National survey of *Batrachochytrium dendrobatidis* infection in UK amphibians, 2008

Final report

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Natterjack toad *Epidalea (Bufo) calamita*

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National survey of amphibian chytridiomycosis

SUMMARY

Almost one third of all known amphibians are threatened with extinction and amphibian populations are declining globally. One of the main drivers of these declines and species extinctions in the fungal disease, amphibian chytridiomycosis, which is caused by infection with the non-hyphal, zoosporic chytrid fungus, *Batrachochytrium dendrobatidis* (Bd). This pathogen was first detected in wild amphibians in Kent in 2004 and in Cumbria in 2006. The primary aim of this project was to determine the broad-scale distribution of the amphibian chytrid fungus in England, Scotland and Wales and to determine if it is present in *Bufo bufo* on the island of Jersey.

Most of the field work was carried out by volunteers, who were recruited and trained largely through voluntary county groups known as Amphibian and Reptile Groups (ARGs). The ARGs carried out cascade training of volunteers to aid recruitment and to raise awareness of the threat of Bd and the biosecurity measures that should be taken when visiting amphibian breeding ponds.

Ponds were visited to sample amphibians (via non-destructive skin swabbing) for Bd during spring and summer 2008. A total of 5,958 amphibians from 121 amphibian breeding ponds were sampled: 96 ponds in England, 7 in Scotland, 16 in Wales and 2 in Jersey. In summary, 1,849 smooth newts, 1,402 palmate newts, 1,214 common toads, 590 great crested newts, 393 common frogs,152 natterjack toads, 135 alpine newts, 64 marsh frogs and 19 pool frogs were caught and swabbed in Great Britain during 2008. In Jersey, the common toad (*Bufo bufo*) was specifically targeted as this species has been declining on the island.

A major result of this survey was finding Bd-positive amphibians at sites across Great Britain, with all native amphibian species (except the great crested newt) testing positive at least once in this survey, confirming a low host specificity of Bd in Great Britain. No infection was found in Jersey. There was a marked difference in the prevalence of Bd-infection between species and, within some species, between seasons. Also, we found a strong association between the presence of non-native amphibian species and the presence of Bd infection. It would be interesting to further investigate potential confounding factors which could impact the apparent Bd-status of an animal using the data collected in this project, therefore a follow-up study to collect more details on each site is recommended.

INTRODUCTION

Amphibian chytridiomycosis, a disease caused by the virulent fungus *Batrachochytrium dendrobatidis* (Bd), has been recognised as the primary cause of global amphibian population declines and extinctions since the late 1990s (Daszak et al. 2003). The disease is known from many parts of the world, including Europe where it is decimating multi-species amphibian assemblages in Spain (Bosch & Martínez-Solano 2006).

In Britain, work funded by English Nature (and later by Natural England) and conducted by the Institute of Zoology, London (IoZ) led to the fungus being found in the wild at two locations: Tunbridge Wells, Kent and several natterjack toad (*Epidalea* (*Bufo) calamita*) sites in Cumbria. At Tunbridge Wells, infection was first detected in 2004 in introduced North American bullfrogs (*Lithobates catesbeianus*) (Cunningham *et al.* 2005), a species known to be a silent carrier of Bd. Following the apparent eradication of bullfrogs from this site in 2004, further surveillance by the IoZ showed Bd infection in common toads (*Bufo bufo*) in 2005 and in 2007 (when it wiped out a captive cohort of metamorphs). Infection was also found in common newts (*Lissotriton* (*Triturus) vulgaris*) at this Tunbridge Wells site in 2007.

In 2006, investigations following chytridiomycosis-associated mortality of natterjack toads in a captive collection associated with the restocking of a wild population at Mawbray, Cumbria, found a high prevalence of Bd infection at the Mawbray site, but in no amphibians at several other natterjack toad breeding sites investigated in 2006 (including one other in Cumbria). Further investigations by the IoZ in 2007 showed Bd infection to be present in at least seven additional natterjack toad breeding sites in Cumbria.

In addition, in 2007, a small number of introduced alpine newts (*Triturus alpestris*) in Canterbury, Kent tested positive for Bd infection at the IoZ. Like the North American bullfrog, this species of newt is known to be a silent carrier of Bd.

Natural England commissioned the IoZ to conduct a survey in 2008 of amphibians from as many ponds as funding would allow in order to better determine the extent of Bd infection of wild amphibians in England. Additional funding was obtained from the Countryside Council for Wales, Scottish Natural Heritage and The States of Jersey to extend this survey to encompass their respective jurisdictions. Knowledge of the true extent of Bd infection in amphibians in Great Britain is required to inform policy on matters such as possible containment and/or eradication measures.

PROJECT AIMS

Primary Aim

To determine the broad-scale distribution of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in England, Scotland and Wales and to determine if it is present in *Bufo bufo* on the island of Jersey.

Additional Aims

(i) To raise awareness of amphibian chytridiomycosis among key stakeholders, including herpetofauna volunteers, NGOs (Non-Governmental Organisations) and landowners.

(ii) To train key workers in biosecurity measures appropriate to limit transmission of amphibian chytridiomycosis.

(iii) To compare infection prevalence in different native species.

(iv) To investigate if there is an association between the presence of *B. dendrobatidis* infection and the presence of non-native amphibian species.

(v) To obtain a clearer view of the level of threat posed by *B. dendrobatidis* to UK amphibians in general and to the natterjack toad (*Epidalea calamita*) in particular.

(vi) To obtain data to inform future sampling strategies.

METHODS

In order to determine the distribution of Bd in Great Britain, we aimed to identify the Bd status of as many ponds as possible with as wide a geographical spread as possible in as statistically meaningful way as possible, given the time and resources available. In order to maximise the number of ponds sampled, a network of volunteers was used in addition to the project staff.

Volunteers were recruited largely through working with voluntary county groups known as Amphibian and Reptile Groups (ARGs). Liaison with the ARGs was through Amphibian & Reptile Groups of the United Kingdom (ARG UK), a national body representing the local groups. IoZ also liaised with The Herpetofauna Conservation Trust in preparing the survey. (The HCT has since merged with Froglife to form a new organisation, Amphibian and Reptile Conservation.) Such recruitment also helped to fulfil the aim of raising awareness of amphibian chytridiomycosis in Great Britain. A presentation was made on the subject at the 2008 annual Herpetofauna Workers Meeting, followed by a workshop on Bd surveillance, field sampling of amphibians for Bd detection and biosecurity procedures. Many volunteers were recruited at, or via, this meeting. Additional workshops were held in Manchester and in Wales, where further volunteers were recruited, and the ARGs carried out cascade training of volunteers to aid recruitment, and to raise awareness of the threat of Bd and the biosecurity measures that should be taken when visiting amphibian breeding ponds.

