bring the virus back to the ponds as adults (Brunner et al. 2004). This is a rather unique system, as *A. tigrinum* at the index site for ATV is the only amphibian species

present and therefore must maintain infection within a single host species for ATV to persist (Brunner et al. 2004). This is unusual because most amphibians exist in multi-species amphibian communities.

Ranaviruses have also been described infecting all potential amphibian hosts in an aquatic amphibian community (Duffus et al. 2008). In this aquatic amphibian community, it is thought that the ranaviruses are transmitted both within and between species through vertical and horizontal transmission (Duffus et al. 2008). Ambystomatid salamanders, of which two different species are present, are presumed to experience single-species disease dynamics as in the *A. tigrinum* – ATV system, but also act as reservoirs for virus, transmitting infections to the anuran species which are present (Duffus et al. 2008). It must be stressed that it is currently unknown if anurans in the system can have the same single-species dynamics as seen in ambystomatid salamanders, and it is unknown if all species are infected with the same ranavirus (Duffus et al. 2008). In North America, there seems to be some host-strain specificity with respect to ranaviruses (Schock et al. 2008), demonstrating the need for more detailed molecular investigations into the identity of North American ranaviruses.

In the UK, ranaviruses infect and are isolated from common frogs (*Rana temporaria*) and common toads (*Bufo bufo*), and there are slight molecular differences between isolates derived from the different hosts that are unlikely to be associated with functional divergence (Hyatt et al. 2000). The isolates from common frogs can cause two different disease syndromes, an ulcerative form and a haemorrhagic form, but the isolates from common toads only appear to elicit the haemorrhagic form of disease (Cunningham et al. 2007a&b). The true extent of native and introduced amphibian species affected by the ranavirus in the UK is currently unknown.

The mortalities thought to be caused by ranavirus infections in the UK are dominated by common frogs. In order to assess the risk posed by ranaviruses to any amphibian host, including frogs, and to ascertain if alternate/reservoir hosts exist, it is necessary not only to determine the extent of species affected but also potential host-strain associations. In North America, ATV-like ranaviruses are more virulent in ambystomatids than frog virus 3 (FV3)-like viruses, which are associated with anurans (Schock et al. 2008). Alternately, FV3-like viruses were more virulent in

anurans than in ambystomatids (Schock et al. 2008). In the case of species that are carriers of the virus, or where individuals carry sub-lethal viral infections (e.g. Brunner et al. 2004), these infections are only detected through molecular analysis. Further molecular analysis of the virus is needed to assess the potential threat that they pose; to determine if reservoir/alternate hosts are present; and to elucidate the routes of transmission that permit viral persistence.

Most studies that examine the molecular variation of ranaviruses have focused solely on the variation of a 500bp segment of the major capsid protein (MCP) for phylogenetic analysis (e.g. Bollinger et al. 1999; Hyatt et al. 2000). However, this region is highly conserved and thus not particularly informative for phylogenetic reconstruction of closely related viruses: more informative variation may be missed if other loci are not used (e.g. Ridenhour and Storfer 2008). Evidence for local adaptation of ranaviruses in ATV from the western USA (Ridenhour and Storfer 2008) was only detected because of a more rigorous phylogenetic approach. The use of coding (with known function) and non-coding DNA sequences were key in determining that local selection pressures were resulting in differentiation between ATV strains from different geographical regions (Ridenhour and Storfer 2008). This illustrates the need for a multiple gene approach for finer scale analyses of ranavirus genetic variation, such as those seen in the UK. Since the ranaviruses in the UK have emerged in approximately the same amount of time, it is important to consider multiple genes for analysis since previous analyses of UK virus isolates have shown little variation (Hyatt et al. 2000).

Here, I examine different species of amphibian, which could be potential reservoir, or alternate hosts of the ranavirus in the UK. Virus isolates from infected animals are subjected to a multi-gene molecular assessment to determine the phylogenetic relationships between them and to evaluate potential host-strain associations. This is the first study of its kind for ranaviruses isolated primarily from anurans.

Methods

Sample Collection

Tissue samples were obtained through several different routes. All amphibians archived at the Institute of Zoology, Zoological Society of London from 2004-2006 and in a good state of preservation were sampled. In 2007 and 2008, amphibian mortality reports received from the public, through either FrogLife or colleagues, were forwarded to me. Whenever possible, I arranged for whole carcasses to be delivered for complete post mortem examination. Samples were collected for ranavirus work from other ongoing projects on amphibian disease, native species and introduced amphibians in the U.K. at the Institute of Zoology. Any animals that were live when they were collected were taken with the site owner's permission and were euthanized according to Home Office Schedule One procedures by an overdose of MS-2,2,2 (tricane methylsulfonate, Thompson and Joseph Ltd, Norwich, UK) buffered to pH 7 with sodium bicarbonate.

All common frogs referred to in this chapter are from the work that was conducted to collect data for Chapter 2 of this thesis.

DNA extractions and Preliminary Ranavirus Screens

DNA from hepatic tissue samples was extracted using the Wizard SV96 Genomic DNA Purification System (Promega, Southampton, UK). DNA was then screened for the presence of ranaviral DNA as follows: Primers to amplify a 500bp region of the major capsid protein (MCP) of frog virus 3 (FV3), originally developed by Mao et al. (1996) were used as the probe for ranavirus DNA (all were sourced from MWG Eurofins Operon, Ebersberg, Germany). All polymerase chain reaction (PCR) reagents used were Multiplex PCR kits (QIAGEN, Crawley, UK). The thermocycler settings were: 95˚C for 45sec, 52˚C for 45sec, 72˚C for 45sec for 35 cycles, then 4˚C for ∞ as per Pearman et al. (2004). All samples were screened twice to ensure repeatability and accuracy of the results. Any ambiguous results were re-screened. Positive PCR and negative extraction controls were also used. The PCR products were quantified on a 1% agarose gel stained with ethidium bromide. The presence of a band at the 500bp level on the gel was considered diagnostic for the presence of ranaviral DNA and was therefore considered to be positive for infection with the virus.

Notes on Ensuring PCR Reliability

The DNA extracted from each sample was screened twice for the presence of the MCP. The positive PCR controls were originally taken from animals that had tested positive for ranavirus and had been extracted using the same method as described above. Alternatively, I used a UK viral isolate, which had been extracted using the QIAGEN DNeasy blood and tissue kit (QIAGEN, Crawley, West Sussex, UK). Positive PCR controls were used on each PCR plate. If the positive PCR controls failed to amplify, the run was determined to be a failure and that plate was re-run. Negative extraction controls were also used, usually six per plate, placed in every second or third row. Only one plate showed any contamination, and in this case, it was detected in four of the six controls. The samples from this plate were re-extracted and analyzed again. In cases where an ambiguous PCR result was found, the sample was also re-run. Ambiguous PCR results were classified as such if the results of the two replicate PCRs were not concordant or a faint band at the 500 base pair region was observed.

Cell Culture and Viral Isolation Methodology

Fathead minnow cells (FHM) (*Pimephales promelus*) were obtained from the European Collection of Cell Cultures (No. 88102401, ECACC, Oxford, UK). The cells were propagated at 25°C in Eagle's Minimum Essential Media (EMEM) (Sigma – Aldrich, Andover, UK), supplemented with 1% L – glutamine (Sigma – Aldrich, Andover, UK), 0.005% Penicillin – Streptomycin (Sigma – Aldrich, Andover, UK), 0.005% Nystatin (Gibco, Invitrogen, Paislely, UK), and 10% Research Grade Foetal Bovine Serum (Hyclone, Perbio Science, Northumberland, UK). Attempts to isolate the ranavirus from all individuals that tested positive for the ranavirus using PCR were done for all samples from 2006 to 2008. Where there were multiple individuals from a single mortality event testing positive using PCR, I selected a maximum of five individuals for isolation to ensure the successful isolation of a virus from at least one animal.

To isolate virus, I first homogenized a small piece of hepatic tissue in 15 to 20mL isolation media (0.01% FBS, 0.01% Penicillin – Streptomycin, 0.005% Nystatin, 5 x 10^{-4} % Gentamycin and 0.01% L-glutamine) using an ultra-Turrax tube drive (IKA-Werke GMBH & Co. KG, Staufen, Germany). I then filtered the homogenate using a sterile 0.22µL syringe filter and syringe or a 50mL Steriflip©

unit with a 0.22µL filter (Millipore, Hertfordshire, England). The filtered homogenate was then added to one 75cm^2 flask of FHM cells or split between two confluent 25cm^2 flasks. I monitored flasks daily for the development of viral plaques. When the cells had detached from the bottom of the flask, the virus was harvested and was filtered with a sterile 0.22µL syringe filter and syringe before being aliquoted into 1.5-2mL cryovials. This was then frozen at -80˚C until the second passage.

I passaged the virus on 75cm^2 confluent flasks of FHM cells. Each flask was inoculated with 100µL to 1mL of the viral isolate, 25mL of maintenance media [Eagle's Minimum Essential Media (EMEM), supplemented with 1% L – glutamine, 0.005% Penicillin – Streptomycin, 0.005% Nystatin, and 1% Research Grade Foetal Bovine Serum] was added and I then transferred each flask to the 25°C incubator. I again monitored all flasks for the formation of viral plaques and harvested virus when no cells were left adhering to the bottom of the flask.

Sequencing

The major capsid protein (MCP) was chosen because it is commonly used by many authors to determine the phylogenic affinities of Iridoviruses on large and small scales (e.g. Tidona et al. 1997; Hyatt et al. 2000). The loci encoding open reading frame (ORF) 57r was chosen because of the availability of comparison sequences and because of its previous use by Ridenhour and Storfer (2008) to examine local adaptation in *Ambystoma tigrinum* viruses (ATV) in the western USA. ORF57r encodes an eIF-2α homologue, which is involved in host immune evasion by keeping the cellular transcriptional and translational machinery active, permitting viral replication, which would have otherwise been shut down as an antiviral defence (Ridenhour and Storfer 2008).

I used the second passage of each isolate to ensure that they all had experienced the same conditions for the same amount of time. I extracted the DNA using DNEasy Blood and Tissue Extraction Kits (QIAGEN, Crawley, West Sussex, UK) however, I modified the protocol in the following ways: I used 300µL of virus and cell suspension; I did not spin down the sample or use PBS. I screened the extracted DNA to make sure that the extraction had worked and also to ensure that what was isolated was a ranavirus. I used the same primers for MCP as for the screens and 50µL PCR reactions: 25µL Multiplex Mix, 5.3uL of each forward and

reverse primer, 12.4μL of distilled water and 2.0μL of template DNA). The Multiplex Mix and distilled water were from the QIAGEN Multiplex PCR kit and all primers were from MWG Eurofins. However, for ORF 57R, I used 25μL reactions. I cleaned the PCR products using a polyethylene glycol (PEG) precipitation (protocol from the Santos Lab, Auburn University). I PEG-precipitated products out on a 1.5% agarose gel (stained with ethidium bromide) to ensure that I had not lost the DNA from the original reaction. I outsourced sequencing to Cogenics (Beckman Coulter Genomics, Essex, UK). In one case, after several attempts to culture the ranavirus from tissue, I was unable to isolate the virus from a PCR positive *Lissotriton vulgaris*. I used the original extraction for the template for the MCP for sequencing, however, the DNA in the original extract was too poor for ORF 57R amplification, since it is a larger fragment.

Phylogenetic Analysis

To build the phylogenetic trees, I first corrected the sequences by sight from the electrophorograms in ChromasLite (Technelysium Pty Ltd, Australia). The sequences were then imported into MEGA 4 (Tamura et al. 2007) and aligned using ClustalW (Larkin et al. 2007) to check for congruency between the forward and reverse sequences for each isolate at both loci. In all cases, the sequence that contained the fewest corrections was used to ensure minimal human error on base calling. To examine both sequences together, I joined the MCP and ORF57r sequences of each isolate end to end. I then aligned these joined sequences and used this to build the phylogenetic trees.

I chose the Jukes and Cantor model of molecular evolution, which assumes that substitutions occur at any site with the same likelihood and frequency (Nei and Kumar 2000). I chose this model because it is semi-conservative and since I did not have any estimates of molecular evolution at these loci in Iridoviruses, a prudent and conservative approach should yield results that are more robust. I built the phylogenetic trees using the neighbour joining (NJ) method in MEGA 4. The NJ method is based on minimum evolution and gives branch lengths based on the differences between the sequences (Nei and Kumar 2000). These methods for developing phylogenetic trees are most appropriate for ranaviruses because of their rigorous and conservative nature. All of the trees presented were built in this manner and were tested for confidence using bootstrap tests of branches in the trees.

Appropriate out groups were selected from GenBank: MCP - ATV Manitoba (Accession No. AY548314); CMTV Spain (Accession No. FM213466); FV3 (Accession No. FJ459783); and Grouper virus (Accession No. EU847414). ORF 57r - ATV ORF 57r (Accession Number: EU512332).

Results

Screening of Animals

One of 73 common toads (*Bufo bufo*) sampled in 2007 tested positive for the ranavirus, while none of the 33 toads sampled in 2008 tested positive for the ranavirus using PCR screens for the presence of ranavirus genomic DNA. No great crested newts (*Triturus cristatus*) tested positive for the ranavirus (n = 2, 2007 and n = 13, 2008), nor did any alpine newts (*Mesotriton alpestris*) test positive for the ranavirus (n = 4, 2007). However, 1 of 17 common or smooth newts (*Lissotrion vulgaris*) sampled in 2007 and 1 of 18 sampled in 2008 tested positive for the ranavirus. One common midwife toad (*Alytes obstetricans*) sampled in 2008 tested positive for the ranavirus (n = 1).

Virus Isolation

I was able to obtain 27 different viruse isolates from the diseased animals that were sent in for necropsy (Table 4.1). Of these, 24 were from common frogs (*R. temporaria*), one was from a midwife toad (*A. obstetricans*) and one was from a common or smooth newt (*L. vulgaris*) (Table 4.1). In addition, five isolates from Hyatt et al. (2000) and Cunningham et al. (2007a&b) were also cultured for sequencing.

Phylogenetic Relationships

MCP:

Twenty-seven new ranavirus isolates and five old isolates appear on the same monophyletic branch of the NJ tree (Figure 4.1). Once the branches with less than 50% support are removed, the same basic branching pattern is seen, however, this reveals three different groups within the large group (Figure 4.2). RT 122 and RT 123 group together and are from the same location (see Table 4.1) and RT 126 is from the next county, however RT 113 also groups with them and it is of unknown origin (Figure 4.2). OS 21 and RT 127 group together on their own branch as does

RT 119 (Figure 4.2). Within the rest of the tree, grouping does not appear to be influenced by the species from with the isolate was obtained.

Table 4.1. List of ranavirus isolates obtained, including the host species of origin, the location of the sample and the area of England that the host was originally collected.

Isolate	Species of Origin	Location	Area of England
RT 5	*Rana temporaria*	Herne Bay	Kent
RT 8	*R. temporaria*	N/A	Unknown
RT 80	*R. temporaria*	Brighton	East Sussex
OS 14	*Alytes obstetricans*		
XT/611/07	*R. temporaria*		
RT 112	*R. temporaria*	N/A	Unknown
RT 113		N/A	Unknown
RT 115		N/A	Unknown
RT 116	*R. temporaria*	Bournemouth	Dorset
RT 119	*R. temporaria*	Plymouth	Devon
RT 120			
RT 122	*R. temporaria*	Wokingham	Berkshire
RT 123			
RT 126	*R. temporaria*	Southampton	Hampshire
RT 127	*R. temporaria*	Wallington	Surrey
RT 128			
RT 129	*R. temporaria*	Preston	Lancashire
RT 130			
RT 131			
RT 132			
RT 133			
RT 134			
RT 135			
RT 137			
RT 138			
TT 216	*R. temporaria* (tadpole)	Deal	Kent
OS 15	*Lissotrion vulgaris*	N/A	Unknown
OS 21			

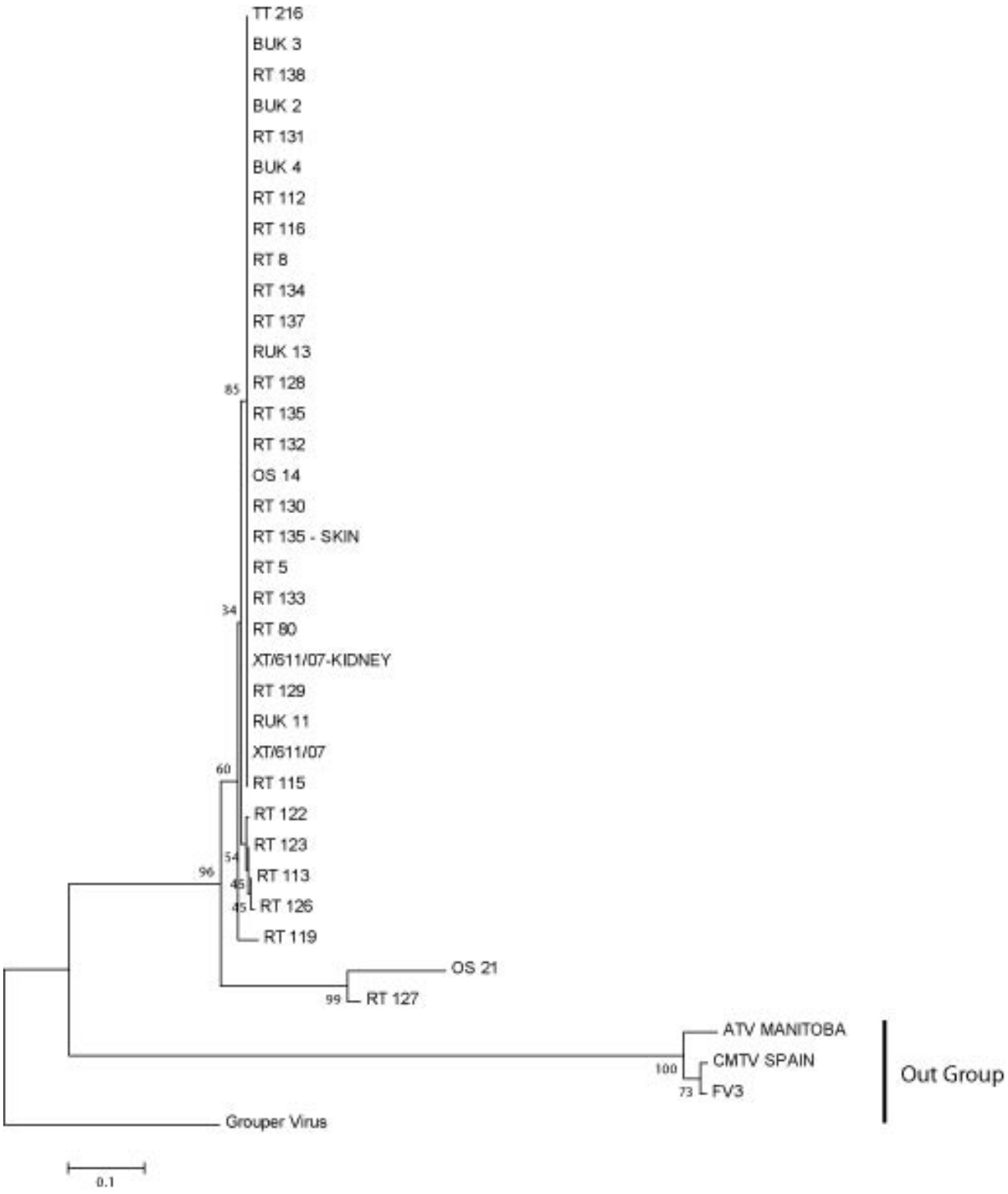

Figure 4.1. Phylogeny of the major capsid protein sequences of different ranavirus isolates from the UK. The tree was made using the neighbour joining method with the Jukes-Cantor model of nucleotide evolution. (ATV Manitoba Accession No. AY548314; CMTV Spain Accession No. FM213466; FV3 Accession No. FJ459783; Grouper virus Accession No. EU847414.). The scale bar indicates nucleotide divergence.

