

**Investigation on the spread of chytridiomycosis  
between UK natterjack toads and other inland  
amphibian populations within Cumbria**

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## ABSTRACT

Chytridiomycosis is an amphibian disease caused by a fungal pathogen, *Batrachochytrium dendrobatidis*, which is known as one of the causative agents of global amphibian declines. *B.d.* has recently been detected in the UK, including six natterjack toad (*Epidalea calamita*) populations in Cumbria in 2006/2007. This study was undertaken in 2008; firstly to survey ten non-natterjack inland sites within a 3km radius away from a *B.d.* positive *E. calamita* site. No *B.d.* positive amphibians were detected from the survey, which suggests little or no movement of pathogen from the infected sand dune habitat toward inland farmland at least within a year. As a second part of the study, five *E. calamita* sites outside Cumbria region were surveyed and two sites in Merseyside region were found *B.d.* positive. Although *E. calamita* may have the ability to spread the pathogen at a small scale through natural migration/dispersal, the detection of *B.d.* at Merseyside sites (10s of kilometers away from infected Cumbria sites) suggests anthropogenic involvement in the spread of *B.d.*. This mode of transmission is further supported due to the species having been a focus of high conservation activity and movement of the animals over a long time period.

Further investigations on these amphibians are required, especially on their susceptibility toward *B.d.* and how they behave when they are infected. Surveillance of *E. calamita* sites which have not yet been surveyed for *B.d.* is also required to obtain a complete picture of *B.d.* distribution amongst the UK *E. calamita* populations. Together with the results from the nationwide chytrid surveillance being conducted in 2008, *B.d.* control measures should then be determined to successfully eradicate or at least terminate the further spread of the pathogen within UK.

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## **ACRONYMS**

ACAP	Amphibian Conservation Action Plan
ANOVA	Analysis of variance
BAP	UK Biodiversity Action Plan
CBD	Convention on Biological Diversity
Ct	Threshold cycle
DEFRA	Department for Environment, Food and Rural Affairs
Fst	Fixation Index-Statistics
GAA	Global Amphibian Assessment
GE	Genomic equivalents
GLMs	Generalized Linear Models
HCT	Herpetological Conservation Trust
IoZ	Institute of Zoology, Zoological Society of London
IUCN	International Union for Conservation of Nature
MDS	Multi Dimensional Scaling
NARRS	National Amphibian and Reptile Recording Scheme
PCR	Polymerase Chain Reaction
Rt-PCR	Real Time PCR
UV-B	Ultraviolet-B
ZSL	Zoological Society of London

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**WORD COUNT**            12,663 words

## **1. INTRODUCTION**

**Chytridiomycosis, the amphibian infectious disease, was first found in the United Kingdom (Kent) in 2005 and newly detected in important natterjack toad sites in Cumbria in the last two years, 2006 - 2007. The extent and mode of the spread of the disease in the UK is not yet well understood. This project was thus carried out to survey other natterjack sites outside the Cumbria region to obtain a better picture of the spread of the disease among the UK natterjack sites. In addition, the study also examined the mode and degree of spread (from a positive natterjack site in South Cumbria) at a much smaller scale at inland non-natterjack sites.**

### **1.1 Introduction to the topic**

Amphibians play an important role in the ecosystem because they feed on insects, including many pest species of agricultural crops. They are also important food sources for many larger animals such as water birds, mammals, reptiles, and even spiders and large insects. They often have economical importance to humans as a food source (Mazzoni et al., 2003; Daszak et al., 2004), medical resource in some regions (i.e., Chinese medicine) (Zhou et al., 2006), and as an important potential source of future pharmaceutical drugs (Clarke, 1997).

Rapid amphibian declines have been reported globally in recent years and have raised concern among scientists (Stuart et al., 2004) The chytrid fungus *Batrachochytrium dendrobatidis* (*B.d*) causes the amphibian infectious disease chytridiomycosis. This disease has been identified as one of the main causes of amphibian declines world-wide (Daszak et al., 1999; Stuart et al., 2004) and has been hypothesised to have caused the extinction of some species, including golden toads (*Bufo periglenes*) in Costa Rica and the global extinction of the only two gastric-brooding frogs in the genus *Rheobatrachidae* in Australia (Pounds et al., 1997; Berger et al., 1998; Berger et al., 2000; Lips, 1998,1999; Daszak et al., 1999). This disease is now known to be infectious to at least 200 species of amphibians globally (Hyatt et al., 2007). Within Europe, *B.d* has been causing mass die-offs of midwife toads (*Alytes obstetricans*) in central Spain since 1997 (Bosch et al., 2001) and has also been detected in 7 other countries (Portugal, Italy, Switzerland, Germany, Denmark, France and the UK) in more recent years (Garner et al., 2005; spatialepidemiology.net, 2008).

The first detection of *B.d.* in the UK was among introduced North American Bullfrogs (*Lithobates catesbeiana*) captured in Kent during the summer of 2004 (Cunningham et al., 2005; Garner et al., 2005). Subsequently, during 2006/2007 the disease was then found amongst four native amphibian species at six natterjack toad (*Epidalea calamita*) sites in Cumbria. Cumbria is recognised as an important region for UK amphibians, especially *E. calamita*, the rarest of the seven amphibian species found in the UK. This species has experienced a major decline in the 20<sup>th</sup> century in the UK (Beebee 1983; Denton et al., 1997)

Now that chytridiomycosis has been found in several native amphibian species in various locations throughout the UK, it is crucial to define the extent of *B.d.* distribution nationwide, especially among the *E. calamita* sites. It is also important to understand the degree and mode of *B.d.* spread around the positive sites in order to implement regional and local control measures for the disease.

This project focus was therefore to look at *B.d.* infection within the UK *E. calamita* sites, and non-natterjack sites at varying distances from an infected population in Cumbria (Sandscale Haws).

## **1.2. Aims and objectives**

### Aims

- 1) To identify the current *B.d.* distribution among an infected *E. calamita* site and surrounding inland amphibian sites in Southern Cumbria.
- 2) To identify factors that might influence the spread of *B.d.* within UK *E. calamita* study sites.
- 3) To identify actions required to stop further spread of the disease within the UK amphibian communities.

### Objectives

- 1) To investigate whether *B.d.* has spread from an infected *E. calamita* site, Sandscale Hawes, to inland non-*E. calamita* sites.
- 2) To investigate the infectious status of *E. calamita* sites in two regions where *B.d.* surveillance has never been carried out.

- 2) To investigate whether the observed distribution of *B.d.* is more consistent with either a) spreading through natural amphibian movement; or b) spreading by anthropogenic factors (i.e., human induced movement of amphibians, movement of herpetologists).
- 3) To investigate other factors, including genetic diversity and geographical/hydrological factors, that may influence the *B.d.* distribution among the study sites.
- 4) To investigate the natterjack conservation activities carried out in the past to see whether these activities may have had a role in *B.d.* dissemination
- 5) To identify what must be done now and in the near future to prevent further spread of *B.d.* in the UK.

#### Hypotheses

- 1) *B.d.* is spreading through natural amphibian dispersal, and therefore *B.d.* distribution shows:
  - geographical structure (spatial correlation)
  - correlation with amphibian population boundaries suggested by genetic information.
- 2) *B.d.* is spreading through anthropogenic introduction, and therefore *B.d.* distribution shows:
  - correlation with frequency of visits by herpetologists and other conservation activities, i.e., monitoring and conservation-related construction activities, translocation, and the captive rearing and releasing of *E. calamita*.

## **2. BACKGROUND**

### **2.1. Global Amphibian Declines**

Global amphibian declines were first recognised at the First World Congress of Herpetology in 1989 (Stuart et al., 2004). In order to identify the scale and nature of amphibian declines globally, the Global Amphibian Assessment (GAA) was conducted on 5918 known species using the IUCN Red List criteria. The assessment suggested that more amphibian species (32%) are threatened globally than mammal (23%) and bird (12%) species, and that there is a high number of rapidly declining species among amphibians, which is not commonly found in other animal groups (GAA, 2008; Stuart et al., 2004).

The greatest threat to amphibians globally is the habitat loss caused by habitat alteration, degradation and fragmentation (e.g., ponds filled in or new roads constructed) (GAA, 2008). Pollution, such as by fertilisers (Hayes et al., 2002) and introduced species that compete with or prey on native amphibians (Lannoo et al. 1994; Fisher and Shaffer 1996; Finlay & Vredenburg, 2007) are also key causes of amphibian declines, as well as human-induced over exploitation for food, medicine, biological research use and the pet trade (Lannoo et al. 1994; Wang et al., 2004).

In addition to these threats, there have been unique cases of rapid disappearance of amphibians in some pristine habitats without any obvious human disturbances taking place (Wake 1991). Several factors are, however, suggested as the causes of these mysterious declines: emerging infectious diseases (e.g. chytridiomycosis and rana virus) (Daszak et al., 1999; Burrowes et al., 2004), increased UV –B radiation (Kiesecker & Blaustein, 1995; Blaustein et al. 2003), and climate change (Burrowes et al., 2004; Pounds et al. 1999; Kiesecker et al. 2001; Carey and Alexander 2003). Several studies have shown evidence of disease involvement in amphibian mass mortalities and rapid declines in different parts of the world (Daszak et al., 1999; Bosch et al., 2001; Berger et al., 1998; Young et al., 2001). Although at present disease seems to play a relatively small role in global amphibian declines in comparison to habitat loss and degradation (GAA, 2008), disease-related declines are often intensive and cause rapid local extinction, sometimes driving species to extinction (Daszak et al., 1999; Daszak et al., 2003; Young et al., 2001). All of the various amphibian threat factors listed above are likely to be interacting with each other in a complex way (Burrowes et al., 2004 ; Blaustein and Kiesecker, 2002), which makes it difficult for us to understand and come

up with effective measures to mitigate the declines. For example, pathogen outbreaks were shown to be linked to increased UV-B radiation (Kiesecker et al. 2001), and some introduced species are shown to act as a vector in spreading emerging infectious diseases globally (i.e., the African clawed frog, *Xenopus laevis* and North American Bull frog, *L. catesbeiana*) (Parker et al., 2002; Mazzoni et al., 2003; Daszak et al., 2004; Garner et al., 2006; Fisher & Garner, 2007).

## **2.2 Chytridiomycosis**

There are a number of amphibian infectious diseases which are shown to be associated with amphibian declines, but the clearest link so far exists for an amphibian fungal disease called chytridiomycosis (Daszak et al., 2003). Chytridiomycosis is caused by a fungal pathogen, *Batrachochytrium dendrobatidis* (*B.d.*), first discovered by Berger and colleagues in 1998. The pathogen was collected from a number of amphibian mass mortalities in Australia and Panama during the 1993 to 1998 period (Berger et al., 1998). Due to the scale of amphibian population declines caused by chytridiomycosis, this disease has been described as the worst known vertebrate infectious disease, infecting vast numbers of species and driving its host to extinction (ACAP 2005; Fisher & Garner, 2007).

### **2.2.1. *B.d.* lifecycle**

*B.d.* only infects keratinised tissue of amphibians, mouth part of tadpoles and skin of post-metamorphic individuals (Berger et al., 1998; Longcore et al., 1999; Green & Converse, 2005). The localized distribution of *B.d.* in tadpoles does not cause mortality, and fatal epidermal infection occurs once their skin become keratinized after metamorphosis (Berger et al., 1998). This pathogen has two life stages: [Stage 1] Sessile, reproductive stage as a zoosporangium where zoospores are asexually reproduced inside the zoosporangium and [Stage 2] Motile, infectious stage as a zoospore with a sperm-like tail which enables it to disperse in water (Johnson & Speare, 2003). Transmission of the disease occurs by a zoospore in water or through direct contact between amphibians (Johnson & Speare, 2003; Green & Converse, 2005). This pathogen is capable of surviving in water and moist sand for a long period of time as a form of dormant zoosporangia without keratinised tissue (Johnson & Speare, 2003; Johnson & Speare, 2005). Laboratory experiments showed that *B.d.* is capable of developing at temperatures of 4-25°C and pH 4-8 (Piotrowski, 2004) and grows optimally at 23°C (Longcore et al., 1999). Its growth stops at 28-29°C and it starts dying when the temperature reaches 30°C (Longcore et al., 1999). When susceptible species are infected,

mortality occurs within 18 - 48 days after infection (Berger et al., 2004; Woodhams et al., 2007a).

### **2.2.2. Origin of *B.d.***

There are two different theories on the origin of this infectious disease (Rachowicz et al., 2005).

- The first theory is that *B.d.* originates from Africa and has recently spread globally through the international trade of amphibians (the global trading of the African clawed frog, *Xenopus laevis*, from southern Africa began in the mid-1930s for use in pregnancy tests and subsequently for laboratory experiments; trading of North American bull frogs, *L. catesbeiana*, also began in the 1930s for food and continued at least until late 1990s) (Weldon et al., 2004; Hanselmann et al., 2004; Rachowicz et al., 2005; Garner et al., 2006; Fisher & Garner 2007).
- The second theory is that *B.d.* already existed globally but that amphibians have become more vulnerable to the disease due to environmental changes such as climate change (Pounds et al., 1999; Kiesecker et al. 2001).

The fact that *B.d.* has been detected in amphibians internationally traded (Fisher&Garner, 2007) for zoo collections (Pessier et al., 1999), as pets (Weldon et al., 2004), for ornamental garden pond stocking (Daszak et al., 1999), for food (Mazzoni et al., 2003) and for laboratory research (Parker et al., 2002) support the first theory (Boyle et al., 2004). DNA sequencing data of *B.d.* collected from infected animals world wide showed a low level of genetic variation, and the genetic relationship among different *B.d.* strains showed no overall geographical structuring, which also supports the theory of the recent human-induced global spread (Morehouse et al., 2003). However, there are other studies showing a significant association between amphibian mass mortality events and environmental changes, such as increased temperature and UV-B radiation (Bosch et al., 2007, Pounds et al., 2006). Therefore, it is likely that both the international trade of amphibians and environmental changes are enhancing the spread and severity of the disease observed in recent years (Fisher & Garner, 2007).

### **2.2.3. Modes of *B.d.* dissemination**

On the global scale, the international trade of amphibians is likely to be the biggest factor causing the spread of the *B.d.* infection (Fisher & Garner, 2007). Some amphibian species,

such as North American bull frog, one of the most commonly internationally traded amphibians, are shown to be less susceptible to the disease than others (Garner et al., 2006). Individuals of such species can carry *B.d.* for a long time without being severely infected. Therefore, these amphibians can act as a vector and disseminate the disease to more vulnerable species. There are many other variable factors that could be associated with *B.d.* dissemination on the local scale. Due to the relatively long lag period from the time of infection until the death of individuals (24 to 220 days) (Berger et al., 2004; Lips et al., 2005), *B.d.* transmission from infected individuals to un-infected individuals of the same population or a nearby population through natural amphibian movement is likely to happen. *B.d.* was shown to stay infectious for up to 7 weeks in lake water (Johnson & Speare, 2003) and 3 months in sterile moist river sand. In addition, it survived 1 to 3 hours of drying in bird feathers (Johnson & Speare, 2005) in laboratory experiments. Therefore, environmental factors and other non-amphibian vectors are likely to play a significant role in *B.d.* dissemination as well as amphibian individuals.

#### **2.2.4. Treatment and control of the disease**

Variable treatments have been shown to be effective in treating *B.d.* infected individuals in captivity. Using various chemicals such as formalin and malachite green as anti-fungal baths have been shown to be effective with African clawed frogs, *Xenopus tropicalis* (Parker et al., 2002) and itraconazole with blue-and-yellow poison dart frogs, *Dendrobates tinctorius* (Nichols & Lamirande, 2000), yet malachite green has been reported to cause deformities (Young et al., 2007). Other chemicals have also been tried, however, they have not cleared the infection successfully or have had a negative impact on the survival of individuals (Nichols & Lamirande, 2001). Exposing infected animals to high temperatures (37°C for 16 hrs) has also been shown to be effective in clearing infection (Woodhams, 2003). This treatment however may not be applicable to those species that are not tolerant to high temperature conditions.

The problem with such treatments is that they are only applicable in captive conditions and it is almost impossible to apply them to treat amphibians in the wild for several reasons. First, it would be very expensive. Second, treating individual amphibians may be possible, but it is almost impossible to treat the habitat these individuals live in, such as ponds, without damaging other organisms living there, including important food sources for amphibians and the habitat. Thirdly, the requirement to capture every infected individual is almost an impossible goal to attain.

Amphibians have naturally well adapted immune systems to tolerate pathogens and other types of diseases, and also to treat injuries in wet conditions (Carey et al., 1999). This is why the amphibian immune system has been studied for potentially new pharmaceutical drugs (Clarke, 1997). Being the primary defensive barrier of infection from the external environment, the skin of amphibians produces host-defensive peptides, including antimicrobial peptides (Carey et al., 1999; Woodhams et al., 2007a), some of which are shown to act against *B.d.* (Woodhams et al., 2006a; Woodhams et al., 2007b). Interspecific variation in the immune resistance against *B.d.* was observed (Blaustein et al., 2005; Woodhams et al., 2007b). Through further investigation in this field, hopefully in the future, the knowledge obtained about the innate immunity variation can be used in conservation decision making, for example to identify the more vulnerable species or lineages that may require urgent conservation actions such as captive breeding program (Garner, 2007).