In order to have a high confidence of disease detection, a minimum number of animals must be sampled from a population. The statistical model used to generate a minimum sample size makes several assumptions, for instance

that the population sampled acts as a discrete population and that the infection status of individuals remains constant. In this case, if we assume the sensitivity of the infection test is 100%, then a sample of 30 amphibians gives a probability of Bd detection of 99% assuming an actual infection prevalence of 15%, and a probability of detection of 79% assuming an actual infection prevalence of 5%.

In the absence of any information on species differences in Bd prevalence in Great Britain, we assumed that each amphibian species is equally likely to be infected with Bd. We also assumed that, if present, Bd infection would be randomly distributed amongst the amphibians present, regardless of species.

To help determine the best time of year for detecting Bd, two sampling visits were proposed for each site: spring (April/May) and summer (June/July). Consequently we aimed to acquire a total of 60 samples from each site during 2008.

Site selection

The standard method for obtaining statistically meaningful results in this type of study would have been to select sampling locations at random. However, amphibians are not distributed randomly across the landscape, and their abundance at ponds is highly variable. Assigning randomly selected ponds to surveyors would have been unfeasible as it would likely result in a high number of zero or very low captures, for a high survey effort. This was a particular concern here as the surveyors were largely volunteers, whose participation depended on goodwill and who might not wish to expend much effort in poor survey locations.

Moreover, as we aimed to sample 30 amphibians at each site in the spring and again in the summer, sites sampled needed to be known amphibian breeding ponds with a large enough population to make sampling visits productive. In addition, we required sites to be accessible to volunteers. In order to assess the presence/absence of Bd over a broad-scale, we allocated approximately equal numbers of sites within each English region. Therefore, the following criteria were used to identify sites for sampling across England: regional location, moderate-large amphibian population, easy access, and practicality of capture. In Scotland, Wales and Jersey, sites for sampling were identified by the respective national conservation agency on the basis of conservation importance of amphibian populations.

To ensure reasonable coverage of natterjack toad sites and of ponds with non-native species in England, a number of known natterjack toad breeding ponds (n = 6) and twelve ponds with known populations of non-native species were selected. The selected natterjack sites were in the North West, whilst the selected non-native sites were more-widely distributed across England. These sites were in addition to any sites containing natterjack toads or non-native amphibians, which were selected for sampling across Great Britain and Jersey (see above). Non-native species sampled were: pool frog *Pelophylax (Rana) lessonae*, marsh frog *Pelophylax ridibundus* (formerly, *Rana ridibunda*) and alpine newt *Mesotriton (Triturus) alpestris*. Note that the pool

frog is now considered a native species in England, and has been reintroduced at a single site in Norfolk using Swedish animals. However, most UK populations are introductions of known non-native and inappropriate origin. The “non-native” classification used here is because these latter, known non-native populations were sampled. The Norfolk reintroduced population in Norfolk has been screened outside this project and is known currently to be negative for Bd.

Additionally, two clusters of sites (one in Cumbria, one in Kent) around ponds where Bd infection already had been established, were identified and sampled as part of this study. These clustered sites were sampled to help address Additional Aims (v) & (vi).

Sampling protocol

Surveyors collected samples by swabbing (using sterile cotton-tipped dry swabs) the ventral pelvic skin, the ventral femoral skin and the plantar aspects of the hind feet (and the tail of newts) of each amphibian caught. Only metamorphosed amphibians were sampled. Surveyors were asked to preferentially sample animals in the aquatic phase, since there is some indication that Bd is less easily detected in terrestrial amphibians. Only surveyors with the appropriate licence were permitted to catch or swab species (*i.e.* natterjack toad and great crested newt) scheduled under the Wildlife and Countryside Act 1981 (as amended) and the Conservation (Natural Habitats &c.) Regulations 1994 (as amended).

All surveyors participating in the work were instructed to observe strict biosecurity guidelines in order to minimise the risk of disease transmission between sites. These guidelines were developed in association with surveyors at the 2008 Herpetofauna Workers Meeting.

qPCR Analyses

Skin swabs were analysed for the presence of Bd DNA at the Institute of Zoology using qPCR: the qPCR analysis of skin swabs is currently the most sensitive test known for the detection of Bd infection in live amphibians (Boyle et al, 2004, Hyatt et al. 2007). The qPCR (also known as real-time PCR) is able to detect the presence of the genome equivalent of one tenth of one Bd organism. To reduce time and costs, extracted DNA samples from swabs taken from the same site were doubled-up (*i.e.* pooled into pairs) for qPCR analysis. Pooling only two swabs at a time does not reduce sensitivity of detection (Hyatt et al. 2007). Each pool was tested in duplicate. If a pooled sample gave a positive signal for Bd on qPCR, extracted DNA from each swab was tested separately in duplicate. Further re-tests were conducted on samples recording very low Bd genome values in order to minimise the risk of false positives.

RESULTS AND DISCUSSION

Number of amphibians and sites sampled

A total of 5,958 amphibians from 121 amphibian breeding ponds were sampled: 96 ponds in England, 7 in Scotland, 16 in Wales and 2 in Jersey. There was good representation of sampling across the English regions. A list of the sites sampled, with details of the location and of the number of amphibians sampled at each site during each sampling period, is shown in Appendix 1. In summary, 1,849 smooth newts, 1,402 palmate newts, 1,214 common toads, 590 great crested newts, 393 common frogs,152 natterjack toads, 135 alpine newts, 64 marsh frogs and 19 pool frogs were caught and swabbed in Great Britain during 2008. In Jersey, the common toad (*Bufo bufo*) was specifically targeted as this species has been declining on the island. Coverage on Jersey, therefore, was limited to the two main breeding sites for this species, at which 97 toads were caught and swabbed. Maps showing the location of the sites sampled in Great Britain (but not Jersey) are presented in Figures 1 & 2.

Results of Bd qPCR analyses

Sixty-six amphibians of seven species from 19 sites gave positive results for the presence of Bd DNA using qPCR. Bd-positive sites were found in Scotland (1 site), Wales (3) and in all regions of England (15), except for the North East and the East of England. No Bd-positive animals were detected from Jersey. The numbers of each species sampled in Great Britain (i.e. omitting Jersey) during each sampling period, and the numbers of these which gave Bd-positive results, are shown in Table 1.

