The epidemiology of the amphibian pathogen
*Batrachochytrium dendrobatidis*

in the UK

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Statement of originality

All ideas presented within this thesis were formulated by the candidate under the guidance of Andrew Cunningham, Mat Fisher and Trent Garner.

I declare that the work presented in this thesis is that of my own and that I have acknowledged the work of others where relevant.
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ABSTRACT

*Batrachochytrium dendrobatidis* (*Bd*) is a recently emerged multihost fungal pathogen, which has rapidly spread globally. It has been detected in 52 different countries, and in over 500 amphibian species. In susceptible hosts, it causes the disease chytridiomycosis. Although globally, *Bd* causes amphibian declines (and in some parts of the world, has resulted in a 40% loss of amphibian species), host responses are inconsistent and *Bd* appears able to coexist with some amphibian species or assemblages of species in a state of endemism.

*Batrachochytrium dendrobatidis* was first detected in wild amphibians in the UK in 2004, in an introduced population of North American bullfrogs (*Lithobates catesbeianus*). Apparent spillover of infection into native species, including deaths in wild common toad (*Bufo bufo*) metamorphs at the index site, and in natterjack toads (*Epidalea calamita*) bred for reintroduction, has led to considerable concern for the conservation status of our native amphibian species.

This thesis reports the results of cross-sectional, longitudinal and experimental investigations into the epidemiology of *Bd* in the UK.

National cross-sectional surveys were conducted in 2008 and 2011, during which skin swabs were collected from almost 9000 amphibians and tested for the presence of *Bd* DNA using real-time PCR. Infection was detected at 30 sites (16% total), in all six native amphibian species tested, and in three of four non-native amphibian species tested. There was no evidence of change in either the distribution or prevalence of infected sites between the two survey years. There was no obvious spatial clustering and intra-site prevalence was almost uniformly low (median 10-12%). The results showed a strong positive association between *Bd* occurrence and the presence of non-native amphibian species, suggesting that co-introduction with non-native amphibians may have played a significant role in the current distribution of infection in the UK. Infection was also associated with sites occupied by natterjack toads, a species that has been the subject of long-term conservation management.
(including translocation events), providing further evidence that human-assisted movement of amphibians has contributed to the current distribution of Bd in the UK.

A longitudinal study conducted in 2010 found a strong seasonal pattern of infection in newt species, with a higher prevalence of infection during the later, warmer months of the breeding season. Seasonal variation in Bd infection (and chytridiomycosis) has been commonly reported. However previous efforts have typically been conducted in tropical rather than temperate climates, where this pattern is reversed. This study also found, in terrestrially sampled common toads, an apparent increase in the prevalence of infection post-breeding suggesting that the breeding period is associated with an increased exposure or susceptibility to Bd (or both).

Finally, two large-scale experiments were conducted to investigate infection in smooth newts (Lissotriton vulgaris) and palmate newts (Lissotriton helveticus). There was no evidence that experimental exposure to Bd was associated with increased mortality, or morbidity. In addition, even at high levels of exposure, infection was both rare and short lived, suggesting that these two native species are unlikely to provide a reservoir for infection in the UK.

Overall, these results show a widespread but patchy distribution of infection, consistent with multiple point-introductions. At infected sites, the prevalence of Bd was low, and infected individuals had low infectious burdens, suggesting that the full expression of chytridiomycosis is not present in the UK at this time. A strong correlation with non-native amphibians and no evidence of broad-scale range expansion between 2008 and 2011 suggest that natural colonisation of ponds may not play a substantial role in the epidemiology of infection in this country. As a result, a combination of strict biosecurity protocols for fieldworkers, avoidance of long-distance translocations of native species and the prevention of future release of non-native species may be sufficient to control further spread of infection in the UK.
DEDICATION

This thesis is dedicated to my parents, who have lived this experience almost as vividly as I.
AKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors, Andrew Cunningham, Mat Fisher and Trent Garner for their skillful guidance throughout this process and under whose studentship I have found challenge, and support in equal measure.

Also to TJ McKinley at Cambridge Infectious Diseases Consortium, the statistician responsible for the design of the first UK national Bd survey, and who held my hand throughout the analysis of the second.

To Jim Foster at Natural England (and now at Amphibian Reptile Conservation), who championed both national surveys and was instrumental in designing the post survey follow-up questionnaire.

To Brian Banks and Gordon Haycock who contributed their time and expertise to the project and played a crucial role in the design of the longitudinal study. To Brian especially, for teaching me lessons in herpetology I will never forget.

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To Ruth Cromie, Michelle O’Brien, Jay Redbond and the rest of the WWT team for unfailing support, conscientious participation in the 2011 national survey and the highest ratio of person hours to number of amphibians sampled!

To the Amphibian and Reptile Groups of the UK and all its members without whom the UK national Bd surveys would simply not have been possible. I consider myself extremely fortunate to have been introduced to this extraordinary community.
To Amphibian and Reptile Conservation, the British Herpetological Society and ARG UK for opportunities to present this research at conferences.

To Jon and Ray Cranfield, but particularly to Ray, for the frequent newt-hunting expeditions, his constant good humour in the face of a challenge and for sharing his vast knowledge of natural history.

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To all those at IoZ and Imperial, including the CBD team at St Mary’s, and RACE who have contributed directly, or indirectly to this thesis. The names are too many to mention, and I am so grateful.

I owe a huge debt of gratitude to my parents. They have supported me through more years of education than I ever thought possible – and it hasn’t always been easy. During the last two months of writing, I moved back home where despite making very little contribution, I was assured that is was lovely to have me there. I owe a special thank you to my mother who in the last few days before submission kindly proofread all but the final chapter (still then in the writing phase), taking on Andrew’s baton as the ‘comma police’.

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<td>AIC</td>
<td>Akaike information criterion</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>ARG-UK</td>
<td>Amphibian and Reptile Groups of the UK</td>
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<td>Bd</td>
<td><em>Batrachochytrium dendrobatidis</em></td>
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<td>BdGPL</td>
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<td>Countryside Council for Wales</td>
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<td>GLM</td>
<td>Generalised linear model</td>
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<td>GLMM</td>
<td>Generalised linear mixed model</td>
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<td>IUCN</td>
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<td>NE</td>
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<td>OIE</td>
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<td>OR</td>
<td>Odds ratio</td>
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<td>PIL</td>
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<td>PSU</td>
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<td>rt-PCR</td>
<td>Real-time Polymerase Chain Reaction</td>
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<td>SNH</td>
<td>Scottish Natural Heritage</td>
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<tr>
<td>SSU</td>
<td>Secondary Sampling Unit</td>
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1 INTRODUCTION

1.1 Amphibian declines

The class Amphibia is believed to have existed for around 250 million years. It is represented by three extant orders: Anura (frogs and toads), Caudata (salamanders) and Gymnophonia (caecilians), of which frogs and toads are the most numerous, and caecilians the least. The total number of known species is currently in excess of 7200 (AmphibiaWeb; http://www.amphibiaweb.org/, accessed February 2014, 2014), almost half of which were described within the last few decades (e.g. Duellman 1999, Lannoo 2005). The rate at which new species are being discovered shows no evidence of slowing down, and in 2012 it was equivalent to one new species being described every 2.5 days (AmphibiaWeb).

However, for this hugely diverse and charismatic taxonomic group, the discovery of new species has been increasingly eclipsed by the sudden decline, and sometimes-complete extinction of others (Blaustein and Wake 1990, Drost and Fellers 1996). In 2004, the first Global Amphibian Assessment (GAA) concluded that 32.5% of all known amphibian species were globally threatened (i.e. falling within IUCN Red List categories of Vulnerable, Endangered or Critically Endangered), (Stuart et al. 2004). Even taken at face value, this estimate identifies amphibian species as being more severely threatened than either birds or mammals. Furthermore, this figure is likely to be heavily confounded by the large number of amphibian species (25% of total, IUCN 2001), for which so little information is available they can only be assessed as Data Deficient (Table 1.1). Data Deficient species are commonly characterised by limited geographic range and/or low population size (such that few species encounters have been recorded). As a result, these species are intrinsically more likely to be threatened. Taking this into account the true proportion of threatened amphibian species is therefore likely to be considerably higher than current estimates suggest (Stuart et al. 2004, Bielby et al. 2006, Mendelson et al. 2006).
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<th>IUCN Red List Categories</th>
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<td><strong>EXTINCT (EX)</strong></td>
<td>A taxon is Extinct when there is no reasonable doubt that the last individual has died. A taxon is presumed Extinct when exhaustive surveys in known and/or expected habitat, at appropriate times (diurnal, seasonal, annual), throughout its historic range have failed to record an individual. Surveys should be over a time frame appropriate to the taxon’s life cycle and life form.</td>
</tr>
<tr>
<td><strong>EXTINCT IN THE WILD (EW)</strong></td>
<td>A taxon is Extinct in the Wild when it is known only to survive in cultivation, in captivity or as a naturalized population (or populations) well outside the past range. A taxon is presumed Extinct in the Wild when exhaustive surveys in known and/or expected habitat, at appropriate times (diurnal, seasonal, annual), throughout its historic range have failed to record an individual. Surveys should be over a time frame appropriate to the taxon’s life cycle and life form.</td>
</tr>
<tr>
<td><strong>CRITICALLY ENDANGERED (CR)</strong></td>
<td>A taxon that meets the IUCN criteria for Critically Endangered is considered to facing an extremely high risk of extinction in the wild.</td>
</tr>
<tr>
<td><strong>ENDANGERED (EN)</strong></td>
<td>A taxon that meets the IUCN criteria for Endangered is considered to be facing a very high risk of extinction in the wild.</td>
</tr>
<tr>
<td><strong>VULNERABLE (VU)</strong></td>
<td>A taxon that meets the IUCN criteria for Vulnerable is considered to be facing a high risk of extinction in the wild.</td>
</tr>
<tr>
<td><strong>NEAR THREATENED (NT)</strong></td>
<td>A taxon is Near Threatened when it has been evaluated against the criteria but does not qualify for Critically Endangered, Endangered or Vulnerable now, but is close to qualifying for or is likely to qualify for a threatened category in the near future.</td>
</tr>
<tr>
<td><strong>LEAST CONCERN (LC)</strong></td>
<td>A taxon is Least Concern when it has been evaluated against the criteria and does not qualify for Critically Endangered, Endangered, Vulnerable or Near Threatened. Widespread and abundant taxa are included in this category.</td>
</tr>
<tr>
<td><strong>DATA DEFICIENT (DD)</strong></td>
<td>A taxon is Data Deficient when there is inadequate information to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status. A taxon in this category may be well studied, and its biology well known, but appropriate data on abundance and/or distribution are lacking.</td>
</tr>
<tr>
<td><strong>NOT EVALUATED (NE)</strong></td>
<td>A taxon is Not Evaluated when it has not yet been evaluated against the criteria</td>
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Please refer to [http://www.iucnredlist.org](http://www.iucnredlist.org) for detail
In addition, the GAA identified a group of 435 ‘rapidly declining species’. This term was used to describe species that in 2004, qualified for a higher IUCN threat category (Table 1.1) than was attributed in 1980 (Stuart et al. 2004). Evidence of rapid decline at species level corroborated a growing number of independent reports documenting the sudden and recent disappearance of amphibians in the wild (e.g. Pounds et al. 1997, Berger et al. 1998, Lips 1999, Bosch et al. 2001).

It is now widely accepted that we are experiencing an amphibian crisis of global proportions. Over the last three decades nine amphibian species have been newly classified as Extinct under the IUCN Red List criteria (Table 1.1) including both species of Australian gastric brooding frog (Rheobatrachus sp.) and the sharp snouted day frog (Taudactylus acutirostris) (Laurance et al. 1996, Schloegel et al. 2006). However, due to the extensive survey requirements necessary to satisfy IUCN extinction criteria (Table 1.1), confirmed cases likely represent a small proportion of actual amphibian extinctions. Already 120 additional species, reported missing, are being investigated (Stuart et al. 2004) and conservative estimates suggest that the current extinction rate of amphibians could be more than 200 times the background level, as informed by the fossil record (McCallum 2007).

A range of threats has been proposed by way of explanation for the current crisis. These include familiar anthropogenic threat processes such as habitat loss or alteration, overexploitation by the commercial trade of living and dead animals, the introduction of invasive species and the toxicity of pesticides and other harmful chemicals (Collins and Storfer 2003).

However, none of these conventional hypotheses provide an adequate explanation for the large number of so called ‘enigmatic’ population declines reported in areas of seemingly pristine habitat (Wake 1991, Stuart et al. 2004). Defining cases include the sudden disappearance of the Eungella gastric-brooding frog (Rheobatrachus vitellinus) from a national park in Northern Australia (see above) (McDonald 1990, McDonald and Alford 1999) and the catastrophic collapse of amphibian populations in the remote cloud forests of
Monteverde, Costa Rica where, in the 1980s, 40% of frog species disappeared in a short period of time (Pounds and Crump 1994, Pounds et al. 1997). Species lost from these forests include the now extinct golden toad (*Incilius periglenes*), known only to science since the 1960s, and the once common Costa Rican variable harlequin toad (*Atelopus varius*) now critically endangered (IUCN 2013).

1.2 *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis

The inadequacy of traditional anthropogenic threats in explaining amphibian declines gave voice to a newly hypothesised threat to amphibian populations: that of infectious disease (Laurance et al. 1996). This hypothesis that infectious disease was driving amphibian declines gained further credibility in 1998, with the results of detailed pathological investigations of dead and dying amphibians collected from sites of population decline in Australia and Central America (Berger et al. 1998). On gross examination of the specimens, the authors found abnormal epidermal skin sloughing, which was variably associated with histological signs of cellular changes to the epidermis, such as irregular cell loss, erosions and marked thickening of the stratum corneum. However, the most important and the only consistent histological finding in these specimens, was the presence of a hitherto unknown non-hyphal fungus of the phylum Chytridiomycota. The disease was termed amphibian chytridiomycosis.

This organism has since been formerly described as *Batrachochytrium dendrobatidis* (hereafter *Bd*), and constitutes not only a new species, but also a new genus within the order Chytridiales (Longcore et al. 1999). The life cycle of *Bd* comprises two known stages: an aquatic flagellated zoospore (the infective stage), and a spherical, intracellular thallus (the reproductive stage) (Longcore et al. 1999, Pessier et al. 1999). Infection develops within the keratinized layers (stratum corneum) of amphibian skin or in tadpole mouthparts (Pessier et al. 1999). Zoospores appear to invade cells via a germ tube (Longcore et al. 1999, Berger et al. 2005), the end of which forms a swelling, which subsequently develops into a thallus (Van Rooij et al. 2012). Zoospores form inside the thallus and once mature, are released, via a discharge papilla, into the environment (Berger et al. 2005, Woodhams et al. 2008). In this
way, infection can spread between hosts, without the need for physical contact to occur. In 
addition, \textit{Bd} has been shown to maintain infection within an existing host, via rhizoid-like 
structures, which penetrate the deeper layers of the skin and give rise to new thalli (Van Rooij 
\textit{et al.} 2012).

\subsection*{1.2.1 Chytridiomycosis as a cause of amphibian declines}

Proposed by Berger \textit{et al} (1998) as the causal agent of disease, the pathogenicity of \textit{Bd} was 
confirmed when pure culture (isolated from a captive collection of dendrobatid frogs at the US 
National Zoo, hence ‘\textit{dendrobatidis}’) killed experimentally-exposed frogs (Nichols \textit{et al}. 2001). 
The fungus was subsequently re-isolated from affected animals, thus satisfying Koch’s 
postulates and establishing a causal pathway between pathogen and disease (Longcore \textit{et al}. 

Demonstrating the impact of a pathogen on wild populations presents a different challenge. 
Research into population declines tends to occur after the event, making it impossible to 
determine whether a pathogen is endemic within a population, or has been recently 
introduced. In the literature relating to \textit{Bd}, there are two key examples that specifically 
address this issue. A now infamous study conducted by Lips \textit{et al}. (2006) took advantage of a 
well-documented wave of amphibian declines and mass mortality events, moving in a 
southeasterly direction through Central America. Mortality was shown to be associated with 
the presence of \textit{Bd} at multiple sites and it was suggested that a causal relationship existed 
between the two. To test this hypothesis, researchers set up permanent transects in El Copé 
National Park, Panama, a location with no prior history of \textit{Bd} infection and ‘ahead’ of the 
aforementioned front of declines. Transects were surveyed between 1998 and 2005 to 
monitor amphibian species richness and abundance, and between 2000 and 2004 for the 
presence of \textit{Bd}. Using standardised surveys over a sustained period of study, researchers 
were able to document not only the arrival of \textit{Bd} to El Copé, and the simultaneous 
appearance of large numbers of dead, infected amphibians, but also a rapid and significant 
decline in amphibian density and diversity at this site (Lips \textit{et al}. 2006).
A second study, featuring the Californian mountain yellow-legged frog (a complex of species comprising *Rana sierra* and *Rana muscosa*), was conducted across a series of three lake basins, in Sequoia-Kings Canyon National Park (Vredenburg et al. 2010). Based on long-term population data and intensive sampling for *Bd*, Vredenburg et al. (2010) reported major population declines and amphibian mortality soon after the first detection of *Bd* within this study area. Furthermore, the authors documented the rapid spread of *Bd* from the point of earliest detection, in a similarly wave-like manner to that seen in Panama, until all populations were infected (Vredenburg et al. 2010).

Both papers outlined above elegantly illustrate the devastating effect that *Bd* can have on wild populations of amphibians. *Bd* is now believed to be primarily responsible for amphibian declines and extinctions previously attributed the label ‘enigmatic’; affecting at least 200 different species world-wide (Skerratt et al. 2007).

1.2.2 Diagnosis of *Bd* infection and amphibian chytridiomycosis

The clinical manifestation of infection caused by *Bd* is known as amphibian chytridiomycosis. It has been described as ‘the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction’ (ACAP 2005) and has recently been listed as globally notifiable by the World Organisation of Animal Health (OIE) (Schloegel et al. 2010). In susceptible animals, progression of disease can be rapid. As a result, death may occur before clinical signs have been observed. Where ante-mortem signs of disease are observed, they are largely non-specific, including skin changes (discolouration, erythema or increased skin shedding), anorexia, lethargy and loss of the righting reflex (Nichols et al. 2001). The absence of *Bd*-specific symptoms of disease means that diagnosis of infection requires laboratory confirmation. Initially, this was achieved using microscopic techniques, principally histopathology, for direct visualisation of the fungus (Berger et al. 1998, Briggs and Burgin 2003). However, this approach has since been largely superseded by molecular detection of the pathogen. Where diagnosis of infection was
previously carried out post mortem, or by invasive sampling of live animals (e.g. toe clipping), diagnostic samples can now be collected by swabbing the skin to harvest \textit{Bd}, where present. Skin swabs are then analysed for the presence of \textit{Bd} DNA using rt-PCR (real-time polymerase chain reaction) (Boyle \textit{et al.} 2004). This methodology has the advantage of being both non-invasive and more sensitive than microscopic techniques (Hyatt \textit{et al.} 2007) and has greatly improved capacity to detect infected populations in the field.

Our ability to diagnose animals infected with \textit{Bd} has been poorly matched by our understanding of the disease it causes. Currently, the best-supported explanation is that pathogenesis is dependent on the unique physiological properties of amphibian skin (post metamorphosis), which, in contrast to all other vertebrate species, is actively involved in the exchange of respiratory gases, water, and electrolytes. As a result, amphibian skin plays a critical role in homeostasis. Voyles \textit{et al} (2009) demonstrated that \textit{Bd} infection in green tree frogs (\textit{Litoria caerulea}) was associated with a reduction in cutaneous electrolyte transfer, possibly due to \textit{Bd}-induced changes to the structure of the epidermis. Diseased animals were shown to have low levels of plasma sodium and potassium and, when mortality occurred, the cause of death was confirmed as cardiac asystole. Although the precise mechanism remains unclear, the epidermal dysfunction hypothesis, resulting in a sometime-fatal electrolyte imbalance is the most widely accepted theory of morbidity and mortality in infected amphibians.

In larval stage anurans, \textit{Bd} infection is limited to the keratinised oral discs. In some species, infection has been shown to compromise grazing efficiency, which manifests as an increased rate of mortality at or around metamorphosis as a result of poor body mass (Parris and Baud 2004, Parris and Cornelius 2004, Garner \textit{et al.} 2009, Skerratt \textit{et al.} 2010). However, larval-stage mortality is rarely reported.
1.3 *Bd* and infection dynamics

The traditional view of wildlife pathogens, even those associated with high levels of mortality, is that they play a secondary role in causing population declines and extinctions (in fact, the presence of pathogens within a host population is generally accepted to be a vital component of healthy ecosystem function see Hudson *et al.* 2006). There are two key assumptions underpinning the belief that wildlife disease is not a primary cause of extinction, (1) that disease transmission is density- rather than frequency-dependent and (2) that there is no reservoir host population. The epidemiology of *Bd* undermines both of these assumptions:

1.3.1 Density- versus frequency-dependent transmission

When transmission of a pathogen is density-dependent, the rate at which new cases become infected is dependent on host density (Anderson and May 1978; May and Anderson 1978). With increasing host concentration, the rate of contact between infected and uninfected individuals increases providing greater opportunity for transmission to occur. When the population density falls below a threshold size, insufficient contacts arise for the maintenance of infection and the pathogen fades out.

There is some evidence that host density impacts the prevalence of *Bd* infected individuals, suggesting that direct, or at least close contact between amphibians has a role in generating new infections (Rachowicz and Briggs 2007). However, because *Bd* zoospores can persist in the environment, there is also potential for transmission to occur in a frequency-dependent manner (Anderson and May 1991), providing that the density of infectious zoospores in the environment is sufficient to compensate for a reduction in host number. This effect can also be observed in vector borne infection dynamics (Antonovics 1995).

By de-coupling transmission and host density, pathogens capable of frequency-dependent transmission are more likely to drive a population to extinction (Ryder *et al.* 2007). The risk of extinction is especially high for pathogens capable of saprophytic reproduction, since this can provide a continued source of infection entirely independent of the host. Although, at present, we are lacking empirical evidence of a saprophytic lifecycle for *Bd* (Longcore *et al.* 1999, 2011).
Piotrowski et al. 2004), the ease with which *Bd* is cultured in the laboratory suggests that reproduction outside the host may well be possible.

The presence of an environmental infectious life stage (the zoospore) provides a pathway for indirect transmission of *Bd* to occur. This process can be evidenced in the successful inoculation of experimental animals with cultured zoospores (e.g. Garner et al. 2009). As a result, a frequency-dependent framework might be considered appropriate and indeed, there is some evidence to support this (Rachowicz and Briggs 2007). However, as *Bd* zoospores have been shown to have a very limited range, travelling less than 2cm distance before encysting (Piotrowski et al. 2004), in the absence of a confirmed saprophytic life stage, successful transmission may, none-the-less, rely heavily on immediate or close range contact between hosts (however, movement of zoospores may be facilitated by water flow, Johnson and Speare 2005).

On balance, transmission of *Bd* is likely to be influenced by both density- and frequency-dependent components. Although the relative contribution of each is currently poorly understood, the results of theoretical modelling show, comprehensively, that in a combined system such as this, even a small amount of frequency-dependent transmission will increase the likelihood of host extinction (Ryder et al. 2007).

### 1.3.2 *Bd* and reservoirs of infection

Even in a system where pathogen transmission is strictly density-dependent, extinction can still occur. This scenario is dependent on the presence of a reservoir host population, defined as one or more epidemiologically connected populations or environments in which the population can be permanently maintained, and from which infection is transmitted to a pre-defined target population (the population of interest) (Haydon et al. 2002). The reservoir population can be made up of individuals from the same species, a different species, or a series of linked populations. The functional population size of a reservoir population must exceed the critical community size (Table 1.2) and there must be sufficient ecological overlap between reservoir and target populations so as to allow for effective contact between
individuals. Sustained transmission of a pathogen from a reservoir population can lead to continued exposure of a non-maintenance population (in which a pathogen would otherwise fade out) and, under certain conditions, this dynamic can lead to pathogen-driven extinctions.

Table 1-2 Terminology of multihost pathogens adapted from Haydon et al. 2002

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RESERVOIR OF INFECTION</td>
<td>one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population.</td>
</tr>
<tr>
<td>TARGET POPULATION</td>
<td>the population of concern or interest.</td>
</tr>
<tr>
<td>NON-TARGET POPULATION</td>
<td>all other potentially susceptible host populations that are epidemiologically connected, whether directly or indirectly, to the target population and which could constitute all or part of the reservoir.</td>
</tr>
<tr>
<td>CRITICAL COMMUNITY SIZE</td>
<td>the minimum size of a closed population within which a pathogen can persist indefinitely.</td>
</tr>
<tr>
<td>NON-MAINTENANCE POPULATION</td>
<td>population smaller than the critical community size.</td>
</tr>
<tr>
<td>MAINTENANCE POPULATION</td>
<td>population larger than the critical community size.</td>
</tr>
<tr>
<td>MAINTENANCE COMMUNITY</td>
<td>complex system in which pathogen transmission between a number of non-maintenance population results in a functional population that exceeds the critical community size.</td>
</tr>
<tr>
<td>SOURCE POPULATION</td>
<td>any population that transmits infection directly to the target population.</td>
</tr>
<tr>
<td>FADEOUT</td>
<td>stochastic pathogen extinction that occurs in populations below critical community size.</td>
</tr>
</tbody>
</table>

The concept of a reservoir host/hosts can easily be applied to *Bd*. Not only does *Bd* have a vast host range (infection has now been detected in over 500 different species, Olson et al. 2013), infection outcome varies widely between species. Some species succumb rapidly to infection and die (e.g. Berger et al. 1998) whereas others, such as the North American bullfrog (*Lithobates catesbeianus*) have been shown to harbour infection with no apparent clinical signs of disease (Daszak et al. 2004). In mixed-species assemblages (extremely common among amphibians), asymptomatic carriers of *Bd*, termed also ‘disease tolerant’ can
provide a functional reservoir of infection (although infections in reservoir hosts are not necessarily nonpathogenic see Haydon et al. 2002).

The concept of infection reservoirs can also be extended to different life-history stages and therefore the presence of intra-specific reservoirs of infection (Narayan et al. 2014). Of particular relevance is the profound physiological transformation that takes place between larval and post-metamorphic life stages in anuran species. In contrast to the generalised skin infection seen in susceptible post-metamorphic animals, pre-metamorphosis, *Bd* is found exclusively in the keratinizing epidermis of tadpole mouthparts. The resulting, localised infection, although not entirely benign (Parris and Baud 2004, Parris and Cornelius 2004, Garner et al. 2009, Skerratt et al. 2010), is rarely fatal, and prevalence can be high (Woodhams and Alford 2005). As a result, larval stage anurans may be an important reservoir for infection (Daszak and Cunningham 1999, Daszak et al. 2003), particularly in species and climates associated with a prolonged larval period (Briggs et al. 2010) where the presence of overwintering tadpoles may play a vital role in maintaining infection between seasons (Narayan et al. 2014).

Lastly, the potential role of non-amphibian hosts in maintaining a source of infection is also deserving of mention. The presence of a non-amphibian host for *Bd* has been postulated by a number of studies reporting (1) that *Bd* can be carried by a range of species including terrestrial reptiles (Kilburn et al. 2011), waterfowl (Garmyn et al. 2012) and nematodes (Shapard et al. 2012) and (2) that *Bd* can be grown on non-amphibian tissues such as boiled snakeskin (Longcore et al. 1999), bird feathers (Johnson and Speare 2005) and toe scales from waterfowl (Garmyn et al. 2012). However, until very recently, there was no evidence of *Bd* growth on living (non-amphibian) hosts, as would be required for long-term persistence of infection in the absence of an amphibian population. In 2013, McMahon et al. published a seminal paper in which they demonstrated (1) the presence of *Bd* in the gastrointestinal tracts of field-sampled crayfish (*Procambarus* spp. and *Orconectes virilis*), (2) that the presence of crayfish at field sites was a positive predictor for *Bd* infections in co-occurring amphibians and (3) that infection in crayfish was maintained for at least 12 weeks and could be transmitted to
amphibians under experimental conditions. This study also found that exposure of crayfish to water that previously held *Bd* was associated with mortality and gill recession suggesting that *Bd* may release a chemical capable of causing pathology, even in the absence of infection.

1.4 Determinants of disease

One of the defining characteristics of *Bd* infection is the variability in outcome following infection. Despite a few popularly cited examples of catastrophic population crashes, *Bd*-linked morbidity, mortality and population declines are, in fact, from being the norm. As a result, an important distinction must be made between *Bd*-infection, and the expression of disease (chytridiomycosis). Common to all host-pathogen relationships, multiple variables (known and unknown) contribute to determining the impact of infection:

1.4.1 Host

Resistance to chytridiomycosis (but not to *Bd* infection) has been demonstrated in a wide range of host species. Frequently cited examples include the North American bullfrog (*Lithobates catesbeianus*) and the African clawed frog, (*Xenopus laevis*), both of which have been shown to harbour infection in the absence of disease (Daszak et al. 2004, Weldon et al. 2004).

A number of factors are believed to contribute to variation in host response at this level. There is good evidence to support a role for the innate immune system in infection-tolerant hosts. Thus far, two innate defense mechanisms have been identified: (1) species-specific anti-microbial secretions produced by granular glands present in the skin (Rollins-Smith and Conlon 2005, Woodhams et al. 2007); (2) anti-*Bd* properties of certain symbiotic bacteria, found within normal amphibian skin flora (Harris et al. 2006, Woodhams et al. 2007). Peptide secretions and bacterial skin flora have been shown to be both intra- and inter-specific (Rollins-Smith and Conlon 2005, Harris et al. 2006, Woodhams et al. 2007) and as a result, differences in susceptibility to chytridiomycosis can be observed both within and between species.
Despite the fact that amphibians have a well-developed immune system, the evidence supporting a role for adaptive immunity in defining host response is less compelling. Two studies investigating Bd infection in Silurana (Xenopus) tropicalis (a species susceptible to chytridiomycosis) found no evidence that genes associated with adaptive immunity were upregulated in infected hosts (Ribas et al. 2009, Rosenblum et al. 2009). A third study used heat-treated Bd to immunise X. laevis and, in this species, successfully demonstrated the presence of Bd-specific immunoglobulins post exposure (Ramsey et al. 2010). However, in this instance, immunisation was carried out by intraperitoneal injection and it is therefore difficult to draw parallels with the reality of amphibians exposed in the wild. Furthermore the decision to model adaptive immunity in an apparently disease-resistant host species such as X. laevis is questionable. Future studies investigating the presence (or absence) of an adaptive immune response to Bd infection might be better targeted towards host species with demonstrated but variable susceptibility to disease such as the Mallorcan midwife toad (Alytes obstetricans, Tobler and Schmidt 2010), using natural exposure pathways, for example, bathing animals in Bd-infected water.