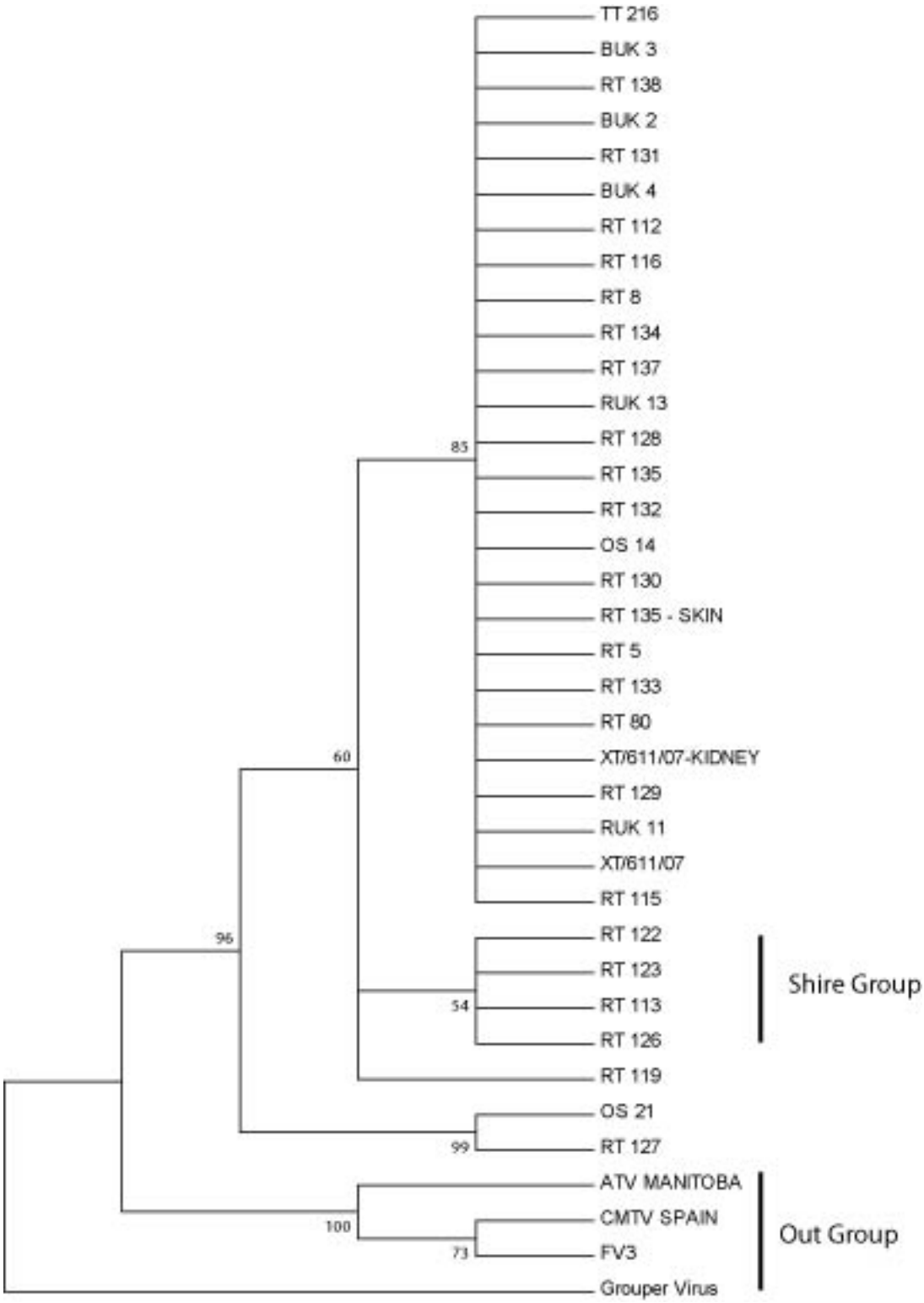

Figure 4.2. Truncated phylogeny of the major capsid protein sequences of different ranavirus isolates from the UK. The tree was made using the neighbour joining method with the Jukes-Cantor model of nucleotide evolution. All branches with less than 50% support were truncated. Note the distinct group of isolates, the Shire group, which are all from Berkshire, Hampshire and an unknown location. (ATV Manitoba Accession No. AY548314; CMTV Spain Accession No. FM213466; FV3 Accession No. FJ459783; Grouper virus Accession No. EU847414.)

ORF 57r:

The majority of the recently isolated viruses group together in a monophyletic lineage that is different from some of the viruses isolated over a decade ago (Figure 4.3). RT 130, 131, 132, 133, 134, and 137 group together, however, RT 135 and RT 135-Skin do not group with them and all of these isolates are from the same mortality event (Figures 4.3, 4.4, and Table 4.1). Again, here, the isolate from the common newt (OS 15) is on a separate branch of the tree from other isolates. The original isolates (RUK 13, BUK 2 and BUK 3) group together at the base of the tree with another isolate from common frogs (RT 119). When the tree is truncated, the same, well supported, branching pattern is seen.

Both Loci:

When both loci are considered together in the same trees, a similar branching pattern to that of ORF57r is seen (Figures 4.4 and 4.5). In this case, RT 119 is the most basal group, RT 130, 131, 132, 133, 134, and 137 again group together but away from other isolates from the same mortality event and are the most divergent group (Figures 4.5 and 4.6).

In the truncated tree, a second monophyletic group becomes apparent (called 'Secondary Clade'; Figure 4.6). This monophyletic group contains ranavirus isolates from common frogs and the isolate from the midwife toad.

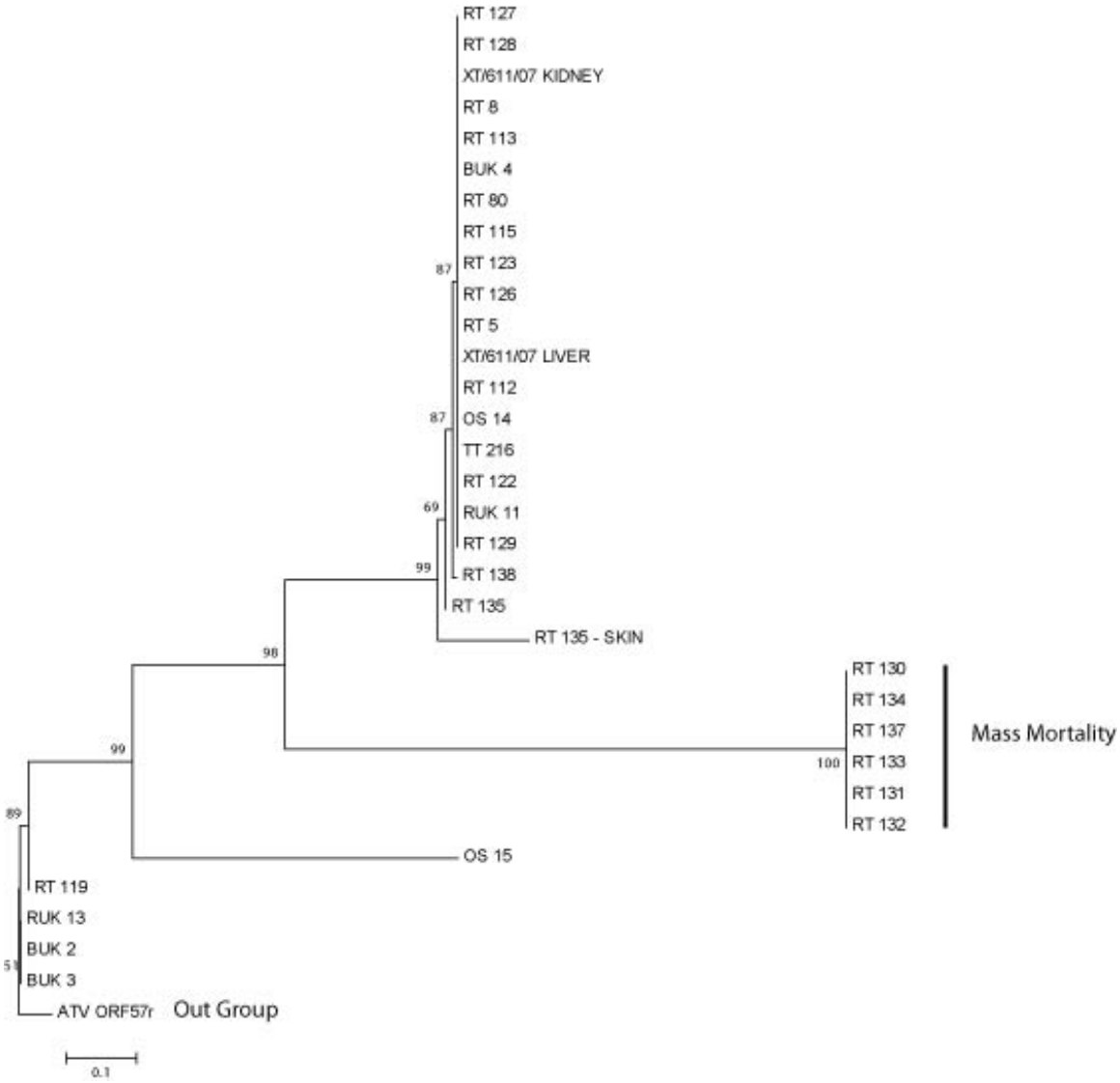

Figure 4.3. Phylogeny of ORF 57r sequences of different ranavirus isolates from the UK. The tree was made using the neighbour joining method with the Jukes-Cantor model of nucleotide evolution. The label of mass mortality indicates that all of the isolates in that group are from the same mortality event. (ATV ORF 57r Accession Number: EU512332) The scale bar indicates nucleotide divergence.

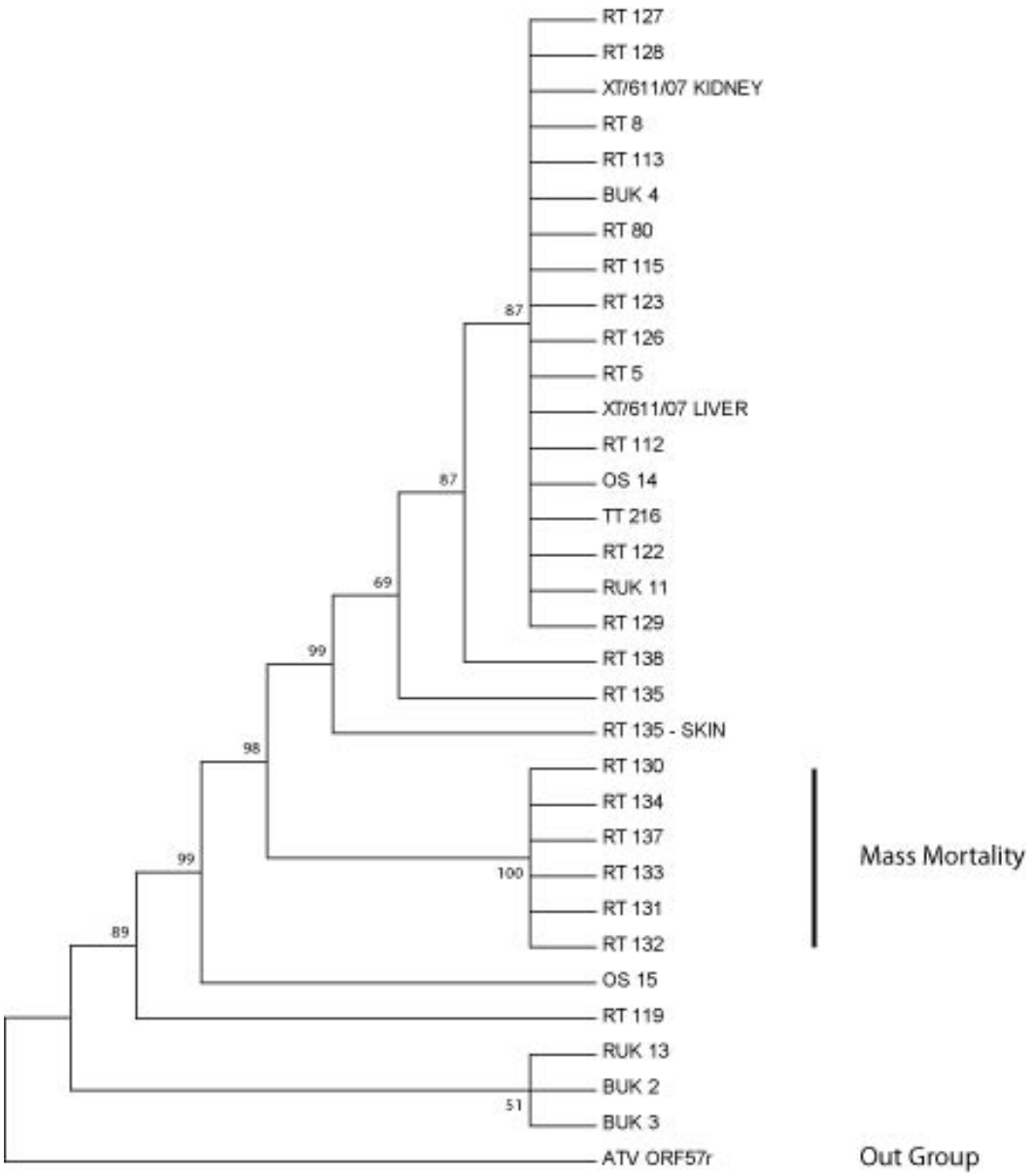

Figure 4.4. Truncated phylogeny of ORF 57r sequences of different ranavirus isolates from the UK. The tree was made using the neighbour joining method with the Jukes-Cantor model of nucleotide evolution. All branches with less than 50% support were truncated. The label of mass mortality indicates that all of the isolates in that group are from the same mortality event. (ATV ORF 57r Accession Number: EU512332)

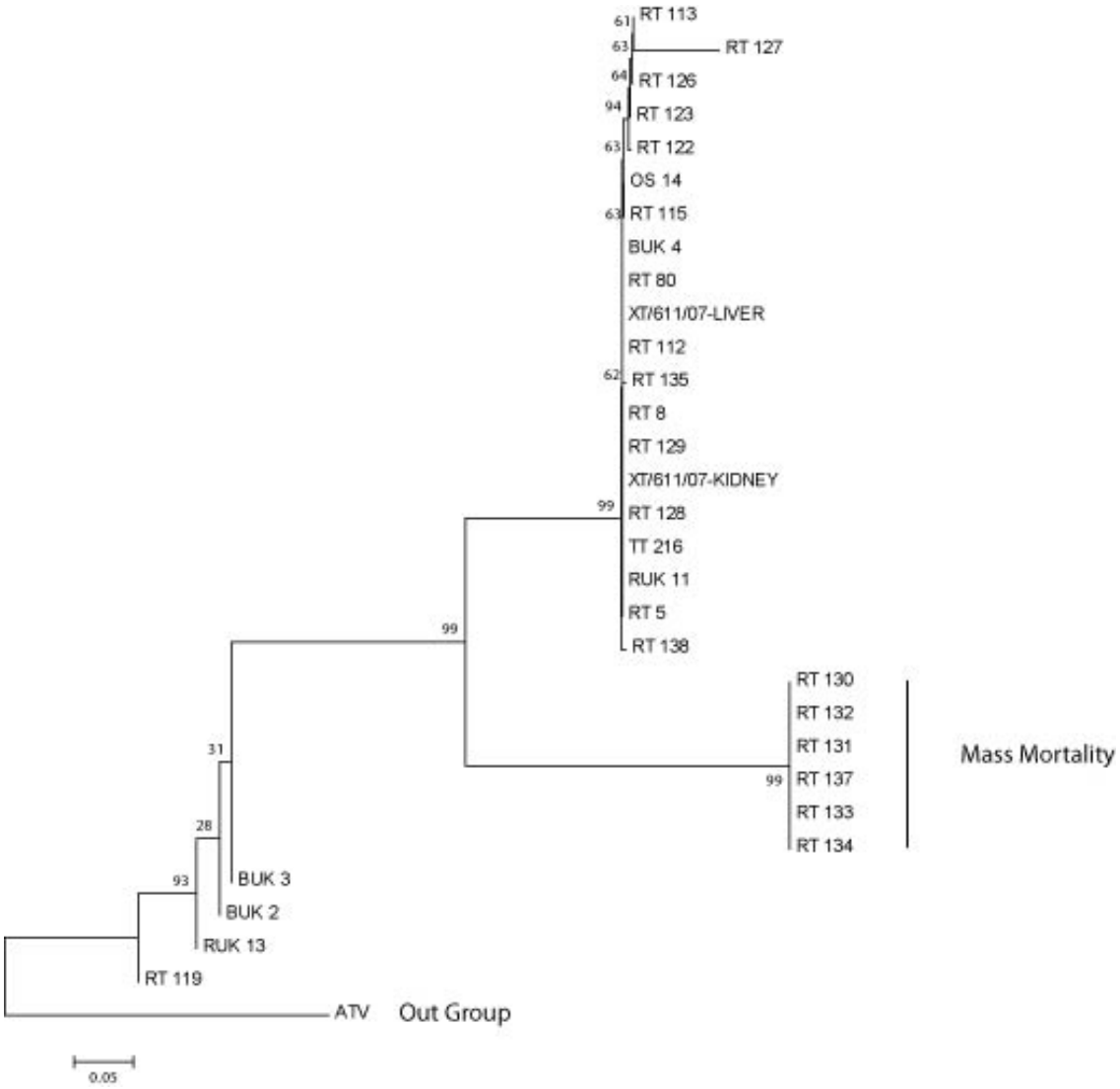

Figure 4.5. Phylogeny of both MCP and ORF 57r sequences of different ranavirus isolates from the UK. The tree was made using the neighbour joining method with the Jukes-Cantor model of nucleotide evolution. The scale bar indicates nucleotide divergence. ATV is a combination of EU512332 and AY548314.

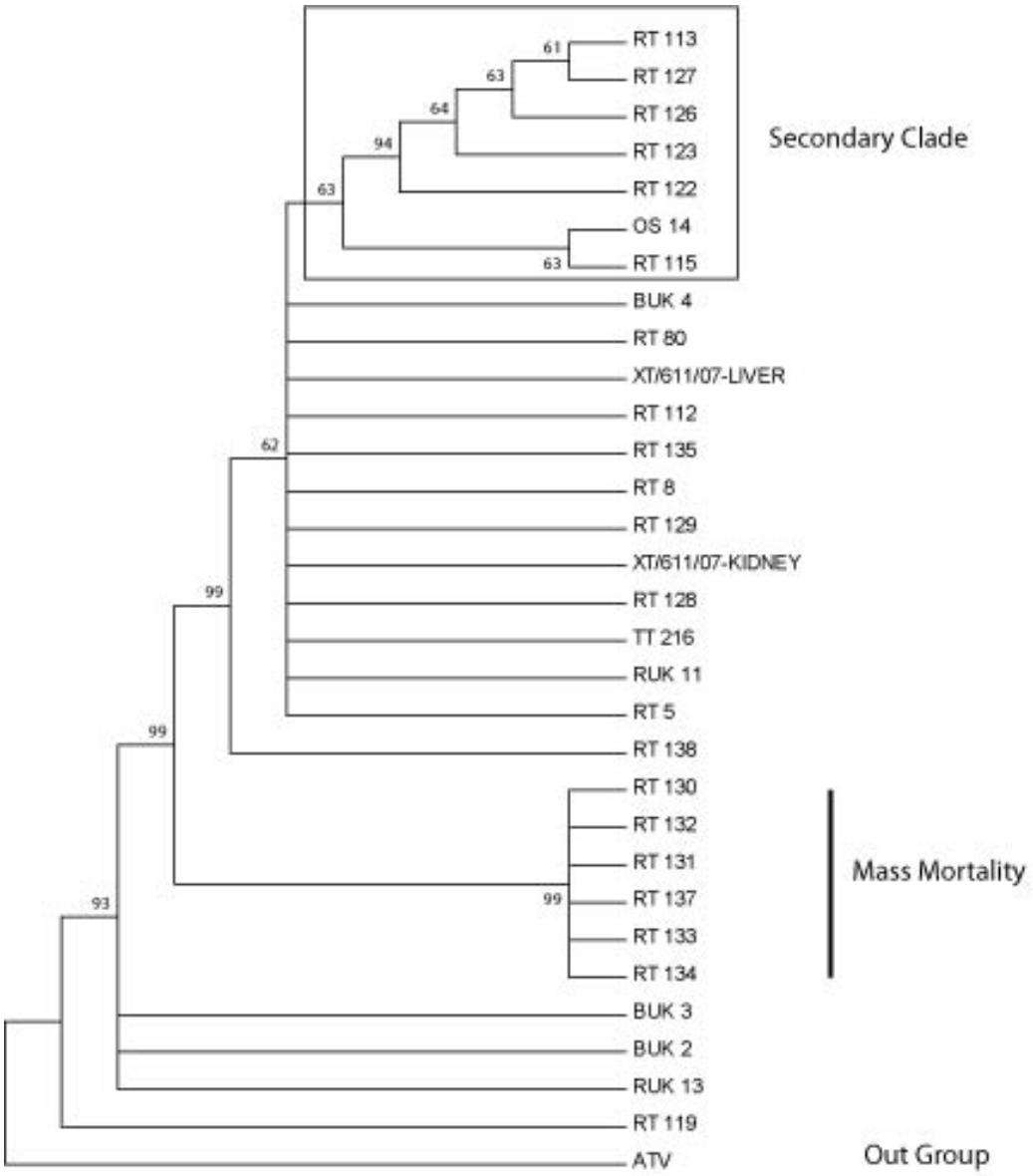

Figure 4.6. Truncated phylogeny of both MCP and ORF 57r sequences of different ranavirus isolates from the UK. The tree was made using the neighbour joining method with the Jukes-Cantor model of nucleotide evolution. The mass mortality label indicates that all of the strains present are from the same mass mortality event. The box labelled 'Secondary Clade' highlights a second monophyletic group of unknown biological significance within the main tree. ATV is a combination of EU512332 and AY548314.