Of the 119 sites sampled in Great Britain, the 30 x 2 target for assessment of a population during the spring and summer sampling periods was achieved for 67. However, 28 or more amphibians were sampled from 105 sites (85 in England, 6 in Scotland, 14 in Wales) during the spring sampling period and from 77 sites (63 in England, 3 in Scotland, 11 in Wales) in the summer sampling period. A sample size of 30 animals gives a 99% probability of detection if the prevalence of Bd infection is around 15%, or a 79%probability of detection if the prevalence of Bd infection is around 5%. Given the necessary assumptions in the sample size calculations, there is little discernible effect of 28 *vs* 30 samples, especially since Bd prevalence is higher than 5% (and is usually higher than 15%) in most published studies. We are, therefore, reasonably confident that we have a high probability of detecting Bd infection in at least 105 sites in the spring and in at least 77 sites in the summer.

During the spring sampling period, 10 Bd-positive ponds were detected in Great Britain: 10 of 105 ponds where at least 28 amphibians had been swabbed compared with none of 13 ponds sampled where fewer than 28 amphibians had been swabbed (one site was not visited for sampling in the spring).

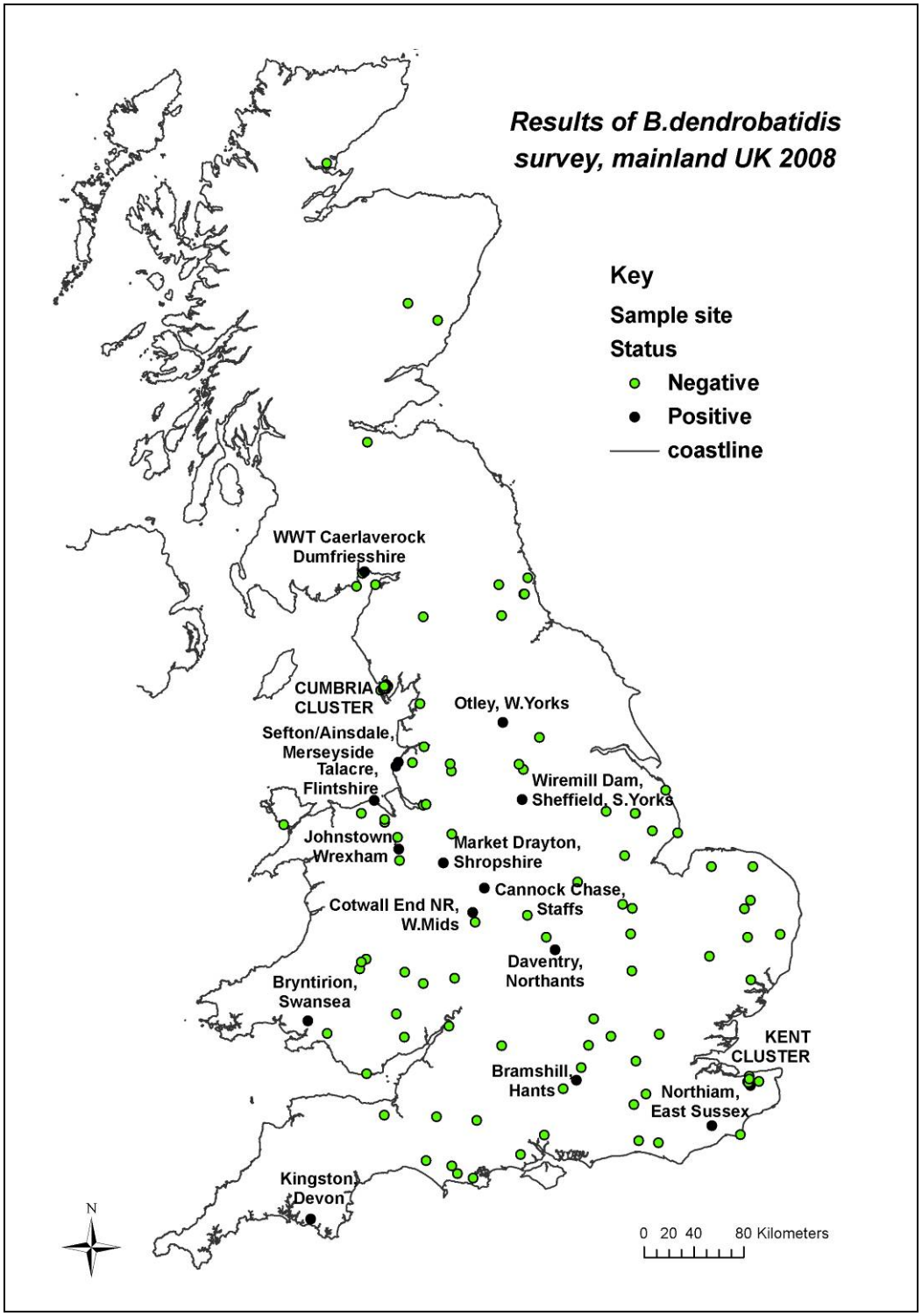

Figure 1. Map of mainland Great Britain depicting the sites sampled in 2008 and differentiating between Bd-positive (black dots) and Bd-negative (green dots) sites. Site names are shown for positive sites only.