There are, of course, fundamental differences in the manifestation of infection in larval and post-metamorphic amphibians (see above). A further distinction can be made between recently metamorphosed and later stage amphibians, with a peak in the incidence of fatal chytridiomycosis during and immediately after metamorphosis (Bosch et al. 2001, Carey et al. 2006, Garner et al. 2009). Metamorphosis is a time of critical transition, triggered by elevated concentrations of certain hormones, principally thyroid hormone and corticosteroids, which involves the reorganisation of tissues and organ systems, including the immune system (Rollins-Smith 1998). As a result, metamorphosis is associated with transient immunosuppression, which may leave animals more vulnerable to developing disease should infection arise. Recently, however, an alternative but related mechanism has been suggested to explain the disproportionately high incidence of chytridiomycosis associated with metamorphosis. Thyroid hormone has actually been shown to induce gene expression in Bd contributing to invasion and colonisation of host tissues (Thekkiniath et al. 2013). Thus, elevated thyroid hormone levels during metamorphosis may have a dual effect on host-
pathogen dynamics, by acting firstly, on host susceptibility and secondly on the pathogenicity of Bd.

Finally, ecological traits and life-history strategies should also be considered. For instance, in areas suffering Bd-associated mortality, population declines have been shown to be most common in amphibians with an aquatic life stage, and species with stream dwelling tadpoles were found to be at greater risk compared with those breeding in ephemeral or isolated ponds (McDonald and Alford 1999, Kriger and Hero 2007), although see Weinstein 2009 and Longo et al. 2013 for examples of chytridiomycosis driven declines in terrestrial amphibians. In addition, experimental evidence supports the use of heat treatments to ‘cure’ infected frogs, and it is possible that thermoregulatory behaviour, such as basking, may be employed by wild amphibians to limit infection, or to delay the onset of disease (Woodhams et al. 2003, Andre et al. 2008).

1.4.2 Environment

Environmental conditions play a critical, though often poorly described role in determining when and where outbreaks of chytridiomycosis might occur. The impact of temperature has thus far received the greatest attention. In the laboratory, Bd growth and survival has been shown to be highly temperature dependent. Piotrowski et al. (2004) found that, whilst isolates of Bd grew and reproduced successfully at temperatures of 4-25°C, optimum growth was achieved between 17-25°C. Furthermore, an incubation temperature of 30°C, when maintained for 8 consecutive days, killed 50% of replicates. Subsequent experimental studies have offered additional insight: lower temperatures appear to be associated with a longer generation time, but an increased fecundity, and zoospores have been shown to have a longer survival time and greater activity under cooler conditions (Woodhams et al. 2008). Thus temperature dependent growth and development of Bd has the potential to significantly impact the density of infectious zoospores, and as a result, the force of infection. Overall, in vitro studies are consistent with the observation that, in the field, outbreaks of chytridiomycosis occur with greater frequency in cooler climates (Berger et al. 2004, Retallick et al. 2004, Ron 2005, Forrest and Schlaepfer 2011, Olson et al. 2013). However, like other
ectotherms, amphibian host fitness and immune function is also sensitive to ambient temperature (Carey et al. 1999). As a result, conclusions based on pathogen optima alone may be misleading.

Certain studies have also identified altitude as an important determinant of outbreaks of chytridiomycosis, reporting more rapid amphibian declines at higher altitudes and latitudes (Kriger and Hero 2006, Skerratt et al. 2007).

However, whilst the outbreak of disease does appear to be limited by certain environmental variables, occurrence of infection is not bound by the same restrictions. Instead, the landscape distribution of Bd is typical of a true generalist and includes highly variable conditions encompassing extremes both of temperature and of altitude (Seimon et al. 2007, Walker et al. 2010).

Most recently, there has been increasing interest in the potential role of non-amphibian organisms in determining infection dynamics. Experimentally, aquatic micro-fauna have been shown to rapidly lower the abundance and density of Bd zoospores with a corresponding decrease in the probability that infection will occur (Schmeller et al. 2014). In the same study, field data revealed a site-specific infection risk which correlated with the microfauna present. These findings support those of earlier works, which demonstrated grazing of chytrid zoospores by daphnia (a keystone zooplankton grazer in fresh water systems) and a strong effect of daphnia on the amount of Bd DNA in fresh water mesocosms (Kagami et al. 2004, Hamilton et al. 2012). Clearly, any reduction on the density of infectious Bd zoospores reduces the force of infection. This affects not only the probability of an infection developing, but also the potential impact of infection (Schmeller et al. 2014).

1.4.3 Pathogen
Genetic variability between isolates (initially believed to be low, James et al. 2009) may also affect the outcome of infection. Berger et al. (2005) provided the first evidence of isolate-specific virulence, by demonstrating variation in time to death following experimental infection of Litoria caerulea by three different isolates. Employing a wider panel of isolates, Fisher et al.
(2009) also found that isolates could be characterised by infection outcome and, crucially, that virulence could be linked to genotype. Most recently, population genetics studies have revealed that *Bd*, once believed to harbour limited genetic diversity (James et al. 2009), is in fact comprised of at least three, deeply divergent lineages (Farrer et al. 2011, Schloegel et al. 2012).

### 1.5 Origin and spread

Two hypotheses (which are not mutually exclusive) have been proposed for the emergence of *Bd* as a global pathogen of amphibians: (1) that it is a newly emerged pathogen (Skerratt et al. 2007), and (2) that it is an endemic pathogen, responding to changing environmental conditions (Rachowicz et al. 2005). Recent advances in *Bd* population genomics, however, show the global emergence of chytridiomycosis to be the result of a recently diverged and virulent lineage, described as the Global Panzootic Lineage (*Bd*GPL) that most probably emerged during the first half of the 20th century (Farrer et al. 2011). It has been suggested that *Bd*GPL is the result of a recombination event that was brought about due to anthropogenic mixing of pre-existing, but previously isolated allopatric lineages of *Bd* (Farrer et al. 2011).

The mechanisms underlying the current distribution of *Bd*GPL are not well understood. Until proven otherwise, the only environmental stage of *Bd* is the zoospore which is known to be both short-lived and of limited dispersal potential (Piotrowski et al. 2004). It has been suggested that migratory water birds may act as a vector for *Bd*. However, although *Bd* DNA has recently been detected on the feet of geese, its viability has not been confirmed (Garmyn et al. 2012). Hence, it is often surmised that long-distance dispersal of *Bd* is achieved solely, (or at least mainly), by the anthropogenic movement of infected amphibians.

Amphibians are traded for a variety of purposes, including the food trade, the pet trade, the supply of animals for zoological collections, biomedical research or for biocontrol purposes. In addition, large numbers of amphibians are inadvertently moved long distances (often between continents) as “stow-aways” in produce and other goods. Although exact figures are not available, the volume of trade is considerable. For example, between 1998 and 2002, the US...
alone imported more than five million kilograms of amphibians (Schlaepfer et al. 2005). Furthermore, the amphibian trade operates across all continents on which amphibians exist, and involves hundreds of different species (Schlaepfer et al. 2005, Jenkins 2007). This provides the opportunity for mixing of species that would not otherwise come into contact, including animals of captive and wild origin. Traded animals can come into contact with wild populations by accidental or intentional release, both of which are common. In a recent analysis, Fisher and Garner (2007) identified 28 species of introduced amphibians known to be carriers of Bd. For the majority of these species, infection is believed to be asymptomatic. Two such species have received particular attention: L. catesbeianus and X. laevis, both of which have been traded in vast numbers, are known to harbour infection, and further, have established feral populations in the Americas, Europe, Australia and Asia affording ample opportunity for spillover of infection into endemic amphibian populations. Trade in X. laevis dates back to the 1930s, when it was first used as a biological assay for human pregnancy testing, and it has since become the most common amphibian species used in biomedical research. The earliest published case of Bd infection in an amphibian is from a 1938 museum specimen of this species (Weldon et al. 2004). This finding is partly responsible for the 'Out of Africa' hypothesis (Weldon et al. 2004) under which Bd originated from and has spread out of Africa. However, biased sampling of archived specimens (by taxon and by country) makes this result and other similar findings difficult to interpret (Fisher et al. 2009). Furthermore, recent advances in Bd genomics (Farrer et al. 2011) show that historical infections cannot be properly evaluated without first determining lineage.

1.6 Amphibian species in the UK

1.6.1 Native amphibian species

The United Kingdom (UK) is home to six extant native amphibians (not including the recently reintroduced northern clade pool frog, Pelophylax lessonae, see below). This includes three caudate species: the smooth, or common, newt (Lissotriton vulgaris), the palmate newt (Lissotriton helveticus), and the great crested newt (Triturus cristatus); and three anuran
species: the common frog (*Rana temporaria*); the common toad (*Bufo bufo*); and the natterjack toad (*Epidalea calamita*). All UK species also occur in northern continental Europe. The ecology of UK amphibians is described by Beebee and Griffiths (2000). All native species are predominantly terrestrial, but return to the water during the spring in order to breed. The timing and duration of breeding season varies between species. Common frogs and toads are considered explosive breeders (sensu Wells 1977), on account of their relatively brief breeding season, whilst natterjack toads and all three newt species have a more drawn out breeding season and are thus described as prolonged breeders (sensu Wells 1977). Breeding periods vary considerably between different parts of the country, but approximate timings are as follows: common frog, February and March; common toad March and April; natterjack toad, April to June; smooth, palmate and great crested newts, March to June.

At mating, eggs are deposited in water and hatch into a short-lived aquatic larval stage. The newly metamorphosed young will typically emerge in summer/early autumn of the same year. However, if breeding has taken place particularly late in the year, larvae may overwinter in water and not emerge until the following year. It normally takes between 2 and 3 years for juveniles to reach sexual maturity.

Outside the breeding season, most metamorphosed amphibians native to the UK can be found on land, hiding in compost heaps, under rocks, wood piles or other suitable refugia. There are, however, two important exceptions to this rule. Firstly, the common frog may overwinter in water, buried into mud and sediment at the bottom of ponds. Secondly, caudate larvae can go through a process of arrested development (neotony) whereby they achieve metamorphosis and may reach adult size, but retain their external gills. These animals (which are sometimes capable of reproduction) may never emerge from the water, providing a continuous aquatic amphibian presence throughout the year. Of the three native species, the smooth newt shows the greatest tendency towards neotony although this is relatively rare occurrence.
During the 1950s and 60s most of the UK’s native amphibian species underwent significant declines. This was the result of widespread habitat destruction primarily due to changes in agricultural practice (Cooke and Scorgie 1983). In response, there followed a period of intensive conservation effort (including large scale pond restoration and a drive to increase the number of garden ponds) which proved very effective at slowing this trend (Cooke and Scorgie 1983).

All native UK amphibian species gained a degree of statutory protection under the Wildlife and Countryside Act of 1981. In the case of common toads, common frogs and the two smaller newt species (smooth and palmate), all of which are widespread in the UK, this is limited to protection against sale or trade (including dead animals or derivatives thereof). Under the same Act, the great crested newt and the natterjack toad are afforded an additional level of protection with the prohibition of reckless killing, injury or disturbance, including damage or destruction of their habitat.

Since the implementation of the Wildlife and Countryside act in 1981, the number of great crested newt populations in the UK has increased dramatically, with estimates ranging between 15,000 and 75,000 occupied sites (Langton 2009). However, this trend has not been observed for natterjack toads, the number of populations of which has remained stable. It is estimated that the total number of occupied sites is fewer than 60, almost all of which are found within just five estuaries along the coast line of north-west England and south-west Scotland (Banks et al. 1994, Buckley and Beebee 2004).

With the exception of the northern clade pool frog (see below) - the natterjack toad is the rarest and most intensively managed amphibian in the UK. Conservation management practices have included headstarting (rearing of spawn or tadpoles in captivity to the metamorphic stage) and translocation events to either boost existing populations or to reintroduce the species to sites of local extirpation.
The common toad is the only native amphibian known to be currently undergoing a decline in the UK. Though still widespread, common toad populations have been declining in lowland England for the last 25 years (Young and Beebee 2004). The cause(s) driving this decline remains unclear. The common toad belongs to the family of true toads, (Bufonidae). Globally this family has been shown to be overthreatened (Bielby et al. 2006), and contains significantly more rapidly declining species than would be expected by chance (Stuart et al. 2004).

The native status of the pool frog (P. lessonae) has undergone much debate over recent years (Snell 1994). For a long time this species was considered exotic, its presence in the UK believed to be the result of multiple introductions dating back to the early 1800s (Smith 1951). However, recent evidence has shown that the northern clade of this species was present in the UK long before any known introductions (Beebee et al. 2005, Snell et al. 2005). Unfortunately the last UK population belonging to this clade is believed to have become extinct in the early 1990s. A programme is now underway to reintroduce the species from a closely related population of northern clade pool frogs in Sweden. The programme is currently restricted to a single protected site in the East of England.

### 1.6.2 Non-native amphibian species in the UK

Southern clade pool frogs can be found in many parts of the UK (Wycherley et al, 2003). However, these populations are the result of introductions from continental Europe and are regarded as a non-native species (Beebee et al. 2005).

Although the release of non-native amphibian species in the UK is illegal under the Wildlife and Countryside Act 1981, a number of other non-native amphibian species can be found living wild in the UK. Introduced species include the marsh frog (Pelophylax ridibundus), the edible frog (Pelophylax esculentus), the midwife toad (Alytes obstetricans), the African clawed frog (X. laevis), the European tree frog (Hyla arborea), the North American bullfrog...
(Lithobates catesbeianus), firebellied toads (Bombina sp.), the Italian crested newt (Triturus camifex) and the alpine newt (Ichthyosaura alpestris) (Beebee and Griffiths 2000, Inns 2009).

The marsh frog, which is highly aquatic and belongs to the European green or water frog complex (which also includes the pool frog and the hybrid edible frog), has been perhaps the most successful alien amphibian species introduced to the UK (Beebee and Griffiths 2000). However, the alpine newt can also be found living in great abundance, all be it in relatively isolated populations (Bell and Bell 1993). The ecology of the alpine newt is similar to our native newts with which it frequently coexists. However, this species has a greater propensity for neotony compared with our native newt species and is therefore more often to be found occupying breeding sites throughout the year.

A number of alien species found in the UK, including the North American bullfrog and African clawed frog have been identified as asymptomatic carriers of Bd (Daszak et al. 2004, Ramsey et al. 2010). The midwife toad, on the other hand, is known to have undergone Bd-related population declines in Spain (Bosch et al. 2001) and there is evidence that at least one Bombina sp. is susceptible to Bd-related mortality (Stagni et al. 2004).

1.7 *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in the UK

The first case of *Bd* infection to be reported in the UK was in 1999, in a captive population of dead and dying exotic dendrobatid frogs (Cunningham, unpublished observations). The organism was not identified in the UK in the wild until 2004, when infection was detected in a population of introduced North American bullfrogs (L. catesbeianus) that had become established at a site in Kent, South East England (Cunningham et al. 2005). Infection at this site persisted following the apparent eradication of bullfrogs and subsequent surveillance in 2005 and 2007 showed infection in common toads (*Bufo bufo*), with associated mortality of recently metamorphosed animals (Cunningham, unpublished observations). Infection was also found in smooth newts (*Lissotriton vulgaris*) at this location, although no mortality was observed in this species.
In 2006, *Bd*-associated mortality was detected in a captive population of natterjack toads (*Epidalea calamita*), bred for the restocking of a wild population in Cumbria, North West England (Cunningham, unpublished observations). Investigations found a high prevalence of *Bd* infection at the receptor site. During further surveys of UK natterjack sites carried out in 2008, *Bd* was shown to be present in at least seven other breeding populations (Arai 2008).

In 2007, *Bd* infection was detected in alpine newts (*Ichthyosaura alpestris*) at a site in Kent, over 40 miles away from the index site. Recent research has shown that infected alpine newts are capable of transmitting infection to common toad tadpoles under experimental conditions (J. Sears, unpublished observations.).

In 2008, the Institute of Zoology carried out a large-scale survey to establish better the distribution of *Bd* in the UK. This work was funded by Natural England, the Countryside Council for Wales, Scottish Natural Heritage and the Institute of Zoology.

### 1.8 Aims, objectives and thesis structure

Amphibian chytridiomycosis is a sometime-fatal disease responsible for catastrophic amphibian declines in many parts of the world, including parts of mainland Europe. The causative agent of chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*) is present in the UK and, furthermore, has been linked to mortality in captive and wild native anuran species.

Despite a concerted global research effort, there remain many gaps in our understanding of the epidemiology of this now notorious pathogen. A common thread throughout the literature, however, is that the both the dynamics and outcome of infection are highly context dependent. As a result, it is not appropriate to generalise conclusions from studies in which conditions are non-identical. Thus, whilst there may be little evidence that *Bd* has induced amphibian declines outside of the well-known outbreak areas (Olson et al. 2013), following the recent detection of infection in UK amphibians, a comprehensive assessment of the
extent and nature of Bd infection in the UK is urgently required. The investigations reported in this thesis aim to make a contribution to this broader goal by attempting to address a number of specific objectives.

1) Determine the current geographical distribution of infection in the UK

2) Identify the amphibian host range of infection in the UK

3) Test the effect of selected site-level characteristics of amphibian breeding ponds on the occurrence of Bd

4) Determine whether there is any evidence of rapid change in the geographical distribution of Bd in the UK

5) Identify seasonal variation in the prevalence of infection in metamorphosed amphibians at mixed-species amphibian breeding sites in the UK

6) Investigate the impact of experimental exposure to Bd in two native UK amphibian species

7) Investigate the potential role of two native amphibian host species in the spread and maintenance of infection in the UK

This thesis is written as a series of 6 Chapters (of which this is the first). The methods and materials are laid out in Chapter 2. This chapter is intended to provide an overview of methodologies used, and to discuss limitations that are more generally applicable. Chapter-specific methodological detail can be found within the relevant chapters.

Chapter 3, which addresses objectives (1) to (4) describes two national cross-sectional pond surveys that were conducted in 2008 and 2011. Data generated from these surveys were used to map the geographical distribution of infection and to identify potential host species. Maps showing spatial epidemiological data can play an important role in assessing the magnitude of the problem and in defining priority areas for control. When data are collected over multiple time points (as in this study, where surveys were conducted in 2008, and in 2011), maps can also be used to monitor change, or progress following an intervention. Furthermore, where pathogen introduction is believed to be recent, visualisation of clustered
infected sites can help to generate hypotheses concerning the source and spread of infection. This chapter also made use of generalised linear modelling to identify associations between infection, and a range of site- and individual-level characteristics and offers a possible explanation for the current pattern of infected sites in the UK.

Chapter 4, which addresses objective (5), draws on epidemiological compartmental modelling to argue a case for seasonal heterogeneity in the prevalence of infection at amphibian breeding sites, based on the natural history of the pathogen, and of the host. It further describes a longitudinal study of known Bd-infected sites aimed at testing this hypothesis. The results are reported and possible explanations are provided.

Chapter 5 addresses objectives (6) and (7) and describes an attempt to experimentally infect smooth (*Lissotriton vulgaris*) and palmate (*Lissotriton helveticus*) newts using cultured *Bd* zoospores. It acknowledges that whilst existing field and experimental data suggest these species have a low susceptibility to *Bd*, they may, on account of their prolonged breeding period, none-the-less play an important role in the maintenance of infection within a mixed-species amphibian community. To investigate this hypothesis, post-exposure infection status was monitored over an extended period of time, incorporating an imposed winter hibernation and at least one transition between aquatic and terrestrial states. The results are presented and possible explanations are provided.

Chapter 6 offers an over-arching discussion of findings, attempting to place these within the greater global context of *Bd* epidemiology. A review of the limitations of this work is provided, together with suggestions for future avenues of research. Policy recommendations are also included.
2 MATERIALS AND METHODS

2.1 Licencing
The project methods were reviewed and approved by the Ethics Committee of the Institute of Zoology at the Zoological Society of London (ZSL). Licences were obtained from the relevant national authorities for the capture and sampling of protected species (natterjack toads \textit{(Epidalea calamita)} and great crested newts \textit{(Triturus cristatus)}, and for the post-sampling release of any non-native species captured. Experimental procedures were licensed by the Home Office (PPL 70/6227, PIL 70/23470).

2.2 Study design

2.2.1 Chapter 3
The national \textit{Bd} surveys, which took place in 2008 and 2011, followed a two-tiered cross-sectional design. Amphibian breeding sites were selected as the primary sampling units (PSU) and animals within each site were selected as the secondary sampling unit (SSU). In 2008, sites were surveyed on two occasions namely (1) spring and (2) summer. The purpose of sampling sites on two occasions in 2008 was to determine whether detection of infection varied between season. This was not found to occur and in 2011, sites were surveyed on only one occasion (spring or summer). The data were used to map the distribution of infection and to compare the distribution of infection between survey years. Covariate data were collected to test for site- and individual-level predictors of \textit{Bd} infection.

2.2.2 Chapter 4
The longitudinal study took place in 2010 and involved repeat sampling of a subset of known \textit{Bd}-infected sites to investigate relative infection (prevalence and infection burden) between species and through time. Each site was surveyed at five time points between March and June, the period of greatest amphibian activity in the UK. Covariate data were collected to investigate additional variation associated with age and sex of amphibians sampled.

2.2.3 Chapter 5
The experimental studies took place between 2010 and 2011 and followed a single factor experimental design, based loosely on the methods of Garner \textit{et al.} (2009). Smooth
(Lissotriton vulgaris) and palmate (Lissotriton helveticus) newts were randomly allocated to one of three treatments: repeated high dose Bd, repeated low dose Bd and repeated sham infection (the control group). The original design of the experiment was for all newts to be exposed during their aquatic phase, and to be housed in mixed sex pairs. This design was necessarily modified due to unforeseen circumstances, as described in full in Chapter 5. Throughout the study, animals were sampled at strategic intervals to monitor infection status. Survival and proportional change in mass were compared between the three treatments both during exposures and over a prolonged follow up period. Sex and starting mass were investigated as covariates.

2.3 Sample size calculations

2.3.1 Chapter 3

2.3.1.1 Sampling to estimate the proportion of infected sites

The target sample size for the number of sites to be surveyed in England during the 2008 national Bd survey was 90. This sample size was calculated as follows:

(1) The confidence interval around a proportion can be approximated to the normal distribution as below, where \(p\) is the observed proportion of infected sites, \(\alpha\) is the significance level and \(z\) is the desired percentile of the standard normal distribution:

\[
p \pm z_{(1-\alpha)} \times \sqrt{p(1-p)/n}
\]

(2) The sample size required in order to estimate the proportion of infected sites can therefore be calculated using:

\[
n = \left( \frac{z_{(1-\alpha)} \times \sqrt{p(1-p)}}{x/100} \right)
\]

where \(x\) is the percentage error on either side of the estimate.

(3) From a total of 30 ponds that were sampled in the UK between 2004 and 2007, 11 (37%) contained Bd-positive animals present on at least one occasion (Cunningham, unpublished
observations). Assuming this to be a true reflection of the prevalence of infected ponds in the UK, for a 95% confidence interval, with 10% error on either side, the required sample size is $89.21 \approx 90$ ponds.

Further detail on site number and allocation for the national *Bd* surveys (including sites in Wales, Scotland and Northern Ireland) can be found in the methods section of Chapter 3.

2.3.1.2 Sampling to detect infection

The target sample size for the number of amphibians to be sampled at each site visit was 30. This sample size was calculated as follows:

(1) The number of samples required to detect at least one infected individual within a population can be calculated using the formula:

$$ p = 1 - (1 - p_p)^n $$

$$ \rightarrow n = \log(1 - p) / \log(1 - p_p) $$

where $p$ is the probability of finding at least one positive result and $p_p$ is the true background prevalence (Cannon and Roe 1982).

(2) Assuming a background prevalence is 15% (this assumption was not based on prior data), for a 99% probability of detection, the required sample size is therefore $28.3 \approx 30$ amphibians. If the actual prevalence of infection is only 5%, a sample of 30 animals provides a detection probability of 79%.

2.3.2 Chapter 4

2.3.2.1 Number of sites

There were eight sites were included in the longitudinal study. This was the maximum number of sites possible, given the resources available and the requirement of the study that each site was surveyed five times.
2.3.2.2 Number of samples

Sampling targets for the number of amphibians swabbed during the longitudinal study were not specified for this work and in fact, it would have been prohibitive to do so. Although seasonal variation in amphibian activity can be broadly characterised (see Chapter 1), in reality, site-specific amphibian abundance is difficult to predict and can vary widely between years. Instead, the only specification given was that survey effort on each occasion should be time-limited (6 person-hours, see above) and that the maximum number of amphibians, across the full range of species, should be caught and sampled within this period. This methodology was considered the best approach for ensuring maximum total sample size across the study.

2.3.3 Chapter 5

2.3.3.1 Number of source populations

Experimental animals were sourced from wild populations. Under optimal conditions, all experimental animals would have been sourced from the same wild population. However, due to the large number of animals required (see below), and the recommendation that no more that 10% animals were removed from any single population to avoid negatively impacting population viability, this was not possible. As a result animals were collected from either four (palmate newts) or five (smooth newts) populations. All populations were located within a 10 km radius.

2.3.3.2 Number of replicates

The experimental design was based on 30 replicates per treatment, for each of the two newt species. As it was intended that newts should be housed in mixed sex pairs, the target sample size was 180 for both species (360 animals in total). A commonly used rule of thumb is that a sample size of 30 is usually sufficiently large as to satisfy an assumption of normality (on which parametric statistical tests rely e.g. to compare mean change in mass between treatment groups). In the absence of prior information relating to experimental exposure of either study species to Bd, the design of this study was guided by this benchmark. The emphasis was on using the minimum possible number of animals whilst maintaining optimal conditions for correctly identifying variation.
2.4 Selection of study sites

2.4.1 Chapter 3

The standard method for determining the distribution of a species (in this case, *Bd*) involves selecting sampling locations at random. However, amphibians are not distributed randomly across the landscape, and their abundance in ponds is highly variable. Assigning randomly selected ponds to surveyors was considered unfeasible as it would likely result in a high number of zero or very low captures for a high amount of effort. This was a particular concern as most surveyors were volunteers (see below), whose participation depended on goodwill and who might not wish to expend much effort in poor survey locations. Instead, and to maximise the number of adequately sampled populations, site selection during the first survey year (2008) was primarily dependent on the following criteria: (1) a moderate-to-large amphibian population, (2) easy access and (3) regional stratification to ensure a reasonable geographical distribution of survey sites. A subset of ponds were also selected specifically on account of the presence of natterjack toads, or because they were occupied by non-native amphibian species. The 2011 survey prioritized the resampling of sites surveyed in 2008. Additional sites were selected based on the criteria (1) to (3) outlined above.

2.4.2 Chapter 4

All sites chosen for inclusion in the longitudinal study tested positive for *Bd* in the 2008 national survey. In addition to prior evidence of infection, there were four further selection criteria: (1) amphibian diversity, to ensure amphibian presence throughout the survey period and to enable sampling across a wide range of species, (2) amphibian abundance, to ensure high catch rates, and therefore large sample sizes, (3) accessibility, including land ownership, and (4) geographic location, to limit travelling time between sites (resources were limited and hence there was a trade off between time spent travelling and time available for sampling). All eight sites were occupied by at least three different amphibian species including a mix of native and non-native species.

2.4.3 Chapter 5

For the experimental component of this project, animals were collected from a series of amphibian breeding ponds in Essex. None of the source populations had previously been
tested for infection with *Bd*. As a result prior exposure to *Bd* could not be precluded. It was considered unfeasible to source animals from known negative populations because (1) demonstrating freedom from infection with a reasonable level of confidence requires extremely large sample sizes (particularly where background prevalence is low as is typically the case for *Bd* in the UK) (2) sites currently testing negative for *Bd* may none-the-less have been exposed in the past and (3) due to large annual fluctuations in newt population sizes, it was difficult to specify donor populations in advance.

2.5 Selection of amphibians

2.5.1 Life history stage

2.5.1.1 Chapters 3 and 4

Throughout this project, samples were collected from metamorphosed amphibians only. Anuran larvae can be infected with *Bd*. However, in tadpoles, the distribution of infection is limited to the keratinized oral discs. In larger species, (e.g. North American bullfrogs), it is possible to swab tadpole mouthparts by carefully rolling a swab (see below) around the inside of the oral discs. However, the larvae of UK native anuran species are too small to sample in this way. Instead collection of samples for *Bd* testing requires the complete removal of larval mouthparts, an invasive procedure that cannot be conducted on live animals. Sampling of larvae was therefore not a suitable activity for volunteers (Chapter 3). A decision was made to exclude tadpoles also from the longitudinal study. Caudate larvae do not possess any keratinized body parts and as a result, there is no justification for sampling this life stage. Adult and juvenile anurans (where the term juvenile refers to the non-reproductive stage after metamorphosis) were distinguished according to size. Juvenile caudate species (newts) were considered too small to sample without inflicting damage.

2.5.1.2 Chapter 5

All experimental animals were adult newts
2.5.2 Species

2.5.2.1 Chapter 3

In 2008 there were no restrictions as to which species should be sampled and swab were submitted from six UK native species and four non-native species. In 2011, volunteers were asked not to target common frogs as *Bd* prevalence in this common species was found to be very low in 2008, and therefore it is not a good indicator species. In addition, as frogs are active early in the year (at a time when few other amphibians are available for sampling), and are frequently encountered in very high numbers, there was a risk of receiving a large proportion of *R. temporaria* samples and much lower numbers from other species which were potentially of greater interest, particularly as in 2011, sampling was restricted to a single time point at each site.