Discussion

Infections in Other Species

The first ranavirus infections in common newts (*Lissotrion vulgaris*) and an adult common midwife toad (*Alytes obstetricans*) are reported here. While midwife toads are an introduced species in the UK, common newts are not, they are regularly found in ponds with common frogs. Since ranaviruses are considered to be infections of amphibian communities (Duffus et al. 2008), finding ranaviral infections in species that are sympatric with common frogs and common toads is not surprising.

The midwife toad with the ranaviral infection was from a site were a mass mortality in both common frogs and common toads was occurring. Unfortunately, I was not able to collect any common toads from this site, but, infections were found in common frogs (see Chapter 2). Here, the ranavirus infection of the midwife toad was most likely the result of pathogen spillover from infections in common frogs and toads. This conclusion is supported by the high sequence homology between the ranavirus isolates from common frogs (*Rana temporaria*) and the isolate from the midwife toad. Midwife toad tadpoles are known to be susceptible to ranavirus infections because two mass mortality events on the Iberian Peninsula (Balsiero et al. in press; Balsiero et al. 2009). However, this is the first ranavirus-associated mortality in adults.

The infections in common newts were unexpected because there were no reports of European urodele species with ranavirus infections when I started the work. Some of the newts that I tested (n = 5) were from a site where ranavirus infections are known in common frogs (Chapter 2; Teacher 2009). One of the newts had a large amount of subcutaneous oedema, which in frogs I have found to be a reliable sign of ranavirus infection. However, I was not able to detect the presence of ranaviral disease in these animals, despite the presence of visibly diseased common frog adults in the pond at the time when the newts were sampled. The ranavirus infection found in the common newt in 2007 was from a mortality event that involved only adult common newts. This is the first ranavirus associated mortality event in common newts. Recently, in Spain, a mass mortality of alpine newt (*Mesotrition alpestirs cyreni*) larvae was associated with the common midwife toad virus, a ranavirus (Balsiero et al. in press). This mortality event also involved common midwife toad tadpoles (Balsiero et al. in press), further demonstrating the

need for a community based approach to understanding ranavirus dynamics in European amphibians.

The great crested newts (*T. cristatus*) that were screened for the presence of ranavirus DNA were from a mass mortality event of unknown causes in northern England. No visible signs of disease were seen and none of the individuals had viral infections. In this case, it is likely that the mortality event was the cause of environmental pollution, not a pathogen.

The presence of a ranavirus infection in a common toad is not unexpected, as ranaviral disease has been previously described in them (Hyatt et al. 2000; Cunningham et al 2007b). Most of the animals screened, from both 2007 and 2008, were part of a preliminary health screen of toads from the Channel Island of Jersey (see Fernández-Loras et al. in review). I was surprised to only find one ranavirus infection in common toads from the UK mainland. Ranaviruses have been implicated in the decline of common toads across the UK (e.g. Cunningham et al. 2007b) and this does not seem to be supported here, as if this were true, I would have found more infections in the toads that were examined. Therefore, more investigations into the role of ranavirus infections in common toads are required before disease can be cited as a cause of the declines in the UK, but my data suggest no such role for ranavirus in UK common toads.

Phylogenetics

The homology between the major capsid protein (MCP) sequences of the different ranavirus isolates was expected. The MCP gene is conserved across all iridoviruses and has been suggested as a target for examining molecular evolution in the Iridoviridae (Tidona et al. 1998). Previously, slight differences between the BUK and RUK isolate MCP sequences have been found; however, they were not thought to result in functional differences (Hyatt et al. 2000). Selection at the MCP was not seen in *Ambystoma tigrinum* virus (ATV) isolates from different regions in the western USA (Ridenhour and Storfer 2008). The highly conserved nature of this gene across geography and host species, despite a constant selection pressure from the host's immune system, suggests that the MCP alone is inadequate to examine evolutionary and phylogenetic relationships between ranaviruses on small geographic scales or in recently introduced viruses.

The close homology of the ORF57r sequences as revealed in the phylogenetic trees was unexpected. However, the topology of the trees was different from those based on the MCP sequence data. ORF 57r in ATV has been found to be under strong selection pressure and sequence divergence was high between different populations because of local selection (Ridenhour and Storfer 2008). Six isolates from the same mortality event all grouped together on a highly supported branch, however, two other isolates from the same mortality event grouped with the rest of the common frog isolates on an equally well supported branch.

When both the MCP and ORF57r genes are considered together in the same phylogenetic tree, the topology is nearly identical to that of ORF57r. The isolate from common newts (OS 15) remains on a separate branch, BUK 2, BUK 3 and RUK 13 remain basal to the UK clade and the group of six isolates from the same mass mortality event still group together.

The grouping of BUK 2, BUK 3 and RUK 13 may be because they are three of the six original isolates from the UK (Hyatt et al. 2000; Cunningham et al. 2007b). When this information is combined with the fact that the modern common frog isolates, the isolate from the midwife toad and the other two original isolates all branch together, it provides evidence of pathogen spillover from a primary host (in this case, common frogs) to alternate hosts.

The ranavirus first emerged in common frogs in the UK, with infections being discovered subsequently in common toads (Cunningham et al. 1996; Hyatt et al. 2000; Cunningham et al. 200b). If ranaviruses are primarily transmitted during the breeding season when contact rates between individuals are extremely high (as per Chapter 2 and 3), the reproductive biology of frogs and toads needs to be considered further in the context of disease transmission between species. Common frogs and common toads breed at approximately the same time. During this period, they are frequently in contact with one another and frogs and toads frequently amplex the wrong species when they are searching for a mate. In fact, common toads that have amplexed a frog will not release it immediately, even if the frog gives an alarm call (Marco and Lizana 2002). This behaviour would lead to prolonged contact between the two species and would facilitate the transmission of a ranavirus infection between them. In this manner, an infected common frog could easily pass the ranavirus to a

common toad, resulting in the spillover of infection from the primary host to an alternate host.

The infections seen in common newts may be the result of a different type of disease spillover event. Ranavirus infections are known to be transmitted between conspecifics through scavenging e.g. wood frogs (*Rana sylvatica*; Harp and Petranka 2006) and *R. latastei* (Pearman and Garner 2005). It is also thought that one mode of transmission between wood frog tadpoles and ambystomatid larvae are through the scavenging of infected tadpole carcasses by the larvae (Duffus 2006). Therefore, if the newts, as either larvae or adults, were scavenging off infected common frog or common toad carcasses that remained in the pond, ingestion of the virus could easily result in the development of a ranavirus infection. Experimental evidence in common frogs suggests that there may be different 'quasi-species' of virus circulating (Cunningham et al. 2007a), which may vary in their infectivity and virulence in newts. However, more research into ranavirus prevalence in newts and more sequence data from different isolates obtained from newts are required before this can be confirmed.

Conclusions

The first ranavirus infections in common newts in Europe and the in common midwife toads in the UK are described. The high homology between the different ranavirus isolates may indicate that the infections in species other than common frogs are the result of pathogen spillover. However, more isolates from other amphibian species infected with the ranavirus from the UK are required if this hypothesis is to be confirmed. It can be concluded that the quasi-species of viral isolates do not occur because of the clean sequences that were obtained. If quasi-species were present, sequences would not have been clean from the isolate DNA and cloning would have been required to obtain good sequence data. Full genome analysis of the viral isolates would help to provide more a more detailed understanding of the relationships between the isolates and with the next generation sequence technologies whole genome sequences of ranaviruses is possible.

CHAPTER 5: EXPERIMENTAL ASSESSMENT OF VIRULUENCE AND HOST SPECIFICITY OF THE RANAVIRUS IN *RANA TEMPORARIA* AND *BUFO BUFO*

Abstract

Ranaviruses are emerging pathogens in amphibians which were first reported in UK common frogs (*Rana temporaria*) in the late 1980s/early 1990s. To date, only preliminary assessments of host-specificity and virulence have been conducted. In this chapter, I assess the relationship of infection, signs of disease, and mortality with viral isolate and dose for four UK ranavirus isolates from UK amphibian hosts in R. *temporaria* and *Bufo bufo* tadpoles. In *R. temporaria* tadpoles exposed to low doses of strains that originated from *R. temporaria,* experienced higher mortality than those exposed to strains from *B. bufo*. There was no such difference at the high dose. This result suggests some degree of host-specificity at low dose. The origin of the isolate had no significant effect on the presence of infection, or signs of disease at death at the high dose. The most common sign of disease was abdominal haemorrhages and/or bloating. In *B. bufo*, reduced survivorship, the presence of infection, and signs of disease at death were all associated with dose, not isolate. The most common sign of disease at death for *B. bufo* tadpoles was skin sloughing. Taken together, these experiments demonstrate host specificity at low doses, which is lost at higher doses; it also suggests that *R. temporaria* is the primary host of the ranavirus in the UK since virulence can be higher in a primary host where secondary hosts exist.

Introduction

Seemingly similar pathogens can have quite different effects on the host [e.g. UK ranaviruses (Cunningham et al. 2007a&b)], as virulence is affected by many aspects of the biology of both the host and pathogen. Conventionally, virulence is assumed to be greater in pathogens that rely solely on horizontal transmission (Lipsitch et al. 1996). However, there are complex interactions between aspects of horizontal and vertical transmission which result in context dependant outcomes (Lipsitch et al. 1996). Host characteristics, such as life history stage/age, are also important determinants of virulence (Day 2003). Therefore, the life history of the host and mode of transmission of the pathogen need to be considered as important factors in pathogen emergence.

Virulence can be defined in many different ways, here, I will use the term in the sense of how infection with a ranavirus causes mortality. When a pathogen is highly virulent, it may kill its host before it can be transmitted to another individual or kill an entire population without being transmitted to a different susceptible population. However, if there is an alternate host that is present, the pathogen can remain, even long after it has driven its primary host to extinction. If a pathogen has the potential to use different hosts, a complex relationship between virulence and transmission in the secondary host is expected (Woolhouse et al. 2001), therefore examining host-specificity and virulence provide important insights into the relationship between a pathogen and a given host.

Investigations into the host-specificity and virulence of most ranaviruses have not been comprehensively performed. This may be due to early reports of an apparent lack of host-specificity: the same ranavirus has been found to infect sympatric fish and amphibian tadpoles (Mao et al. 1999). More recent evidence has suggested that some degree of host-specificity does exist. Experiments examining the host-specificity and relative virulence of two different 'groups' of amphibian ranaviruses in North America has provided some interesting insights into host-strain relationships. *Ambystoma tigrinum* virus (ATV) isolated from ambystomatid salamanders elicited higher rates of mortality (i.e. it was more virulent) in ambystomatid larvae than in anuran metamorphs and, similarly, frog virus 3 (FV3)-like virus isolates from anurans caused higher mortality rates in anuran metamorphs than in ambystomatid larvae (Schock et al. 2008). Although the sample sizes in these

experiments were small, this does provide evidence of host-specific virulence at the stage of recent metamorphs (Schock et al. 2008). While Shock et al (2008) addressed ranavirus host-strain associations from different branches of the amphibian tree, evidence of geographical host-strain associations are emerging for ranaviruses. This is seen in ATV from the western US, where there is molecular evidence of regional selection on the virus (Ridenhour and Storfer 2008).

In the UK, ranaviruses began to emerge approximately twenty years ago, originally affecting common frogs (*Rana temporaria*) and causing mass mortality events across the SE of England (Cunningham et al. 1996). Ranaviruses were subsequently isolated from naturally diseased common toads (*Bufo bufo*) (Hyatt et al. 2000; Previous Chapter) and common newts (*Lissotriton vulgaris*; Previous Chapter). Ranavirus isolates from *R. temporaria* and *B. bufo* differ slightly on the molecular level (Hyatt et al. 2000) and in the disease syndromes that they cause (Cunningham et al. 2007a&b). In *R. temporaria*, two different disease syndromes have been associated with ranavirus infections: the ulcerative form and the haemorrhagic form (Cunningham et al. 1996). *B. bufo*, however, only appear to suffer from the haemorrhagic form of ranaviral disease in the UK (Cunningham et al. 2007b). This difference indicates that there is some host-specificity in response to the viruses present in the UK.

To investigate the importance of host-strain relationships, a series of experiments were performed by Cunningham et al. (2007a&b). Due to limited replication and absence of negative controls, the conclusions must be provisional but do permit inferences about potential trends in host-strain associations. When adult *R. temporaria* were exposed via immersion to a tissue homogenate made from lesions of naturally diseased frogs 30% of those exposed to the homogenate derived from an ulcerative lesion developed ulcerations. None of the frogs exposed to the homogenate derived from haemorrhagic tissue developed the haemorrhagic syndrome, or any other signs of disease (Cunningham et al. 2007a).

To assess how the condition of the frog influenced the development of disease, animals with wounds created on their thigh were exposed to the same treatments as described above (Cunningham et al. 2007a) Seventy percent of the wounded frogs that were exposed to the tissue homogenate derived from haemorrhagic tissue developed the haemorrhagic syndrome (Cunningham et al.

2007a). Only 20% of wounded frogs exposed to the ulcerative homogenate developed ulcerations (Cunningham et al. 2007b). From the results of these experiments, both the source of the virus and the condition of the frog appear to affect the development of disease (Cunningham et al. 2007a). Further experiments demonstrated that a ranavirus isolated from a naturally diseased *B. bufo* suffering from the haemorrhagic syndrome caused the same disease in *R. temporaria* adults (Cunningham et al. 2007b). This is an unsurprising result since *B. bufo* is only known to be affected by the haemorrhagic form in the wild (Cunningham et al. 2007b). Mock infected frogs, which underwent the same treatment without the virus, were used and no disease developed (Cunningham et al. 2007b). Unfortunately, the reciprocal experiment was not performed, so no host-strain associations can be inferred and further experimental evidence is necessary.

Overall, the ranavirus-amphibian system in the UK is poorly understood in terms of host-strain specificity. In the first chapter, I presented a study that showed adults were the only life history stage of *R. temporaria* that was consistently infected with ranavirus in the wild. However, the lack of detectable infection in earlier life history stages in the wild does not necessarily imply a lack of susceptibility to infection at these stages. Further investigations into the susceptibility of other *R. temporaria* life history stages are necessary to more fully understand the dynamics of the system. With the discovery of potential alternative hosts for ranaviruses present in the UK (*B. bufo,* Hyatt et al. 2000; *Lissotriton vulgaris*; Previous Chapter) and the demonstration that a ranavirus from *B. bufo* can infect *R. temporaria* (Cunningham et al. 2007b), it is important to assess the potential for interspecies transmission of the virus. Since ranaviruses are thought not to exhibit strong host-specificity, it is necessary to determine if *R. temporaria* is susceptible to the ranavirus strains that sympatric species carry and vice versa. This is especially pressing since the emergence of the ranavirus has been linked to declines in *R. temporaria* in England (Teacher 2009).

An ideal model system exists to assess host-specificity and the susceptibility of both *R. temporaria* and *B. bufo* as virus isolates from both species exist and experimental exposure of tadpoles to ranaviruses is a well established technique (e.g. Pearman et al. 2004; Pearman and Garner 2005; Duffus et al. 2008). Importantly, isolates from both disease syndromes from *R. temporaria* are available (Cunningham

et al. 2007a&b), so strain-disease specificity can also be explored. In this chapter, I examine the effect of different ranavirus isolates in two different anuran hosts, *R. temporaria* and *B. bufo*. Using isolates from both disease syndromes in *R. temporaria* and two isolates from *B. bufo* adults, the relationships between host, strain and virulence were examined in the first statistically sound ranavirus experimental exposures that examine host-strain relationships in anurans. These experiments also permited me to determine if tadpoles of *R. temporaria* and *B. bufo* are susceptible to ranavirus infections and if susceptibilities are dose-dependent.

Methods

Viral Culture Methodology

I used four viral isolates for my experiments: BUK 2, BUK 3, RUK 11 and RUK 13 (Hyatt et al. 2000, Cunningham et al. 2007a&b): these four were isolated from different hosts (*Bufo bufo*: BUK 2, 3, *Rana temporaria*: RUK 11, 13) and isolated from infected wild animals found in the UK in the early 1990s (Cunningham et al. 2007a&b). Furthermore, the two RUK isolates were associated with different disease syndromes: RUK 11 was isolated from a *R. temporaria* adult suffering from the haemorrhagic syndrome, whereas RUK 13 was isolated from a frog exhibiting the ulcerative syndrome (Cunningham et al. 2007a).

Fathead minnow cells (FHM) (*Pimephales promelus*) were obtained from the European Collection of Cell Cultures (No. 88102401, ECACC, Oxford, UK). The cells were propagated at 25°C in Eagle's Minimum Essential Media (EMEM) (Sigma – Aldrich, Andover, UK), supplemented with 1% L – glutamine (Sigma – Aldrich, Andover, UK), 0.005% Penicillin – Streptomycin (Sigma – Aldrich, Andover, UK), 0.005% Nystatin (Gibco, Invitrogen, Paislely, UK), and 10% Research Grade Foetal Bovine Serum (Hyclone, Perbio Science, Northumberland, UK). All viruses were grown on confluent flasks of FHM cells by inoculating flasks with 1000µL of a given isolate. Twenty-five millilitres of maintenance media [Eagle's Minimum Essential Media (EMEM), supplemented with 1% L – glutamine, 0.005% Penicillin – Streptomycim, 0.005% Nystatin, and 1% Research Grade Fetal Bovine Serum, all suppliers as above] was added to each flask and all flasks were then incubated at 25°C. The flasks were monitored daily for the formation of viral plaques. When no cells where adhering to the flask, the virus from a given flask was

harvested and stored at -80˚C. Each isolate was standardized to cell culture before titration by passaging three times before challenge experiments.

Mock cells for negative controls were obtained in the following manner. Confluent flasks of FHM cells had their media changed to maintenance media and were left in the media for 3 days, the average amount of time that the isolates took to grow. The cells were then scraped into the media and harvested. The cell solution was then frozen at -80˚C, then thawed and refrozen to ensure that they went through the same number of freeze-thaw cycles as the isolates.

Viral Titration Methodology

To determine the number of plaque forming units per millilitre (PFU/mL) for each virus, I used serial dilutions of harvested virus (10^{-3} to 10^{-8}, e.g. Duffus et al. 2008) and titrated the respective concentrations into 6 well flasks with confluent FHM cells. One millilitre of each dilution for each isolate was added to a single well in a single plate (one isolate per plate) and all plates were incubated at 25°C for 24 hours. An additional 2mL of maintenance media was then added to each well. The plates were then monitored daily for the formation of plaques. When plaques were first detected in the well with the highest concentration virus, media was removed from all wells and the cells were fixed in 100% methanol. The methanol was removed after 10 minutes and cells were then stained in a 0.05% crystal violet-20% methanol solution (20 minutes, then washed with water to removed excess stain) and plaques counted after staining was completed. Each titration for each isolate was performed in duplicate and I averaged the number of plaques for each dilution for each isolate to determine the PFU/mL for each isolate harvest.

Animal Husbandry

R. temporaria tadpoles were collected from a site in Faversham, Kent, England in March 2009. This site is known to be ranavirus free (see Chapter 2) and contains a large, apparently healthy population of adults. Eggs were brought back to the Institute of Zoology, Zoological Society of London, in water from the pond. Upon arrival, the eggs were transferred into ~ 45L of aged tap water (aged at least 48hrs) in an 84L plastic box (Really Useful Box Company, Normanton, UK). After the tadpoles had hatched and reached the free swimming stage, they were fed Tetra Tabimin pellets (Tetra Fish, Southampton, UK) *ad libitum*. Water was changed every second day, using water that had been aged for a minimum of 48hrs to ensure that the

vast majority of the chlorine had evaporated. *B. bufo* tadpoles were collected from Cowden, Sussex, England in late March 2009. They were cared for in the same manner.

Rana temporaria *Tadpole Exposures*

When the tadpoles had reached Gosner stage 25 (Gosner 1960), I began the experimental exposures. Tadpoles from different tanks were mixed and a subset was removed for the experiment. The total sample size for each treatment was 35 individuals for a total of 315 animals. Tadpoles were exposed in groups of 5 in Petri dishes (Nunc, Roskilde, Denmark) via bath exposure to one of nine different treatments: control (30mL); BUK 2 10^4 or 10^6 PFU in 30mL, BUK 3 10^4 or 10^6 10^4 or 10^6 PFU in 30mL, RUK 11 10^4 or 10^6 PFU in 30mL or RUK 13 10^4 or 10^6 PFU in 30mL. The amount of cellular debris was kept constant across all treatments. Tadpole groups were exposed for 18hrs, after which tadpoles were transferred into their own Petri dishes with 30mL aged tap water. Each was fed 125µL of a dilution of Tabimin pellets (6 finely ground pellets suspended in 50mL aged tap water) every second day. The water in each Petri dish was changed every third day along with the position of each treatment block on shelving. I also rotated tadpole positions each day within their treatment group: rotation of block and tadpole positions were done to avoid any block effects. During the peak of mortality, the tadpoles were checked twice a day for mortality, and when the rate of mortality declined they were checked once a day. When I found a dead individual, I examined the carcass carefully for signs of disease such as bloated and/or haemorrhaged abdomens (commonly seen in ranavirus infected tadpoles e.g. Greer et al. 2005, Table 5.1). The experiment lasted for a total of 30 days and survivors were euthanized using an overdose of MS-2,2,2 (1g/L tricane methylsulphonate (Thompson and Joseph Ltd., Norwich, UK) buffered to pH 7 with sodium bicarbonate). All carcasses were stored in 2mL microcentrifuge tubes in 100% ethanol for molecular analysis.