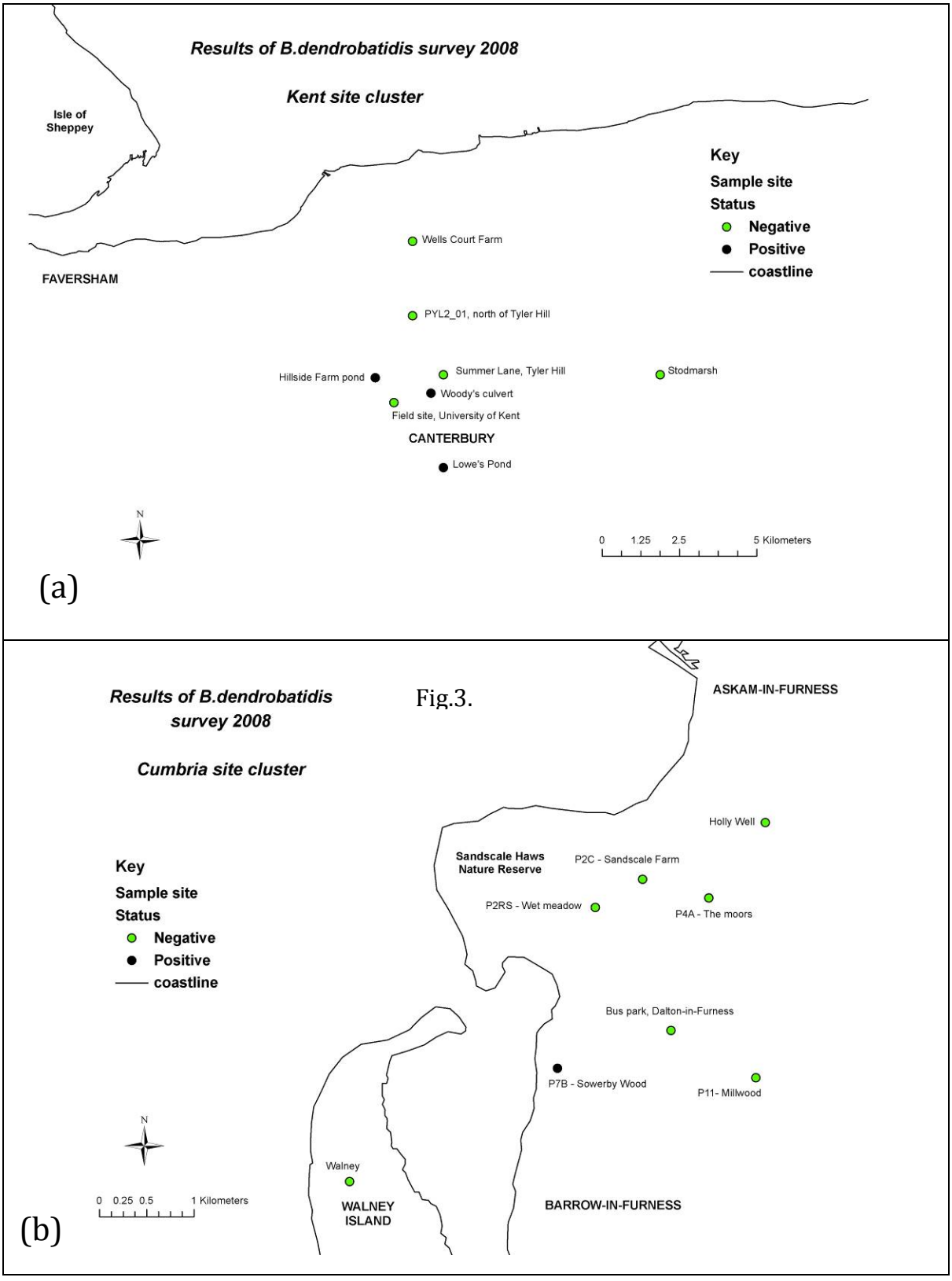

Fig.3.

Figure 2. Maps showing sites where clusters of ponds were sampled: (a) East Kent and (b) South Cumbria. Sites with Bd-positive amphibians are depicted by black dots; Bd-negative sites are depicted by green dots.

During the summer sampling period, nine Bd-positive ponds were detected on mainland Great Britain: 8 of 77 ponds where at least 28 amphibians had been swabbed, and 1 of 13 ponds sampled where fewer than 28 amphibians had been swabbed. In this latter pond, eight of 14 animals sampled were Bd-positive; all animals sampled in this pond were natterjack toads. 29 sites were not visited for sampling in the summer period.

Both sites in Jersey were sampled only in the spring. At one site, 86 common toads were swabbed, 11 at the other. All animals tested from Jersey were negative for Bd. The former site was likely adequately sampled to detect Bd if it was present. The latter site likely was not adequately sampled to have confidence in the negative result, unless this comprised a large proportion of the amphibian population at this site.

The percentage of sites testing positive across countries was remarkably consistent. Across Great Britain 19 of 119 (16%) sites tested were Bd-positive: 1 of 7 (14%) in Scotland, 3 of 16 (19%) in Wales and 15 of 96 (16%) in England. A list of all infected and uninfected sites from the national 2008 survey, including details of the amphibians testing positive, is shown in Appendix 2.

These findings demonstrate that the distribution of Bd infection is much wider throughout Great Britain than had previously been realised. Previously, Bd infection was known only from a small number of sites in Cumbria and Kent. It is worth emphasizing, however, that the selection of sites in this survey was not random but 'semi-stratified random', i.e. sites were chosen with some selection bias (see Methods). In addition, two clusters of sites (in Kent and Cumbria) were tested due to their proximity to known positive sites. This non-random aspect of the study could result in an overestimation of the extent of the distribution of Bd throughout Great Britain.

Comparison of infection between species

In order to investigate possible differences between the infection prevalence of Bd in different species following exposure to Bd, only data from known Bd-positive ponds was used (Table 2). Ponds that tested positive during one sampling period only were assumed to be Bd positive for both sampling periods. It was also assumed that, if at least one animal tested positive from a site, that all animals tested at that site (for both sampling periods) were equally likely to have been exposed to the pathogen.

This analysis showed that natterjack toads were the most likely species to be Bd-positive in the spring, but that common toads were the most likely species to be positive in the summer. Common frogs and smooth newts and palmate newts gave much lower prevalences for Bd infection. Of 43 great crested newts examined from Bd-positive ponds, none was positive for infection.

Of the non-native species examined, 19 pool frogs were tested across 2 sites, 64 marsh frogs from four sites and 135 alpine newts from 11 sites. Pool frogs tested positive from one site, alpine newts tested positive from six sites and no marsh frogs tested positive.

Table 1. Results of Bd qPCR analyses showing the numbers of each species of amphibian caught in Great Britain in each sampling period with the results of Bd-qPCR for each species. Results for non-native species are presented in the shaded rows.

species	seasonal comparison							total*		
	spring				summer					
	N$^{o.}$ swabbed	N$^{o.}$ Bd +ve	% Bd +ve		N$^{o.}$ swabbed	N$^{o.}$ Bd +ve	% Bd +ve		N$^{o.}$ swabbed	N$^{o.}$ Bd +ve	% Bd +ve
Common toad *Bufo bufo*	997	7	0.7		217	11	5.1		1214	18	1.5
Common frog *Rana temporaria*	241	1	0.4		152	0	0		393	1	0.3
Natterjack toad *Epidalea calamita*	116	20	17.2		36	1	2.8		152	21	13.8
Great crested newt *Triturus cristatus*	294	0	0		296	0	0		590	0	0
Palmate newt *Lissotriton helveticus*	682	1	0.1		720	0	0		1402	1	0.1
Smooth newt *Lissotriton vulgaris*	950	5	0.5		899	12	1.3		1849	17	0.9
Pool frog *Pelophylax lessonae*	0				19	3	15.8		19	3	15.8
Marsh frog *Pelophylax ridibundus*	28	0	0		36	0	0		64	0	0
Alpine newt *Mesotriton alpestris*	68	1	1.5		67	4	6.0		135	5	3.7

*In addition, one unidentified newt was sampled in the spring and 42 unidentified newts were sampled in the summer. None of the unidentified newts was positive for Bd.