2.5.2.2 Chapter 4

During the longitudinal study, samples were collected from all amphibian species present. This included six native amphibian species and two non-native species.

2.5.2.3 Chapter 5

The experimental studies involved two native amphibian species, the smooth newt and the palmate newt. These species were chosen because (1) they are widely distributed in the UK and can therefore be sourced in high numbers from wild populations (2) they are capable of becoming infected in the wild (3) there are marked differences in the structure of the epidermis between aquatic and terrestrial phases with potential implications for infection and (4) at the time, there were no experimental studies investigating *Bd* infection in either species. The natterjack toad and the common toad (*Bufo bufo*) would have made more obvious models for infection because prevalence in these species in the UK is higher (Chapter 3) and both species have been shown to be susceptible to fatal chytridiomycosis (Bosch and Martinez-Solano, Minting 2012). However, a parallel PhD project was tasked with investigating the impact of infection in natterjack toads (Minting 2012) and there is considerable existing data concerning the experimental infection of common toads (Garner *et al.* 2009, Garner *et al.* 2011).
2.5.3 Location

2.5.3.1 Chapter 3
During the national *Bd* surveys, surveyors were asked to sampling amphibians exclusively from the water, and not on land. This methodology was decided upon in order to (1) standardise sampling between sites and (2) sample amphibians at greatest (perceived) risk of being infected.

2.5.3.2 Chapter 4
During the longitudinal study, sampling of terrestrial amphibians was permitted although in the event, only common toads were sampled on land. The location of sampled common toads was considered as a covariate in the analysis.

2.5.3.3 Chapter 5
For the experimental studies, it was the intention that study animals be exposed during their aquatic phase. As such, all study animals were collected during the breeding season, and all individuals were collected from the water.

2.5.4 Timing

2.5.4.1 Chapter 3
The national surveys took place in 2008 and in 2011. A longer time period between surveys would not have been possible within the time frame of this PhD. In 2008, the aim was to sample each pond twice (once in spring and once in summer) in order to capture different species cohorts during the periods of greatest amphibian activity. For the purpose of this study, spring was loosely defined as the months of March, April and the first two weeks of May and summer was defined as the last two weeks of May until the end of June. There was however, a degree of flexibility permitted to account for regional variation in amphibian activity and, ultimately, the survey relied on volunteers to determine dates which best defined local spring/summer conditions. This meant that some spring visits in the north of the country took place in June and a few summer visits in the south took place in early May.

The aim of capturing two time points was to determine whether detection of infection was significantly more likely in either season. This was not found to be the case in 2008 and
therefore in 2011 we requested that volunteers collect samples on one occasion only, during the period March-June, the timing of the visit being informed by the species present at the pond.

2.5.4.2 Chapter 4

During the longitudinal study, each site was surveyed on five occasions between March and June (again, the period of greatest amphibian activity in the UK). The first three visits were scheduled to take place at two-weekly intervals (mid-March, late March/early April and mid-April). This is typically the time at which common toads are most likely to be encountered in ponds. This species remains at breeding sites for a short period of time before dispersing on land. Hence, frequent surveys are required to maximise the likelihood of visits coinciding with common toads in any great number. It was also important that, if possible, common toad populations should be sampled across more than one time point (e.g. to compare Bd-infection prevalence at the beginning and end of breeding).

The remaining two surveys took place monthly, in mid-May and mid-June, during which it was anticipated that the four newt species (smooth, palmate, great-crested and alpine newts) would be the most commonly sampled amphibians. However, the natterjack toad and the southern clade pool frog are also active during these months.

The common frog, the earliest of UK amphibians to emerge from hibernation, is the only species for which peak breeding fell outside the survey period. Targeting this species would have required visiting ponds very early in the year, when all other species are either absent, or present in very low numbers. Hence, both diversity and sample size may have been low. Further, previous studies suggest that infection is not common in this species (Balaz 2013) and thus sampling of common frogs was not considered a priority.

As above, the exact timing of site visits was informed by regional variation in peak amphibian activity. Consequently, at each of the five time points, surveys at more southerly sites tended to be carried out slightly earlier than at sites further north.
2.6 Procedures

2.6.1 Capture of amphibians
Animals were caught primarily by hand, or by sweep netting of ponds. Bottle traps, for catching newts, were also permitted.

2.6.2 Collection of samples
Amphibians were sampled by swabbing the skin using sterile rayon-tipped swabs; MW100 dry swab, Medical Wire & Equipment Ltd. Swabbing followed a standardised methodology (Figure 2.1) which included 3 strokes each of the ventral femoral skin, the plantar aspects of both hind feet and the ventral pelvic skin of each anuran caught. In newts, the tail was also swabbed. This methodology is recommended by Hyatt et al. (2007) who found that swabbing provided better DNA recovery than either toe clipping or bathing.
Figure 2-1 Swabbing protocol followed for 2008 and 2011 UK Bd surveys. For all species, each stage should be repeated three times and should include, in anurans (a) the ventral femoral skin, (b) the lower hind limb, (c) the plantar aspect of the foot and digits and (d) the drink patch. In caudates the hind limb should be swabbed in a single motion (e), and the base of the tail should also be swabbed (f).
2.6.3 Biosecurity

2.6.3.1 Barriers to the spread of infection between sites

This project followed the biosecurity guidelines agreed by Natural England, Amphibian and Reptile Conservation, (formerly Herpetological Conservation Trust), Froglife and the Institute of Zoology (Appendix 1), which were issued to all those participating in field work.

2.6.3.2 Biosecurity between amphibians sampled in the field

In 2008 it was recommended that surveyors collect 30 amphibians into a shared container, for example a bucket, prior to the onset of swabbing. The advantage of this strategy was that animals could be released immediately after swabbing, with no risk of sampling the same individual more than once. In addition, the procedure was relatively simple for volunteers to follow in order to obtain data on whether or not Bd infection was present at any given site. The disadvantage of cohousing animals in this way, even temporarily, is that it may result in false positives, due to skin-to-skin contamination between infected and uninfected animals.

To address this limitation, during subsequent survey work (the 2011 national survey, Chapter 3 and the longitudinal study, Chapter 4), an additional level of biosecurity was introduced. Instead of collecting animals into a shared container, prior to swabbing, animals were temporarily held individually within clear plastic bags, which also contained water, vegetation and trapped air. Animals were contained in this way, away from direct sunlight, for a maximum of 6 hours. There were no reports of mortality under these conditions. Furthermore, disposable vinyl gloves were worn for swabbing and clean gloves were used for handing each animal.

2.6.3.3 Biosecurity between amphibians under laboratory conditions

For the reasons outlined above, all experimental animals were handled using vinyl gloves, which were changed between animals.

2.6.4 Detection and quantification of Bd DNA using real-time PCR

All swab samples were returned to the Institute of Zoology where they were stored at 4°C prior to analysis. There was a maximum delay between collection and analysis of samples of 18 months. Storage of samples for this period of time, even at room temperature, has been
shown to have no effect on zoospore recovery (Hyatt et al. 2007). Swabs were analysed using real-time PCR (rt-PCR) Taqman assay, following the protocol set out by Boyle et al. (2004). The analytical sensitivity of this assay is 0.1 zoospore equivalents and, relative to a panel of 21 Australian chytridiomycetes from the orders of Chytridiales, Blastocladiales and Spizellomycetales, the specificity is 100% (Hyatt et al. 2007).

2.6.4.1 Extraction of DNA
To extract the DNA from the samples each swab was transferred to a 1.5 ml Eppendorf containing PrepMan Ultra and silicon beads. These tubes were shaken for 90 seconds using a beadbeater and then centrifuged for 30 seconds at 14 500 rpm. The tubes were subsequently heated for 10 minutes at 100° C, cooled, and then centrifuged again for a further 30 seconds before removing the supernatant (the extracted DNA).

2.6.4.2 Real-time PCR
Prior to PCR, 4 µl supernatant was diluted using 36 µl molecular grate distilled water to create a 1:10 dilution to minimise the risk of inhibition posed by high PrepMan concentration. In preparation for rt-PCR, 96-well plates were filled with a reagent mixture comprising Taqman Universal MasterMix, forward and reverse primers (ITS-1 Chytr3 and 5.8S Chytr) and a fluorescent Taqman Chytr MGB2 probe (Applied Biosystems). Each plate also included a set of four reference standards (prepared from cultured Bd using a haemocytometer to determine concentrations) equivalent to 100, 10, 1 and 0.1 genomic equivalents (GE) of Bd DNA and, in addition, a negative control (water only). All samples, including the standards and negative control were run in duplicate. There were therefore 43 samples tested per plate. Plates were analysed using an ABI Systems rt-PCR machine. Successful amplification of target DNA was detected by a change in signal from the probe.

2.6.4.3 Quantification of Bd DNA
The number of PCR cycles (Ct value) required to detect amplification was used to estimate the quantity of Bd DNA in each well, based on a linear regression of Ct values for reference standards and using a cut off of $R^2 > 0.95$ as a quality control (plates for which $R^2 < 0.95$ were re-analysed until desired results were achieved).
2.6.4.4 Interpretation of results

Interpretation of rt-PCR results varied between chapters. In Chapter 3, samples collected during the cross-sectional surveys were classified as being infected providing both replicates were shown to contain \textit{Bd} DNA. Positive results were confirmed by checking that amplification curves were sigmoidal. It was not appropriate to use quantitative results (the number of genomic equivalents) for samples collected in 2008, because analysis of samples took place over a prolonged period of time during which a new set of standards were introduced which may have affected GE estimates and could, therefore, confound the results if quantitative results were relied upon. Interpretation of samples collected in 2011 necessarily follow the same methodology, although quantitative data are presented.

In Chapter 4, 0.1 GE, after correcting for dilution was considered the minimum acceptable value indicative of infection, following the guidelines recommended by RACE (Risk Assessment of Chytridiomycosis to European amphibian diversity). This threshold is intended to increase the specificity of rt-PCR as a diagnostic test for \textit{Bd}.

In Chapter 5, as in Chapter 3, no minimum acceptable value was applied. This strategy is considered appropriate under laboratory conditions where the risk of contamination (which may result in false positive results) is deemed to be minimal.

2.6.5 Culture of \textit{Bd} for experimental studies

Isolate SFBC 009 was chosen for the exposure treatments in Chapter 5. This isolate was isolated from a toe clip taken from an infected natterjack toad sampled in Cumbria, North West England in 2011, using the methods described by Longore (2000). Although the genotype of this isolate is undetermined, all UK isolates genotyped to date (eight in total) fall within the hypervirulent global panzootic lineage (Farrer \textit{et al.} 2011). This includes SFBC 014, isolated from the same host species sampled at the same location and isolated on the same date. Cultures were grown in TGHL broth (8 g tryptone, 2 g gelatin and 4 g lactose/1000 ml distilled water), again, following the methods set out by Longcore (2000). On the day of exposure an aliquot of culture was counted using a haemocytometer to determine the concentration of active zoospores. The culture was subsequently diluted down (using TGHL
broth) to achieve the desired concentration. Sterile TGhL broth was used for exposing control animals.

2.7 Acquisition of variable data

Individual-level characteristics were recorded at time of sampling. In Chapter 3, site-level information was collected via questionnaire, completed by surveyors either at the time of sampling (2011) or in the following year. Detail is provided in the methods section of Chapter 3.

2.8 Statistical methods

2.8.1 Models

2.8.1.1 Chapters 3 and 4

For the national *Bd* surveys and the longitudinal study, infection was modelled using generalised linear models (GLMs) and generalised linear mixed models (GLMMs) with a binomial error structure. Generalised linear models were used when all explanatory variables were fixed effects e.g. for modelling the binary infection status of a site based on a single survey result. Generalised linear mixed models were used to model hierarchical data e.g. to control for the effect of site on observations collected at the individual amphibian-level.

In Chapter 4, infection burden (the number of genomic equivalents detected by rt-PCR) was also modelled, using linear mixed models, with a normal error structure. The raw data were positively skewed. Logarithmic transformation (base e) was used to remove this skew (confirmed using histogram plots).

2.8.1.2 Chapter 5

For the experimental studies, survival data were plotted using the Kaplan-Meier survivorship function. Cox proportional hazards models were used to examine whether treatment influenced survival. A key assumption of Cox proportional models is that the hazard (e.g. risk of mortality), remains constant throughout the period of interest. This may not be an appropriate model for an experiment during which cumulative exposure to a pathogen might
be expected to result in cumulative risk of mortality. However, in this study, there was no evidence of non-proportionality.

Sex and starting mass were investigated as covariates and models were compared using Analysis of Variance (ANOVA).

Generalized linear modelling with normal errors was used to assess the effect of exposure on proportional change in mass with sex and starting mass included as covariates.

Prevalence of infection was calculated, together with 95% confidence intervals and comparisons between treatments and between time points were made using Fisher’s exact tests.

2.8.2 Model building

Model building followed a four-step process which included:

1. Identification of a limited set of explanatory variables chosen for their perceived biological relevance to the outcome of interest (individual- or site-level infection status)
2. Unconditional analyses of relationships between explanatory variables and the outcome of interest using a ‘liberal’ $P$-value to identify a short list of potentially important predictors.
3. Multivariable model building
4. Model selection using Akaike’s information criterion (AIC, see below)

Where the number of potential predictors (2) was relatively high (site-level analyses, Chapter 3), model building was automated and followed a process of forwards stepwise selection, a process by which the computer first fits a model with only the intercept and then selectively adds terms according to the chosen selection criterion (in this instance, AIC). Where the
number of potential predictors was low (individual-level analyses), model building was manual and all possible models were evaluated.

2.8.2.1 Selection of variables
A P-value of <0.2 was chosen to identify predictors that should be carried forward into the multivariable modelling stage. This cut-off is essentially arbitrary, but was chosen as a reasonable compromise between shortlisting unnecessary variables and missing out important predictors.

2.8.2.2 Selection of models
Selection of models was based on Akaike’s information criterion, first introduced by Akaike in 1973 (cited by Burnham and Anderson 2001) and defined by the equation:

\[
AIC = -2\ln L + 2(k + 1)
\]

where \( L \) is the maximised log-likelihood and \( k \) is the number of predictors in the model. These two components of the model provide a trade-off between model fit (maximum likelihood) and the number of parameters included in the model. As a result, this approach prevents overfitting of models (i.e. models so tightly fitted to the dataset that they have limited application to similar but non-identical datasets). The smaller the value of the AIC, the better the model. If two models have comparable log-likelihoods, the more parsimonious model (i.e. the one with fewer parameters) will have the smaller AIC. This method of model selection is more flexible than hypothesis testing as, providing the set of observations remains constant, AIC can be used to compare models regardless of whether they are nested (where the predictors in one model are a subset of the predictors in the other model).

2.8.2.2.1 Terminology
In relation to model selection, the term ‘full model’ refers to the model contain the full range of predictors identified, through univariable analyses as being potentially important and includes, where appropriate, any interactions between these variables. The optimal is the model associated with the lowest AIC value.
2.9 Potential sources of error

2.9.1 Random error

2.9.1.1 Sample size

The presence or absence of infection at site-level was inferred from a sample of amphibians from each population. Therefore, even when present, infection may have been missed (see section 2.3.1.2 on sampling to detect infection), purely on account of the fact that not all individuals were sampled.

2.9.1.2 Detection of Bd

No diagnostic test is 100% accurate. Thus it is possible that some infected animals were sampled, but no infection was detected. Real-time PCR can detect quantities of Bd as low as 0.1 zoospore equivalents, and as a result, can be considered to be an extremely sensitive assay. However, the sensitivity of swabbing amphibians in order to collect a diagnostic sample that truly represents the infection status of that animal is less easy to quantify. Thus, it may be that some infected amphibians were sampled, yet no Bd DNA was collected.

With regard to specificity, relative to a panel of 21 Australian chytridiomycetes from the orders of Chytridiales, Blastocladiales and Spizellomycetales, the specificity or rt-PCR is 100% (Hyatt et al. 2007) and thus the specificity of this assay would also appear to be extremely high. It is however, possible for false positive results to be generated prior to sample analysis whether as a result of contamination in the laboratory, during sample collection or due to cross-contamination between animals prior to sampling (see note on biosecurity).

2.9.2 Bias

Bias is defined as a systematic (non-random) deviation from the true value. Biased estimates can result from poor sampling methods such that certain populations or individuals are more likely to be selected than others.

The criteria used for selection of sites during the national Bd surveys are likely to have resulted in a biased sample in which easily accessible ponds with large amphibian population
sizes are over-represented. Furthermore, in 2008, a number of sites were selected specifically on account of being occupied by non-native species, or the native but comparatively rare natterjack toad. If the probability of Bd occurrence varies according to the presence or absence of these characteristics, estimates of prevalence may not be representative. For example, easily accessible sites are likely to be subject to a higher volume of human traffic. If human traffic is positively correlated with infection risk, based on the sites sampled in this study, the prevalence of infected sites in the UK is likely to be overestimated. In addition, associations between explanatory and response variables that appear important at sample level may no longer be applicable at national level. These limitations should be taken into consideration when interpreting the results of this work.

Bias can also be introduced when selecting which amphibians to sample. There is an abundant literature documenting that capture probability of wild animals varies according to disease status (e.g. Conner et al. 2000). If Bd infection makes capture of infected amphibians either more or less likely than uninfected individuals, this too could result in an over- or under-estimation of prevalence and spurious associations with covariates. There is also a growing body of research documenting variable infection according to life-history stage. In the current study, samples were collected exclusively from metamorphosed amphibians and thus all findings entirely overlook the contribution of larval stage amphibians to infection prevalence and dynamics in the UK.
2.10 Contributions to the work presented in this thesis

In Chapter 3, the 2008 UK national *Bd* survey was coordinated by Eddie Brede at the Institute of Zoology (IoZ) and was completed prior to the onset of this PhD. The samples were analysed by staff and students at IoZ. Statistical analysis of the results from the 2008 national survey and prior to this, the collection of additional site-level data from surveyors via a telephone questionnaire, were completed by the candidate. The candidate was responsible for all aspects of the design, training of volunteers, data collection and statistical analyses of the 2011 UK national *Bd* survey. Processing of samples using rt-PCR was carried out by the candidate or by technicians Gaby Peniche and Chris Durrant at IoZ. In both survey years, samples were collected almost entirely by volunteers from the Amphibian and Reptile Groups of the United Kingdom (ARG-UK).

In Chapter 4 samples were collected by two contracted consultants, Brian Banks and Gordon Haycock (the candidate was, at this stage, already engaged in the work of Chapter 5). Processing of samples using rt-PCR was carried out by the candidate or by technicians Gaby Peniche and Chris Durrant at IoZ. Statistical analyses were carried out by the candidate.

In Chapter 5, the candidate was responsible for all aspects of the work. Technicians Gaby Peniche and Chris Durrant assisted with animal husbandry, sample collection and analysis of samples using rt-PCR.
3 NATIONAL CROSS-SECTIONAL STUDIES

3.1 Introduction

*Batrachochytrium dendrobatidis* is a fungal pathogen first described in 1998 (Berger et al.). In susceptible hosts, it causes amphibian chytridiomycosis, a sometime fatal disease that has been implicated in global amphibian declines.

The earliest detected case of *Batrachochytrium dendrobatidis* (*Bd*) infection in wild amphibians in the UK, was in 2004 in North American bullfrogs (*Lithobates catesbeianus*) established at a site in Kent, South East England (Cunningham et al. 2005). This species, a known carrier of *Bd*, is suspected as having contributed to the spread of infection globally (Daszak et al. 2004, Garner et al. 2006, Schloegel et al. 2009). Infection at the Kent site persisted following the eradication of the bullfrogs, and subsequent surveillance at this site in 2005 and 2007 identified infection in common toads, with associated mortality of recently metamorphosed animals (A. Cunningham, unpublished observations). Infection was also found in smooth newts (*Lissotriton vulgaris*) at this site, although no mortality was observed in this species.

In 2006, chytridiomycosis-associated mortality was detected in a captive population of natterjack toads (*Epidalea calamita*) reared for the restocking of the species in Cumbria, North West England (Arai 2008). A high prevalence of *Bd* infection was found at the receptor site. During further surveys of UK natterjack sites carried out in 2006 and 2007, *Bd* was found in at least six natterjack breeding populations (Arai 2008). Natterjack toads are protected throughout Europe, but are particularly threatened in the UK where their range is restricted to fragmented sand dune and heathland. Thus, in the UK, this species is particularly vulnerable to stochastic events such as the introduction of pathogens.
In 2007, *Bd* infection was detected in alpine newts (*Ichthyosaura alpestris*) at a second site in Kent, over 60 km from the index site. The alpine newt is an invasive species in the UK with a long history of being kept by hobbyists and of being sold in garden centres and pet shops.

Further to the results of opportunistic sampling of amphibians for *Bd* infection during the period 2004-2007, this chapter concerns two national *Batrachochytrium dendrobatidis* (*Bd*) surveys that were carried out in the UK in 2008 and in 2011. There were four main objectives:

1. To map the current geographical distribution of *Bd* in the UK.
2. To determine the amphibian host range of *Bd* in the UK.
3. To test specific and biologically appropriate individual- and site-level variables for an association with infection.
4. To identify change in the prevalence and distribution of infection between survey years.

### 3.2 Materials and methods

Both surveys were based on a two-tiered cross-sectional survey design. The first stage in the sampling process involved identifying suitable survey sites (ponds). Within each site amphibians were swabbed for the presence of *Bd* DNA.

#### 3.2.1 Site selection

Sampling of ponds in the UK between 2004 and 2007 found that 37% of 30 ponds contained *Bd*-positive animals present on at least one occasion (Cunningham, unpublished observations). Assuming this to be an accurate representation of background infection prevalence, with random sampling of sites and 100% diagnostic sensitivity and specificity, a sample size of 90 ponds across the UK should provide an estimate of prevalence of infected ponds with 95% confidence and an accuracy of +/−10% (see Chapter 2 for a description of how this sample size was calculated). For the 2008 survey this figure was used to guide the target number of survey sites within England, the first country for which funding (by Natural England, NE) was secured. Funding from Scottish Natural Heritage (SNH, Scotland) and the
Countryside Council for Wales (CCW, Wales) was not confirmed until shortly before the survey, and after the planning stages. The total number of survey sites in Scotland and Wales (essentially ‘bonus’ sites) was defined by resource availability.

Site selection was dependent on the following criteria: (1) a moderate-to-large amphibian population, (2) easy access and (3) practicality of capture. In addition, regional stratification was employed to ensure a reasonable distribution of survey sites. In England, the aim was to recruit approximately equal numbers of sites across eight government regions (North West, North East, Yorkshire and the Humber, West Midlands, East Midlands, East of England, South East and South West). In Scotland and Wales, sites for sampling were identified by the respective national conservation agency. Additionally, in England, 18 sites were chosen because of the presence of natterjack toads (6 sites) or because they were known to contain non-native amphibians (12 sites), in order to help evaluate possible associations between the presence of these species and Bd. In both Kent and Cumbria, a cluster of sites was sampled as part of separate amphibian studies.

For the 2011 survey, site selection prioritised the resampling of as many 2008 sites as possible. The secondary aim was to increase coverage in previously underrepresented areas of the UK. This included Northern Ireland, which had not previously been sampled. Thus this survey involved the UK in its entirety, whilst the 2008 survey was limited to Great Britain (GB). In order to achieve a reasonably consistent level of coverage across the whole of the UK, sampling was stratified not by region, but by county with a target density of 1 site/1000km². This target density was based on the maximum county-level density achieved during the 2008 survey.

3.2.2 Amphibian sampling

At each survey the target number of amphibians sampled was 30 individuals. This number was deemed to be both feasible (with regard to catching and processing time as well as likely population size) and statistically robust. For example, given random sampling and a 100% sensitivity of detection, a sample size of 30 amphibians gives a 99% probability of detecting Bd, assuming an actual intra-site prevalence of 15%. Alternatively, if the actual infection
prevalence is only 5%, a sample of 30 randomly selected amphibians provides a detection probability of 79% (see Chapter 2 for sample size calculations).

3.2.3 Volunteer involvement

In both years surveys were carried out almost exclusively by volunteers. This approach meant that resources could be allocated to laboratory costs, rather than fieldwork, and as a result, greatly increased survey capacity. Volunteers were recruited primarily via Amphibian & Reptile Groups of the UK (ARG - http://www.arguk.org). This is a network of volunteer groups which undertake herpetofauna conservation work throughout the UK. Training was provided at national and regional meetings and members attending these events were subsequently able to carry out cascade training to further aid recruitment. In addition to carrying out training sessions at ARG UK meetings, preparation for the 2011 survey included a series of workshops with independent conservation groups in Northern Ireland. This was particularly important on two counts (1) N. Ireland had not previously been sampled (and there were therefore no existing sites) and (2) there are no active Amphibian & Reptile Groups in N. Ireland and as a result, no easily accessible network of volunteers with herpetological experience.

3.2.4 Timing of survey visits

In 2008, the aim was to sample each pond twice (once in spring and once in summer) in order to capture different species cohorts during the periods of greatest amphibian activity. Volunteers were given guidelines that spring visits should take place in March, April and the first two weeks of May, and summer visits should take place in the last two weeks of May, June and July. There was however, a degree of flexibility permitted to account for regional variation in amphibian activity and, ultimately, the survey relied on volunteers to determine dates which best defined local spring/summer conditions. This meant that some spring visits in the north of the country took place in June and a few summer visits in the south took place in early May.

The aim of capturing two time points was to determine whether detection of infection was significantly more likely in either season. This was not found to be the case in 2008 (see
below) and therefore in 2011 we requested that volunteers collect samples on one occasion only, during the period March-June, the timing of the visit being informed by the species present at the pond.

3.2.5 Sample collection

Surveyors collected samples by swabbing (using sterile rayon-tipped swabs; MW100 dry swab, Medical Wire & Equipment Ltd) the ventral femoral skin, the plantar aspects of the hind feet and the ventral pelvic skin of each anuran caught. In newts, the tail was also swabbed. Each area was swabbed three times and volunteers were issued with an illustrated protocol to ensure, as far as possible, a standardised technique (see Chapter 2). Only metamorphosed amphibians were sampled. At the time of sampling, records were made of the species sampled, its age (adult/juvenile), sex (male/female/unknown), and sampling date. In 2008 there were no restrictions as to which species should be sampled. However in 2011, volunteers were asked not to sample common frogs as *Bd* prevalence in this common species was found to be very low in 2008, and therefore it is not a good indicator species.

3.2.6 Survey packs

Survey packs included a survey protocol (Appendices 2 and 3) and a set of 60 (2008) or 30 (2011) swabs. In 2011, volunteers were, in addition, issued with a box of 100 disposable vinyl gloves, a roll of 50 sandwich bags, a pre-paid first class return envelope and two recording sheets (one for site details, another for recording swab details).

3.2.7 Inter-site biosecurity

All surveyors participating in the work were trained to observe strict biosecurity guidelines in order to minimise the risk of spread of pathogens (*Bd*, ranavirus, etc.) or invasive species (e.g. *Crassula helmsii*) between sites, as recommended by the Amphibian & Reptile Groups of the UK (ARG advice note 4, Appendix 1). Footwear and equipment were cleaned, disinfected (using 1% solution of DuPont™ Virkon®S or a 1:10 dilution of household bleach)
and thoroughly dried between sites. Disposable vinyl gloves were worn for handling animals, or for any activity involving contact with pond water, and these were changed between sites.

3.2.8 Covariate data collection

During the 2008 survey, data collection was limited to site name and grid reference, survey date, and species sampled. In some cases, volunteers provided sex/age data, although this was not consistent. In collaboration with Natural England, the Institute of Zoology compiled a list of key questions relating to biologically plausible site-level infection risk factors, which 2008 volunteers might be able to answer. These questions were formatted into a questionnaire by the candidate and completed via telephone interviews with volunteers during the period October 2009 to January 2010.

In 2011, a similar questionnaire was distributed via the survey pack, to be completed at the time of survey, and returned with the swabs. Certain questions were rephrased to make them more specific. For example, rather than asking whether dead or moribund amphibians had been observed at the site, surveyors were asked whether they had observed dead or moribund amphibians at the site that could not reasonably be explained by cold snap, predation or road mortality. The interpretation of reported morbidity/mortality is therefore survey year specific. Further, whilst in 2008 volunteers were asked for details of all known introductions of non-native amphibian species, in 2011, this line of questioning was limited to non-native species currently existing at a site. In hindsight, this modification was poorly judged, as historical introductions of non-native species may not be represented by present-day species composition. Thus, following the 2011 survey, telephone interviews were conducted during which surveyors were asked to report any previously unstated non-native amphibian introductions and the circumstances surrounding these introductions. Due to time constraints, interviews were held exclusively with volunteers who had surveyed Bd-positive sites. Therefore these data are not included in the analysis and have been provided for interest only. To standardise between years, the analysis utilises current non-native species presence only. In 2008, volunteers were also asked about historical introduction of native species. This question was retained in 2011 because it is not otherwise possible to distinguish between naturally occurring and introduced (or partly introduced) populations.
In summary, site-level covariates included in the analysis were as follows: (1) current presence/absence of each amphibian species, (2) current presence of non-native species (collectively) (3) known introduction of native amphibian species from at least 1km distance, (4) known introduction of fish, (5) observation of moribund or dead amphibians at any time (and in 2011, limited to morbidity/mortality not explained by a cold snap, predation or road mortality) and (6) permanence of the water body (permanent of ephemeral). For the 2008 survey, seasonal classification (spring, summer) of surveys was determined as described above and was used to inform data analyses. In 2011, sites were surveyed on one occasion only.

3.2.9 Real-time PCR analyses

DNA was extracted from swabs and processed using Bd-specific rt-PCR according to the methods of (Boyle et al. 2004) as set out in Chapter 2. There was an initial screening round during which pairs of samples were pooled. Pooling extracted DNA from two swabs has been shown not to significantly reduce the sensitivity of detection (Hyatt et al. 2007). Each pool was tested in duplicate. If a pooled sample was positive for Bd, the component samples were analysed individually.