Bufo bufo *Tadpole Exposures*

The tadpoles used for the *Bufo* experiment were slightly older (Gosner Stage 29-30). I used the experimental design for the *R. temporaria* exposures, but after the exposures were complete tadpoles were transferred to individual 75cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) containing 140mL aged tap water. The tadpoles were fed as per *R. temporaria* until the 10^{th} day, when I then reduced food

concentration to 3 pellets in 50mL and pipetted 100µL of food suspension into each flask once every 2 days. I did this to retard growth rate as forelimbs had begun to emerge on some animals. I changed water every fourth day by pouring off 120-140mL of flask water, leaving 20-40mL of the pre-change water to reduce the osmotic stress associated with total water changes and to reduce the risk of disposing of the tadpole with the water. I then filled each flask with aged tap water to 140-160mL. I rotated both treatment and tadpole position at each water change. The tadpoles were monitored for mortality and dead animals examined for signs of disease in the same manner as for the *R. temporaria* tadpoles. In this experiment animals reaching Gosner Stage 43-44 were euthanized and counted as survivors. This was done to ensure that the animals did not drown and also to avoid potential post-metamorphic effects, since the goal was to examine pre-metamorphic disease dynamics. The experiment lasted for 30 days and again the survivors were euthanized using an overdose of MS-2,2,2 (1g/L tricane methylsulphonate buffered to pH 7 with sodium bicarbonate). All carcasses were stored in 2mL microcentrifuge tubes in 100% ethanol for molecular analysis.

All experiments were performed with the required Home Office licenses and with the approval of the Zoological Society of London's Ethics Committee.

Molecular Analysis

The first 10 animals that died and the first 5 euthanized at the end of the experiment (n=15) were selected from each treatment for screening for the presence of the ranavirus. In treatments where 10 animals did not die over the course of the experiment, all animals that died were included and the rest of the sample was made up of euthanized individuals. A small triangular section of the left side of the tadpole was aseptically sampled, to avoid cross-contamination and frozen at -20˚C until extraction. DNA from tadpole tissues was extracted using the Wizard SV96 Genomic DNA Purification System (Promega, Southampton, UK). The extracted DNA was then screened for the presence of ranaviral DNA as follows: Primers to amplify a 500bp region of the major capsid protein (MCP) of frog virus 3 (FV3), originally developed by Mao et al. (1996) were used to amplify ranavirus DNA (all were sourced from MWG Eurofins Operon, Ebersberg, Germany). All polymerase chain reaction (PCR) reagents used were Multiplex PCR kits (QIAGEN, Crawley, UK). The thermocycler settings were: 95˚C for 45sec, 52˚C for 45sec, 72˚C for 45sec for

35 cycles, then 4˚C for ∞ as per Pearman et al., (2004). Positive controls and negative extraction controls were used. All samples were screened at least twice to ensure repeatability and accuracy of the results. Any ambiguous screens or screens where positive PCR controls failed were re-screened: ambiguous or failed screens were exceptionally rare. The PCR products were quantified on a 1% agarose gel stained with ethidium bromide. The presence of a band at the 500bp level on the gel was considered diagnostic for the presence of ranaviral DNA and was therefore considered to be positive for infection with the virus.

Statistics

Survivorship was examined using both Log-Rank analysis and Wilcoxon tests. When a significant difference was detected, I used Proportional Hazard Models to determine what the significant contributing factors were (Kleinbaum and Klein 2005; Machin et al. 2006). I used a Log-Rank test because of the multiple group comparison that was necessary (Kleinbaum and Klein 2005; Machin et al. 2006). The Wilcoxon test was also used because of the increased weight on mortality that occurred early on in the trial (Kleinbaum and Klein 2005). I used a proportional hazard models because the hazard was continuous and the model allows the inclusion of categorical variables (Machin et al. 2006). When building the models, I included treatment, dose and isolate. Models were deconstructed to significant variables only. Relationships between infection prevalence and signs of disease were examined with the controls removed to determine the factor that was driving the differences, the direction of the differences was established using a Fisher's Exact Test. All statistics were performed with JMP 8.0 (SAS Institute, North Carolina, USA).

Results

Rana temporaria *Experiment*

I detected a significant difference between the different treatments with respect to survivorship (n = 35/treatment, Log-Rank: $p < 0.0001$, Wilcoxon: $p < 0.0001$, df = 8; Figure 5.1). This was primarily driven by the dose, with individuals exposed to a concentration of virus of 10^6 experiencing significantly higher mortality (Proportional Hazards, n = 315, df = 2, $p < 0.0001$; Figure 5.2).

Further analysis on low dose treatments revealed a significant difference, with RUK isolates experiencing significantly more mortality than BUK isolates (n = 35/treatment, Log-Rank: p = 0.0320, Wilcoxon: p = 0.0835, df = 1; Figure 5.3).

Infection prevalence was not significantly different between treatments (n = 120, df = 7, χ^2 = 12.540, p = 0.0841), nor was it different between isolate (Likelihood Ratio: n = 120, df = 1, χ^2 = 3.249, p = 0.3548). I detected a significant difference in infection prevalence between doses (Likelihood Ratio: n = 120, df = 1, χ^2 = 4.923, p = 0.0265), with individuals exposed to a concentration of 10^6 PFU with significantly more infections than those exposed to 10^4 PFUs (Fisher's Exact Test, n = 120, p = 0.0211; Figure 5.4)

The signs of disease that *R. temporaria* tadpoles developed can be found in Table 5.1. The most common signs of ranaviral disease developed were abdominal haemorrhages with or without abdominal bloat. The presence of signs of disease at death was significantly different between treatments (Likelihood Ratio: n = 276, df = 14, χ^2 = 91.0349, p < 0.0001). I detected a significant difference in the presence of signs between doses (Likelihood Ratio: n = 276, df = 1, χ^2 = 68.559, p < 0.0001), with individuals exposed to a concentration of 10^6 PFU with significantly more infections than those exposed to 10^4 PFUs. There was no difference between the isolates (Likelihood Ratio: n = 276, df = 1, χ^2 = 5.047, p = 0.5378; Figure 5.5).

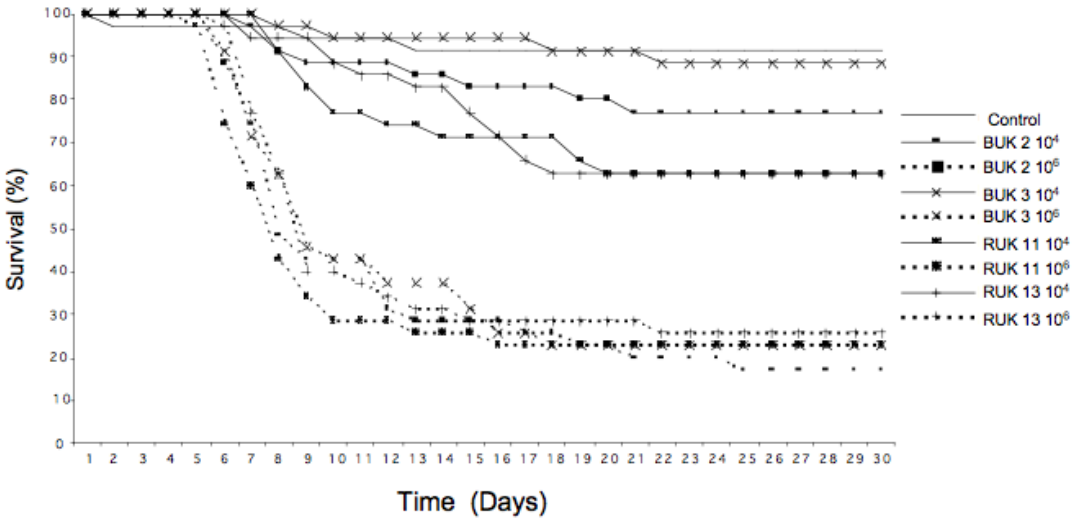

Figure 5.1. Survivorship curves for all treatment groups of *R. temporaria* tadpoles exposed to ranavirus isolates.

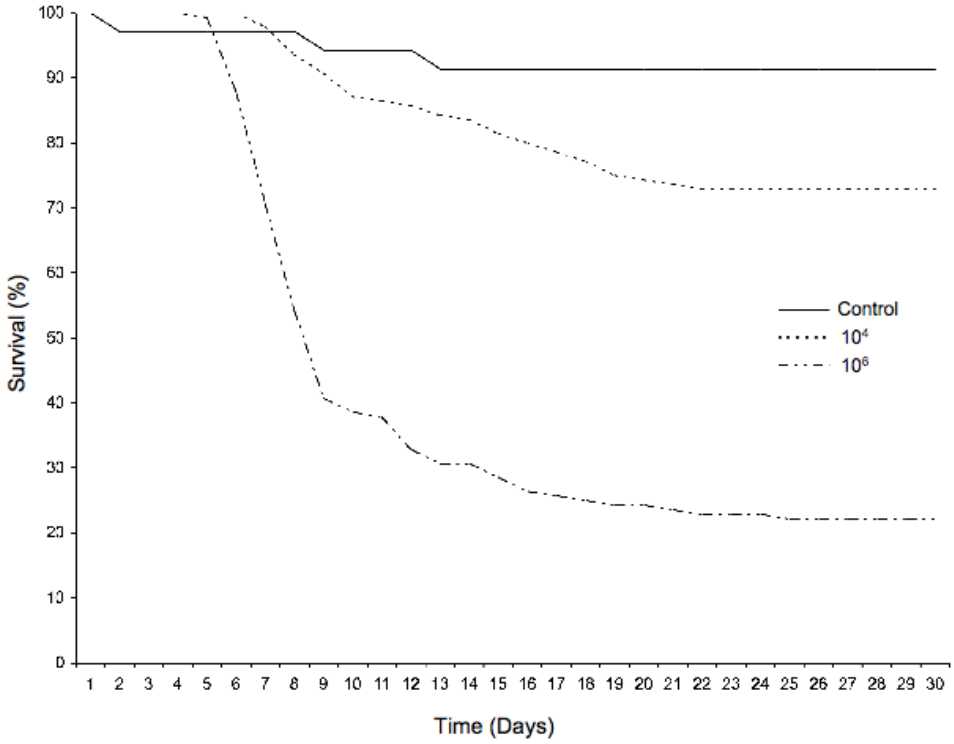

Figure 5.2. Dose dependant mortality in *R. temporaria* tadpoles exposed to ranavirus isolates. Tadpoles in the 10^6 treatment were significantly more likely to die than either the 10^4 or control treatments. Each dose is an average of all strains used.

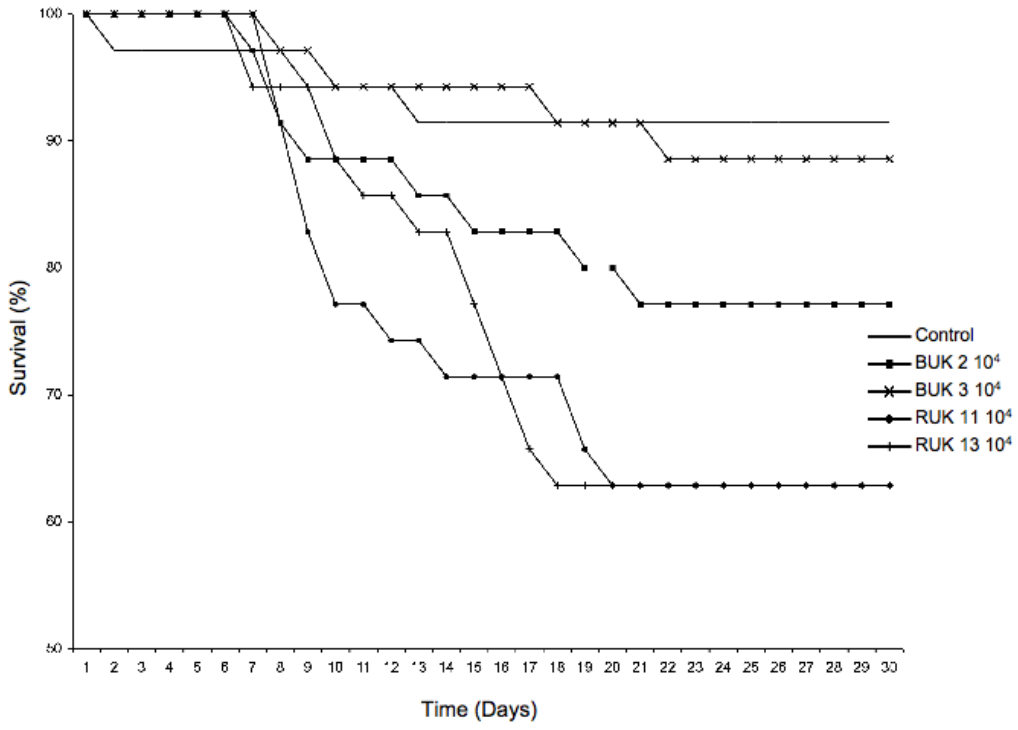

Figure 5.3. Survivorship for *R. temporaria* tadpoles exposed to 10^4 and control treatments. Tadpoles exposed to the BUK isolates are significantly more likely to survive than those exposed to RUK isolates at low viral concentrations. Note: The survival axis is broken and starts at 50%.

Table 5.1. Summary of the signs of disease exhibited by individuals by treatment for *R. temporaria* tadpoles exposed to different ranavirus isolates. The number euthanized is the number of animals that survived until the end of the experiment.

	Control	BUK 2		BUK 3		RUK 11		RUK 13	
		10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6
No Signs	1	0	0	0	0	0	9	1	0
Bloat	1	3	5	1	3	2	1	3	2
Abdominal Haemorrhage	0	1	7	1	9	4	4	2	12
Abdominal Haemorrhage and Bloat	0	2	9	1	13	4	9	3	7
Other Haemorrhage	0	0	2	0	0	0	0	0	1
Other Haemorrhage and Bloat	0	1	2	0	0	1	3	2	2
Other	0	0	1	0	2	2	2	1	1
Humane Euthanasia	1	1	1	1	0	0	0	1	1
Metamorphosed	0	0	0	0	0	0	0	0	0
No. Died	2	27	27	3	27	11	28	13	24
No. Euthanized	32	7	6	29	8	22	7	22	9
Total	35	35	35	35	35	35	35	35	35

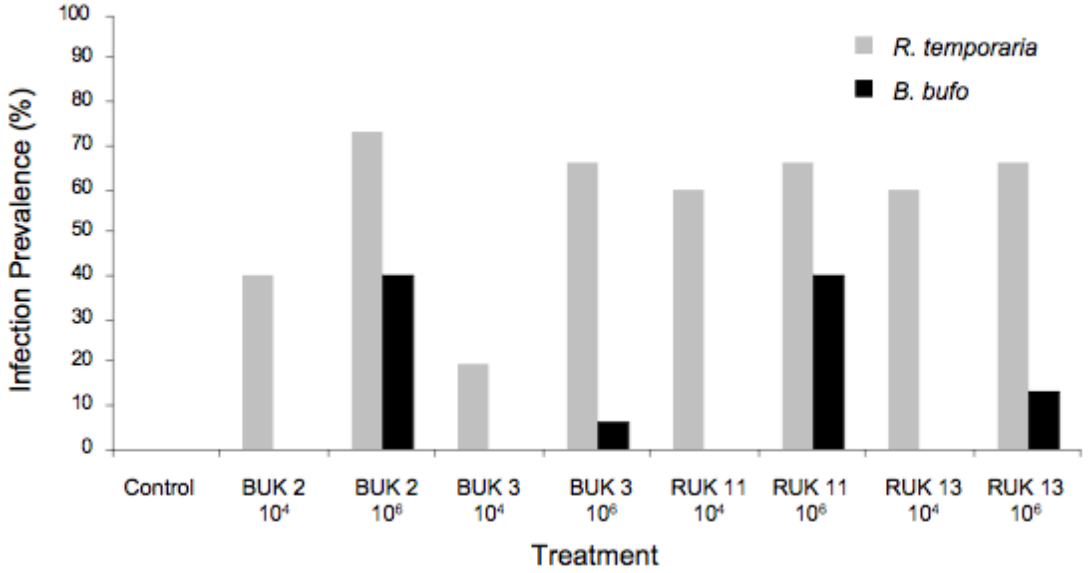

Figure 5.4 Infection prevalence in *R. temporaria* and *B. bufo* tadpoles exposed to different treatments. Differences are primarily due to dose, not isolate, with individuals exposed to the 10^6 PFU dose significantly more likely to have an infection at death than those at the 10^4 PFU dose. Controls show no infections.

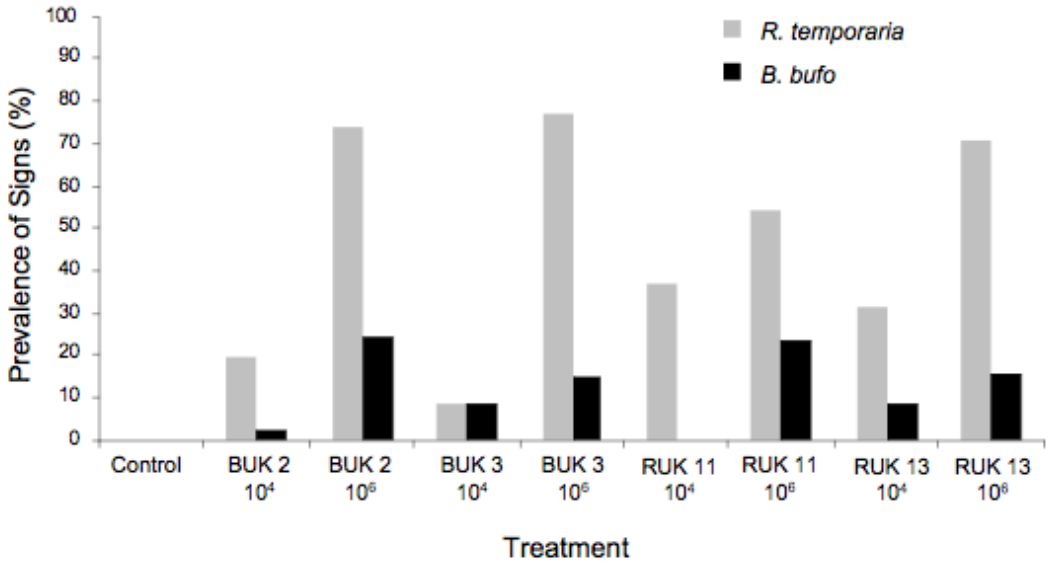

Figure 5.5. Presence of signs of disease in *R. temporaria* and *B. bufo* tadpoles exposed to different treatments. Differences are primarily due to dose, not isolate, with individuals exposed to the 10^6 PFU dose significantly more likely to have signs at death than those at the 10^4 PFU dose. Controls show no signs of disease at death.

Bufo bufo *Experiments*

I detected a significant difference in survivorship in the *B. bufo* exposures (n = 32-35/treatment, Log-Rank $p < 0.0001$, Wilcoxon $p = 0.0001$, df = 8; Figure 5.6). The difference was due to dose (Log-Rank $p < 0.0001$, df = 3; Figure 5.7).