Table 2. Bd prevalence in each species of amphibian sampled from Bd-positive ponds, showing a comparison between spring and summer. Results for non-native species are presented in the shaded rows.

species	seasonal comparison									total			
	spring*				summer*								
	No. ponds	N$^{o.}$ swabbed	N$^{o.}$ Bd +ve	% Bd +ve	No. ponds	N$^{o.}$ swabbed	N$^{o.}$ Bd +ve	% Bd +ve		No. ponds	N$^{o.}$ swabbed	N$^{o.}$ Bd +ve	% Bd +ve
Common toad *Bufo bufo*	9	193	7	3.6	4	23	11	47.8		10	216	18	8.3
Common frog *Rana temporaria*	8	21	1	4.8	2	23	0	0		9	44	1	2.3
Natterjack toad *Epidalea calamita*	4	64	20	31.2	1	28	1	3.6		4	92	21	22.8
Great crested newt *Triturus cristatus*	3	25	0	0	4	18	0	0		5	43	0	0
Palmate newt *Lissotriton helveticus*	6	73	1	1.4	9	127	0	0		10	200	1	0.5
Smooth newt *Lissotriton vulgaris*	10	139	5	3.6	11	153	12	7.8		14	292	17	5.8
Pool frog *Pelophylax lessonae*	0				1	14	3	21.4		1	14	3	21.4
Marsh frog *Pelophylax ridibundus*	0				0					0			
Alpine newt *Mesotriton alpestris*	4	51	1	2.0	6	55	4	7.3		6	106	5	4.7

Comparison of infection between site types

Sites with non-native species
A site was considered to contain non-native species if (a) it had been deliberately chosen for testing because of the known presence of at least one non-native species of amphibian, or (b) at least one of the amphibians swabbed at that site during the current study had been identified as a non-native species. In addition, the site at Northiam was considered a non-native site because North American bullfrogs were known to have been present at this site in the recent past. The data for mainland Great Britain were then examined for a possible association between the presence of non-native species and the presence of Bd using the Pearson chi-square test.

This analysis found that a site is significantly (X^2 = 11.5, p < 0.001) more likely to be positive for Bd if it contains non-native amphibians (8 of 19 sites) than if only native amphibian species are present (11 of 100 sites).

Natterjack toad sites
Known natterjack toad breeding sites (regardless of whether natterjacks formed part of the sample) showed an increased likelihood of testing positive for the presence of Bd. Four out of 10 natterjack sites tested positive, compared to 15 of 109 sites without natterjacks, X^2 = 4.7, p = 0.03.

There is no association in this dataset between non-native sites and natterjack sites (X^2 = 2.1, p < 0.15), i.e. results of this survey do not suggest that non-natives and natterjacks are more likely to occur in the same place than would be expected by chance. It is possible that direct or indirect contact with non-native species, current or historic, might be a factor and this, amongst other possibilities, requires investigation. All of the Bd-positive natterjack sites recorded so far in the UK are in north-west England, south-west Scotland or north Wales. No natterjack sites or natterjack toads have tested positive in southern or eastern England, either in this study or in previous ones.

SUMMARY OF MAIN FINDINGS AND CONCLUSIONS

This survey has been a very useful exploratory study, in terms of defining the likely extent of Bd infection of amphibians in Great Britain. Although possibly the largest national survey yet undertaken for Bd infection anywhere in the world, the study does have its limitations and the results should be examined only for evidence of trends rather than to provide exact data on the Bd-status of any given species, site or region.

Major findings from this survey are:

(1) The presence of Bd-positive amphibians at sites across Great Britain, albeit in low numbers (prior to this survey Bd infection was known in GB only

from Kent and Cumbria). Bd therefore seems to be widely distributed at the broad scale.

(2) A strong association between the presence of non-native amphibian species and the presence of Bd infection. This finding supports an hypothesis that non-native species are linked to the introduction and spread of Bd, but further work is required to verify if this is the case.

(3) All native amphibian species (except the great crested newt) tested positive at least once in this survey, confirming a low host specificity of Bd in Great Britain. Any future research on the impacts of Bd on British amphibians and any attempts to contain or control the spread of Bd will need to take this into account.

(4) There is a marked difference in the prevalence of Bd-infection between species and, within some species, between seasons. These data indicate that, in order to maximise the chance of detecting the presence of Bd infection, natterjack toads should be sampled at natterjack breeding ponds in the spring, whilst common toads should be skin-swabbed in the summer.

The study raises some important areas for future research. It would be interesting to investigate potential confounding factors which could impact the apparent Bd-status of an animal (and hence the apparent Bd-prevalence in a species). From work elsewhere, it appears that animals captured from water at a Bd-positive site are more likely to have detectable infection that those caught on land. It is possible, for example, that natterjack toads were more likely to be aquatic in the spring and terrestrial in the summer. For future surveys, it should be recorded on the swab whether an animal was collected from water or from land.

The study did not collect detailed information on the site-specific factors that might predispose populations to infection (and maintenance of infection). The information on non-native species occurrence, for example, is incomplete and needs further exploration. A follow-up study collecting more details on each site is recommended.

The current survey was designed only to assess if Bd was present at a site and was not designed to assess disease prevalence within sites. At some sites several amphibians in the same sample tested positive, suggesting a high prevalence of chytrid infection. However, the protocol allowed surveyors to collect amphibians into the same bucket before sampling, so there is a possibility that infection was transmitted between individuals at the time of capture. A requirement to collect amphibians into different containers and prevent transmission between individuals would be onerous given the constraints of working in the field. It is therefore suggested that the protocol for volunteers is not altered in this respect, especially if the focus of the study remains disease detection, not prevalence.

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We thank the funders and our colleagues at other NGOs, but most of all we thank the many volunteers who made this study possible. Volunteers were crucial to the implementation of this survey and their contribution is much appreciated. Thanks are also due to the landowners who allowed access to the amphibians on their land.