Stopping the movement of infected animals and contaminated materials from infected sites to uninfected sites is likely to be the most effective way to control the disease in the wild, and that is the common strategy taken to control other known major epidemics, such as foot-and-mouth disease or bird flu. Controlling disease between countries may be achievable as the point of entry is more or less limited and clear. Strict quarantine systems or banning the international trade of amphibians would be required to control *B.d.* among countries. DEFRA is undergoing a public consultation regarding a proposed ban on the sale of specified non-native amphibian species, including the African clawed frog, *Xenopus laevis*, and the American bullfrog (*L. catesbeiana*), which are the major carriers of *B.d.* (DEFRA, 2007). On the other hand, controlling the spread of *B.d.* within a country where it has already spread to various locations would be more complicated, as there are a number of potential factors likely to influence the spread of the disease. The fact that amphibians have a complex life cycle using both aquatic and terrestrial habitats, and that the disease can survive in the environment without its host animals for a long period of time (Mitchell et al., 2007), makes eradication programs for this disease difficult. One option to eradicate *B.d.* at sites that are already infected may be to remove infected animals, either euthanize or treat them, fill in the pond and create a new habitat elsewhere. However, destroying the ponds would have unexpected consequences for other organisms and creating new suitable ponds with good vegetation, invertebrates or algae to feed on may be labour intensive, costly and time consuming. As *B.d.* is not harmful to human health or economically important, only limited support might be

available from the government to carry out such nation-wide disease eradication projects, this in comparison to other notifiable diseases such as foot-and-mouth or bird flu.

## 2.3 Situation in the UK

### 2.3.1 Amphibians in the UK

There are 7 native amphibian species found in the UK, which are listed in Table 2.1. All these species are globally listed as Least Concern on the IUCN Red List, but within the country some have declined dramatically (Buckley & Beebee, 2004), mainly due to habitat loss and alteration. The UK Biodiversity Action Plan (BAP) has been established by the UK government in response to the Convention on Biological Diversity (CBD), signed in 1992. Out of 7 native amphibian species, 4 species are listed as priority species.

**Table 2.1.** List of the native UK amphibian species and their current conservation status. Ecological information and illustration for these species, except for *E. calamita*, are shown in Appendix 2.1. Detailed information on *E. calamita* is described in the next chapter (2.3.2)

Scientific name	Common name	Legal protection	UK BAP status*	Action categories
<i>Epidalea calamita</i>	Natterjack toad	Yes	Priority species	- Site specific action - Priority habitat based action (expansion) - Priority habitat based action (condition)
<i>Bufo bufo</i>	Common Toad		Priority species	- Research into conservation action - Survey known sites - Landscape/regional strategic planning - Landscape/regional level projects
<i>Rana temporaria</i>	Common Frog			
<i>Triturus cristatus</i>	Great Crested Newt	Yes	Priority species	- Measures to control/regulate agro-chemicals - Priority habitat based action (condition) - Landscape/regional strategic planning
<i>Lissotriton vulgaris</i>	Smooth Newt			
<i>Lissotriton helveticus</i>	Palmate Newt			
<i>Pelophylax lessonae</i>	Pool frog		Priority species	- Site specific action - Management actions to benefit single species - Priority habitat based action (condition)

\*The UK Biodiversity Action Plan status

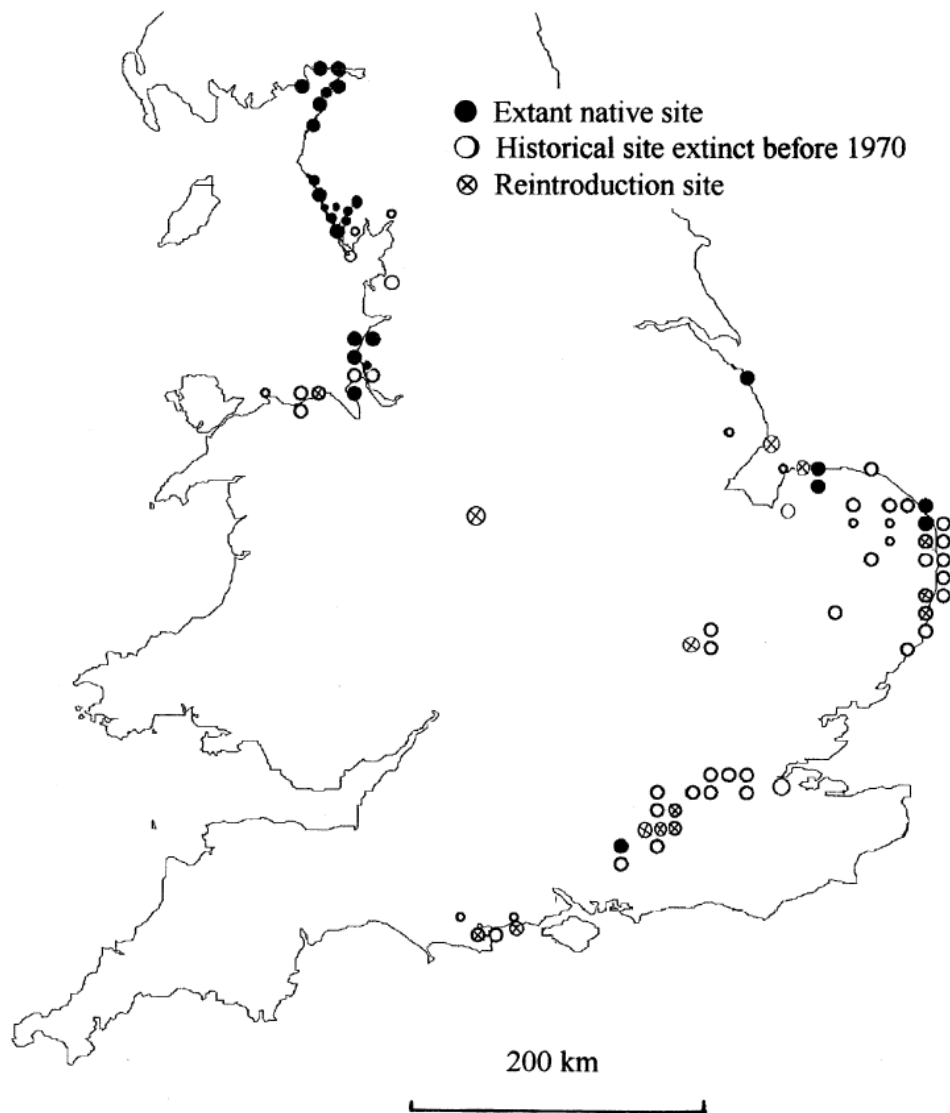
UK BAP (2007)

### 2.3.2 Natterjack toad



**Fig. 2.1.** Picture of an adult *E. calamita* © Surrey Amphibian and Reptile Group

*Epidalia calamita*, commonly known as the natterjack toad, is distributed throughout western and central Europe. The UK is the northwestern edge of its distribution, isolated from the mainland European populations since around 8000 BP when the sea level rose and the land connected to the main land was submerged (Hitchings & Beebee, 1996). This species has declined dramatically within the UK and has disappeared from 70-80% of its former distribution range in the period between the late 1800s and 1970, mainly due to habitat loss and alteration, i.e., loss of open heathland and dunes through successional changes and anthropogenic acidification of breeding sites (Denton et al., 1997). The current known distribution of *E. calamita* is restricted to several sites in south and east England, northwest England and southwest Scotland, as shown in Fig. 2.2. Many current existing populations are small, with only tens or several hundreds of adult individuals, and there is a concern regarding the fragmentation of these populations (Hitchings & Beebee, 1996). *E. calamita* has been legally protected since 1975, and population trends have been monitored nationally since the 1970s (Buckley & Beebee, 2004). Other conservation actions such as translocation and reintroduction have also been carried out as a part of a species recovery programme (Fig. 2.2.) (Denton et al., 1997).



**Fig. 2.2.** Map of *E. calamita* distribution in the UK. (Denton et al., 1997)

In the UK, *E. calamita* is usually found in coastal dune systems, lowland heaths and upper saltmarshes (Beebee & Denton., 1996). In spring, breeding occurs in shallow, sunny, ephemeral pools where small larvae grow rapidly and metamorphose within 6 to 8 weeks (Beebee, 1983; Banks & Beebee, 1988).

### 2.3.3 Non-native amphibians found in the UK

Like many other countries, several introduced amphibians are found in the UK and are imposing various threats to the native amphibian species. Information on these amphibians is summarized in Table 2.2.

**Table 2.2.** List of non-native amphibian species found in the UK, their breeding status and threats they impose

Scientific name	Common name	Breeding	Threat
<i>Hyla arborea</i>	European tree frog	Yes	
<i>Lithobates catesbeiana</i>	American Bullfrog		- Vector of <i>B.d.</i> - Predation and competition.
<i>Pelophylax esculenta</i>	Edible Frog	Yes	- Vector of <i>B.d.</i> & ranavirus.
<i>Pelophylax perezi</i>	Iberian water frog	Yes	
<i>Pelophylax ridibundus</i>	Marsh Frog	Yes	- Vector of <i>B.d.</i> & ranavirus. - Predation and competition.
<i>Alytes obstetricans</i>	Midwife toad	Yes	- Vector of <i>B.d.</i>
<i>Bombina bombina</i>	Fire-bellied toad		
<i>Bombina variegata</i>	Yellow-bellied toad	Yes	
<i>Xenopus laevis</i>	African Clawed Toad	Yes	- Vector of <i>B.d.</i> - Predation and competition.
<i>Mesotriton alpestris</i>	Alpine newt	Yes	- Vector of <i>B.d.</i>
<i>Triturus carnifex</i>	Italian Crested Newt	Yes	- Hybridization with great crested newt. - Competition.

(Fisher & Garner, 2007; DEFRA, 2007; NARRS, 2008)

### 2.3.3. *B.d.* in the UK

*B.d.* was first recorded in 2004 among a population of introduced North American bullfrogs, *L. catesbeiana*, which had established a colony in Cowden, Kent, southeast England (Cunningham et al., 2005; Feltrer & Cunningham, 2006). Further, in 2007, a surveillance study was carried out on imported exotic amphibians at Heathrow airport and *B.d.* was detected in 4% of tested amphibians, indicating that more infected amphibians are still coming into the country (Peel, 2007). From this evidence, it seems reasonable to assume that *B.d.* introduction into the UK might have been through the international amphibian trade (i.e., North American Bull frogs) (Fisher & Garner, 2007).

Another worrying trend was found during the winter of 2003/2004, when a volunteer involved with the Herpetological Conservation Trust (HCT)'s captive rearing programme at a site in Cumbria (Mawbray) recognised and reported unexplained mortality in wild-caught and captive reared *E. calamita* toadlets, after already releasing many of them back into the wild. These amphibians kept in captivity were diagnosed and found positive for *B.d.* infection. (Feltrer & Cunningham, 2006). In response to this event, the Mawbray site and several other *E. calamita* sites in the Cumbria region were surveyed for *B.d.* in 2006 and 2007, which led

to the discovery of 6 infected sites, including Mawbray (Cunningham, pers. comm.; HCT, 2008).

*B.d.* was also detected in Canterbury in Kent (ZSL, 2008), however, *B.d.* distribution in other regions is not yet known, as other regions have not been surveyed. The UK Chytrid Survey 2008 was set up by ZSL and is being carried out this year to identify the extent of *B.d.* spread in the country.

#### **2.4 Chytrid surveillance methodology**

Until recently, most studies on *B.d.* have used histological testing of stained skin tissues (Kinger et al., 2006a). The limitations of this technique are that it requires expert knowledge and experience to correctly identify the fungus, and is time consuming (Boyle et al., 2004). Use of skin tissues of live animals, such as toes, is also problematic, not only because it is an invasive method, but also because the level of infection varies among different toes on the same individual (Boyle et al., 2004).

In recent years, the method of real-time PCR using a Taqman assay has been developed and is increasingly being applied for chytrid diagnostic tests using swab samples (Boyle et al., 2004). This technique has been proven to be more accurate and reliable than histological techniques (Boyle et al., 2004; Kinger et al., 2006a). In addition, it is a rapid, non-invasive, highly sensitive and specific method which enables detection of *B.d.* quantitatively, at levels as low as a single zoospore (Boyle et al., 2004). With the conventional PCR method, it is not possible to detect *B.d.* quantitatively and it has a lower detection limit of approximately ten zoospores and processing takes longer (Boyle et al., 2004). The main constraint of the PCR technique, both conventional and real-time PCR, is the high cost associated with the laboratory analysis, but the costs are becoming lower (Kinger et al., 2006b).

A study carried out by Kinger and Hero (2006) showed large-scale seasonal variation in the *B.d.* prevalence level within a single amphibian population in Queensland, Australia. Within the population, the highest prevalence level was observed during the cooler season (<19.4°C). The seasonal variation pattern in the *B.d.* prevalence level may differ in other localities and with different amphibian species, but the study demonstrated that the seasonal variation must be taken into account when designing *B.d.* surveillance methodology and data analysis.

### **3. METHODS**

#### **3.1 Surveillance methodology selection**

Swab sampling for real-time PCR using the Taqman assay (Boyle et al., 2004) was set as a protocol for the UK nationwide surveillance 2008. Use of the swabbing technique made it easier to involve volunteers as it is less invasive, more practical, and easier for non-expert volunteers to perform after a short training period. In addition, it does not require a Home Office licence. At each site, the aim was to collect 30 adult individuals of any native amphibian species. Thirty individuals was the standard procedure set for the UK nationwide surveillance 2008, as it gives 99% confidence to detect 15% prevalence infection (DiGiacomo & Koepsell, 1986). The surveillance was designed to be carried out in two sessions. The first surveillance took place in the spring, as it is the season when most amphibians gather in ponds to breed, which enables the capture of enough individuals to swab. The second surveillance was carried out in the summer when an increase in *B.d.* prevalence level is expected; as optimal growth temperature of *B.d.* is at 23°C (Longcore et al., 1999) and the temperature is most likely to happen in the summer in the UK.

The purpose of the nationwide surveillance was to find out the extent of *B.d.* infected sites nationwide, where a site could include more than one pond if they were within approximately 100m of each other (Brede et al., 2008).

#### **3.2 Fieldwork/sampling**

##### **3.2.1 Study sites**

###### Experiment 1.

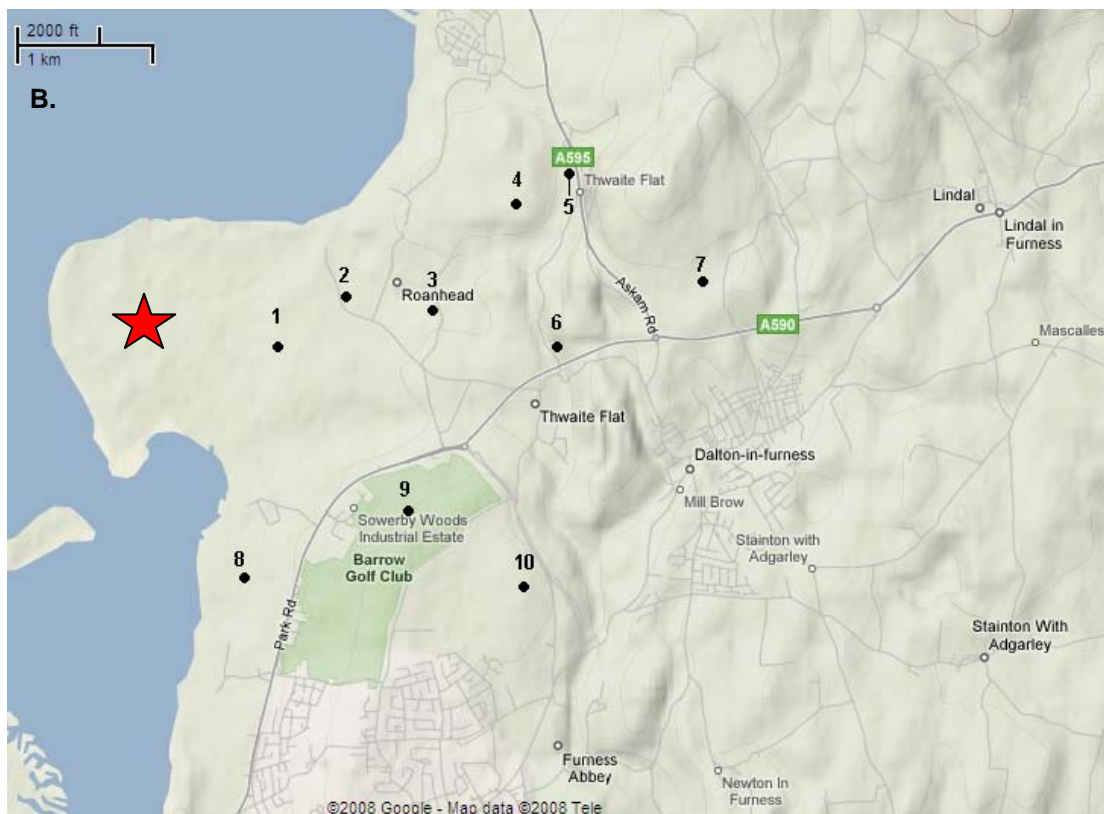
Surveillances of non-natterjack inland amphibian sites in South Cumbria adjacent to Sandscale Hawes (Fig.1), the *B.d.* positive *E. calamita* site identified in 2007, were carried out in the spring period (31<sup>st</sup> of March – 11<sup>th</sup> of April) and in the summer period (29<sup>th</sup> of May- 4<sup>th</sup> of June). Due to budget and time constraints, the aim was to survey 10-20 ponds within a 3km radius away from Sandscale. Starting the first survey within a 3km radius from Sandscale was thought to be appropriate, as the area includes variable geographical barriers and structures such as minor roads, major roads, railways, stream, public footpath, farmland boundaries, etc., and because the maximum migratory distance of amphibians was expected to be about 2-3km per generation. We planned to carry out the second survey on the same sites, but if all distant sites turned out positive on the first survey, surveys of ponds further away from these would

be chosen for the second survey.

