An investigation into the effects of *Batrachochytrium dendrobatidis* (*Bd*) on natterjack toad (*Bufo calamita*) populations in the UK

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Contents

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Summary

The chytridiomycete *Batrachochytrium dendrobatidis (Bd)* is a parasite which has been blamed for amphibian declines across the world. This study was designed to investigate the effects of *Bd* on natterjack toads (*Bufo calamita*), following the discovery of *Bd* in populations of this species in the UK.

The effect of *Bd* on natterjack toads was assessed by fieldwork and experiments. Wild adult natterjacks were tagged and repeatedly tested for *Bd* during 2009-2011. Captive adults and juveniles from infected populations were also tested in response to changes in environmental conditions. Swabs were used to collect *Bd* DNA from the skin of study animals. Swabbing did not reliably diagnose infection but the quantity of *Bd* DNA in swabs (*Bd* score) provided an indication of infection activity. Immersion in water appeared to trigger *Bd* zoospore emergence from the skin, resulting in an increase in the likelihood of *Bd* detection and increases in *Bd* score.

Bd dynamics in natterjack populations were also influenced by salinity. Natterjacks in the UK are found mainly in coastal habitat, where ponds are often inundated by high tides. Adults captured in brackish water were less likely to test positive than those caught in fresh water. *Bd* isolated from coastal natterjacks was killed *in vitro* by a salinity equivalent to 50% seawater. The isolate grew fastest at low salinities, suggesting that it may have become adapted to brackish conditions. Despite this adaptation, tidal inundation may be sufficient to disinfect ponds and limit *Bd* transmission.

Capture-mark-recapture (CMR) data from adult natterjacks revealed a weak negative correlation between *Bd* score and survival in the wild. Males had higher *Bd* scores than females but survival did not differ between sexes and there was no correlation between *Bd* score and growth. An experiment showed *Bd* could kill natterjacks if infection activity was boosted by wet conditions. However, only 6% of wild adults recorded *Bd* scores in excess of a mortality threshold derived from this experiment. Many adult and juvenile natterjacks can tolerate *Bd* infection and act as reservoirs of this pathogen.

Despite detection of *Bd* in at least 14 UK natterjack populations by 2011, no mass mortalities of adult natterjacks have been recorded and spawning has continued at all sites. *Bd* does not appear to have a major effect on natterjacks but this situation may not persist and vigilance should be maintained. *Bd* isolated from natterjack toads in this study belongs to a global panzootic lineage (GPL) of *Bd* which Farrer *et al* (2011) claim has achieved a global distribution as a result of human activities.

Chapter 1.

Introduction

I began this project in 2008, following detection of *Batrachochytrium dendrobatidis (Bd)* in samples from natterjack toads (*Bufo calamita*) in Cumbria (Feltrer & Cunningham 2006). Discovery of *Bd* in this species was a cause for concern, as natterjacks are endangered in the UK (Buckley & Beebee, 2004). The government agency Natural England (NE) provided the majority of funding for this project, which was designed to investigate the impact of *Bd* on natterjack toad populations. In this chapter I provide some background information on *Bd* and natterjack toads, a summary of the project aims and an explanation of the chapter order.

***Batrachochytrium dendrobatidis (Bd)* – a global scourge of amphibians?**

The chytridiomycete *Batrachochytrium dendrobatidis (Bd*) has been blamed for the decline of many amphibian species (Skerratt *et al*, 2007) since its discovery by Longcore & Pessier (1999). *Bd* is a successful parasite of amphibians which may also infect birds (Garmyn *et al* 2011) and crustaceans (Rowley *et al* 2006, Rohr *et al* 2012). *Bd* has been recorded from amphibian populations on all five continents where amphibians are known to occur.

The infectious life-stage of *Bd* is a motile, aquatic zoospore (Berger *et al*, 2005). Once attached to the skin of an amphibian, zoospores encyst in the keratinised epidermis and develop into mature zoosporangia (Fig. 1.1). Zoosporangia produce more zoospores, which emerge from the skin via discharge tubules. Evidence suggests that *Bd* infection can cause death in amphibians by inhibiting the cutaneous uptake of electrolytes, particularly sodium, which are essential for circulatory function (Voyles *et al*, 2009).

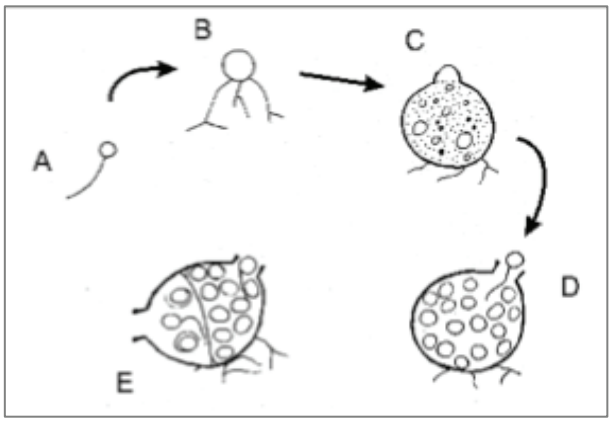


Fig. 1.1 Lifecycle of *Bd* in culture (reproduced with permission from Berger *et al*, 2005).

A = motile zoospore
B = germling
C = developing zoosporangium
D = monocentric zoosporangium
E = colonial thallus

A mechanism which results in death from *Bd* infection has been described but the factors leading to outbreaks of chytridiomycosis (the disease caused by *Bd*) in populations are poorly understood. According to the novel pathogen hypothesis, outbreaks have resulted from arrival of *Bd* in areas where the pathogen is not endemic and amphibians have not evolved an effective immune response (Lips *et al*, 2006). Others have suggested *Bd* is endemic to many regions, with chytridiomycosis triggered by environmental factors (Rachowicz *et al*, 2005).

Farrer *et al* (2011) describe a global panzootic lineage (GPL) of *Bd*, which they claim has recently evolved, is hypervirulent and has been spread by human activities. However, the importance of the GPL may have been overstated, since the strains sequenced were largely collected in response to a test developed by Boyle *et al* (2004), which does not detect all extant lineages (see Goka *et al*, 2009). Despite self-referential bias in genetic studies, it is likely international trade has accelerated the introduction of novel strains to naïve populations.

Bd is just one of several pathogens which are known to infect amphibians. Several iridoviruses, including *Ranavirus*, have been linked to mass-mortalities of amphibians (see Mavian *et al*, 2012). The body of evidence suggests *Bd* has had a greater global impact than *Ranavirus,* but the relative importance of different amphibian diseases is difficult to gauge (see Duffus, 2011). Governments are now required to report detection of *Bd* and *Ranavirus*, which are globally notifiable diseases according to the World Organization for Animal Health (Schloegel *et al* 2010).

There are options for controlling the spread of amphibian diseases. Stricter control of the trade in farmed and wild amphibians might reduce the rate of pathogen spread and the likelihood of newly evolved, virulent strains achieving global distributions. However, Garner *et al* (2009a) claim it would be difficult to control the amphibian food trade in many countries and a ban on pets could drive trade underground. Quarantine of live imports and treatment for *Bd* is a possibility, as infected amphibians can be treated in several ways, including the use of fungicides. Schlaepfer *et al* (2005) report that 15 million wild-caught amphibians entered the U.S.A. legally from 1998-2002, so even this would present a major challenge.

Attempts to control pathogens such as *Bd* in wild amphibian populations are unlikely to succeed, unless the population has a very restricted distribution (for instance, on a small island) and all hosts present in the ecosystem can be identified and treated. Attempts have been made to control *Bd* in wild populations of Mallorcan midwife toads (*Alytes muletensis)* using fungicides (see Lubick, 2010) but with limited success (Bosch *et al*, 2010).

_Bd_ in mainland Europe and the UK

Bd was first detected in samples from amphibians in Europe in 1997 (Walker *et al*, 2010) and 2004 in the UK (Cunningham *et al*, 2004). Without prior monitoring, detection does not imply arrival and it is unlikely *Bd* arrived on these dates. No monitoring for *Bd* was carried out in the UK before 2004. Only two strains of *Bd* have been isolated from wild amphibians in the UK, both of which belong to the GPL (Farrer, *pers.comm* 2011). Although the GPL may be a recent introduction, different (and possibly endemic) strains have been found in mainland Europe (Farrer *et al*, 2011). More work is required to determine if endemic strains exist in the UK.

Mass-mortalities of amphibians have been attributed to *Bd* in mainland Europe, mainly in species not native to the UK, such as midwife toads *Alytes obstetricans* (Fig 1.2.) and fire salamanders *Salamandra salamandra* but also in common toads *Bufo bufo* (Bosch & Martinez-Solano, 2006). To date, no mass-mortalities have been linked to *Bd* in the UK but the impacts of *Bd* may have gone unnoticed, especially if *Bd* can have more subtle effects. It is possible natural selection in response to *Bd* has already occurred. May *et al* (2011) report unusual variation in the frequency of MHC alleles in natterjack toad (*Bufo calamita*) populations in the UK, which may have resulted from the introduction of a novel pathogen.

Two national surveys of the distribution of *Bd* in the UK have been completed, in 2008 (Cunningham & Minting, 2008) and 2011 (unpublished data). In 2008 *Bd* was detected in most native and non-native amphibian species sampled and was found to be fairly widespread. *Bd* has now been found at many other UK sites, in addition to those shown in Fig. 1.3.

Fig. 1.2. Mass-mortality of European midwife toadlets (*Alytes obstetricans).*
Reproduced with permission of M. Fisher.

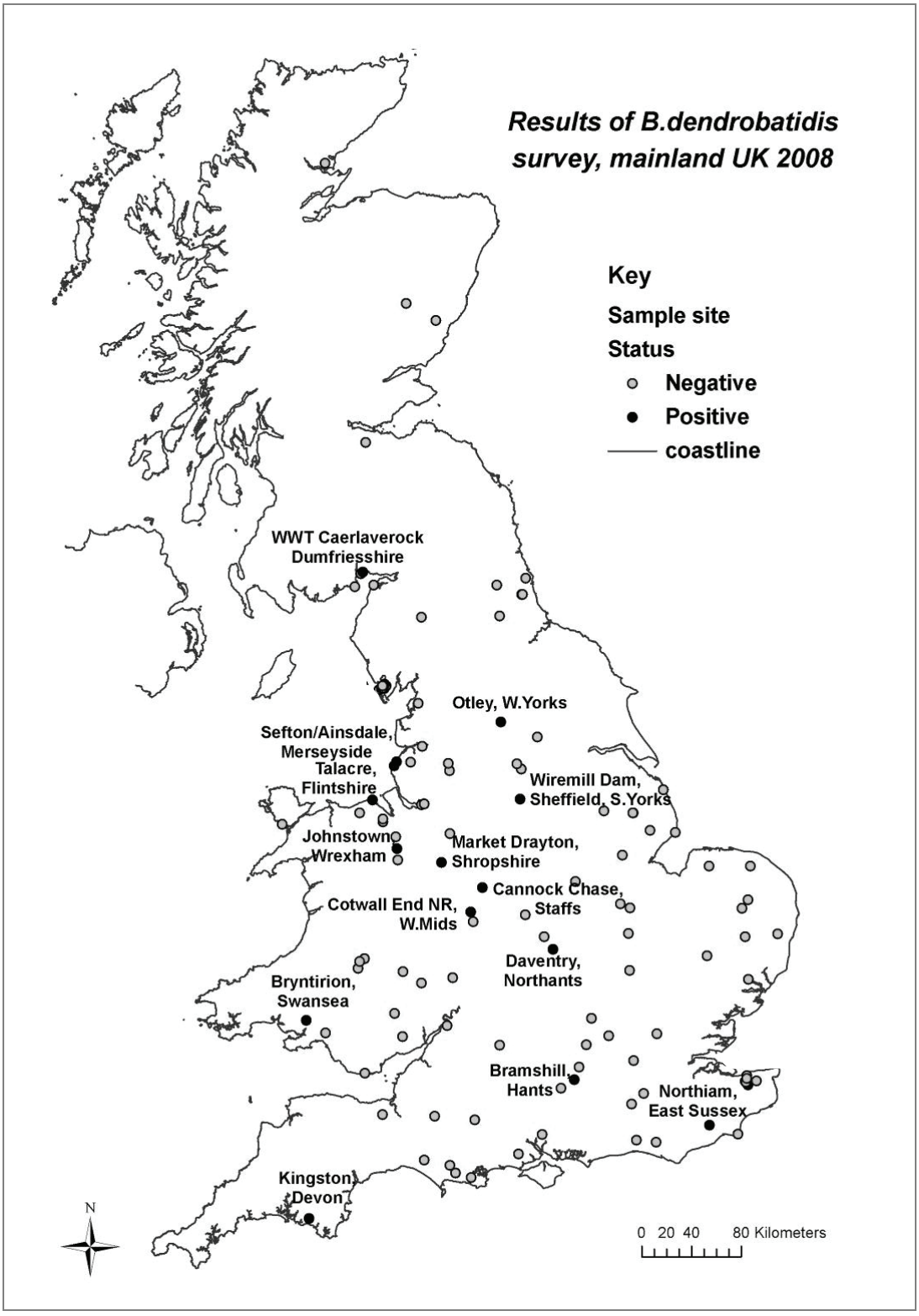


Fig. 1.3. Results of the 2008 survey for *Bd* in the UK (positive = *Bd* detected). Results are from all amphibians captured. Names shown for positive sites only. Reproduced with permission from Cunningham & Minting (2008).

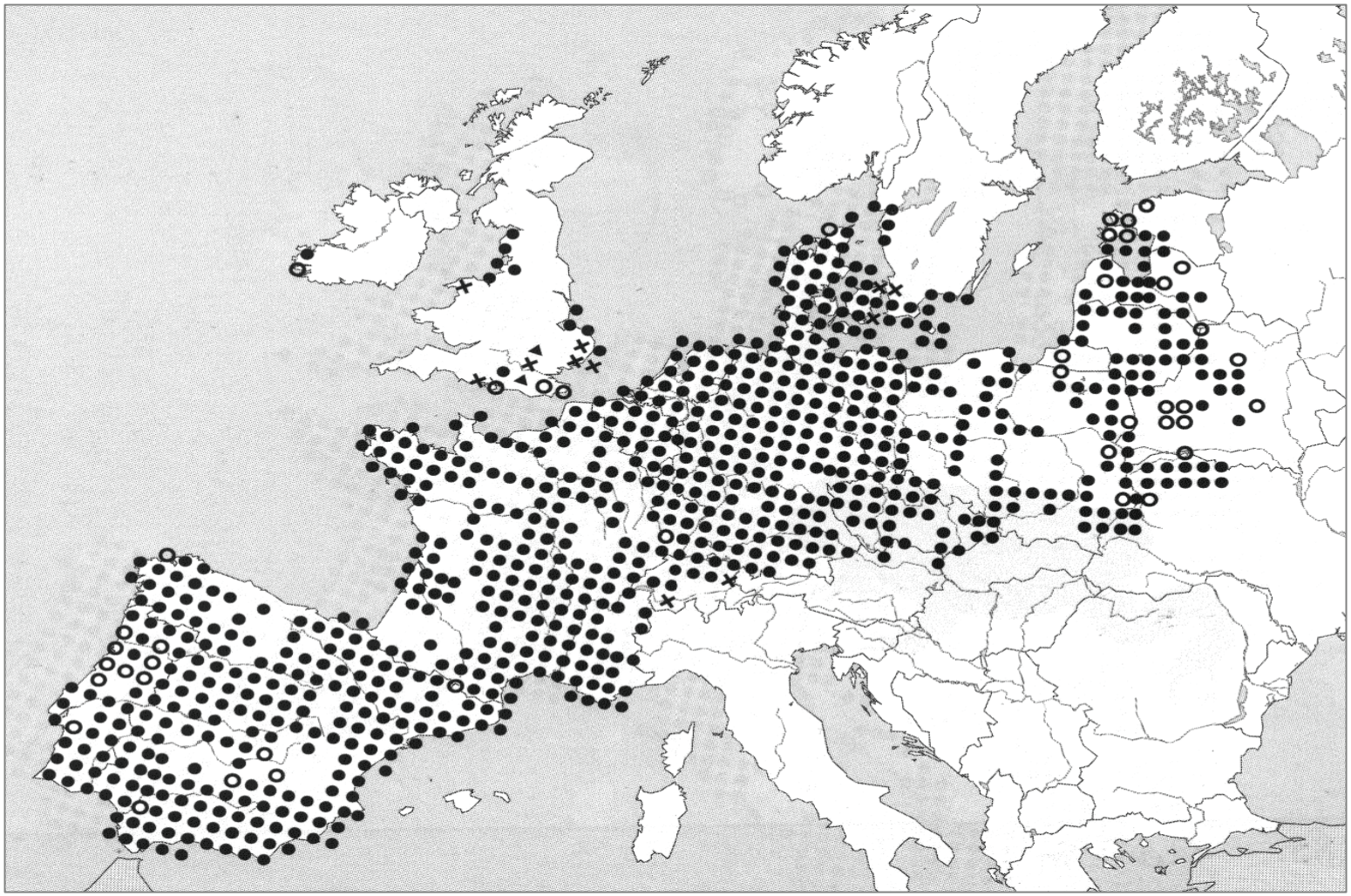
The natterjack toad in the UK; native biodiversity under threat

The natterjack toad (*Bufo calamita*) is endangered in the UK (Buckley & Beebee, 2004), where most of its habitat has been lost as a result of land drainage, development or changes in grazing practices. The natterjack is common in mainland Europe north of the Alps (Fig. 1.4.) and is classified as a 'species of least concern' by the International Union for the Conservation of Nature (IUCN).

Although the natterjack is not considered endangered in Europe, it is a protected species under the EC Habitats Directive 1992 (Annex IV) and Bern Convention 1979 (Appendix 2). In the UK it is protected under regulation 39 of the Conservation (Natural Habitats) Regulations 1994 (schedule 2, as amended), where it is also protected by the Wildlife and Countryside Act (1981) (section 9, schedule 5, as amended) and Natural Environment and Rural Communities Act (section 41, 2006). As a signatory to the Rio Convention on Biodiversity (1992), the UK government is required to take action to protect biodiversity. Attempts at compliance include Local Biodiversity Action Plans (LBAPs), under which natterjack populations (or their habitat) are listed as a management priority. In England these plans will soon be replaced by the England Biodiversity Strategy (Edgar,*pers.comm* 2012).

There are only seven native species of amphibian in the UK, compared to at least 56 in mainland Europe (Arnold & Ovenden, 2002). Many species failed to recolonise the UK before the formation of the English Channel after the last ice age. In the 1990s the last native population of pool frogs (*Pelophylax/Rana lessonae*) in the UK became extinct. It is possible other species will be lost, including the natterjack, if conservation effort is not maintained. Effort is being made to conserve the remaining UK natterjack populations, including habitat management, long-term monitoring and translocations. Licensed movements of tadpoles or spawn have helped to establish (or re-establish) many populations (Beebee & Buckley, 2004) in the UK in addition to those shown in Fig.1.4.

The ecology of this charismatic species is described in detail by Griffiths & Beebee (2000). Adults and juveniles are easily identified by their yellow dorsal stripe and the calls of adult males can be heard for several kilometres. In the UK, natterjacks hibernate on land from November until April, with spawning peaking in May. Natterjacks breed most successfully in ephemeral ponds in the absence of common species which tend to dominate in large, permanent ponds (Griffiths *et al*, 1991). Tadpoles metamorphose by early August, with toadlets dispersing away from ponds during heavy rain. Most adults move away from ponds in June and feed nocturnally on invertebrates until hibernation. In Europe, natterjacks are found in many habitats up to an altitude of around 2000m but in the UK few inland populations have persisted (Fig. 1.5.), with the majority found in coastal sand dunes and saltmarshes.



Key: • – present after 1970; o – before 1970; x – extinct; ▲ – introduced

Fig. 1.4. European distribution of the natterjack toad (*Bufo calamita*). Reproduced with permission from Gasc *et al* (1997).

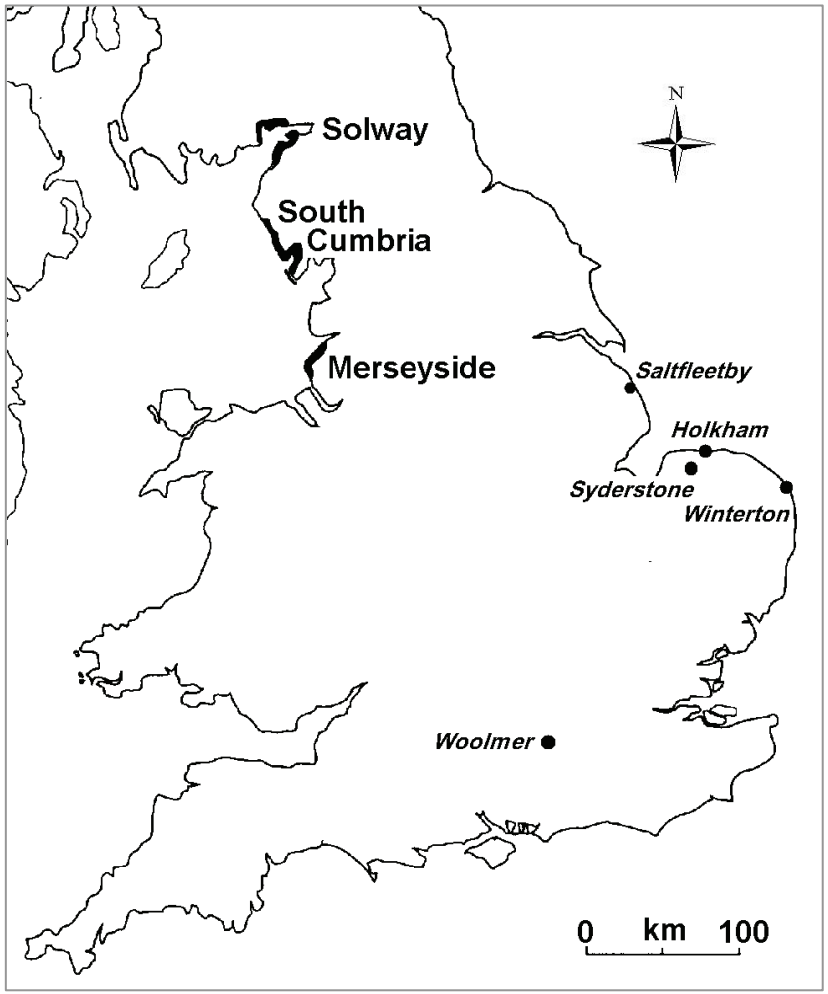


Fig. 1.5. Natterjack distribution in the UK (native sites).
Reproduced with permission from Rowe & Beebee (2007).