There was a significant difference in the presence of an infection at the time of death between treatments (Likelihood Ratio: n = 120, df = 7, $\chi^2 = 30.381$, $p < 0.0001$). I determined that this difference was due to dose and individuals exposed to a concentration of 10^6 PFU had significantly more infections than those exposed to 10^4 PFUs (Fisher's Exact Test, n = 120, $p < 0.0001$). There was no effect of isolate on the presence of an infection at death (Likelihood Ratio: n = 120, df = 3, $\chi^2 = 6.912$, $p = 0.748$; Figure 5.4)

The signs of disease that *B. bufo* tadpoles developed can be found in Table 5.2. The most common sign of ranaviral disease seen was skin sloughing. I detected a significant difference between treatments with respect to the presence of signs at death (Likelihood Ratio: n = 269, df = 7, $\chi^2 = 23.523$, $p = 0.0014$). This difference was due to dose, with individuals exposed to a concentration of 10^6 PFU with significantly more infections than those exposed to 10^4 PFUs (Fisher's Exact Test, n = 269, $p < 0.0001$). There was no difference between the isolates (Likelihood Ratio: n = 269, df = 3, $\chi^2 = 0.337$, $p = 0.9529$; Figure 5.5)

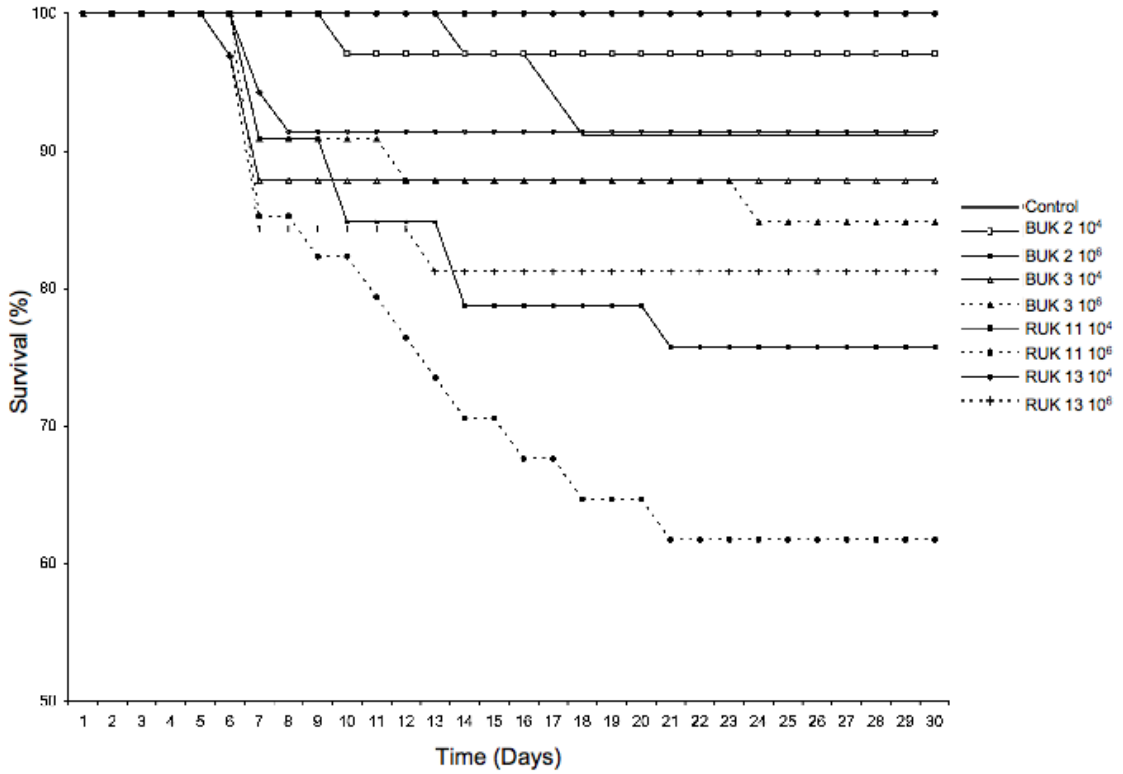

Figure 5.6. Survivorship curves for all treatment groups of *B. bufo* tadpoles exposed to ranavirus isolates. Please note that the y-axis is broken and starts at 50%.

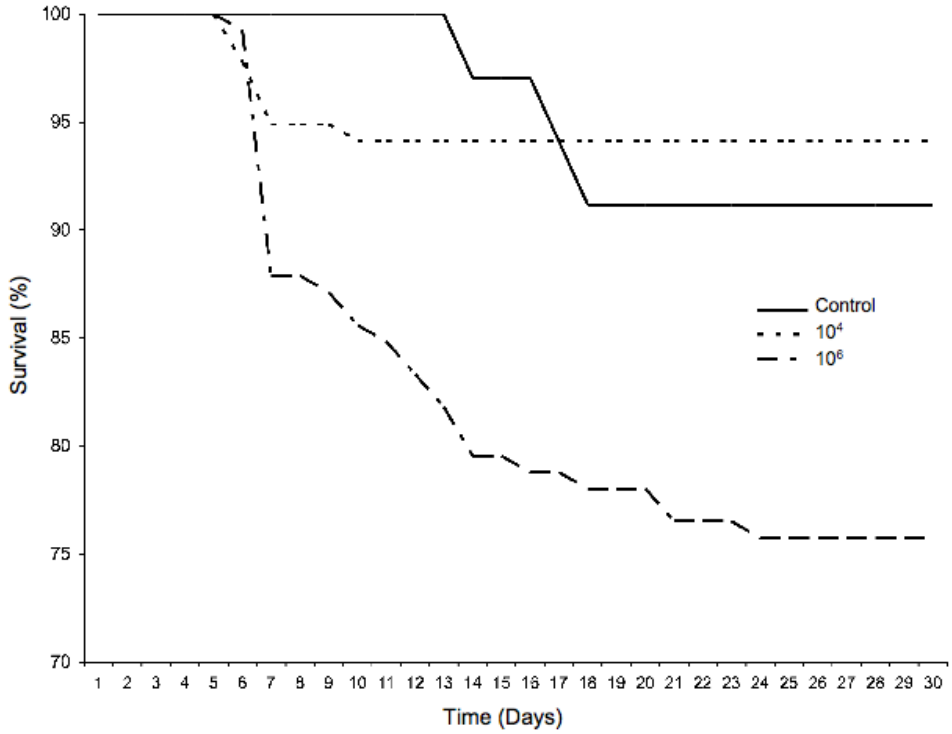

Figure 5.7. Dose dependant mortality in *B. bufo* tadpoles exposed to ranavirus isolates. Tadpoles in the 10^6 treatments showed significantly higher mortality than either the 10^4 PFU or control treatments. Each dose is an average of all strains used.

Table 5.2. The number of *B. bufo* tadpoles per treatment that exhibited signs of disease and the type of signs.

	Control	BUK 2		BUK 3		RUK 11		RUK 13	
		10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6
No Signs	3	0	0	1	0	0	4	1	1
Skin Sloughing	0	1	5	3	3	0	5	1	3
Bloat	0	0	1	0	0	0	3	0	0
Skin Sloughing and Bloat	0	0	2	0	2	0	1	1	2
Metamorphosed	8	15	12	20	16	14	13	22	18
No. Died	3	1	8	4	5	0	13	3	6
No. Euthanized	23	18	13	9	12	21	8	10	8
No Data*	1	1	2	2	2	0	1	0	3
Total	35	35	35	35	35	35	35	35	35

* Individuals with no data are those that were lost during the experiment and no information was collected on disease status or death date as no carcass was recovered.

Discussion

Survivorship

In *R. temporaria* tadpoles exposed to low concentrations of virus, isolate was an important predictor of death. Tadpoles that were exposed to RUK isolates were more likely to die that those that were exposed to the BUK isolates. The RUK isolates were from common frogs and the BUK isolates were from toads (Hyatt et al. 2000; Cunningham et al. 2007a&b). This shows that there is some host-strain association in the ranaviruses present in the UK.

In *B. bufo* tadpoles, survivorship was associated with dose, not isolate. Dose dependent mortality is well documented in ranavirus exposure experiments. For example, higher exposure concentrations of ATV resulted in larger mortality rates in *A. tigrinum* larvae (Brunner et al. 2005). In anurans, *R. latastei* tadpoles exposed to higher concentrations of FV3 not only experienced higher mortality rates, but the onset of mortality occurred more quickly at higher doses (Pearman et al. 2004).

Infection Prevalence

In both experimental exposures, the prevalence of infection in *R. temporaria* and *B. bufo* tadpoles was associated with the dose of ranavirus used and not the

isolate. Dose-dependant infection prevalence has been reported in other anuran species. In *R. sylvatica* tadpoles which were exposed to difference concentrations of FV3, tadpoles in the low concentration treatments did not have as many infections as those in higher doses (Duffus et al. 2008).

Although statistical comparisons between the *R. temporaria* and *B. bufo* experiments would be invalid since the experiments did not occur at the same time or use tadpoles of the same developmental stage, it is interesting to observe the differences in infection rates between them. In *B. bufo* tadpoles exposed to 10^4 PFU of virus, none developed detectible infections, where as infections were much more common at the same concentration in *R. temporaria* tadpoles. In the 10^6 PFU treatments comparatively few *B. bufo* tadpoles developed infections, where as most *R. temporaria* tadpoles developed infections. This maybe due to the difference in age of *R. temporaria* vs *B. bufo* tadpoles, which were Gosner stage 25 vs Gosner stages 29-30 respectively.

Alternatively, the apparent differences in infection rates between the two species could indicate that *R. temporaria* is the primary host of the ranavirus in the UK. The increased virulence in *R. temporaria* could be the result of viral adaptation to its primary host (Woolhouse et al 2001). There is a complex relationship in multi-host pathogens between virulence and transmission dynamics in secondary hosts the outcome of which is dependant on many factors (Woolhouse et al. 2001).

Signs of Disease

The presence of signs of ranaviral disease at death in both experiments was again driven by dose. Brunner et al. (2005) found a similar trend in ambystomatid larvae-ATV exposures. Larvae that were exposed to higher concentrations of ATV had a higher proportion of individuals showing signs of disease than those at lower concentrations (Brunner et al. 2005). Therefore, the observed patterns of signs of disease in my experiments are not unexpected.

R. temporaria tadpoles showed a variety of different signs of disease (see Table 5.1) however the most common signs were abdominal haemorrhage with or without bloating. This occurred across all isolates and concentrations. The consistency of signs seen in one species was not unexpected, especially the presence of abdominal haemorrhages. Abdominal haemorrhages have been observed in association with ranavirus outbreaks in wild *R. sylvatica* tadpoles (Greer et al. 2005)

and in experimental exposures of *R. sylvatica* (Duffus et al. 2008) and *R. latastei* (Pearman et al. 2004). It is important to contrast this to the signs that are seen in adult *R. temporaria* where different isolates are associated with different disease syndromes (Cunningham et al. 2007a&b). In my experiments, I used the same isolates as Cunningham et al. (2007 a&b), however, the isolates had been passaged in cell culture several more times which may have resulted in the elimination of competing virus 'strains' from the same infections

The *B. bufo* tadpoles also exhibited homogeneity in the signs of disease (Table 5.2), with the most common sign being the sloughing of skin. Since the examinations of signs of disease were superficial, it is possible that due to the dark pigmentation of the tadpoles, deeper signs such as abdominal haemorrhages could have been missed. Different signs of disease developed in each species, which is probably not due to the differences in age between *R. temporaria* and *B. bufo* tadpoles, but more to with the difference between primary and secondary hosts, as previously proposed.

Conclusions

The combination of isolate specific mortality in *R. temporaria* tadpoles at low doses and a lack of association between isolate and mortality in *B. bufo* suggests that *R. temporaria* is the primary host for the ranavirus in the UK. This is the expectation when a multi-host pathogen is more virulent in its primary host than alternate hosts (Woolhouse et al. 2001). Importantly, the amount of ranavirus that an individual would be exposed to in the wild would be much lower than the concentrations seen in this experiment. Hence the isolate-specific virulence (which was observed at the lower concentrations) may be even more important for wild populations.

There does appear to be a host-specific response to infection with different signs of disease being displayed in different species. The degree to which this is associated with viral host-specificity is unknown. In the wild, adult *B. bufo* are only known to suffer from the haemorrhagic form of ranaviral disease where as *R. temporaria* adults suffer from both haemorrhagic and ulcerative forms (Cunningham et al. 2007 a & b). Therefore, it appears that the signs of disease are likely to be host-specific rather than virus-isolates specific or environmental.

Importantly, the presence of infections in *R. temporaria* tadpoles which have been exposed to ranavirus isolates from the UK demonstrates that they are susceptible to infection. In Chapter 2 only one ranavirus infection was found in 'wild' *R. temporaria* tadpoles, which was unexpected since other anuran tadpoles are known to be infected with the ranavirus in the wild (e.g. Duffus et al. 2008). This enforces the conclusion that the dynamics of the ranavirus in *R. temporaria* is primarily limited to adults, since no significant infections are found in tadpoles, which are clearly susceptible to ranavirus infections.

CHAPTER 6: CONCLUDING REMARKS

In this thesis, I have attempted to adhere to a hypothesis based approach to understanding an emerging infectious agent, a method which is seldom applied by researchers who study amphibian infections and diseases. Despite the presence of a long term epidemiological data set that spans nearly twenty years, little effort had been expended to understand the basic biology of the ranavirus(es) responsible. At first, research was descriptive and provided important insight into ranaviral disease and mass mortality events. These descriptions remain the most complete of ranaviral disease and mass mortality events in any amphibian species. However, the research expanded into a series of poorly designed experiments that examined ranaviral transmission and disease. The effect of ranavirus emergence on common frog populations has been rigorously and informatively performed (e.g. Teacher 2009, Teacher et al. 2009a&b), however, the biology of the pathogen had been all but forgotten. To adequately understand the potential impact that a pathogen may have on a host population, we need to understand the biology of the pathogen.

In an attempt to rectify the lack of information about the biology of the ranavirus(es) that are present in the UK, I went back to basic ecological and experimental methodologies to investigate the ranavirus present in UK common frogs. As in any scientific investigation, I identified more questions that require answers before a full understanding of the ecology of the ranavirus present in the UK can be obtained. We need to understand the biology of the ranavirus, especially in common frogs, which are declining in some areas due to the emergence of the ranavirus (Teacher 2009), so we can make good conservation and management decisions.

Ranavirus Ecology and Transmission Dynamics in Common Frogs

In Chapters 2 and 3, I investigated the ecology and transmission dynamics of the ranavirus present in common frogs (*Rana temporaria*) in the 'wild' in the UK. Evidence from North America suggested that the larval stages of amphibians were the most affected by ranavirus infections (e.g. Brunner et al. 2004; Greer et al. 2005; Duffus et al. 2008). I expected to find comparable ranavirus infection rates in common frog tadpoles, however, only one of the 288 tadpoles screened positively for the presence of ranavirus DNA. This led me to test the two models of virus transmission and maintenance from North America (Brunner et al. 2004 and Duffus

et al. 2008). I didn't detect any infections in the common frog eggs (n = 740), which was unexpected because there is evidence of vertical/pseudovertical transmission in North American ranids (e.g. *R. sylvatica*; Duffus et al. 2008). With the vast majority of infections present only in adult common frogs, the North American models could not be used to explain the transmission and maintenance of the ranavirus in the UK.

The infection rate of 1 in 20 for one of the sites is comparatively low for ranaviruses (e.g. >90% in some cases). The ranavirus in UK common frogs is unique as the life history stage that appears to be paramount in the maintenance and transmission of the virus are the adults. (In both North American models, the larval stages are key.) This finding lead to the more theoretical question of 'Can the ranavirus be maintained when only adult-to-adult transmission occurs?' Using mathematical models, I explore the conditions under which the ranavirus could persist assuming only adult-to-adult horizontal transmission of the virus. The model predicted not only that the virus could be maintained in this manner, but also population declines similar to those described by Teacher (2009) in populations of common frogs where the ranavirus had emerged.

I also used models to account for the presence of the two different disease syndromes seen in common frogs, which are not necessarily mutually exclusive (e.g. Cunningham et al. 1996; Cunningham et al. 2007a&b). I assumed that each disease syndrome was caused by a different strain of ranavirus. Since the models developed were complex, I chose to focus on the basic reproductive rates (R_o) for each strain. The models indicated that the ulcerative from of the disease needed to be present for the establishment of the haemorrhagic form to take hold. However, when more individuals had the ulcerative form when the haemorrhagic form was introduced, the model showed that the haemorrhagic form would need to be more virulent to be maintained, indicating competition for hosts between the two forms. This finding was unexpected since in many cases, diseased common frogs show signs of both forms of ranaviral disease. Because of the presence of open wounds associated with the ulcerative syndrome, I thought that it would be easier for the haemorrhagic form to infiltrate the population (based on data presented by Cunningham et al. 2007a).

Another possible explanation of the presence of two disease syndromes in common frog populations maybe due to an infection-re-infection scenario. This situation would also fit the developed models. When the common frog develops the

ulcerative form of the disease, the open wounds permit a route of entry for the virions into the deeper tissues. In this way, the frog actually could re-infect itself, if we assume that the initial infection that caused the ulcerative form of disease is strictly limited to the epithelium and dermal layers. The erosion of the first layer of defence permits the virus to become a systemic infection and the haemorrhagic form of ranaviral disease develops. This is a plausible situation because some animals show evidence of healed ulcerations and present the haemorrhagic form of ranaviral disease.

With adult common frogs being the primary life history stage affected by ranaviral disease, it may be possible to used the model to predict scenarios where the pathogen can be successfully eliminated from the population. However, before this is possible, further investigations into both the biology of the ranavirus and common frog are required. For example, better estimates of contact rates between individuals are needed to model transmission accurately. Additionally, the likelihood of transmission per contact needs to be established. Investigations used established methodologies would provide the necessary information for better models to be built. When better estimates of the parameters of the model are obtained, then the models have the potential to be useful tools for conservation and management. An example of this is to determine if a cull of adult frogs at a ranavirus infected site would permit the eradication of the disease and the number of times that this would have to be done.

Host-Specificity and Virulence of UK Ranavirus Isolates

In Chapters 4 and 5, I investigated potential host-strain associations of UK ranavirus isolates. Ranaviruses can be considered in many cases to be infections of amphibian communities (Duffus et al. 2008) and the presence of naturally diseased common frogs and common toads (*Bufo bufo*) (Cunningham et al. 2007a&b) suggested that the same might be true for ranaviruses in the UK. The presence of multiple susceptible/infected species in an amphibian community could have large effects on the transmission dynamics and maintenance of ranavirus infections in the common frogs present.

I found ranavirus infections in two species of amphibians which were previously unknown to harbour the pathogen, the common newt (*Lissotriton vulgaris*) and the common midwife toad (*Alytes obstetricans*). The discovery of

ranavirus infections in common newts is important because they are often sympatric with common frogs. In a pond where the common frogs had been affected by ranavirus associated mortality for over a decade, the newt population was rather large and did not show signs of ranaviral disease, while the frogs did. I wish that I had had the forethought to sample the newts in this pond, because if the newts were in fact infected they could have acted as a reservoir of the virus for the common frogs.

Phylogenetic analysis of the virus isolates showed that there was little divergence in either of the two loci that were examined. High homology of the major capsid protein region used was expected, however, for ORF 57r, it was not. ORF57r has previously been used to show strong local selection in the *Ambystom tigrinum* virus present in the western USA (Ridenhour and Storfer 2008). There are several plausible reasons for the high homology seen in the UK ranavirus isolates. Firstly, directional selection may be responsible. Certain MHC II haplotypes have been selected in populations of common frogs where the ranavirus has emerged (Teacher et al. 2009a) and this could be the viral response. Or the topography of the trees made from the ranavirus isolates could be due to a bottleneck. This seems like the most likely case for the ranaviruses in the UK. Since the pathogen began to emerge in and spread from the south east of England, it is likely that the ranavirus was only introduced a few times and that the current 'strains' are the ones that have successfully become established. However, since the two loci examined make up less than 1% of the complete ranavirus genome, more information is needed before conclusions of this nature have been made. Newly available sequencing technology will make rapid sequencing of the ranavirus genome financially viable and permit for the examination of the whole genome which will eliminate the hit-and-miss nature of single loci sequencing and hopefully allow for finer scale patterns of divergence to be observed.

When comparing the differences between ranavirus isolates from different amphibian species, this high homology is also seen. Based on the fact that nearly all ranavirus associated mortalities have been reported in adult common frogs, it is a safe assumption that they are the primary host of the infection in the UK. If the ranavirus-associated mortality from which the common midwife toad was obtained is examined more closely, we find that the majority of the animals affected were common frogs. This combined with the high homology of all the isolates suggests

that infections in other species could be the result of pathogen spill over from common frogs. However, experimental evidence that examines the direction of transmission would be needed to confirm this. In any case, further experimental investigations into the susceptibility of other amphibian species in the UK is needed to understand the dynamics of the ranavirus.

In an attempt to gain a preliminary understanding of host-strain associations and virulence in UK ranavirus isolates, I performed a series of experiments. These are the first statistically sound experiments which examine the relationship between, host, isolate and virulence for amphibian ranaviruses. Using four different ranavirus isolates from naturally infected amphibians (two from common frogs and two from common toads) at two different concentrations, I looked at these relationships in both common frog and common toad tadpoles. I found that in common frogs, when exposed to low doses, host-specific virulence occurred. The isolates originally from common frogs had higher mortality rates than those from common toads in the common frog tadpoles. This relationship eroded at the high dose and was not seen in the common toad tadpole exposures, where virulence was does dependant. This is an interesting result because other investigations have suggested that ranaviruses are more virulent in the host from which the isolate was derived (Schock et al. 2008).

The isolate dependant mortality at low doses in common frogs is important, since in the wild, animals would be exposed to low concentrations of a ranavirus. If the infection is primarily spread between adults during the breeding season ranavirus infections are more likely to become established and persist. The lack of host-specificity in common toad tadpoles exposed to the same viral isolates as common frog tadpoles, in combination with the number of infections seen in adult common frogs, further supporting the conclusion that common frogs are the primary host of ranaviruses in the UK.