3.2.10 Statistical analyses

The distribution of infected sites was visualised using Quantum GIS version 1.8.0 (Quantum GIS Development Team 2012). All statistical analyses were performed using R version 2.15.26 (R Core Team 2012).

Infection status was modelled as a binary variable at individual and site levels (0 = no positive samples detected, 1 = at least one positive sample detected) using logistic regression with binomial errors to examine univariable associations between response and explanatory variables. Any variables with $P$-values < 0.2 (see Chapter 2) were carried forward into the multivariable modelling phase. For the individual-level risk factors, multivariable analysis consisted of generalised linear mixed modelling (GLMMs, using the ‘lme4’ (Bates et al. 2012))
and `effects’ (Fox 2003) packages in R, with random effects corresponding to each individual site. The dataset was restricted to include samples from positive sites only.

For the 2008 site-level investigations, spring and summer datasets were analysed separately, thus eliminating repeat measures and negating the need for any additional random effect terms (which could not, in any case be fitted, because some sites only received a single visit). For the 2011 site-level investigations, all visits were included in a single model as sites were only visited on one occasion in this survey year. For both 2008 and 2011 site-level analyses, visits for which fewer than 26 individuals were sampled were removed to minimise variation in detection probability (a sample size of 26 gives a 98.5% confidence level for the detection of infection assuming a minimum prevalence of 15%). The site-level analysis of 2008 and 2011 data combined, was conducted using generalised linear mixed modelling, as above, with random effects corresponding to each site. To enable the use of random effect terms, the data were restricted to sites that had been visited at least twice (including site visits conducted in 2008 and 2011). To allow for the inclusion of a seasonal effect, 2011 site visits were classified as either spring, or summer as defined during depending on the seasonal classification of visits at that site in 2008, or, if the site had not previously been surveyed, the nearest site. As above, visits at which fewer than 26 samples were collected were not included.

For all individual- and site-level analyses, samples with missing data for any variable contributing to the full model were removed from the working dataset, together with any variables for which there was no variation in the corresponding response values. Final models were selected by forwards stepwise model selection using AIC.

Bootstrapping was used to compare survey results between years using 1000 resamples, (selected randomly, with replacement) to calculate 95% confidence intervals for the proportion of positive site visits in each survey year, and the proportion of positive samples in each survey year.
3.3 Results

3.3.1 Number of sites surveyed

In 2008, samples were collected from 125 sites across England (102), Scotland (7) and Wales (16). A total of 92 of these sites were successfully allocated to volunteers for repeat sampling 2011. However, in the event, only 63 site surveys were completed. In addition, a further 74 new sites were allocated to volunteers for sampling in 2011. Surveys were successfully completed at 59 of 74 new sites. Therefore the number of sites surveyed in 2011 was 122 (78%), of which 63 (52%) were also surveyed in 2008. Therefore 59 (48%) of sites surveyed in 2011 had not previously been sampled for Bd.

Volunteers reported two main reasons for failing to survey allocated sites namely conditions (17 sites) and personal circumstances (17 sites). Sampling conditions were a particular problem because 2011 was an unusually dry year. This meant that migration patterns were less predictable, and that at some sites, ponds had dried up before sampling was attempted.

3.3.2 Number of surveys conducted

The total number of surveys carried out in 2008 was therefore 227, of which 129 took place during the spring, and 98 took place during the summer. In some cases, where surveyors were unable to collect the full 30 samples on a single occasion, they attempted to make up the sample over a series of dates. In this instance, each visit was treated separately. The only exception to this rule applies to a subset of samples collected from the Kent cluster of sites, where samples were collected over a maximum of three consecutive dates and where animals were marked to avoid resampling. In 2011, all 122 sites were surveyed on one occasion only.

3.3.3 Number of samples collected

In total, 5776 amphibians were sampled in 2008. A sample size of ≥ 26 swabs was achieved during 178 individual surveys (103 in the spring and 75 in the summer).

In total, 3106 amphibians were sampled in 2011. A sample size of ≥ 26 swabs was achieved at 91 sites.
3.3.4 Summary of real-time PCR results

In 2008 (Figure 3.1), *Bd* DNA was detected at 25 sites (20% of total) in England (21/102, 21%), Scotland (1/7, 14%), and Wales (3/16, 19%). In 2011 (Figure 3.2), *Bd* DNA was detected at 14 sites (11% of total), in England (13/91, 14%) and Wales (1/12, 8%). No *Bd* DNA was detected at sites sampled in Scotland or Northern Ireland, where the sample sizes were 16 and 3 respectively.

In 2008 161 of 5776 samples (3%) tested positive for *Bd* DNA. Infection was detected in all native amphibian species and in two non-native species: the pool frog (*Pelophylax lessonae*) and the alpine newt (Table 1). The only species sampled that did not test positive was the marsh frog, *Pelophylax ridibundus*, but this species was only sampled at three sites (and only one sample was collected from a positive site). Infection prevalence at positive sites ranged from 3% to 97% in the spring (lower quartile [Q1] 3%, median 7%, upper quartile [Q3] 27%), and from 3% to 86% in the summer (Q1 5%, median 10%, Q3 17%). In the spring, five sites had a prevalence of *Bd* infection > 20%. These included two sites from which samples were taken almost entirely from natterjack toads (*Epidalea calamita*) (97% and 33% prevalence) and two sites at which the samples were collected exclusively from common toads (40% and 27% prevalence). The fifth site with a particularly high proportion of positive samples in the spring (73%) contained multiple species of non-native amphibians, although the samples collected were predominantly from smooth newts. In the summer three sites had a prevalence of *Bd* infection > 20%. Two of these sites contained non-native amphibian species (27% and 23% prevalence), while the third site, with an infection prevalence of 86%, was a common toad site. No site had a prevalence of > 20% in both spring and summer.

In 2011 51 of 3106 samples (2%) tested positive for *Bd* DNA. Infection was detected in all native amphibian species apart from the common frog and the great crested newt. Common frogs were only sampled at two sites and only one of these locations was shown to harbour infection. However, great crested newts were sampled at 43 different sites, seven of which returned positive samples in either 2008, or 2011. Three of the four non-native species tested positive on at least one occasion. These were the alpine newt, the southern clade pool frog,
and the marsh frog. There were no positive samples from edible frogs. However, this species was not well represented: only three individuals were sampled, all from the same (infected) site. Infection prevalence at positive sites ranged from 3% to 30% (Q1 3%, median 12%, Q3 17%). There were just three sites at which prevalence was shown to exceed 20%. The site with the highest prevalence (30%) was a non-native amphibian site, where multiple introduced amphibian species are found, living wild. A second site, at which the prevalence was 22%, was situated within the grounds of a zoological collection which includes exotic amphibians, although non-native species are not known to be present in the wild at this site. The third site, at which the prevalence of infection was 23%, was a natterjack toad site, and samples were taken almost exclusively from this species.
Figure 3-1 (a) Map of the United Kingdom showing the distribution of sites sampled for Bd (Batrachochytrium dendrobatidis) in 2008 during a national survey. Sites from which at least 26 samples were collected in both spring and summer sampling periods are represented by triangles (n = 69). Sites from which fewer than 26 samples were collected are represented by triangles (n = 56). In both cases, sites at which Bd DNA was detected are shown in red (n = 25) and sites at which Bd DNA was not detected are shown in blue (n = 100). In two counties, Cumbria and Kent, multiple sites were sampled in close proximity. These areas have been enlarged to show individual sites (insets (b) and (c) respectively).
Figure 3-2 (a) Map of the United Kingdom showing the distribution of sites sampled for Bd (*Batrachochytrium dendrobatidis*) in 2011 during a national survey. Sites from which at least 26 samples were collected are represented by triangles (n = 91). Sites from which fewer than 26 samples were collected are represented by circles (n = 31). In both cases, sites at which Bd DNA was detected are shown in red (n = 14) and sites at which Bd DNA was not detected are shown in blue (n = 108). In two counties, Devon and Kent, multiple sites were sampled in close proximity. These areas have been enlarged to show individual sites (insets (b) and (c) respectively).
Table 3-1 Results of real-time PCR analyses for samples collected in England, Scotland and Wales during a national survey for *Bd* (*Batrachochytrium dendrobatidis*) in 2008 (total samples = 5776). Results have been stratified by species, age, sex and survey period. Results for non-native species are shown in shaded rows. Percentages are given in parentheses.

| Species       | Spring |   |   | Summ  |   |   |   | Total  |   |   |   |
|---------------|--------|---------------|---------------|--------|---------------|---------------|---------------|--------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
|               | *n*    | *Bd+ve*       | *n*           | *Bd+ve* | *n*           | *Bd+ve*       |               | *n*    | *Bd+ve*       | *n*           | *Bd+ve*       | *n*           | *Bd+ve*       |               |               |               |               |
| **B. bufo**   | 966    | 29 (3.0)      | 216           | 18 (8.3) | 1182          | 47 (4.0)      |               |        |               |               |               |               |               |               |               |               |
| **R. temporaria** | 241  | 2 (0.8)       | 153           | 0 (0)    | 394           | 2 (0.5)       |               |        |               |               |               |               |               |               |               |               |
| **E. calamita** | 64   | 39 (60.9)     | 88            | 3 (3.4)  | 152           | 42 (27.6)     |               |        |               |               |               |               |               |               |               |               |
| **L. helveticus** | 675  | 2 (0.3)       | 713           | 2 (0.3)  | 1388          | 4 (0.3)       |               |        |               |               |               |               |               |               |               |               |
| **L. vulgaris** | 932  | 29 (3.1)      | 892           | 24 (2.7) | 1824          | 53 (2.9)      |               |        |               |               |               |               |               |               |               |               |
| **T. cristatus** | 293  | 0 (0)         | 284           | 1 (0.4)  | 577           | 1 (0.2)       |               |        |               |               |               |               |               |               |               |               |
| **I. alpestris** | 68   | 5 (7.4)       | 66            | 4 (6.1)  | 134           | 9 (6.7)       |               |        |               |               |               |               |               |               |               |               |
| **P. lessonae** | 0    | 0 (0)         | 19            | 3 (15.8) | 19            | 3 (15.8)      |               |        |               |               |               |               |               |               |               |               |
| **P. ridibundus** | 28   | 0 (0)         | 36            | 0 (0)    | 64            | 0 (0)         |               |        |               |               |               |               |               |               |               |               |
| unknown       | 1      | 0 (0)         | 31            | 0 (0)    | 32            | 0 (0)         |               |        |               |               |               |               |               |               |               |               |
| **TOTAL**     | 3288   | 106 (3.2)     | 2498          | 55 (2.2) | 5776          | 161 (2.8)     |               |        |               |               |               |               |               |               |               |               |
| **Sex**       |        |               |               |         |               |               |               |        |               |               |               |               |               |               |               |               |
| Male          | 1772   | 64 (3.6)      | 966           | 18 (1.9) | 2738          | 82 (3.0)      |               |        |               |               |               |               |               |               |               |               |
| Female        | 1118   | 25 (2.2)      | 914           | 22 (2.4) | 2032          | 47 (2.3)      |               |        |               |               |               |               |               |               |               |               |
| Unknown       | 378    | 17 (4.5)      | 618           | 15 (2.4) | 996           | 32 (3.2)      |               |        |               |               |               |               |               |               |               |               |
| **Age**       |        |               |               |         |               |               |               |        |               |               |               |               |               |               |               |               |
| Adult         | 2891   | 89 (3.1)      | 1929          | 40 (2.1) | 4820          | 129 (2.7)     |               |        |               |               |               |               |               |               |               |               |
| Juvenile      | 152    | 5 (3.3)       | 419           | 12 (2.9) | 571           | 17 (3.0)      |               |        |               |               |               |               |               |               |               |               |
| Unknown       | 225    | 12 (5.3)      | 150           | 3 (2.0)  | 375           | 15 (4.0)      |               |        |               |               |               |               |               |               |               |               |
Table 3-2 Results of real-time PCR analyses for samples collected in the United Kingdom during two national surveys for *Bd* (*Batrachochytrium dendrobatidis*) conducted in 2008 (n = 5776) and 2011 (n = 3106). Results have been stratified by species, age, sex and year. Results for non-native species are shown in shaded rows. Percentages are given in parentheses.

<table>
<thead>
<tr>
<th>Species</th>
<th>2008</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Bd+ve</td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. bufo</em></td>
<td>1182</td>
<td>47 (4.0)</td>
</tr>
<tr>
<td><em>R. temporaria</em></td>
<td>394</td>
<td>2 (0.5)</td>
</tr>
<tr>
<td><em>E. calamita</em></td>
<td>152</td>
<td>42 (27.6)</td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>1388</td>
<td>4 (0.3)</td>
</tr>
<tr>
<td><em>L. vulgaris</em></td>
<td>1824</td>
<td>53 (2.9)</td>
</tr>
<tr>
<td><em>T. cristatus</em></td>
<td>577</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td><em>I. alpestris</em></td>
<td>134</td>
<td>9 (6.7)</td>
</tr>
<tr>
<td><em>P. lessonae</em></td>
<td>19</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td><em>P. ridibundus</em></td>
<td>64</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>P. esculenta</em></td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>unknown</strong></td>
<td>32</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>5776</td>
<td>161 (2.8)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2738</td>
<td>82 (3.0)</td>
</tr>
<tr>
<td>Female</td>
<td>2032</td>
<td>47 (2.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>996</td>
<td>32 (3.2)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>4820</td>
<td>129 (2.7)</td>
</tr>
<tr>
<td>Juvenile</td>
<td>571</td>
<td>17 (3.0)</td>
</tr>
<tr>
<td>Unknown</td>
<td>375</td>
<td>15 (4.0)</td>
</tr>
</tbody>
</table>
3.3.5 Comparison of results between spring and sampling survey periods in 2008

There were 69 sites at which a sample size of ≥ 26 swabs was achieved in both the spring and the summer. Of these 69 sites, 17 (25%) yielded at least one positive sample: five sites tested positive only in spring, six only in summer, and six returned positive samples in both seasons.

3.3.6 Comparison of results from 2008 and 2011

In 2008, 25 (20%) of 125 sites returned at least one positive sample. In 2011, the equivalent figure was just 14 (11%) from a total of 122 sites surveyed. However, sampling effort varied between years, averaging 46 swabs per site in 2008 and 25 swabs per site in 2011. There were 63 sites at which sampling took place in both years. This included 18 sites at which Bd was detected in 2008. Half of these sites (n = 9) were also found to test positive when resurveyed in 2011. There were 45 sites that tested negative in 2008 and which were retested in 2011. Only one of these sites tested positive in 2011. In total, 184 sites were surveyed across 2008 and 2011 and infection was identified at 30 (16%) of these sites (25 from 2008 and 5 new sites identified in 2011).

Excluding visits where fewer than 26 samples were collected, 28 of 178 site visits returned positive samples in 2008 (16%, bootstrapped 95% CI 11–21%) and 13 of 91 site visits returned positive samples in 2011 (14%, bootstrapped 95% CI 8–22%).

3.3.7 Individual-level analyses

The protocol followed during the 2008 survey included co-housing of amphibians prior to sampling introducing an unquantifiable risk of cross-contamination between individuals. As a result, it would not have been appropriate to draw individual-level inferences from these data. The protocol followed during the 2011 national survey was modified to avoid contact occurring between individuals during sampling. As a result, individual-level analyses were conducted using data generated during this survey year only.
Individual-level analyses for the 2011 national survey were based on a restricted data set containing samples from positive sites only (n = 412). All three variables tested (species, age and sex) met the selection criterion imposed at univariable screening ($P < 0.2$). It was necessary to remove five of ten species levels due to a complete absence of positive test results for common frogs, great-crested newts, edible frogs, and an absence of negative results for marsh frogs and southern clade pool frogs. The resulting dataset comprised 397 observations. Once these species were discounted, it was no longer possible to fit age as a covariate, because the only positive samples from juveniles were taken from marsh frog (1) and pool frog (4). A further 17 samples were removed due to missing sex data. Therefore, model selection was based on 380 observations. In addition to random effects corresponding to each site, the optimal model retained both available fixed effects (species and sex, AIC 259.1). However, an interaction term was not supported (AIC 265.4). The results of this model are given in Table 3.3, and a matrix of species level odds ratios is given in Table 3.4.

The odds of *Bd* occurrence were statistically significantly lower in male amphibians, when compared to females (OR 0.39 95%CI 0.19 - 0.84). In addition, the odds associated with *Bd* occurrence in palmate newts were significantly lower than in either smooth newts OR 0.19 (95%CI 0.05 - 0.78), or alpine newts OR 0.13 (95%CI 0.03 - 0.61). There were no other statistically significant differences between species.
Table 3-3 Results of best individual-level multivariable model 2011. Parameters and tests are based on 380 observations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Species ¹</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species ¹</td>
<td>E. calamita</td>
<td>1.76</td>
<td>0.30</td>
<td>10.22</td>
<td>0.531</td>
</tr>
<tr>
<td></td>
<td>L. helveticus</td>
<td>0.31</td>
<td>0.06</td>
<td>1.62</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>L. vulgaris</td>
<td>1.59</td>
<td>0.39</td>
<td>6.48</td>
<td>0.514</td>
</tr>
<tr>
<td></td>
<td>I. alpestris</td>
<td>2.32</td>
<td>0.46</td>
<td>11.78</td>
<td>0.311</td>
</tr>
<tr>
<td>Sex ²</td>
<td>Male</td>
<td>0.39</td>
<td>0.19</td>
<td>0.84</td>
<td>0.015</td>
</tr>
</tbody>
</table>

¹ Reference category B. bufo, ² Reference category Female

Table 3-4 Results of best individual-level multivariable model, species levels in 2011. Parameters and tests are based on 380 observations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species (2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. bufo</td>
<td>B. bufo REFERENCE 1.76 (0.30-10.22) E. calamita 0.57 (0.10-3.31) L. helveticus 3.25 (0.62-17.02) L. vulgaris 0.63 (0.15-2.55) I. alpestris 0.43 (0.08-2.19)</td>
</tr>
<tr>
<td>E. calamita</td>
<td>REFERENCE 0.31 (0.06-1.62)</td>
</tr>
<tr>
<td>L. helveticus</td>
<td>5.70 (0.85-39.38) REFERENCE 5.17* (1.28-20.84)</td>
</tr>
<tr>
<td>L. vulgaris</td>
<td>1.10 (0.20-5.92) 0.19* (0.05-0.78) REFERENCE 1.45 (0.41-5.17)</td>
</tr>
<tr>
<td>I. alpestris</td>
<td>0.76 (0.12-4.81) 0.13* (0.03-0.61) 0.69 (0.19-2.45) REFERENCE</td>
</tr>
</tbody>
</table>
### 3.3.8 Site level analysis

Site-level analyses were carried out on a restricted dataset comprising surveys for which the number of animals sampled was \( \geq 26 \) (\( n = 178 \) for 2008 and \( n = 91 \) for 2011).

#### 3.3.8.1 2008 survey

For the 2008 site-level analyses, spring (\( n=103 \) sites) and summer (\( n=75 \)) datasets were analysed separately, thus eliminating repeat measures and negating the need for any additional random effect terms. There were five variables available for multivariable modelling (\( P < 0.2 \) at univariable screening) of the spring data, and six variables available for multivariable modelling of the summer data. For both spring and summer, it was necessary to run two parallel full models to allow the effect of non-native amphibians to be investigated both as individual species and collectively (Table 3.5, spring and Table 3.6, summer). Interaction effects were not included as there was no biological justification for doing so.

**Table 3-5 Model comparison for site-level analyses, spring sampling period.** All variables are fixed effects. Results are based on 82 observations and performed using logistic regression with binomial error structure.

<table>
<thead>
<tr>
<th>Full model</th>
<th>Optimal model</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1a) ( E. ) cala + ( L. ) vulg + ( T. ) cris + ( I. ) alp</td>
<td>( L. ) vulg + ( E. ) cala + ( I. ) alp</td>
<td>58.70</td>
</tr>
<tr>
<td>(2a) ( E. ) cala + ( L. ) vulg + ( T. ) cris + non-native amphibians</td>
<td>( L. ) vulg + ( E. ) cala + non-native amphibians</td>
<td>60.61</td>
</tr>
</tbody>
</table>

**Table 3-6 Model comparison for site-level analyses, summer 2008.** All variables are fixed effects. Results are based on 57 observations and performed using logistic regression with binomial error structure.

<table>
<thead>
<tr>
<th>Full model</th>
<th>Optimal model</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1a) ( L. ) helv + ( T. ) cris + ( I. ) alp + ( P. ) less + morbidity/mortality</td>
<td>( I. ) alp + morbidity/mortality</td>
<td>46.76</td>
</tr>
<tr>
<td>(2a) ( L. ) helv + ( T. ) cris + non-native amphibians + morbidity/mortality</td>
<td>non-native amphibians + morbidity/mortality</td>
<td>51.22</td>
</tr>
</tbody>
</table>
The results of the optimal models (i.e. with the lowest AIC values) are shown in Table 3.7 (spring) and Table 3.8 (summer). The only variable retained by both models was the presence of alpine newts (*I. alpestris*). In both cases, the occurrence of alpine newts was positively associated with detection of *Bd*, and in both cases this result was strongly statistically significant. Analysis of the spring data showed the presence of natterjack toads (*E. calamita*) to be strongly associated with the presence of infection, whilst the presence of smooth newts (*L. vulgaris*) was associated with a lower probability of detecting *Bd*. These results were not repeated in the summer. Apart from the presence of alpine newts, the only variable included in the summer model was the reported observation of dead/moribund amphibians and this was positively associated with infection (OR 5.0, 95%CI 1.0 - 37.7).

### Table 3-7 Results of optimal spring 2008 model. Parameters and tests are based on 82 observations and performed using generalised linear modelling and forwards stepwise model selection using AIC.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of <em>E. calamita</em></td>
<td>36.75</td>
<td>3.79</td>
<td>851.41</td>
<td>0.004</td>
</tr>
<tr>
<td>Presence of <em>L. vulgaris</em></td>
<td>0.04</td>
<td>&lt;0.01</td>
<td>0.31</td>
<td>0.007</td>
</tr>
<tr>
<td>Presence of <em>I. alpestris</em></td>
<td>24.50</td>
<td>2.68</td>
<td>542.89</td>
<td>0.009</td>
</tr>
</tbody>
</table>

### Table 3-8 Results of optimal summer 2008 model. Parameters and tests are based on 57 observations and performed using generalised linear modelling and forwards stepwise model selection using AIC.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of <em>I. alpestris</em></td>
<td>22.73</td>
<td>3.07</td>
<td>267.32</td>
<td>0.004</td>
</tr>
<tr>
<td>Observed morbidity/mortality</td>
<td>5.04</td>
<td>1.00</td>
<td>37.85</td>
<td>0.067</td>
</tr>
</tbody>
</table>

3.3.8.1.1 Kent and Cumbrian clusters

In 2008 clusters of sites were sampled in Cumbria (n=11) and Kent (n=7). Within each cluster, the maximum distance between any two sites was 7.5km (Kent) and 4.3km (Cumbria). No site
was further than 2.5km from the nearest neighbouring site, and many sites were less than 1km apart. At such close range, the result may be confounded by spatial autocorrelation. Therefore spring and summer models were re-run, excluding data from all sites falling within these two clusters. The results from the spring model (Table 3.9) are comparable of those achieved using the full dataset (Table 3.7). However, in the summer, once data from these clusters had been removed, there were only two records from sites at which alpine newts were present. As Bd had been found to be present at both of these sites, it was not possible to fit the summer model to this dataset.

### Table 3.9 Results of optimal spring 2008 model, excluding Kent and Cumbria clusters.

Parameters and tests are based on 71 observations and performed using generalised linear modelling

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of E. calamita</td>
<td>50.00</td>
<td>4.78</td>
<td>1190.02</td>
<td>0.003</td>
</tr>
<tr>
<td>Presence of I. alpestris</td>
<td>50.00</td>
<td>2.85</td>
<td>1402.79</td>
<td>0.006</td>
</tr>
<tr>
<td>Presence of L. vulgaris</td>
<td>0.05</td>
<td>&lt;0.01</td>
<td>0.48</td>
<td>0.017</td>
</tr>
</tbody>
</table>

3.3.8.2 2011 survey

There were four variables available for multivariable modelling after univariable screening namely the presence of natterjack toads, the presence of great crested newts, the presence of non-native amphibians (collectively), and reported morbidity/mortality. For the purposes of the 2011 survey, morbidity/mortality was only included if it was not consistent with predation, mortality or cold snap.

The results of univariable screening meant that non-native amphibians were only considered collectively and not by individual species. Therefore it was sufficient to build a single full model.

The optimal model (AIC 47.6 against AIC 49.4 for the full model) included three fixed effects: the presence of natterjack toads, great crested newts and non-native amphibians. The results of this model are given in Table 3.10. Presence of non-native species and of natterjack toads
was associated with a higher odds of infection. Great crested newt presence was negatively associated with infection, although this result was not statistically significant.

**Table 3-10** Results of optimal 2011 model. Parameters and tests are based on 76 observations and performed using generalised linear modelling and forwards stepwise model selection using AIC.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of non-native amphibians</td>
<td>19.57</td>
<td>3.62</td>
<td>137.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Presence of <em>E. calamita</em></td>
<td>22.48</td>
<td>0.69</td>
<td>798.55</td>
<td>0.054</td>
</tr>
<tr>
<td>Presence of <em>T. cristatus</em></td>
<td>0.27</td>
<td>0.04</td>
<td>1.39</td>
<td>0.137</td>
</tr>
</tbody>
</table>

**3.3.8.2.1 Kent and Cumbrian clusters**

In 2011, the Cumbrian cluster of sites was not resampled. However, five sites within the 2008 Kent cluster were resampled in 2011. As before, due to potential confounding which may have occurred as a result of spatially correlated characteristics associated with these sites, the optimal 2011 model was re-run, excluding surveys from these sites. Similar results were obtained (Table 3.11).

**Table 3-11** Results of optimal 2011 model, excluding Kent cluster. Parameters and tests are based on 72 observations and performed using generalised linear modelling.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of non-native amphibians</td>
<td>33.00</td>
<td>4.77</td>
<td>775.59</td>
<td>0.001</td>
</tr>
<tr>
<td>Presence of <em>E. calamita</em></td>
<td>22.00</td>
<td>0.69</td>
<td>775.59</td>
<td>0.055</td>
</tr>
<tr>
<td>Presence of <em>T. cristatus</em></td>
<td>0.30</td>
<td>0.03</td>
<td>1.80</td>
<td>0.211</td>
</tr>
</tbody>
</table>
3.3.8.3 Combined site-level analysis (2008 and 2011)

For the purposes of a combined 2008 and 2011 analysis, the dataset was restricted to sites which had been surveyed at least twice (185 site visits total, of which 145 took place in 2008 and 40 took place in 2011). This permitted site-specific random effect terms to be included in the model. Five variables met with the univariable selection criterion of $P < 0.2$. The presence of the alpine newt, the southern clade pool frog and the marsh frog were all positively associated with infection, as was the presence of non-native species collectively. The presence of great crested newt was negatively associated with infection. I was also interested in investigating two a priori confounders: survey season (defined by surveyors in 2008, and by proxy in 2011), and survey year.

Once missing values pertaining to each of the selected variables had been excluded, the dataset comprised 160 observations. In this sample, there were no surveys from pool frog sites at which Bd had not been detected and as a result, the effect of pool frog presence on the occurrence of Bd could not be modelled. There were two parallel full models (for modelling non-native amphibians as individual species and collectively (Table 3.12).

<table>
<thead>
<tr>
<th>Full model</th>
<th>Optimal model</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1a) $T. cristatus + I. alpestris + P. ridibundus + Season + Site$</td>
<td>$T. cristatus + I. alpestris$</td>
<td>117.6</td>
</tr>
<tr>
<td>(2a) $T. cristatus + non-native + Season + Site$</td>
<td>$T. cristatus + non-native$</td>
<td>119.7</td>
</tr>
</tbody>
</table>

Table 3-12 Model comparison for site-level analyses, both years. Results are based on 160 observations and performed using generalised linear modelling with binomial error structure.

The fixed effects are shown in normal type and the random effects in italics.
The optimal model contained just two fixed effects (Table 3.13). The presence of alpine newts (*I. alpestris*) was positively associated with infection, and this result was strongly statistically significant. Occupancy of a site by great-crested newts (*T. cristatus*) was negatively associated with infection. This result was moderately statistically significant.

**Table 3-13 Results of optimal model for 2008 and 2011 combined.** Parameters and tests are based on 160 observations and performed using generalised linear modelling and forwards stepwise model selection using AIC.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of <em>I. alpestris</em></td>
<td>41.78</td>
<td>2.78</td>
<td>626.68</td>
<td>0.007</td>
</tr>
<tr>
<td>Presence of <em>T. cristatus</em></td>
<td>0.085</td>
<td>0.01</td>
<td>1.03</td>
<td>0.053</td>
</tr>
</tbody>
</table>

### 3.3.9 Non-native species introductions

Of the 30 sites at which *Bd* was detected during the 2008 and 2011 national surveys, 12 can be linked to non-native introductions due to (1) the current presence of non-native amphibians or (2) historical records of non-native introductions. Concerning these sites, further information is provided below (Table 13.14). There was, in addition, one high-risk site, within the grounds of a zoo (founded 1959) where, although there were no known naturalised populations, non-native amphibians were present on site, in captivity.
Table 3.14 Information concerning non-native amphibian introductions that have taken place at Bd-positive sites. Sites at which non-native species are no longer present are in shaded rows.