A.



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**Fig. 3.1.** Maps showing the location of the study sites in two different scales.

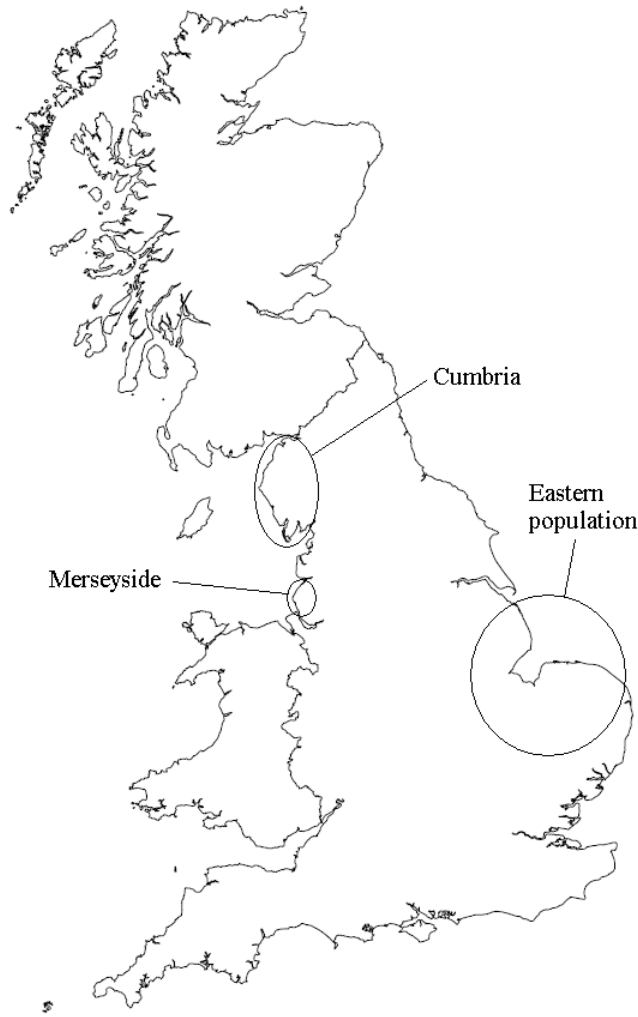
**A.** A map of Britain, showing the location of south Cumbria - ●.

**B.** Locations of inland non-natterjack sites sampled for *B.d.* in south Cumbria. Exact locality is shown on a map in Appendix 3.1. and the numbers refer to site names as detailed in Appendix 3.2. ★ - Sandscale Haws (*B.d.* positive *E. calamita* site)

During the first surveillance visit, potential ponds were selected on a map (Ordnance Survey Explorer Map OL6, 2005, shown in Appendix 3.1.) and after obtaining permission to survey the ponds from the owners, these ponds were visited. Pond suitability for this survey (i.e., finding 30 amphibian individuals) was identified by looking for suitable emergent vegetation, sign of spawning (e.g. spawn strings, calling, and dead/live individuals) and actual attempts to dip-net in the pond. Nine sites had enough amphibians, and swabbing was carried out. In addition to these 9 sites, another site (no. 3) where only 12 individuals were captured was still surveyed, as it was at a good intermediate location among the other sites. The locations of these sites are shown in Fig.3.1. B. It was not possible to carry out the second summer survey at sites 1, 3, 6 and 10. Sites 1 and 3 were dried up. During the spring survey, sites 6 and 10 had only *Bufo bufo* aggregating to spawn, and had no newt species to be surveyed in the summer period.

### Experiment 2

Among the UK *E.calamita* populations, the *B.d.* survey had been carried out only in the Cumbria region in the past two years. This year, two sites in Merseyside (Birkdale and Ainsdale) and 3 sites among the eastern population (Saltfleetby, Sandy and Syderstone) (Fig. 3.2.) were newly surveyed by trained UK volunteers. Surveyor instructions (Brede et al., 2008) and sampling kits were provided to the volunteers by the Institute of Zoology (IoZ). The *E. calamita* site at North Walney, south Cumbria, where *B.d.* surveillance was carried out and found negative in 2007 was additionally surveyed by the author during the two periods of fieldwork for experiment 1 as it was close by.



**Fig. 3.2.** Map of Britain showing regions of *E. calamita* sites sampled for *B.d.* surveillance.

### **3.2.2 Field methods**

To prevent spreading *B.d.* infection among the study sites, all the equipment (dip-net, wellingtons, waders, buckets, and bottle traps) were thoroughly disinfected using Virkon (Antec International Limited, UK) (1% concentration) prior to visiting different sites. Either new or disinfected gloves were worn when different sites were visited.

Dip-netting and/or bottle traps were used to capture the animals. Bottle traps were made by using a 2L plastic bottle cut in half (with the top forming an inverted funnel) and held at an approximately 30 degree angle by piercing with a garden cane. These were set up partially submerged (with an air gap) among or around the pond vegetation the night before and left overnight to trap newts. Collected individuals were placed in a plastic bucket with 10mm of their pond water prior to swabbing until 30 animals were collected. Sterile cotton swabs with

supplied plastic containers were used for swabbing (Medical Wire & Equipment, UK). The hind legs and abdomen of each individual were swabbed, and then details of the animal was recorded (i.e. species, sex and any other additional information, such as signs of deformity). The animals were then immediately released back into the original pond.

All the swabs collected were kept in a refrigerator (4°C) from the day of sample collection (Experiment 1) or as soon as they arrived at the IoZ (volunteers collectors).

### **3.3 Laboratory methods**

To avoid cross contamination of the samples, which may cause false positive results, various degrees of precautionary approaches were taken. Clean gloves and a lab coat were constantly worn, and sterile (either newly opened or autoclaved) Eppendorf tubes and beads were used. Pipette tips were changed each time different samples and chemicals were used. Cutting pliers/blades were disinfected using Virkon (1% concentration) and ethanol (70% concentration), and a new sterile Petri dish was used each time different swab samples were handled. The surface of the fume cabinet was carefully disinfected with ethanol each time different populations were handled.

#### **3.3.1 DNA extraction**

For each sample, a 1.5ml centrifuge tube was prepared with 0.03 to 0.04g of 0.5mm Zirconium/silica Microbeads (BioSpec Products, USA) and 60ul PrepMan Ultra (Applied Biosystems, USA). The tip of the swab was cut using the pliers and placed into the prepared centrifuge tube. The contents of these tubes were then homogenized using a Mini-Beadbeater 8 (BioSpec Products, USA) for 45 seconds, and then centrifuged for 30 seconds at 14500 rpm (Personal Microcentrifuge MiniSpin Plus, Eppendorf, Germany). After repeating the process of homogenization and centrifugation, the samples were placed in a 100°C water bath for 10 minutes and then removed to cool. The samples were then centrifuged for another 3 minutes at 14500 rpm prior to the supernatant being pipetted and stored in labelled 1.5ml sterilised Eppendorf tubes at -17°C.

#### **3.3.2 Quantitative real-time Taqman PCR**

To diagnose the absence/presence of *B.d.* infection and quantify the level of infection, real-time polymerase chain reaction (Rt-PCR) using a Taqman assay (Boyle et al., 2004) was used.

PCR reaction master mix containing ITS-1/5.8S DNA primers (eurofins, Germany) Chytr MGB2 probe (MGB, Germany), H<sub>2</sub>O were prepared according to Boyle et al. (2004). Two DNA samples were pooled together to reduce the laboratory cost. To make up a 1/10 dilution of the two pooled samples, 4 µl of previously collected supernatant from each sample was pipette into a 1.5ml sterilised Eppendorf tube with 72ul of double processed tissue culture water. The reaction master mix (20 µl) was put in each well of a 96 well microtitre plate (Applied Biosystems, USA) together with the DNA dillution (5 µl). A negative control and two standards (containing 1 and 10 genome equivalents of *B.d.* zoospore DNA) were placed in each plate to obtain genomic equivalents (GE) for potential positives. All samples were duplicated in order to increase the accuracy of the test by averaging the results and obtaining standard deviations. A clear PCR seal was fitted on the plate and centrifuged in Centrifuge 5804R (Eppendorf, Germany) at 4000rpm at 12<sup>o</sup>C for 3 minutes.

The plate was then set up in a 7300 Real-Time PCR System (Applied Biosystems, USA), where the amplification cycle was set as: Stage 1: 50<sup>o</sup>C for 2 minutes, Stage 2: 95<sup>o</sup>C for 10 minutes, Stage 3: 95<sup>o</sup>C at 15 seconds and then 60<sup>o</sup>C for one minute (repeated 60 times). The Rt-PCR system software (Version 1.4) calculated genomic equivalents (GE) of *B.d.* zoospores for positive samples from the Ct number, defined as the threshold cycle number at which the fluorescent signal produced by the labelled probe crosses a threshold, in comparison to a standard curve produced per plate. As the sample extractions were diluted 1/10 before PCR, 0.1 GE was considered as the minimum acceptable value for positive infection (Garner et al., 2006). Positive samples were re-analysed individually to double check if the result was truly positive and to see which one of the pooled individuals was positive.

### **3.4 Data analysis**

#### **3.4.1. Data extraction and arrangement**

The final PCR results were gathered in a Microsoft Excel spreadsheet together with the *B.d.* surveillance results from the Cumbria sites produced by Andrew Cunningham, Yedra Feltrer and Judit Hidalgo-Vila in 2006 and 2007. Monitoring data on the UK *E. calamita* sites were extracted from the Natterjack Site Register (Beebee & Buckley, 2001), provided by John Buckley, Amphibian Conservation Officer at the HCT. Genetic information on the UK *E. calamita* was obtained through the published literature (Rowe et al., 1998; Rowe & Beebee, 2007).

### 3.4.2. Statistical analysis

All of the following statistical analyses were carried out using program R (The R Foundation for Statistical Computing, R version 2.5.1., <http://www.r-project.org>).

#### Experiment 1.

Due to negative results in all of the sites surveyed in Experiment 1, no statistical analysis was possible on that data set.

#### Experiment 2

For experiment 2 the data set was analysed in four ways:-

1). Monitoring data in the Natterjack Site Register (Beebee & Buckley, 2001) was analyzed to see whether carrying out two different count methods (Adult&Juvenile count and Spawn count) was necessary. 2). The observed *B.d.* infectious levels among amphibians at 17 *E.calamita* sites were analyzed firstly to see whether infectious level varies among species and secondly to investigate whether higher infectious level is observed where high conservation activity has been going on. 3). Spatial autocorrelation analysis was carried out to see whether there is a spatial structuring (i.e. clumping or dispersed) in the geographical location of disease positive sites and negative sites. 4). Finally, genetic distances, suggested from published Fst values, were used to investigate whether genetically well connected sites have the same disease status (positive or negative).

1. Population count data for the 17 *E. calamita* sites, where B.d. survey has been carried out, was available in the Natterjack Site Register (Beebee & Buckley, 2001). Population count data using two different methods (adult & juvenile count and spawn count) over 30 to 40 years was extracted from the register and used for the analysis. Firstly, the data was plotted on graphs. Then, in order to investigate whether different count types produce different population trend, analysis of deviance with generalized linear models (GLMs) was carried out to test count number (n) against two explanatory variables, years (yr) and the different count types (CT). Model simplification was carried out using analysis of variance (ANOVA).
2. Data from site 1, 3 and 4 in 2006 contained large number tadpole and metamorph data, as well as adult data. Out of 180 tadpole and metamorph individuals, only one tadpole from site 4 was positive. As it is shown that *B.d.* infects different life stages of

amphibians differently, it was judged that using these large negative tadpole/metamorph data together with the adult data and comparing infectious level among sites was not appropriate, as surveys in 2007 and 2008 were carried out only on adult amphibians. If the positive tadpole was found in a site where no other positive results were found, removing the positive data would have been a problematic. However, as the positive tadpole was found in the site where 9 adults were also found positive, removing it from the data was not seen as a problem. Therefore tadpole and metamorph data from these 3 sites was removed.

Then, GLMs analysis for proportional data (Crawley, 2007) was firstly used to test the observed *B.d.* infection rates (infected/uninfected) against “species” as an explanatory variable, to see whether there is a variability in infection rate among species. Secondly, GLMs analysis for proportional data was used to test whether different levels of conservation activities carried out in the past have influenced the infection rates observed at different sites. Conservation activities at each site have been recorded in the Natterjack Site Register (Beebee & Buckley, 2001). The activities were classed in four different categories, “monitoring”, “conservation related construction work” (such as creating new ponds and removing competitive species’ spawns), “ex-situ captive rearing and releasing” (of either tadpoles or toadlets), and “translocation” (of any *E.calamita* individuals, or spawns from different populations). From the register, annual records from each site listed and the total number of years when each activity was carried out was calculated. Then the infection rates (infected/uninfected) were tested against the 4 explanatory variables, “monitoring”, “construction”, “rearing”, and “translocation”. For this analysis, an assumption was made that heavily visited sites for conservation activities have higher chance of *B.d.* introduction, possibly by herpetologists.

3. Spatial autocorrelation analysis on the 17 *B. calamita* sites were carried out using autologistic regression, which is an autocovariate regression created for binary data (Dormann et al., 2007). The function is called the distance-weighted autocovariate regression in R (autocov\_dist), and was used for the analysis. The geographical co-ordinates of each location was obtained using an online geocoding service, Simple Geocoder (Map Channels, 2008). Using the co-ordinates, program R measured the straight line distances among sites and spatial autocorrelation of the two disease states

(positive or negative) was tested with the variable neighbourhood radius, to see whether spatial structure (such as clumping of positive sites) can be observed at various scale; small scale looking at locations of positive and negative sites at local scale (e.g. within Cumbria), intermediate scale looking at regional scale (e.g. N.W. Britain) and large scale looking at the national scale (e.g. whole Britain).

4. The correlation between the *B.d.* infection state (positive or negative) and genetic distances between the *E. calamita* populations were tested in order to investigate if genetically well connected populations tend to show the same infectious state, which may indicate *B.d.* dissemination through natural *E. calamita* movement. Pairwise  $F_{st}$  estimates published previously (Rowe & Beebee, 2007) were used as a measure of genetic distances between sites with the assumption that these values reflected the current population connectivity through natural migration/dispersal. Pairwise  $F_{st}$  values were only comparative among the 12 sites in Cumbrian region, as no pairwise comparison was made between different regions (e.g. Cumbria sites and Merseyside sites). Therefore, this analysis was carried out only on sites in Cumbria region. First of all, as the available  $F_{st}$  values only compared a site to another, the information was converted into coordinates using Metric Multi Dimensional Scaling (MDS) which spatially reflect the genetic distances among sites and make comparison between all the sites with variable different status possible. It also enables us to visibly see the genetic relationship between these sites. However, MDS produces as many numbers of coordinates as  $n-1$  ( $n$ =number of samples) producing  $n-1$  dimensions, which include trivial dimensions which derived from random noise (Anderson et al., 2003). In order to separate coordinates which represent meaningful patterns from trivial coordinates derived from random noise, the broken-stick method was used (Jackson, 1993) and principal coordinates necessary for the analysis were identified. According to these principal coordinates, a 3-D graph expressing the genetic distances between all the sites was plotted using Graph-R (2008). Finally, in order to investigate spatial relationship between positive and negative sites based on genetic distances, the correlation between the disease states (positive or negative) of sites and their positions (principal coordinates) were tested using GLMs.

## **4. RESULTS**

### **4.1. Experiment 1. South Cumbria non-natterjack sites**

#### **4.1.1. *B.d.* surveillance results**

*B.d.* surveillance results of 10 inland non-natterjack sites located at various distances away from Sandscale Haws are shown in Table 4.1. No positive infection was found in any of the samples collected in these sites, either in the spring or summer periods. A summary of the species compositions of swabbed amphibians at each site are shown in Appendix 4.1.