Eddie Brede co-ordinated the survey, organised and trained many of the volunteers, conducted swabbing at several sites and analysed many of the swabs. Matthew Perkins, Judith Hidalgo-Vila and Jennifer Sears also carried out swabbing at many sites and analysed many thousands of swabs. TJ McKinley, University of Cambridge, provided statistical advice, including advice on the study design.

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Appendix 1. Details of sites where amphibians were sampled for Bd infection, including the number of animals sampled and the number positive for Bd.

Country	Region	Site Name	Easting	Northing	NJ* site	alien species present	Number amphibians swabbed (number Bd-positive)			site Bd-positive
							spring	summer	total	
England	**North West**	Darcy Lever gravel pits	374100	407700	no	no	30 (0)	29 (0)	59 (0)	no
		WWT Martin Mere	342800	414300	no	no	30 (0)	30 (0)	60 (0)	no
		Broadfields, Winterley	374500	357500	no	no	30 (0)	30 (0)	60 (0)	no
		Williamson Park	348900	461300	no	no	28 (0)	14 (0)	42 (0)	no
		Bolton garden pond, Bromley Cross, Lancs.	373100	413300	no	no	30 (0)	29 (0)	59 (0)	no
		Marsh Way pond, Penwortham	352100	426900	no	no	4 (0)	0	4 (0)	no
		Rocksavage, Runcorn	351500	380500	no	no	32 (0)	22 (0)	54 (0)	no
		Ineos Ltd., Runcorn	353800	381200	no	no	28 (0)	27 (0)	55 (0)	no
		P4a - The Moors, Dalton, Cumbria	320800	474900	no	no	12 (0)	0	12 (0)	no
		P11 - Millwood, Dalton, Cumbria	321300	473000	no	no	30 (0)	0	30 (0)	no
		PX1 - Chapel Hills, Dalton, Cumbria	321600	474700	no	no	30 (0)	0	30 (0)	no
		P2RS - Wet meadow, Dalton, Cumbria	319600	474800	no	no	31 (0)	0	31 (0)	no
		P7b - Sowerby Wood, Dalton, Cumbria	319200	473100	no	no	30 (1)	30 (0)	60 (1)	yes

Country	Region	Site Name	Easting	Northing	NJ* site	alien species present	Number amphibians swabbed (number Bd positive)			Site Bd-positive
							spring	summer	total	
		P29 - Rakes Cottage, Dalton, Cumbria	322900	475300	no	no	30 (0)	30 (0)	60 (0)	no
		Business park, Cumbria	320400	473500	no	no	30 (0)	30 (0)	60 (0)	no
		Long Pond, Cumbria	321700	476400	no	no	30 (0)	30 (0)	60 (0)	no
		Holly Well, Cumbria	321400	475700	no	no	30 (0)	30 (0)	60 (0)	no
		P2c - Sandscale Farm, Dalton, Cumbria	320100	475100	no	no	30 (0)	30 (0)	60 (0)	no
		North Walney, Cumbria	317000	471900	yes	no	18 (0)	30 (0)	48 (0)	no
		Birkdale, Sefton, Merseyside	331400	414800	yes	no	30 (16)	0	30 (16)	yes
		Ainsdale, Sefton, Merseyside	329300	411500	yes	no	30 (1)	0	30 (1)	yes
		Grune, Skinburness, Cumbria	313000	556000	yes	no	22 (0)	0	22 (0)	no
		Nichol Hill, Penrith, Cumbria	351500	530500	no	yes	30 (0)	8 (0)	38 (0)	no
	North East	Tyne & Wear	435500	561500	no	no	30 (0)	0	30 (0)	no
		Rainton Meadows Pond, Co. Durham	432300	548500	no	no	30 (0)	0	30 (0)	no
		Joe's Pond, Co. Durham	432800	548700	no	no	30 (0)	0	30 (0)	no
		Gibside, Derwent Valley	412200	556000	no	no	30 (0)	30 (0)	60 (0)	no
		Bishop Auckland	414500	531500	no	no	30 (0)	0	30 (0)	no
	Yorkshire	Coburn Hill Wood, Leeds	444800	434500	no	no	30 (0)	30 (0)	60 (0)	no

Country	Region	Site Name	Easting	Northing	NJ* site	alien species present	Number amphibians swabbed (number Bd-positive)			Site Bd-positive
							spring	summer	total	
		Wiremill Dam	431000	385000	no	no	30 (5)	0	30 (5)	yes
		Barugh Green, Barnsley	431800	408800	no	no	30 (0)	0	30 (0)	no
		Yorkshire sculpture park, near Barnsley	428400	413000	no	no	0	17 (0)	17 (0)	no
		Sun Lane, Otley, W.Yorks	415500	446600	no	yes	30 (0)	30 (1)	60 (1)	yes
	West Midlands	Colwall, Malvern	376470	242760	no	no	30 (0)	30 (0)	60 (0)	no
		Milford Quay, Cannock Chase, Staffs	400500	314500	no	no	30 (0)	30 (2)	60 (2)	yes
		Cotwall End NR	391100	295200	no	yes	30 (0)	30 (2)	60 (2)	yes
		Begwyns, Herefordshire	351500	238500	no	no	30 (0)	30 (0)	60 (0)	no
		Nuneaton, Warwickshire	435100	292800	no	no	30 (0)	30 (0)	60 (0)	no
		Darkley, Herefordshire	336600	247600	no	no	30 (0)	0	30 (0)	no
		Saltwells NR, Quarry Bank	393100	287200	no	no	30 (0)	30 (0)	60 (0)	no
		Rugby, Warwickshire	450500	275500	no	no	30 (0)	30 (0)	60 (0)	no
		Market Drayton, Shropshire	367500	334500	no	yes	30 (0)	30 (3)	60 (3)	yes
	East Midlands	Riseholm, Lincoln	498500	375500	no	no	20 (0)	0	20 (0)	no
		Baumber, Lincolnshire	522000	374000	no	no	30 (0)	30 (0)	60 (0)	no
		Gibraltar Point,	556100	358600	no	no	30 (0)	30 (0)	60 (0)	no