<table>
<thead>
<tr>
<th>Location</th>
<th>Date</th>
<th>Species</th>
<th>Detail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penrith, Cumbria</td>
<td>1990s</td>
<td><em>I. alpestris</em></td>
<td>Existing population. Origin unknown</td>
</tr>
<tr>
<td>Otley, West Yorkshire</td>
<td>1990s</td>
<td><em>I. alpestris</em></td>
<td>Existing population. Captive population at a local school, released by school children</td>
</tr>
<tr>
<td>Market Drayton, Shropshire</td>
<td>1970</td>
<td><em>I. alpestris</em></td>
<td>Existing population. Animals supplied by Dutch retailer de Rover. Intentional release into nearby garden pond in 1970. Individuals from this population introduced to survey site in 1974.¹</td>
</tr>
<tr>
<td>Barnes, London</td>
<td>1950s</td>
<td><em>P. ridibundus</em></td>
<td>Existing population. Origin unknown</td>
</tr>
<tr>
<td>Canterbury, Kent (Woody’s Culvert, Hillside Farm and Lowe’s pond)</td>
<td>1990s</td>
<td><em>I. alpestris</em></td>
<td>Three sites in close proximity. Alpine newt populations at these sites are believed to be the result of natural colonisation from a nearby garden pond introduction site.</td>
</tr>
</tbody>
</table>
| Newdigate, Surrey          | 1903 onwards | Multiple, including *I. alpestris*  
T. carnifex  
P. lessonae (S. clade)  
P. esculenta | Site occupied by Beam Brook Aquatics Farm, established in 1903 and associated with the importation and breeding of non-native amphibian species until 1959. At this time, the site was taken over by L. Haig and Co Ltd, who used the site for breeding amphibians for scientific research until at least the mid-1970s. Both businesses used outdoor concrete pits for breeding animals. Newdigate is also the site of the first known alpine newt introduction in the UK (1920s).² |
| Nutfield, Surrey           | 1980s    | Multiple, including *X. laevis*  
*I. alpestris*  
T. carnifex  
P. lessonae (S. clade)  
P. esculenta | Site occupied by *Xenopus* Ltd from 1980-1989 and *Xenopus* reported breeding at and around this site during this time period. Alpine newts introduced from Newdigate in the 1980s. In addition, Italian crested newts and at least two species of the water frog complex have been introduced. The main source for these species is also believed to be Newdigate.² |
| Bramshill, Hants           | 1980s    | *P. lessonae* (S. clade)  | Origin unknown                                                          |
| Newton Abbot, Devon        | 1960s    | *B. variegata* &      
P. ridibundus        | Historical breeding site for fire-bellied toads (1960s), origin unknown. Existing population of marsh frogs, origin unknown |
| Northiam, East Sussex      | 1999 & 2004 | *I. alpestris* &      
L. catesbeianus        | Historical. Single record of an alpine newt, in 1999. Assumed to have originated from nearby, as yet unidentified breeding site. Bull frog from Cowden in Kent, the index site for *Bd* in the UK, 2004. |

¹ (Bell and Bell 1993)  
² (Langton et al. 2011)
3.4 Discussion

In 2008, infection was detected at 20% of all sites tested. In 2011, when sampling was limited to a single survey per site, infection was demonstrated at approximately 12% of sites surveyed.

The sites sampled in both years were not randomly selected. In order to obtain a sufficiently robust sample of animals at each survey, it was necessary to select sites at which large numbers of amphibians could reliably be encountered and furthermore, sites to which volunteers could safely and easily gain access. In addition, in 2008, 18 sites were chosen specifically because of the presence of natterjack toads (6 sites) or because they were known to contain non-native amphibians (12 sites). By attempting to re-survey as many of the 2008 sites in 2011 as possible, this selection bias was carried forward. Thus, these results may not be entirely representative of the true prevalence of infection in the UK. However, the results of both surveys clearly demonstrate that Bd infection in the UK is widespread.

Infection was detected in all eight English government regions, with the exception of the East of England and the North East of England and also in Scotland and Wales. Prior to his study, Bd had been detected in amphibian populations in just two British counties: Kent in South East England and Cumbria in North West England. Infection was not detected in Northern Ireland, although samples were collected from just three sites and in one year only. It was not possible to test sensibly for spatial clustering at the national level due to non-random sampling and over-representation of highly sampled areas. However, visualisation of the distribution of survey sites in both years shows that whilst Bd has been detected across a wide geographic range, infection is patchily distributed.

There was no evidence that the occurrence of Bd (at site-level) changed between survey years. Therefore, at least within this timescale, there was no indication of a broad-scale expansion of range. In addition, of the 44 sites at which Bd was not detected in 2008, and which were retested in 2011, only one was reclassified as being infected. Thus, with just a
single exception, sites from which infected samples were returned in 2011 were either from newly recruited sites, or known sites of infection.

Intra-site prevalence was generally low. This was in spite of a survey protocol which, in 2008, advocated co-housing of amphibians before they were sampled, introducing a real risk of cross-contamination which could have elevated the perceived prevalence. In 2008, 50% of Bd-positive site visits yielded an infection prevalence ≤ 10% (i.e. less than 3 positive animals out of 26 sampled). In 2011, the median prevalence at positive surveys was similarly low, at 12%. Furthermore, a quarter of all Bd-positive surveys were associated with an infection prevalence ≤ 5%. At such low levels of detectable infection, it is likely that infection was missed at some sites, even where the target sample size was met. For example, in 2008, 69 sites were successfully surveyed (at least 26 samples collected) in both the spring, and the summer. Seventeen of these sites were shown to be infected with Bd, but only six consistently tested positive in both the spring and the summer.

On account of the risk of cross-contamination between individuals during sampling, investigation of individual-level variables was only carried out for the 2011 dataset. The results of these analyses show a statistically significant difference in prevalence between males (lower) and females (higher). Similar results were obtained from a longitudinal study (Chapter 4). These results are surprising, since male amphibians tend to return to breeding ponds earlier than females and thus might be expected to be exposed to Bd for a longer period of time. Following these observations, further investigation is warranted.

The only statistically significantly inter-species difference in 2011 was that palmate newts were significantly less likely to test positive relative to smooth and alpine newts. This result is surprising considering that both the smooth and palmate newts (in particular) share comparable ecology and frequently coexist in ponds in the wild. Variation in host susceptibility to Bd is, however, a well-known feature of the epidemiology of this pathogen. Resistance to infection is most commonly credited to two innate mechanisms of immunity: (1) antimicrobial skin secretions (Rollins-Smith and Conlon 2005, Woodhams et al. 2007) and (2) presence of skin bacterial flora that produce anti-Bd substances (Harris et al. 2006, Woodhams et al.
It is possible that the palmate newt is benefiting from one or both of these defense mechanisms and this would be an interesting avenue of further investigation.

In 2008 the optimal model for the spring survey period predicted a strong negative association between presence of smooth newts and \( Bd \), and a strong positive association between the presence of \( Bd \) and both natterjack toads and alpine newts. The optimal model for the summer data included just two variables: presence of alpine newts and observed morbidity/mortality. Both variables were positively associated with the presence of \( Bd \). In 2011 the optimal model contained three variables, and predicted a higher odds of infection at sites where non-native species had been introduced (strongly significant) or where natterjack toads were present (moderately significant). The presence of great crested newts was negatively associated with infection, although this result was not significant.

In the combined analysis of 2008 and 2011 survey sites, the optimal model contained just two variables. The presence of alpine newts was strongly associated with infection whilst the presence of great crested newts was negatively associated with infection. This result was moderately significant. There was no support for the inclusion of natterjack toad presence in this model. However this analysis was performed using data from sites that had been surveyed at least twice. As a result, six of 12 possible natterjack sites were excluded.

In both 2008 and 2011, the presence of at least one non-native species was strongly associated with the presence of \( Bd \). The alpine newt was repeatedly identified as being associated with \( Bd \) infection: in both the spring and summer models for 2008, and in the overall (2008 and 2011) model.

Although the alpine newt is a native species in many parts of mainland Europe, it is an invasive species in the UK and its introduction to the wild in this country was made illegal in 1982 under schedule 9 of the Wildlife and Countryside Act 1981. There is, however, a long history of this species being sold in British garden centres and pet shops and naturalised populations are known to have existed since at least the 1920s (Gillett 1991). As a consequence, the alpine newt is now widespread across much of UK, apparently following multiple introduction events. In this study amphibians were sampled in ponds containing
alpine newts in Kent, Surrey, Hertfordshire, Shropshire, Cumbria, Staffordshire and Yorkshire, and infection was detected in alpine newt populations in all of these counties apart from Hertfordshire.

The other species positively associated with infection at the site level was the natterjack toad (spring sampling period only). Unlike the alpine newt, this species is native to the UK but is subject to a high level of human involvement associated with its conservation management. This includes translocation events either to boost existing populations, or to reintroduce the species to sites of local extirpation. As a result, animals have been moved over distances considerably greater than would be achieved through natural dispersal. The absence of a natterjack toad 'effect' in the summer may be attributed to lower water exposure which has been shown to influence infection status (as determined by swab sampling) in this species (Minting 2012). Of the four sites at which natterjack toads were tested in the summer, and at which natterjack toads were the most frequently sampled amphibian, the animals were found terrestrially at three of these. This observation is also relevant to the individual-level results, which show a statistically significant difference in prevalence of infection in natterjack toads between spring (high) and summer (low).

In the summer of 2008, there was a higher probability of detecting Bd at sites from which volunteers had observed dead or morbid amphibians. This result was marginally significant. However, as this information was collected retrospectively, no clinical or post-mortem data were obtained from these animals, so it was not possible to differentiate deaths resulting from other causes (e.g. predation, breeding mortality) and any that might have been due to chytridiomycosis.

Interestingly, this study found a negative association between Bd infection and the presence of smooth newts, although this effect was only observed in the spring. Although this result is difficult to reason, it may also be related to water exposure. Assuming infection risk increases with duration of aquatic period, it is reasonable to expect that prevalence will increase during the course of the breeding season (and this was found to be the case during a longitudinal
Another species found to be negatively associated with the presence of *Bd* at a site was the great crested newt, although this was only significant for the overall model. The reasons for a negative association between *Bd* presence and these newt species are unclear and require further investigation (e.g. see Chapter 4).

The positive association between the presence of non-native amphibians and *Bd* and also between the presence of natterjack toads and *Bd* could be driven by the same mechanism: co-introduction of the pathogen with human-assisted amphibian movements. Presence of non-native species at a site, by definition, is the result of an anthropogenic introduction event. While natterjack toads are native to the UK, conservation management efforts have included the rearing of tadpoles in captivity to the metamorphic stage, followed by release to the wild. On at least one occasion, natterjack toadlets were reared alongside a captive collection of exotic amphibian species (which included alpine newts). This process therefore provides the opportunity for direct or indirect contact with non-native species. It is possible therefore, that one or more *Bd* introductions to the wild in the UK occurred inadvertently via such conservation actions. In addition, translocation of natterjack toads between sites is common and, due to the patchy distribution of this species in the UK, translocation distances can be considerable, providing further opportunities for pathogen dissemination. For example, one population of natterjack toads, which in 2008 was shown to be infected with *Bd*, had been used a donor site for translocations to at least three other sites, and over distances of up to 100km. Of these three known receptor sites, two were sampled in 2008 and infected samples were returned from both.

Although widespread, the current distribution of *Bd* in the UK is patchy with low intra-site prevalence. This pattern and similarities in results between survey years are consistent either with multiple point introductions without epidemic spread, or with post-epidemic recovery and endemic persistence. In the UK, *Bd* was first described in wild amphibians in Kent in 2004, but due to a lack of sampling it is unknown if the pathogen was present in the UK prior to this date. These results are most consistent with multiple introductions associated with non-native species. Non-native introductions to the UK have occurred since at least the 1830s (Langton...
et al. 2011) and it is possible that Bd has been introduced multiple times over this period. Detailed molecular analyses may elucidate this further (see Chapter 6).
4 LONGITUDINAL STUDIES

4.1 Introduction

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) was first described in 1998 (Berger *et al.*). In susceptible hosts, it causes the disease chytridiomycosis. Infection has been linked to global amphibian population declines, although infection outcome has been found to vary widely between species and the full expression of fatal chytridiomycosis has rarely been reported outside of a few well describes outbreak areas (Olson *et al.* 2013).

The life cycle of *Bd* comprises two known stages: an aquatic flagellated zoospore (the infective stage), and a spherical, intracellular thallus (the reproductive stage) (Longcore *et al.* 1999, Pessier *et al.* 1999). Infection develops within the keratinized layers (stratum corneum) of amphibian skin or in tadpole mouthparts (Pessier *et al.* 1999). Zoospores appear to invade cells via a germ tube (Longcore *et al.* 1999, Berger *et al.* 2005), the end of which forms a swelling, which subsequently develops into a thallus (Van Rooij *et al.* 2012). Zoospores form inside the thallus and once mature, are released, via a discharge papilla, into the environment (Berger *et al.* 2005, Woodhams *et al.* 2008).

*Batrachochytrium dendrobatidis* was first detected in the wild in the United Kingdom (UK) in 2004, in a population of introduced North American bullfrogs (*Rana catesbeiana*) that had become established at a site in Kent, South-East England. Subsequent to this event, national surveys for the presence of *Bd* have shown infection to occur in all native amphibian species and furthermore, across a wide geographical distribution of sites (Chapter 3). Given the catastrophic impact this pathogen has had on amphibian population in some parts of the world (Chapter 1), there is now considerable interest in investigating the epidemiology of *Bd* in a UK setting, to better understand the potential threat posed by this now notorious pathogen to UK native wildlife, and to inform any attempts to mitigate against the further spread of infection.
The use of compartmental mathematical models is one of the cornerstones of infectious disease epidemiology (Anderson and May 1991). At their most simple, they consist of just two classes, or compartments, where individuals can either susceptible to becoming infected (S), or infected (I), which infers that they are also infectious (Figure 4.1). An uninfected individual's risk of becoming infected is often referred to as the force of infection. At a population level, this is a product of three key factors, (1) the rate at which contacts occur between individuals, c, (2) the probability that when an infected individual comes into contact with a susceptible individual transmission actually occurs, p and (3) the proportion of the population (N) that is actually infected, I/N. Changes to anyone of these three components will affect the rate of new infections.

Figure 4-1 SI compartmental model showing S (susceptible individuals), I (infected individuals), p (probability of transmission), c (contact rate and N (population size).
The UK is home to six native amphibian species: the common frog (*Rana temporaria*), the common toad (*Bufo bufo*), the natterjack toad (*Epidalea calamita*), the smooth newt (*Lissotriton vulgaris*), the palmate newt (*Lissotriton helveticus*) and the great crested newt (*Triturus cristatus*). The northern clade pool frog (*Pelophylax lessonae*), which became extinct in the UK in the 1990s, has recently been re-introduced to this country, at a single location in Norfolk, in the East of England.

All native species are predominantly terrestrial. However, breeding for all UK species occurs in the water, permanent or otherwise. The breeding period (which varies between species, see below) is associated with large congregations of amphibians, often in relatively small ponds and as a result, at very high densities. From an epidemiological perspective, the breeding season therefore represents a period of exceptionally high contact rates between hosts during which transmission of infection can occur.

The timing and duration of breeding season varies between species. Common frogs and toads are considered explosive breeders (sensu Wells 1977), on account of their relatively brief breeding season, whilst natterjack toads and all three newt species have a more drawn out breeding season and are thus described as prolonged breeders (sensu Wells 1977). Breeding periods vary considerably between different parts of the country, but approximate timings are as follows: common frog, February and March; common toad March and April; natterjack toad, April to June; smooth, palmate and great crested newts, March to June. As most amphibians exist in mixed-species assemblages in the UK, the result is an aquatic amphibian population that is constantly shifting. There are a number of important epidemiological consequences of this chain of events.

1. Overall host density is affected by migration of amphibians into and out of the pond according to their breeding patterns, and, by the contribution of developing young as eggs hatch into larvae and subsequently undergo metamorphosis.

2. The type and intensity of contact between hosts is affected by species, and life history. For example, for anuran species, prolonged physical contact between male and female breeding pairs is essential for fertilisation. Furthermore, fierce contact
may occur between males in competition for a mate. For the UK’s newt species, physical contact is not necessary for fertilisation and may only occur in passing, during courtship.

3. Host susceptibility varies between species, and life stages.

4. Given the nature of Bd, a pathogen whose pathophysiology is synonymous with its own reproduction, infectiousness can also be assumed to vary between hosts also.

5. The proportion of infected animals is affected not only by the rate at which susceptible individuals become infected (all of the above), but also by pre- and post-breeding migration, the hatching of eggs and the emergence of metamorphosed young.

Therefore, even at a very crude level, during the course of a single breeding season, the rate of contact between hosts (1 and 2), the likelihood of transmission (3 and 4) and the proportion of infected animals (5) can be expected to be extremely dynamic.

For Bd, an additional and crucially important contribution to the force of infection is the density of free-living zoospores, the infectious life stage of this pathogen. The pool of free-living zoospores is affected by both pathogen fecundity (the rate at which new zoospores are introduced to the pond), and zoospore longevity (the rate at which existing zoospores are lost from the population) (Briggs et al. 2005, Tunstall 2012).

Seasonal variation in the prevalence of Bd has been frequently attributed to a direct effect of changes in environmental conditions on each of these two components. At present, the strongest argument for an effect of environmental conditions on Bd growth and survival rests with temperature. In the laboratory, Bd growth and survival has been shown to be highly temperature dependent. Piotrowski et al. (2004) found that, whilst isolates of Bd grew and reproduced successfully at temperatures of 4-25°C, optimum growth was achieved between 17-25°C. Furthermore, an incubation temperature of 30°C, when maintained for 8 consecutive days, killed 50% of replicates. Subsequent experimental studies have offered additional insight: lower temperatures appear to be associated with a longer generation time, but an
increased fecundity, and zoospores have been shown to have a longer survival time and greater activity under cooler conditions (Woodhams et al. 2008). Thus temperature dependent growth and development of *Bd* has the potential to significantly impact the density of infectious zoospores, and as a result, the force of infection. Overall, in vitro studies are consistent with field observations that show, in tropical countries a decrease in infection prevalence with increasing temperatures (e.g. Woodhams et al. 2008, Rowley and Alford 2013). However, in colder climates such as the UK, where the average air temperature is 13°C in the summer, and only 6°C in the winter (UK Department of Energy and Climate Change 2013), *Bd* growth may be limited primarily by low temperatures, which, while they may not kill *Bd*, could hinder its growth (Woodhams et al. 2003, Kriger and Hero 2007).

The discussion thus far serves two main purposes, (1) to illustrate that transmission of *Bd* in a seasonally breeding mixed-species population of amphibians is highly dynamic and (2) that the factors contributing to this dynamic and many, varied and complex.

Any attempt to disentangle the various components of *Bd* transmission is beyond the scope of this chapter. Instead, the aim of this study was to describe the collective outcome of these processes, namely the prevalence of infection, during the course of a single breeding season.

To achieve this aim, eight sites of known *Bd* infection were surveys on five occasions between the months of March and June (the period of greatest amphibian activity in the UK). On each occasion, amphibians were caught and sampled (across the full range of species present) and samples were subsequently analysed for the presence of *Bd*. Samples were collected from metamorphosed amphibians only. The data were analysed for overall and species-specific trends in infection, and to compare infection between species. All native amphibians were sampled and, in addition, two non-native amphibians – the alpine newt and the s. clade pool frog.
4.2 Methods

4.2.1 Site selection

All sites chosen for inclusion in this study were found to be infected with *Bd* during a national cross-sectional *Bd* survey that took place in 2008 (Chapter 3). In addition to prior evidence of infection, there were four further selection criteria: (1) amphibian diversity, to ensure amphibian presence throughout the survey period and to enable sampling across a wide range of species, (2) amphibian abundance, to ensure high catch rates, and therefore large sample sizes, (3) accessibility, including land ownership, and (4) geographic location, to limit travelling time between sites. Details of the eight sites that were selected are given in Table 4.1. Results from the 2008 national survey are also provided. All eight sites were occupied by at least three different amphibian species. With the exception of the northern clade pool all native amphibians were represented. In addition, non-native species were found at three sites, alpine newts at Sunlane and Market Drayton, and pool frogs at Bramshill.
Table 4-1 Details of the eight survey sites selected for longitudinal sampling. Location and species composition are provided, together with the results from the 2008 national *Bd* survey. Non-native sites are shaded in grey.

<table>
<thead>
<tr>
<th>Site name</th>
<th>Country</th>
<th>County</th>
<th>British National Grid</th>
<th>R. temp</th>
<th>B. bufo</th>
<th>E. caia</th>
<th>L. helv</th>
<th>L. vulg</th>
<th>T. cris</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Merseyside</td>
<td>SD 293 115</td>
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<td>present</td>
<td>present</td>
<td>absent</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
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<td>Hampshire</td>
<td>SU 752 621</td>
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<td>absent</td>
<td>absent</td>
<td>present</td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
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<td>West Midlands</td>
<td>SO 907 913</td>
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<td>absent</td>
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<td>present</td>
</tr>
<tr>
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<td>Wrexham</td>
<td>SJ 299 461</td>
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<td>absent</td>
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</tr>
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<td>present</td>
</tr>
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<td>absent</td>
</tr>
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<td>Grid Reference</td>
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<td>Summer Present</td>
<td>Summer Absent</td>
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</tr>
<tr>
<td>-------------------</td>
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<tr>
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<tr>
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<td>present</td>
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<td>absent</td>
</tr>
</tbody>
</table>
4.2.2 Timing of survey visits

Each site was surveyed on five occasions between March and June, which is the period of greatest amphibian activity in the UK. The first three visits were scheduled to take place at two-weekly intervals (mid-March, late March/early April and mid-April). This is typically the time at which common toads are most likely to be encountered in ponds. This species remains at breeding sites for a short period of time before dispersing on land. Hence, frequent surveys are required to maximise the likelihood of visits coinciding with common toads in any great number. It was also important that, if possible, common toad populations should be sampled across more than one time point (e.g. to compare Bd-infection prevalence at the beginning and end of breeding). The remaining two surveys took place monthly, in mid-May and mid-June, during which it was anticipated that the four newt species (smooth, palmate, great-crested and alpine newts) would be the most commonly sampled amphibians. However, the natterjack toad and the s. clade pool frog are also active during these months. The common frog which is the earliest of UK amphibians to emerge from hibernation, is the only species for which peak breeding fell outside the survey period. Targeting this species would have required visiting ponds in early spring, when all other species were either absent, or present in very low numbers. Hence, both diversity and sample size may have been low. Further, previous studies suggest that infection is not common in this species (Balaz 2013) and thus sampling of common frogs was not considered a priority.

The exact timing of site visits was informed by regional variation in peak amphibian activity. As a result, at each of the five time points, surveys at more southerly sites tended to be carried out slightly earlier than at sites further north.

4.2.3 Amphibian sampling

The current study undertook an intensive sampling approach. Each site was surveyed on five occasions. Six person-hours were dedicated to each survey, with the aim of catching and sampling as many metamorphosed amphibians as possible, across the full range of species present. Animals were captured primarily by sweep netting of ponds. Bottle traps, for catching newt, were also permitted. Because bottle traps are left in situ overnight, and multiple animals can be caught within
the same trap (and may not have the same infection status), surveyors were requested to record trap number so that animals caught within the same trap could be identified and if necessary, an effect of catch method could be controlled for in the analysis.

Samples were collected by non-invasive skin swabbing, as has previously been described (Chapter 2). All animals were held separately between capture and swabbing and a clean pair of disposable vinyl gloves was used for the handling of each animal.

4.2.4 Real-time PCR analyses

DNA was extracted and processed as previously described and largely according to the methods of Boyle et al. (2004). However, in the current study, bovine serum albumin (BSA) was included in the Taqman mastermix to minimise inhibition of the PCR. A change in protocol relative to the national surveys (Chapter 2) is justifiable because there was no intention to compare results directly. An additional modification of the methods described in Chapter 2, is that samples that amplified in duplicate were only provisionally classified as being positive. Genomic equivalents (GE) for all samples in this category were derived from standard curves and I considered a GE of 0.1, after correcting for dilution, as the minimum acceptable value indicative of infection. This threshold value for the classification of rt-PCR results for Bd follows the recommendations of RACE (Risk Assessment of Chytridiomycosis to European amphibian biodiversity) for samples collected outside of a lab-based controlled experiment. All provisionally positive samples averaging less than 0.1 were defined as negative. Prevalence was calculated as the proportion of individuals testing positive at 0.1 GE or greater.

4.2.5 Covariate data collection

Species, sex and age were recorded for all animals sampled. In addition, surveyors were asked to record whether the animals were found terrestrially, or in the water (described as state), to provide details of any physical abnormalities observed and, for anuran species, to assess whether females were pre- or post-spawning.
4.2.6 Statistical analyses

All statistical analyses were performed using R version 2.15.26 (R Core Team 2012).

4.2.6.1 Modelling infection probability

Infection was coded as a binary response variable (0 = negative, 1 = positive). Time (visit number), species, age, sex, state and breeding status were identified as possible explanatory variables (all categorical). Due to differences in the onset and duration of the breeding period, a model that averages the effect of time across all species may be misleading. Further, the effect of age, sex, state and breeding status may also be associated with a species-specific effect.

The standard approach for modelling relationships of this nature would be to fit an interaction term between species, and any of the other covariates that may be of interest. However, in this study there were not enough infected samples to make this a viable option. For example, with respect to time, not all species were sampled during all five visits and, even when a species was sampled, it was not uncommon for all swabs to test negative for Bd DNA.

Instead, the data were subdivided into three taxon-specific groups (to reflect the onset and duration of the breeding period), and considered each group separately. Only samples from species with at least one infected sample were included. Further, samples from sites at which no infection was detected were excluded from the analysis. When subdividing the data, samples from caudate species were pooled, because all UK caudates (newts) share similar ecology. Return to breeding ponds begins early in the year and is a gradual process. Breeding itself takes place over several months and newts can remain at breeding sites late into the summer. Common toads and pool frogs (the only anuran species for which infected samples were obtained) were considered independently of one another because they have a different ecology: common toads migrate to the water en masse, during the spring, breed, and then return to land after only a short period of time. For pool frogs, breeding takes place much later, around mid-summer, and, unlike the common toad (which is almost impossible to sample in meaningful numbers outside its breeding season), individuals can be encountered at a breeding site for several months before and after breeding, mostly living terrestrially.
Unfortunately, subdividing the data in this way resulted in groups with few data points and hence it was not possible to investigate all variables. The largest group was that of the three newt species combined. These data were modelled using logistic regression with binomial errors to model infection status and generalised linear mixed modelling (GLMMs, using the ‘lme4’ (Bates et al. 2012) and ‘effects’ (Fox 2003) packages in R) with random effects corresponding to each site. Fixed explanatory variables consisted of time (visit number), species and sex. Age was not relevant to this model because only adult individuals were sampled. Likewise, because all individuals were captured on land, “state” was not used as a parameter in this model. Breeding status was not assessed in newt species.

For the common toad, all infected samples were collected from terrestrial adults from the same site and at a single time point. Generalised linear modelling was used, only, to compare infection rates between male and female toads.

For the pool frog, all infected samples were collected from juvenile aquatic animals and from a single site. Generalised linear modelling was used to compare infection rates between visits.

A GLMM was used to compare the prevalence of *Bd* across all species (not within groups) for which infected samples had been collected. Species was the only fixed variable. Random effect terms were included to account for the effect of site.

**4.2.6.2 Modelling infection intensity**

The infection intensity results were highly right-skewed. Log$_e$ transformation was used to remove this skew. Linear mixed models, with a normal error structure (using the ‘nlme’ (Pinheiro et al. 2013) package in R), were used to investigate the effect of species, with random effect terms relating to each site. Within the newt sub-group, the effect of sex and time (visit number), was also investigated because these variables were shown to be important in predicting infection status.
4.3 Results

4.3.1 Overview

All 8 sites were successfully surveyed on five occasions, between the 73rd and 172nd day of the year. Surveys were closely clustered in time (Figure 4.2), and at no point was there any overlap between the timing of site visit number across sites.

Figure 4-2 Scatter plot of survey dates (day of year) for all eight sites sampled during a longitudinal study of *Bd* infection in UK amphibians.

From a total of 2597 amphibians sampled, just 48 (1.8%) were shown to be infected. Tables 4.2 and 4.3 show a summary of rt-PCR results for each site, stratified by species (Table 4.2), and visit number (Table 4.3). Infection was found at six of eight sites (Figure 4.2), and in five of eight species.
### Table 4-2 Percentage of infected samples collected during longitudinal sampling: species breakdown.