General Conclusions and Future Directions

Ranavirus ecology and evolution will continue to be an important area of research in the face of global amphibian declines, even if it is overshadowed by other pathogens which seem to provide a one-size-fits-all explanation of disease associated mortality and decline. Ranaviruses can and do play important roles in host population dynamics (e.g. Teacher 2009). They will also continue to cause disease and

mortality in areas of the world where the general public will be able to observe the results in their own back yards, leading, in all likelihood to the desire for a solution.

Despite the visible role ranaviruses may play, they also provide an interesting system to explore host-pathogen interactions. There is a large breadth of questions, both applied and theoretical, which can be explored experimentally using amphibians and ranaviruses. Not only can host-strain interactions be examined, but if developed into a model laboratory system, long term investigations into host-pathogen dynamics can be performed and questions about evolutionary assumptions can be broached. This system has a lot of potential and truly deserves a greater amount of attention by researchers in many areas of biology than it currently receives.

There is so much left to explore and investigate with respect to ranavirus emergence in the UK. The epidemiology of ranaviruses in the UK remains relatively unexplored. Currently, the distribution of the virus in England has not been totally traced, nor is the total number of amphibian species that suffer from natural ranavirus infections. Little to no effort has been put into examining amphibians for the presence of the ranavirus in either Scotland or Wales. Basic pathogenesis also needs to be explored and disease development in different species of amphibians should be compared.

From this theses, the following general conclusions can be made about the ranavirus present in the UK: the primary host of the virus are common frogs; the virus is most likely maintained in populations of common frogs by adult to adult transmission during the breeding season; and infections in other amphibian species are probably the result of disease spill over from diseased common frogs. However, it is important to note that more research is necessary into the potential roles of other amphibian species in the dynamics of the ranavirus. This thesis is just a starting point for examining the biology of ranaviruses in the UK, there is still much exciting work left to be done.

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APPENDIX A – PEG PURIFICATION PROTOCOL

PEG Precipitation of PCR products

Assumes a 50µL PCR reaction volume, for 25µL reactions all volumes are halved.

1 – Add 50µL PEG solution to the PCR product. Mix via pipetting up and down.
2 – Incubate at 37°C for 15min.
Place a bottle of 80% EtOH in the fridge to keep it cool.
3 – Centrifuge at high speed for 15 min. at room temp.
4 – Remove the supernatant and discard.
5 – Add 125µL of 80% EtOH
6 – Centrifuge at max for 2min.
7 – Remove the supernatant and discard.
8 – Dry off the pellet by centrifuging for 10min. Make sure there is no trace of EtOH.
9 – Dissolve the pellet in 25µL water. Pipette up and down,.
10 – Run 2-4µL out on a 1.5% agarose gel to make sure that it worked.

20% PEG Solution

10.0 g Polyethylene glycol 6000MW
7.3 g NaCl
45mL ddH_2O
Shake and let the components go into solution. After, top volume up to 50mL.

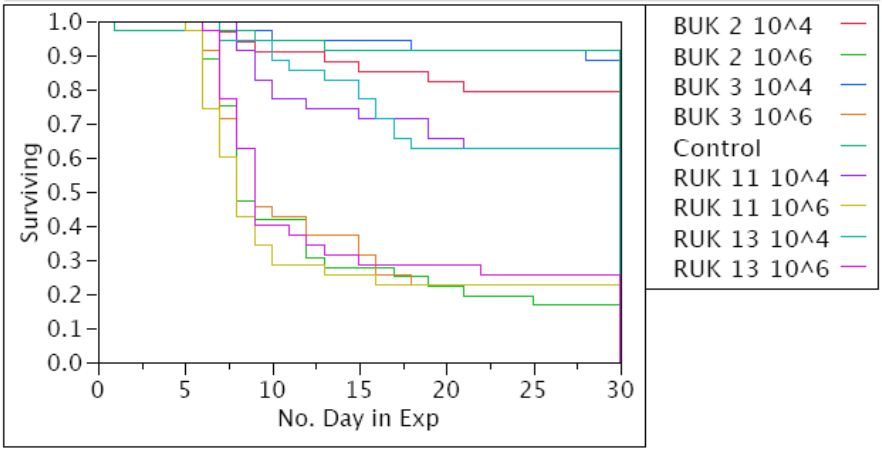
Protocol from:

The Santos Lab, Department of Biological Sciences, Auburn University, Auburn, Alabama, United States of America.
http://www.auburn.edu/~santosr/protocols/PEGTAEProtocol.pdf

APPENDIX B – SAMPLE OF SURVIVAL ANALYSIS

Product-Limit Survival Fit

Survival Plot



Time to event: No. Day in Exp
Grouped by Treatment

Summary

Group	Number failed	Number censored	Mean	Std Error
BUK 2 10^4	34	0	26.5294	1.25262
BUK 2 10^6	36	0	13.3333	1.45078
BUK 3 10^4	35	0	28.4	0.88991
BUK 3 10^6	35	0	14.2571	1.55803
Control	35	0	28.0857	1.10109
RUK 11 10^4	35	0	23.3429	1.55997
RUK 11 10^6	35	0	12.9143	1.63284
RUK 13 10^4	35	0	23.7714	1.44192
RUK 13 10^6	35	0	14.5714	1.62919
Combined	315	0	20.5365	0.58575

Quantiles

Group	Median Time	Lower 95%	Upper 95%	25% Failures	75% Failures
BUK 2 10^4	30	.	.	30	30
BUK 2 10^6	8	8	9	7.5	18
BUK 3 10^4	30	.	.	30	30
BUK 3 10^6	9	8	12	7	18
Control	30	.	.	30	30
RUK 11 10^4	30	19	21	12	30
RUK 11 10^6	8	7	8	6	16
RUK 13 10^4	30	17	18	16	30
RUK 13 10^6	9	8	11	8	30
Combined	30	18	28	9	30

Tests Between Groups

RANA modified: Survival of No. Day in Exp Page 2 of 2

Product-Limit Survival Fit

Tests Between Groups

Test	ChiSquare	DF	Prob>ChiSq
Log-Rank	131.1671	8	<.0001*
Wilcoxon	131.5818	8	<.0001*

APPENDIX C – SAMPLE OF PROPORTIONAL HAZARDS ANALYSIS

RANA modified: Fit Proportional Hazards Page 1 of 1

Proportional Hazards Fit

Whole Model

Number of Events	315
Number of Censorings	0
Total Number	315

Model	-LogLikelihood	ChiSquare	DF	Prob>Chisq
Difference	28.152	56.3046	2	<.0001*
Full	1639.182			
Reduced	1667.334			

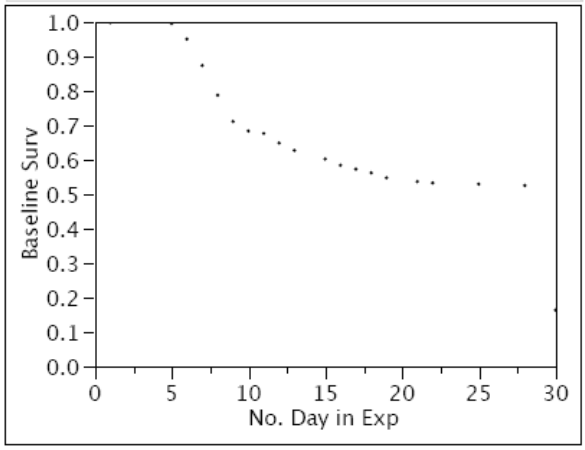
Parameter Estimates

Term	Estimate	Std Error	Lower CL	Upper CL
Dose[10^4]	-0.2337122	0.08478	-0.398694	-0.065665
Dose[10^6]	0.62560792	0.0864096	0.4573419	0.7967256

Effect Likelihood Ratio Tests

Source	Nparm	DF	L-R ChiSquare	Prob>ChiSq
Dose	2	2	56.3046492	<.0001*

Baseline Survival at mean



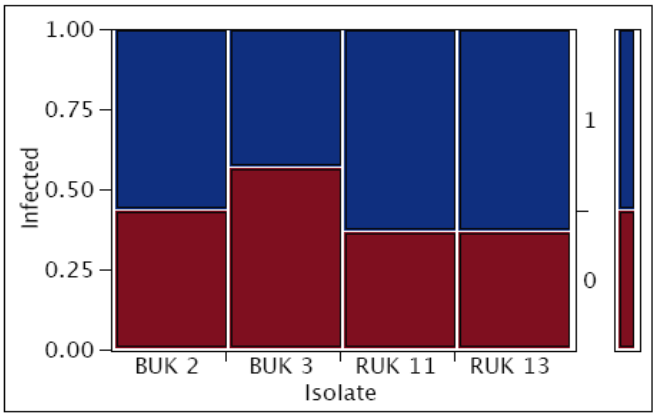
APPENDIX D – SAMPLE OF CONTINGENCY ANALYSIS

subset: Contingency of Infected Page 1 of 3

Fit Y by X Group

Contingency Analysis of Infected By Isolate

Mosaic Plot



Contingency Table

Isolate	Count / Total % / Col % / Row %	Infected 0	Infected 1	
BUK 2		13 10.83 25.00 43.33	17 14.17 25.00 56.67	30 25.00
BUK 3		17 14.17 32.69 56.67	13 10.83 19.12 43.33	30 25.00
RUK 11		11 9.17 21.15 36.67	19 15.83 27.94 63.33	30 25.00
RUK 13		11 9.17 21.15 36.67	19 15.83 27.94 63.33	30 25.00
		52 43.33	68 56.67	120

Tests

subset: Contingency of Infected Page 2 of 3

Fit Y by X Group

Contingency Analysis of Infected By Isolate

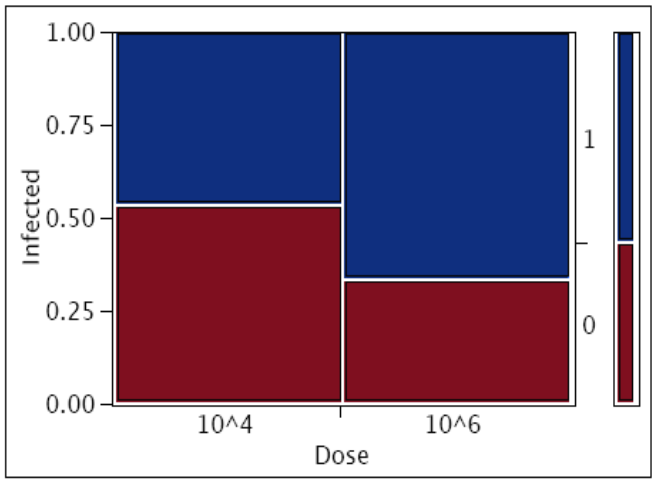
Tests

N	DF	-LogLike	RSquare (U)
120	3	1.6244402	0.0198

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	3.249	0.3548
Pearson	3.258	0.3535

Contingency Analysis of Infected By Dose

Mosaic Plot



Contingency Table

Dose	Infected	0	1	
	Count	0	1	
	Total %			
	Col %			
	Row %			
	10^4	32	28	60
		26.67	23.33	50.00
		61.54	41.18	
		53.33	46.67	
	10^6	20	40	60
		16.67	33.33	50.00
		38.46	58.82	
		33.33	66.67	
		52	68	120
		43.33	56.67	

Tests

subset: Contingency of Infected Page 3 of 3

Fit Y by X Group

Contingency Analysis of Infected By Dose

Tests

N	DF	-LogLike	RSquare (U)
120	1	2.4615632	0.0300

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	4.923	0.0265*
Pearson	4.887	0.0271*

Fisher's Exact Test	Prob	Alternative Hypothesis
Left	0.9919	Prob(Infected=1) is greater for Dose=10^4 than 10^6
Right	0.0211*	Prob(Infected=1) is greater for Dose=10^6 than 10^4
2-Tail	0.0422*	Prob(Infected=1) is different across Dose

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PhD Candidate,
Wildlife Epidemiology, Institute of Zoology, Zoological Society of London
and
School of Biological and Chemical Sciences, Queen Mary University of London

Nationality: Canadian **Date of Birth**: December 5, 1981

Academic Background

2006-Present	PhD Candidate, Wildlife Epidemiology, Institute of Zoology, Zoological Society of London and School of Biological and Chemical Sciences, Queen Mary University of London. Supervisors: Dr. Trent Garner and Prof. Richard Nichols
2006	Instructor of Herpetology, Department of Biology, Trent University, Peterborough, ON, Canada.
2004-2006	Master of Science (MSc.), Watershed Ecosystems Graduate Program, Trent University, Peterborough, ON, Canada Supervisor: Prof. Michael Berrill
2000-2004	BSc.H. (Subject of Specialization, Biology), Queen's University, Kingston, ON, Canada.

Research Interests

The ecology, evolution, transmission dynamics and host-pathogen interactions, specifically of the amphibian-ranavirus system. I am especially interested in the community level interactions of viral dynamics and the effects of infection on the fitness of the host, including the resultant red queen effect. My research has focused on the ecology, transmission dynamics and host-specificity of ranaviruses and their amphibian hosts in North America and the UK. Uniquely, I use a hypothesis based approached that combines ecological and experimental data to develop mathematical models of host-pathogen interactions in this system.

Honours, Awards and Grants

Current	**Queen Mary, University of London Research Studentship** (≈ £ 14,500 *per annum* plus tuition remainder, Date of first award: October 2006, International Award) **Overseas Research Studentship**, Queen Mary, University of London (£10,000 *per annum*, Date of first award: October 2006, International Competition) **Natural Science and Engineering Council of Canada (NSERC) 3 Year Doctoral Award** ($21,000 *per annum*, Date of first award: January 2008, National Canadian Competition)
2008	Convocation Research Trust, University of London (£4,800)
2006 - 2007	Amphibian Conservation Research Trust Student Research Grant (£2,000) British Wildlife Health Association Grant (£500) British Ecological Society (BES) Travel Grant for BES Student Members (£300) Prize for the Best Student Oral Presentation, Ecology and Management of Wildlife Diseases, York, England (€400)
2005-2006	**Natural Science and Engineering Council of Canada (NSERC) Postgraduate Scholarship** (PGS-M) ($17,500 CDN) (National Competition) The Richard Ivey Memorial Scholarship in Forest Biodiversity ($7,500 CDN) Teaching Assistantship, Trent University ($3,000 CND)
2004-2005	Research Assistantship, Trent University, ($5,200 CND) Teaching Assistantship, Trent University ($3,000 CND)
Prior to 2004	**Dean's List**, Queen's University (minimum 80% academic average) held **2002-2004** Ontario Scholar (80% or above academic average) held 1997-2000 Lieutenant Governor General of Ontario's Award for Community Volunteerism (2000) Principal's Award of Distinction (Academic, Athletic and Volunteering Excellence) (2000)

Teaching Experience

2008 and 2009	Problem Based Learning Facilitator for the Wildlife Animal Health/Biology MSc. Program, Institute of Zoology/Royal Veterinary College, University of London
2007 - 2008	Demonstrator and Marker for Foundations Courses, School of Biological and Chemical Sciences, Queen Mary, University of London
2006 - 2007	Demonstrator and Marker for Fundamentals of Ecology and Evolution, Foundations Course, School of Biological and Chemical Sciences, Queen Mary, University of London
	Graduate Teaching Assistant, University College London, University of London
May – June 2006	Instructor (Herpetology), Department of Biology, Trent University, Peterborough, Ontario, Canada
2004 - 2006	Graduate Teaching Assistant, Department of Biology, Trent University, Peterborough, Ontario, Canada

Additional Research Positions and Relevant Experience

2009	Supervision of an MSc. Project for the Wild Animal Health/Biology Program at the Institute of Zoology/Royal Veterinary College, University of London. Fontdecaba i Baig, Àngels, 2009. Examination of interspecies transmission of a ranavirus between two sympatric species. WAH MSc. Thesis, Royal Veterinary College, University of London and Institute of Zoology, Zoological Society of London. (She won the prize for the best WAH project.)
2008	Co-Supervision of an MSc. Project with Drs. T. Garner and K. Acevedo-Whitehouse for the Wild Animal Health/Biology Program at the Institute of Zoology/Royal Veterinary College, University of London.
2006 – 2009	Aid in the training of various students, technicians, and postgraduate/doctoral researchers in the care of amphibians, amphibian post mortem examinations, histology and various molecular methodologies.
	Responsible for the set up (2006/07) of a Cell and Virus Culture facility at the Institute of Zoology, Zoological Society of London and currently responsible for its day to day running and requirements.
Summer 2004	Research Assistant for Prof. M. Berrill, Department of Biology, Trent University, Peterborough, Ontario, Canada.
Summer 2003	Research Assistant for Dr. C. Eckert and Dr. K. Samis, Department of Biology, Queen's University, Kingston, Ontario, Canada.
2002 - 2003	Aquatic Ecology Laboratory Assistant (Voluntary) for Dr. S. Arnott, Department of Biology, Queen's University, Kingston, Ontario, Canada.

Selected Volunteer Activities and Committee Memberships

2006 - Current	UK Cetacean Strandings Project – Assists Dr. P. Jepson and R. Deaville with post mortem examinations of cetaceans and sea turtles.
2006 - 2007	London Evolutionary Research Network (LERN) Representative for the Institute of Zoology, Zoological Society of London, London, England.
2005 – 2006	Graduate Student Representative on the Special Appeals Committee, Trent University, Peterborough, Ontario, Canada.
2002- 2004	Departmental Student Council Representative, Department of Biology, Queen's University, Kingston, Ontario, Canada.

Contributions

Articles in Refereed Journals

Duffus, A.L.J. In Press. The Chytrid Blinders: What Other Disease Risks to Amphibians are we Missing? EcoHealth. Submission No. ECH-09-0072.R1

Acevedo-Whitehouse, K. and **A.L.J. Duffus**. 2009. Effects of Environmental Change on Wildlife Health. Philosophical Transactions of the Royal Society B: Biological Sciences. 364:3429-3438; doi:10.1098/rstb.2009.0128

Duffus, A.L.J. and A.A. Cunningham. In Review. Disease Threats to Amphibians in Europe. Herpetological Journal (Invited Review)

Duffus, A.L.J., Nichols, R.A., and T.W.J. Garner. In Review. Intraspecies Ranavirus Dynamics in UK Common Frogs (*Rana temporaria*). Journal of Wildlife Disease. (Submission No. 2009-10-245)

Fernández Loras, A., Hidalgo-Vila, J., Hermosilla, C., García, G., López, J., **Duffus, A. L. J.**, Cunningham, A. A., and V. Roca. Preliminary health screening and possible pathogen determination in a *Bufo bufo* population. Journal of Natural History. Submission No. TNAH-2009-0231

González-Hernádez, M., Denoël, M., **Duffus, A.L.J.,** Garner, T.W.J., Cunningham, A.A., and K. Acevedo-Whitehouse. Submitted. Dermocystid infections and associated skin lesions in free living palmate newts (*Lissotrition helveticus*) from Southern France. Parasitology International.

Duffus, A.L.J., Pauli, B.D., Wozney, K., Brunetti, C.R., and M. Berrill. 2008. FV3-like Infections in Aquatic Amphibian Communities. Journal of Wildlife Diseases, 44(1): 109-120.

Duffus, A.L.J. and D.H. Ireland. 2006. *Rana sylvatica* (Wood Frog) Terrestrially Amplexing Pairs. Herpetological Review, 37(4):449.

Duffus, A.L.J. In Press. *Pseudacris triseriata* (Western Chorus Frog) Egg Predation by Fairy Shrimp (Anostraca)? Herpetological Review. (Submission No. AN07-41)

Theses

Duffus, A.L.J. 2006. Field Monitoring, Transmission and Influences of Immunosuppression on Ranaviral Infections in Native North American Amphibian species. MSc. Thesis, Watershed Ecosystems Graduate Program, Trent University, Peterborough, ON

Duffus, A.L.J. 2004. A Comparison of Resting Egg Densities and Hatching Success in Acidified and Reference Lakes. BSc.H. Thesis, Department of Biology, Queen's University, Kingston, ON, Canada.

Conference Presentations

Duffus, A.L.J. and K. Acevedo-Whitehouse. 2009. Effects of Environmental Change on Wildlife Health. Impacts of Environmental Change on Reproduction and Development in Wildlife. Zoological Society of London, London, England. October 15th and 16th.

Duffus, A.L.J., Nichols, R.A., Cunningham, A.A., Garner, T.W.J. and M. Berrill. 2007. Ranavirus Ecology: A Canadian Example for the UK? Ecology and Management of Wildlife Diseases, York, England, November 12-16, 2007. **(Won Prize for Best Student Oral Presentation)**

Duffus, A.L.J., Brunetti, C.R., Pauli, B.D., and M. Berrill. 2005. Interactive Effects of Malathion Exposure and Ranaviral Infection in Wood Frogs (*Rana sylvatica*). Canadian Amphibian and Reptile Conservation Network Annual Meeting, Ottawa, Ontario, Canada, September 12-15, 2005.