Country	Region	Site Name	Easting	Northing	NJ* Site	alien species present	Number amphibians swabbed (number Bd-positive)			Site Bd-positive
							spring	summer	total	
		Skegness, Lincs								
		Stickford, Boston, Lincs	535700	360100	no	no	30 (0)	30 (0)	60 (0)	no
		Daventry Lang Farm	457500	265500	no	no	30 (0)	14 (8)	44 (8)	yes
		Helpringham	513500	340500	no	no	30 (0)	30 (0)	60 (0)	no
		Melton Mawbray	475500	319500	no	no	30 (0)	30 (0)	60 (0)	no
		Saltfleetby, Lincs	546500	392400	yes	no	30 (0)	30 (0)	60 (0)	no
	East of England	Romford	unknown	unknown	no	no	6 (0)	4 (0)	10 (0)	no
		Peterborough Canal	519400	298200	no	no	35 (0)	0	35 (0)	no
		Goswold Farm, Thrandeston, Suffolk	612500	275500	no	no	30 (0)	0	30 (0)	no
		Syderstone Common	583300	331500	no	no	30 (0)	30 (0)	60 (0)	no
		Castor Hanglands NNR	511800	301700	no	no	30 (0)	30 (0)	60 (0)	no
		Oak Plain, Loughton, Essex	541200	198200	no	no	30 (0)	30 (0)	60 (0)	no
		Halesworth	638500	277500	no	no	29 (0)	19 (0)	48 (0)	no
		London Wetland Centre	522600	176800	no	yes	31 (0)	30 (0)	61 (0)	no
		Fairy Lake, Ickworth Park, Suffolk	581800	260300	no	no	30 (0)	30 (0)	60 (0)	no
		Water Meadows, Bobbits Lane, Ipswich	614800	241400	no	no	30 (0)	30 (0)	60 (0)	no
		Alconbury Hill, Godmanchester	518400	277900	no	no	28 (0)	30 (0)	58 (0)	no
		Sandy, Bedfordshire	519100	248500	yes	no	30 (0)	30 (0)	60 (0)	no
		Wolterton, Norfolk	616500	331500	no	yes	12 (0)	0	12 (0)	no

Country	Region	Site Name	Easting	Northing	NJ* site	alien species present	Number amphibians swabbed (number Bd-positive)			Site Bd-positive
							spring	summer	total	
	South East England	Aston Clinton, Bucks	488500	210300	no	no	30 (0)	30 (0)	60 (0)	no
		Badgerwood House, Fulking	524800	113400	no	no	30 (0)	0	30 (0)	no
		Marlow Bottom, High Wycombe	484300	189300	no	no	30 (0)	30 (0)	60 (0)	no
		Quobleigh Pond, Fair Oaks, Hants	448800	117800	no	no	30 (0)	30 (0)	60 (0)	no
		Northiam, East Sussex	583500	125300	no	no	30 (0)	30 (3)	60 (3)	yes
		Popley Fields, Basingstoke	463900	154600	no	no	30 (0)	30 (0)	60 (0)	no
		Puddletown Forest, Dorset	374400	093200	no	no	30 (0)	30 (0)	60 (0)	no
		Dinton Pastures Country Park, Hurst, Berks	478400	171700	no	no	30 (0)	30 (0)	60 (0)	no
		Moorfield Drainage Dyke	unknown	unknown	no	no	30 (0)	30 (0)	60 (0)	no
		Lowes Pond, Canterbury	614500	157500	no	yes	29 (0)	29 (1)	58 (1)	yes
		Woody's Culvert, Canterbury	614100	159900	no	yes	30 (1)	29 (0)	59 (1)	yes
		Summer Lane, Tyler Hill, Canterbury	614500	160500	no	yes	13 (0)	23 (0)	36 (0)	no
		Hillside Farm, Canterbury	612300	160400	no	yes	30 (0)	31 (2)	61 (2)	yes
		University Field Site, Canterbury	612900	159600	no	yes	30 (0)	30 (0)	60 (0)	no
		Garden Ponds, Wells Court Farm, Canterbury	613500	164800	no	yes	30 (0)	29 (0)	59 (0)	no

Country	Region	Site Name	Easting	Northing	NJ* site	alien species present	Number amphibians swabbed (number Bd-positive)			Site Bd-positive
							spring	summer	total	
		PYL2, near Tyler Hill, Canterbury	613500	162400	no	yes	23 (0)	0	23 (0)	no
		Chorleywood Common, Herts	502500	196500	no	yes	30 (0)	30 (0)	60 (0)	no
		Stodmarsh, Kent	621500	160500	no	yes	30 (0)	0	30 (0)	no
		Dungeness RSPB, Kent	606700	118200	no	yes	30 (0)	30 (0)	60 (0)	no
		Nutfield, Surrey	530500	150500	no	yes	30 (0)	30 (0)	60 (0)	no
		Beambrook, Surrey	521000	142000	no	yes	30 (0)	30 (0)	60 (0)	no
		Offham marsh, Offham, E.Sussex	540700	111900	no	yes	30 (0)	30 (0)	60 (0)	no
	South West England	Slimbridge	372200	204700	no	no	30 (0)	30 (0)	60 (0)	no
		Abbey Meads School, Swindon	414300	188900	no	no	30 (0)	30 (0)	60 (0)	no
		Powerstock Common, Dorset	353800	097400	no	no	30 (0)	30 (0)	60 (0)	no
		Brockenhurst, Hants	429500	102500	no	no	30 (0)	30 (0)	60 (0)	no
		Tisbury, Wilts	394500	129500	no	no	30 (0)	0	30 (0)	no
		Wells, Somerset	362000	132300	no	no	30 (0)	30 (0)	60 (0)	no
		Little Pond, Creech, Dorset	391500	083500	no	no	30 (0)	30 (0)	60 (0)	no
		Scobbiscombe Farm, Kingston, Devon	260800	050800	no	no	30 (1)	30 (1)	60 (2)	yes
		Gibb Hill, Bridgewater	319900	133700	no	no	26 (0)	30 (0)	56 (0)	no
		Bramshill, Hants	474500	161500	no	yes	30 (0)	30 (7)	60 (7)	yes
		Galton Bog, Dorset	379000	087300	no	yes	30 (0)	0	30 (0)	no
Scotland		Pumpherston,	306565	669475	no	no	30 (0)	30 (0)	60 (0)	no