**Table 4.1.** List of sampling sites in south Cumbria and PCR results for *B.d.* infection in 2008.

<b>Site no.</b>	<b>PCR results (Infected/T)**</b>		<b><i>B.d.</i> infection (-/+)</b>
	<b>Spring</b>	<b>Summer</b>	
1	0/30	N/A	-
2	0/30	0/30	-
3	0/12	N/A	-
4	0/30	0/30	-
5	0/30	0/30	-
6	0/30	N/A	-
7	0/30	0/30	-
8	0/30	0/30	-
9	0/30	0/30	-
10	0/30	N/A	-

\*Site no. refers to site locations shown in a map in Fig. 3.1.

\*\*PCR results are shown as number of infected individuals/total number sampled at each site.

As a result of all the sites providing negative results, no further analysis of this data set was required.

### **4.2. Experiment 2. The UK natterjack sites**

#### **4.2.1. *B.d.* surveillance results**

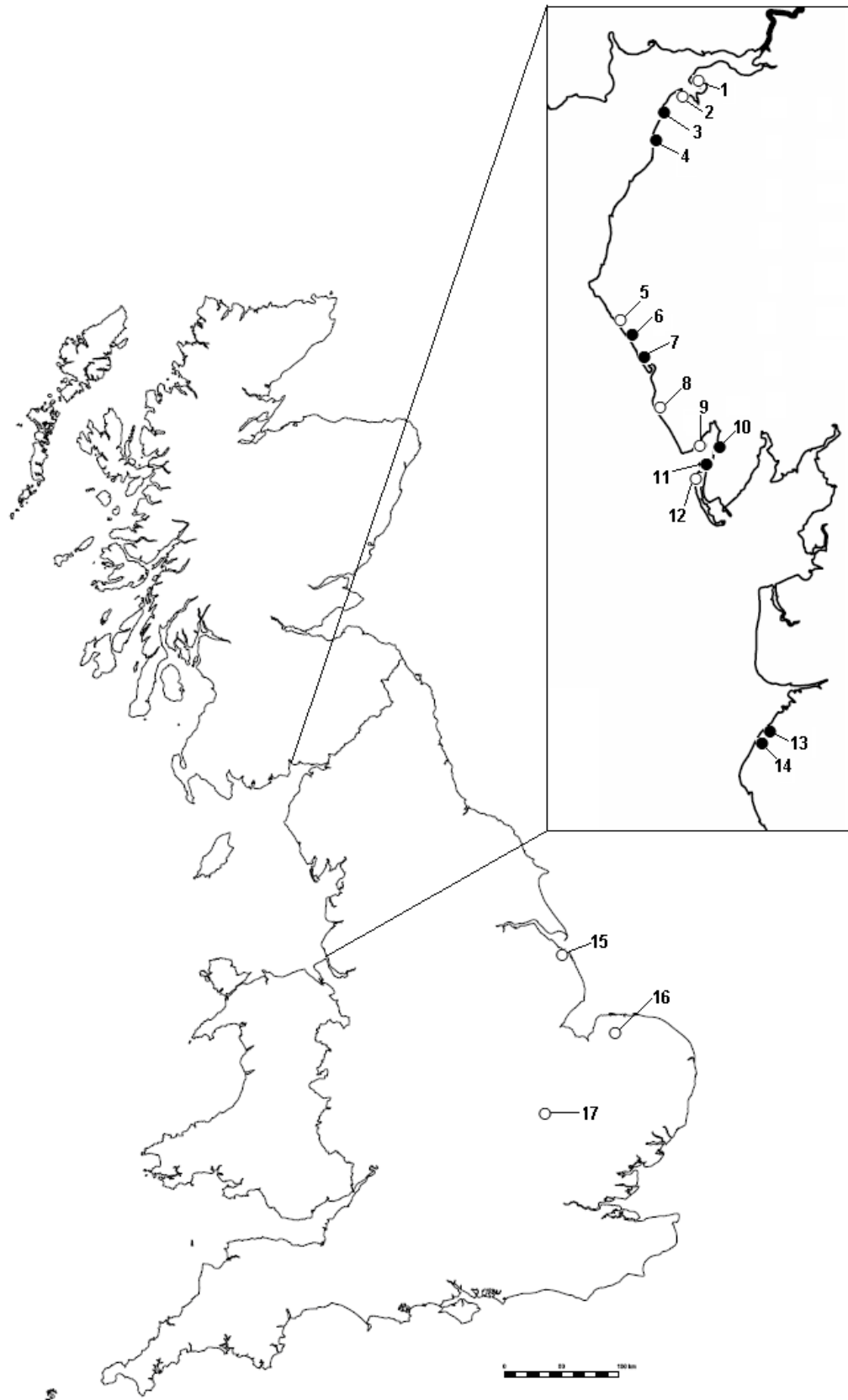
Surveillance results of Merseyside sites, eastern sites and North Walney in 2008 are summarized in Table 4.2, together with the surveillance results of Cumbrian sites from the year 2006 and 2007. Two sites from Birkdale and Ainsdale in the Merseyside region were found to be positive for *B.d.*. No positive result was found among the three sites from the eastern population. North Walney, was again found negative. Locations and infectious status of all 17 *E. calamita* sites surveyed for *B.d.* are shown in Fig.4.1.

**Table4.2.** Names of *E. calamita* sites sampled for *B.d.* infection and the surveillance results.

Site no.	Location	PCR results (Infected/T)**				<i>B.d.</i> infection (-/+)
		2006	2007	2008-1*	2008-2*	
1	Anthorn	0/62				-
2	Grune		0/14		0/22	-
3	Silloth	0/21	<b>3/27</b>			+
4	Mawbray	<b>9/140</b>	0/3			+
5	Braystones		0/14			-
6	Sellafield		<b>7/10</b>			+
7	Drigg		<b>1/81</b>			+
8	Annaside		0/16			-
9	Millom		0/36			-
10	Dunnerholme		<b>8/43</b>			+
11	Sandscale Haws		<b>15/59</b>			+
12	North Walney		0/19	0/18	0/30	-
13	Birkdale			<b>29/30</b>		+
14	Ainsdale			<b>1/30</b>		+
15	Saltfleetby			0/30	0/30	-
16	Sandy			0/30	0/30	-
17	Syderstone			0/30		-

\* Two sample periods were planned in 2008 (1-Spring: March-mid May, 2- Summer: mid May -July), not all were successful.

\*\*Infected=number of positive individuals, T=total number of individuals sampled. Details of positive samples (species and PCR results) are listed in Appendix 4.2.



**Fig.4.1.** Locations of *Epidalea calamita* sites sampled for *B.d.* in Britain.  
 ● - *B.d.* detected sites, ○ - No *B.d.* detected sites. Numbers refer to sites as detailed in Table 4.2 and details of surveillance results are also shown in Table 4.2. and Appendix 4.2.

#### 4.2.2. Monitoring data

Monitoring data on the 17 *E. calamita* sites collected using two different counting methods (adult and juvenile count and spawn count) was extracted from the monitoring data in the Natterjack Site Register (Beebee & Buckley, 2001). The count data at each site was plotted on a graph shown in Appendix. 4.3.

Summary of the final GLMs explaining the monitoring data at the 17 site is shown in Table 4.3.

1. Monitoring data at 6 sites (35.3%) were explained by year alone with count type differences having no influence, indicating that the two different count methods showing the same trend. This category contained 3 *B.d.* positive sites and 3 negative sites. Except for site 1, which is a negative site, notable drop in population size in the past 10-20 years can be seen, while site 1 has increased in the past 10 years.
2. Data from 2 sites (11.8%) showed that the count types had significant influence while year had no influence, which indicates that there was no significant trend in population size over years in these sites, using either method. The sale of numbers constantly differed between the count types. Site 5 had larger adult and juvenile count numbers while spawn number always stayed low. On the other hand, site 16 had large spawn counts and always small adult and juvenile counts. The reason for this observed trend in site 5 is probably because the population size is already very small (maximum adult & juvenile count = 32) and success in reproduction is constantly poor (reflected in low spawn counts). On the other hand, it is not clear why relatively large numbers of spawn counts were made, yet numbers of observed adults and juveniles were always low at site 16. One potential explanation may be due to site manager's preference over the different counting methods.
3. One site (5.88%) (site 15) showed a significant interaction between year and count type, indicating that the trend detected through the different count methods differed. In addition, three other sites (2, 9 & 12) also showed a similar effect but at a lower level of significance with *p* values close to 0.05 (between 0.053-0.059). All the four sites, which showed interactions, were *B.d.* negative sites.
4. For 5 sites (29.4%), none of the GLM models were able to explain the data pattern. (4, 6, 11, 13 and 14) and all the sites are *B.d.* This is probably due to the outliers of unusually large counts made over a couple of years in the past, which may have caused the model unable to predict a significant trend or to make a comparison

between the count methods.

35.3% of sites demonstrated that the two different counting methods showed the same trends and 11.8% showed that the sale of numbers produced by the different count methods differed but trends did not differ. Therefore, carrying out two counting methods may not be necessary, especially if we are only interested in finding a general trend in population sizes.

**Table 4.3.** Summary of analysis of deviance using GLMs for natterjack monitoring data, collected over the past 30 - 40 years (yr) using two different count types (CT). n~yr: Only year explained the model and different count types had no influence. n~CT: Two different count types explained model with year having no influence. n~yr\*CT: Interaction between the two variables (count method/year) explained the model.

Model	Site no.	GLMs		ANOVA		<i>B.d. infection</i> (-/+)
		<i>p</i>	<i>t</i>	<i>p</i>	<b>F</b>	
1. n~yr	1	0.00055 ***	3.87			-
	3	0.0087 **	-2.78			+
	7	0.0013 **	-3.45			+
	8	0.022 *	-2.44			-
	10	0.00023 ***				+
	17	0.00012 ***	-4.20			-
2. n~CT	5	0.021 *	-2.42			-
	16	0.0013 **	3.47			-
3. n~yr*CT	15	0.016 *	2.50	0.015*	6.40	-
	2	0.058 .	1.99	0.0088**	8.15	-
	9	0.053 .	-1.99	0.045 *	4.25	-
	12	0.058 .	-1.95	0.050 *	4.09	-
4. Non-significant	4	-				+
	6	-				+
	11	-				+
	13	-				+
	14	-				+

#### 4.2.3. *B.d. infection* + species and conservation activities

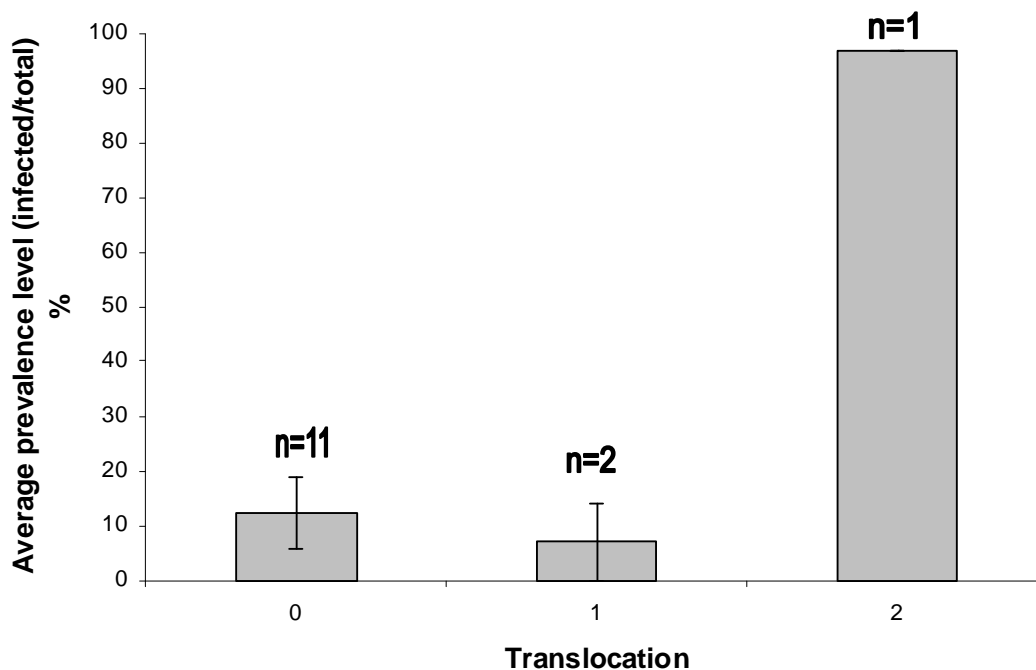
Infection rates among amphibian species was first compared and shown to have no significant correlation (Table 4.4.). Therefore, species was removed from the model and *B.d. infection* level at 17 sites were then tested against the 4 different conservation. A summary of the frequency of the 4 monitoring activities at each site is shown in Appendix 4.4. None of the natterjack conservation activities were also shown to have significant influence at 95% confidence level ( $p>0.05$ ) (Table 4.4.).

**Table 4.4.** Summary of GLMs analysis on species composition and conservation activities at 17 *E. calamita* sites

Tested explanatory variables	F	p
Species <sup>a</sup>	1.46	0.23
Monitoring <sup>b</sup>	0.047	0.83
Conservation related construction work <sup>b</sup>	3.50	0.07
Captive rearing and releasing <sup>b</sup>	2.99	0.093
Translocation (in to the population) <sup>b</sup>	1.14	0.29

<sup>a</sup>ANOVA against null model for species. <sup>b</sup>ANOVA against a model with itself removed. Family=quasibinomial.

The same analysis was then carried excluding the three uninfected eastern sites, which are separated with large geographical distances (>200km). The result showed that “translocation” had a significant influence on the level of the level of infection (F=7.71, p=0.017) and other three activities had no significant influence. The average observed prevalence level among sites where translocation was carried out in the same number of years was calculated and plotted in Fig. 4.2. Although there was an inequality in the number of sites in each category, the highest prevalence level was observed in the site where most translocation events took place.



**Fig.4.2.** Average observed *B.d.* infectious level and number of translocation events in the past in *E. calamita* sites in the north west (Cumbria and Merseyside region). n=Number of sites.

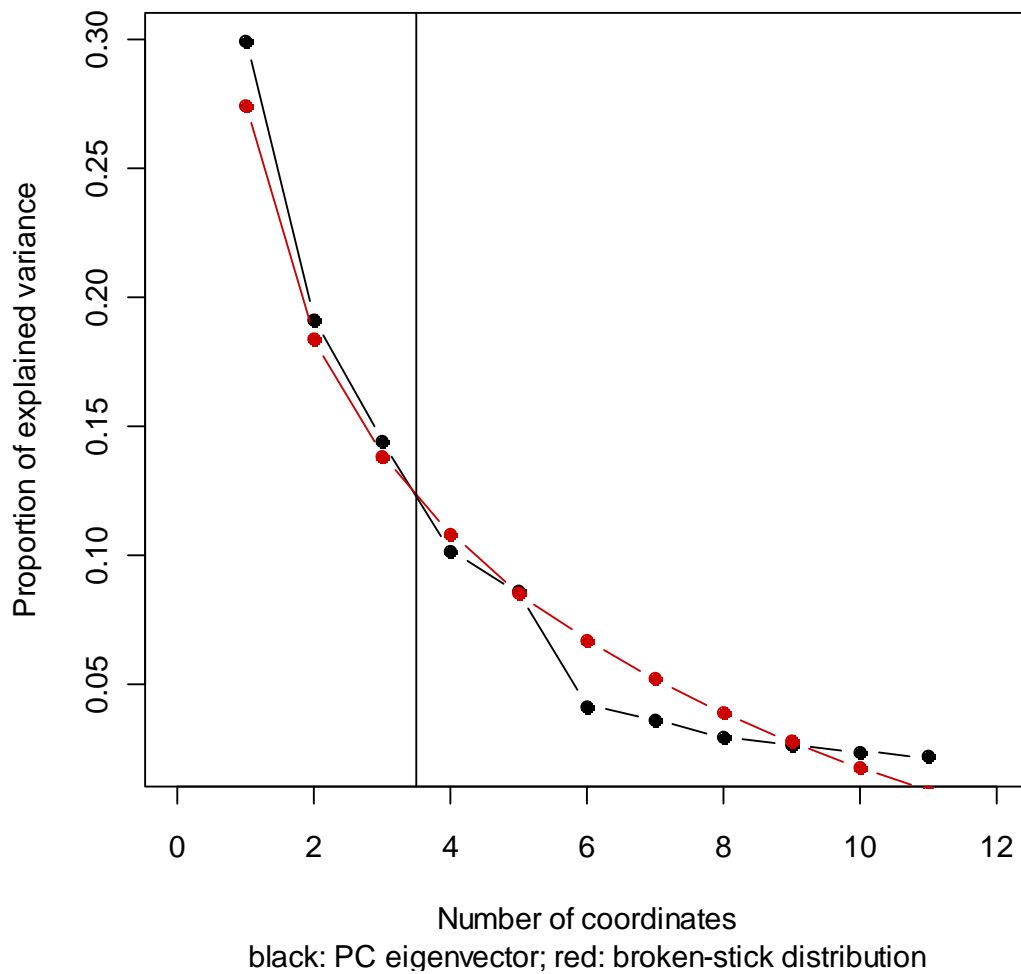
#### **4.2.4. Spatial analysis of *B.d.* distribution**

Geographical co-ordinates for each site, listed in Appendix 4.5., were used to test spatial autocorrelation among sites with different disease status (positive or negative), showing no significant spatial correlation at the 95% confidence level at any neighbourhood distances ( $p > 0.05$ , d.f.=16), indicating that no obvious clustering of positive sites or negative sites can be observed in any geographical scale. The same analysis was carried out using only the 14 western sites, removing the three eastern sites isolated with large geographical distances, however, no spatial autocorrelation was observed ( $p > 0.05$ , d.f.=13).