<table>
<thead>
<tr>
<th>SITE</th>
<th>TOTAL</th>
<th>B. bufo</th>
<th>R. temp</th>
<th>E. cala</th>
<th>L. helv</th>
<th>L. vulg</th>
<th>T. cris</th>
<th>I. alp</th>
<th>P. less</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ainsdale</td>
<td>0% (0/245)</td>
<td>0% (0/110)</td>
<td>0% (0/26)</td>
<td>-</td>
<td>-</td>
<td>0% (0/89)</td>
<td>0% (0/20)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bramshill</td>
<td>5% (11/237)</td>
<td>0% (0/20)</td>
<td>0% (0/7)</td>
<td>-</td>
<td>0% (0/53)</td>
<td>3% (3/116)</td>
<td>-</td>
<td>-</td>
<td>21% (8/39)</td>
</tr>
<tr>
<td>Cotwall</td>
<td>0% (0/344)</td>
<td>0% (0/65)</td>
<td>0% (0/17)</td>
<td>-</td>
<td>-</td>
<td>0% (0/231)</td>
<td>0% (0/31)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hafod</td>
<td>&lt;1% (1/505)</td>
<td>0% (0/1)</td>
<td>-</td>
<td>-</td>
<td>&lt;1% (1/284)</td>
<td>0% (0/185)</td>
<td>0% (0/35)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Market Drayton</td>
<td>2% (5/312)</td>
<td>0% (0/10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1% (1/138)</td>
<td>-</td>
<td>2% (4/164)</td>
<td>-</td>
</tr>
<tr>
<td>Northiam</td>
<td>2% (8/440)</td>
<td>-</td>
<td>0% (0/2)</td>
<td>-</td>
<td>0% (0/19)</td>
<td>2% (8/419)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sunlane</td>
<td>7% (22/310)</td>
<td>4% (5/142)</td>
<td>0% (0/1)</td>
<td>-</td>
<td>1% (1/68)</td>
<td>0% (0/10)</td>
<td>-</td>
<td>18% (16/89)</td>
<td>-</td>
</tr>
<tr>
<td>Talacre</td>
<td>&lt;1% (1/204)</td>
<td>0% (0/65)</td>
<td>0% (0/6)</td>
<td>0% (0/28)</td>
<td>-</td>
<td>1% (1/105)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TOTAL</td>
<td>2% (48/2597)</td>
<td>1% (5/405)</td>
<td>0% (0/70)</td>
<td>0% (0/28)</td>
<td>&lt;1% (2/426)</td>
<td>1% (13/1291)</td>
<td>0% (0/86)</td>
<td>8% (20/253)</td>
<td>21% (8/39)</td>
</tr>
</tbody>
</table>

### Table 4-3 Percentage of infected samples collected during longitudinal sampling: visit breakdown.

<table>
<thead>
<tr>
<th>SITE</th>
<th>VISIT NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOTAL</td>
</tr>
<tr>
<td>Ainsdale</td>
<td>0% (0/245)</td>
</tr>
<tr>
<td>Bramshill</td>
<td>5% (11/237)</td>
</tr>
<tr>
<td>Cotwall</td>
<td>0% (0/344)</td>
</tr>
<tr>
<td>Hafod</td>
<td>&lt;1% (1/505)</td>
</tr>
<tr>
<td>Market Drayton</td>
<td>2% (5/312)</td>
</tr>
<tr>
<td>Northiam</td>
<td>2% (8/440)</td>
</tr>
<tr>
<td>Sunlane</td>
<td>7% (22/310)</td>
</tr>
<tr>
<td>Talacre</td>
<td>&lt;1% (1/204)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>2% (48/2597)</td>
</tr>
</tbody>
</table>
Figure 4-3 Map of England and Wales showing the distribution of sites surveyed during longitudinal sampling for the presence of *Bd*. Sites at which infection was detected are shown in red. Sites at which no infection was detected are shown in blue.
At sites where *Bd* was detected, there was no infection detected in common frogs (*n* = 26), natterjack toads (*n* = 28), or great crested newts (*n* = 35). However, these species were not well represented at these sites. At these six sites, during each of the first two visits (mid-March to early April), infection was only detected in one animal was collected. Over the remaining three visits, there was an increase in overall prevalence, reaching a peak of 5% prevalence during visit 5 (mid-June). The majority of samples were obtained from adult amphibians (98%) and, largely, from aquatic animals (93%). Abnormalities were recorded in approximately 3% of amphibians, although only one of these animals was shown to be infected (GE 5.0). This was an alpine newt which presented with inflamed digits and moderate skin sloughing.
4.3.2 Sampling distributions and the presence of infection

This study failed to detect infection in natterjack toads, common frogs and great crested newts. As a result, samples from these species were excluded from the analysis. Similarly, all samples from Ainsdale and Cotwall were excluded because it was not possible to confirm infection at these sites. Figure 4.4 shows the frequency and infection status of the remaining 1919 observations (note that y-axis scale varies). All three newt species were encountered at each of the five time points, with relatively constant frequency. However, there was considerable and contrasting temporal variation in the frequency of samples collected from the two anuran species (common toad, and the non-native s. clade pool frog).

Figure 4-4 Number and infection status (dark tones represent infection) of samples collected during a longitudinal study of Bd in UK amphibians. Surveys dates are approximately (1) Mid-March, (2) Early-April, (3) Mid-April (4) Mid-May and (5) Mid-June. Graphs (a) to (e) correspond to individual species. Graph (f) shows data across all six sites at which infection was detected.
4.3.3 Comparison of infection between species

Figure 4.5 shows the results of a generalised linear mixed model (GLMM) composed of one fixed effect (species), and one random effect (site). All 1919 observations were included in this model. Reference categories are given along the x-axis, and permit direct comparisons to be made between species. The odds of infection in the two non-native species (red and orange), was higher relative to the three native species in the analysis (green). This result was statistically significant. Relative to alpine newts, there was a higher odds of infection associated with pool frogs, but this was not statistically significant (OR 3.0, 95%CI 0.7 – 13.7). Of the non-native species, the palmate newt was associated with a lower probability of infection relative to the smooth newt (OR 0.3, 95%CI 0.1 – 1.7) and the common toad (OR 0.4, 95%CI 0.1 – 2.4). However, this result was also non significant.

Figure 4-5 Inter-species odds ratios for infection with Bd (shown on log₁₀ scale). Error bars represent 95% confidence intervals. Reference categories are given along the x-axis. Native species are shown in green. Non-native species are shown in orange and red.
4.3.4 Comparison of infection intensity between species

Infection intensity values (genomic equivalents, GE) were generally very low, ranging from 0.1 to 77.2 GE (lower quartile [Q1] 0.3, median 0.7, upper quartile [Q3] 3.8). There were only 17 samples for which the estimated quantity of Bd DNA exceeded 1.0 GE. Of this subset, 16 (94%) samples were from non-native species. Figure 4.6 shows the distribution of GE by species. Three high value outliers are not shown but relate to the alpine newt (one sample at 45 GE, and a second at 77 GE) and the pool frog (a single sample at 59 GE).

Figure 4-6 Box and whisker plot showing Batrachochytrium dendrobatidis genomic equivalents (GE) from samples collected during a longitudinal study of infection in UK amphibians. The top and bottom of each box indicate 1st and 3rd quartiles of the distribution whilst the middle line represents the median (2nd quartile). The ends of the whiskers indicate maximum and minimum values. Genomic equivalents for three samples exceeded 12 GE. These values are not shown due to scale.
Controlling for site as a random effect, the log\(_e\) transformed GE was significantly higher for both the pool frog (\(P.\) lessonae) and the alpine newt (\(I.\) alpestris) relative to two of three native species (the common toad, \(B.\) bufo and the smooth newt, \(L.\) vulgaris), Table 4.4. There were no significant differences between the palmate newt (\(L.\) helveticus) and either of the two non-native species. However, results for the palmate newt are based on just \(Bd\)-infected samples.

Table 4-4 Results of linear regression for log\(_e\) GE. Parameters are based on 1919 observations and results are shown relative to non-native species.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Level</th>
<th>Est.</th>
<th>SE</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I.) alpestris</td>
<td>(B.) bufo</td>
<td>-1.84</td>
<td>0.76</td>
<td>-2.41</td>
<td>0.021</td>
</tr>
<tr>
<td>(L.) helveticus</td>
<td></td>
<td>-0.64</td>
<td>1.13</td>
<td>-0.57</td>
<td>0.572</td>
</tr>
<tr>
<td>(L.) vulgaris</td>
<td></td>
<td>-1.93</td>
<td>0.54</td>
<td>-3.55</td>
<td>0.001</td>
</tr>
<tr>
<td>(P.) lessonae</td>
<td></td>
<td>-0.04</td>
<td>0.64</td>
<td>-0.07</td>
<td>0.947</td>
</tr>
<tr>
<td>(P.) lessonae</td>
<td>(B.) bufo</td>
<td>-1.80</td>
<td>0.87</td>
<td>-2.07</td>
<td>0.046</td>
</tr>
<tr>
<td>(L.) helveticus</td>
<td></td>
<td>-0.60</td>
<td>1.21</td>
<td>-0.50</td>
<td>0.620</td>
</tr>
<tr>
<td>(L.) vulgaris</td>
<td></td>
<td>-1.89</td>
<td>0.69</td>
<td>-2.75</td>
<td>0.009</td>
</tr>
<tr>
<td>(I.) alpestris</td>
<td></td>
<td>0.04</td>
<td>0.64</td>
<td>0.07</td>
<td>0.947</td>
</tr>
</tbody>
</table>
4.3.5 Infection in newts

At the six sites shown to be infected with *Bd*, samples were collected from 253 alpine newts, 973 smooth newts and 423 palmate newts (1649 total). No infection was detected in great-crested newts and hence this species was not included in the analysis. The best model, based on AIC (Akaike's information criterion), was also the full model (Table 4.5), and consisted of three fixed effects (visit number, species and sex) and one random effect (site). It was not able to evaluate interactions between fixed variables because there were insufficient data.

**Table 4-5 Model selection for newt multivariable modelling**

<table>
<thead>
<tr>
<th>Full model</th>
<th>Optimal model</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1a) Species + Visit number  + Sex + Site</td>
<td>Species + Visit number  + Sex + Site</td>
<td>366.8</td>
</tr>
<tr>
<td>(1b) Species + Visit number  + Age + Site</td>
<td>Species + Visit number  + Age + Site</td>
<td>370.5</td>
</tr>
<tr>
<td>(1c) State + Visit number  + Sex + Site</td>
<td>State + Visit number  + Sex + Site</td>
<td>374.8</td>
</tr>
<tr>
<td>(1d) State + Visit number  + Age + Site</td>
<td>State + Visit number  + Age + Site</td>
<td>378.7</td>
</tr>
</tbody>
</table>

The results of the full model are given in Table 4.6. The odds of infection were significantly lower in smooth and palmate newts relative to the non-native alpine newt (by a factor of more than five in smooth newts and a factor of almost 20 in palmate newts). However, there was no statistically significant difference between the two native species (OR 2.8, 95%CI 0.6 – 13.9 for smooth vs palmate, not shown in table). The third, fourth and fifth visits were associated with increasing odds of infection relative to visit one, and there was a strongly significant linear relationship of increasing prevalence throughout the survey period (Figure 4.7.a). This model also predicted a significantly lower probability of infection in male newts relative to females (Figure 4.7.b).
Table 4-6 Odds ratios for optimal newt GLMM. Parameters are based on 1649 observations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level</th>
<th>Odds ratio</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>L. helveticus</td>
<td>0.06</td>
<td>0.01</td>
<td>0.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>L. vulgaris</td>
<td>0.17</td>
<td>0.07</td>
<td>0.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Visit number</td>
<td>Visit 2</td>
<td>0.63</td>
<td>0.04</td>
<td>10.59</td>
<td>0.748</td>
</tr>
<tr>
<td></td>
<td>Visit 3</td>
<td>1.16</td>
<td>0.10</td>
<td>13.48</td>
<td>0.905</td>
</tr>
<tr>
<td></td>
<td>Visit 4</td>
<td>6.06</td>
<td>0.76</td>
<td>48.57</td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td>Visit 5</td>
<td>9.05</td>
<td>1.12</td>
<td>72.97</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>Per visit</td>
<td>8.25</td>
<td>1.87</td>
<td>36.23</td>
<td>0.005</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>0.40</td>
<td>0.19</td>
<td>0.84</td>
<td>0.016</td>
</tr>
</tbody>
</table>

1Reference category I. alpestris; 2Reference category Visit 1; 3Reference category Female

Figure 4-7 Fitted prevalence (%) for visit number (a) and sex (b), based on best newt GLMM. Parameters are based on 1649 observations. Note that the x-axis scale differs between (a) and (b).
4.3.6 Infection intensity in newts

Generalised linear models with normal errors to test whether species, sex or visit number had an effect on the burden of infection in infected animals. The full model comprised three fixed effects (species, sex and visit number), and a single random effect (site). However, there was no evidence that the addition of sex improved model fit (based on AIC) and thus this variable was dropped. The results of the final model are given in Table 4.7. There was a statistically significant difference in log_e transformed GE between alpine newts (\textit{I. alpestris}) and smooth newts (\textit{L. vulgaris}), but no significant differences between visit number.

Table 4.7 Results of newt only linear model of linear regression for log_e GE. Parameters are based on 35 observations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Level</th>
<th>Est.</th>
<th>SE</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{I. alpestris}</td>
<td>\textit{L. helveticus}</td>
<td>-0.25</td>
<td>1.69</td>
<td>0.15</td>
<td>0.885</td>
</tr>
<tr>
<td>\textit{L. vulgaris}</td>
<td>-1.75</td>
<td>0.67</td>
<td>-2.60</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Visit 1</td>
<td>Visit 2</td>
<td>-2.27</td>
<td>2.83</td>
<td>-0.80</td>
<td>0.468</td>
</tr>
<tr>
<td>Visit 3</td>
<td>-1.21</td>
<td>1.97</td>
<td>-0.61</td>
<td>0.545</td>
<td></td>
</tr>
<tr>
<td>Visit 4</td>
<td>-0.61</td>
<td>1.76</td>
<td>-0.34</td>
<td>0.731</td>
<td></td>
</tr>
<tr>
<td>Visit 5</td>
<td>-1.23</td>
<td>1.69</td>
<td>-0.73</td>
<td>0.473</td>
<td></td>
</tr>
</tbody>
</table>

4.3.7 Infection in common toads

In this study, common toads were sampled at four sites shown to be infected with \textit{Bd}: Bramshill (\textit{n} = 22), Hafod (\textit{n} = 1), Sunlane (\textit{n} = 142) and Talacre (\textit{n} = 65). However, \textit{Bd} was only detected in toads from one site, Sunlane. This site was occupied by four native amphibian species, (common toads, common frogs, palmate newts and smooth newts), and one non-native species, the alpine newt. At Sunlane, toads were adequately sampled during the second and third site visits to give meaningful results, but infection was only detected during the third visit, in mid-April, when five (9.3\%) animals tested positive for \textit{Bd}. There was little to distinguish this third visit from the previous one: neither visit coincided with peak breeding. The majority of samples, including all of the \textit{Bd}-positive samples were collected from terrestrial animals. Only adult toads were sampled, and males outnumbered females.
(1:19 in the second visit; 1:6 in the third visit). However, during visit two, all four of the female toads sampled were carrying eggs, whilst on visit three, seven of the eight females sampled, appeared to have spawned. Of the five Bd-positive toads, there were four males and one, post-breeding female. There was no evidence that male toads were any more, or less, likely to test positive than female toads (OR 0.52, 95%CI 0.06 – 4.86).

Sample sizes at the remaining three toad sites were considerably lower than those obtained from Sunlane. At Hafod, only one individual was sampled. At Bramshill, a site occupied by the s. clade pool frog, sampling took place in mid-March (n = 2 common toads sampled) and early April (n = 20). All samples were from male toads, and all but two samples were taken from aquatic animals. Finally, at Talacre (n = 65 common toads sampled), toads were sampled, in equal numbers, during each of the first three visits (n = 22, 22 and 21 respectively), but not during visits 4 or 5. At this site female toads were sampled both pre- and post-spawning and all but four individuals were aquatic.

4.3.8 Infection in pool frogs

Pool frogs were sampled during the third, fourth and fifth visits, but at one site only (Bramshill). All individuals sampled were aquatic. There were no infected samples detected during the third visit, when five adult common toads were skin-swabbed. During the fourth and fifth visits, the majority of samples were from juvenile animals (10 of 11 in visit four and 20 of 23 in visit five). All positive pool frogs detected were in this age group. Prevalence was high during visit four (8 of 10 juveniles, 80%), but low during visit five (1 of 20 juveniles, 5%) and this difference was significant (OR 0.02, 95%CI <0.01-0.25).
4.4 Discussion

All of the sites included in this study had a history of infection with *Bd* (Chapter 3). It was assumed, therefore, that sites would be detected at all sites over the course of the current study, during which sampling was considerably more rigorous. This was not the case. At two sites, Cotwall and Ainsdale, there was no infection detected. Further, no provisional positives at GE < 0.1 were identified.

In 2008, prevalence of infection at these sites was low (7% at Cotwall, and 3% at Ainsdale). At Cotwall, all four positive samples collected in 2008 came from juvenile common toads (although interestingly, not from juvenile common frogs which were sampled in greater number). This species, but not this age class, was sampled in the current study. At Ainsdale, the only positive sample in 2008 came from a natterjack toad. Overall, this species appears to be associated with a relatively high prevalence of infection. In the current study, there were no natterjack toads sampled at Ainsdale. Thus, clustering of infection within species or age cohorts could explain the lack of *Bd*-positive samples at these sites.

It is possible that there was, in fact, no infection present. However, for Ainsdale, at least, this seems unlikely. At this site, when resampled during the 2011 national survey, infection was once more detected in a single adult male natterjack toad.

From each of two further sites, Talacre and Hafod, there was only one *Bd*-infected sample returned (from a single smooth newt at Talacre, and palmate newt at Hafod, <1% prevalence overall). As above, there were no provisional positives at <0.1 GE from either site. In 2008, the prevalence of infection was very low at Hafod (one of 37 samples, just less than 3%). By contrast, at Talacre, prevalence was relatively high (12 of 58 samples), although all swabs were collected from natterjack toads, a species that was not well sampled in the current study (28 individuals, all from Talacre, over four of five survey intervals).
It may also be significant that these four sites (Cotwall, Ainsdale, Hafod and Talacre), where infection was either not detected, or only detected in one animal, encompass multiple water bodies. This is particularly true of Ainsdale and Talacre, both of which are coastal dune sites within which amphibians exist in a network of small, often temporary, pools. The presence of spatially defined subpopulations of amphibians, and/or local clearing of infection when ephemeral water bodies dry out, may also help to explain the shortage of Bd-positive results at these locations.

At the remaining four sites, infection prevalence ranged from 2-7%, and positive samples spanned at least two of five sampling periods. Non-native amphibians are present at three sites (Bramshill, Market Drayton and Sunlane). At the fourth site, Northiam, the population is composed of almost entirely of smooth newts, and there are no non-native amphibian species present. This site consists of a single water body: a small garden pond at which water levels can become very low, and hence population density is high, particularly during the later stages of the breeding season.

Excluding data from the two Bd-negative sites (Cotwall and Ainsdale), fewer than 2% of amphibians sampled tested positive for Bd. The low number of positive samples made it impossible to investigate different patterns of infection within the same model. As a result, the data were subdivided into taxonomic groups, based on ecological characteristics.

The largest dataset available included samples from the three Bd-positive newt species: the smooth newt, the palmate newt and the non-native alpine newt. Despite the study aims, this was the only group for which it was possible to investigate temporal variation. All UK newts (native and non-native) share a similar ecology. Females lay eggs singly, usually on the leaves of water plants, which are used to wrap the eggs and protect them from predators (Beebee and Griffiths 2000). All three Bd-positive species have a total clutch size of approximately 200-300 eggs, with females laying an average of around three to seven eggs per day (Beebee and Griffiths 2000, Garner and Schmidt 2003). The breeding period extends
over several months, typically March to June, with some regional variation. In the animals sampled in this study I found a strongly significant linear association between visit number (time) and the prevalence of Bd, with the modelled prevalence of infection only exceeding 0.5% in the fourth and fifth sampling periods (corresponding to mid-May and mid-June). *Batrachochytrium dendrobatidis* zoospores have been shown to survive for up to seven weeks in water (Johnson and Speare 2003). The terrestrial phase for UK amphibians is considerably longer than this period. Further, a resting, or resistant stage has not been identified for Bd (Longcore *et al.* 1999). Thus, based on existing knowledge, and in a system in which larvae metamorphose within a single season, the most parsimonious explanation for the persistence of Bd within a population is that amphibians can maintain infection over the hibernation period. The results of the current study suggest that assuming that newts do arrive at breeding sites already carrying infection, the prevalence is either extremely low, or for some reason, not detectable. As a pathogen with an aquatic infectious life stage, it is possible that there is little opportunity for transmission to occur on land. However, infection, and Bd-associated declines do occur in non-aquatic amphibians (e.g. Weinstein 2009), and thus, this explanation may not be entirely satisfactory. The increase in prevalence observed over the study period may be the result of a number of contributing factors. Given that newts were present in the water throughout the survey period, increasing infection may be the result of cumulative exposure and/or compromised immune function associated with loss of condition towards the end of an energetically costly breeding season. Further, host density, known to be an important determinant of the spread of infectious organisms within a population (Anderson and May 1991), may increase during the summer months, when water levels can fall and large numbers of anuran tadpoles are present. In addition to all of the above, which implicate the host, these results could reflect the thermal requirements of the pathogen. In culture, Bd grows best between 17° and 25°C (Piotrowski *et al.* 2004). In this study, the maximum water temperature for ponds during the first two visits was 13°C, well below the growth optima. During the third, fourth and fifth visits, water temperatures reached 17°C at some sites, but never exceeded 22°C. Minimum temperatures, on the other hand were as low as 6°C (visit 1) and 8°C (visit 2) and at these temperatures, Bd growth may be limited (Woodhams *et al.* 2003, Kriger and Hero 2007). It is unfortunate that in this study
water temperature was not measured in a standardised way (and at some site visits, not measured at all) and thus could not be included as a covariate in the analyses.

With the newt subgroup, there was a significant difference in prevalence between the sexes, with female newts more than twice as likely to be infected than males. This result is somewhat surprising. For all three species examined, males return to breeding ponds earlier than females, and often remain in the water after the females have left (Griffiths 1984). Further, male breeding characteristics are energetically costly and competition for females is intense (Beebee and Griffiths 2000). As far as I am aware, this finding of a female sex bias of Bd infection has not previously been reported. This is an area that warrants further investigation.

The common toad and the pool frog make up the remaining two ecological subgroups. Each species was considered separately because, while they are both explosive breeders, pool frogs breed considerably later in the year, and remain active for a much longer period of time. In common toads, infection was only identified at one site, Sunlane. Here, toads were sampled in greatest number at the end of March. However, infection was not detected until two weeks later, when infection was detected in five of 54 animals sampled. The total number of infected samples was low, hence it may be dangerous to read too much into these results. However, it might be interesting to assume, momentarily, that these data reflect the true status of the population. During both visits, toads were encountered en masse, but on land. Given that toads only congregate in this manner during breeding, it is not unlikely that these animals were sampled both on their migration to the water and, subsequently, on their return. The survey dates, and the two-week period between them, fit well with these events. Further, females sampled on the second of the two visits had spawned, whilst those sampled on the first, had not. If this interpretation is correct, the breeding period which takes place in water and affords many opportunities for direct contact, may account for the observed increase in infection prevalence. Unlike the gradual development of infection observed in newt populations, infection prevalence in toads appears to have increased much more quickly. Breeding in common toads is a fiercely physical activity. Because males out number females,
there is considerable competition for a mate, frequently resulting in ‘toad balls’ as male toads fight to displace one another from a sought-after female (Beebee and Griffiths 2000). As a result, contact rates are high. This may afford a more efficient route of transmission than via water alone. The common toad has been shown to be highly susceptible to infection and, in some instances, has been found to succumb to disease (Bosch and Martinez-Solano 2006). Hence, the rapid development of infection may be due to the inherent vulnerability of this species. Further, it is possible that rapid development of infection might result if Bd is already active within the water, perhaps due to an earlier arriving amphibian host. Alpine newts, a species shown to be associated with Bd infection are also present at this site. Although only four individuals were sampled at this time (all negative), given the dates of the two surveys, this may not reflect low alpine newt numbers, but rather, the relative abundance of common toads.

There were very few samples obtained from pool frogs, which were present at just one survey site, Bramshill. It may be significant that only juvenile animals were found to be infected, although given the late breeding season of this species, any juveniles encountered would have hatched out and metamorphosed during the previous year. Hence, the transient period of immunosuppression that occurs during metamorphosis may not be relevant to this result. It may also be of interest that the prevalence of infection was significantly lower in mid-June relative to the previous visit, which took place in mid-May. This is the opposite of the pattern observed in newts, but there may be different processes driving the infection dynamics in these two groups. Unlike the other species in this study, pool frog presence at a breeding site is not necessarily aquatic. During the period of the study, adults and juveniles could be found terrestrially, several metres from the water’s edge, including basking on rocks. Temperatures > 25°C have been used to experimentally clear Bd infection from amphibians (Woodhams et al. 2003). If pool frogs are able to raise their body temperature to a sufficient level by this basking behaviour, they may be able to suppress, or clear, Bd infection naturally (Richards-Zawacki 2010).

One of the main outcomes of the two national surveys (Chapter 3) was an apparent association between the presence of non-native amphibians and the presence of Bd. When
prevalence of infection was compared between species, both non-native amphibian species were associated with a statistically higher odds of infection relative to the native species. Further, whilst infections in native species rarely exceeded 1 GE, *Bd* loads associated with non-native species were considerably higher. Spill-over of *Bd* from non-native hosts could, therefore, result in far greater levels of exposure than would naturally occur in native populations in the UK. These results suggest that, not only should non-native species be considered a risk factor for introducing *Bd* to a population, they may play an important role in the persistence of infection in native amphibian populations. However, as almost all samples from pool frogs were collected from juveniles, at least for this species, the apparent effect of ‘non-native’ may be better explained by life history stage.

This study found a statistically significant temporal variation in the prevalence of *Bd* in newts, which shows that surveying ponds early in the breeding season is unlikely to detect *Bd* infection even if it is present at a site. This observation may also be applicable to surveying newts in other temperate countries. For common toads, sampling may be more effective after breeding. However, further temporal studies (which would benefit from the use of radio-tracking devices) would be helpful to establish patterns of infection in this species (and in the three remaining native species for which I was unable to obtain a large enough sample size from known-infected ponds). Finally, this study suggests that at least non-native species (the alpine newt) is associated with a higher prevalence and intensity of infection than the native species tested. Further investigation into the potential impact of *Bd* in the UK should focus on sites at which native species are syntopic with introduced species, as these cohorts of native amphibians may be at the highest risk of suffering *Bd*-related declines.
5 EXPERIMENTAL STUDIES

5.1 Introduction

The amphibian pathogen Batrachochytrium dendrobatidis (Bd) is notorious for the wide range of hosts it can infect and the variation present within host response (Fisher et al. 2009, Olson et al. 2013). Understandably, research relating to Bd has typically been focused on host species that easily succumb to infection, especially where infection leads to the development of disease (chytridiomycosis). However, more resistant species should not be overlooked. By acting as reservoir or spillover hosts, these amphibians may play an important role in the maintenance or spread of infection within a multi-host system. Further, if resistance to infection is energetically costly, exposure of these species to Bd may have implications for long-term population viability (Garner et al. 2009, Luquet et al. 2012).

In the UK, Bd is widely distributed and all native species are capable of becoming infected (Chapter 3). This includes the smooth newt (Lissotriton vulgaris), and the palmate newt (Lissotriton helveticus). The intensity and the prevalence of infection in these two species are consistently low (Chapters 3 and 4). This could suggest a level of resistance to infection attributable to host immunity (Rollins-Smith and Conlon 2005, Harris et al. 2006). However, the results of a longitudinal study suggest that extrinsic factors may also be important (Chapter 3). For newts, the most dramatic environmental change occurs with the transition between land and water. Given that Bd has an aquatic infective life stage (Piotrowski et al. 2001, Berger et al. 2005), this shift is likely to be associated with a change in the risk of exposure to Bd. Furthermore, there are marked differences in the structure of the epidermis between aquatic and terrestrial newts (Warburg and Rosenberg 1997), and this may affect susceptibility to infection. Based on these factors, an appreciation of how infection dynamics vary between aquatic and terrestrial environments is likely to form an important step in understanding the role of these species in the epidemiology of Bd in the UK.
Experimental studies provide a controlled environment in which to study a limited number of variables. As such, they can form a useful step in understanding the mechanisms underpinning events in the natural world.

In the current study, smooth and palmate newts were exposed to $Bd$ zoospores under experimental conditions. Animals were subjected to an intensive, four-week dosing regimen, followed by lengthy period of observation including four months of hibernation and at least one transition between land and water.

Following the findings of Chapter 4, which showed a strong temporal trend in the prevalence of infected newt species, the objectives of this study were to investigate

1. How readily study animals succumb to infection under experimental conditions.
2. Whether infection status is maintained, or varies over time, particularly in relation to the transition between aquatic and terrestrial states.
3. Whether exposure to $Bd$ (irrespective of infection status) is associated with a cost, either in terms of mortality, or change in mass.
5.2 Methods

5.2.1 Study animals

Study animals were collected in April and May of 2011, from eight breeding sites in Essex, South East England. Only adult newts were collected. Male and female newts were collected in equal number, and all animals were in their aquatic phase. Source populations were identified by a local herpetologist. The maximum number of animals collected from each site was 10% of the total estimated population size.

As all animals were collected from the wild, it was not possible to preclude prior exposure to Bd. Pre-exposure treatment of animals with the antifungal drug itraconazole (to clear newts of pre-existing Bd infections) was considered, but decided against. There are no published trials for the use of itraconazole in either species (or indeed, in any caudate amphibian). Thus, efficacy is untested, and side effects (which are described in other amphibians (Garner et al. 2009)) have not been investigated. Further, treatment may affect the outcome of experimental exposure to Bd, should active residues remain in the skin after treatment.

5.2.2 Husbandry

On collection, animals were housed in a temporary open-air holding facility. After a maximum of 28 days, they were transferred to Imperial College where they remained for the duration of the experiment. Starting from the first dose of Bd (day 0 in Figures 5.2 and 5.3 below), the study period lasted for approximately 11 months, from May 2011 to April 2012. Animals were overwintered between November 2010 and March 2011, and during this time, were held in a 4°C cold room with zero light exposure. During the active period, newts were maintained at 18 ± 2°C, with a 12:12 hour day/night cycle.

Three different tank set-ups were implemented over the course of this study. Fully aquatic animals were housed in mixed-sex pairs, to provide opportunity for courtship. Tanks consisted of a 1.6L Really Useful Box® (Really Useful Products Ltd) containing 1L aged tap water, and weighted black plastic strips to provide cover and egg laying material (Figure
5.1a). Fully terrestrial animals were housed individually in 0.6 L Really Useful Boxes®, lined with damp paper towel, with a small plastic pot to provide cover (Figure 5.1b). In the last phase of the experiment, animals were group-housed (maximum 12 animals per tank, Figure 5.1c, equal sex ratio), to amplify the likelihood of detecting infection. The group enclosures were semi-aquatic and consisted of a Macrolon® 1500cm² rodent cage (Tecniplast, Italy) filled with 6L aged tap water. A central island was constructed out of a 1.6L Really Useful Box®, weighted with gravel and topped with coconut coir substrate (Plantation Soil, Exo Terra®), filled with 6L aged tap water. A central island was constructed out of a 1.6L Really Useful Box®, weighted with gravel and topped with coconut coir substrate (Plantation Soil, Exo Terra®). A resin cover object (Small Reptile Cave, Exo Terra®) was also provided.