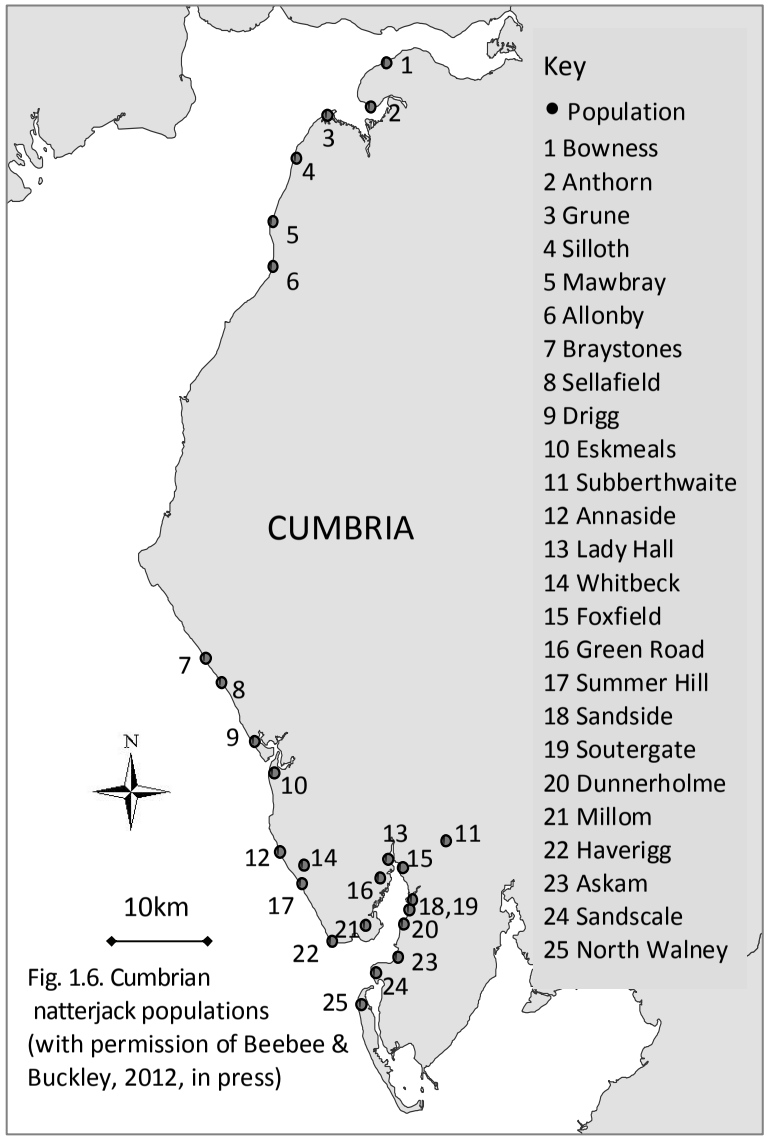


Fig. 1.6. Cumbrian natterjack populations (with permission of Beebee & Buckley, 2012, in press)

Project aims and explanation of chapter order

The principal aim of this project was to assess the impact of *Bd* upon natterjack toad populations in the UK. I did not aim to predict the future impacts of *Bd*, as host-pathogen relationships are often unstable. However, I did aim to determine the current impacts of *Bd* on a small number of populations and captive individuals in controlled conditions. The project was largely field-based, to allow intensive monitoring of populations and maximise opportunities for the detection of any negative impacts of *Bd* in the wild. Experiments were used to try and answer questions which could not easily be resolved using field data.

In order to achieve the main project aim, I tried to answer several key questions;

- How reliable is the standard diagnostic swab test for *Bd* infection?
- Where and when does *Bd* activity (and transmission) peak?
- Do environmental factors have a significant influence on *Bd* in this system?
- Can *Bd* kill natterjack toads, when all other factors are held constant?
- Is *Bd* linked to a reduction in survival of natterjack toads in the wild?
- Has *Bd* caused the local extinction of any natterjack populations?

I address the first two questions in Chapter 2, where I evaluate the ability of an epidermal swab test to diagnose *Bd* infection in natterjack toads and describe seasonal variation in the detection of *Bd* in adults and juveniles. My interpretation of results in subsequent chapters is guided by my findings in Chapter 2, which illustrate the practical limitations of the swab test.

In Chapter 3, I investigate the relationship between an environmental factor (salinity) and *Bd*. I also carry out an experiment to see if *Bd* kills natterjacks when existing infections acquired in the wild are allowed to progress. This is a novel approach – in most experimental studies of *Bd* infection and survival, captive amphibians are dosed with cultured strains of *Bd*.

In chapter 4, I report the results of a capture-mark-recapture (CMR) study to see if *Bd* scores from swab tests correlate negatively with the survival and growth of adult natterjacks. I also present spawn count data from natterjack populations where *Bd* has been present for several years.

Selection of study populations

When this project began, eight natterjack populations in the UK had tested '*Bd*-positive'. As six of these populations were in Cumbria (Arai, 2008), which also has the most natterjack populations of any UK county (Beebee & Buckley, 2012, in press), I chose Cumbria as the base for this project. My original plan was to intensively monitor and compare a small number of *Bd*-positive and *Bd*-negative populations. I initially selected four populations in northern Cumbria (Mawbray, Silloth, Grune and Anthorn). *Bd* had been detected at Mawbray and Silloth (Feltrer & Cunningham, 2006) but not at Grune or Anthorn (Arai, 2008). These populations were also separated by unsuitable habitat and thought unlikely to interact, unlike many in southern Cumbria which are poorly separated and may act as a large metapopulation.

The original plan was altered when it became apparent in 2009 that natterjacks were too scarce at Silloth for sufficient sample collection. Access to Anthorn was denied due to landowner concerns about transfer of *Bd* between sites. Alternative populations were selected to replace Silloth (Sellafield) and Anthorn (Bowness-on-Solway). Natterjacks at Sellafield had tested *Bd*-positive in 2007 and the population at Bowness was a recent introduction from Anthorn, which tested negative in 2006 (Arai, 2008). Sellafield was too distant from the project base to allow capture-mark-recapture (CMR) work on adults (Chapter 4) but I was able to collect other useful data from this site. The CMR study was successful at three sites where adult natterjacks toads were intensively monitored during 2009-2011; Mawbray, Grune and Bowness-on-Solway (Fig. 1.7.). The latter is also known as Campfield Marsh (see Beebee & Buckley, 2012, in press). Additional populations were sampled by Bramwell (2011), as part of a study of the relationship between salinity and *Bd* which is covered in Chapter 3.

Mawbray Grune Bowness-on-Solway

Fig. 1.7. Examples of ponds used for breeding by natterjacks at intensively-monitored sites

Project licensing and ethical review

I obtained appropriate licences for carrying out the work described in this project from the Home Office (Ref: PIL70/21773) and Natural England (Refs: 20083687, 20101286, 20111178). The project (Ref: WLE 0505) methods were reviewed and approved by the Ethics Committee of the Institute of Zoology at the Zoological Society of London (ZSL).

Biosecurity precautions

I followed strict biosecurity guidelines agreed by Natural England, Amphibian and Reptile Conservation (ARC; formerly the Herpetological Conservation Trust, HCT), Froglife and the Institute of Zoology at the Zoological Society of London (ZSL). These guidelines are available from the Amphibian and Reptile Groups of the UK (ARG UK, 2008).

My project included activities with a high risk of disease transfer, according to the ARG guidelines, such as transportation of live natterjacks from *Bd*-positive sites to experimental facilities several kilometres away (see Chapters 2 & 3). Captive natterjacks from different populations were housed in separate vivaria and released at their capture locations. All equipment and footwear were disinfected before and after use with Virkon (10mg/ml). To minimise the risk of disease transfer during fieldwork, no more than one population was visited per day. New disposable gloves were used when sampling each animal.

A note on taxonomy; *Bufo* versus *Epidalea*

Speybroeck *et al* (2010) have argued against many of the revisions to nomenclature proposed by Frost *et al* (2006), including the placement of the natterjack toad (Fig. 1.8.) in a new genus (*Epidalea*). As the new genus had not been widely accepted, I retained the original genus (*Bufo*).

Fig. 1.8. Life-stages of the natterjack toad (left to right; spawn, tadpoles, toadlet, adults).

Chapter 2.

Infection of amphibians by *Batrachochytrium dendrobatidis* (*Bd*) can be cryptic during terrestrial phases; evidence from the natterjack toad (*Bufo calamita*)

Abstract

Batrachochytrium dendrobatidis (*Bd*) is a widespread amphibian parasite which produces a free-swimming zoospore to infect new hosts. Evidence from natterjack toads (*Bufo calamita*) suggests the activity and detectability of *Bd* infections is boosted by immersion in water. Adult natterjacks were more likely to test positive when captured in water during the spawning season and there was an increase in *Bd* detection and *Bd* scores in toadlets following rain. Infected individuals subjected to wet and dry cycles in captivity were more likely to test negative during dry phases. Before hibernation, toadlets kept on land tested negative but tested positive following immersion in the spring. Consequently, swab tests for *Bd* DNA did not accurately reflect infection prevalence in this species. *Bd* may respond to conditions external to the host, in order to maximise the efficiency of zoospore production and transmission. Tolerance of cryptic or dormant infections could explain persistence of this pathogen in amphibians which rarely visit water.

Introduction

Diseases often display cyclic patterns, in terms of infection prevalence and timing of outbreaks. These cycles may be linked to environmental factors but even in well-studied systems, it is difficult to predict when outbreaks will occur (Dowell, 2012). It is known that pathogens can only persist in host populations if a threshold level of transmission is achieved. Although recently discovered (Longcore & Pessier, 1999) and poorly understood, the chytridiomycete *Batrachochytrium dendrobatidis* (*Bd*) is a parasite of amphibians and must obey this rule. As a widely-distributed pathogen, *Bd* must have efficient strategies for transmission.

Increases in infection levels within populations tend to coincide with peaks in disease transmission. Consequently, monitoring of seasonal infection dynamics can provide clues as to how a disease is being maintained within a population. This study was undertaken as part of a project to try and assess the effect of *Bd* upon natterjack toads (*Bufo calamita*). In order to develop an understanding of the dynamics of *Bd* infection in this species, I monitored levels of *Bd* detection and infection activity in natterjack populations for *Bd* three years.

Prior to this research, information on seasonal variation in *Bd* detection in natterjacks was very limited. A survey of the distribution of *Bd* in the UK (Cunningham & Minting, 2008) suggested positive results were more likely from natterjack adults during spring than summer. A sample of natterjack toadlets had tested positive for *Bd* in the autumn after collection from the wild in the spring, but they might have been infected by other captive amphibians (Cunningham & Feltrer, 2006). There were no records of tests for *Bd* among natterjack spawn or tadpoles.

While investigating seasonal dynamics of *Bd* in natterjack toads, I attempted to evaluate the effectiveness of the swab test which is widely used to diagnose *Bd* infection. Most studies of *Bd* infection in amphibian populations, including this one, have relied heavily upon swab tests. This is the most sensitive method available for *Bd* detection, which has been widely-adopted by researchers since development by Boyle *et al* (2004) but like most diagnostic tests, it is not 100% reliable. Even when surveys include histological examination of tissue samples, false negatives can occur, resulting in underestimates of true infection prevalence.

One potential limitation of the swab test is variation in sensitivity. Most researchers have attributed variation in levels of *Bd* detection, assessed using swabs, to changes in infection status. The proportion of animals testing positive is often described as '*Bd* prevalence'. This is a dangerous assumption, since there may be other reasons for an increase in the number of positive results, such as a change in infection activity or load. Swab tests work by detecting *Bd* DNA on the surface of the skin. The infectious stage of *Bd* is a zoospore, which may not continuously emerge from infected animals. Swabbing may not detect the reproductive life-stages of *Bd* (zoosporangia) which are encysted within the epidermis.

I had reason to suspect *Bd* would be able to respond to conditions external to the host and for zoospore emergence to increase upon contact with water. According to Gleason and Lilje (2009), it is possible for zoospores to encyst within zoosporangia before release, in order to avoid unfavourable environments. *Bd* zoospores are aquatic and killed by drying (Johnson *et al*, 2003), so emergence in dry conditions would be a maladaptive waste of resources.

To test my hypothesis that immersion in water would trigger the activity of *Bd* infections, I exposed captive adult natterjacks to cycles of wet and dry conditions. To test for persistence of infections across years, I tested captive natterjack toadlets for *Bd* before and after hibernation. Although natterjack spawn and tadpoles do not overwinter, these life-stages were also sampled to test for involvement in *Bd* dynamics within years.

Methods

Detection and quantitative measurement of *Bd* DNA by real-time PCR

Skin swabbing followed by real-time PCR (rt-PCR) analysis of the swabs was adopted as the standard method for detection and measurement of the quantity of *Bd* DNA on the skin of amphibians sampled. A limited number of tissue samples (tadpoles and spawn) were also collected and analysed using the same rt-PCR test. Swab and tissue samples were stored in a fridge at 4-5°C until transport to the laboratory for analysis.

Sample analysis was completed using the method developed by Boyle *et al* (2004). Boyle *et al* (2004) identified a sequence of DNA within the ITS-1 region which is specific to *Bd*. Use of this target resulted in a very sensitive test, as the ITS-1 region has a high copy number. An extraction prepared from one *Bd* zoospore would be expected to contain several copies of the ITS-1 region sequence. According to Boyle *et al* (2004), the test is capable of detecting 0.1 genomic equivalents (GE) of *Bd* DNA.

DNA was extracted from samples using the following method. Each swab (or tissue sample) was transferred into a 1.5ml Eppendorf containing PrepMan Ultra and zirconium/silica microbeads. The tubes were shaken for 90s using a beadbeater to macerate the samples and centrifuged for 30s at 14,500 rpm. Tubes were then heated to 100°C for 10min, allowed to cool, centrifuged again and the supernatant (extraction) collected. In order to perform the rt-PCR tests, 4µl of each extraction were pipetted into a 1.5ml Eppendorf containing 36µl of microbiology-grade, nuclease-free distilled water (dH_20). This 10x dilution was performed on all samples to minimise the risk of PCR-inhibition by a high concentration of Prepman Ultra.

A 96 (x50µl) well optical PCR plate was prepared to receive the extractions. First, a reagent mix was added to each well. This contained Taqman Universal MasterMix, forward and reverse primers ITS-1 Chytr3 and 5.8S Chytr and fluorescent Taqman Chytr MGB2 probe (Applied Biosystems) to locate the sequence of *Bd* DNA described by Boyle *et al* (2004). Four reference standards prepared from *Bd* cultures (using a haemocytometer) were included in each plate. These contained the equivalent of 100, 10, 1 and 0.1 GE of *Bd* DNA. Two wells were assigned to each of the four standards, plus two for a blank dH_20 control. Inclusion of standards and controls left 86 wells free to test 43 samples twice in each plate.

Analysis of the plate was completed using an ABI Systems rt-PCR machine, programmed to calculate *Bd* DNA quantity for each sample from the reference standards. Successful amplification of target DNA was detected by a change in signal from the fluorescent-labelled probe above baseline levels. The minimum number of PCR cycles (*Ct*) required to detect amplification was approximately 26-27 *Ct* for the 100 GE *Bd* DNA standard and 38-39 *Ct* for 0.1 GE. A linear regression and estimate of R^2 was calculated from the standard results and any plates with sub-standard regressions (<0.9 R^2) repeated. Satisfactory results were used to make a final calculation of *Bd* score for each sample from the mean result of the two wells (multiplied x10 to account for post-extraction dilution). In cases where amplification was recorded in only one of the two sample wells, the sample was repeated. If amplification was then recorded in both wells, the sample was classed as positive.

Estimates of *Bd* DNA quantity were derived from all samples showing evidence of amplification. Most *Bd* studies to date, including this one, have used a threshold of 0.1 GE to classify samples as positive or negative. In this study, the estimated quantity of *Bd* DNA was also analysed as a continuous variable. This '*Bd* score' was log-transformed before analysis to achieve a normal data distribution (see Chapter 3, p41), so some inaccuracy in estimates derived from *Ct* values was not considered problematic.

Sub-sets of samples were re-tested with the addition of internal positive controls (IPCs) to test for the presence of PCR-inhibition. These samples were selected to check that observed patterns in *Bd* detection data were not spuriously generated by environmental contaminants such as salts or humic acids, which are known PCR inhibitors. To test for PCR-inhibition, samples were tested as described above but with 1µl of 10x Exo IPC and 0.5µl of 50x Exo IPC DNA added to one of each duplicate well. Exo IPC DNA is unlikely to amplify in the presence of PCR inhibitors. The rt-PCR machine was re-programmed to detect amplification of Exo IPC DNA in these wells, in addition to *Bd* DNA. If the IPC and *Bd* DNA failed to amplify (<50Ct), samples were classified as inhibited. Wells testing positive for IPC DNA were classified as non-inhibited. An experiment was also undertaken to assess the effectiveness of Environmental Taqman (Applied Biosystems) as an alternative reagent to the standard Taqman recommended by Boyle *et al* (2004). The results of these tests are included in Chapter 3, where the factor under investigation (salinity) warranted a special assessment of bias due to PCR-inhibition.

Calculation of sampling targets for detection of *Bd* in populations

Sample targets were set before attempting to detect *Bd* in wild amphibian populations. According to the epidemiological program Epi Info (Dean *et al*, 2004) at least 60 samples (*n*) are necessary to provide 95% confidence of pathogen (*Bd*) detection if population prevalence (p_p) is 5% and the test has a sensitivity of 100%. Epi Info uses the following equation to derive estimates, where *p* is the probability of finding at least one positive result;

$$p = 1 - (1 - p_p)^n \quad \Longrightarrow \quad n = \frac{\log(1-p)}{\log(1-p_p)}$$

The proportion of natterjacks testing positive in earlier surveys was variable (Cunningham & Minting, 2008) and the ability of the swab test to determine prevalence was questionable. Consequently a conservative target for each life-stage was set at a minimum of 60 samples.

Seasonal variation in the detection of *Bd* in wild adult natterjacks

Three natterjack populations in north Cumbria were selected for intensive monitoring during 2009-2011. These populations (Mawbray, Grune and Bowness-on-Solway) were considered unlikely to interact as they were separated by unsuitable habitat (for locations, see Chapter 1, Fig. 1.6.). Field data from these populations were used to complete many other analyses during this project, including those described in Chapters 3 and 4.

Toads were captured during standardised night-searches which were carried out at intervals of no greater than 14 days at each site. The searches included visits to all known breeding ponds. Searches were completed from April to September during 2009 and 2010 and April to May in 2011, achieving effective coverage of three spawning seasons.

All adult natterjack toads (>38 mm snout-to-vent length, SVL) seen during searches were captured and sampled, except amplectant pairs which were only sampled if this could be achieved rapidly without disrupting spawning. Each adult toad was first rinsed with dechlorinated tapwater to ensure the skin surface was wet and free of debris. A swab for *Bd* DNA was then taken using a sterile cotton swab. The method was standardised with 35 strokes of the swab across the hind feet, drink patch, front feet and dorsal epidermis. I swabbed most areas of the animal to try and maximise the probability of *Bd* detection, as I had no information about the distribution of infection in natterjacks.

Detection of *Bd* in adult natterjacks captured in ponds versus on land

I also noted location of capture for each toad; either in a pond or > 5m from water. This was to see if *Bd* was more frequently detected in toads captured in water, regardless of the season. If this prediction was confirmed it would provide support for the immersion hypothesis.

Seasonal variation in levels of *Bd* detection in natterjack toadlets

Samples from two Cumbrian populations (Mawbray and Sellafield) were used to describe seasonal variation in *Bd* detection and *Bd* scores in natterjack toadlets. To increase opportunities for sampling toadlets in this study, natural refugia at these sites were augmented prior to toadlet emergence by laying concrete tiles on land at intervals around the edges of ponds.

To assess temporal variation in *Bd* results from toadlets, swab samples were taken (using the same method as for adults, except that only 10 swab strokes were used) at two-week intervals in 2009 from toadlets found beneath the pondside refugia, until the majority of toadlets had dispersed. This was the only year in which toadlets were systematically sampled for *Bd*. I tested wild toadlets for *Bd* for as long as possible after emergence.

Experiment to assess the effect of immersion on *Bd* activity and detection

I suspected many adult natterjacks which tested negative in the wild after the spawning season (or in dry conditions) were still infected with *Bd* but tested negative due to a decline in *Bd* infection activity. To test this hypothesis, I carried out an experiment where natterjack adults from known *Bd*-positive populations were captured and exposed to cycles of wet and dry conditions. I measured the frequency with which swabs failed to detect infection in each condition by housing toads singly and collecting a series of swabs from each animal.

Adult natterjacks (n = 6) were collected from populations (Grune and Sellafield) where *Bd* had been recorded elsewhere in this study and by Arai (2008). Strict biosecurity precautions (see Chapter 1, p19) were undertaken to prevent cross-contamination between toads and equipment used during transport and captivity. Toads were transported to a secure, roofed outdoor enclosure and individually housed in plastic vivaria (320 mm x 400 mm x 180 mm). These vivaria were raised on pallets to prevent any opportunity for cross-contamination by surface drainage.

Each toad was allocated a pair of vivaria set up to simulate aquatic and terrestrial conditions. Aquatic vivaria were filled to a depth of 30 mm with de-chlorinated tapwater. A piece of roof tile, plus an inverted water dish and plastic pipe section, provided toads with an opportunity to avoid continuous immersion but ensured contact with wet surfaces at all times. Terrestrial vivaria were filled with dry builder's sand to a depth of 80 mm. Refugia were provided in the form of a piece of roof tile and a pipe section. A water dish was kept filled with dechlorinated tapwater and toads were fed *ad libitum* with mealworms *Tenebrio molitor* during dry phases.

Toads were transferred between aquatic and terrestrial vivaria according to the treatment phase. Experimental start dates for each toad were staggered to control for other factors such as temperature. When not in use, vivaria were cleaned and disinfected using Virkon.

Each toad was subjected to three cycles of wet versus dry conditions (see Table 2.3). Two day wet phases were separated by four-day dry phases, to mimic short visits to breeding ponds. Toads were swab-tested for *Bd* on both wet phase days and the first and last days of each dry phase. The first test for each toad was taken upon capture (all toads were captured in ponds) and classified as a wet phase test. All surviving toads were released at their capture locations.

Experiment to test for the persistence of *Bd* infections during hibernation

I also carried out an experiment where toadlets from a *Bd*-positive population were kept captive from summer 2010 until spring 2011, to see if *Bd* infections persisted over winter. In spring the toadlets were subjected to wet conditions in an attempt to trigger *Bd* activity.

A large sample of natterjack toadlets (n = 90) was collected from Sellafield. The toadlets were kept in groups (n = 15) in 1 m^2, open-topped enclosures in coastal dune habitat away from ponds. Enclosures were furnished with artificial refugia and water dishes filled with dechlorinated tapwater. Toadlets were fed *ad libitum* with hatchling crickets (*Acheta domesticus)*. Before hibernation, swab samples were taken from toadlets on six regularly-spaced occasions, from capture in June to October. Identities were tracked using photographs to record the unique dorsal wart patterns of individuals.

The groups of toadlets were overwintered from October to April in plastic vivaria (320 mm x 400 mm x 180 mm), raised up on bricks inside enclosures. Each vivarium was filled to a depth of 150 mm with builder's sand and covered with a ventilated lid. Holes were drilled in the bases of the

vivaria to allow free drainage. Each enclosure was also roofed with mesh. In April 2011 the vivaria were searched for survivors. The majority of toadlets had died and decomposed. The few survivors (n = 6) were removed and placed in individual plastic vivaria (0.7 litre R-U boxes) inside an unheated building. They were then subjected to a six day dry phase, during which they were kept on a 5 mm layer of dry sand and provided with a small water dish. This phase was included to see if the toadlets would test positive for *Bd* on land following hibernation, without entry into water. The dry phase was followed by a three day wet phase, to see if wet conditions would trigger an increase in *Bd* activity and detection. During this wet phase the sand was replaced with a 5mm depth of water. The toadlets were swabbed on days two, four and six of the dry phase and on all three days of the wet phase. All six toadlets survived and were released at their capture location.

Detection of *Bd* in natterjack spawn and tadpole samples

In the UK, natterjack toads typically spawn from April to June. Natterjack tadpoles do not overwinter and the majority metamorphose by late July. Although pre-metamorphic life-stages could not act as long-term reservoirs of *Bd* infection, samples were tested for *Bd* DNA to see if they were involved in *Bd* dynamics during the summer.

Natterjack spawn and tadpoles from four sites (Sellafield, Mawbray, Grune and Bowness-on-Solway) were sampled for *Bd* DNA in 2009. Three stages of pre-metamorphic development (see Gosner, 1960) were sampled in an attempt to detect and measure the quantity of *Bd* DNA present; spawn, well-developed tadpoles with keratinised mouthparts (Gosner stage 36) and metamorphosing tadpoles with all four limbs and a tail stub (Gosner stage 45). These tadpole stages were chosen as *Bd* is usually found in keratinised tissues (Pessier *et al*, 1999), which do not exist in spawn but are found in the mouthparts of late-stage tadpoles and skin of metamorphs.