#### **4.2.5. Population structure based on genetic data and *B.d.* distribution**

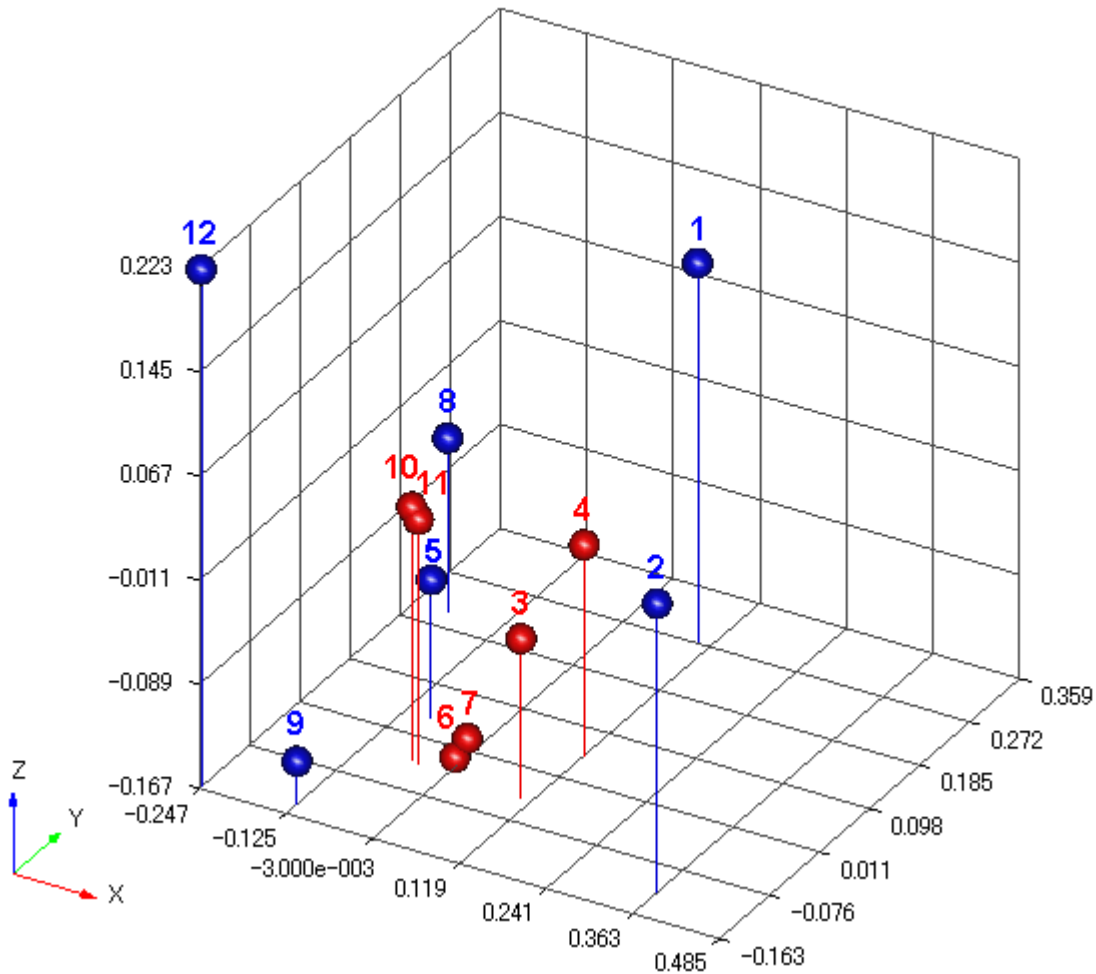
Metric MDS was carried out on to convert the pairwise  $F_{st}$  estimates between 13 western *E.calamita* sites into coordinates which represent all the genetic information among them. It initially produced 12 coordinates. According to the Broken-stick method (Fig.4.3.), the first three coordinates represented the genetic information meaningfully and the rest of the coordinates are trivial coordinates derived from random noise. Therefore, the first three coordinates were chosen to be used as principal, explaining 63.46% of the total explained variance (coordinate 1 explains 29.90%, 2 explains 19.14%, 3 explains 14.41%), and sites were plotted in 3 dimensional scale according to the coordinates (Fig.4.4.).

## Broken-Stick and the number of principal coordinates



**Fig. 4.3.** Comparison between the amount of variances explained by coordinates produced by MDS (red) and Broken-stick method (black). The first intersect of two lines (represented by the vertical line) indicate where trivial coordinates derived from random noise start. The first 3 coordinates, therefore, represented meaningful genetic information.

### Metric MDS



**Fig.4.4.** Twelve *B. calamita* sites plotted using the 3 coordinates produced by Metric MDS. X axis = 1<sup>st</sup> coordinate, Y axis = 2<sup>nd</sup> coordinate, Z axis = 3<sup>rd</sup> coordinate. Numbers refer listed in Table 4.2. *B. d.* detected sites are shown in blue, no *B. d.* detected sites are shown in red. Numbers refer to sites numbers as detailed in Table 4.2. and locations shown in Fig. 4.1.

GLMs analysis was carried out using the three principal coordinates. GLMs showed no significant interaction between the principal coordinates and the disease states at the 12 sites ( $p > 0.05$ ), however, two pairs of sites with similar  $F_{st}$  values (6 & 7, 10 & 11) are clustered and have the same disease positive status (Fig. 4.4.). All the negative sites were plotted at an appreciable distant away from the positive sites.

## **5. DISCUSSION**

### **5.1. Role of amphibians in the natural dispersal of *B.d.***

#### **5.1.1 Non-natterjack species at in-land sites**

The surveillance at inland non-natterjack sites within 3km radius from Sandscale Hawes found no *B.d.* infection. There are several possible explanations for this result. Firstly, habitat types between the positive site (sand dunes) and inland (farmland) were different, with this likely to be acting as an ecological boundary preventing migration of amphibians between the two habitat types. There are a lot of unknowns regarding the migratory patterns of amphibian species, however, a study on migratory activity of adult *B. bufo* observed that it stayed within its small home ranges (55 to 1600m) around its breeding pond (Sinsch, 1988) and its strong pond fidelity is suggested (Reading et al., 1991). Therefore, the non-natterjack amphibian movement between sites, especially between different habitat types, may actually have been a rare event. Even less is known about the movement of infected amphibians with the possibility that an individuals' migratory ability may be lowered (or sedentary) in comparison to that of a healthy individual.

Semlitsch (2008) suggests that there are two different types of movement among pond-breeding amphibians, "migration" and "dispersal". He defines "migration" as "movements, primarily by resident adults, toward and away from aquatic breeding sites" and "dispersal" as "unidirectional movements from natal sites to breeding sites that are not pond of birth and not part of the local population". He suggests that that long dispersal event (>1km) to non-natal pond is more likely to be under taken by juveniles over the first several years of their lives while they achieve reproductive maturity. This leads to a second explanation that no spread of *B.d.* was observed in the inland sites because *B.d.* has only recently been introduced to Sandscale and insufficient time has passed for it to spread by amphibian juvenile dispersal.

A third explanation might be that both adult and juvenile amphibians have the ability to move between these site, however, the last few years have simply been bad years for amphibians around this region and subsequent migration/dispersal events to/from Sandscale. As it is common to observe large fluctuations in numbers of amphibians, often due to variable climatic condition, this explanation is quite plausible.

A similar survey to this one was carried out at two non-natterjack sites within 2-3km from Mawbray in 2007, and they were both found to be negative as well. This said, as only six (4 *R. temporaria* and 2 *B. bufo*) and nine (all *B. bufo*) individuals were surveyed during the study the power to detect infected individuals was low. However, taken together with this project's result in south Cumbria, this may also suggest that *B.d.* has not spread in-land to non-natterjack site, at least within a year.

### **5.1.2. Natterjack toad (*E. calamita*) sites**

Interestingly, the pairwise  $F_{st}$  estimate between 3 and 4 was also 0.01, not significantly different from 0 (Rowe & Beebee, 2007), however they were plotted slightly further apart from each other in comparison to the other two positive pairs with 0.01  $F_{st}$  estimates (6 & 7, 10 & 11). This may be due to a model specific effect (MDS considers the genetic distances between all sites), where the  $F_{st}$  relationship between sites 3/1 ( $F_{st}=0.19$ ) and 4/1 ( $F_{st}=0.1$ ) results in site 4 being 'pulled' closer to site 1 than to site 3 (for  $F_{st}$  values see Appendix 4.6). The reason for this notable difference is not evident from the data set as site 3 is geographically closer to site 1 than site 4. It could be an artefact from genetic analysis carried out or it could also be something genetically interesting happened in the past. It may represent the route of colonization or dispersal pathways of *E. calamita* in the past which may not correlate with geographical locations, or there may have been unrecorded translocation events happened in the past from site 1 to 4, which made site 4 genetically closer to site 1 than site 3.

Inter-site distances along coastal dune habitat were all approximately 4km between the three pairs of positive sites (3 & 4, 6 & 7, 10 & 11) and no geographical barriers such as stream or road were detected on satellite image on Google Earth. One road was detected between site 10 and 11, however it seems to be a quiet single lane road and it is bridged at one point, this helping to maintain the habitat connectivity. Therefore natural amphibian movement between these three pairs of sites would seem likely.

Looking at Fig. 4.4., it can be seen that site 5 is plotted further away from sites 6 and 7. Site 5 is, however, geographically very close to site 6 (2.5km along the coast) and site 7 is geographically further away from site 6 (4 km). Upon checking the satellite image, it became apparent that there is a wide stream (>5m width) running between sites 5 and 6, separating it from the southern *E. calamita* sites 6 and 7. This suggests that genetic information (via  $F_{st}$ ) do appear to reflect habitat connectivity and separation quite well at a small scale. As site 5 is

currently uninfected and site 6 and 7 are infected, the stream may be preventing the spread of *B.d.* via natural (amphibian dispersal) or anthropogenic means (the movement of people). This said, however, as a public pathway (Cumbria Coastal Way) goes through 5 and 6 (via a bridge), and all the way to site 7 the likelihood of the stream acting as a barrier to the human spread of *B.d.* appears unlikely..

The fact that three pairs of sites (3 & 4, 6 & 7, 10 & 11) which are well connected to each other are all positive and a site (5) which is separated from its adjacent infected sites(6) by a river is still uninfected seems to suggest that *E. calamita*'s natural dispersal might be partly responsible for the spread of *B.d.* among sites at a small scale. It is suggested that *E. calamita* has a greater dispersal capacity than other anurans, such as *R. temporaria* and *B. bufo* (Miaud et al, 2000.), which also seem to support a theory based upon superior dispersal ability. The migratory range of female natterjacks was observed to be between 1.2 and 2.6 km in Germany (Sinsch, 1992b), up to 1.2km in Spain (Miaud et al, 2000), and toadlets were found to disperse up to 2km away (Sinsch, 1997). Among UK natterjacks, dispersal among populations separated by 2-3 km in coastal dunes and estuaries was also suggested to happen quite frequently (Rowe et al., 2000b). However, nothing is known about the behaviour of *B.d.* infected natterjacks, therefore it is not known whether infected natterjacks are physically able to migrate between these sites.

It is also difficult to distinguish whether the observed pattern of *B.d.* infection in the Cumbrian sites is truly due to amphibian movements between sites or due to the coastal sites being good locations for the public to walk along. On the other hand, it is evident when comparing the Cumbria and Merseyside regions, that *B.d.* has not been spread at a large scale by natural amphibian movements due to the great distances involved (10s of kilometres with no natterjack sites in between).

## **5.2. Role of human factors in the spread of *B.d.***

Human-induced factors appear to be the most likely cause of *B.d.* spread between the *E. calamita* sites separated by long geographical distances. From this study, it was found that "translocation" might be a likely influencing factor in the distribution of *B.d.* amongst sites in the North West region. As conservation activity on *E. calamita* has been high for a long time period, through the movement of animal translocations, the subsequent movement of herpetologists when monitoring populations and the noted incident of a mass mortality in a

captively reared population (Penrith), it is highly likely that conservation activity has played a role in the spread of *B.d.* among the UK natterjack sites. A similar scenario has been reported for the Mallorcan midwife toad, *Alytes muletensis*, a focus of conservation activity through an intensive captive rearing program. A mass mortality event occurred in 2005 and after investigation, it was found that *B.d.* was likely to have been introduced into native wild populations via captively reared individuals (M. C. Fisher, pers.comm.; G. Garcia, pers.comm.)

Although this specific factor has not been tested during this study, it seems likely that both herpetologists and the general public may have played a role in the spread of *B.d.* between sites via the movement of infected individuals and/or contaminated equipment (i.e. boots).

### **5.3. Other potential vectors**

Beside amphibian and human movement, there are other potential vectors which could possibly aid in the spread of *B.d.* between sites. During fieldwork visit of Sandscale Haws region, lots of dog walkers were seen. Dogs may walk into infected ponds and/or sandy areas and thus through carrying zoospores on their coat could potentially act as a vector in its spread. In addition, a lot of cattle and sheep were also observed freely stepping into water bodies and moving between them. When they are rotated to different stock enclosures, it is likely that they still have contaminated water and mud on their body, and so may act as a mode of transmission to the next pond that they visit.

Finally, waterfowl are also potential vectors in this region, being a coastal region famous for its bird life, with several RSPB/Wildlife Trust Nature Reserves. As a birds migratory capacity is high, it has the potential to spread *B.d.* on both at a small and large scale. Johnson & Speare (2005) demonstrated in a laboratory environment that *B.d.* survives 1 to 3 hours of drying on bird feathers, however, it may survive for less time in a natural habitat that is subject to local conditions i.e. alkaline sea water.

### **5.4. Monitoring method**

Although more sites suggested that the different monitoring methods show same trend, however, data from other sites suggest population counts collected using different counting methods do show differences, and uncertainty exists in some other sites. Therefore, although it seems there is definitely a potential in implementing just a single counting method, further

investigation is required.

### **5.5. Critical examination of the limitations and biases**

A number of factors should be considered when interpreting the results from previous studies that formed the data set for this study.

The number of individuals swabbed at some sites was small, therefore, it may have limited ability to detect infection. For example, only 14 individuals were surveyed at Braystones and 16 individuals at Annaside in 2007. With these numbers, a prevalence level below 20% is not likely to be detected at a 95% confidence level (DiGiacomo & Koepsell, 1986). Therefore, although no positive animals were detected, there is only a limited level of confidence when suggesting that they are truly negative. Also, because there has not been repeated sampling at those sites initially found to be negative in 2006 or 2007, there is no guarantee that these sites are still negative this year, in 2008.

Among sites and/or years, there has been a variability in the quality of monitoring data. Due to this, there is no way to determine whether low numbers are due to a lower monitoring effort or because the population number has actually declined. This can be particularly apparent when one considers the natural variation in counting ability that would occur between different sites (and their managers) and/or when there is a change of personnel at a given site. There was also some variability due to the monitoring method used, where at some sites counts were made via by the number of spawn strings whereas others used the number of adult and juvenile as a counting method. Counts made by estimation (mark and recapture method) were also recorded at site 14 (Ainsdale) for two years in 1970s. Because no actual counts were made around that period, there is no way to tell whether this site actually had that greater number of amphibians or whether it was just due to the different counting method used.

An assumption was made when using number of years when conservation activities classed in the four different activities, that these numbers represent the intensity of these activities at each site and therefore represent the chance of *B.d.* introduction through these activities. However, *B.d.* introduction can actually happen with a single event (e.g. single visit by a herpetologist or public with contaminated equipment, or translocation of an animal from an infected site).

Spatial analysis was limited by the small sampling size (16 locations). Therefore, it was not clear whether the non-significant result was obtained due to the small sample size or due to no actual spatial structuring in the distribution of *B.d.* Use of  $F_{st}$  value as an indicator of current genetic connectivity between sites also had a limitation. It does not perfectly show current natural amphibian migratory connectivity and so may not reflect recent changes in migratory pattern (i.e. habitat separation caused by a recent formation of a geographical barrier, such as roads). Therefore, care had to be taken when interpreting these results.