Selected Symposia Presentations, Seminars, Invited Talks and Lectures

Duffus, A. 2009. Disease and Extinction: The Case of the Ranaviruses. Parasites and Infectious Diseases, School of Biological and Chemical Sciences, Queen Mary, University of London.

Duffus, A. 2009**.** Ranavirus Ecology: Investigating Disease and Making A Splash, Edexcel Biology Program, Education Department, Zoological Society of London.

Duffus, A. 2008. Ranavirus Ecology: Different Dynamics in Different Systems. Department of Ecology and Evolutionary Biology, University of Glasgow.

Duffus, A. 2008. Disease and Declines in Amphibians. Department of Ecology and Evolutionary Biology, University of Glasgow.

Duffus, A. 2008. Ranaviruses: An Emerging Infectious Disease in UK Amphibians. Zoology Graduate Student Symposium, Department of Zoology, University of Cambridge, Cambridge England.

Duffus, A. 2007. Amphibian Declines and Emerging Disease: The Ranavirus as an Agent of Extinction, Evolution Seminar Series, Department of Biology, University College London, University of London

Duffus, A. 2007. Ranavirus Ecology in the UK: Why is it so Different? Research Seminar Series,

Institute of Zoology, Zoological Society of London.

Selected Institutional Conference Presentations

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Review

Effects of environmental change on wildlife health

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Environmental change has negatively affected most biological systems on our planet and is becoming of increasing concern for the well-being and survival of many species. At an organism level, effects encompass not only endocrine disruptions, sex-ratio changes and decreased reproductive parameters, but also include teratogenic and genotoxic effects, immunosuppression and other immune-system impairments that can lead directly to disease or increase the risk of acquiring disease. Living organisms will strive to maintain health by recognizing and resolving abnormal situations, such as the presence of invading microorganisms or harmful peptides, abnormal cell replication and deleterious mutations. However, fast-paced environmental changes may pose additional pressure on immunocompetence and health maintenance, which may seriously impact population viability and persistence. Here, we outline the importance of a functional immune system for survival and examine the effects that exposure to a rapidly changing environment might exert on immunocompetence. We then address the various levels at which anthropogenic environmental change might affect wildlife health and identify potential deficits in reproductive parameters that might arise owing to new immune challenges in the context of a rapidly changing environment. Throughout the paper, a series of examples and case studies are used to illustrate the impact of environmental change on wildlife health.

Keywords: anthropogenic stressors; environmental change; health; immunocompetence; survival; wildlife

1. INTRODUCTION

Our planet is currently suffering a staggering rate of dramatic environmental change. Around the world, ecosystems are increasingly subjected to the negative effects of human population growth and its expanding ecological footprint (Jackson *et al.* 2001; Hughes *et al.* 2003). Be it in the form of habitat loss or alteration, the introduction of invasive species, pathogen spillover, accumulation of persistent pollutants, climate change or stratospheric ozone depletion, global environmental change has altered physical and biological systems and is becoming of increasing concern for the well-being and survival of many species (Thomas *et al.* 2004; Hoffmann & Willi 2008).

Predicting the consequences of global environmental change on biodiversity is a complex task mainly because the effects encompass multiple and complex dynamic processes that rarely have single and clear-cut actions. Rather, the effects appear to interact and can even have additive costs, and these can manifest at several levels. For instance, habitat degradation and fragmentation not only may decrease food availability and restrict the movement of animals, thus impairing nutritional status and limiting gene flow, but also may increase the opportunity for contact among humans, domestic livestock and wildlife (Deem *et al.* 2001), potentially enhancing disease transmission rates (Smith *et al.* 2009). Furthermore, pollutants can alter habitat quality, reduce nutrient availability and encourage toxic algae blooms along coastlines (Smith 2003; Havens 2008; Paul 2008), all of which can indirectly affect the survival of sensitive species; furthermore, pollutants can directly impact reproductive parameters (Sonne *et al.* 2006, 2007), sex ratios (Reusch & Wood 2007) and immunocompetence (Selgrade 2007). Because of this very complexity, environmental change is likely to seriously impair the viability of wildlife.

It could be argued that living organisms have long been subject to a myriad of evolutionary pressures arising from the environment (Reusch & Wood 2007) and are consequently well adapted to respond to such pressures. However, the current pace of environmental change is unprecedented (Thomas *et al.* 2004) and it is unknown whether the capacity of species to adapt to such changes and counteract their harmful and often combined effects may be exceeded. Regrettably, published data on this subject are still extremely limited, making it difficult to understand the full extent of the effects of environmental change on wildlife health.

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In the hope that this paper will stimulate the research much needed for assessing wildlife health in the context of a rapidly changing environment, we (i) review the key role of an optimally functional immune system for survival, (ii) examine the effects that exposure to fast-paced change might exert on immunocompetence, (iii) discuss the direct consequences that various drivers of environmental change can exert on health and (iv) identify some of the levels at which these effects might impinge on reproductive parameters. We conclude the paper by proposing avenues of research that we consider to be necessary for a more complete understanding of the effects of environmental change on wildlife health.

2. THE IMMUNE SYSTEM AND ITS ROLE IN SURVIVAL

All organisms are constantly exposed to a wide and changing array of pathogens, foreign peptides, abnormal cell replication and occurrence of deleterious mutations. Under normal circumstances, organisms will strive to maintain health by recognizing and resolving abnormal situations. Responses comprise a complex and interactive network of specific and non-specific humoral and cell-mediated components, broadly defined as immune responses (Tizard 2002), which will largely depend upon opportune recognition of the antigen, the presence and structure of antigen cell membrane receptors, the intensity of exposure to the antigen, a timely activation of containment and destruction measures and, ultimately, the generation of a specific and definitive adaptive immune response (Nizet 2006). The optimal functioning of these responses is known collectively as immunocompetence.

Presenting a detailed description of the components and mechanisms of action of the immune system is well beyond the scope of this paper, and there exist several excellent up-to-date reviews on the immune system of vertebrates (e.g. Borghesi & Milcarek 2007; Kvell *et al.* 2007) which can be consulted to obtain a better understanding of the immune repertoire and its mechanisms of action. What is of relevance here is to underscore the importance of immunocompetence for survival (Lochmiller 1996). This crucial role has been demonstrated by clinical and experimental studies of humans and laboratory animals that show that immune-deficient or -suppressed organisms tend to have defective wound healing (Uba *et al.* 2004), higher mortality rates, shorter lifespans and develop more severe infectious diseases and cancer (Brandau & Gilbert 2007; Norlin *et al.* 2008).

While there are few studies of the relative importance of different immune effectors in terms of wildlife survival, most likely owing to the difficulty of controlling for all confounding factors (including variation in exposure to pathogens and nutritional differences), research on a number of bird species has demonstrated that even simple estimates of non-specific immune responses can reliably predict a large and significant amount of variation in survivorship (e.g. Moller & Saino 2004; Haussmann *et al.* 2005), even when accounting for differences in body condition (Hanssen *et al.* 2003).

3. ENVIRONMENTAL STRESS AND IMMUNE COMPETENCE

In order to function adequately, the immune system is dependent on numerous regulatory interactions between organs, in a complex set of hormone and neuropeptide connections involving the nervous and endocrine systems. When an organism is faced with a stressor—broadly defined as 'any aversive condition, be it a predator or an aggressive conspecific, disturbance to established social hierarchies, overcrowding, an infective parasite or foreign peptide or thermal extremes' (Husband & Bryden 1996) that would affect fitness if not avoided or successfully tolerated (Martin 2009)—various body systems act together to activate and coordinate responses. This cascade of events involves synergistic, agonistic or antagonistic interactions between the sympathetic adrenomedullary system, the hypothalamic–pituitary–adrenocortical axis and the hypothalamic–pituitary–gonadal axis (Lawrence & Kim 2000). Although such stress responses are essential to survival, inadequate, excessive or unremitting adrenocortical and autonomic function is detrimental for reproduction and survival and can have dramatic effects on immunocompetence (Leonard 2006), thereby increasing susceptibility and vulnerability to disease (Friedman & Lawrence 2002).

There are a number of mechanisms that animals use to counteract or mitigate negative effects of stress responses, such as seasonal modulation of responses (Romero 2002), acclimatization (French *et al.* 2008) and reduction in HPA activity (Cyr *et al.* 2007). These mechanisms should, in principle, allow individuals to survive, despite environmental changes. However, it is possible that co-occurring or unpredictable stressors may exceed those mechanisms (Romero 2002). The consequences of facing and dealing with unpredictable stressors arising from erratic environments may pose additional pressures on the optimal functioning of the immune system and ultimately harm the health and survival of wild populations (Martin 2009).

To fully understand the impacts of anthropogenic environmental change on wildlife health, potential immunosuppressive effects of chronic and unpredictable stressors must be taken into account. Environmental changes have led to the emergence of over 40 infectious diseases since 1970, such as HIV/AIDS, Ebola and other viral haemorrhagic diseases, new strains of cholera, ranavirus and chytrodiomycosis in amphibians and antibiotic-resistant tuberculosis, and this trend is predicted to increase in the future (Aguirre & Tabor 2008; Jones *et al.* 2008; Smith *et al.* 2009). Such a trend implies that an optimal immune system will be essential to ensure the viability and persistence of individuals and populations.

(a) *Stress-induced immunosuppression*

During periods of sustained stress, circulating levels of glucocorticoids are known to increase owing to activation of the hypothalamic–pituitary–adrenocortical axis (Shanks *et al.* 1994). These adrenal hormones have powerful anti-inflammatory and immunosuppressive properties that can modulate all steps of the

immune response, including the maturation, selection and proliferation of lymphocytes and the activation of inflammatory cells (Griffin 1989). Glucocorticoids also inhibit the production of various cytokines (e.g. IL (Interleukin)-1, IL-6 and TNF (Tumoral Necrosis Factor)-α) (Tait *et al.* 2008) and promote downregulation of lymphocyte function, particularly of pro-inflammatory and cellular responses (Elenkov 2004).

Various studies have measured circulating levels of glucocorticoids in a number of wildlife species, although few have examined the effects that the released stress hormones can exert on immune parameters (Berger *et al.* 2005). These studies have shown that glucocorticoid levels can increase significantly due to various stressors, including human disturbances (e.g. Arlettaz *et al.* 2007; Schmidt *et al.* 2009), increased predator presence (e.g. Polednik *et al.* 2008) and climate fluctuations (e.g. Hangalapura *et al.* 2003; Romero *et al.* 2006; Shultz & Kitaysky 2008). Nonetheless, a few studies have failed to find an association between glucocorticoid levels and human proximity (e.g. von der Ohe *et al.* 2004). In one case, it was found that animals (lizards) from urban sites had lower baseline and stress-induced corticosterone blood levels than those from rural areas, suggestive of adaptive mechanisms for counteracting stress responses (French *et al.* 2008). These conflicting results highlight the difficulty of making generalizations concerning the role of environmental change and stress responses. They also underscore the need for further studies to increase our comprehension of potential additive effects and of the relative importance of long term and concurrent stressors on immunocompetence, health and survival of wild populations.

(b) *Pollutant-induced immunosuppression*

Assessing the health risk posed by environmental change is further complicated because, in addition to potential alterations caused by overwhelmed responses to unpredictable stressors, immune responses may also be affected directly by anthropogenic drivers of change, such as the pollutants that have greatly accumulated in both terrestrial and aquatic ecosystems in the last several decades (Boon *et al.* 2002; Fairbrother *et al.* 2004; Noyes *et al.* 2009) and are currently considered a threat to a large number of species (Schipper *et al.* 2008).

Even taking into account the difficulty of determining lethal exposure levels for wildlife and elucidating the variations in species' sensitivities to different contaminants (Raimondo *et al.* 2007), there is growing evidence that common environmental pollutants, such as organochlorines and heavy metals, may impair immunocompetence and health in a wide range of animal taxa (Selgrade 2007). This effect will potentially increase susceptibility to infectious and non-infectious disease. Polychlorinated biphenyls (PCBs) are one of the contaminants that have received much attention owing to their immunotoxic effects (Fisk *et al.* 2005). Moreover, PCBs and other pollutants (mainly aromatic and hydrophobic compounds) can cause DNA strand breakage or bind covalently to nucleotides (i.e. adduct formation) (Luch 2005). While such genotoxic effects remain mostly unexplored for most wildlife taxa, studies in laboratory animals have shown that unrepaired DNA damage can lead to gamete loss, lethal embryonic mutations, abnormal development and cancer (Hinton *et al.* 2005) and it is likely that exposed wildlife will face similar consequences.

Among other higher vertebrates, marine mammals appear to be particularly susceptible to persistent pollutants, mainly owing to their trophic position, large adipose reserves and long life spans (Aguilar *et al.* 1999). For example, high PCB concentrations in harbour porpoises have been related to elevated nematode burdens (Bull *et al.* 2006), impaired health (Jepson *et al.* 2005) and infectious disease outbreaks (Hall *et al.* 2006). Moreover, there is evidence from belugas from heavily polluted areas (Martineau *et al.* 1988) that exposure to persistent contaminants can also induce detectable DNA damage and increase the risk of developing cancer (Martineau *et al.* 1994).

Associations between anthropogenic pollutants and immunocompetence have also been reported for amphibians and birds, where exposure to heavy metals, pesticides (e.g. DDT and malathion) and herbicides has been correlated with poor immune responses (Gilbertson *et al.* 2003; Snoeijs *et al.* 2005; Koprivnikar *et al.* 2007), viral, fungal and helminth infections (Forson & Storfer 2006; Davidson *et al.* 2007; Rohr *et al.* 2008) and mortality events (Daszak *et al.* 1999). Amphibians appear to be particularly sensitive to the effects of anthropogenic pollutants (Hopkins 2007), probably owing to their intimate association with the aquatic environment during key life-stages (i.e. larval development and breeding) and their permeable skin (Pessier 2002). For amphibians, the population-level consequences of exposure to agrochemicals extend beyond immunosuppression and disease risk, because these pollutants can also alter reproductive parameters and hamper development. For instance, the widely used agrochemical atrazine has not only been found to reduce white blood cell counts and phagocytic activity of the northern leopard frog, *Lithobates* (formerly *Rana*) *pipiens* (Brodkin *et al.* 2007), but can also alter sex ratios and reduce the testicular content of spermatids (Orton *et al.* 2006). Furthermore, limb deformities and infections caused by parasitic infectious diseases have increasingly been associated with agrochemicals (Johnson *et al.* 2007). Taken together, these observations strongly suggest that agrochemicals are likely to impact health and reproductive success of exposed populations, particularly when in conjunction with other environmental stressors.

4. ANTHROPOGENIC DRIVERS OF WILDLIFE DISEASE

Identifying the cause of wildlife diseases is difficult because rarely can a single factor be identified as responsible, a concept commonly termed the 'epidemiological triad' (figure 1). In addition to immune suppression related to exceeded stress responses and pollutant exposure, environmental change can

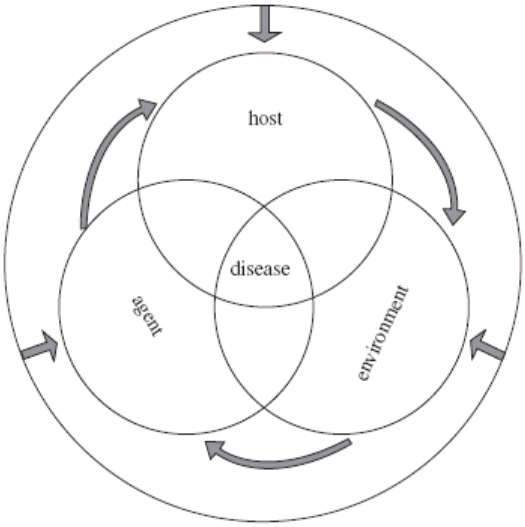

Figure 1. Epidemiological triad. Occurrence of disease results from the interaction between host, environment and disease-agent components (e.g. pathogen or a chemical, UV radiation). Critical host components might be age, sex, genetic background, nutritional and physiological status or prior immune exposure to a pathogen. Environmental host components include climate, interaction with other species, densities and aggregation indexes. Each of the three components can alter the others (e.g. a particular climate regime may decrease food availability, thus changing the nutritional status of an individual or may allow a pathogen to be established in a new area or host). In the context of a rapidly changing environment (black circle), all three components will be subject to changes which will, in turn, alter the epidemiological triad.

impinge directly on wildlife health and survival and, consequently, affect the viability of their populations in various intricate ways (figure 2). For example, climate-related shifts in pathogen and host ranges and pathogen spillover from humans and domestic animals can both increase exposure to new diseases (reviewed in Smith *et al.* 2009). Similarly, changes in habitat size or quality might lead to a reduction in prey population sizes and increased competition for resources (Ryall & Fahrig 2006), which in turn might augment starvation and lead to disease and/or death. Effects will be further complicated if the genetic makeup of the affected populations has been compromised owing to reduced gene flow or inbreeding, as low levels of genetic diversity tend to be correlated with reduced fitness and lowered evolutionary potential (Spielman *et al.* 2004). Furthermore, some environmental stressors can directly compromise health by inducing genotoxicity, developmental abnormalities or systemic alterations. In this section, we present three cases of known and potential health problems caused by interacting factors related to anthropogenic environmental change.

(a) *Climate change, droughts, starvation and disease*

Climate change has altered physical and biological components of the environment, causing shifts in temperature ranges and rainfall indexes and altering the abundance and distribution of predator and prey species, as well as of pathogens and hosts (MacLeod *et al.* 2007; Tibbetts 2007; Patz *et al.* 2008). Droughts and scarcity of food associated with climate change are regular occurrences and are expected to become more frequent, particularly in arid and semi-arid ecosystems (Easterling *et al.* 2000).

Threatened or vulnerable large mammal populations that inhabit such critical ecosystems are likely to be severely affected by these climatic changes. For instance, an extended period of severe drought in Tanzania was related to unusually high mortality of young elephants, particularly of males (Foley *et al.* 2008). During these extreme climatic events, poor nutrition (i.e. suboptimal levels of protein, vitamins and other essential nutrients) and dehydration will lead to depletion of fat reserves, poor body condition (Beldomenico *et al.* 2008) and may decrease innate and acquired immune responses. This will reduce resistance to infection, which in turn can impair nutrient absorption owing to altered gut permeability and inflammation, leading to aggravation of the nutritional status (Katona & Katona-Apte 2008) and further decreasing the chances of survival (Matthews *et al.* 2006; Beldomenico *et al.* 2008). A classic example of this positive feedback system is seen in humans, in which malnutrition is the primary cause of immunodeficiency worldwide and which in turn is strongly related to the prevalence of infectious diseases and infant mortality (Katona & Katona-Apte 2008). For wildlife, such events could have disastrous consequences for already depleted populations.

Meeting the energetic demands placed by nutritional or hydric stress in order to survive is likely to impact upon the demands of other physiological processes, such as immunity, growth, maintenance and reproduction (Houston *et al.* 2007), even though this resource allocation might result in decreased population fitness. Under this assumption, animals faced with nutritional or hydric stress will 'take the risk' of investing less in reproduction or in maintaining optimal immune responses because in that situation it is more important to reduce the immediate risk of death from starvation, malnutrition or dehydration. This has important implications for the health of wildlife, as a population impacted by poor nutrition or drought will be at a higher risk of acquiring endemic or novel infections.