Samples were taken from 12 different spawn strings at each study site during May. Natterjack spawn was identifed by its single strand of eggs. 60 tadpoles of each Gosner stage described above were also collected from each population during late May or early June. It is difficult to differentiate between natterjack and common tadpoles but samples were taken from ponds where only natterjack spawn had been recorded. Tadpoles were euthanized using MS-222 upon capture. Spawn and tadpole samples were stored in 70% ethanol. Short sections of each spawn sample (containing 5-10 eggs), the mouthparts from Gosner stage 36 tadpoles and limbs from stage 45 tadpoles were removed and tested for *Bd* DNA.

Results

Seasonal variation in detection of *Bd* in wild adult natterjacks

A spring peak in positive *Bd* tests occurred in all three natterjack populations studied, in all three years (Table 2.1 and Fig 2.1). Although the monitoring period was brief, tests for a significant annual cycle were completed. Sample sizes were small for individual populations during late summer. However, it was possible to pool data before testing for the presence of a significant cycle, as peaks in *Bd* detection occurred simultaneously across populations.

No samples were collected from October to March as toads could not be captured and tested during hibernation. Natterjack toads hibernate on land (Leskovar & Sinsch, 2001) and there was evidence to suggest (see below) that *Bd* detection rates, recorded by swab tests, would remain close to zero when toads were on land away from ponds. I assumed that the detection rate for *Bd* from October to March would be zero, when performing tests for cyclicity.

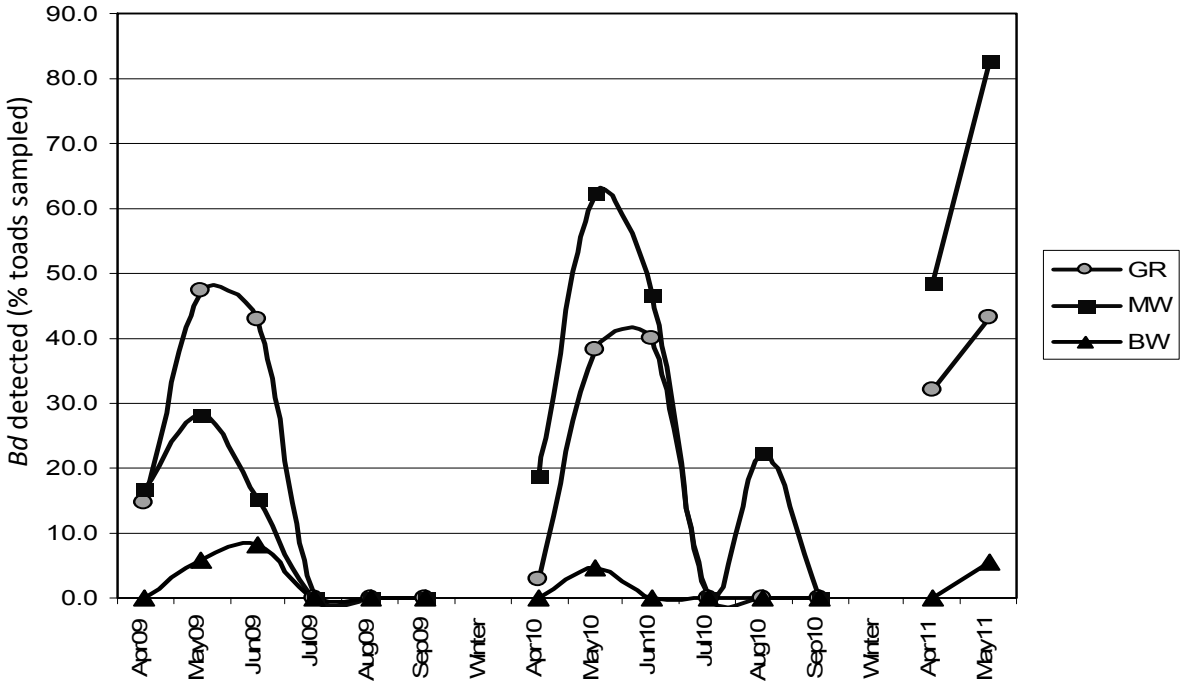
Table 2.1. Detection of *Bd* in adult natterjack toads by month and survey population

(Detection = ≥ 0.1 GE *Bd* DNA in test swab by rt-PCR)

		Grune		Mawbray		Bowness		ALL	
Year	Month	n	%	n	%	n	%	n	%
2009	Apr	34	14.7	6	16.7	0	NA	40	15.0
2009	May	40	47.5	39	28.2	34	5.9	113	28.3
2009	Jun	7	42.9	13	15.4	12	8.3	32	18.8
2009	Jul	19	0.0	9	0.0	28	0.0	56	0.0
2009	Aug	6	0.0	19	0.0	7	0.0	32	0.0
2009	Sep	6	0.0	9	0.0	1	0.0	16	0.0
2010	Apr	33	3.0	37	18.9	8	0.0	78	10.3
2010	May	21	38.1	24	62.5	21	4.8	66	36.4
2010	Jun	10	40.0	15	46.7	3	0.0	28	39.3
2010	Jul	12	0.0	15	0.0	13	0.0	40	0.0
2010	Aug	3	0.0	9	22.2	5	0.0	18	11.1
2010	Sep	1	0.0	2	0.0	2	0.0	4	0.0
2011	Apr	28	32.1	31	48.4	4	0.0	63	38.1
2011	May	37	43.2	29	82.8	18	5.6	84	48.8

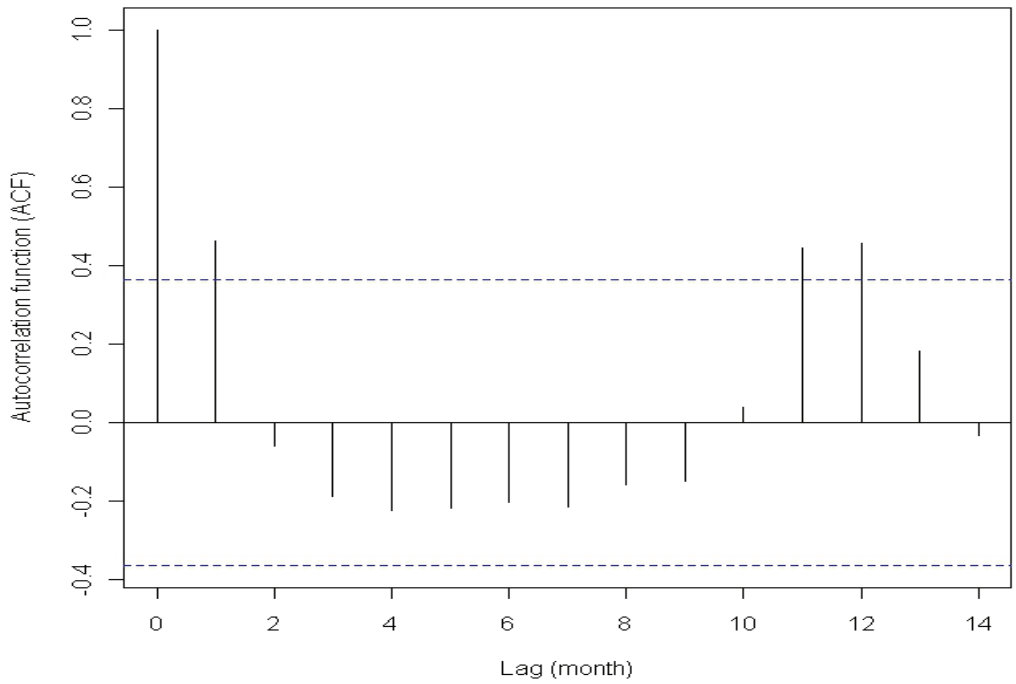
I used the autocorrelation function (ACF) in R (see Crawley, 2007) to test for a significant cycle in *Bd* detection across time intervals (months). A plot of ACF values with 95% confidence interval bands (dashed lines) confirmed the existence of a cycle with significant positive effects (increases in *Bd* detection) occurring in months (lags) 1 and 12 (Fig. 2.2). The first ACF value is always 1, but other significant ACF values are evidence of cyclicity. There was evidence for an annual cycle of increased *Bd* detection in spring.

Fig. 2.1. Detection of *Bd* in adult natterjack toads by month and survey population



GR - Grune, MW - Mawbray, BW - Bowness. Detection = ≥0.1 GE *Bd* DNA

Fig. 2.2. Evidence for an annual cycle of *Bd* detection in adult natterjacks (--- 95% C.I.)



A trigonometric linear model was also used to test for a cyclic pattern in the data (Table 2.2). A significant p-value for the cos or sin parameter in this type of model is evidence for a waveform. The cos parameter was significant, providing additional support for a cyclic pattern.

Table 2.2 Linear model of *Bd* detection in adult natterjacks by month (all populations, 2009-2011)

Linear model	Detection ~ sin(time * 2 * pi) + cos(time * 2 * pi))			
Coefficients	Estimate	S.E.	t-value	p<t-value
Intercept	7.86	2.10	3.75	0.0009
sin(time*2*pi)	4.85	2.91	1.66	0.1081
cos(time*2*pi)	-13.83	2.97	-4.66	0.0001
	Residual S.E. = 11.1 on 26 df, adjusted R^2 = 0.45			
	F-statistic = 12.2 on 2 and 26 df, p<0.0002			

Detection of *Bd* in adult natterjacks captured in ponds versus on land

During spawning (April-June inclusive) the proportion of *Bd* positives was higher among pond (78/269) than land (10/88) samples (Chi^2 = 10.17, df = 1, p<0.001) when samples from all populations were combined. Natterjack toads were more likely to test positive for *Bd* if they were captured in water during the spawning season. However, this pattern did not persist after June, when there were very few *Bd* positives among pond (2/77) or land (0/89) samples.

Seasonal variation in levels of *Bd* detection in natterjack toadlets

In 2009, large numbers of natterjack toadlets emerged from ponds 14 at Mawbray and 1 and 2 at Sellafield (for pond locations, see Beebee & Buckley, 2012, in press). I collected sufficient samples of toadlets from pondside refugia to monitor *Bd* detection rates at two-week intervals (Mawbray n = 10-36, Sellafield n = 23-27), until toadlets began dispersing after rain.

Bd was detected among toadlets from both populations, along with rapid increases in *Bd* detection several weeks after emergence (Figs. 2.3 & 2.4). Increases in detection and quantitative *Bd* scores were observed following the first prolonged rainfall since emergence at each site. Rain occurred on 24th July at Mawbray and 8th August at Sellafield. Following these dates, *Bd* detection rates increased from close to zero at both sites to at least 70% at Mawbray and 42% at Sellafield. Small numbers of dead toadlets were seen under refugia following rain.

Changes in conditions linked to rainfall included a reduction in daytime temperature, pond water conductivity and toadlet density beneath refugia. The majority of refugia, previously on dry land, were partially flooded as a result of rising pond levels.

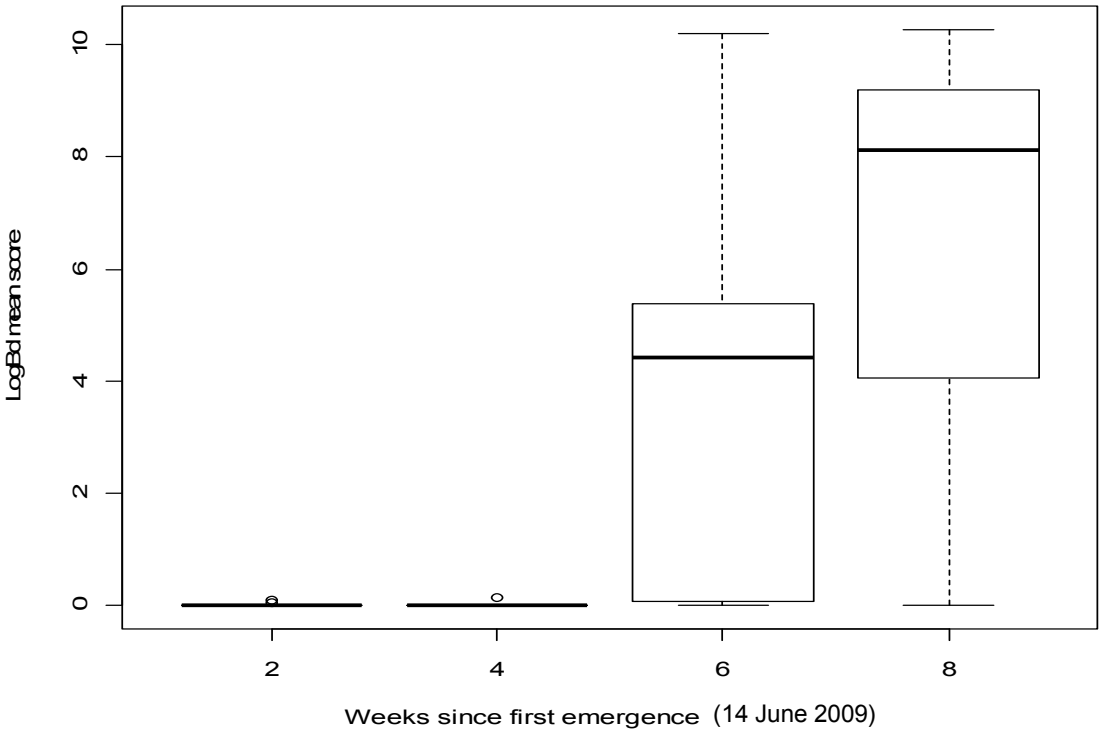


Fig. 2.3. *Bd* scores of natterjack toadlets after metamorphosis (Mawbray)

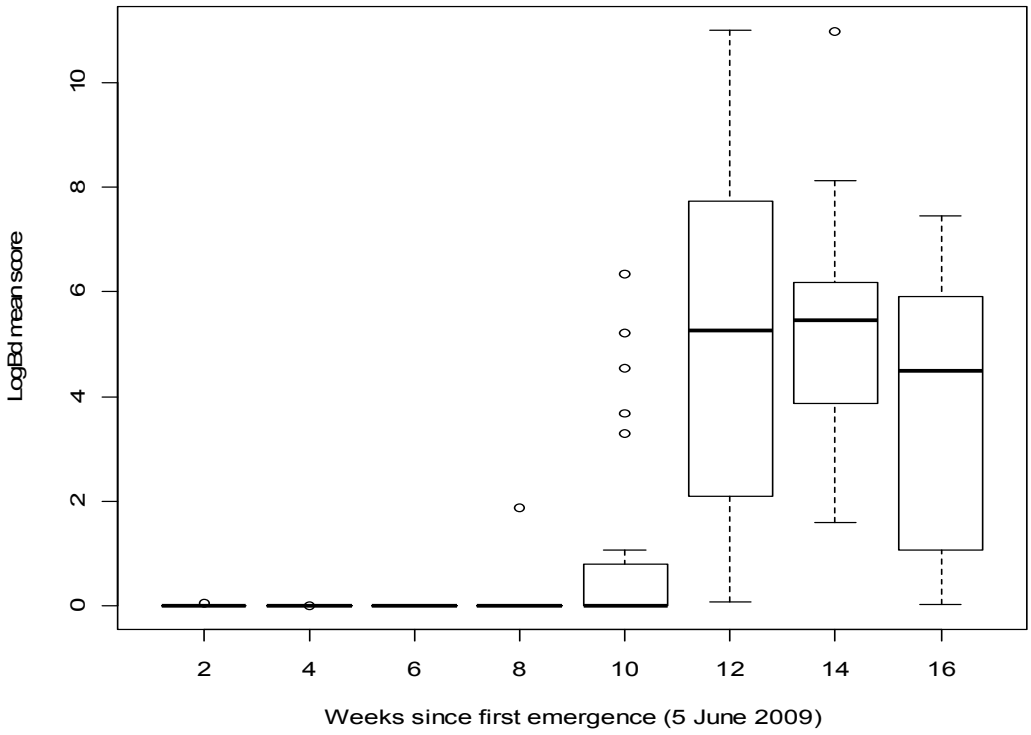


Fig. 2.4. *Bd* scores of natterjack toadlets after metamorphosis (Sellafield)

Key to Figs 2.3 & 2.4: Horizontal line = median value. Box = 25th-75th percentile (inter-quartile range from 1st to 3rd quartile). Whisker = whisker ends denote maximum and minimum values, or 1.5x the interquartile range, whichever is greatest. Circle = outlier (point >1.5x less than upper limit of 1st quartile or >1.5x more than that of 3rd quartile).

Experiment to assess the effect of immersion on *Bd* activity and detection

Bd was detected in samples from all six adult toads collected for this experiment. The results were analysed for an effect of wet versus dry treatment on likelihood of *Bd* detection, using a generalised linear mixed effect model to take into account repeated measures on individuals.

Table 2.3. *Bd* detection and scores in response to wet versus dry conditions

Shaded cells – *Bd* detected (positive) using standard threshold (0.1 GE)

Numerical values – *Bd* scores in genomic equivalents (GE) per swab

Day	1	2	5	6	7	8	11	12	13	14	17
Toad	Wet	Dry	Dry	Wet	Wet	Dry	Dry	Wet	Wet	Dry	Dry
1	241.3	0.0	156.3	38.4	82.4	0.0	0.0	141.3	229.0	329.8	0.0
2	0.6	0.0	0.0	1.0	3.6	0.0	0.0	37.1	29.6	0.0	0.0
3	0.0	0.0	0.0	3.9	4.1	1.0	0.0	4.5	0.0	0.0	0.0
4	0.0	0.3	4.0	104.6	85.5	0.0	42.1	14.6	20.9	3.8	1.7
5	902.1	14.0	0.0	1157.8	3942.4	26117.4	6629.3	3953.2	1091.1	398.1	DEAD
6	10.6	2799.1	20314.0	6823.6	2882.8	0.0	1247.2	3061.9	2055.4	9835.0	17380.5

Table 2.4. The effect of wet versus dry treatment (WD) on likelihood of *Bd* detection

GLMER: Generalized linear mixed model, fit by the Laplace approximation

	GLMER: detection ~ WD + (WD | Toad ID), binomial			
Fixed effects	Estimate	S.E.	z-value	p<z-value
Intercept	0.205	0.734	0.279	0.780
WD	2.344	0.925	2.535	0.011
	Number of observations: 65 Groups: Toad ID, 6			

There was a significant effect of wet versus dry treatment on likelihood of *Bd* detection. The model was used to estimate the mean likelihood of detection during wet (0.927) and dry conditions (0.551). Toads were more likely to test positive for *Bd* when sampled in wet conditions. The majority of negative results recorded were false, since the same toads usually tested positive again at a later stage during the experiment.

The two toads with the highest *Bd* scores both tested negative on one occasion during a dry phase, suggesting that very active or heavy infections can rapidly become cryptic with respect to the swab test when toads move onto dry land. These two toads died, one during the experiment (toad 5) and one shortly after (toad 6).

Experiment to test for persistence of *Bd* infections during hibernation

None of the six toadlets which survived hibernation tested positive for *Bd* when kept on land during the previous summer. Four of these toadlets subsequently tested positive for *Bd* after exposure to wet conditions (Table 2.5). Infections had remained cryptic in these toadlets for nearly a year, from capture in June 2010 until May 2011.

Table 2.5. *Bd* scores of overwintered toadlets in response to wet conditions

Shaded cells – *Bd* positive using standard detection threshold (0.1 GE)

Numerical values – *Bd* scores in genomic equivalents (GE) per swab

Sample dates	Jun-Oct 2010	April 30 2011	May 2 2011	May 4 2011	May 5 2011	May 6 2011	May 7 2011
Toadlet	Dry	Dry	Dry	Dry	Wet	Wet	Wet
1	(0.0 x6)	0.0	0.0	0.0	0.0	0.0	0.0
2	(0.0 x6)	0.0	0.0	0.0	0.0	1.1	19.5
3	(0.0 x6)	0.0	0.0	0.0	0.0	0.4	2.6
4	(0.0 x6)	0.0	0.0	0.0	0.0	0.0	0.0
5	(0.0 x6)	0.0	0.0	0.0	0.1	0.0	5.1
6	(0.0 x6)	0.0	0.0	0.0	0.0	0.2	0.0

Detection of *Bd* in natterjack spawn and tadpole samples

Bd DNA was detected in spawn and tadpole samples (Table 2.6) but quantitative *Bd* scores were consistently low in comparison with those of adult natterjacks or toadlets. Maximum *Bd* scores recorded from pre-metamorphic stages (all sites) were 0.4 GE for spawn, 0.3 GE for Gosner stage 36 tadpoles and 0.6 GE for stage 45 tadpoles.

Table 2.6. Detection of *Bd* in natterjack spawn and tadpole samples

pos/n = positive results (standard 0.1 GE threshold) / total sample

% = % samples *Bd* detected G36, G45 = Gosner tadpole stages 36, 45

	Sellafield		Mawbray		Grune		Bowness	
Sample	pos/n	%	pos/n	%	pos/n	%	pos/n	%
Spawn	4/12	33.3	3/12	25.0	4/12	33.3	1/12	8.3
G36	1/60	1.7	3/60	5.0	2/60	3.3	0/35	0.0
G45	5/53	9.4	4/51	7.8	1/12	8.3	1/10	10.0

Discussion

Seasonal peaks in *Bd* detection have been reported in other species attending waterbodies to spawn, such as the leopard frog *Rana pipiens* (Voordouw *et al*, 2010) and stony creek frog *Litoria wilcoxii* (Kriger & Hero, 2007). This study was the first to report a significant annual cycle of *Bd* detection. The cycle suggested natterjack populations could survive *Bd* infection across years and that reservoirs of infection were present at all study sites.

A lower rate of detection in natterjacks captured on land compared to ponds could be explained by avoidance of the infectious life-stage of *Bd* (aquatic zoospores). However, if the swab test is an accurate indicator of infection prevalence, some toads gained and lost infections on a weekly basis (see Appendix 1). Although pond entry probably increases infection risk, not all of these transitions were likely to reflect true infection status. Adults found in water after June rarely tested positive, an inconsistent result but not if pond visits after spawning were too brief to boost *Bd* activity.

Increases in *Bd* detection and scores in wild natterjack toadlets following heavy rain were consistent with my immersion hypothesis but could also be explained by an increase in transmission rates. Wet conditions under pondside refugia provided ideal conditions for *Bd* transmission. The results of the experiment to test for maintenance of infections in overwintered toadlets suggest many natterjacks gain *Bd* infections at this stage. I was unable to determine the impact of *Bd* on toadlets in the wild.

Wet versus dry treatment of captive adults generated results consistent with the immersion hypothesis which could not be explained by an increase in transmission, as toads were individually housed. The simplest explanation for the difference in *Bd* detection rates between wet and dry conditions was a change in *Bd* activity. Although *Bd* scores could also represent infection load, toads which frequently recorded high *Bd* scores (and therefore appeared to be heavily infected) tested negative at least once during dry phases.

There is evidence for seasonal variation in infection load in other species. Retallick *et al* (2004) found increased *Bd* infection load in Eungella torrent frogs (*Taudactylus eungellensis*) during the aquatic breeding phase. But variation in infection load could not explain my observations of individually housed natterjack toads switching rapidly between negative and positive *Bd* ‘status’, results which were more consistent with a short-term change in infection activity.

It was not possible to overwinter adults in captivity but it is likely they will retain infections over winter in a similar way to toadlets. Other data (Chapter 3) suggest infection prevalence in wild adults can reach 100%, with infections only detectable after extended exposure to wet conditions. Adults frequently tested positive for *Bd* on first capture in the spring (see Appendix 1), even when found alone in ephemeral ponds. Natterjack adults and juveniles may act as long-term reservoirs of *Bd* infection.

Natterjack spawn and tadpoles also tested positive for *Bd* but scores did not exceed the maximum (1.82 GE) recorded from water by Stockwell *et al* (2010), in an attempt to differentiate between background contamination and infection. Rachowicz & Vredenburg (2004) claim *Bd* infection occurs in *Rana muscosa* tadpoles, but natterjack tadpoles and spawn seem unlikely to play a major role in *Bd* transmission.