## **5.6. Recommendations – Where do we go from here?**

### **5.6.1. Recommendations for future research**

1. First of all, variability in susceptibility to *B.d.* among all the UK amphibian species must be tested. So far, susceptibility tests have only been carried out on *B. bufo* in this country, (M. C. Fisher, pers. comm.) and nothing is clearly known about how other species react to the pathogen.
2. A study similar to experiment 1 should be repeated in other locations in different years, to be more certain about *B.d.* spread between inland non-natterjack sites at a small scale. Repeating the study at sites around Mawbray may be beneficial as we know that the site has been infected for at least a few years, possibly more than 4 years (since the mass mortality events detected at Penrith), assuming that the site has remained infected. As no spawning has yet been observed at the ponds where *B.d.* positive samples were collected (J. Buckley, pers.comm.), it is likely that the site is still infected. This study will help to inform the likelihood of non-natterjack amphibians in the spread of the disease. However, this will also be dependent on whether there are many ponds suitable to be surveyed around that area. Sandscale was ideal in that aspect, as there were many ponds in the region. Repeating the survey in non-natterjack sites close to Sandscale within a few years time may also be useful.
3. All other *E. calamita* sites in the UK must be surveyed for *B.d.* to ascertain their status in regard to *B.d.* distribution/infection in *E. calamita* populations. This will also increase the statistical power for the analysis and so may enable more statistically significant results to be obtained.
4. As translocation of competitor's spawn (*B. bufo* and *R. temporaria*) from *E. calamita* sites to inland non-natterjack sites has been taking place in some locations for many years

(including *E. calamita* sites found positive for *B.d.*), surveillance must be carried out on these inland sites to see whether *B.d.* has spread to these locations through this activity.

## **5.6.2. Recommendations for management implication**

### **1. Improvement needed on monitoring methodology**

Pond-breeding amphibian species are known to have large natural fluctuations in numbers (Marsh, 2001; Storfer, 2003), therefore to be able to detect the real trends in a population long-term monitoring is required. As many of the UK *E. calamita* sites have been monitored for more than 30 years, providing a precious data set likely to cover these natural fluctuations. This said however, monitoring of these populations is labour intensive, especially as this species has a long breeding season [lasting for 4-5 months (April to August) (Buckley, pers. comm.)]. Use of a single monitoring method would reduce the time and labour required to monitor a single site, which would potentially enable more sites to be monitored thoroughly or the effort saved could be spent on other conservation activities required on site. Counting spawn strings is suggested as it is easier to find, less sensitive to weather and can be searched during the day time, and the suggested protocol for monitoring is to visit the site once a week during the breeding season (Buckley, pers. comm.). A simple guideline to standardize the method for monitoring also exists (HCT, 2003). However, because the breeding period is so long, the current protocol is hard to achieve and the quality of reporting shows much variation. In addition, personal preference also seems to exist over counting methodology, a practice that adds further variance. It is very important to find a way to standardize the technique so that data can be compared properly over the years and between sites with more confidence. It is suggested that a meeting with site managers should be convened to standardize monitoring methodology through involving them in the decision making process.

### **2. *B.d.* control among natterjack sites**

The *B.d.* problem within the UK natterjacks has to be tackled urgently, as their disappearance at infected sites has already been observed and those sites that are infected on the whole have had the larger population sizes in the NW region (Ainsdale, Drigg, Sandscale). In the worst case scenario, there is a possibility that *B.d.* could wipe out all the NW sites leaving only several sites in the east where population sizes are generally small, fragmented and genetically poor.

Conservation activity has to be revised to counter this new threat, as it is a completely different type of threat to that previously encountered in traditional conservation scenarios. For example, in the past as isolated small populations are more likely to be suffering from low genetic variation common practice would involve boosting their genetic variation and increasing numbers through translocation. However, in the case of *B.d.*, translocation of animals is possibly the greatest factor increasing the risk of the pathogen dissemination and resultant population extirpation.

#### Distribution of information and network

It is difficult to decide who and when to inform regarding the detection of *B.d.* at *E. calamita* sites. It is deemed unproductive to create a scare (and its resultant negative impact), but ignorance amongst individuals could result in the spread of *B.d.* to uninfected sites. For example, some of the positive sites are located among golf course, informing the public at these locations may upset the public and cause economical damage locally, however, at the same time there is a danger of acting too slow and spreading the disease. At least among the herpetologists involved in *E. calamita* conservation, the dissemination of correct knowledge regarding the pathogen and its status at sites is important. This could be achieved through stronger communication network, possibly through meetings, seminars and dissemination of newsletters.

#### Herpetologist movement

Currently within the Cumbria region, site managers are advised to stay within their own sites and not to move between sites (Buckley, pers. comm.). There are several sites that are managed by the same people. If these are geographically close with well connected habitat, and natural amphibian migration is highly likely, then there is probably not a problem. However, care should be taken in such scenarios such as sites 5 and 6, where site 5 is still uninfected whereas site 6 is infected; both sites are geographically very close but natural amphibian migration appears not to occur due to a natural barrier. Here, extra caution should be taken by the site manager to ensure that there is no translocation of *B.d.* between the sites i.e. decontamination of boots and equipment between sites. It may be even better to prepare two sets of equipment, however care has to be taken not to mix them up.

### Translocation

Currently, to carry out a translocation of *E. calamita* a license is required, however, the license remains valid for several years (J. Buckley, pers. comm.). If the infection status of an acceptor/donor site changes during this period and the person responsible for the translocation is unaware of the situation, a transmission event can occur. The licensing system needs to be improved so that it responds to such sudden changes in circumstances. The person issuing the license must be well informed about the pathogen and ideally good levels of communication should be developed between them and epidemiologists. In addition, period of validity must be reduced to 1 season or 1 year. In the short term, the termination of all translocation events among *E. calamita* sites is desirable, as translocation is likely to be one of the factors causing the dissemination of *B.d.* Translocation of competitor's spawn from *E. calamita* sites to inland non-natterjack sites must also be reconsidered especially from sites which are already *B.d.* positive.

### Captive rearing

It is also suggested that the practice off-site captive rearing should be avoided as *B.d.* mediated mass deaths were observed among captively reared *E. calamita*, however it may be necessary in some sites when low recruitment has been observed over several consecutive years. An alternative deemed more suitable is that practiced at Saltfleetby, where the rearing takes place on-site, in an artificial pond (J. Buckley, pers. Comm.). If in-situ rearing is not feasible and ex-situ captive rearing is necessary, it must be ensured that all precautions against *B.d.* are taken i.e. the person carrying out the rearing does not have any other amphibians, such as exotic amphibian pets, in captivity.

Finally, to cut down the spread of *B.d.* all the herpetologists involved in *E. calamita* conservation should be made aware of the pathogen and should be encouraged to separate their field equipment and/or follow the recommended disinfection protocols.

### **3. Controlling the further introduction of *B.d.***

Although *E. calamita* is legally protected and handling the animals without a license is not allowed, this does not ensure that the public i.e. children, might take specimens home for a while and then release them back, possibly to another locations. In addition, amateur herpetologists very often have other exotic amphibians at home. Therefore, to successfully

manage the transmission of *B.d.* a revision of information made available to the public should be made.

#### **4. What do we do with sites that are already infected?**

It is difficult to determine what would be the best way to deal with those sites that are already infected. A number of treatment methods in captivity are already available (Parker et al., 2002; Nichols et al., 2001; Woodhams et al., 2003), however, this approach might be deemed financially impracticable to apply to all the non-captive UK infected amphibian populations. Further, the approach taken will depend on the results from a current UK chytrid surveillance. If *B.d.* is mainly contained among the known *E. calamita* sites, it may be possible to cure the rare natterjacks in captivity, fill in the infected ponds and create a new clean ponds for natterjacks. However, as long as there are infected animals that remained uncaptured or if other UK amphibians are acting as vectors, this method may have limited success. This requires further investigation, however, it is also important to act quickly before *B.d.* spreads further.

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## 7. APPENDICES

**Appendix 2.1:** Ecological information of UK native amphibian species (Crown, 2008; Sterry, 1997).

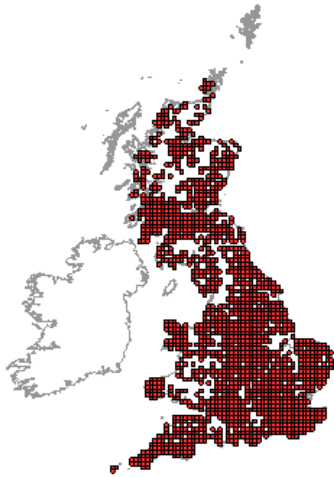
### 10km square legend

 Present in 10km squares

### **Common toad, *Bufo bufo***

Ecology: Visits ponds and lakes to breed in spring but at other times often found well away from water, sometimes in surprisingly dry areas. Often spawn in same ponds as common frog but usually active a few weeks after that species. On land, feeds on wide range of invertebrates including insects and slugs. Hibernates under logs and stones.

Distribution: Common and widespread throughout mainland Britain but absent from Ireland.

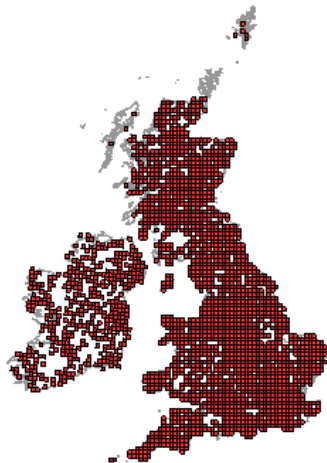


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### **Common frog, *Rana temporaria***

Ecology: Spawn in December or January in West Country but not until March or April in North or upland districts.

UK Distribution: Common and widespread in mainland Britain, introduced to Ireland (distribution rather patchy).

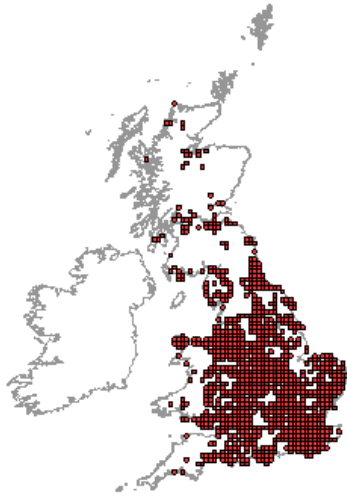


© Sayako Arai

**Great Crested Newt, *Triturus cristatus***

Ecology: Largest newt found in the UK, protected by law. Often found in large, weedy ponds and small lakes, sometimes in dew ponds a considerable distance from other water bodies. Spawns in spring, may remain in water throughout year.

UK Distribution: Widespread but extremely local in mainland Britain, absent from Ireland.

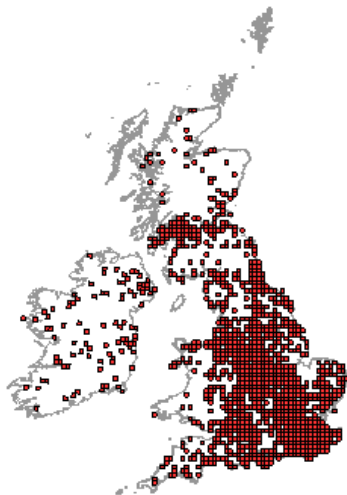


Male © John Cancalosi, naturepl.com

**Smooth Newt, *Lissotriton vulgaris***

Ecology: Found in ponds, ditches and lakes from March to September; leaves water and hibernates during winter under fallen logs.

UK Distribution: Widespread and common across most of mainland Britain; the only newt species in Ireland but rather scarce.

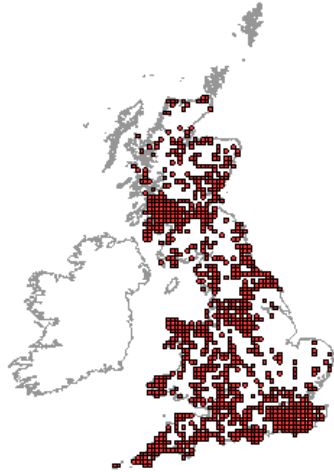


Male (up) and female (bottom) © www.osfimages.com

**Palmate Newt, *Lissotriton helveticus***

Ecology: Found in lakes, ponds and canals; often in fairly acid pools on upland moors or near to coast. Much of year in water but hibernates on land between November and March, under logs or stones. Feeds on invertebrates but also frog tadpoles.

UK Distribution: Widespread in mainland Great Britain.



Male © Sayako Arai

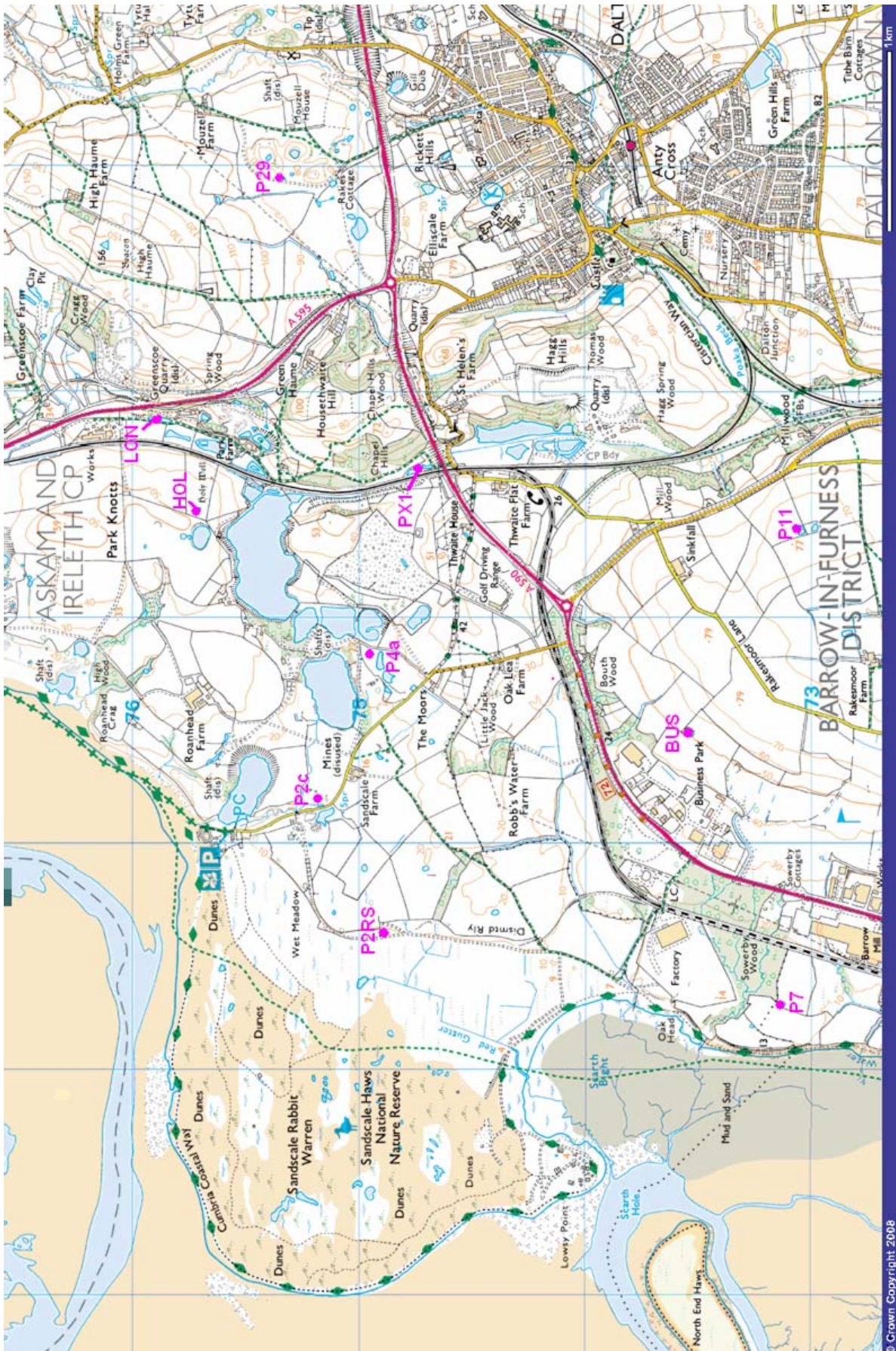
**Pool frog *Pelophylax lessonae***

Ecology: Pool frogs are often found in permanent, open ponds. It also spends part of year in grassland and woodland. It feeds on invertebrates.

UK Distribution: After becoming extinct in the wild, this species was reintroduced to a Norfolk site in 2005. Apart from the native pool frog, imported populations are known to occur in Hampshire, Surrey, Essex and north Norfolk.



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**Appendix 3.1.** Map of exact locations of 10 sampling sites in south Cumbria, inland non- *B. calamita* sites. (Ordnance Survey Explorer Map OL6, 2005)

**Appendix 3.2.** List of site no. referring to the names given to each site during the project, shown in Appendix 3.1.

Site no.	Site names
1	P2RS
2	P2c
3	P4a
4	Holly Well
5	Long Pond
6	PX1
7	P29
8	P7b
9	Business Park
10	P11

**Appendix 4.1.** List of species swabbed for *B.d.* at 10 south Cumbria, non-*B. calamita* sites. Site 1& 3 had dried up and 6&10 had no amphibians in the summer.