(b) *Urogenital cancer: a complex disease of California sea lions*

Until recently, the reported occurrence of cancer in wildlife has been rare. Although it is likely that the malignant tumours reported for wildlife only account for a fraction of the real cases, the striking difference in incidence rates (4.5% or less in captive and free-ranging wildlife compared with 30% or less in humans; Nagy *et al.* 2007) suggests that cancer is not normally a significant health problem for wildlife. However, exceptions to this have become apparent: since the mid-1990s, an unusually high prevalence (18% of all animals examined; Lipscomb *et al.* 2000)

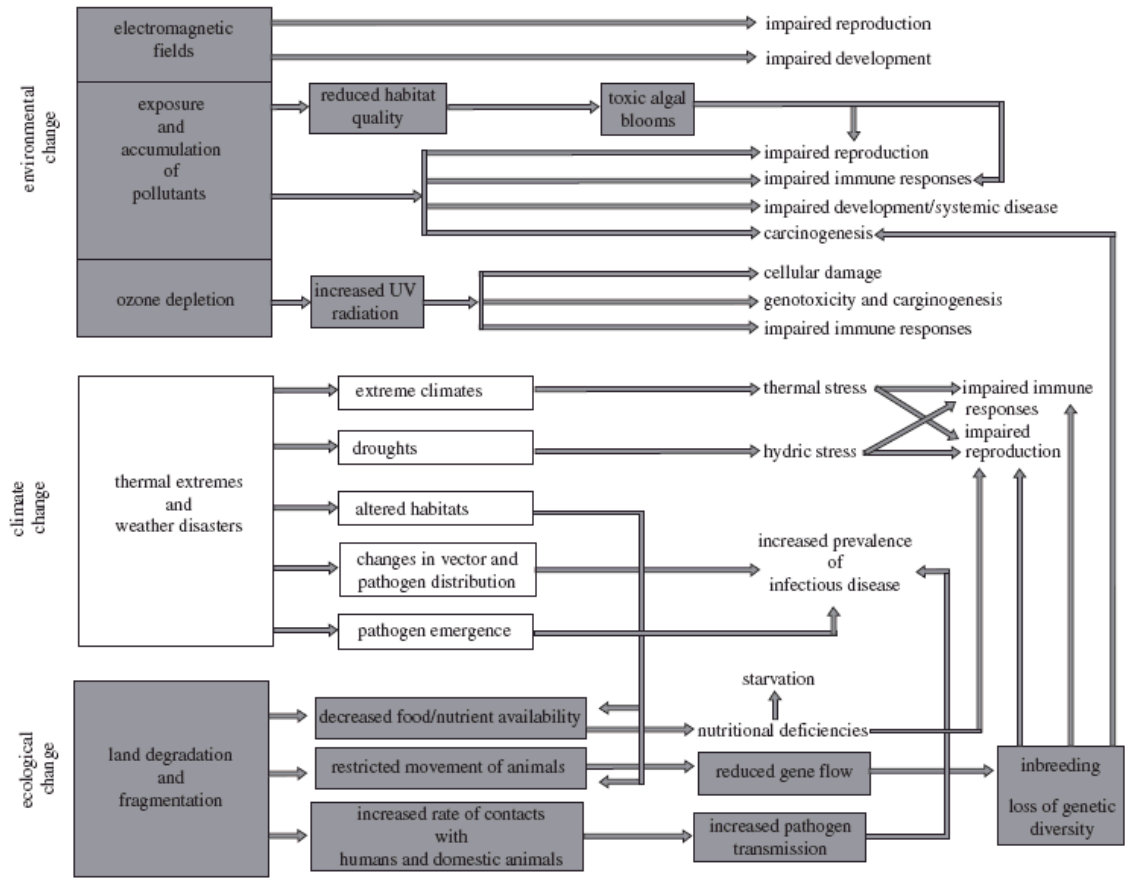

Figure 2. Potential effects of anthropogenic environmental change on wildlife health. The illustration intends to depict the intricate and multiple ways by which changes to the environment can affect health. Regardless of the level at which the changes occur (environmental, climatic and ecological), these will in turn alter other physical and biological processes, eventually increasing the risk of starvation, and exposure to diseases. At an individual level, environmental changes will affect health by weakening immune responses, impairing development and causing systemic disease or cancer.

of epithelial-cell carcinomas of urogenital origin have been observed in adult California sea lions, *Zalophus californianus* stranded along the central California coast (Gulland *et al.* 1996; Buckles *et al.* 2006).

The cause of the problem remains unclear although recent studies have shown that various factors are involved with occurrence of this type of cancer in sea lions, including infection with a novel ottarine gammaherpesvirus (Buckles *et al.* 2006), high organochlorine concentrations (Ylitalo *et al.* 2005) and endogenous sex hormones (Colegrove *et al.* 2009). Genetic factors also seem to play a role in the disease, with high levels of inbreeding (Acevedo-Whitehouse *et al.* 2003) and specific MHC alleles found to be associated with an increased risk for cancer (Bowen *et al.* 2005).

The California sea lion is not an endangered species and, regardless of its clinical severity, it is unlikely that urogenital cancer will cause a significant health problem at a population level, particularly since the disease affects mostly sexually mature adults (Gulland *et al.* 1996; Lipscomb *et al.* 2000). However, the emergence of this condition is an excellent example of the intricate ways in which anthropogenic environmental change can impact upon wildlife health.

(c) *UV radiation and health*

In the past decades, there has been an increase in the amount of harmful UV radiation that reaches the biosphere. This increase has been caused by the reduction in the stratospheric ozone layer which protects the planet from UV radiation. UV radiation is known to cause DNA, cellular and structural damage, which can lead to skin cancer (Situm *et al.* 2008) and can also impair cell-mediated immune responses (Marrot & Meunier 2008). In contrast to the many studies conducted in humans (reviewed in Marrot & Meunier 2008) and marine invertebrates (e.g. Hader *et al.* 2007; Pruski *et al.* 2009), there have been strikingly few efforts to investigate the effects of UV exposure in wildlife, and thus, increased UV radiation is rarely taken into consideration when assessing the health of a species or population.

One notable exception to this paucity of studies is the research conducted in amphibians. Several empirical and experimental studies have shown that UV radiation can be detrimental to amphibian development (Hakkinen *et al.* 2001; Ankley *et al.* 2002), hatching success (Blaustein *et al.* 1997), susceptibility to infection (e.g. Kiesecker & Blaustein 1995;

Kiesecker *et al.* 2001, but see Garcia *et al.* 2006) and survival (Formicki *et al.* 2008). The magnitude of the effect appears to vary between life stages (being stronger for developing individuals; Ankley *et al.* 2002) and between species. For instance, while ambient exposure to UV radiation decreases hatching success and survival in *Rana arvalis*, no effects were detected in *R. temporaria* and *Bufo bufo* (Hakkinen *et al.* 2001), raising the possibility that there may be genetic differences in susceptibility and/or avoidance behaviour between species.

The effects of UV radiation on health are particularly relevant for amphibians because various other disruptors (e.g. habitat loss, climate change, disease emergence and eutrophication) are already associated with the current global decline of this taxon (Sodhi *et al.* 2008; Rovito *et al.* 2009), and it is likely that this stressor might increase the risk by negatively affecting the health of their populations. Other taxonomic groups living in regions with high exposure to UV radiation (e.g. the Antarctic), at high altitudes or those lacking anatomical or behavioural adaptations to UV exposure (e.g. marine mammals) might also be at risk from UV damaging effects.

5. THE IMMUNE-REPRODUCTIVE LINK

In the context of a rapidly changing environment, one further complication for wildlife health arises from the link between the immune and reproductive systems. One of the proposed explanations for this association relates to resource partitioning. Under this hypothesis, maintaining a competent immune system will incur an energetic cost; thus, the resources required to preserve a functional system and to mount specific immune responses may be drawn away from other key physiological processes, such as growth and reproduction (reviewed in Norris & Evans 2000; Sheldon & Verhulst 1996). In a high-risk disease scenario, devoting resources for reproduction might decrease future reproductive success through the consequential effects of low investment in immune responses (Gustafsson *et al.* 1994). If the energetic demands to cover an immune response were not met, the likely consequence would be the occurrence of disease, which might then lead to reduced host performance and increased mortality (Hanssen *et al.* 2003). The trade-off between key physiological processes works in both directions: during times when energetic demands are high (e.g. during reproduction) immune function may be decreased to allow an individual to maximize its reproductive effort, thereby increasing the likelihood of successful survival of offspring (Norris & Evans 2000) but also potentially increasing susceptibility to infection.

While the energetic trade-off hypothesis is not without detractors (reviewed in Lochmiller & Deerenberg 2000), a growing number of experimental studies have shown that increased immune activity diverts resources from traits such as development of sexual ornamentation (e.g. Zuk & Johnsen 2000), clutch size (e.g. Martin *et al.* 2001), nestling provisioning (e.g. Ilmonen *et al.* 2000) and offspring growth rates (e.g. Fair *et al.* 1999). If we are to generalize from these observations in the context of fast-paced environmental changes that directly and indirectly pose additional pressures on the immune system of wildlife species, it is likely that there will be population-level costs in terms of reproductive parameters and other physiological traits. As yet, this remains to be explored fully.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Ecosystems are currently undergoing rapid rates of change, which have the potential to inflict severe damage on the health of wildlife and humans. Repercussions are not only important at an individual level (e.g. cancer and immunosuppression), but can have important population level implications (e.g. reduced reproduction = population decline; reduced immune responses = increased infectious disease). The increasing rates of disease (both infectious and non-infectious) in wildlife is of great concern since disease may be an indication that populations are approaching a state of stress which is negatively affecting immune function, and it is unknown how close this is to the upper limits of their tolerance. When examining the impacts of environmental change on wildlife health, multiple layers of complexity need to be examined and the interactions between different factors need to be considered. Because of the complexity of immune responses, trade-offs between key physiological processes, as well as the potential additive effects of some environmental disruptors on both the immune response and reproductive systems, this wide approach has seldom, if ever, been used to assess potential impacts on population health.

This is an exciting time in terms of research possibilities. The abundant data and laboratory tools developed for studies of both humans and model organisms can be extremely useful to attempt addressing key questions on wildlife health. This approach, in addition to the use of a rigorous and 'environmentally explicit' framework that considers all levels of environmental change will undoubtedly allow researchers to examine the root causes of existing health conditions and potential future diseases for wildlife in the context of a rapidly changing environment.

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PUBLICATION B

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Forum

Chytrid Blinders: What Other Disease Risks to Amphibians Are We Missing?

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Abstract: Amphibian declines are occurring on a global scale, and infectious disease has been implicated as a factor in some species. *Batrachochytrium dendrobatidis* (*Bd*) has been associated with amphibian declines and/or extinctions in many locations, however, few of the studies have actually performed detailed pathological investigations to link the emergence of the disease with mortality rates large enough to cause the declines. Many studies are based solely on the presence of infection, not disease, because of the reliance on molecular tests for *Bd*. The emphasis of the importance of *Bd* combined with easy molecular tests has resulted in poor investigations into amphibian mortality and declines in many areas. The line between infection and disease has been blurred, and a step back to basic pathological and biological investigations is needed as other disease risks to amphibians, such as ranaviruses, are likely being missed. In this article, starting points for proper investigative techniques for amphibian mortalities and declines are identified and areas that need to be improved, especially communication between biologist and veterinarians involved in amphibian disease research, are suggested. It is hoped that this will start a much needed discussion in the area and lead to some consensus building about methodologies used in amphibian disease research.

Keywords: amphibian declines, disease, *Batrachochytrium dendrobatidis*, ranavirus, pathology, disease investigation

Amphibians are currently considered by some to be the most threatened vertebrate taxon on the planet (Stuart et al., 2004). The decline and extinction of many species has resulted in a significant amount of research directed at understanding the causes behind them. For some species, it has been determined that disease is a major factor contributing to their decline (e.g., Berger et al., 1998; Daszak et al., 1999). However, much of the focus has been placed on one pathogen, *Batrachochytrium dendrobatidis* (*Bd*). *Bd* is a chytridiomycete fungus, which is the causative agent of chytridiomycosis in amphibians (Berger et al., 1998). It has been associated with the decline and/or extinction of many amphibian species around the globe (e.g., Australia and Central America: Berger et al., 1998; Panama: Lips, 1999; Spain: Bosch et al., 2001). In Central America, *Bd* appears to be advancing in a wave-like pattern and is associated with mass mortalities and declines when it arrives in a new area (Lips et al., 2006; but see Parmesan and Singer, 2008; Bickford et al., 2008). Mass mortalities and declines in populations of the common midwife toad (*Alytes obstetricans*), common toads (*Bufo bufo*), and fire salamanders

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(*Salamandra salamandra*) in Spain, and mortalities of Tyrrhenian painted frogs (*Discoglossus sardus*) on Sardinia, have been associated with the emergence of *Bd* (Bosch et al., 2001; Bosch and Martínez-Solano, 2006; Garner et al., 2009; Bielby et al., 2009). This emphasizes the impact that *Bd* can have on amphibian populations, but are there other diseases that may be at play in these systems?

Unfortunately, most studies that have associated *Bd* with amphibian mortality events or declines have not included thorough diagnostic investigations. In most cases, postmortem examinations were not performed or, if done, they were done so on a limited and uninformative scale by inadequately trained personnel. A large problem with pathological investigations into amphibian mortalities is a lack of trained veterinary pathologists or amphibian biologists who can accurately perform necropsies on amphibians. With the advancement of *Bd* screening methods to include quantitative PCR (Boyle et al., 2007; see Hyatt et al., 2007 for alternative methodologies), most research examining *Bd* has become associated with a "swab and run" mentality, and the need for a full pathological investigation to enable an accurate diagnosis to be obtained seems to have been forgotten. The line between what constitutes an infection versus a disease has been blurred with the advent of highly sensitive molecular tests. Just because an infection is present in an individual does not mean that disease will necessarily be present or even develop later on (e.g., Daszak et al., 2004; Hanselmann et al., 2004). Infection has been defined as the presence of a pathogenic agent in an individual (Scott, 1988), whereas disease has been defined as an infection that is causing a measurable clinical effect on an animal, the severity dependant upon the results of an examination of the affected individual (Scott, 1988). Although the distinction between infection and disease are subtle, it is extremely important that delineation between the two is made.

It is necessary for researchers to remember that there are other amphibian pathogens that can contribute to mortality events and population declines. One such group are ranaviruses (Daszak et al., 1999; Hyatt et al., 2000), which have been responsible for many large mortality events in North America (e.g., *Ambystoma tigrinum*, Bollinger et al., 1999; multiple species, Green et al., 2002) and in Europe (*Rana temporaria*, Cunningham et al., 1996; *Bufo bufo*, Cunningham et al., 2007; *Alytes obstericans*, Balsiero et al., 2009). In England, ranavirus emergence has been associated with persistent population declines in *R. temporaria* (Teacher, 2009). Other costly pathogens potentially capable of causing host population declines include nematodes, protozoa, bacteria, and ectoparasites (Densmore and Green, 2007). However, the true extent of the involvement of these pathogens in amphibian mortality events and general amphibian population dynamics is largely unknown, arguably due to the lack of proper pathological investigations in amphibian mortality events. This is further compounded by the general lack of knowledge with respect to what could be termed the natural disease-induced mortality rate in amphibians and what could be considered their natural parasite/pathogen loads. Several studies have performed full pathological examinations with respect to amphibian mortality events, and include: Cunningham et al. (1996), Berger et al. (1998), and Green and Sherman (2001) (preserved and archived samples). In each of the studies cited above, at least one veterinary pathologist, familiar with amphibian pathology was involved. Rarely is a trained amphibian pathologist involved in current investigations into mortality events and declines thought to be due to the emergence of disease.

It has been hypothesized that *Bd* originated in Africa and was introduced into the amphibian trade by *Xenopus* spp. (Weldon et al., 2004), followed by further expansion in the trade in other species, notably the North American bullfrog (*Rana catesbeiana*) (Garner et al., 2006). But what other potential pathogens are also present in the amphibian trade? For example, in places where the North American bullfrog has been imported, evidence of ranavirus infection in other amphibian species has been found (e.g., ranavirus antibodies in *Bufo marinus* in Venezuela: Zupanovic et al., 1998) or have been associated with mass mortality and/or decline of other species (e.g., Great Britain: Cunningham et al., 1996; Cunningham, 2001). Ranavirus(es) found in UK amphibian populations have similar genetic sequences to those from the USA and are likely to have originated there (Hyatt et al., 2000). Even North American bullfrogs that are imported back into the USA commercially have been found to be infected with both *Bd* and the ranavirus (Schloegel et al., 2009). These animals were imported from Asia (mostly from Taiwan) or South America (primarily Brazil and Ecuador) (Schloegel et al., 2009). Although the prevalence of *Bd* in these animals was 62% and the ranavirus was considerably lower at 8.5%, it does demonstrate the potential for disease to be spread by the amphibian trade. However, the conditions (e.g., high density, little water, unhygienic conditions) that some of these animals were held in were ideal for the development of, for instance, bacterial disease (see photo in Schloegel et al., 2009). There

are also other viral threats that may be circulating via the commercial trade of amphibians (e.g., herpes-like viruses: Hipolito et al., 2003; *Rana gyrilo* virus: Zhang et al., 2001), again with the limited knowledge that we possess with respect to the diseases of amphibians, there are likely many more potential pathogens that pass without notice.

Research into amphibian disease, decline, and mortality is in dire need of improvement. Those of us who have undertaken research that examines aspects of amphibian infectious disease need to communicate effectively not only with each other, but also include input from both the veterinary and other biological sciences. Without the participation of suitably trained veterinary pathologists, infectious disease biologists, epidemiologists, and amphibian biologists/ecologists, investigations into mortality events and amphibian declines will continue to remain, for the most part, painfully incomplete. Daszak et al. (2003) set out guidelines for linking amphibian declines with disease, starting with fulfilling Koch's postulates. In their most basic form, these postulates are as follows: (1) The pathogen occurs in all cases of the disease. (2) The pathogen is responsible for the clinical signs of the disease, as well as the pathological changes associated with the disease. (3) The pathogen is not associated with another disease. (4) The pathogen, when isolated and introduced into a naive individual, causes the same disease (modified from Evans, 1976). Unfortunately, Koch's postulates may not be possible to fulfill in some circumstances because of the nature of the pathogen (Evans, 1976). However, this does not mean that the postulates should be disregarded. In all cases where disease is suspected, every attempt should be made to fulfill Koch's postulates. There are modifications of the postulates (e.g., The Rivers Conditions for Viruses), which detail how the link between a pathogen and a disease can be made when the pathogen is resistant to pure culture (Evans, 1976).

Once Koch's postulates have been supported, the pathogen then has to be confirmed as the cause of the majority of the mortality in a mortality event (Daszak et al., 2003). Furthermore, the establishment of disease-induced mortality as the actual cause of the population decline is essential if the link between disease and decline is to be made (Daszak et al., 2003), illustrating the need for a significant amount of pathological data from multiple individuals. While the link between disease, mortality, and decline are difficult to make, it is very important to do so. A good example of the need to establish a link between disease and decline is the presence of *Bd* in the Savannah River site in South Carolina, where infections but not disease were found in historical samples, and the decline of amphibians in the area was associated with climatic change and reproductive failure (Daszak et al., 2005). This example also illustrates the importance of field data for pathological investigations. Again, the need for effective communication and collaboration between different disciplines to unearth the true causes of a mortality event or decline is shown. If one discipline alone had addressed the problem without the other, it is possible that the true cause may not have been properly elucidated.

A vital component of the study of amphibian infectious disease is the performance of a proper postmortem examination of dead individuals and clinical observations/examinations of "sick" animals, if possible. When a mass mortality is discovered, as many animals as possible need to be collected and preserved properly for full necropsies, including larval stages, as this is necessary to determine the cause(s) accurately. An excellent starting point for establishing a necropsy procedure can be found in Pessier and Pinkerton (2003). Although some suggestions may not be possible in all situations, every effort should be made to collect as much information as possible about the animals, as well as the environment from which they were obtained. The collection of samples for histopathology is of utmost importance, especially when lesions are present, since changes at the tissue and cellular levels can be extremely important in the proper diagnosis of a disease. A major problem, however, is that veterinary pathologists or histologists who are trained or knowledgeable with respect to amphibians are in extremely short supply, and there are currently no widely available texts on normal amphibian histology, let alone histopathology.

When performing a postmortem examination, it is important to make detailed records of any abnormalities that are found. This is extremely helpful if sensitive molecular tests are used to confirm a diagnosis. If there are no pathological findings or signs of disease, even if the DNA or RNA of a pathogen is present, then it is more difficult to say that the pathogen present has been the ultimate cause of death. As previously discussed, the differences between infection and disease are extremely important.

The amount of information that can be obtained from an animal will depend on its state of decomposition and on what form of preservation is used. In the field, animals can be preserved in 70–100% ethanol or 10% buffered formalin, however, it is imperative that the proper volumes of

fluid be used. There is no point preserving an animal if they are going to rot in the solution because there was not enough present to fix the animal properly in the first place. A good rule of thumb for volume is usually 10× that of the specimen. The use of ethanol as a preservation agent permits for some molecular tests (e.g., PCR, RT-PCR) to be performed, but eliminates the possibility of bacteriology or virology done through culture techniques.

The purpose of this article is to begin an open discussion, among researchers and veterinarians who are involved in amphibian research, about how to approach investigations into amphibian mortality events and population declines. Both researchers and veterinarians need to work together so that as much information as possible is gathered through the use of their complementary skills. It is hoped that a discussion of a standard practice for investigating amphibian mortality and decline events can be initiated. Protocols for collecting and preserving samples from the field, how they are examined, what samples are collected and tests performed are all desperately in need of consensus between researchers. We need to maximize the information that we obtain, ensure that important evidence is not missed, and that other evidence is not overemphasized in order to properly address the causes of amphibian mortality and decline (including non-disease causes).

The global decline of amphibians has been associated to pathogens mainly though extremely poor investigative techniques, as a result of improvements in molecular diagnostics. In many cases, investigations into the presence of disease was not performed, only the presence of an infection detected (e.g., Garner et al., 2005). Since the actual role of potential pathogens is poorly understood in the case of most amphibian declines, we need to start back with basic pathology and disease investigations, using the newer molecular methodologies to support the findings, instead of dictating them. We as researchers need to understand host–parasite interactions through the fulfillment of Koch's postulates, identification of the pathogen from mortality events using proper pathological methodologies, including the confirmation that the pathogen was responsible for a large proportion of the mortalities seen and, importantly, that the mortality caused by the presence of the pathogen is actually the cause of the population decline (as per Daszak et al., 2003). It is only if we do this, will we be able to accurately assess the potential threat of disease in potentially the most threatened vertebrate taxon, and only then will we have a chance at mitigating the effects.

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