The results of this study demonstrated *Bd* was well-established in natterjack populations and supported my hypothesis that immersion boosts *Bd* activity and detection. I could not determine the proportion of toads which remained infected from year to year. Either infection loss was rare, or *Bd* achieved transmission very rapidly each spring, via a small number of infected individuals. Although annual peaks in *Bd* detection occurred in all study populations, detection rates were much lower at one site (Bowness-on-Solway). The reasons for this are explored in more detail in Chapter 3.

Detection of *Bd* in species which rarely visit water, such as the Jemez mountain salamander *Plethodon neomexicanus* (Cummer *et al*, 2005), has raised questions about how *Bd* achieves transmission. Since the only known infectious life-stage of *Bd* is aquatic, the salamander must enter water or transmission must occur via an unidentified process on land. If *Bd* remains dormant or cryptic with respect to diagnostic tests for long periods but rapidly becomes active in response to immersion, there may be no need for a terrestrial mode of transmission.

Wild amphibians collected for *Bd* experiments are often assigned negative status on the basis of single swab tests and excluded from analyses due to 'contamination' (e.g. Weinstein, 2009). Field studies (e.g. Van Sluys & Hero, 2009) have reported lower *Bd* 'prevalence' in amphibians found in adjacent areas of wet and dry habitat. These conclusions now appear questionable. I rejected swabbing as a method for estimating infection prevalence but retained it as a technique for assessing levels of *Bd* activity. Despite variation in sensitivity, swab tests could provide a useful indication of *Bd* activity or load. For the purposes of this project, I have assumed that swab results will correlate with *Bd* infection activity and rates of transmission.

Chapter 3.

Salinity limits *Batrachochytrium dendrobatidis (Bd)* infection in the natterjack toad *(Bufo calamita)*

Abstract

Batrachochytrium dendrobatidis (*Bd*) is a parasite of many amphibians, including the natterjack toad (*Bufo calamita*). Natterjacks in the UK are found mainly in coastal habitat, where ponds are often inundated by high tides. Salinity has a strong influence on *Bd* dynamics in this ecosystem. Positive *Bd* results were less common from natterjacks sampled in brackish compared to freshwater ponds. Use of internal positive controls (IPCs) and Environmental Taqman confirmed the pattern was not due to PCR-inhibition by salts. *Bd* isolated from natterjacks was killed *in vitro* by a salinity equivalent to 50% seawater, a salinity which adult natterjacks can tolerate. This isolate grew fastest at low salinities, raising the possibility that *Bd* can also adapt to salinity. Nevertheless, tidal inundation may be sufficient to disinfect ponds and reduce *Bd* transmission at saline sites. The implications of these findings are discussed with respect to the conservation of amphibians.

Introduction

Environmental factors have a major influence on the ecology of disease (Collinge & Ray, 2006). Extreme or unusual conditions can de-stabilise host-parasite relationships, resulting in disease outbreaks, declines and possibly extinction of host populations (Pounds *et al* 2006). A wide range of environmental factors have been shown to trigger or limit outbreaks of disease. It is unlikely variation in one environmental factor will explain the occurrence of a pathogen within a population, but it may be possible to identify influential factors.

The chytridiomycete *Batrachochytrium dendrobatidis* is a widespread parasite of amphibians. Several environmental factors which have been shown to influence the probability of *Bd* detection or disease (chytridiomycosis) in amphibian populations. These include temperature (Berger *et al*, 2004), the presence of water (Murphy *et al* 2011), urban pollution (Lane & Burgin, 2008), pesticides (Rollins-Smith & Davidson, 2010), herbicides (Gahl *et al*, 2011) and ultraviolet (UV-B) radiation (Ortiz-Santaliestra *et al* 2011). In some studies, adjacent populations of the same species (e.g. *Rana muscosa*, Briggs *et al*, 2010) have persisted following arrival of *Bd* while others have disappeared. Such patterns might be explained by local variation in disease resistance but environmental factors could be just as important.

One environmental factor which has not been effectively explored with respect to *Bd* dynamics is salinity. Salinity influences the survival and transmission of many pathogens in coastal environments, including enteric viruses (Lipp *et al*, 2001) and trematodes (Lei & Poulin, 2011). Salts are often used to treat fungal infections (Edgell *et al*, 2003). Few studies have measured the effects of salinity on *Bd* in the wild. Lane & Burgin (2008) suggested salts present in urban wastewaters could be limiting the spread of *Bd* in southern Australia. White (2006) claimed addition of sodium chloride (NaCl) to a pond resulted in a reduction in *Bd* infection and mortality in green and golden bell frogs (*Litoria aurea*). During development of disinfection methods for equipment, Johnson *et al* (2003) found a 5% NaCl solution killed 100% of *Bd* in culture in 5 min. Lower concentrations were not lethal in 5 min, but this did not rule out an effect of low levels of salinity on *Bd* infection in wild amphibians.

In order to investigate the effect of salinity on *Bd* dynamics in the wild I needed to identify a suitable host-pathogen system. Several amphibians found in coastal habitat can tolerate elevated salinity, including the green and golden bell frog *Litoria aurea*, natterjack toad (*Bufo calamita*), green toad *Pseudepidalea/Bufo viridis*, crab-eating frog *Fejervarya*/*Rana cancrivora*, African clawed toad *Xenopus laevis* and Couch's spadefoot toad *Scaphiopus couchii* (Sinsch, 1992).

In the UK, most natterjack populations are found in coastal habitats. The ponds in which they breed are often subjected to variation in salinity as a result of tidal inundation. As several coastal populations of this species in the UK are infected with *Bd* (Feltrer & Cunningham 2006, Arai 2008), they presented an ideal opportunity for a study of the effects of salinity on *Bd*.

Three approaches were used to investigate the relationship between salinity and *Bd* in natterjack toads. Firstly, I explored field data to see if the proportion of toads testing positive for *Bd* varied with respect to the salinity of ponds in which they were captured. Secondly, I tested the effects of fungicidal treatments, including salinity, on *Bd* swab test scores and survival in captive natterjacks. Thirdly, I collected an isolate of *Bd* from natterjacks and measured its growth and survival *in vitro* in response to increasing salinity.

Other researchers have assumed the proportion of positive swab tests provides a reliable indication of infection prevalence but my evidence suggests it does not (Chapter 2). However, swab tests may still provide a useful indication of *Bd* activity and correlate with transmission rates (and possibly infection load). Consequently, the swab test was retained as a method for assessing levels of *Bd* activity in this study but I avoided use of the term prevalence.

Methods

Salinity and *Bd* detection in wild adult natterjack toads

I collected swab samples from adult natterjacks (for method, see Chapter 2, p24-25) at four coastal sites in northern Cumbria (Mawbray, Grune, Bowness-on-Solway and Anthorn) during 2009-2011. Nine populations in southern Cumbria were also sampled by Bramwell (2011) in 2011 using the same method (Annaside, Braystones, Haverigg, Drigg, Dunnerholme, Sandscale, Sandside, Askam and Soutergate). Samples were analysed by rt-PCR (Chapter 2, p22) using the method developed by Boyle *et al* (2004) and classified as positive or negative using a standard threshold for *Bd* detection (0.1 GE *Bd* DNA). For site locations, see Chapter 1 (Fig. 1.6.).

The salinity of ponds in which toads were captured was measured using a conductivity meter. Conductivity correlates closely with sodium and chloride levels in pond water (Karraker *et al*, 2008) and can be used as a surrogate for salinity. If more than one toad was captured during a sampling event, all samples were assigned the same conductivity reading. A salinity threshold was used to categorise ponds as fresh or brackish. The linear relationship between salinity and conductivity (Fofonoff & Millard, 1983) can be used to predict seawater content. Undiluted seawater around the UK coast has a typical salinity of 32 ppm and an approximate conductivity of 35,000 µS (log µS 10.5). Most inland ponds in this study had a conductivity of 60-150 µS following rain but no more than 400 µS during droughts, suggesting marine salts were present in ponds with higher conductivities. A threshold was set with all samples exceeding 750 µS (>log µS 6.6) classified as brackish. Assuming a background conductivity of 250 µS, a pond with a conductivity of 2000 µS would have a salinity of 0.16% and contain around 5% seawater.

I compared the proportion of toads testing positive for *Bd* in fresh versus brackish ponds. Samples from toads captured on land (>5 m from a pond) were excluded from the analysis, as evidence (see Chapter 2) suggested cryptic infection would be common in such samples, resulting in zero inflation of the data. At three sites (Grune, Mawbray and Sandscale) enough samples were collected to compare *Bd* detection rates in fresh versus brackish ponds within populations (Table 3.2.). Results from all populations were combined to illustrate the relationship between *Bd* detection and salinity (Fig. 3.2.). Tests were completed to check if results were biased by an increased rate of PCR-inhibition among brackish samples.

Effects of fungicidal treatment on *Bd* scores and mortality

An experiment was carried out to assess the effects of a standard fungicidal treatment (itraconazole) and salinity (as a potential fungicide) on *Bd* scores and mortality in adult natterjacks. Use of quantitative *Bd* score was intended to provide improved analytical resolution, compared to the more widely used 'negative versus positive' classification. *Bd* scores were generated by measuring the amount of *Bd* DNA in swab samples using rt-PCR (see Chapter 2, p22).

The experiment was designed to serve two purposes. Firstly, to test for a causal link between *Bd* and mortality in adult natterjacks. I recorded a weak correlation between adult natterjack survival and *Bd* score in the wild (see Chapter 4) but this did not identify *Bd* as the cause of mortality. Experiments on other species (e.g. Stockwell *et al*, 2010) have suggested a negative correlation between *Bd* score and survival in captivity. I tested cause and effect in captivity by creating a '*Bd*-negative' group (using itraconazole) for comparison with mortality in a control group (fresh water) where pre-existing *Bd* infections were allowed to progress.

Secondly, to test for an effect of salinity on pre-existing *Bd* infections in a controlled environment. Field observations linked salinity to a reduction in *Bd* detection (Table 3.2.) and other studies had reported a negative effect of sodium chloride on *Bd*. If salinity had a negative effect upon *Bd*, this might help explain the reduction in *Bd* detection seen in brackish conditions in the wild.

The hypotheses under test in this experiment were;

- Itraconazole and salinity would reduce *Bd* scores, compared to fresh water.
- Itraconazole and salinity would reduce mortality, compared to fresh water.
- Higher *Bd* scores would be associated with an increase in mortality

I tested these hypotheses by subjecting individually-housed natterjack toads (with pre-existing *Bd* infections acquired in the wild) to one of three treatments; itraconazole, saline and a fresh water control (see Table 3.1.). The experiment included a wet phase for all toads followed by a dry phase. The wet phase was included to allow *Bd* to become more active in toads which were not subjected to an effective fungicidal treatment. Other experiments (Chapter 2) had suggested wet conditions would boost *Bd* activity and *Bd* scores. Treatments were applied during the wet phase. A dry phase was included to allow feeding and recovery before release.

Table 3.1. Sampling regime, fungicidal treatment experiment (Shaded cells = toads swabbed for *Bd*)

Day (wet phase)									Day (dry phase)									
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Itraconazole (n = 15)									Treatment identical for all groups									
Saline (n = 16)																		
Control (n = 16)																		

Toads were collected from two natterjack populations, Sandscale (n = 30) and Grune (n = 18). Only males were collected to minimise the impact upon breeding success. I predicted a high proportion of these toads would be infected with *Bd* if collected during the spring, based on previous results from these two sites (see Chapter 2. and Arai, 2008).

Upon capture all toads were placed in separate containers containing dechlorinated tapwater to prevent dehydration. They were then transported to the experimental facility. Precautions were undertaken to prevent transfer of *Bd* between toads and equipment during transport and captivity. Toads were individually housed in vivaria (200 mm x 120 mm x 120 mm) inside a mesh-sided enclosure which ensured a natural temperature regime.

I standardised the effect of population by distributing toads evenly across treatment groups (n = 16 per treatment). All toads were swab-tested for *Bd* before treatments began. During the experiment all toads were swabbed a total of five times, with the exception of those which died before the end of the experiment. One toad died before treatments started and was not included in the experiment (consequently the itraconazole group size was reduced to 15).

During the wet phase all vivaria were kept filled with dechlorinated tapwater to a depth of 10 mm. This was the standard treatment for control group toads, which were treated exactly the same as the other two groups, except that they were not subjected to any fungicidal treatment. All toads were provided with a platform to allow avoidance of continuous immersion. Each vivarium was emptied, disinfected and re-filled twice during the wet phase.

A treatment regime for the itraconazole group was approved by a veterinarian. Toads were bathed in 0.01% itraconazole ($C_{35}H_{38}Cl_2N_8O_4$) for 5min on each of the nine wet phase days. Sham itraconazole treatments were applied to saline and control group toads.

Saline group toads were exposed to a salinity of 1% throughout the wet phase, by dissolving sea salt (1g/100 ml) in the tapwater used to fill the vivaria. I selected this salinity to be within the range (<1.7%) tolerated by adult natterjacks (see Sinsch, 1992 and Mathias, 1971) but with the potential to influence *Bd* detection rates and scores, based on my field observations.

At the start of the dry phase on day ten I emptied, disinfected and refilled each vivarium with dry builder's sand to a depth of 80 mm. Each toad was provided with a water dish filled with dechlorinated tapwater, a plastic refugium and fed *ad libitum* with house crickets *Acheta domesticus*. Survivors were released at capture locations on day nineteen.

Log-transformation of quantitative *Bd* scores

The amount of *Bd* DNA in samples is estimated in genomic equivalents (GE) when using real-time PCR (see Chapter 2, p22). *Bd* detection is often assessed with reference to a standard 0.1 GE threshold for a positive result, but use of quantitative *Bd* score can improve analytical resolution. *Bd* scores tend to be non-normally distributed, with a positive skew. According to Crawley (2007), such distributions are common in parasitological data. Log-transformation (base e^x) was fairly effective at removing skew from *Bd* score data in this study (Fig. 3.1.). Searle *et al* (2011) added one to all *Bd* scores to avoid generation of negative values from zeros. All *Bd* scores in this project were transformed using this method, which was universally adopted to facilitate comparisons between *Bd* score data collected in a variety of contexts.

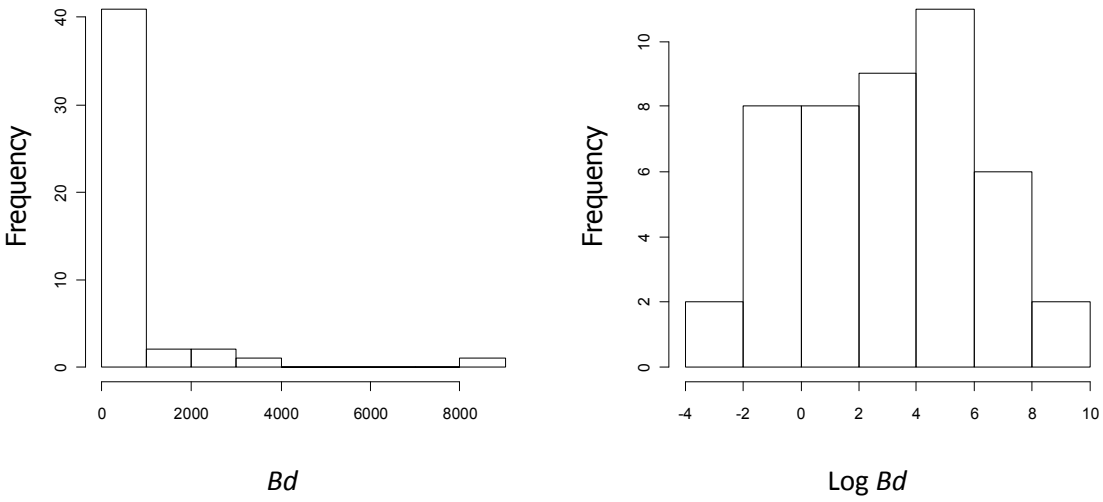


Fig. 3.1. Raw and log-transformed *Bd* scores (fungicidal treatment experiment, on capture)

Effect of salinity upon *Bd* growth *in vitro*

Finally, an *in vitro* experiment was carried out to find out if;

- *Bd* could survive and grow in salinities tolerated by natterjack toads
- A reduction in growth occurred at salinities linked to a decline in *Bd* detection in the wild

To complete this experiment it was necessary to find a suitable isolate of *Bd*. A number of *Bd* isolates have been collected from amphibians and maintained in the laboratory since the discovery of *Bd* by Longcore *et al* (1999) but it was preferable to obtain an isolate from one of the natterjack study populations. Different strains of *Bd* can vary in virulence (Farrer *et al*, 2011) and may be locally adapted to hosts and environmental conditions.

Bd isolates were collected from natterjack toads at Sellafield (for site location, see Chapter 2.) during the breeding season of 2010, using the method described by Longcore *et al* (1999). Isolation was attempted using samples from a total of 12 toads (eight males and four females). *Bd* was successfully isolated from five toads; four males and one female. The toads were swab-tested for *Bd* DNA and two individuals which produced isolates tested negative using swabs; additional evidence for cryptic infection (see Chapter 2).

Isolates were cultured in the laboratory using the method described by Longcore *et al* (1999). One isolate (SFBC009) was selected at random and used to measure *Bd* growth in a range of salinities. Ideally, this experiment would have been completed using several isolates to control for individual effects but the risk of significant variation between isolates was considered low, as they were all collected from natterjack toads in the same pond on the same day.

Using aseptic techniques, 4 µl of isolate were transferred to a sterile 250 ml flask containing 100 ml of TG (1% tryptone, 0.3% glucose) growth media. This culture was incubated at 18 °C for four days. The concentration of zoospores (zsp) was measured using a haemocytometer. A solution of 200 zsp/µl was created by dilution with additional growth media. 100 µl of the 200 zsp/µl solution were added to each wells of a 96-well optical plate. Growth media with salinities ranging from 0.0-7.0% were added (100 µl) to test growth in salinities 0.0, 0.11, 0.22, 0.44, 0.88, 1.75 and 3.5% (plus blanks, with 200 µl of plain growth media per well). The plate was incubated at 18°C and growth measured at intervals for 19 days on a refractometer set to detect light absorbance (abs) at 450 nm. Two rows were contaminated by mould but results from ten wells per salinity were available for analysis.

Results

Salinity and *Bd* detection in wild adult natterjack toads

Positive *Bd* results were significantly less common in brackish compared to fresh samples (Table 3.2.). This pattern persisted when samples from all populations were combined (Fig. 3.2.).

Table 3.2. Proportions of positive *Bd* results among fresh versus brackish samples

µS = mean conductivity of pond water (all sampling events). Fresh/Brackish threshold = 750µS (log 6.6 µS)

pos/n = number of positive tests for *Bd* using standard (0.1 GE) threshold / total sample

Chi ^{2} = two-sample tests for equality of proportions (Fresh pos/n, Brackish pos/n) with continuity correction

NA = expected frequencies too low (<5) for Chi ^{2} tests

Population	Fresh µS	Brackish µS	Fresh pos/n	Brackish pos/n	Chi ^{2}	df	p<value
Grune	119	1217	63/165	9/51	6.5	1	0.011
Mawbray	151	982	73/134	5/31	13.4	1	0.001
Bowness	-	1120	0/0	6/141	NA	NA	NA
Anthorn	-	3687	0/0	0/62	NA	NA	NA
Annaside	275	-	1/6	0/0	NA	NA	NA
Braystones	169	-	1/8	0/0	NA	NA	NA
Drigg	242	-	5/30	0/0	NA	NA	NA
Dunnerholme	246	1538	1/7	3/26	NA	NA	NA
Haverigg	-	5124	0/0	0/26	NA	NA	NA
Sandscale	415	925	13/18	8/24	4.76	1	0.029
Sandside	-	3020	0/0	3/25	NA	NA	NA
Askam	441	-	2/8	0/0	NA	NA	NA
Soutergate	-	3795	0/0	0/5	NA	NA	NA
ALL	256	2372	159/376	34/391	113.1	1	0.001

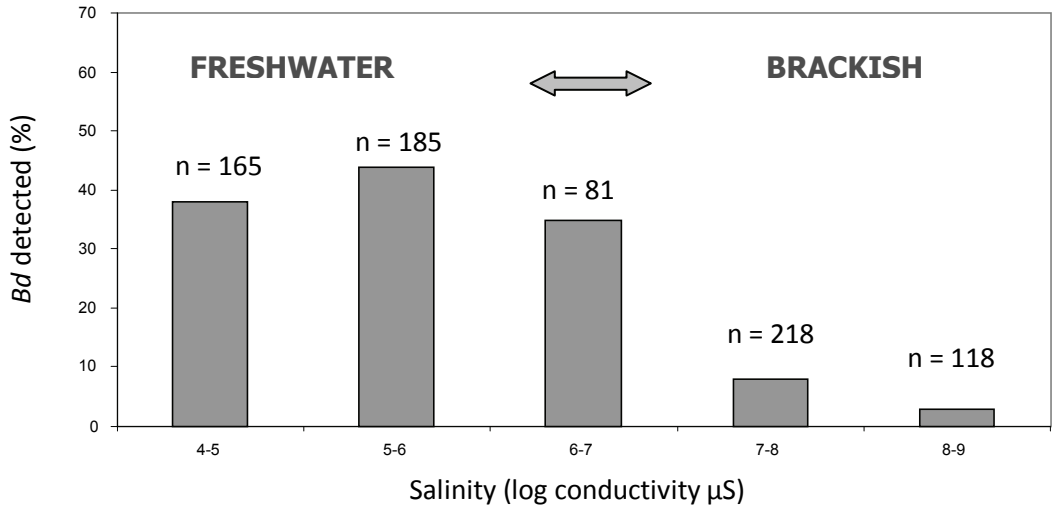


Fig. 3.2. Pond salinity and *Bd* detection in natterjack toads (all samples)

Quantitative assessment of sampling bias caused by PCR inhibition

Marine salts including sodium chloride (NaCl) can inhibit amplification of target DNA during PCR (Davalieva & Efremov, 2010). PCR inhibition can be detected using internal positive control (IPC) DNA which will not amplify in the presence of inhibitors (see Chapter 2, p23). To assess inhibition levels, a random selection of samples from toads found in fresh and brackish conditions were re-tested with IPCs. A pond versus land comparison was also made, as positive *Bd* results were less common among samples from toads captured on land (see Chapter 2).

The results suggested PCR inhibition could mask the presence of *Bd* DNA in many samples when using standard reagents. To test this, the samples were re-tested using Environmental Taqman (EQ) and IPCs. Unlike standard Taqman (TQ), EQ is designed to minimise PCR inhibition by contaminants including salts. When samples were processed with EQ the IPC DNA consistently amplified, so any *Bd* DNA present should also have amplified.

Table 3.3. Detection of *Bd* using standard Taqman (TQ) and Environmental Taqman (EQ)
Inhibition – percentage of total sample (n) with evidence of PCR inhibition
pos – number of positive tests for *Bd* DNA using standard (0.1 GE) threshold
Chi ^{2} – two-sample tests for equality of proportions (TQ pos/n, EQ pos/n) with continuity correction
NA – expected frequencies too low (<5) for Chi ^{2} tests

Sample	n	Inhibition TQ	Inhibition EQ	TQ pos	EQ pos	Chi ^{2}	df	p<value
Brackish, pond	87	60%	0%	8	14	1.30	1	0.254
Fresh, pond	47	15%	0%	23	26	0.11	1	0.736
Brackish, land	30	63%	0%	0	2	NA	NA	NA
Fresh, land	28	29%	0%	2	4	NA	NA	NA
ALL	192	45%	0%	33	46	2.29	1	0.130

Use of EQ resulted in an increased rate of *Bd* detection in all groups but these differences were statistically insignificant (Table 3.3.). The proportion of positive *Bd* results remained lower in brackish (16/117) compared to fresh (30/75) samples when re-tested with EQ (Chi ^{2} 51.20, df = 1, $p<0.001$). The lower rate of *Bd* detection in samples from brackish ponds was unlikely to be fully explained by PCR inhibition. Similarly, detection remained lower in land (6/58) compared to pond samples (40/134) when analysed using EQ (Chi ^{2} 7.42, df = 1, $p<0.006$). I used these findings to justify an ecological interpretation of the patterns recorded in field data.