Site no.	Spring				Summer		
	<i>B.bufo</i>	<i>T.cristatus</i>	<i>L.vulgaris</i>	<i>L.helveticus</i>	<i>T.cristatus</i>	<i>L.vulgaris</i>	<i>L.helveticus</i>
1		1	21	9			
2	1	2	16	11	2	15	13
3			12				
4		3	13	14	7	12	11
5			11	19		6	24
6	30						
7	11		19			20	10
8	30					5	25
9	15		9	6	1	18	11
10	30						

**Appendix 4.2.** Details of Details of positive samples (species and PCR results).

**4.2.1.** Species composition and numbers of positive results.

Site no.	Location	Year	Species	sex	sample	No. infected
3	Silloth	2007	<i>B.bufo</i>	M	S	2
			<i>L.vulgaris</i>	F	S	1
4	Mawbray	2006	<i>B.bufo</i>	F	S	1
			<i>E.calamita</i>	M	S	2
				J	S	5
				Tadpole	T	1
6	Sellafield	2007	<i>E.calamita</i>	N/A	S	7
7	Drigg	2007	<i>E.calamita</i>	M	S	1
10	Sandscale Haws	2007	<i>B.bufo</i>	M	S	1
			<i>E.calamita</i>	F	S	2
				J	S	3
				M	S	4
			<i>R.temporaria</i>	J	S	5
11	Dunnerholme	2007	<i>B.bufo</i>	M	S	1
			<i>E.calamita</i>	F	S	1
				M	S	6
13	Birkdale	2008	<i>E. calamita</i>	F	S	4
				M	S	24
			<i>R.temporaria</i>	M	S	1
14	Ainsdale	2008	<i>E. calamita</i>	M	S	1

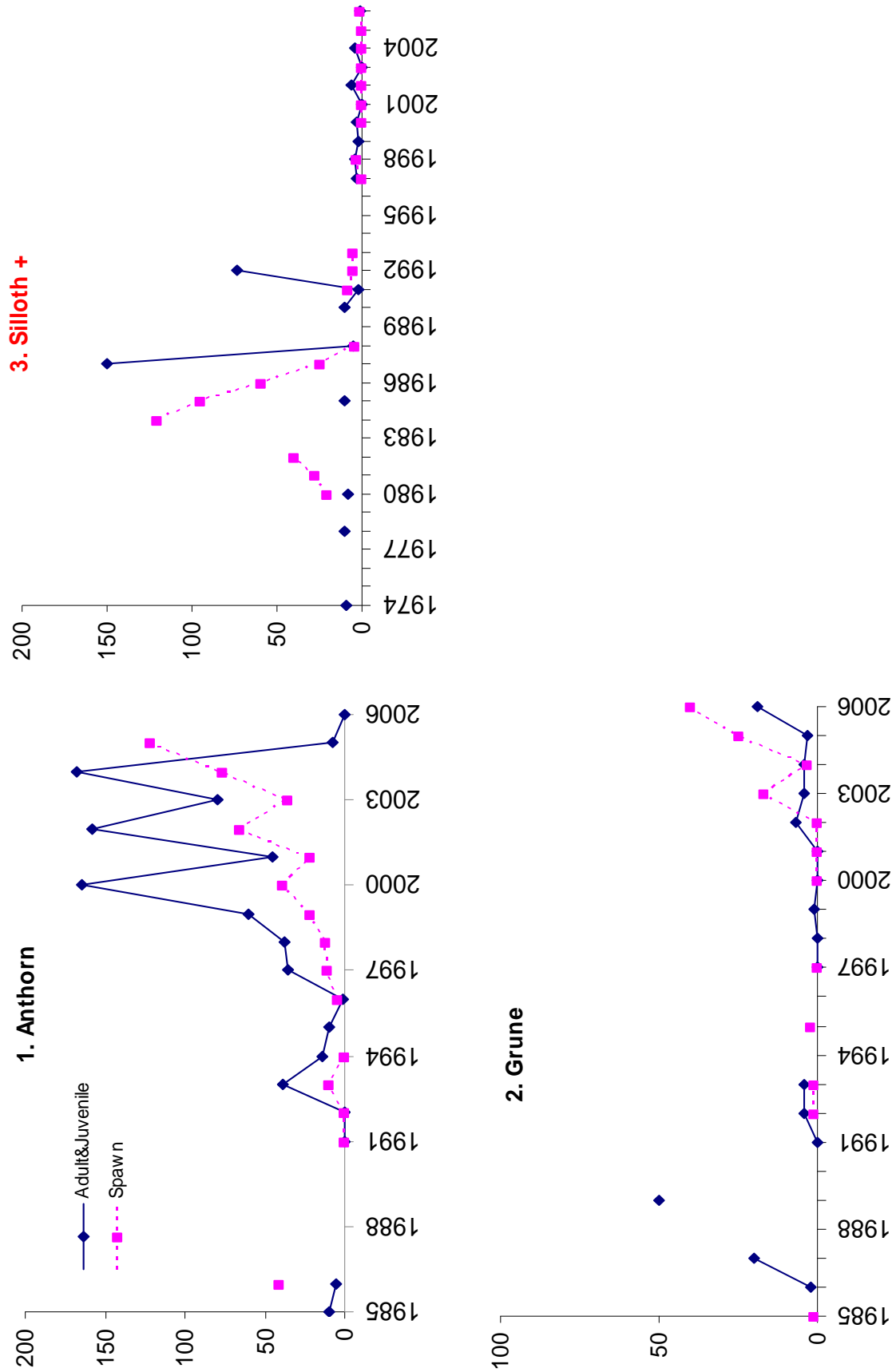
4.2.2. Rt-PCR results for positive samples from Birkdale and Ainsdale, 2008.

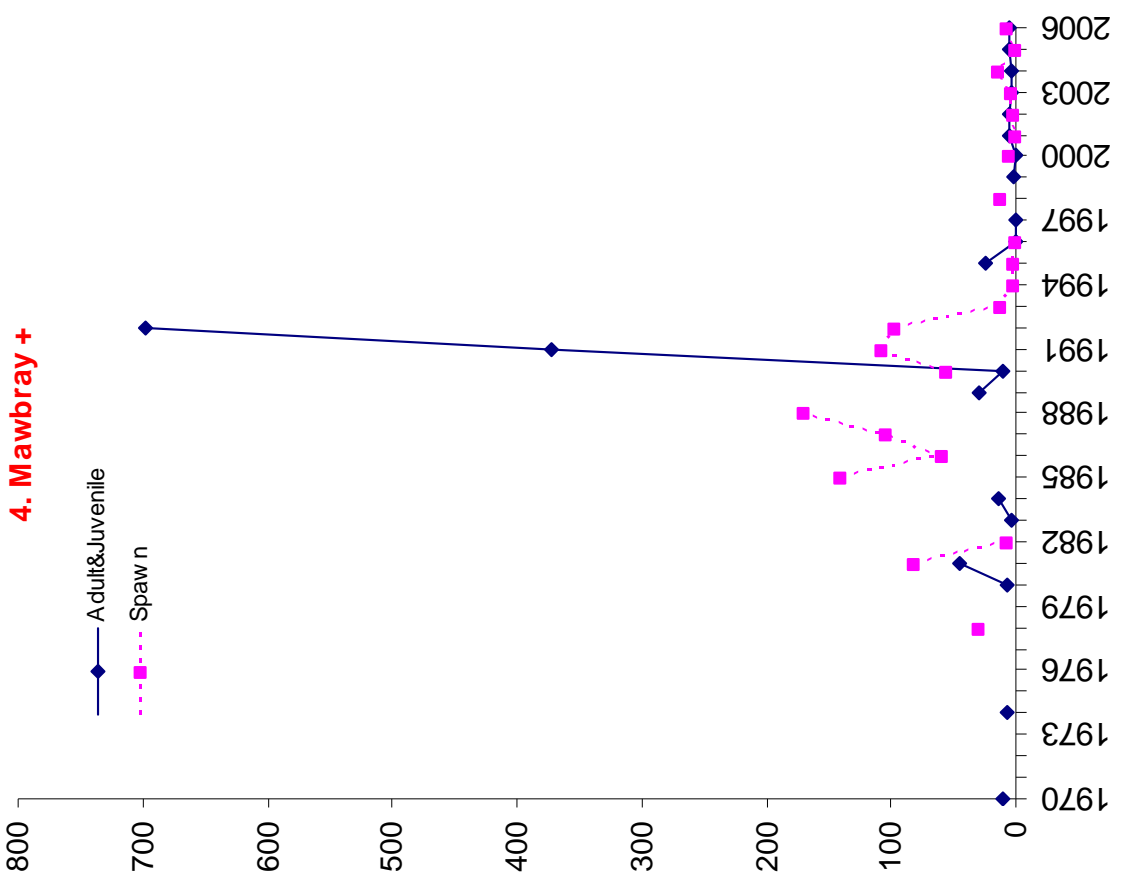
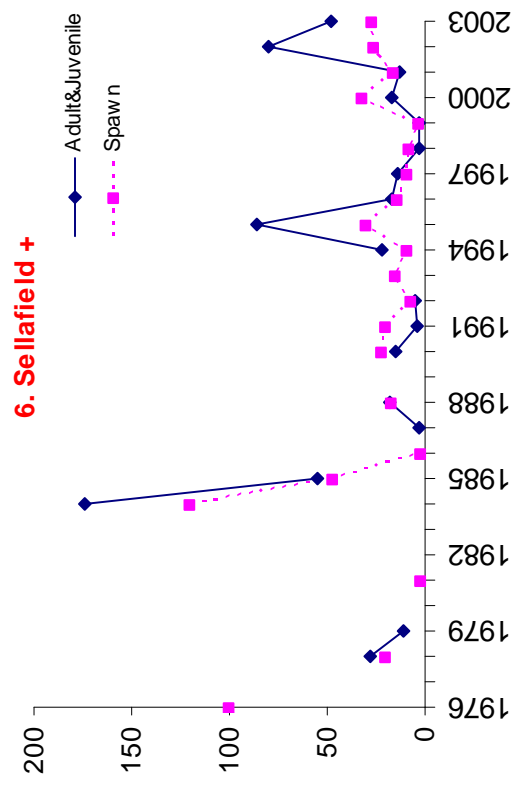
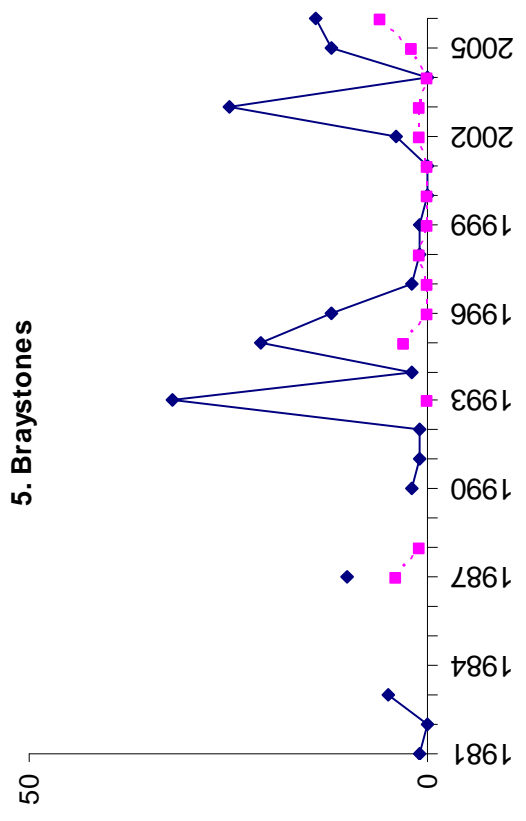
Site no.	Location	Species	sex	Zoospore*
13	Birkdale	<i>R. temporaria</i>	M	3.65
		<i>E. calamita</i>	F	0.99
				1.20
				4.18
				1.33
			M	2.85
				11.48
				20.02
				1.56
				1.02
				3.98
				1.86
				318.16
				0.77
				8.13
				2.29
				1.80
				2.92
				1.94
				17.87
		0.20		
		5.46		
		2.09		
		3.81		
		22.33		
		6.00		
		2.06		
		29.26		
		1.55		
14	Ainsdale	<i>E. calamita</i>	M	3.75

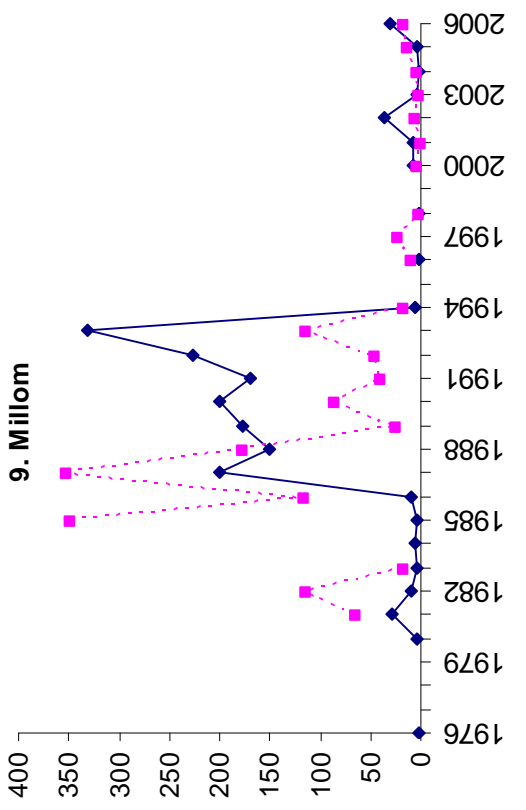
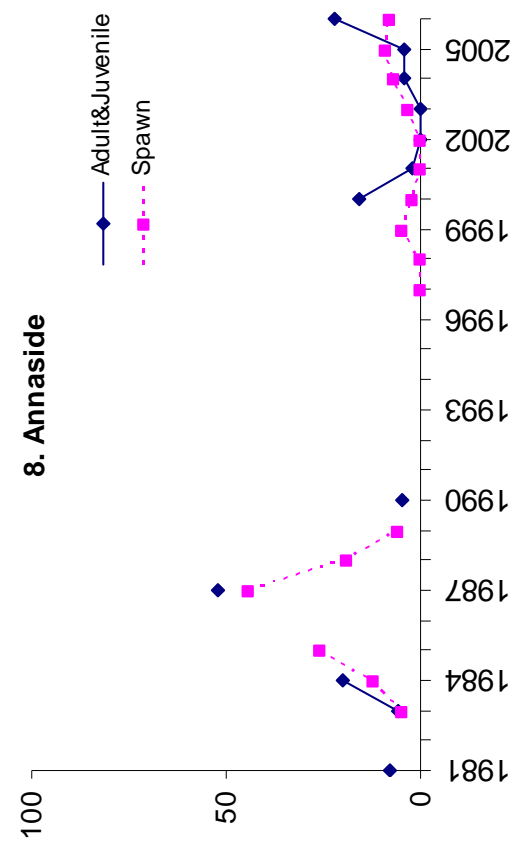
\* Genomic equivalents(GE) of zoospore numbers calculated using standards placed on the same PCR plate.