Figure 5-1 Tanks used over the course of the experiment including (a) aquatic tank: 1.6L Really Useful Box (RUB) which contains 1L aged tap water and plastic bin liner substrate for egg laying, (b) terrestrial tank: 0.6L RUB with plastic cover object and damp paper towel substrate and (c) semi-aquatic tank which contains 6L aged tap water and an island composed of coir substrate with Exo Terra cover object within 1.6 L RUB.
During the active period, animals were inspected daily for general condition, and the position of each tank rotated, to avoid unwanted environmental effects due to, for example, gradients in light or temperature. Feeding took place twice a week. Aquatic animals were supplied with *Daphnia spp.*, harvested with permission from Regent’s Park, London (under the management of Royal Parks). On the day of feeding, *Daphnia spp.*, were collected directly into a bucket of aged tap water. Once transferred to Imperial College London, *Daphnia spp.*, were diluted as required, and subdivided by treatment. In each treatment, every tank received a 30ml volume. I used a magnetic stirrer, on the lowest setting, to ensure feed was equally distributed. Terrestrial animals received a diet of crickets (1st instar, supplied by Livefood UK Ltd), approximately 15 crickets per tank. Again, feed was subdivided by treatment prior to distribution. Animals in semi-aquatic tanks received crickets and daphnia, in equal quantity, and proportional to group size. Tanks were cleaned once a week. For aquatic and semi-aquatic tanks, this consisted of a half water change, replacing the discarded water with clean aged tap water. For terrestrial tanks, the paper towel lining was replaced, and cover objects were cleaned and disinfected. During hibernation there was no feeding or cleaning, and to keep disturbance to a minimum, animals were checked just once every two weeks. In preparation for hibernation (4°C), animals first spent one week at 8°C. Emergence from hibernation followed the same two-step process (one week at 8°C before return to 18 ± 2°C).

### 5.2.3 Exposure of study animals to *Bd*

In this experiment palmate newts were exposed in water, but smooth newts were exposed on land. The intention had been to conduct all exposures using aquatic animals, because this phase of their life cycle is most likely to be associated with exposure to *Bd* zoospores in the wild. However, pre-exposure, I observed a rapid deterioration in the health of some aquatic smooth newts (Appendix 4). These animals were euthanased as specified by the Home Office licence and the defined humane endpoints listed therein, with an over dose of MS222 (0.2% for 3 hours minimum), followed by pithing, using a 23 gauge needle. To prevent further loss of animals, the remaining smooth newts were transferred to terrestrial tanks. There were
no further pre-exposure signs of disease and it was therefore possible to continue with the experiment.

Tanks of newts were randomly assigned to one of three possible treatments (Garner et al. 2009): (1) repeated high dose (50 000 zoospores), (2) repeated low dose (5 000 zoospores), and (3) repeated sham infection (sterile culture medium). Palmate newt treatments were replicated 25 times (one male and one female per replicate) and smooth newt treatments were replicated 44 times (one male or female per replicate). I exposed replicates twice a week, over four weeks (Figures 5.2 and 5.3). I used isolate SFBC 009 of Bd, which was isolated from a toe clip taken from an infected natterjack toad sampled in Cumbria, North West England in 2011. Although the genotype of this isolate is undetermined, all UK isolates genotyped to date (eight in total) fall within the hypervirulent global panzootic lineage (Farrer et al. 2011). This includes SFBC 014, isolated from the same host species sampled at the same location and isolated on the same date. Cultures were grown in TGhL broth (8 g tryptone, 2 g gelatin and 4 g lactose/1000 ml distilled water). On the day of exposure an aliquot of culture was counted using a haemocytometer to determine the concentration of active zoospores. The culture was subsequently diluted down (using TGhL broth) to achieve the desired concentration. Sterile TGhL broth was used for exposing control animals. To expose the palmate newts, I added 1 ml of culture/sterile broth to the existing tank water. To expose the smooth newts, the same dose was administered directly onto the ventral surface of each animal.
Figure 5-2 Timeline for the palmate newt experiment for high dose, low dose and control groups. Day 0 corresponds to the day of the first exposure. Animals were paired and placed in aquatic tanks during the exposure period. Swab 1 was collected ten days before the first exposure. Swab 2 was collected mid-way through the exposure period (specifically, on the same day as and immediately preceding the fourth exposure). Swabs 3a 3b and 3c were collected at one, two and three weeks after the final exposure. Swabs 4 and 5 were collected at the onset of and emergence from hibernation. Swab 6, which corresponds to the last day of the experiment, took place one week after transfer to group housing.
Figure 5-3 Timeline for the smooth newt experiment for high dose, low dose and control groups.

Day 0 corresponds to the day of the first exposure. Animals were individually housed in terrestrial tanks during the exposure period. Swab 1 was collected on day 0, prior to exposure. Swab 2 was collected mid-way through the exposure period (specifically, on the same day as and immediately preceding the fifth exposure). Swab 3 was collected one week after the final exposure. Swabs 4 and 5 were collected at the onset of and emergence from hibernation. Swab 6, which corresponds to the last day of the experiment, took place four weeks after transfer to group housing.
5.2.4 Measurements

Animals were weighed to the nearest 0.01g at the start of the experiment, one week post exposure and at the onset of hibernation. Mortality was recorded daily. To monitor infection status, I collected swab samples at strategic intervals over the course of the experiment (Figures 5.2 and 5.3). For both species, swabs were taken before exposures, mid-way through exposures, and one week after the eighth and final exposure. Palmate newts were also swabbed at two and three weeks after the final exposure to assess the impact of (1) a full water change and (2) transfer to terrestrial tanks, on infection status. To investigate whether infection could be maintained overwinter, animals were swabbed at the beginning and end of hibernation, and also following a period of group housing in semi-aquatic tanks. It had been the intention to record the number of eggs laid on a weekly basis to compare between treatments. However as egg laying ceased before the first exposure, this was not possible.

5.2.5 Real-time PCR analyses

DNA extraction and processing was conducted according to the methods outlined in Chapter 4 (i.e. including bovine serum albumin, BSA), except that all samples were analysed individually (i.e. there was no initial screening of pooled samples). Genomic equivalents (GE) for all samples were derived from standard curves. Because samples were collected during a lab-based controlled experiment, I did not impose a minimum acceptable GE for positive samples. Therefore, where amplification occurred in duplicate, all values were considered positive.

5.2.6 Statistics

I assessed individual infection status as a binary variable (infected vs uninfected). Smooth newts were considered individually. A pair of palmate newts was considered a single unit and was classified as positive when one or both individuals had a positive test result. Prevalence levels were calculated, together with their 95% confidence intervals and comparisons between treatments and between time points were made using Fisher's exact tests.
I compared infection intensity (GE) of infected study animals with data from a longitudinal study (Chapter 4), in which samples were analysed according to the same methodology used in the current study.

Survival data were visualised by plotting a Kaplan-Meier survivorship function to illustrate the effect of treatment on survival to hibernation. Data were censored for animals that survived beyond this point. I used Cox proportional hazards models to examine whether treatment influenced survival probability. For the smooth newt experiment, I examined two a priori confounders, sex and starting mass and compared models using Analysis of Variance (ANOVA). Because animals were paired in the palmate newt experiment, it was not possible to explore the effect of sex or start weight in this species.

Generalized linear modelling with normal errors was used to assess the effect of exposure on proportional change in mass between the start of the experiment (day 0) and one week after the final exposure (day 33 or 31, depending on species). Proportion change in mass was also compared between the start of the experiment and the onset of hibernation (day 171 or 157). Sex and start weight were included as a priori confounders of sex and start weight. Because there were so few candidate models, all models were evaluated. Model selection was based on AIC.

5.3 Results

5.3.1 Infection status

Pre-exposure sampling confirmed low levels of existing infection in both species, and in animals from five of eight collection sites. Two (1%) of 150 palmate newts and 8 (4%) of 181 smooth newts tested positive for Bd. These samples were collected from aquatic animals. After transfer of smooth newts to terrestrial tanks, only one animal (1%) tested positive, from a total of 131 animals for which aquatic comparison data were available. Thus, pre-exposure smooth newts were likely to be detectably infected with Bd when terrestrial, than when they were aquatic. This difference was statistically significant (Fisher’s exact test, p = 0.007).
Despite using large quantities of *Bd* zoospores, animals rarely tested positive after the onset of exposures. In the palmate newts, the greatest number of positives was detected from swabs taken one week after the final exposure (day 33, Figure 5.4). At this time, the proportion of pairs testing positive was significantly higher in the high dose group, relative to both the low dose group (Fisher’s exact test, $p = 0.005$) and the control group (Fisher’s exact test, $p < 0.001$). However, seven days and full water change later (day 39), there were no positive swabs from any of the three treatment groups. In the smooth newts, the greatest number of positives was detected from swabs taken mid-way through the exposure period (day 13, Figure 5.5). At this time, the number of animals testing positive was significantly higher in the high dose group, relative to the control group (Fisher’s exact test, $p = 0.002$). However, there was no significant difference between high and low dose treatment groups (Fisher’s exact test, $p = 0.192$). One week after the last exposure (day 31), of 120 remaining animals, only two individuals tested positive for *Bd*, including one animal from the control group. Thus the probability of a positive result was comparable to pre-exposure testing at day 0 (Fisher’s exact test, $p = 0.470$).

Swabs 4, 5 and 6 from both species tested negative. Swabs 4 and 5 (before and after hibernation) were collected from terrestrial animals. Swab 6 was taken from group-housed animals with access to water. During this phase of the experiment, in both species, approximately 50% of individuals were aquatic. The remaining animals continued to live terrestrially.
Figure 5-4 Prevalence (%) of *Bd*-positive palmate newt pairs (where infection was detected in one or both individuals). Animals were aquatic for all but the fifth swabbing date, day 46.

Figure 5-5 Prevalence (%) of *Bd*-positive smooth newts. Newts were terrestrial throughout.
5.3.2 Infection intensity

After the onset of exposures, the mean GE score for positive swabs taken from palmate newts was 1.1 (95% CI 0.5 – 1.7), with a median value of 0.7 zoospore equivalents. For smooth newts, the mean GE score was just 0.5 (95% CI 0.1 – 0.9), with a median value of 0.2 zoospore equivalents. These figures are comparable with results observed in naturally infected smooth and palmate newts in the UK (Figure 5.6).

(a) 
(b)

Figure 5-6 Genomic equivalents from swabs collected from (a) palmate newts and (b) smooth newts. 
Samples collected from experimental animals, after the onset of exposures, are shown in light grey. 
Samples collected from wild newts during a longitudinal survey (see Chapter 4) are shown in dark grey.
5.3.3 Survival analysis

Between the first exposure, and the onset of hibernation, 42 (56%) of 75 palmate newt pairs were removed from the experiment due to mortality in one or both individuals (Figure 5.8). Survival was higher in the smooth newt experiment (Figure 5.9), where mortality over the same period was just 23% (31 of 132). Cox proportional hazards regression showed no effect of treatment on mortality in either species. In the palmate newt experiment, relative to unexposed animals, the mortality hazard ratio was 1.3 (95% CI 0.58-2.66) for animals exposed to low dose *Bd*, and 1.2 (95% CI 0.53-2.48) for animals exposed to high dose *Bd*. For the smooth newt experiment, Two *a priori* confounders were investigated, namely, sex, and start weight. There was no significant difference in explanatory power after including these variables and as a result, the simpler model was accepted. Relative to unexposed animals, the mortality hazard ratio was 1.3 (95% CI 0.53-3.07) for animals exposed to low dose *Bd*, and also 1.3 (95% CI 0.53-3.07) for animals exposed to high dose *Bd*. In the palmate newt experiment, mortality was particularly high during the two weeks immediately post exposure, when 24 of 66 remaining aquatic pairs were removed (days 26 to 39, Figure 5.8). This included 14 tanks where one or both animals were found dead, with no ante mortem signs of disease, and ten tanks where animals were euthanased due to the appearance of clinical signs. Symptoms included anorexia, lethargy, abnormal posture, submandibular pustules (Figure 5.7a) and generalised discolouration of the skin (Figure 5.7b). However, there was no evidence that mortality or morbidity was associated with infection status (Fisher’s exact test, $p = 0.269$).

![Figure 5-7 Presentation of aquatic palmate newts showing (a) submandibular pustule and (b) generalised discolouration of the skin](image-url)
Figure 5-8 Effect of treatment on survival of palmate newts. Animals were moved to individual terrestrial tanks on day 39. Prior to this date a pair of newts is considered a single unit.

Figure 5-9 Effect of treatment on survival of smooth newts. Animals were housed in individual terrestrial tanks throughout.
5.3.4 Change in mass

On average, palmate newts lost mass between days 0 and 33 (mean proportional change in mass = 0.73, 95% CI 0.71 – 0.76), Figure 5.10a. The optimal model for this period contained treatment group and starting mass as explanatory variables, although neither was statistically significant. Results for the longer period, day 0 to day 171, were very similar to the day 0 to day 33 period (mean proportional change in mass = 0.76, 95% CI 0.71 – 0.80), Figure 5.10b. The optimal model for the day 0 - 171 period contained treatment group and starting mass, as before. There was no effect of group on proportional change in mass. As expected, animals with a lower starting mass were associated with a proportionally lower end mass (effect ± SE = 0.206 ± 0.077; $F_{3,59} = 2.79$, $p = 0.010$).

On average, smooth newts lost mass between days 0 and 31 (mean 0.89, 95% CI 0.88 – 0.91), and gained mass between days 0 and 157 (1.14, 1.08 – 1.20), Figure 5.11. In both cases, proportional to starting mass, animals in the high dose group were more likely to lose mass, and less likely to gain mass, relative to the control group (effect ± SE = -0.051 ± 0.020; $F_{2,118} = 4.26$, $p = 0.015$ at day 31; -0.138 ± 0.066; $F_{3,95} = 7.42$, $p = 0.040$ at day 171. The optimal model for change in mass at day 171 included starting mass as an additional explanatory variable. As expected, there was a significantly lower proportional increase in mass in larger animals (-0.324 ± 0.073; $F_{3,95} = 7.42$, $p < 0.001$ at day 171).
Figure 5-10 Boxplot showing the effect of treatment on proportional change in mass in palmate newts between (a) days 0 and 33 and (b) days 0 and 171.

Figure 5-11 Boxplot showing the effect of treatment on proportional change in mass in smooth newts between (a) days 0 and 33 and (b) days 0 and 171.
5.4 Discussion

This chapter reports a study in which two amphibian species, the smooth newt and the palmate newt, were exposed to *Bd* zoospores under experimental conditions. Both species are native to the UK, and both are capable of becoming naturally infected (Chapters 3 and 4). A low level of background infection was detected in study animals of both species and from multiple collection sites. Therefore, at least some (and maybe all) of the study animals had previously been exposed to *Bd*. This may have affected the result.

The dosing protocol involved repeated exposure to large quantities of *Bd* zoospores at, what was believed to be relatively high concentrations. Despite these conditions, very few samples were found to test positive for *Bd* DNA. Furthermore, where infections did appear to develop, they were short-lived, disappearing not long after the conclusion of the exposure period. These results suggest that smooth and palmate newts are an unlikely reservoir for *Bd* (Cleaveland and Dye 1995), and that infections detected in wild newts may be the product of spillover from a more suitable maintenance host.

The results of two national surveys (see Chapter 3) and a longitudinal study (Chapter 4), which found prevalence and infection burden to be low in both species, are consistent with this hypothesis. However, it would be interesting to repeat the smooth newt experiment with aquatic animals, as originally intended. Data from UK newts sampled during the breeding season show a higher prevalence of infection in this species relative to palmate newts (Chapter 3) and thus smooth newts may play a more significant role in maintaining infection within *Bd*-positive amphibian assemblages.

In addition, this study was limited to investigating infection in adult newts. In many anuran species, metamorphs and sub-adults are associated with considerably higher levels of infection (e.g. Briggs *et al.* 2010, Russell *et al.* 2010) and therefore, these age classes should be investigated as potential intraspecific reservoirs of *Bd* (Brunner *et al.* 2004).
Mortality was recorded between the first day of exposure, and the onset of hibernation. Whilst in smooth newts the number of deaths was relatively constant throughout this period, in aquatic palmate newts there was a sharp increase in mortality (which included the implementation of humane end points) during the two weeks after the final exposure (approximately four to six weeks after the first dose exposure to *Bd*). Mortality due to chytridiomycosis has been observed in other experimentally exposed animals at a comparable number of days post exposure (e.g. Berger *et al.* 2005, Carey *et al.* 2006, Garner *et al.* 2011). However, this study found no evidence that mortality was associated with infection, or treatment, and thus these deaths cannot be attributed to *Bd*. Deterioration of tank water quality may offer a more plausible explanation, and may have impacted host immunity to the extent that they were vulnerable to infection by opportunistic pathogens such as *Saprolegnia* spp, the causal organisms of a generalised infection known as Saprolegniasis. Clinical signs of this disease included the appearance of cotton-wool-like fungal colonies typically around the tail and hind limbs, sometime leading to ulceration of the skin. This presentation is consistent with the pathology recorded in aquatic newts during the current study, although diagnosis was not confirmed.

The two-week period associated with increased mortality in palmate newts was the final two weeks of the aquatic phase of this experiment. Although tanks were cleaned weekly, only half water changes were carried out. This is likely to have led to a cumulative build up of debris and an increasing concentration of nutrient rich TgHL broth, which may have resulted in an increased burden of aquatic microorganisms, including but not limited to *Saprolegnia* spp.

In this study, proportional change in mass was compared between treatment groups. In the palmate newt, there was no statistically significant variation between treatments. However, in smooth newts there was a small but significant increase in proportional mass lost (or decrease in mass gained) in the animals exposed to high doses of *Bd* relative to the low dose and control groups. This suggests, that although the smooth newt appears to be relatively resistant to *Bd*, there may be a cost involved in maintaining this state.
A cost to host fitness, in the absence of detectable infection, has been reported in at least two other studies where amphibians were experimentally exposed to *Bd* (Garner et al. 2009, Luquet et al. 2012). Both resisting infection, and clearing existing infection are likely to require additional investment of resources in immune defenses e.g. for the production of anti-*Bd* skin peptides (Woodhams et al. 2007) and this may detract from investment in growth.

One of the aims of this experiment was to investigate infection in newts during the transition between an aquatic and terrestrial existence. This change in external conditions has important ramifications for the host, and the pathogen. It was anticipated that these changes would be reflected in the prevalence of infection. Unfortunately, because *Bd* was only detected over a short time span, which did not coincide with transition events, it was not possible to test this hypothesis in exposed animals. However, in smooth newts pre-exposure, there was a significant reduction in prevalence when animals were transferred from aquatic to terrestrial environments. This may reflect a real change in infection status. If newts lose their infections rapidly after returning to land, this would support a role for newts as spillover- rather than reservoir hosts for *Bd*. Alternatively, this result may be better explained by a change in the detectability of infection.

A recent study in natterjack toads found that, based on swab results, infection status regularly fluctuated between positive, in wet conditions and negative, in dry conditions, in the absence of external sources of re-infection (Minting 2012). The author suggests that this effect may be the result of changes in the reproductive rate of *Bd*, in response to a more or less hostile environment, and that this may make it more difficult to detect the presence of infection using a swab.

In addition to this point, in newts, the transition between water and land is accompanied by marked changes in skin structure. During the aquatic stage, the skin has a slimy texture. However, in terrestrial animals the skin takes on a velvety appearance, which is characterized histologically by an increase in the abundance of tubercles in the stratum corneum (Warburg and Rosenberg 1997). Supposing this change presents a physical barrier between the swab
and Bd present in the skin, this could also affect diagnostic sensitivity. With these considerations in mind, in the final stages of this study, overwintered study animals, apparently devoid of infection, were subjected to a period of group housing. During this time, animals had access to water, to facilitate a return to an aquatic existence. The aim was to maximise the probability of generating detectable infections at the population level, to demonstrate the maintenance of ‘cryptic’ infections overwinter. However, there was no infection detected in these individuals, in spite of a successful return to water of almost 50% of animals. It is possible that the period of follow-up was not sufficient to allow overwintered Bd to reach detectable levels (the palmate newts were observed for just one week before the experiment was terminated).

Throughout this study, the infection burden of Bd-positive samples was low. Thus it is possible that Bd-positive samples were the result of contamination with environmental Bd DNA (e.g. in the water or in terrestrial animals, remaining on the skin after dosing). The striking similarity between experimental swab results and those from wild caught animals lends some biological plausibility to my measure of prevalence in experimental animals. However, a follow-up investigation, comparing swab results from animals treated with both live and heat-treaded Bd zoospores would be valuable.

In conclusion, despite repeated exposure to high quantities of Bd zoospores, there was no detectability of exposure on survival in either smooth or palmate newts. As the doses used are likely to far exceed those encountered in nature, this suggests that adult smooth and palmate newts are unlikely to experience infection-based mortality in the wild. However, the animals used for this study were sourced from populations with existing infection, and hence a level of immunity can not be excluded. In addition, the high rates of apparently non-Bd related mortality may have confounded results.

An effect of exposure on mass was detected with greater weight loss evident in the high dose smooth newts group, relative to the control and low dose animals. This suggests that, at least in this species, there may still be a cost associated with exposure to Bd.
Throughout the study, infection burdens were low, though consistent with samples from wild newts in the UK.

Although the number of Bd-positive samples in exposed groups increased during and immediately after dosing, this signal was short lived, suggesting that adult smooth and palmate newts are not a suitable reservoir host for Bd. Thus infection detected in wild animals maybe the result of spillover from a more suitable host.
6 DISCUSSION

*Batrachochytrium dendrobatidis* (*Bd*) is an archetypal emerging pathogen that is highly infectious, has a broad host range and is pathogenic across a wide diversity of species. First identified in 1998 (Berger *et al.* 1998), *Bd* has rapidly achieved a global spread, facilitated by the international trade of amphibians for food, pet trade, zoological collections, biocontrol, and for use in biomedical research.

This pathogen has now been detected in over 50 countries and across five continents (Olson *et al.* 2013) (Figure 6.1). Outbreaks of fatal chytridiomycosis (the disease caused by *Bd*) have been attributed with causing the greatest disease-driven loss of biodiversity ever recorded (Fisher *et al.* 2012) and in some parts of the world, infection-based mortality has resulted in the loss of 40% of all amphibian species (Crawford *et al.* 2010).

However, although globally, *Bd* causes amphibian declines, host responses are inconsistent and *Bd* appears able to coexist with some amphibian species or assemblages of species in a state of endemism. In Northern European countries, although infection is widespread (and Figure 6.2), and *Bd*-linked mortality has been documented (Bosch *et al.* 2001, Bosch and Martinez-Solano 2006, Bielby *et al.* 2009, Pasmans *et al.* 2010, Walker *et al.* 2010) chytridiomycosis-driven declines have not yet been reported.

The spread of *Bd* in some parts of the world has occurred in a wave-like fashion, a pattern that can be explained by natural movement of infected hosts, following an initial introduction event (Lips *et al.* 2008, Walker *et al.* 2010, Farrer *et al.* 2011). However, the results of this thesis suggest that this pattern of spread is not a good fit for the UK.

In the UK, as in much of Europe, infection is widespread, but patchy, with no obvious spatial clustering indicating a single point of introduction or direction of spread (Chapter 3). Furthermore, where *Bd* does occur, prevalence is low (see Chapters 3 and 4).
Figure 6-1 The global distribution of \textit{Bd}

Figure 6-2 The distribution of \textit{Bd} in Europe
The incidence of *Bd* has been demonstrated to have a strong seasonal component. The processes underpinning this observation are complex and may include seasonally heightened host susceptibility, seasonal changes in contact rate (e.g., due to increased host density during the amphibian breeding season) or changes in pathogen growth and survival resulting in a variable force of infection. Ultimately, these components result in a transmission rate that varies throughout the year. During seasons where conditions for transmission are favourable (e.g., high host density, host susceptibility, and optimal environmental conditions for pathogen growth), the number of new cases per infection (the basic reproduction number, $R_0$) may be high. However, during sub-optimal conditions, the number of new cases can become significantly reduced. If there is a very strong effect of seasonality on transmission, $R_0$ may even fall below 1, leaving the pathogen vulnerable to stochastic events and fade out which may occur before the new 'high' season ensues.

This dynamic offers a possible explanation for the patchy distribution of *Bd*-infected sites in the UK (Chapter 3). However, under these circumstances, the distribution of infection might be expected to be more homogeneous. Instead, a strong association was identified between the occurrence of *Bd* and sites of known non-native amphibian introductions.

One explanation for this result, and the explanation that has been most strongly advocated in this thesis is that the current distribution of infection in the UK is at least partly the result of co-introduction with exotic amphibian species. Data gathered and analysed during this thesis support this hypothesis. At one of the *Bd*-positive sites included in two national surveys (Chapter 3), non-native amphibian introductions date back to the 1900s (Langton *et al.* 2011), but continued into the 1960s (this site was an aquatics nursery), providing a wide window of opportunity for the co-introduction of *Bd*. At the remaining *Bd*-positive sites where non-native amphibians had been introduced, introduction dates ranged from between 1950 and 2000. Histories varied, not only by date, but also by species and circumstances, suggesting multiple introduction events. This may mean that there are multiple origins of genetically distinct isolates in the UK.
However, an alternative explanation does exist: that *Bd* is an endemic pathogen in the UK, to which native species have developed a degree of resistance. Non-native amphibians, however, have more recently been exposed to the pathogen and are therefore more susceptible to becoming infected. This results in a higher prevalence of infection at non-native amphibian sites, and hence a greater probability of detecting infection when sampling these populations.

The only native species whose occurrence was positively associated with that of *Bd* was the natterjack toad. As above, it is possible that this correlation simply reflects a higher level of susceptibility to infection, relative to other native species. Susceptibility to *Bd*, including high burdens of infection and the incidence of chytridiomycosis in the wild in the UK has been documented for natterjack toads (Minting, 2012). However, this species is also unusual in the UK on account of being the rarest (with the exception of the recently re-introduced northern clade pool frog) and most intensively managed of the UK native species. Conservation of natterjack toads has included a process known as headstarting, which involves the captive rearing of natterjack toad tadpoles, to metamorph stage, a practice which is not carried out for any other of the UK’s native species. In some cases, this practice has been conducted in premises occupied by captive collections of exotic amphibian species, potentially providing opportunities for the spread of infection between non-native and native hosts. It is possible therefore that one or more *Bd* introductions to the wild in the UK occurred inadvertently via such conservation actions. Further opportunities for human assisted pathogen dispersal could have occurred due to translocation of natterjack toads between breeding sites (e.g. to boost population sizes) or to reintroduce the species at sites of local extirpation. Again, these activities are particular to the management of natterjack toads in the UK and could, in part, be responsible for the correlation observed in this study between *Bd* infection and natterjack toad sites.

Molecular epidemiology techniques could be usefully implemented to disentangle these hypotheses. Whole genome sequencing of a global set of *Bd* isolates has already revealed that *Bd*, as we know it, is comprised of at least three deeply divergent lineages (Farrer et al.)
The only lineage that has thus far been implicated in declining amphibian populations is the Global Panzootic Lineage (BdGPL). To date, eight UK isolates have been sequenced, all of which fall within the Global Panzootic Lineage (BdGPL). This result is consistent with Bd being introduced to the UK during the 20th century, which is when BdGPL is believed to have emerged. However, efforts to isolate Bd from UK sites have been biased. All isolates thus far were collected from natterjack toads, or from sites of non-native amphibian introductions, in both cases, targeting populations particularly associated with anthropogenic activity. Thus, this result may not be representative and does not rule out the possibility of an endemic genotype of Bd in the UK. Future work should therefore aim to redress this balance, incorporating isolates from as many and as varied UK sites as possible.

Whole genome sequencing is not the only tool that can be employed to identify genetic variation between isolates. Multilocus sequence typing (MLST), a process aimed at identifying polymorphisms in the sequences of so-called housekeeping genes (genes that are essential for the maintenance of basic cellular function and therefore less susceptible to selection), can be also be used to match closely related or clonal isolates and, under the assumption that more recently introduced pathogens are associated with lower overall genetic diversity.

The results of the national surveys (Chapter 3), suggest that the distribution of Bd in the UK is relatively stable. Under the hypothesis that the arrival of Bd in the UK is at least partly the result of co-introduction with non-native amphibians, a strong association between amphibian introduction events and the presence of infection may indicate that natural colonization of new sites in not a major component of the epidemiology of this pathogen in the UK. This is supported by the lack of evidence of any expansion in the range of infection distribution between 2008 and 2011.

Intra-site prevalence in the UK was shown to be almost uniformly low. A low prevalence of infection does not, in itself, rule out a significant population-level impact of disease. For example, low infection prevalence can occur in situations where the onset of infection leads, rapidly, to host mortality. However, in this study, both the prevalence and, in infected hosts,
the burden of infection, were extremely low. In addition, reports of mortality at survey sites (which were rare) related to isolated cases that were more consistent with predation or road mortality than infectious disease. Based on these observations, it would appear that the full expression of chytridiomycosis is not manifest in the UK.

Gahl et al. (2012) propose four hypotheses for the absence of amphibian decline in Bd-infected populations:

1. Declines are occurring, but have not been detected
   Amphibian mortality can easily go unnoticed due to a combination of predation, rapid decomposition of carcasses, a limited active period and remote or challenging habitat type. Furthermore amphibian populations fluctuate widely in size and detecting declines can therefore be problematic. However, in the UK, there is an extensive network of enthusiastic herpetologists (including the volunteer groups that form part of the Amphibian and Reptile Groups of the UK, ARG UK). This means that many ponds are surveyed on a yearly basis and hence population data are available. There are also at least two annual conferences (ARG UK and the British Herpetology Society annual conferences) at which concerns over population declines would normally be raised.