Effects of fungicidal treatment on *Bd* scores and mortality

Bd DNA was detected in at least one sample from all of the toads in this experiment (see Table 3.4.). Negative tests from 9/48 (19%) of individuals before treatments began provided additional evidence of cryptic infection with respect to the swab test (see Chapter 2).

As predicted, *Bd* scores rose during the wet phase but did not appear to decrease in dry conditions, in contrast to the results of a similar experiment described in Chapter 2. A comparison of the log *Bd* mean scores of toads sampled in wet versus dry phases (n = 43, as five toads had died by the first day of the dry phase) confirmed that scores did not decrease (Welch's two-sample paired t-test, t= 0.028, df = 42, p<0.978, Fisher's F-test for homogeneity of variance, F = 0.8462, num df = 42, denom df = 42, p<0.591). This justified simplification of the analysis, by using the post-treatment log *Bd* mean scores for toads across wet and dry phases when testing for treatment effects. These scores were assumed to represent the level of *Bd* infection activity experienced by each toad. Completed swabs were used to generate mean values for toads which died during the experiment.

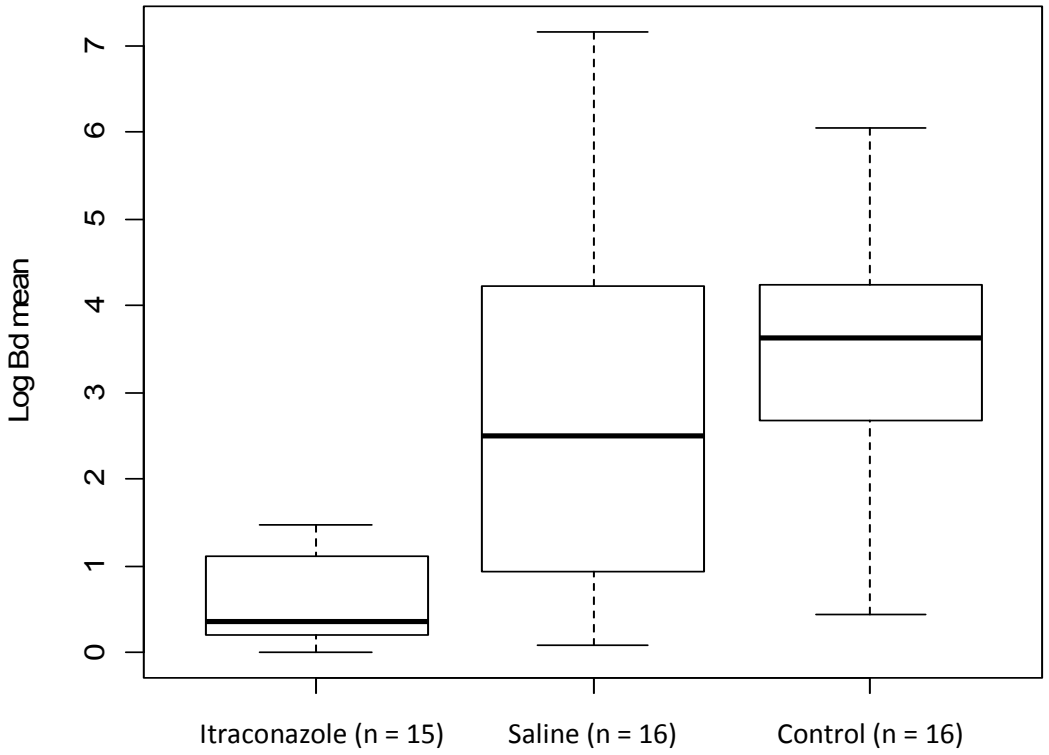


Fig. 3.3. Differences in post-treatment *Bd* score by treatment group

Key to Fig. 3.3: Horizontal line = median value. Box = 25th-75th percentile (interquartile range from 1st to 3rd quartile). Whisker = ends denote maximum and minimum values, or 1.5x interquartile range, whichever is greatest.

Table 3.4. *Bd* scores on capture and in response to experimental treatment

Bd – *Bd* genomic equivalent (GE) score; Log *Bd* – log *Bd* GE score +1 (post-treatment)

Toad	Treatment	Capture Wet	Day 3 Wet	Day 6 Wet	Day 9 Wet	Day 13 Dry	Day 17 Dry	Log *Bd* mean
1	Itraconazole	0.0	0.5	0.0	0.6	0.0	1.6	0.366
2	Itraconazole	0.6	0.2	0.0	0.0	1.2	0.0	0.194
3	Itraconazole	1.6	DEAD	NA	NA	NA	NA	NA
4	Itraconazole	6.6	1.2	0.2	0.0	0.0	0.0	0.195
5	Itraconazole	5.2	0.5	0.0	0.0	0.7	0.6	0.281
6	Itraconazole	10.6	0.0	0.4	16.1	0.3	15.8	1.252
7	Itraconazole	5.3	0.0	0.0	0.0	0.0	0.0	0.004
8	Itraconazole	5.2	1.1	0.0	0.0	0.0	0.0	0.150
9	Itraconazole	123.9	87.3	0.0	0.0	0.1	1.3	1.074
10	Itraconazole	0.9	0.6	0.6	0.0	1.0	0.8	0.444
11	Itraconazole	10.5	0.0	0.0	0.3	0.0	0.0	0.052
12	Itraconazole	0.0	0.0	0.0	0.0	0.5	2.5	0.332
13	Itraconazole	3.1	3.5	0.0	32.9	0.1	8.6	1.471
14	Itraconazole	0.0	0.3	0.2	7.3	0.0	0.0	0.512
15	Itraconazole	0.0	5.5	6.5	5.2	0.0	0.0	1.142
16	Itraconazole	2077.0	0.0	0.2	13.9	17.2	0.0	1.157
17	Saline	6.5	0.0	0.1	4.7	1.1	0.8	0.633
18	Saline	1.2	0.4	0.9	4.7	37.5	272.8	2.396
19	Saline	0.3	0.4	0.0	1.1	2.2	0.0	0.448
20	Saline	0.0	0.0	0.4	99.7	0.5	65.2	1.909
21	Saline	1.0	0.0	0.4	10.1	0.4	0.0	0.616
22	Saline	270.4	105.0	2.2	1662.8	74.7	550.2	4.776
23	Saline	0.4	0.0	0.5	0.0	0.0	0.0	0.091
24	Saline	0.2	0.6	2.8	114.1	105.6	14.7	2.795
25	Saline	95.9	0.6	11.7	126.4	39.4	800.0	3.649
26	Saline	23.1	2.4	0.0	94.1	4.7	103.9	2.434
27	Saline	10.5	0.9	1538.5	144.1	75.8	8161.7	5.261
28	Saline	3.1	25.8	0.0	2.8	0.6	2.3	1.257
29	Saline	0.3	8.1	22.3	442.1	15.8	145.1	3.851
30	Saline	0.0	13.0	1.2	490.5	0.0	21.0	2.547
31	Saline	52.8	396.9	206.3	25518.0	DEAD	NA	7.156
32	Saline	750.1	3.2	88.6	976.0	254.2	91.2	4.576
33	Control	0.0	0.0	0.0	8.0	0.0	0.0	0.439
34	Control	91.1	0.7	3.3	283.1	31.8	DEAD	2.782
35	Control	183.4	14.4	6.2	7632.4	81.7	DEAD	4.516
36	Control	0.7	0.2	0.9	1431.4	NA	1279.7	3.812
37	Control	1.6	7.0	43.5	DEAD	NA	NA	2.937
38	Control	25.3	1.5	0.8	352.0	152.7	9.8	2.957
39	Control	13.6	67.5	13.8	458.5	DEAD	NA	4.351
40	Control	1.9	0.0	0.5	12365.1	86.5	630.1	4.149
41	Control	1.3	1.3	0.2	2.2	169.6	3.0	1.741
42	Control	4.3	1.0	1.1	543.0	265.9	62.3	3.494
43	Control	0.0	0.4	4.5	573.2	1205.5	26.3	3.759
44	Control	1.4	4.9	903.4	721.8	11969.4	228.9	5.999
45	Control	135.5	25.3	6786.1	DEAD	NA	NA	6.046
46	Control	0.7	0.0	1.8	30.0	50.3	87.8	2.578
47	Control	0.0	0.0	3.1	121.2	609.0	558.7	3.792
48	Control	0.4	0.0	0.0	41.5	0.1	8.9	1.229

Post-treatment log *Bd* mean scores differed across treatments (Fig. 3.3.). The variances of the scores differed across the three treatment groups (Bartlett's test for homogeneity of variance, Chi ^{2} = 20.86, df = 2, p<0.001). A non-parametric one-way analysis of variance found a significant difference between the ranks of the scores in each group (Kruskall-Wallis statistic = 22.08, n = 47, p<0.001). Post-hoc comparisons (critical z value = 2.39, p<0.05) of the mean ranks of the scores in each group showed itraconazole group scores were lower than those of the other two groups but the *Bd* scores of toads in the saline group were not significantly lower than those of the controls. As predicted, itraconazole had a significant negative effect on *Bd* but 1% salinity did not appear to be an effective treatment for *Bd*.

Most mortalities (n = 6/47) occurred near the end of the experiment (Fig. 3.4.). As the majority of toads (87%) survived, it was impossible to assess the effect of treatment on survival time, as insufficient data were available to estimate survival curves. However, it was possible to test the effect of treatment and *Bd* score on mortality, using generalized linear models (GLMs).

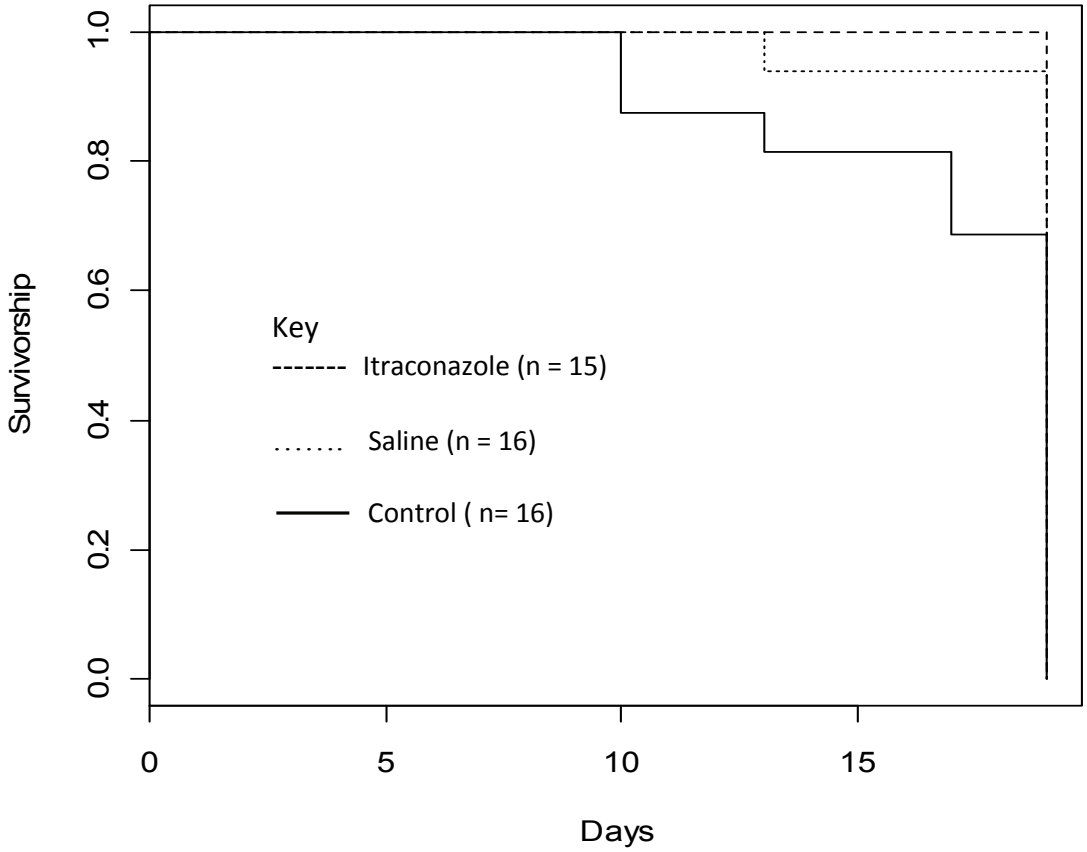


Fig. 3.4. Kaplan-Meier survivorship curves by treatment group

Table 3.5. The effect of treatment group on mortality

	GLM: mortality ~ group, binomial			
Coefficients	Estimate	S.E.	z-value	p<z-value
Intercept	-7.248	2.922	-2.480	0.013
group	2.162	1.037	2.085	0.037
	Residual deviance = 27.58 on 45 df, AIC: 31.58			

Table 3.6. The effect of post-treatment *Bd* score on mortality

	GLM: mortality ~ log *Bd* mean, binomial			
Coefficients	Estimate	S.E.	z-value	p<z-value
Intercept	4.71	1.41	3.35	0.001
log *Bd* mean	-0.85	0.33	-2.60	0.009
	Residual deviance = 25.30 on 45 df, AIC: 29.31			

There were significant effects of treatment and *Bd* score on mortality. Post-treatment log *Bd* mean score was the best predictor of mortality, which was associated with higher *Bd* scores.

Estimation of a mortality threshold from *Bd* score

An attempt was also made to define a *Bd* score threshold for mortality using these data. Other researchers have linked *Bd* score thresholds to chytridiomycosis and mortality in amphibians. Vredenburg *et al* (2010) reported population declines in mountain yellow-legged frogs *Rana muscosa* when mean *Bd* scores reached 10,000 GE (log*Bd* 9.2 GE). This was subsequently referred to as 'Vredenburg's 10,000 zoospore rule' by Kinney *et al* (2011), who found a similar pattern in crawfish frogs (*Lithobates areolatus*).

The threshold was derived using a similar method to Stockwell *et al* (2010) in a study where *Bd* scores were compared to mortality in green and golden bell frogs (*Litoria aurea*) and striped marsh frogs (*Limnodynastes peronii*). In my analysis I used the logistic function in R (Crawley, 2007) to identify the *Bd* score above which most (>50%) natterjacks died (Fig. 3.5.). A threshold of log*Bd* 5.6 GE was identified but this was poorly defined, due to a small sample size and the fact that in my study mortalities occurred over a wide range of *Bd* scores.

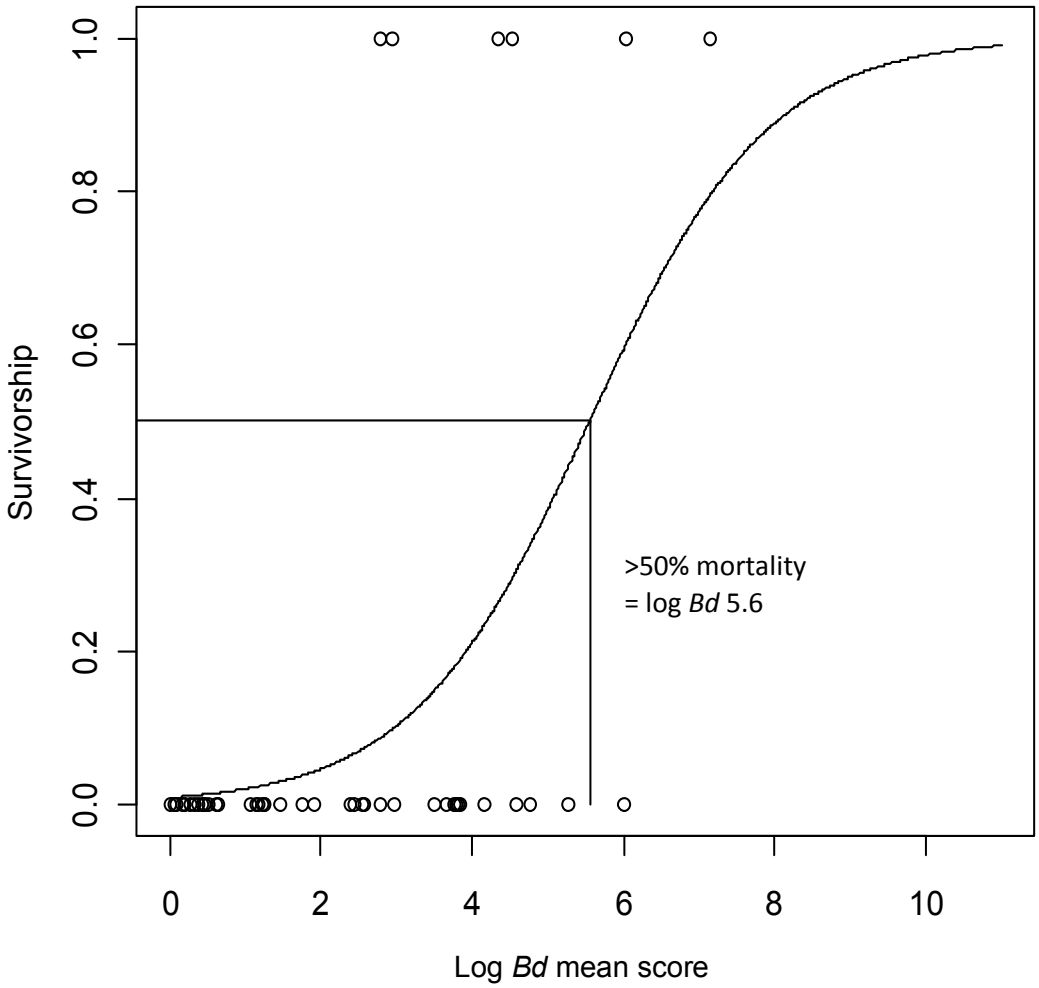


Fig. 3.5. Estimation of a mortality threshold for adult natterjacks from *Bd* score

Effect of salinity upon *Bd* growth *in vitro*

Bd growth *in vitro* (Fig. 3.6) became resource limited (see Fig. 3.7. for an example of convergent growth curves). Since the aim was to assess growth in the absence of density-dependence, I only analysed data from the first seven days. Growth rates were compared using absorbance readings (see Fig. 3.8.). There was zero growth in ≥1.75% salinity (or blanks). No movement of *Bd* zoospores was detected after seven days in 1.75% and three days in 3.5% salinity. Zoospores remained active and zoosporangia grew in all inoculated wells with a salinity of up to 0.88%. A lethal salinity threshold for *Bd* of between 0.88% and 1.75% suggested *Bd* was unlikely to survive in water with a salinity of 1.7%, the maximum tolerated by adult natterjacks (Mathias, 1971), or in ponds with higher salinities as a result of tidal inundation.

A reduction in growth did not occur at salinities linked to a reduction in positive *Bd* results. In the wild, positive *Bd* results were rare above a salinity of 0.08% (log conductivity 7µS, Fig. 3.2.). In artificial media, *Bd* grew in salinities of up to 0.88% and fastest growth occurred in 0.44% (see Fig. 3.8.). I compared mean growth readings on day seven across the five salinities (n = 10 per salinity) where growth occurred (0.00%, 0.11%, 0.22%, 0.44% and 0.88%). Variance did not differ across these salinities (Bartlett's test for homogeneity of variance, $Chi^2 = 5.00$, df = 4, $p<0.287$). A parametric one-way analysis of variance detected differences in growth (Fisher's F statistic = 23.58, n = 50, $p<0.001$). Post-hoc comparisons showed *Bd* growth by day seven was significantly higher in 0.44% than in any of the other salinities tested and growth in 0.11%, 0.22% and 0.88% was also higher than in 0.00% salinity (Tukey's HSD, n = 50, critical q-value = 4.02, $p<0.05$).

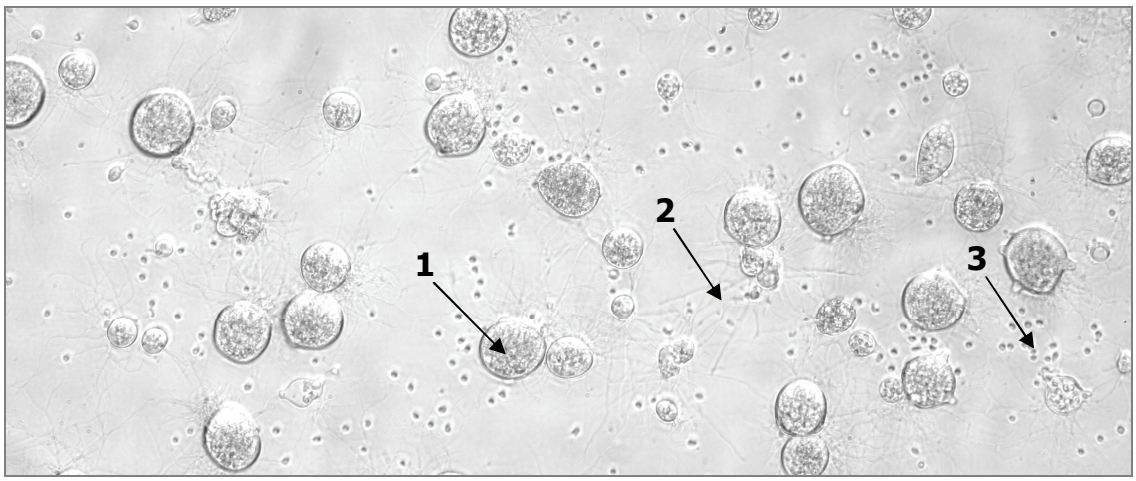


Fig. 3.6. *Bd* growing in tryptone-glucose (TG) growth media (0.22% salinity, day five).

Key: 1 Zoosporangium 2 Filamentous rhizoids 3 Emerging zoospores

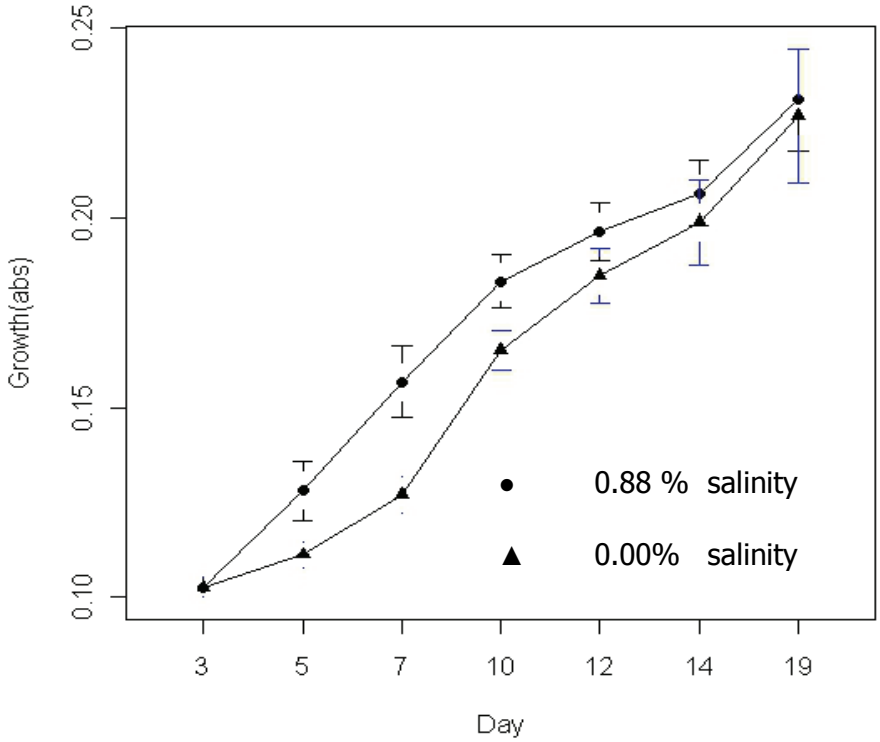


Fig. 3.7. Density-dependent *Bd* growth *in vitro* (means and 95% C.I.)