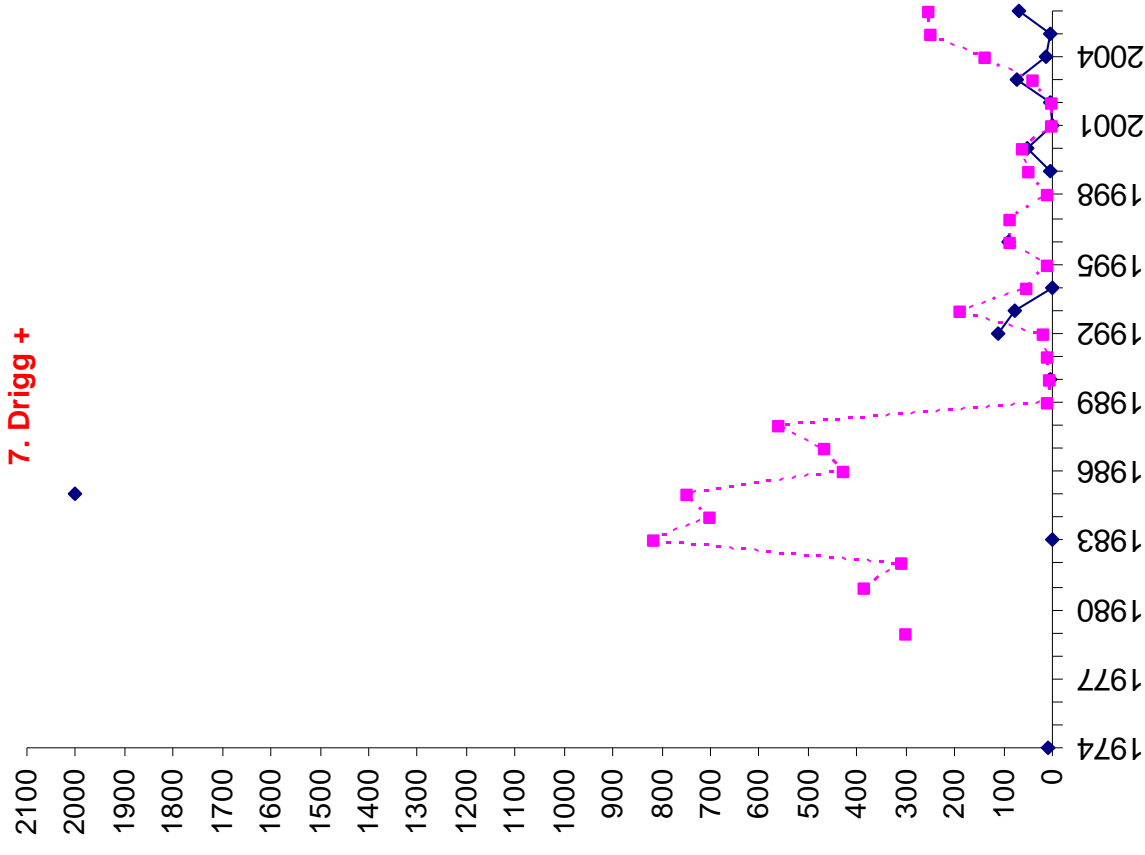
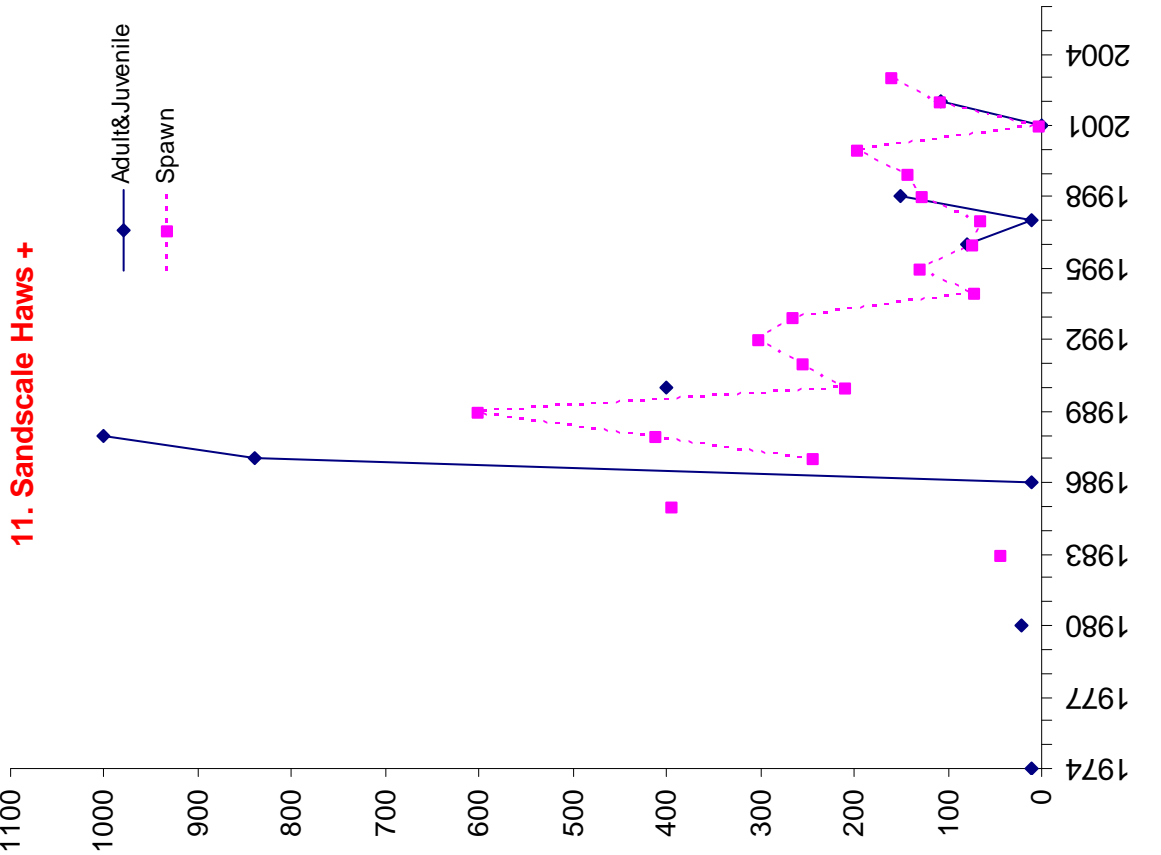
(GE>0.1 was judged as positive as the extractions were diluted 1/10 before PCR.)

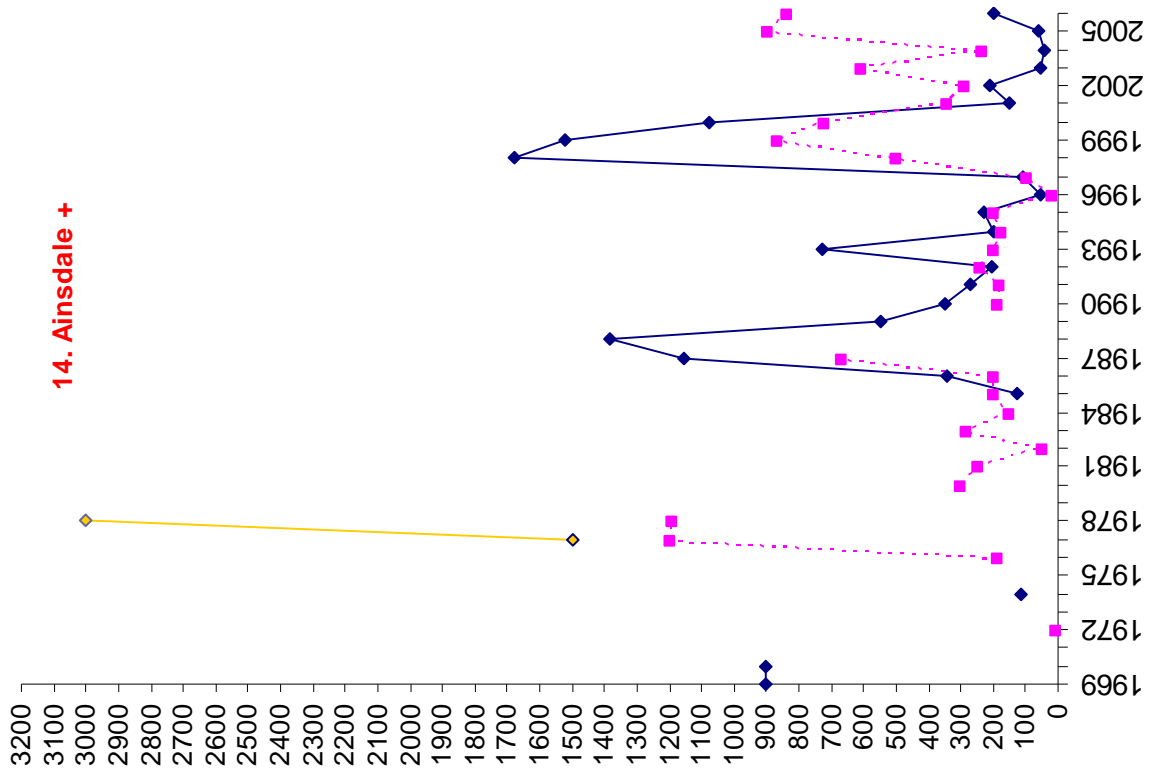
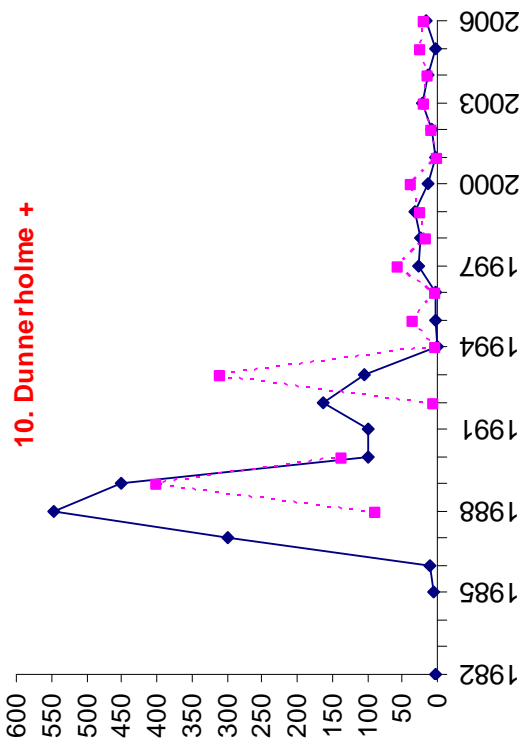
**Appendix 4.3.** Graphs of adult/juvenile counts and spawn counts at 17 *E. calamita* sites. Data extracted from the Natterjack Site Register (Beebee & Buckley, 2001). Sites where *B.d.* positive results are obtained are shown with + sign beside their name.



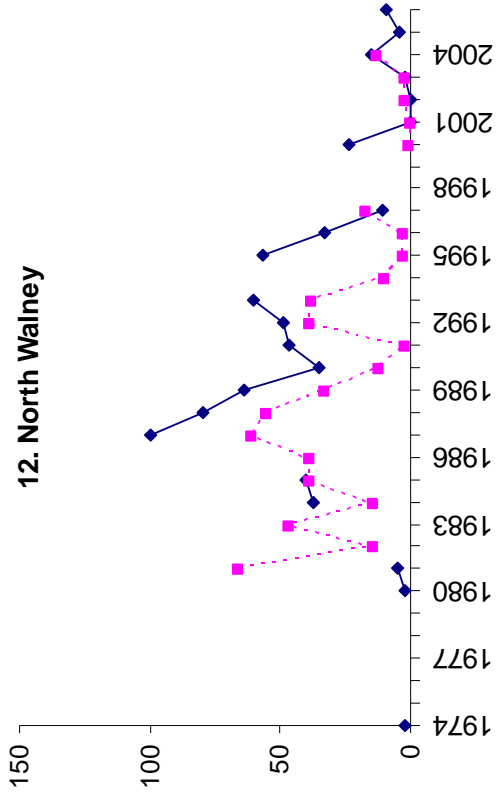




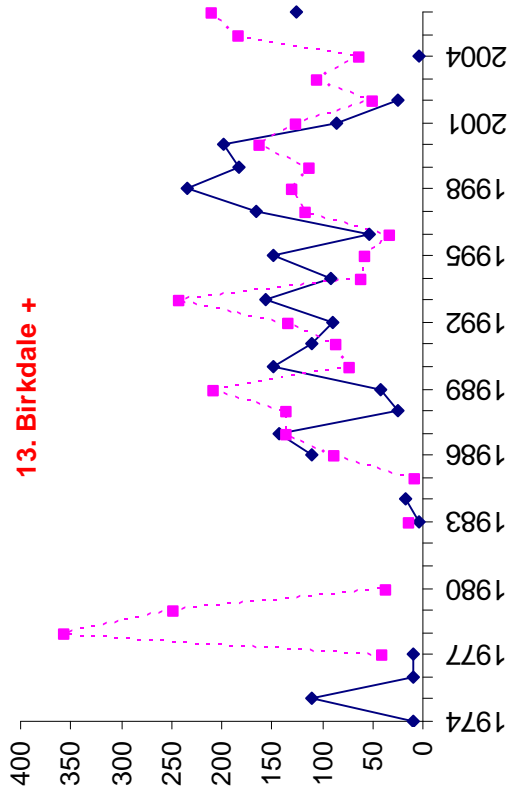


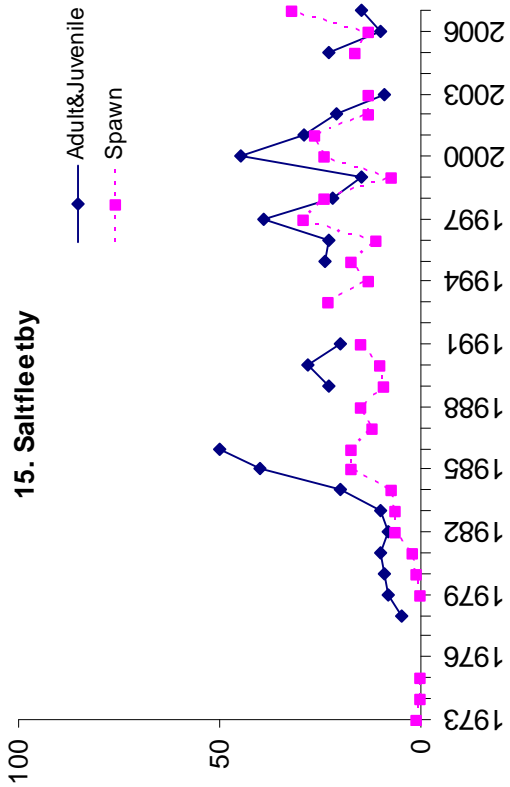
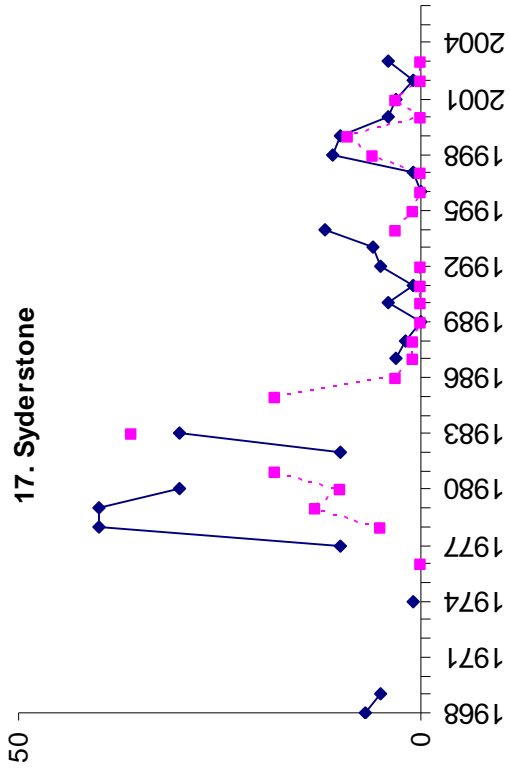


12. North Walney



13. Birkdale +





**Appendix 4.4.** Summary of the 4 conservation activities used as explanatory variables.

Data extracted from the Natterjack Site Register (Beebee & Buckley, 2001).

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<b>Site no.</b>	<b>Location</b>	<b>Monitoring</b>	<b>Construction</b>	<b>Rearing</b>	<b>Translocation</b>
1	Anthorn	20	11	0	0
2	Grune	20	5	1	0
3	Silloth	27	11	4	0
4	Mawbray	33	6	1	1
5	Braystones	24	3	0	1
6	Sellafield	32	22	0	0
7	Drigg	38	7	0	0
8	Annaside	23	6	1	0
9	Millom	30	15	1	0
10	Sandscale Haws	27	10	0	0
11	Dunnerholme	25	6	0	0
12	North Walney	27	11	1	0
13	Birkdale	35	26	0	2
14	Ainsdale	42	32	2	0
15	Saltfleetby	34	21	0	0
16	Sandy	27	14	0	2
17	Syderstone	33	23	7	6

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**Appendix 4.5.** Geographical coordinates of *B. calamita* sites. Coordinates obtained using Simple Geocoder (Map Channels, 2008)

<b>Site</b>			
<b>no.</b>	<b>Location</b>	<b>Latitude</b>	<b>Longitude</b>
1	Anthorn	54.90351	-3.27407
2	Grune	54.87863	-3.32697
3	Silloth	54.85915	-3.3962
4	Mawbray	54.80425	-3.43342
5	Braystones	54.43049	-3.52977
6	Sellafield	54.40922	-3.5032
7	Drigg	54.36568	-3.45341
8	Annaside	54.27348	-3.40859
9	Millom	54.20871	-3.25273
10	Dunnerholme	54.20586	-3.20686
11	Sandscale Haws	54.16555	-3.24391
12	North Walney	54.13611	-3.27268
13	Birkdale	53.60936	-3.05203
14	Ainsdale	53.59924	-3.06413
15	Saltfleetby	53.4036	0.207602
16	Sandy	52.11582	-0.26004
17	Syderstone	52.8557	0.714728

**Appendix 4.6.** Table of pairwise Fst values between the 12 natterjack sites from the west coast. Site numbers refer to the locations listed in Appendix 5. \* = not significantly different from 0. Fst estimates extracted from Rowe & Beebee (2007).

Site no.	1	2	3	4	5	6	7	8	9	10	11	12
1	0	0.28	0.19	0.10	0.19	0.21	0.20	0.16	0.32	0.18	0.16	0.35
2	0.28	0	0.20	0.17	0.28	0.22	0.22	0.43	0.33	0.22	0.21	0.43
3	0.19	0.20	0	0.01*	0.17	0.11	0.10	0.25	0.16	0.16	0.11	0.26
4	0.10	0.17	0.01*	0	0.16	0.12	0.10	0.20	0.18	0.13	0.09	0.27
5	0.19	0.28	0.17	0.16	0	0.05	0.08	0.10	0.15	0.12	0.11	0.19
6	0.21	0.22	0.11	0.12	0.05	0	0.01*	0.13	0.10	0.11	0.10	0.24
7	0.2	0.22	0.10	0.10	0.08	0.01*	0	0.13	0.11	0.11	0.09	0.24
8	0.16	0.43	0.25	0.20	0.10	0.13	0.13	0	0.20	0.12	0.12	0.25
9	0.32	0.33	0.16	0.18	0.15	0.10	0.11	0.20	0	0.09	0.07	0.21
10	0.18	0.22	0.16	0.13	0.12	0.11	0.11	0.12	0.09	0	0.01*	0.16
11	0.16	0.21	0.11	0.09	0.11	0.10	0.09	0.12	0.07	0.01*	0	0.14
12	0.35	0.43	0.26	0.27	0.19	0.24	0.24	0.25	0.21	0.16	0.14	0