2. Species are not susceptible
   One of the defining features of Bd is the variability in outcome that has been observed between species, and between populations of the same species (Daszak et al. 2004, Blaustein et al. 2005, Rollins-Smith and Conlon 2005). Although empirical evidence of adaptive immunity remains absent, innate immune defenses including the presence of antimicrobial skin peptides and anti-Bd skin flora have been shown to increase host resistance to Bd (Harris et al. 2006, Woodhams et al. 2007). These, or other, as yet unknown, mechanisms of resistance, may be operating in UK species. Experimentally (Chapter 5), smooth and palmate newts were not easily infected, and there was no evidence that mortality was associated with exposure. Of the remaining four native species, field data showed the prevalence of infection to be consistently very low (or absent) in common frogs and great-
crested newts. However at certain survey sites there was a high prevalence of infection in natterjack toads and common toads. Both species have been shown to die from fatal chytridiomycosis and common toad populations have declined in parts of Spain.

In other amphibian systems, long-lived tadpoles play an important role in maintaining infection over the winter months (when metamorphosed animals leave the water), in the UK, anuran larvae only overwinter in exceptional circumstances (very cold temperatures or periods of extreme food scarcity). As a result, for approximately six moths of the year, UK ponds may be completely devoid of amphibian hosts. As Bd has an aquatic infectious life stage, this period of time, when amphibians live terrestrially, may result in lower rates of transmission and this annual cycle may prevent infection burdens from reaching the threshold required to cause disease.

(3) Bd strain is not virulent

This hypothesis seems unlikely as all eight UK Bd isolates (from native and non-native amphibians) that have been sequenced and all fall within BdGPL (M. Fisher unpublished data). However, the presence of BdGPL in UK populations does not rule out the presence of a less virulent endemic isolate of Bd that as yet, has not been isolated.

(4) Environmental factors limit growth.

Both the prevalence of Bd infection and severity of disease are influenced by environmental factors (Fisher et al. 2009). Temperature has been frequently cited as a determinant of Bd growth and survival. In tropical climates, high temperatures appear to limit the prevalence of infection, in temperature countries such as the UK, it is possible that Bd growth is constrained primarily by low temperatures (Woodhams et al. 2003, Kriger and Hero 2007). If cold temperatures limit growth, Bd zoospores may not reach densities sufficient for infection to be lethal (Vredenburg et al. 2010). However, temperature is by no means the only environmental parameter that should be considered. Pond ecosystems are characterised by a complex of interacting abiotic and biotic factors (e.g. Leibold and Wilbur 1992), many of which may influence Bd growth and reproduction and the longevity of zoospores. This is especially true
of ponds in lowland, agricultural areas were run-off of fertilisers into pond systems can result in capable of supporting a high burden of microorganisms. Experimentally, aquatic microfauna have been shown to rapidly lower the abundance and density of Bd zoospores with a corresponding decrease in the probability that infection will occur (Schmeller et al. 2014). In the same study, field data revealed a site-specific infection risk which correlated with the microfauna present. These findings support those of earlier works, which demonstrated grazing of chytrid zoospores by daphnia (a keystone zooplankton grazer in fresh water systems) and a strong effect of daphnia on the amount of Bd DNA in fresh water mesocosms (Kagami et al. 2004, Hamilton et al. 2012).

However, the presence of Bd in UK amphibian populations may not be entirely benign. In smooth newts, evidence of a cost associated with exposure to Bd was found, which may reflect a trade-off between investment in immunity and investment in growth (Garner et al. 2009, Luquet et al. 2012). This effect may also be present in great crested newts and common toads, two other native species that appear to experience very low levels of infection. Thus even in the absence of infection, sub-lethal costs associated with resistance may occur and this may have an effect on long-term population viability.

The biggest challenge facing the international response to Bd is our lack of understanding of the epidemiology of Bd. The relative contribution of direct and indirect transmission between hosts is, at best, poorly understood. There is no known saprophytic life stage of Bd, although the fact that Bd is readily culture in liquid media, in the absence of keratin suggests that such a stage may yet be identified. The various roles of reservoir, spillover and dead end hosts have been poorly characterised. A variety of explanations for seasonal patterns of infection and disease have been suggested, but rarely tested. The known genetic diversity between strains is informative, but is likely to represent only a fraction of the full extent of variation and hence unknown strains of Bd with uncharted virulence and physiological constraints almost certainly exist.
Therefore, while the current situation in the UK (or more broadly, Europe) appears to be stable, we do not know what changes may shift the balance or indeed, how co-infection with other parasites might affect infection outcome. This should be considered in light of the fact is currently no known method for eradiating Bd from areas in which it has been established.

However, perhaps of greatest significance is the fact that we are dealing with a still-emerging pathogen, as has recently been documented in the form of *Batrachochytrium salamandrivorans* (Bs). This organism is a novel species of chytrid fungus capable of parasitizing amphibians, and causing mortality (Martel et al. 2013). *Batrachochytrium salamandrivorans* has been shown to be highly virulent. The epidemiological characteristics of this species may make it more pathogenic to the caudata species of the UK, including the European protected great crested newt.

Perhaps, then, the greatest contribution this thesis can make is to help raise the profile of amphibian infectious disease, and wildlife disease more generally. Although causality cannot be inferred from the results of this study, the current distribution of Bd in the UK is at least consistent with the hypothesis that people have assisted with the dissemination of infection. The practice of moving amphibians freely between populations, once widely practiced by keen herpetologists and curious school children is less common. In the UK, volunteering herpetologists are overwhelmingly well informed about the risk posed by infectious disease and personal responsibility for biosecurity is high. The same, however, cannot be said of wildlife trade which operates over a much wider geographic scale and deals in tens of billions of animals each year, most of which is unregulated (Smith et al. 2009).

Early detection in wildlife trade is vital to control the further spread of emerging pathogens such as Bd (in all its known and unknown variants) and perhaps more pertinently, Bs. It is therefore essential that biosecurity, particularly with regard to the trade in live amphibians is properly addressed. This may include implementing screening surveillance, quarantine procedures or the use of certified suppliers.
6.1 Limitations and further work

The findings of this study should be interpreted in light of a number of sources of potential bias.

Detection of infection was determined by analysing skin swab samples for the presence of *Bd* DNA. Whilst the assay, in itself, is extremely sensitive, the sensitivity of skin swabbing, as a method of collecting *Bd* from infected animals, is harder to quantify and may vary between samplers, and according to host characteristics.

The distribution of sites surveyed during the national cross-section surveys (Chapter 3) were chosen non-randomly. Certain characteristics were preferentially selected for, namely the presence of large, mixed species amphibian populations that were easily accessible by volunteer surveyors. In addition, a number of sites were selected based on the presence of particular species (natterjack toads and non-native amphibian species). As a result, the reported prevalence and distribution of infected sites, together with any risk factors identified, might not be representative of the UK as a whole.

Sampling of amphibians was opportunistic and may have resulted in preferential selection of infected or uninfected individuals (assuming an effect of infection on catch probability). Samples were also collected exclusively from metamorphosed amphibians and almost entirely from adults. *Batrachochytrium dendrobatidis* is known to affect hosts of different life stages in different ways. As a result, low levels of infection observed in the adult population may hugely underestimate the overall prevalence of *Bd*.

Exclusion of larvae and, for the most part, juvenile amphibians from this study has resulted in a significant knowledge gap. Firstly, an effect of infection on either of these two age classes might not be detected at the adult population level. Furthermore, an appreciation of the extent and nature of infection in larval anurans could be vital to understanding seasonal patterns of infection in metamorphosed amphibians.
Of the native species, common toads and natterjack toads were associated with the highest infection prevalences. This is consistent with existing reports of infection-based mortality in both species (Bosch et al. 2001, Walker et al., 2010, Minting 2012). In the UK, a population study, using capture-mark-recapture (CMR) methods has now been completed for a series of natterjack toad populations in North West England (Minting 2012). A similar study has not yet been carried out for common toads in the UK. In England, common toad populations have been declining for the last 25 years with the cause(s) remaining unclear (Young and Beebee 2004), despite the fact that the ecological requirements of common toads in the UK are well understood. The potential for Bd to drive population declines in this species should be a cause for concern and further investigations, incorporating the use of CMR should now be conducted.
REFERENCES


Arai, S. (2008). Investigation on the spread of chytridiomycosis between UK natterjack toads and other inland amphibian populations within Cumbria, MSc thesis, Imperial College


Bates, D., M. Maechler and B. Bolker. (2012). "lme4: Linear mixed-effects models using S4 classes. R package version 0.999999-2." from http://CRAN.R-project.org/package=lme4


Bosch, J., I. Martinez-Solano and M. Gargia-Paris (2001). "Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (Alytes obstetricans) in protected areas of central Spain." Biological Conservation 97: 331-337


Minting, P. (2012). An investigation into the effects of *Batrachochytrium dendrobatidis* on natterjack toad populations in the UK. Doctor of Philosophy, University of Sussex


Quantum GIS Development Team (2012). Quantum GIS Geographic Information System. Open Source Geospatial Foundation Project


Smith, M. (1951). The British Amphibians and Reptiles, Collins


8 APPENDIX
ARG-UK Advice Note 4

Amphibian disease precautions: a guide for UK fieldworkers

Version 1: February 2008
Advice for fieldworkers

Notes:

A “site” in this section refers to an amphibian breeding site separated from others by more than 1km. The basis of this definition is that if you are doing work on several ponds all within 1km of each other, you are unlikely to spread infection beyond the areas it already occurs, or could occur. If you are doing high risk activities (see below), then additional precautions may be required.

Here we set out general guidelines, and then guidance for activities that carry a higher risk follow.

General guidance

- Handle amphibians only if necessary. All common survey activities are allowable so long as reasonable precautions, set out here, are followed. Common methods are: dip-netting, egg searching, torch surveying, bottle-trapping, refuge searching and pitfall trapping.

- Amphibians should only be released only at the point of capture, unless absolutely necessary. If they need to be moved >2km, see high risk guidance below.

- One pair of disposable vinyl gloves should be used for each site sampled, if your hands will be in contact with amphibians or pond water. Do not use latex gloves as they may be harmful to amphibians. If your hands will not be in contact with amphibians or ponds, there is no need to use gloves.

- Containers and equipment used should be disinfected (see guidance below) between each site sampled.

- If entering the water, footwear should be washed & disinfected (see guidance below) immediately after the site visit. If you do not enter the water, there is no need to disinfect footwear unless it is a high risk activity (see below).

- Although there is no evidence of the spread of Bd by vehicles, it is good practice to park on hard standing (rather than vegetated areas) and walk to the pond.

- Dead/sick amphibians should be regarded as a high infection risk and not touched unless you have agreed collection and submission with a suitable authority (e.g. Froglife see http://www.froglife.org/Disease.htm for guidance). Note that there is no general request for dead or sick amphibians, and submission will only be agreed in limited cases.
**Guidance on high risk activities and ways of working**

Some activities or ways of working carry a potentially higher risk of transmitting chytrid fungus beyond the background level of spread. These are listed below, with additional precautions to be taken in addition to those above.

<table>
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<tr>
<th>Activity or way of working</th>
<th>Additional precautions</th>
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| Amphibian survey work at many sites in different parts of the country. This is most common among ecological consultants. | Ensure all field staff are aware of chytrid issues and this note. Follow the general precautions above, and in addition:  
  - Footwear to be disinfected between sites  
  - Consider allocating each set of field gear to a particular site within a season, rather than selecting from a common set used at many different sites. Where appropriate, consider having two sets of field gear, so that one can be in the disinfection and drying process while the other is in use. |
| Translocating (moving) amphibians. As mentioned above this should be avoided where possible, but occasionally it is desirable for conservation, research or mitigation purposes. | Translocation of amphibians >3km from point of capture would only be acceptable where (a) a very strong case is made for the benefits of the translocation, (b) there is no satisfactory alternative, and (c) strenuous efforts to analyse and minimise disease risks are taken. Regarding (c), the donor population and – if appropriate, any receptor population – must be demonstrated to be negative for chytrid with a high confidence. Typically this would involve samples of 60 individuals per site using a recognised diagnostic technique. |
| Fieldwork at sites supporting non-native amphibian species. | Take particular care to avoid capturing and moving non-native amphibians. Clean and disinfect footwear immediately after site visit. |
| Fieldwork by persons who keep non-native amphibian species in captivity. | Implement rigorous barrier methods (gloves, minimal handling, disinfection, etc) to minimise the risk of transmitting pathogens from captive stock to wild sites. Do not bring any native amphibians into captivity. Consider limiting fieldwork that involves handling amphibians at sites supporting important native populations. If fieldwork is essential, do not use any equipment previously used for non-natives. |
| Fieldwork at a population known to be infected with chytrid, or suspected to be infected (from distance to known infection, or other information). | In general amphibian fieldwork at such sites should only be done where essential. This could include research to track the progress of infection, and to assess amphibian population status. Work should:  
  - follow general precautions as above, except where extended below  
  - minimise visits to the site, and to the minimum number of workers  
  - where feasible, use a single set of field gear for each site, and store field gear on site  
  - disinfect field gear between each pond separated by >1km  
  - rigorously disinfect gear and footwear on exit of the site  
  - liaise closely with landowners to minimise |
Disinfection procedure

- **Disinfect boots, waders, nets, bottle-traps, canes, and anything else that would be in contact with amphibians or pond water.** When disinfecting gear during fieldwork the following will be required: bucket, brush, disinfectant, disposable or washing up gloves (to wear while disinfecting), bin bags for waste. Note that when making up bleach or Virkon solutions, pond water can be used so long as it contains little or no organic matter (as this reduces disinfectant effectiveness).

- **Follow this procedure:**
  - Use a brush to scrub off any debris, plant fragments, mud etc.
  - Rinse with water (pond water will suffice)
  - Disinfect using one of the following methods:
    - Soak in a bleach solution (1 measure of household bleach to 9 measures water) for 15 minutes; OR
    - Virkon (10 mg/mL, as per suppliers instructions) for 1 minute; OR
    - Fabrics including those worn while doing amphibian fieldwork can be washed on a 60°C cycle (with detergent, ensuring sufficient rinsing). Nets should be boiled for 10 minutes or if the fabric allows disinfected with spray bleach in a well ventilated area.
  - Rinse with clean water and if possible allow to dry for before next use.
  - Keep field gear (traps, net frames etc) inside plastic bags during transit and storage to reduce the chance of transmitting chytrid.

- **Ideally all used disinfectant solutions should be poured directly into a sink/drain and flushed with clean water.** In the field, pour onto an area of hard-standing, or similar unvegetated area well away from the pond. Used gloves can be disposed as domestic rubbish.
Survey to assess the distribution of amphibian chytrid fungus in England: Surveyor instructions

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1. Welcome to the chytrid survey!

Many thanks for agreeing to help with this important survey. Your help in surveying amphibians for the chytrid fungus is invaluable. Please follow the guidance in this document, which sets out exactly what should be done. If you have any queries or you have not already signed up please contact me (Edward.Brede@ioz.ac.uk) as we need to keep track of who will be doing the survey.

For more background on chytrid fungus and this survey, please see www.zsl.org/ukchytrid

2. Selecting a survey site

We need to achieve a good geographic spread of amphibian sites across England, and are aiming to have around 8 sites per English region assessed. Within your region, you are free to select any site you wish, using the following criteria:

- not known currently to have chytrid infection (as at Feb 2008, only 6 sites in Cumbria and 2 in Kent are known to be infected).

- ideally have both anurans (frogs/toads) and caudata (newts) as you will need to capture 2x30 individual amphibians in two periods (February/March, Mid May/June) and some amphibian species may not be around in sufficient numbers in either of the sampling periods. For the purpose of this survey a population can encompass more than one pond if they are within around 100m of each other. Note that you can capture amphibians of more than one species for the survey if they are available at the time of sampling (eg you do not need to catch either 30 frogs, or 30 toads; it could be 10 frogs + 10 toads + 10 newts).

- have easy access, with the permission of the landowner.

3. Preparing for your survey

Kit list: net, buckets with lids, pen, disposable vinyl gloves, and disinfectant solution if necessary (see ARG UK Note 4 available from www.zsl.org/ukchytrid for details of biosecurity)

Watch the weather, and go on a day when you are reasonably sure there will be good numbers. Visit with at least one helper.
4. Practicalities

a) Catch the amphibians. This should all be done within the same day or right. You should aim to catch 30 adult individuals per sample period of any native UK species (there can be a mix of species within these 30 individuals). If adult numbers are low, and you encounter juveniles these can also be swabbed to make up the 30 individuals. You should not catch eggs or larvae.
b) Place them in secure container such as buckets with lids
c) Swab (see details below)
d) Mark each swab – best done by a scribe
e) Release amphibian at point of capture
f) Keep swabs in fridge until sending off (details of address at the end of this document). Please submit as soon as possible.

5. How to swab your amphibian

Please refer to the ARG-UK Advice Note 4 “Amphibian disease precautions: a guide for fieldworkers” to help you reduce disease risks when doing this work.

Note: a video of this procedure is shown on the project website: www.zsl.org/ukchytrid

1. Prior to catching ensure gloves are moistened with pond water to avoid damaging the amphibian. The swabbing can be done by one or two people (one holding the amphibian whilst the other swabs). Both must wear gloves.
2. Take a firm but careful hold of the amphibian, holding its throat/head region with your thumb/index finger, this allows you to manipulate the lower limbs with your other fingers.
   NOTE: Amphibians do not have a rib cage so care should be taken not to damage internal organs by pressing the abdomen.

3. Swab the inner thigh of the hind leg with a firm continuous action. The aim is to dislodge loose skin, sporangia or zoospores. Repeat three times.
6. Repeat the swabbing procedure in 3 above but this time concentrating on the ‘drift patch’, this found just above the pelvis. Repeat three times. Place in bucket or other secure container.

7. For newts a similar approach is taken. Swab the rear limb in one action, ensuring that all of the inner leg and toes are covered. Repeat three times.
8. For newts the base of the tail and lower abdomen should also be swabbed. Repeat three times. Place in bucket or other secure container.

9. Prior to returning the amphibian to the capture location, observe it a few minutes after the swabbing procedure, to ensure that it is not harmed.
8.3 Surveyor instructions for participation in the 2011 UK national Bd survey

The 2011 UK Chytrid Survey
(aka The Big Swab 2011)

Protocol for Surveyors

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Welcome to the chytrid survey 2011!

Firstly, a huge thank you for offering your time, experience and energy to this project.

The amphibian chytrid fungus (or, to give it its proper name, *Batrachochytrium dendrobatidis*), has been much talked about since it was first identified, little more than a decade ago.

It has also been subject to considerable research effort.

We now know that this remarkable organism is capable of infecting a huge range of amphibian hosts (almost 450 different species at last count), and has achieved a global distribution.

We also know that the effects of infection vary greatly from species to species. Some species appear capable of carrying infection with no evidence of clinical disease, whilst other species experience mortality rates of up to 100%.

We do not know, at the moment, where our own native species lie within this spectrum.

However, whilst parallel research projects grapple with this question, it is vital that we continue to build our understanding of the distribution of *Bd* in the UK in order to minimise further spread of infection and, if necessary, to allow effectively targeted mitigation.

Through the highly successful 2008 chytrid survey, we have already identified a number of chytrid-positive sites in the UK.

The basic design of this year’s survey will be very similar: collection of skin swabs from amphibian populations around the UK, in order to detect chytrid, where present.

Repeating the survey this year is a really exciting opportunity, as it allows us to ask the question ‘Where are you now?’. Is distribution increasing? Or decreasing? Or staying the same? And if it’s not staying the same, where is it going?

In addition, by increasing coverage in previously underrepresented areas of the UK we can also gain a more accurate picture of chytrid distribution across the UK as a whole.

Furthermore, by slightly modifying the protocol (here come the sandwich bags), we can have greater confidence in species-specific data which will help us to identify those species most susceptible to infection.

I hope that being a chytrid swabber will be an enjoyable experience. I will be your lead contact throughout, so please, if you have any questions, comments or observations, do not hesitate to drop me a line.

Freya.smith@toc.ac.uk

With regards to the all important results, I can assure you that I will get these to you as soon as possible. However, do please be aware that with a survey of this magnitude, it may take up to a year before we can get through all the samples. Keep an eye on my page on the ARG-UK website (www.arguk.org) for updates (follow projects, 2nd national chytrid survey 2011).
Survey protocol

The protocol this year has been slightly updated from that used in 2008 (based on results, feedback and lessons learnt in 2008). So, even if you have swabbed before, please take a moment to read through the instructions carefully.

The key points that I would like you to take away with you are:

- **30 amphibians** to be sampled per site (can be a mix of species)
- Single survey between March and June (you can start as soon as you have your swab packs).
- All samples collected on a single day/evening to avoid resampling.
- **No frogs!**
- No tadpoles! (post metamorphic animals only please)
- Capture from the water (i.e. not migrating toads en route to the breeding ponds)
- **Avoid contamination between animals.** Prior to swabbing this can be achieved by temporarily housing amphibians in individual sandwich bags. During swabbing, there are two options to avoid passing infection between individuals: changing gloves between amphibians, or handling amphibians through their sandwich bags (so that they do not come into direct contact with your gloves)

The basic run of events, for the skim readers, should be

- Get to your site on an amphibian friendly day/night
- Catch 30 amphibians (from the water) and put them in individual sandwich bags.
- Swab hind legs, drink patch/lower abdomen and tail of newts (according to illustrated protocol).
- Label swabs clearly and complete recording forms
- Release animals at point of capture
- Make sure you haven’t left any rubbish behind

Swabs, sandwich bags, gloves and a stamped return envelope (for return of samples) will all be provided

The text/illustrations below will explain in detail. If you have any questions after reading through all of the below, let me know and we’ll sort it out.
1) Choose a site

By this stage, most sites will already be confirmed. However, previously, there has been some confusion as to the exact definition of a 'site'. When you collect your 30 samples, all animals sampled should be from the same population. Therefore if you have several adjacent ponds, as long as there are no physical barriers preventing movement between the ponds, and all ponds are within a 500 m radius (i.e. the two most distant ponds are no more than 1km apart), the complex of ponds can be considered a single 'site'. If your site does consist of more than one pond, it would be helpful if you could provide a quick sketch, numbering the ponds (there is a space for this on the Site Recording Sheet). On the Sample Recording Sheet, there is a space for noting down the origin of each sample.

2) Preparing for your survey

If you are surveying after dark, please make sure that you are familiar with the site, and any potential hazards by making a visit during daylight.

The equipment you require will include: net(s) (and/or bottle traps for newts), pen(s) (a marker pen is useful for numbering swab tubes, biro for recording sheets), disposable vinyl gloves (at least one pair each per site), swabs, sandwich bags, several buckets for storing bagged amphibians, and disinfectant solution (see ARG Advice Note 4).

Watch the weather and go on a day when you are reasonably sure there will be good numbers. Visit with at least one other person.

3) Timing the survey

Surveys should take place, for the most part, between April and June. However, some of you may wish to start sampling in March, if you are hoping to catch toads or early-starting newts. If you are planning to survey in March, you might want to drop me an email to make sure I send out your kit in good time.

All samples should be collected on the same night. If samples are collected over a period of different sampling dates, some animals may get swabbed more than once, which muddles up the data.

4) Numbers

We are asking surveyors to collect 30 samples per site. This number is based on the need to make statistical inferences and means that, assuming the background prevalence of infection is at least 15%, we can be 99% confident of sampling at least one positive animal. So, if at all possible, do try and stick to this sample size. There is no need to collect more than 30 samples.

However, if you have completely exhausted the site, and you haven’t managed to collect 30 samples, please do send in your swabs anyway. They will still be useful.

5) Species

The results of the 2006 survey suggest that all UK native species can carry infection. However, prevalence in the common frog (*Rana temporaria*) was very low, and this finding is supported by chytrid surveillance elsewhere in Europe. As a result, even at infected sites, the common frog is unlikely to test positive, meaning that we are more
likely to miss infection if we are sampling this species. We are therefore excluding the common frog from this survey.

The 30 samples can be made up of all other UK species as well as any non-native species you come across. If you are sampling great crested newts (Triturus cristatus) or natterjack toads (Epidalea calamita) you will need your licences amended to include swabbing. If you need assistance with this, please let me know. If you are sampling non-native species this will also require a licence to allow you to re-release animals after swabbing (even if they are going back to exactly the same location) so please make sure you have discussed this with me.

6) Pre sampling segregation

Prior to swabbing, we are asking people to keep individuals separately in order to avoid cross contamination between animals. This is a new addition to the 2008 protocol. The advised method is as follows:

Using a plastic sandwich bag to glove your hand, pick up the amphibian. Pull the bag over your hand (now gently clasping the amphibian) to turn the bag inside out (as though you were cleaning up after your dog...). Add a few damp leaves, or a small quantity of pond water and seal the bag with a tight knot, being careful to leave a pocket of air. Once you have collected the full 30 animals, you can start swabbing. Gently untie or, if necessary, break open the bag at the neck. Drain the water/remove leaves and, handling the amphibian through the bag (so that it doesn’t come into direct contact with your gloves), swab according to the illustrated method below. If you prefer, rather than handling animals through their sandwich bags, you can simply change gloves between individuals. Do whatever feels easiest. The main thing is that we avoid passing infection from one animal to another. The amphibian can then be released directly at point of capture.

Pre-swabbing, you will need something solid to store the bagged animals in to prevent escape of the hamster-ball variety (toads, in particular, can make an impressive bid for freedom even within the confines of a plastic bag). Two or three large buckets should suffice. If surveying during the day, ensure that animals are kept out of direct sunlight so that they do not overheat. Please ensure that you remove all rubbish when you leave.

7) Swabbing

Swabbing methodology is best explained by illustration (see below). The method has not changed from 2008.

8) Recording

This year there are two recording sheets. One for recording site information, and one for recording swab details. Please label all swabs clearly (with pen, not pencil) with the first three letters of your site name, followed by the swab number. e.g. Lon 1 (for swab number 1 from London Zoo)

9) Sending in swabs

On returning from the field please post the swabs to:
UK Chytrid Survey, Wellcome Building, Institute of Zoology, Regent’s Park, London NW1 4RY.

If you are unable to post the swabs straight away, please keep in the fridge for the interim period.
Swabbing by pictures

The sandwich bag technique – for keeping individuals separate prior to swabbing

When you have caught the amphibian, gently pick it up using a plastic, water tight sandwich bag. Invert the bag so that the amphibian is now enclosed within it. Add a small amount of water or some damp leaves for the amphibian to hide in, before sealing the bag with a knot. Please be sure to trap a pocket of air within the bag, before you seal it. Provided the animals are kept in a cool place (i.e. out of the sun if you are surveying during the day) they should be perfectly happy for the duration of your catch period. Once you have assimilated your 30 individuals, you can start swabbing (see below). You will need to take several buckets with you to store the bagged animals in to prevent runaways (amphibians can propel themselves even inside a plastic bag!).

Please also ensure that you have accounted for all bags used at the end of the survey and have left no litter behind you.
How to swab

The purpose of swabbing is to dislodge loose skin, chytrid sporangia or zoospores. This means that you will need to be quite firm with your swabbing.

When handling animals during swabbing, it is important to avoid cross contamination between animals (or swabs). This can be achieved either by handling animals through the sandwich bag (which means that the amphibian does not come in direct contact with your gloves), or, if you prefer, by changing gloves between individuals. A sufficient supply of gloves will be provided. Soil and other organic matter can make it more difficult to detect chytrid. If you have a particularly muddy specimen please give it a quick watery dunk (pond water is fine) to clean off the worst of the mud.

The pictures below show the technique that should be used for swabbing.

1. Take a firm but careful hold of the amphibian, on its back, holding its throat/head region with your thumb/index finger. This allows you to manipulate the lower limbs with your other fingers. Amphibians do not have a rib cage so care should be taken not to damage internal organs by pressing the abdomen.

2. Start by swabbing the inner thigh of the hind leg with a firm continuous action. Repeat three times on each leg.
3 Repeat the swabbing procedure in 2 above but this time concentrating on the lower hind leg. Repeat three times.

4 Repeat the swabbing procedure in 3 above but this time concentrating on the underside of the foot/toes. Repeat three times.

5. Repeat the swabbing procedure in 3 above but this time concentrating on the ‘drink patch’, this found just above the pelvis.
6. For newts a similar approach is taken. Swab the rear limb in one action, ensuring that all of the inner leg and toes are covered. Repeat three times.

7. For newts the base of the tail and lower abdomen should also be swabbed. Repeat three times.

8. After swabbing, the amphibian can either be released directly, or stored in a secure container until release. However, prior to returning it to its capture location, please observe the animal for a few moments to ensure that it is unharmed.

**Dead amphibians.** Please do keep your eyes peeled for any dead amphibians you might encounter during your survey. If you find a carcass, please freeze immediately and contact me ((troya.smith@cez.ac.uk, 02074498621)) to discuss whether submission for post mortem examination is appropriate. To store the carcass safely, seal within two plastic bags (one inside the other) and put in a rigid, crush proof container (e.g. margarine tub) before placing in the freezer.
Extras

Although the primary aim of this study is to investigate the distribution of chytrid fungus in the UK, we are also very interested to find out more about another amphibian pathogen, amphibiocystidium, which affects newts.

Unlike chytrid, amphibiocystidium infection results in very obvious skin lesions as shown below. Should you encounter any newts showing these lesions during your survey, please do jot down your findings on the swab record sheet (under ‘reporting of abnormal individuals’). It would be helpful if you could mark your comments as ‘possible amphibiocystidium’.
8.4 Pre-exposure mortality observed in experimental animals

Pre-exposure, I observed a rapid deterioration in some aquatic newts. Most commonly, newts presented with a halo of infection surrounding one or more digits (Figures 1a and b). Left alone, rather than resolving, these initially superficial infections quickly became necrotic, leading to the complete loss of digits (Figure 1c) and in some cases, the entire foot (not shown). Swabs showed no evidence of pathogenic bacteria, and this condition was not associated with pre-exposure *Bd* infection. At the same time, animals also presented with anorexia, lethargy and abnormal posturing. In total 23 of 181 animals were euthanased. A further 26 animals were found dead, frequently in the absence of any *ante mortem* signs of disease. The remaining animals were moved into terrestrial tanks and there were no further pre-exposure signs of disease/mortality.

Figure 1 Pre-exposure lesions in aquatic smooth newts including (a) digit of left fore foot, (b) digit of right hind foot and (c) left fore, with shortened digits due to infection.