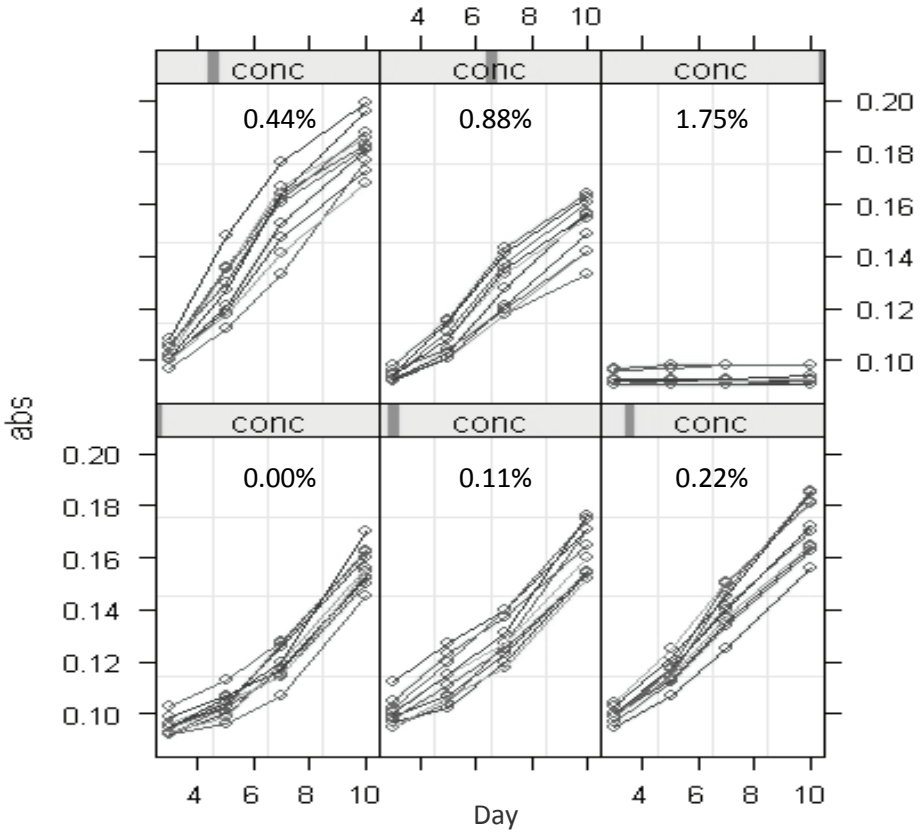


Fig. 3.8. *Bd* growth *in vitro* by salinity (n=10 per salinity)

Discussion

Natterjacks rarely tested positive for *Bd* if there was any evidence of salinity in the capture pond. This suggested that, if seawater was the cause, a barely detectable input was required. Although positive *Bd* results were rare in samples from toads captured in brackish ponds, *Bd* was detected in all populations except Haverigg and Anthorn. Two positive samples were later recorded from Anthorn. This suggests the levels of salinity found in natterjack habitat may not be sufficient to eliminate *Bd* but it was difficult to detect the pathogen at the most saline sites.

The pattern might be due to a decrease in infection prevalence, or chemotaxis, if *Bd* zoospores prefer to emerge into relatively pure water. According to Gleason & Lilje (2009), it is possible for zoospores to encyst within zoosporangia before release, in order to avoid unfavourable conditions. Longcore *et al* (1999) recommend flushing agar plates with distilled water to facilitate harvesting of zoospores from *Bd* cultures. *Bd* may show a range of chemotactic responses. There is evidence of chemotaxis in *Bd* in other studies. Lam *et al* (2012) report that *Bd* zoospores actively avoid antifungal metabolites produced by bacteria. Buck *et al* (2011) report predation of *Bd* zoospores by zooplankton (*Daphnia magna*), another reason why emergence into pure water might be an adaptive strategy.

All toads collected for the fungicidal treatment experiment were infected with *Bd* but several of them tested negative on capture. This cast further doubt upon the reliability of the swab test as a method for determining *Bd* prevalence. It did not, however, undermine the use of swab tests to determine levels of *Bd* infection activity (see Chapter 2). Although a change from wet to dry conditions resulted in a drop in *Bd* scores in other experiments it did not occur here. The reason for this was unclear but an extended wet phase may have boosted infections to levels where recovery was no longer possible, or a longer period of recovery was required.

As predicted, itraconazole was an effective fungicidal treatment and removal of *Bd* had a positive effect on survival. *Bd* scores were linked to mortality, although the number of mortalities was small. This was an important finding which identified *Bd* as the likely cause of death in toads with high *Bd* scores. The results were used to derive a mortality threshold for *Bd* score in natterjacks, for comparison with results from other species and field observations in this project (see Chapter 4.). Additional support for the mortality threshold would have been provided by data collected during another experiment to assess the effect of wet versus dry conditions upon *Bd* scores (see Chapter 2), if these data had been included in the model. In

that experiment, two of six toads died, both of which had mean scores in excess of log *Bd* 5.6, in contrast to four survivors. However, even with a much larger sample, individual variation in response and sampling error could prevent derivation of a clearly-defined mortality threshold.

The results of the *in vitro* experiment were not directly comparable with field data, as *Bd* growth and survival were only assessed in artificial media. In the wild, *Bd* detection declined at a lower salinity than was seen to limit growth of *Bd* in culture. Fastest growth at 0.44% salinity suggested salts were an important nutrient for *Bd*. This finding was of interest to *Bd* researchers because salts were not included in the nutrient broth recipes recommended for *Bd* culture by Longcore *et al* (1999). I collected *Bd* from amphibians in brackish habitat, so this particular strain may be salt-adapted. This could be tested by repeating the experiment with isolates of *Bd* collected from amphibians found only in freshwater habitat.

Despite some discordance between field and experimental results, salinity influenced *Bd* detection levels in the wild and *Bd* survival in culture. A salinity of ≥1.75% (equivalent to 50% seawater in most coastal ponds) kills *Bd* zoospores and will probably result in a reduction in transmission rates in the wild. For this reason, I conclude that salinity will have a negative effect on *Bd* infection prevalence. Amphibians found in more saline habitats, such as natterjacks in haline quarries in Spain (Gomez-Mestre & Tejedo, 2003), coastal populations of green toads and crab-eating frogs in mangrove swamps, may be less likely to suffer from chytridiomycosis. According to Voyles *et al* (2009), the cause of death in amphibians killed by *Bd* is sodium and potassium depletion, with skin damage by *Bd* preventing normal sodium uptake. This depletion may be accelerated if *Bd* normally uses salts for growth and zoospore emergence. Recovery from electrolyte depletion may be easier to achieve in brackish environments, where salts are more abundant. If salinity has a protective effect, it may be focused on the life-stages or species with greatest tolerance of salinity. Adult amphibians usually tolerate higher salinities. Natterjack spawn and tadpoles rarely survive a salinity of >0.7% in the UK (Beebee, 1985) but adults can tolerate 1.7% (Mathias, 1971).

Further research would help determine the extent to which environmental chemistry influences the physiology, behaviour and ecological impact of *Bd*. Although several environmental factors have been identified, many other contenders (such as pH) deserve further investigation. Identification of the factors which control transmission and trigger disease can help explain why some populations decline in response to infection by a pathogen, while others do not.

Chapter 4.

Batrachochytrium dendrobatidis (*Bd*) does not have a significant impact on the survival or growth of adult natterjack toads (*Bufo calamita*) in the wild

Abstract

Batrachochytrium dendrobatidis (Bd) is a parasite of many amphibians including the natterjack toad (*Bufo calamita*), which is an endangered species in the UK. Analysis of capture-mark-recapture (CMR) data from three natterjack populations demonstrated a weak negative correlation between *Bd* swab test scores and survival. *Bd* scores also correlated with age, which was strongly linked to survival, suggesting other age-dependent factors may be more important. Males had higher *Bd* scores than females but survival did not differ between the sexes and there was no correlation between *Bd* score and growth rate. Only 6% of wild adult natterjacks recorded *Bd* scores in excess of a mortality threshold derived from experiments. No mass-mortalities of adult natterjacks were observed during this study and natterjack spawning has continued at several sites where *Bd* has been present for at least four years.

Introduction

Diseases can have major impacts on naïve populations, including mass mortalities and rapid declines in host abundance. The spread of a recently-evolved (Farrer *et al*, 2011), global panzootic lineage (GPL) of the parasite *Batrachochytrium dendrobatidis (Bd)* has been linked to the decline of many amphibian species (Skerratt *et al*, 2007).

Several researchers (Murray *et al* 2009, Pilliod *et al* 2010 and Muths *et al* 2011) have reported a negative correlation between *Bd* detection and the survival of wild adult amphibians, using capture-mark-recapture (CMR) data. Others have reported no such link (Retallick *et al*, 2004, Kriger & Hero, 2006). Briggs *et al* (2010) found no link between *Bd* swab test scores and the survival of mountain yellow-legged frogs (*Rana sierrae)* at some sites, but blamed *Bd* for the disappearance of this species at other sites. Although *Bd* can cause epidemics, more subtle effects can occur if the risk of disease is limited by environmental factors (see Chapter 3) or there is genetic variation in disease resistance (see Savage & Zamudio, 2011).

I investigated the effect of *Bd* on the survival of natterjack toads (*Bufo calamita*), following detection of *Bd* in samples from natterjacks in the UK (Feltrer & Cunningham 2006, Arai 2008). Discovery of *Bd* in natterjack populations was a cause for concern, as this species is endangered in the UK (Buckley & Beebee, 2004).

I assessed the effect of *Bd* on natterjack toads using three approaches. Firstly, I looked for evidence of declines in populations which had tested positive for *Bd*. Spawning activity is recorded in the Natterjack Toad Site Register for the UK (Beebee & Buckley, 2012, in press). Spawn string counts are thought to provide an indication of the number of females (Buckley & Beebee, 2004) and breeding population size. I expected to see a decline or cessation of spawning activity, if *Bd* had a major negative impact on adult natterjack survival.

Secondly, I carried out a capture-mark-recapture (CMR) study of three populations in a region of the UK (Cumbria) where *Bd* had been detected in natterjacks (Arai, 2008). The study included measurement of variables which I expected to correlate with adult survival, including quantitative *Bd* score (from swab tests). I predicted survival would correlate negatively with *Bd* score. Male natterjacks were predicted to have lower survival rates than females due to risks associated with pond attendance. The males of many pool-breeding amphibians, including natterjacks (Tejedo, 1993), spend more time in ponds to anticipate female arrival and maximise breeding opportunities. I predicted males would have higher *Bd* scores, due to increased infection risk and *Bd* activity in response to immersion (see Chapter 2). Carey *et al* (2006) suggested *Bd* might be responsible for reduced male longevity in boreal toads *Bufo boreas*, based on observations of population structure and estimated *Bd* arrival time.

Thirdly, I tested for a negative correlation between quantitative *Bd* score and adult natterjack growth. This may be the first study to compare *Bd* scores with the annual growth of amphibians in the wild. In experiments, *Bd* has been linked to a reduction in tadpole growth. Garner *et al* (2009b) found exposure to *Bd* resulted in a reduction in mass at metamorphosis in common toad (*Bufo bufo*) tadpoles. Captive adult chorus frogs *Pseudacris triseriata* lost more weight if they had tested *Bd*-positive (Retallick & Miera, 2007), suggesting *Bd* might have a negative effect on the growth of adult natterjacks.

Methods

Bd detection in natterjack populations and spawning activity

I compared records of *Bd* detection in UK natterjack populations with records of spawning activity in the Natterjack Toad Site Register for the UK (Beebee & Buckley, 2012, in press). I planned to compare spawning activity between infected and uninfected populations and before and after arrival of *Bd*, if true infection status and the time of arrival could be identified.

Capture-mark-recapture (CMR) study of the effect of *Bd* on survival

Capture-mark-recapture **(**CMR) data were collected from three natterjack populations in northern Cumbria; Mawbray, Grune and Bowness-on-Solway. For locations, see Chapter 1, Fig. 1.6. I set a target for sampling of at least 60 toads per population, in order to have a high level of confidence that *Bd* would be detected (see Chapter 2, p24). Toads were captured during standardised night-searches which were carried out at intervals of no greater than 14 days at each site (total searches at Mawbray = 53, Grune = 47, Bowness = 30). These searches included visits to all known breeding ponds. Searches were completed from April to September during 2009 and 2010 and April to May in 2011, effectively covering three spawning seasons.

All adult natterjacks captured (>38mm snout-to-vent length, SVL) were marked using passive-inert-transponder (PIT) tags (AVID). Toads were identified on recapture using a tag reader. Photographs of unique dorsal wart patterns were also taken to allow identification in the event of tag loss. Toads were sampled for *Bd* on all capture occasions using swabs analysed by rt-PCR (Boyle *et al*, 2004) a method which is described in detail in Chapter 2, p22-23. Sex was determined by checking for the presence of dark thumb pads in males.

The hypotheses under test in this study were as follows;

- Survival rates would be higher in populations with lower *Bd* incidence
- Males would have a lower probability of survival and higher *Bd* scores than females
- Individual survival probability would decrease with increasing *Bd* score

I also collected data to see if *Bd* scores or survival varied with age or size (SVL, mm). Responses to pathogen exposure and survival can vary significantly between age and size classes in animal populations but I did not attempt to predict the nature of these responses.

Estimation of natterjack toad age by skeletochronology

Skeletochronology can often be used to estimate the age of amphibians. This method is similar to dendrochronology, where tree age is estimated by counting growth rings resulting from cyclic changes in growth rate. In temperate amphibians, growth rings or 'lines of arrested growth' (LAGs) form in bone tissues as a result of lower temperature and metabolic rate during hibernation. Multiple LAGs can form when hibernation is interrupted by warm spells or growth is curbed by aestivation. According to Sinsch *et al* (2007), natterjacks in northern Europe which experience cold winters and moderate summers usually only develop one LAG per year. Denton & Beebee (1993) found a few examples of UK natterjacks with multiple within-year LAGs but I assumed the number of LAGs would provide a reasonable indication of age.

In order to estimate toad age I took a toe clip sample from each toad, containing the medial and distal phalanges. There was potential for toe-clipping to affect recapture and survival rates. In studies of several species, McCarthy & Parris (2004) found removal of one toe resulted in a decrease in survival of 4-11%. The ethics of toe-clipping have been questioned by May (2004) but Funk *et al* (2005) argue that the information gained can provide conservation benefits which outweigh the costs. In my study, clipping of a single toe had multiple benefits; information about age, rapid identification of toads which lost PIT-tags and a source of tissue from which *Bd* was isolated for experiments and genomic sequencing. In order to ensure effects on survival were consistent, I removed one toe from all adults captured (the longest toe on the right hind foot) using surgical scissors. Samples were stored in 70% ethanol.

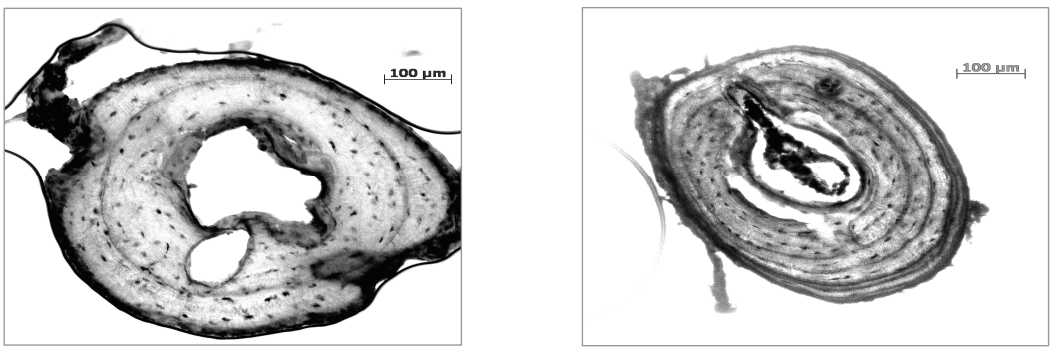
A method for sample processing was developed with reference to Hemelaar (1983), Denton (1991) and Rozenblut and Ogielska (2005). Hemelaar (1983) suggested the first two LAGs could be obscured by endosteal bone resorption and remodelling during growth, so true age might exceed the number of visible LAGs. However, Rozenblut and Ogielska (2005) showed LAG1 (corresponding to first hibernation) was consistently visible in the bones of adult water frogs, provided the mid-length of each bone (diaphysis) was sampled. Tissue from the first year of growth was retained within the diaphyses but not the ends of the bones (epiphyses). To ensure detection of LAG1, I sectioned the mid-length of each phalange.

Each toe sample was first removed from storage in ethanol and boiled in water (20 min) to soften the tissues around each phalange and facilitate their removal with forceps. Decalcification was also necessary to soften the bones before sectioning. Phalanges were

decalcified in formic acid (5%) for 45-75 min. Larger phalanges required longer treatment. Sufficient decalcification was confirmed by an increase in bone flexibility. Upon removal from the acid, each phalange was rinsed in distilled water to remove any traces of acid. After extraction from soft tissues and decalcification, the proximal or distal epiphysis of each phalange was removed using a scalpel. This saved time, as the epiphyses were unlikely to include LAG1 and sectioning using a microtome began with one-third of the bone length removed. Each phalange was frozen vertically in Lamb-Oct embedding matrix on a Peltier-type freezing platform, with the cut end of the phalange protruding from the matrix. Transverse sections were cut at 40 µm intervals with the microtome blade set at 5° from the horizontal.

Sections (approximately 30 per phalange) were transferred from the blade to a watchglass containing distilled water and lightly stained using Delafield's hematoxylin. At this stage it was possible to identify 5-12 sections of sufficient quality for age estimation. These sections were transferred onto microscope slides, set in Immunomount gel and protected with coverslips. The fixed sections were viewed under a light microscope with a 10x magnification lens. The best two or three sections for each phalange were photographed at 300 dpi using an Axio-Vision 4.6 imaging system connected to the microscope and a computer. The photographs were used to generate an age estimate for each toad.

A useful benchmark was provided by samples from Bowness, as natterjacks were reintroduced to this site in 2005 and 2006. Consequently, toads captured in 2009 could only be 3-4 years old, as there was insufficient time for recruitment of a second generation. The sample from the male toad in Fig. 4.1. was collected in 2009. One LAG is visible adjacent to the marrow cavity, another is clearly visible halfway through the bone and one must also exist at the outer bone margin. In this study, most toads were sampled in spring before summer foraging had resulted in the addition of new bone, so in many cases the most recent LAG was invisible.



Male, 51mm, 15.2 g, age estimate = 3 *Female, 68mm, 40.8 g, age estimate = 8*

Fig. 4.1. Transverse sections through the phalanges of natterjack toads

Analysis of capture-mark-recapture (CMR) data using program MARK

Differences in recapture rate can create illusory differences in survival, so it is necessary to calculate separate estimates of survival and recapture probability. This is possible using a maximum likelihood approach in program MARK (White & Burnham, 1999). A range of models can be used to analyse CMR data in MARK. The most powerful 'robust' models can only be justified if there is zero net migration during the study (Kendall *et al* 1997). I could not make this assumption as natterjacks are explosive breeders and I could not identify any period within or across years during which it was safe to assume zero emigration or immigration. Other researchers (Schmidt, 2010) have questioned CMR analyses where within-year comparisons have been made between *Bd* test data and survival (Murray *et al*, 2009, 2010), due to the possibility of permanent emigration following spawning activity.

Standard Cormack-Jolly-Seber (CJS) models assume an open rather than closed population, so individuals can enter or leave the study population at any time (Lebreton *et al*, 1992). Although it is not essential for recapture intervals to be evenly spaced, model specification and interpretation of the results is much simpler if they are. Investigation of within-season effects was not essential, so I decided to use the standard CJS model. I used a three-event encounter history, with each toad coded as seen or not seen in 2009, 2010 and 2011. Estimation of survival and recapture was possible across two time intervals (2009-2010 and 2010-2011).

Program MARK was used to produce estimates of survival (ϕ) and recapture (ρ) probability and test predictions. I used maximum *Bd* score (log-transformed, see Chapter 3, p41) to represent the maximum level of *Bd* infection activity experienced by each animal. Use of a time-invariant covariate is unusual but may be justified by my earlier findings (see Chapter 2) which suggest *Bd* scores derived from skin swabs can only provide an indication of infection activity or load when amphibians are in water, or have been recently immersed.

Comparison of growth rates with *Bd* scores and detection

I tested for links between annual growth rates and *Bd* test results using two approaches; firstly by comparing annual growth with the *Bd* scores of individuals. Secondly, by comparing the size structure of populations with different incidences of *Bd* detection. Toads were measured with a ruler from snout to vent (SVL, mm) on all capture occasions. Annual growth rates were calculated as log (final SVL / initial SVL) / maximum years sampled -1.

Results

***Bd* detection in natterjack populations and spawning activity**

Bd has been detected in samples from natterjacks across most of their UK range; in southern England at Sandy and Frensham (Bramwell, 2011); in Wales at Talacre (Cunningham & Minting, 2008); on Merseyside at Ainsdale and Birkdale (Cunningham & Minting, 2008); in Cumbria at Mawbray (Feltrer & Cunningham, 2006), Drigg, Dunnerholme, Sandscale, Sellafield, Silloth (Arai, 2008), Annaside, Braystones, Haverigg, Subberthwaite (Bramwell, 2011), Grune and Bowness-on-Solway (this study); and in Scotland, where *Bd* was first recorded at Caerlaverock in smooth newts *Triturus vulgaris* (Cunningham & Minting, 2008) and subsequently in natterjacks (Perkins, *pers.comm*, 2010). At two sites (Frensham, Subberthwaite) *Bd* DNA was detected in single samples, so positive *Bd* status for these populations may be unjustified without further tests. I also detected *Bd* DNA in preserved natterjacks from Talacre which were collected in 2006 and in samples from two natterjacks at Anthorn (Cumbria) in 2011.

I have presented these records alongside spawn counts for well-monitored populations (<3yrs missing data since 2000) in the Natterjack Toad Site Register for the UK (Beebee & Buckley, 2012, in press). Detection was unlikely to coincide with *Bd* arrival. *Bd* was detected on the first sampling events at all of the sites in Table 4.1. As I had no confidence in arrival time, I did not compare spawn counts before and after detection of *Bd*. As *Bd* was detected at all effectively-sampled sites (>60 samples, see Chapter 2, p24), I could not compare infected with uninfected populations. There was no evidence of a cessation in spawning activity.

Table 4.1. Spawn counts from natterjack populations where *Bd* has been detected
Shading - dark = *Bd* detected; light = *Bd* detected in earlier tests NA - no data

Year	Ainsdale	Birkdale	Talacre	Caerlav.	Drigg	Sandscale	Sellafield	Mawbray	Grune
2000	834	162	11	19	59	195	9	5	0
2001	899	125	50	76	0	NA	8	0	0
2002	232	50	71	61	2	108	3	1	0
2003	610	104	140	145	40	160	32	4	17
2004	341	62	23	135	136	105	16	13	3
2005	719	183	59	187	248	247	26	0	25
2006	290	210	172	6	251	NA	27	7	40
2007	232	239	137	12	105	NA	14	NA	29
2008	281	285	198	2	108	473	24	2	11
2009	205	235	106	5	176	197	23	14	23
2010	140	167	79	2	161	494	17	24	29
2011	NA	NA	50	3	323	527	22	22	47

Capture-mark-recapture (CMR) study of the effect of *Bd* on survival

From 2009 to 2010 a total of 227 adults were captured at the three intensively-monitored sites. Population sub-totals were; Grune (92), Mawbray (75), Bowness (60). Data from these toads were used to estimate survival and recapture probabilities using the standard CJS model in MARK (Table 4.2.) Age and size were strongly correlated (Pearson's product-moment, $t = 19.90$, $p<0.001$, correlation coefficient = 0.799; linear model F-stat: 395.9 on 1 and 225 df, $R^2 = 0.64$) so I did not include these variables in the same models, to avoid confounding effects.

Dispersion was calculated from the most general model possible (see 'global for ĉ' model, Table 4.2) using the median ĉ test in program MARK. *Bd* score, age or size were not included in this model, as continuous covariates cannot be included in calculations of ĉ (Cooch & White, 2009). Overdispersion results in overestimates of model support if ĉ is set at 1 (zero dispersion), whereas underdispersion can result in overly conservative estimates. I adjusted ĉ to account for underdispersion in the data (median ĉ = 0.748, S.E. = 0.148). When ĉ is adjusted program MARK generates quasi-Akaike Information Criterion (QAICc) instead of AICc values. AICc is a default modification of the AIC in program MARK which allows for small samples.

I first evaluated models of recapture (ρ) to prevent bias in survival estimates (Lebreton *et al*, 1992). I made no hypotheses about relationships between ρ and explanatory variables (time, population, sex, age, size and *Bd* score). To identify the best model of ρ, I compared models of ρ against a global model, using the same method as Pilliod *et al* (2010). The best model of ρ was provided by age (model 3, Table 4.2). Recapture probability declined with age (Fig. 4.2).

Having controlled for recapture probability (ρ) by keeping the structure of recapture confined to age, I compared models of survival (ϕ). I was primarily interested in variables which affected survival. Time (interval), population, sex and *Bd* score had little influence (models 4-7) but survival increased with age (model 1). Older toads had better survival prospects (Fig. 4.3). As I could not include size and age in the same models, I checked if size was more informative by replacing age with size (model 2). Age was more closely linked to ρ and ϕ than size.

Other studies of *Bd* and survival in the wild have not included size or age as explanatory variables. I removed these variables to see if this would generate a different result. When age was not available and *Bd* score was used to model ρ and ϕ, *Bd* did appear to influence survival, generating a model (model 8) which competed with the null model, using the standard

criterion of ≥2 (Q)AICc units (Burnham & Anderson, 2002). Survival decreased with increasing *Bd* score (Fig. 4.4). If *Bd* score had a separate effect on survival to age, model 4 should have competed with model 3. This conflict suggested a correlation between *Bd* score and age or size. There was a weak negative correlation between age and *Bd* score (Pearson's product-moment, t = 3.33, p<0.001, correlation coefficient = -0.217; linear model F-stat: 11.09 on 1 and 225 df, R^2 = 0.04) but not size (Pearson's product-moment, t = 1.62, p<0.107, correlation coefficient = -0.11, F-stat: 2.62 on 1 and 225 df, R^2 = 0.007). The decline in survival with increasing *Bd* score may be due to the correlation between *Bd* score and age (Fig. 4.5).

The observed correlation between age and *Bd* score was too weak to justify exclusion of these variables from the same models. However, variation in the sensitivity of the swab test resulting in zero-inflation (see Fig. 4.5) of the *Bd* score data may have masked a stronger correlation between age and true *Bd* infection load. The swab test often fails to detect infections in individuals which have spent time on land, possibly as a result of a decline in zoospore emergence (see Chapter 2). Zero inflation often implies non-participation with respect to the variable of interest (Gurmu & Dagne, 2012). It was not possible to identify *Bd* negative toads, or determine how much of the variation in *Bd* score was due to changes in infection activity.

Table 4.2. Models used to estimate the survival (ϕ) probability of natterjack toads

QAICc – quasi-Akaike information criterion, c

ΔQAICc – difference in ΔQAICc between the current and the best model

w – QAICc weight; Q/Deviance - $^{-}$2 log-likelihood/ĉ ; K – number of parameters

ϕ(.),ρ(.) – null model; pop – population of origin; time – sampling interval (annual)

Bd – log*Bd* maximum score in genomic equivalents (2009-2010)

Rank	Model name	QAICc	ΔQAICc	w	Likelihood	K	QDeviance
1	ϕ(age),ρ(age)	929.64	0	1	1	4	921.5669
2	ϕ(size),ρ(size)	966.38	36.7418	0	0	4	958.3089
3	ϕ(.),ρ(age)	971.17	41.5304	0	0	3	965.1263
4	ϕ(*Bd*),ρ(age)	972.39	42.7488	0	0	4	964.3159
5	ϕ(sex),ρ(age)	973.01	43.3711	0	0	4	964.9382
6	ϕ(time),ρ(age)	973.11	43.4682	0	0	4	965.0353
7	ϕ(pop),ρ(age)	975.12	45.4857	0	0	5	965.0165
8	ϕ(*Bd*),ρ(*Bd*)	976.29	46.6506	0	0	4	968.2177
Null	ϕ(.),ρ(.)	978.89	49.2536	0	0	2	974.8712
Global for ĉ	ϕ(time*pop*sex), ρ(time*pop*sex)	1002.6943	73.0552	0	0	20	961.1358

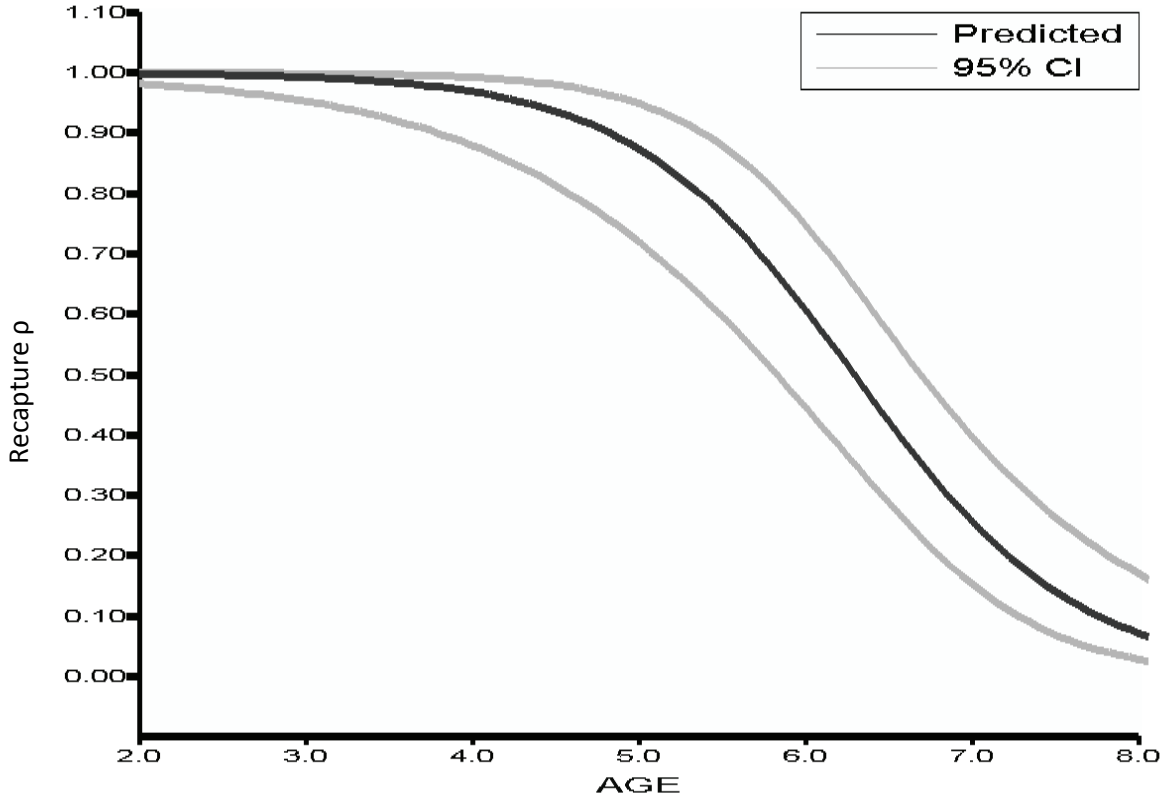


Fig.4.2. Age and estimated recapture probability

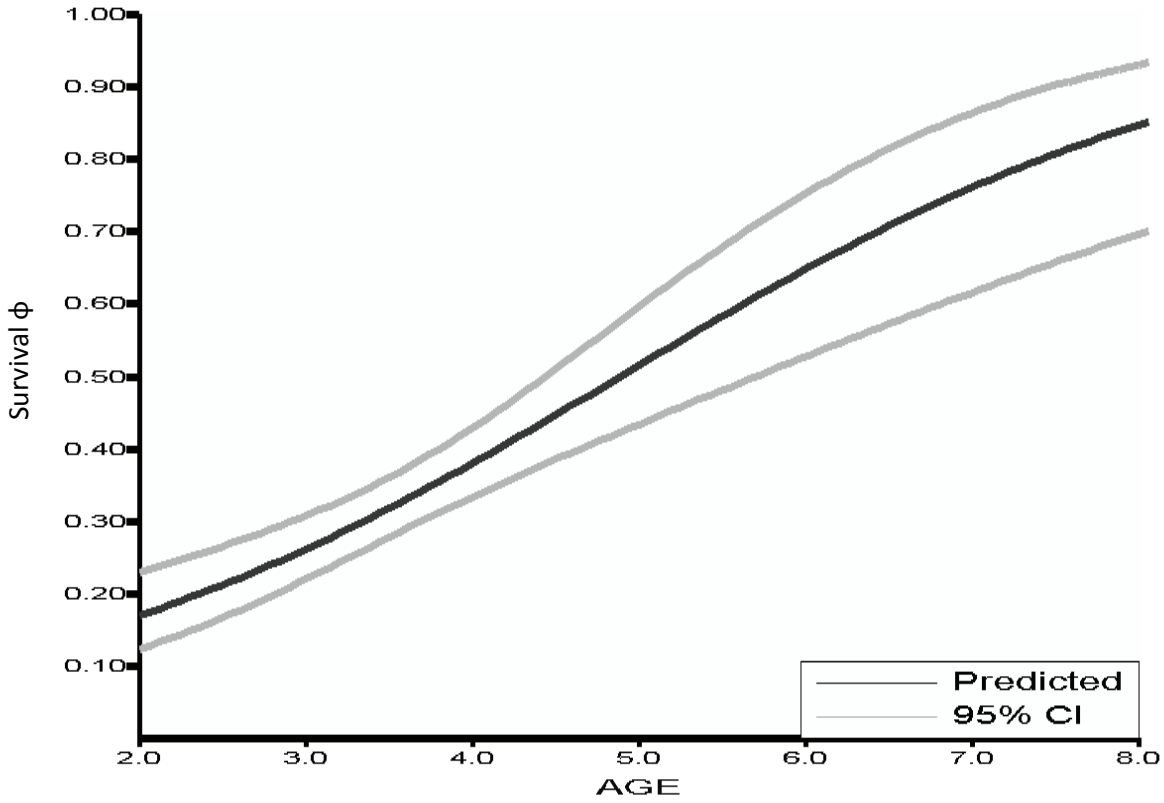


Fig.4.3. Age and estimated survival probability

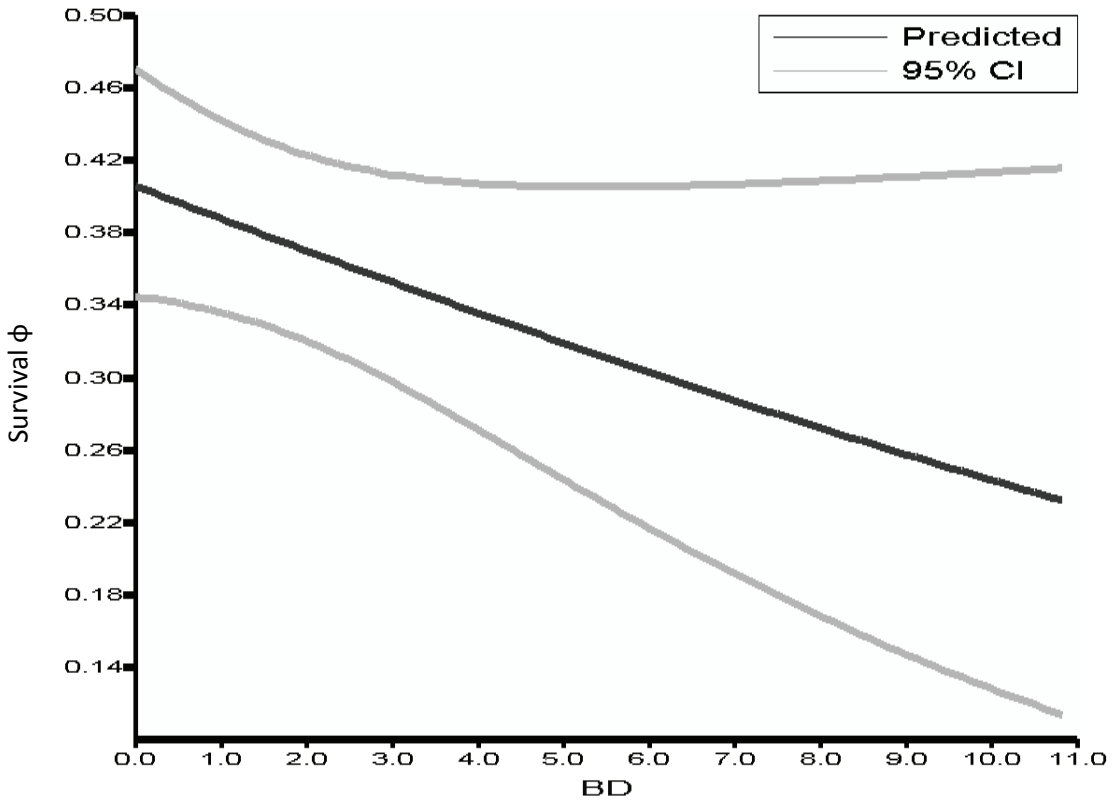


Fig.4.4. Maximum (log-transformed) *Bd* score and estimated survival

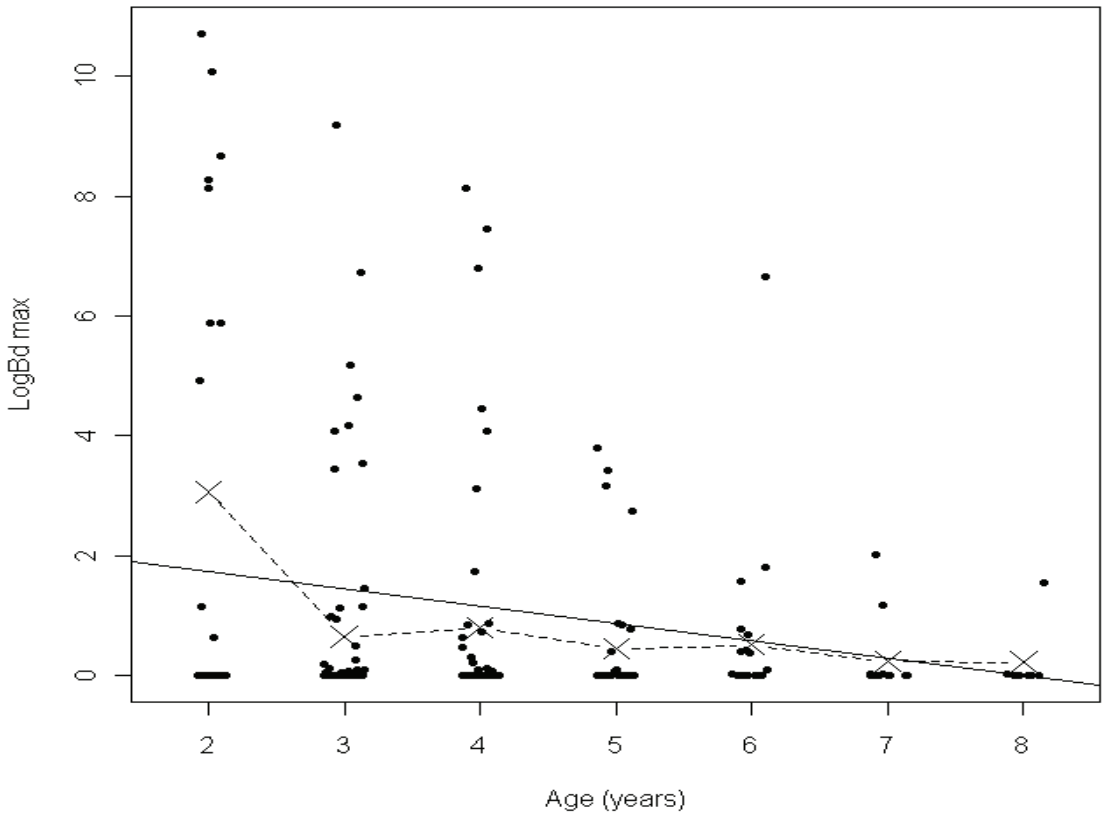


Fig.4.5. Correlation between age and maximum *Bd* score (fitted line and empirical means)

Male and female survival rates were not significantly different. There was overlap in the confidence intervals around the survival estimates for each sex (Table 4.3.) and sex did not generate competitive models. As predicted, males had higher *Bd* scores at Grune (Wilcoxon, W = 1324.5, p<0.010) and Mawbray (Wilcoxon, W = 1201, p<0.001) (Figs 4.6 & 4.7). Insufficient females (n = 6) were sampled for a comparison of *Bd* scores between sexes at Bowness.

Table 4.3. Estimates of survival and recapture (2009-2011) by sex and population

Population	Survival ϕ (95% C.I.)		Recapture ρ (95% C.I.)	
	♂	♀	♂	♀
Grune	0.45 (0.28-0.64)	0.45 (0.13-0.82)	0.69 (0.32-0.92)	0.51 (0.09-0.91)
Mawbray	0.22 (0.11-0.38)	0.40 (0.28-0.53)	1.00 (-)	1.00 (-)
Bowness	0.39 (0.27-0.51)	0.17 (0.04-0.48)	1.00 (-)	1.00 (-)
(All)	0.38 (0.29-0.47)	0.38 (0.31-0.44)	0.85 (0.56-0.96)	0.84 (0.66-0.93)

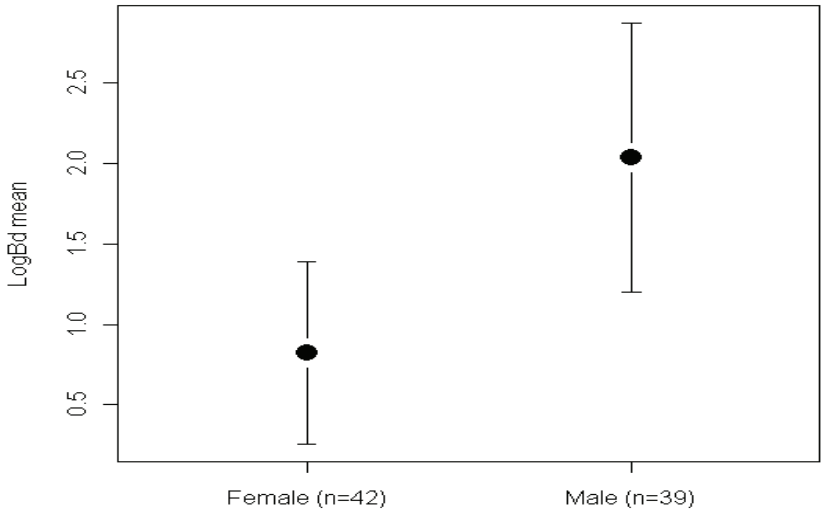


Fig.4.6. Sex and *Bd* score, Mawbray (means and 95% C.I.)

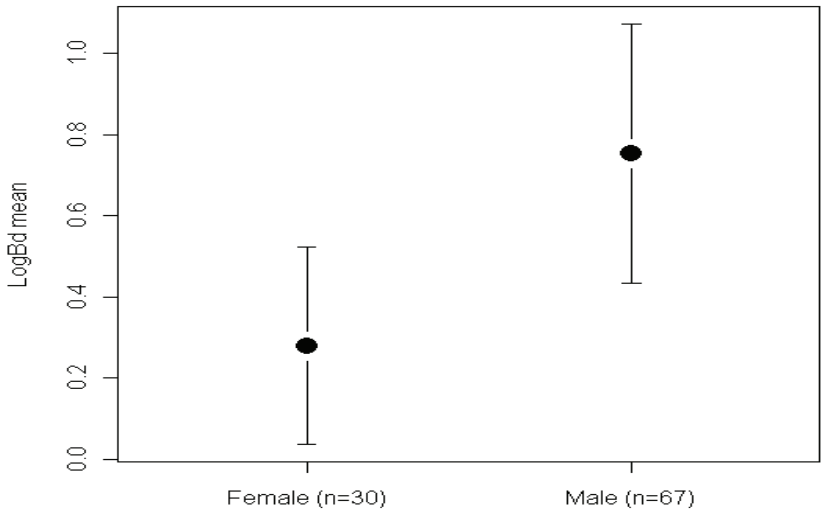


Fig.4.7. Sex and *Bd* score, Grune (means and 95% C.I.)

Comparison of growth rates with *Bd* scores and detection

Data from natterjacks which were measured and swab tested in multiple years (males n = 42, females n = 22) were used to test for a correlation between growth and *Bd* score. There was no correlation between the annual growth rate (log (final SVL / initial SVL) / maximum years sampled -1) and maximum (log-transformed) *Bd* score of male (Pearson's product-moment, t = 0.29, p<0.773, correlation coefficient = -0.045; linear model F-stat: 0.08 on 1 and 41 df, R^2 = 0.002) or female natterjacks (Pearson's product-moment, t = 0.45, p<0.660, correlation coefficient = 0.097; linear model F-stat: 0.20 on 1 and 21 df, R^2 = 0.009). Contrary to predictions, the growth rates of individual toads did not correlate negatively with *Bd* score.

Fitting of Michaelis-Menten growth curves (using the SSmicmen function in R, Crawley 2007) to age-size distribution data (on first capture) suggested growth rates were slower at BW (Figs 4.8-4.9). To test if toads were smaller at Bowness, I compared toad size for the most abundant age classes for each sex (age three for males, age five for females) across all three populations.

Variance in male size differed between the three populations (Bartlett's test for homogeneity of variance, Chi ^{2} = 19.27, df = 2, p<0.001). A non-parametric one-way analysis of variance found a significant difference between the size of males in each population (Kruskall-Wallis statistic = 46.91, total n = 113, p<0.001). Post-hoc comparisons of the mean ranks of male sizes showed that Bowness males (n = 29) were smaller (critical z value = 2.39, p<0.05) than those at Mawbray (n = 26) and Grune (n = 58), which were not significantly different in size.

Variance in female size also differed between populations (Bartlett's test for homogeneity of variance, Chi ^{2} = 10.19, df = 2, p<0.006). A non-parametric one-way analysis of variance also found a significant difference between the size of females in each population (Kruskall-Wallis statistic = 11.15, total n = 26, p<0.004). Post-hoc comparisons of the mean ranks of female sizes showed that Bowness females (n = 7), like males, were smaller (critical z value = 2.39, p<0.05) than those at Mawbray (n = 12) and Grune (n = 7), which again did not differ in size.

The smaller size of natterjack toads at Bowness did not support a negative impact of *Bd* on growth rate. *Bd* was only detected in 7% of natterjacks sampled at Bowness, compared to 29% at Grune and 44% at Mawbray, using the standard 0.1 GE threshold (see Chapter 2).

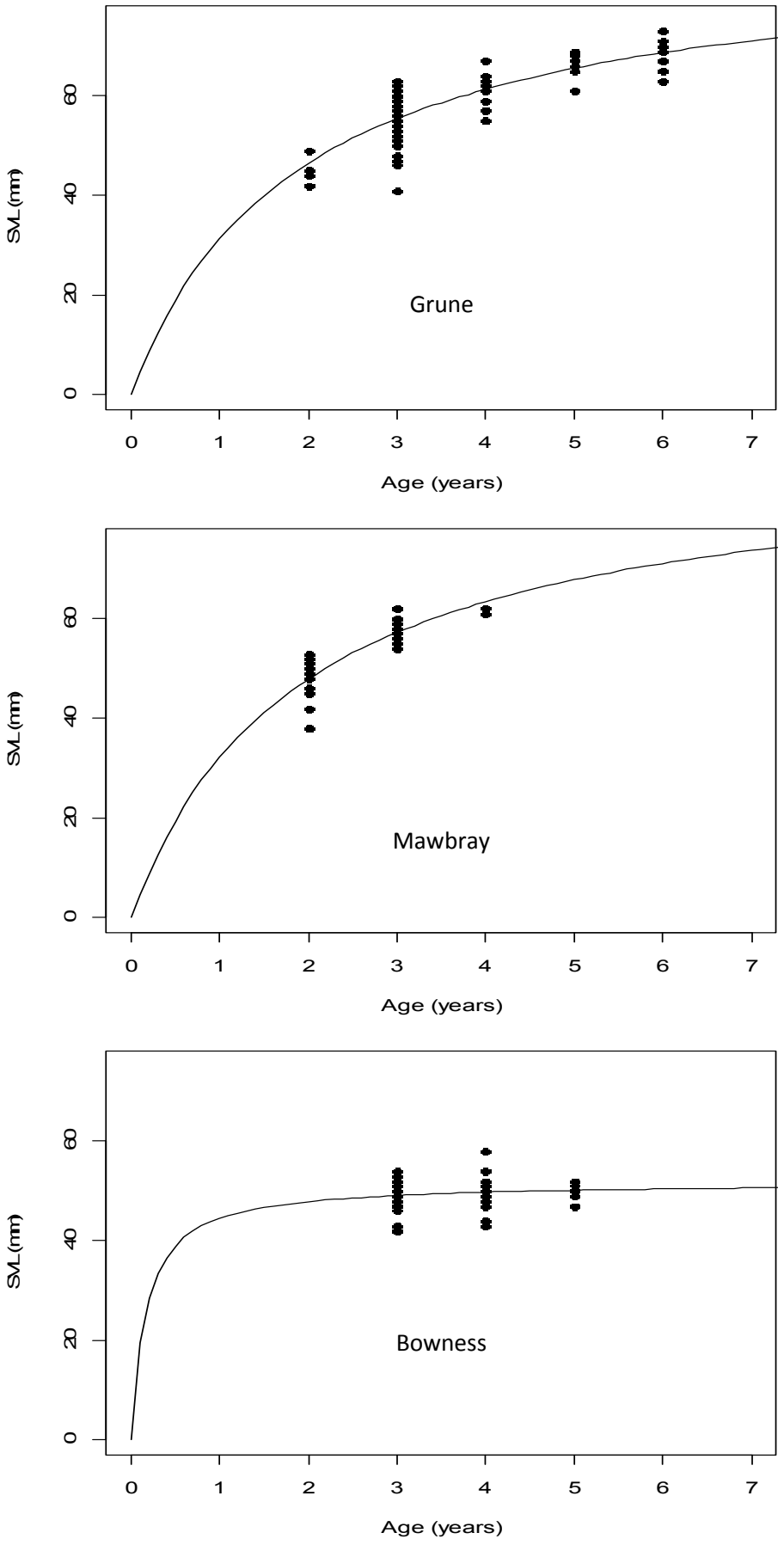


Fig. 4.8. Age-size distributions, male natterjack toads

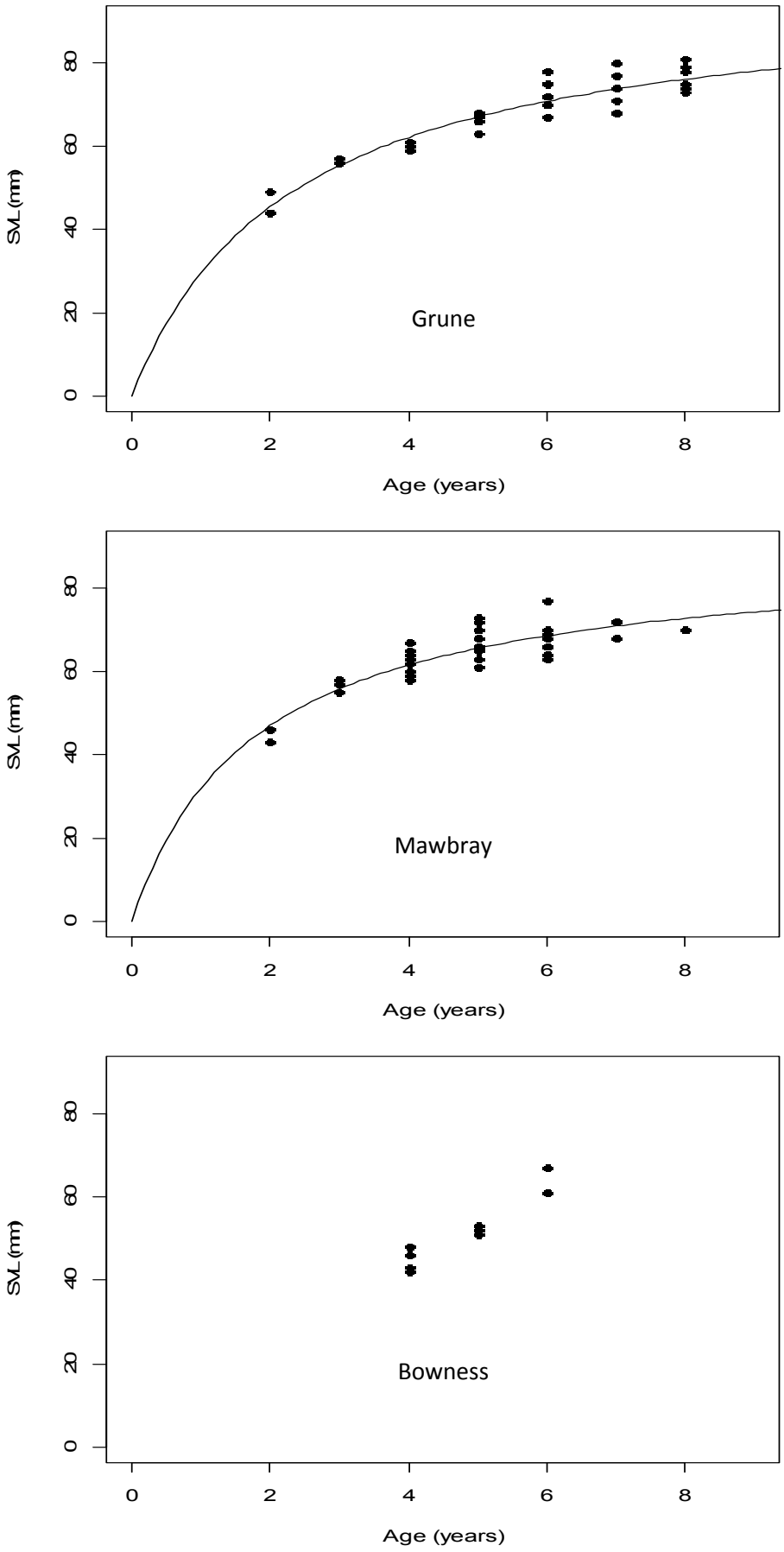


Fig. 4.9. Age-size distributions, female natterjack toads

Growth models using the Michaelis-Menten equation (y = ax / 1 + bx) provided a good fit to data from Grune and Mawbray, with reliable estimation of intercept (a) and slope (b) parameters (Tables 4.4 & 4.5). Data from males at Bowness did not provide an accurate estimate of slope, as there was little evidence of growth after recruitment. A small sample size (n=12) prevented accurate estimation of both parameters (Table 4.6) for Bowness females and plotting of a growth curve was not appropriate in this case (Fig. 4.9.).

Table 4.4. Estimates of growth parameters for adult natterjacks at Grune

	Parameter	Estimate	Std.Error	t value	Pr(>|t|)	Resid.SE, df
Male	a	90.23	3.57	25.30	0.001	3.97,
(n = 93)	b	1.89	0.21	8.85	0.001	df = 91
Female	a	97.98	2.96	33.10	0.001	2.89,
(n = 39)	b	2.31	0.24	9.67	0.001	df = 37

Table 4.5. Estimates of growth parameters for adult natterjacks at Mawbray

	Parameter	Estimate	Std.Error	t value	Pr(>|t|)	Resid.SE, df
Male	a	93.91	6.34	14.82	0.001	2.95,
(n = 45)	b	1.93	0.31	6.21	0.001	df = 43
Female	a	88.98	3.25	27.42	0.001	3.19,
(n = 46)	b	1.78	0.24	7.48	0.001	df = 44

Table 4.6. Estimates of growth parameters for adult natterjacks at Bowness

	Parameter	Estimate	Std.Error	t value	Pr(>|t|)	Resid.SE, df
Male	a	51.77	2.99	17.33	0.001	3.37,
(n = 52)	b	0.17	0.21	0.80	0.427	df = 50
Female	a	440.46	371.59	1.19	0.263	2.53,
(n = 12)	b	36.37	34.89	1.04	0.322	df = 10

Discussion

Bd has been detected in many natterjack populations, suggesting this species, like many others, is highly susceptible to *Bd* infection. In other species, *Bd* infection has been linked to the disappearance of well-monitored populations but in the UK and Spain (Bosch, *pers.comm* 2010) natterjack toads at many sites have tolerated *Bd* and continued to breed. I saw no evidence of mass-mortalities of natterjack toads during my studies. At one site in the UK (Caerlaverock) the reserve manager (Hutchins, *pers.comm* 2010) has expressed concern about a decline in the natterjack population but *Bd* may not be responsible.

The CMR study revealed a negative correlation between *Bd* score and survival but *Bd* score also correlated with age, which had a much stronger link to survival. Other researchers have concluded an effect of *Bd* from similar data but without testing for correlations between *Bd* score and other life-history traits. Age is probably irrelevant in the case of short-lived frogs such as *Litoria pearsoniana* (Murray *et al*, 2009) but not when studying long-lived species. Pilliod *et al* (2010) found a negative correlation between *Bd* detection and survival in boreal toads (*Bufo boreas*) but did not model the effects of age or size. In that study, support for a negative effect of *Bd* was provided by higher (or less variable) survival in populations where *Bd* was not detected.

In natterjacks, reduced survival with increasing *Bd* score could be explained by correlations between *Bd* score and other age-dependent variables. Although younger toads may be less able to tolerate *Bd* infection and suffer a higher mortality rate, the pattern could also be explained by younger toads spending more time in water. I have no evidence to support the latter but experiments (see Chapters 2 and 3) show that *Bd* scores rise in response to immersion. Pond attendance (i.e. immersion) will incur several costs (Sullivan & Kwiatrowski, 2007) in addition to the risks of *Bd* infection or increased infection activity, including increased exposure to predation, loss of foraging opportunities and the energetic costs of breeding.

Schmidt (2010) commented on one of the first CMR studies of *Bd* and survival in the wild by Murray *et al* (2009). In this study, Murray *et al* (2009) claimed male Australian treefrogs *Litoria pearsoniana* which tested positive for *Bd* had a lower survival rate than those which tested negative. Schmidt (2010) argued that *Bd* could have affected within-season survival or behaviour, with *Bd*-positive frogs emigrating from the study area. As this species is short-lived and the study only covered one spawning season, emigrating frogs were not expected to be recaptured and may not have experienced increased mortality. If *Litoria pearsoniana* is, like

Bufo calamita, highly susceptible to *Bd* infection, *Bd*-positive individuals may simply represent the most actively-breeding part of the population. Frogs which have already spent time in water may be more likely to test *Bd*-positive, but they may also be more likely to die or emigrate for reasons which are completely unrelated to *Bd*. It is important to emphasize that estimates derived from CJS models only represent apparent survival because mortality is confounded with emigration. In my study, the positive correlation between age and survival might be due to an increase in spawning site fidelity with age.

If I ignore this possibility and the fact that *Bd* scores probably correlate with other costs associated with pond attendance, the effect of *Bd* score on adult natterjack survival was still weak. Survival probability (over a two-year period) was only 15% lower for high compared to zero-scoring toads (Fig. 4.4.). Very few wild natterjacks (13/227 = 6%) recorded *Bd* scores in excess of a mortality threshold (log *Bd* GE 5.6) derived from experiments (see Chapter 3.). Other studies of survival in relation to *Bd* swab test results have compared survival of 'negative' versus 'positive' individuals, using a standard threshold for *Bd* detection of 0.1 GE *Bd* DNA (see Chapter 2). My use of quantitative *Bd* score should have provided additional power, but I still did not detect a strong link between *Bd* score and survival. I also did not find any evidence for a negative link between *Bd* score (or detection) and growth.

Chapter 5. General discussion

Wider implications for *Bd* research

My findings have wider implications. For instance, variation in the sensitivity of the swab test for *Bd* according to conditions raises an important question. Can animals be assumed to be *Bd* negative on the basis of swab tests? Zero *Bd* infection prevalence in the Alpine salamander *Salamander atra* was reported by Loetters *et al* (2012), in populations which were only sampled once, with one swab per salamander. Others have claimed infection loss on the basis of single swabs following experimental treatments (e.g. Geiger *et al*, 2011). Although swab results may indicate variation in *Bd* activity or infection load, there is often insufficient evidence to support negative infection status. Problems will occur if populations or individuals are incorrectly assumed to be *Bd* negative. Experiments requiring negative controls will be flawed and attempts to control the spread of *Bd* via the amphibian trade or translocation projects may fail. The methods used to assess *Bd* infection status should be reviewed. Accuracy would probably be improved by taking a series of samples during conditions suitable for *Bd* activity. If this cannot be achieved, assumptions of negative status should be avoided.

The discovery that natterjacks can tolerate *Bd* infection and act as reservoir hosts also has wider implications. In mainland Europe, the natterjack is sympatric with other species which are significantly affected by *Bd*. The presence of tolerant hosts could increase the likelihood of extinction or decline of less tolerant species (Fisher *et al* 2012, Reeder *et al* 2012). This may explain the findings of Bosch (*pers.comm*, 2010) that natterjacks in Spain have increased in abundance in Peñalara National Park, while other amphibians (such as midwife toads *Alytes obstetricans*) have declined. The annual cycle of *Bd* detection recorded in my study suggests that *Bd*-tolerant natterjacks will repeatedly release *Bd* zoospores into ponds each spring, with potentially negative consequences for other species.

Evidence that salinity influences *Bd* dynamics in natterjacks complements work in Australia which shows similar effects in the wild (White, 2006) and captivity (Stockwell *et al*, 2012). If there is a slight effect of *Bd* on natterjack survival in the wild, tidal inputs may offer some protection. Other amphibian species which live in haline environments may benefit to a greater extent. Stockwell *et al* (2012) suggest addition of salt to ponds could reduce the risk of chytridiomycosis but such actions might have negative effects on other wildlife.

Suggestions for further research

Immersion in water appeared to trigger *Bd* zoospore release from the skin of infected natterjacks but this hypothesis needs to be confirmed by other methods, such as real-time microscopy. The natterjack could be a suitable study species, as it is susceptible to *Bd* infection, frequently naturally infected and can tolerate infection for extended periods. It would be useful to test other partly-terrestrial species, to see if a similar effect occurs and how the response is influenced by variables such as pH, salinity and temperature.

Unfortunately some of my work was limited by the availability of toads. I would like to repeat some experiments with larger sample sizes, using toadlets or adult natterjacks from populations in mainland Europe where they are more common. The results of the fungicidal treatment experiment (see Chapter 3) suggested salinity might have a positive effect on survival but the difference was statistically insignificant. Stockwell *et al* (2012) have reported a positive effect of salinity on survival in a larger sample of Peron's treefrogs (*Litoria peronii*). Similarly I would like to repeat the wet versus dry cycle experiment (see Chapter 2) with a larger sample, as although the results were significant it might be unwise to make firm conclusions from a sample of just six toads. Larger samples would probably also help to improve the definition of a mortality threshold for natterjacks in relation to *Bd* score.

I would also like to repeat the *in vitro* experiment (see Chapter 3) with other *Bd* isolates collected from coastal natterjacks and amphibians from freshwater environments. Stockwell *et al* (2012) performed a similar *in vitro* experiment with a *Bd* isolate collected from Lesueur's frog (*Litoria lesueuri*) but in contrast to my study, did not find that low levels of salinity boosted *Bd* growth. If the isolate I have tested belongs to a recently-introduced strain, this might show that *Bd* can adapt rapidly to local environmental conditions.

It would be interesting to investigate further the possible link between natterjack population genetics and *Bd* described by May *et al* (2011). It might be possible to identify genes which influence survival in relation to *Bd* infection in natterjacks in captivity. Such studies could be carried out in parallel with that of other closely-related species, such as the common toad *Bufo bufo*, in order to compare responses and help identify functional loci. Savage & Zamudio (2011) have already reported an association between host genetics (MHC class IIb genes) and survival of *Bd* infection in the lowland leopard frog *Lithobates yavapaiensis*.

_Bd_ in a broader context

The main aim of this project was to assess the effect of *Bd* on natterjack toads in the UK. However, it seems appropriate to mention other factors which might interact with *Bd* and have a negative effect on natterjack survival. *Bd* did not appear to be a major problem for the natterjack populations in my study but this situation might change, for instance if pollution or climate change can trigger outbreaks of chytridiomycosis.

Pollutants can interact with diseases, with unpredictable results. Other researchers have demonstrated an interaction between pesticides and the effects of other amphibian diseases. Kerby & Storfer (2009) found that exposure to chlorpyrifos increased the susceptibility of tiger salamanders (*Ambystoma tigrinum*) to *Ambystoma tigrinum* virus (ATV). I did not test for such effects but chlorpyrifos is used at some sites in the UK where natterjacks occur (on golf courses). Negative impacts of pollution on natterjacks in the UK have previously been recorded, for example in response to acidification (Beebee *et al*, 1990).

I found that adult natterjacks were killed by *Bd*, if they were exposed to wet conditions for a long period of time. This might be an unusual situation for adult natterjacks in the wild but if climate change predictions are correct, increases in summer rainfall and extreme weather events might trigger chytridiomycosis outbreaks. There is evidence of a positive correlation between climatic variability and outbreaks of chytridiomycosis (Rohr & Raffel, 2010). Warmer, wetter winters have a negative effect on the condition and survival of other UK species including *Bufo bufo* (Reading, 2007) and great crested newts *Triturus cristatus cristatus* (Griffiths *et al* 2010). Climate change presents a general threat to natterjack toads in the UK, as the majority of surviving populations are in habitat which will be lost if sea level rises.

According to Buckley & Beebee (2004), natterjacks disappeared from >70% of their range in the UK by 1970, largely as a result of habitat change. The consequences of habitat loss or fragmentation may not be obvious. For instance, the separation of populations can result in inbreeding. This may be the case in the UK, where a negative correlation between the reproductive fitness and genetic diversity of natterjacks has been reported (Rowe & Beebee, 2003). Others have shown that persistence of amphibian populations, including natterjacks, is dependent on habitat connectivity (Becker *et al*, 2007) and opportunities for dispersal (Stevens & Baguette, 2008). Translocation projects (see May *et al*, 2011) and attempts to reconnect isolated natterjack populations are a feature of natterjack conservation in the UK.

There is potential for a conflict between these efforts to boost connectivity and genetic diversity and measures designed to prevent the spread of disease. My data suggest translocations of natterjacks within the UK, even spawn or tadpoles, could result in the transfer of *Bd* to new sites. Although *Bd* might not be a major issue for natterjacks, it could have a negative impact on other species. It would be possible to treat spawn or tadpoles with a fungicide such as itraconazole before translocation to sites where *Bd* has not been detected. Hopefully my work will provide a focus for rational debate about disease control. Ideally, control measures should be effective but simple enough for conservation to continue.

In conclusion, natterjack toads in the UK (and mainland Europe) do not appear to be strongly affected by *Bd* in comparison with other species. This may not always be the case, as host-pathogen relationships are notoriously unpredictable. I would suggest a cautious approach with respect to *Bd* and 'pathogen pollution' in general. If natterjacks in the UK have been infected with a non-native strain of *Bd*, they will probably be vulnerable to the introduction of other diseases, which may have a greater impact in the future.

Appendix 1. Detection of *Bd* in adult natterjack toads during 2009 at Grune

Results from toads captured at least once during the spawning season (Apr-Jun inclusive).
Dark cells = positive result for *Bd* DNA using standard 0.1 GE threshold
Light cells = 'sub-threshold' positives (<0.1 GE but evidence of DNA amplification).

	APR		MAY					JUN					JUL				SEP
2009	**08**	**22**	**01**	**02**	**05**	**09**	**19**	**14**	**19**	**21**	**23**	**30**	**04**	**13**	**18**	**26**	**06**
Toad																	
1	0.0						1.0										
2	0.0						0.1										
3	0.0					6.5											
4	0.0													0.0			
5	0.0					0.5	1.2							0.0			
6	0.0																
7	0.0																
8	0.0									0.0					0.0		
9	0.0					2.1											
10	0.4						57.7										
11	0.0					0.0											
12	0.0																
13	0.0																
14	0.0	0.0	33.4														
15	0.0																
16	0.2																
17	0.0	0.0															
18	0.3	0.0	0.1				835.7										
19		0.1															
20		0.0															
21		0.0															
22		2.3															
23		0.0	0.1					0.0	0.7			0.0	0.0				
24		0.0	0.0														
25		0.0															
26		0.0															
27		0.0				57.8											
28		0.0															
29		0.0				0.0											
30			0.0			2.0	0.0		0.0			0.0	0.0				
31			0.0				22.7							0.0		0.0	
32			0.0														
33			0.0														
34			0.0														
35			1.1														
36				0.0													
37				0.0		2.8	43.7										
38					40.4	1.0	64.4										
39						0.0											
40						0.1											
41						0.0											
42						0.0											
43						0.2	1.2										0.0
44						0.0											
45							2.2										
46							0.0							0.0			
47							0.0										
48							0.5										
49									0.0								
50										1.4							
51										175.8		0.0	0.0				

Appendix 2. Details of programs used to complete statistical analysis

The majority of analysis and data plotting was completed using the statistical functions in program R. Software and package libraries are available from http://cran.r-project.org

I have provided page references to the statistical methods I have used from the guide to R by Crawley (2007), along with a page reference to the first time they appear in my thesis.

Thesis	Statistical method	Crawley 2007
27	Time series analysis using the autocorrelation function (ACF)	705
28	Trigonometric linear model to test for a waveform	711
29	Two-sample Chi^2 test for equality of proportions	301
31	Generalized linear mixed effect model to include random effects	630
40	Log-transformation of non-normal data	287
44	Welch's two-sample paired t-test to compare means	299
44	Fisher's F-test for homogeneity of variance	289
46	Kaplan-Meier survival curves	797
47	Generalized linear model with binomial errors	513
48	Rug plot of survivorship	596
49	Bartlett's test for homogeneity of variance	291
49	Tukey's honest significant difference (HSD)	483
60	Pearson's product-moment test for correlation	313
60	Linear model to calculate R^2	399
65	Modelling and plotting growth using the Michaelis-Menten equation	674

I also used Statistix 7.0 (©2000 Statistical Software) to perform statistical tests and program MARK to model survival and recapture probabilities. Program MARK is available from http://www.phidot.org/software/mark along with the 832pp guide 'Program Mark; a gentle introduction' (Cooch & White, 8th edition). Details of how to perform the median ĉ test in MARK are provided in Chapter 5.7 (p28) of this guide. I built the models in Table 4.2. using the design matrix in MARK, checking to ensure the number of parameters was correct for each model, as MARK does not automatically correct for changes in the number of parameters. I used the in-built graphical function in MARK to plot the estimates of survival and recapture in Figs. 4.2.-4.3.

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