Country	Region	Site Name	Easting	Northing	NJ* site	alien species present	Number amphibians swabbed (number Bd-positive)			Site Bd-positive
							spring	summer	total	
		Livingston, W.Lothian								
		Astle, Sutherland	273900	891800	no	no	30 (0)	2 (0)	32 (0)	no
		Capo Plantation, Angus	363100	766700	no	no	30 (0)	30 (0)	60 (0)	no
		Glen Lee, Angus	339000	780300	no	no	30 (0)	0	30 (0)	no
		Southerness, Dumfriesshire	297700	554800	yes	no	30 (0)	0	30 (0)	no
		Caerlaverock NNR, Dumfriesshire	302600	565000	yes	no	23 (0)	0	23 (0)	no
		WWT Caerlaverock, Dumfriesshire	304100	566500	yes	no	30 (4)	30 (0)	60 (4)	yes
Wales		Llangybi, Monmouthshire	336190	195960	no	no	30 (0)	5 (0)	35 (0)	no
		Abergavenny, Monmouthshire	329800	214300	no	no	30 (0)	30 (0)	60 (0)	no
		Brookhill, near Oswestry	332300	336500	no	no	30 (0)	30 (0)	60 (0)	no
		Bryntirion, Llanedi, Swansea	258500	208800	no	no	30 (1)	30 (0)	60 (1)	yes
		Waunceirch, Neath, Port Talbot	274100	198700	no	no	30 (0)	30 (0)	60 (0)	no
		Mold Road, Wrexham, Denbighshire	330800	354800	no	no	30 (0)	15 (0)	45 (0)	no
		Canada Pool, Newborough Forest, Anglesey	239200	364800	no	no	30 (0)	30 (0)	60 (0)	no
		Builth Wells, Powys	300400	250200	no	no	30 (0)	30 (0)	60 (0)	no
		Talacre, Flintshire	312000	384300	yes	no	30 (4)	28 (1)	58 (5)	yes
		Rhydymwyn Valley	320500	366800	no	no	30 (0)	0	30 (0)	no

Country	Region	Site Name	Easting	Northing	NJ* site	alien species present	Number amphibians swabbed (number Bd-positive)			Site Bd-positive
							spring	summer	total	
		NR, Flintshire								
		Rhoose, Vale of Glamorgan	305900	166600	no	no	20 (0)	30 (0)	50 (0)	no
		Pen-y-Bane Pond, Powys	305420	258050	no	no	30 (0)	30 (0)	60 (0)	no
		Craig Goch/Cors y Lyn, Powys	301620	255620	no	no	30 (0)	30 (0)	60 (0)	no
		Saint Asaph Business Park, Clwyd	301600	373900	no	no	30 (0)	0	30 (0)	no
		Johnstown, Wrexham	331600	345400	no	no	27 (1)	10 (0)	37 (1)	yes
		Halkyn Mountain SAC, Flintshire	320400	369300	no	no	30 (0)	30 (0)	60 (0)	no
Channel Islands	**Jersey**	Grosnez, Jersey	unknown	unknown	no	no	86 (0)	0	86 (0)	no
		Les Landes, Jersey	unknown	unknown	no	no	11 (0)	0	11 (0)	no

*NJ = natterjack toad

Appendix 2. Bd-positive sites showing the number and species of animals tested and those that tested positive for Bd.

LOCATION	alien species present	NJ†	Total N°. amphibians tested	Amphibians tested for Bd	Bd-positive amphibians
Scobbiscombe Farm, Kingston, Devon	No	No	60	Spring: 30 palmate newt Summer: 25 palmate newt 5 common toad	Spring: 1 palmate newt Summer: 1 common toad
Northiam, E.Sussex	No	No	60	Spring: 27 smooth newt 3 common frog Summer: 30 smooth newt	Summer: 3 smooth newt
Lowe's Pond, Canterbury, Kent	Yes	No	58	Spring: 22 alpine newt 3 palmate newt 1 smooth newt 3 common frog Summer: 15 alpine newt 14 palmate newt	Summer: 1 alpine newt
Woody's Culvert, University of Kent, Kent	Yes	No	59	Spring: 7 alpine newt 21 palmate newt 2 smooth newt Summer: 8 alpine newt 17 palmate newt 4 smooth newt	Spring: 1 alpine newt
Hillside Farm Toad Pond, Canterbury, Kent	Yes	No	61	Spring: 30 common toad Summer: 14 alpine newt 3 palmate newt 12 smooth newt 2 great crested newt	Summer: 1 alpine newt 1 smooth newt

LOCATION	alien species present	NJ†	Total No. amphibians tested	Amphibians tested for Bd	Bd-positive amphibians
Bramshill, Hants	Yes	No	60	Spring: 29 smooth newt 1 palmate newt Summer: 16 smooth newt 14 pool frog	Summer: 3 pool frog
Bryntirion, Llanedi, Swansea	No	No	60	Spring: 30 common toad Summer: 30 palmate newt	Spring: 1 common toad
Lang Farm, Daventry, Northants	No	No	44	Spring: 26 common toad 4 common frog Summer: 13 common toad 1 smooth newt	Summer: 8 common toad
Cotwall End NR, W.Mids	No	No	60	Spring: 30 common toad Summer: 2 alpine newt 2 smooth newt 3 great crested newt 19 common frog 4 common toad	Summer: 2 common toad
Milford Quay, Cannock Chase, Staffs	No	No	60	Spring: 5 smooth newt 10 great crested newt 5 common frog 10 common toad Summer: 20 smooth newt 1 palmate newt 9 great crested newt	Summer: 2 smooth newt

LOCATION	alien species present	NJ†	Total N°. amphibians tested	Amphibians tested for Bd	Bd-positive amphibians
Market Drayton, Shropshire	Yes	No	60	Spring: 11 alpine newt 19 smooth newt Summer: 3 alpine newt 26 smooth newt 1 common frog	Summer: 2 smooth newt 1 alpine newt
Johnstown, Wrexham, Denbighshire	No	No	37	Spring: 7 smooth newt 10 palmate newt 10 great crested newt Summer: 6 palmate newt 4 great crested newt	Spring: 1 smooth newt
Talacre, Flintshire	No	Yes	58	Spring: 30 natterjack toad Summer: 28 natterjack toad	Spring: 4 natterjack toad Summer: 1 natterjack toad
Wiremill Dam, Sheffield, S.Yorks	No	No	30	Spring: 30 common toad	Spring: 5 common toad
Ainsdale, Sefton, Merseyside	No	Yes	30	Spring: 20 smooth newt 5 great crested newt 4 natterjack toad 1 common frog	Spring: 1 natterjack toad
Birkdale, Sefton, Merseyside	No	Yes	46	Spring: 29 natterjack toad 1 common frog	Spring: 15 natterjack toad 1 common frog
Sun Lane, Otley, W.Yorks	Yes	No	60	Spring: 11 alpine newt 3 smooth newt 8 palmate newt 2 common frog 6 common toad Summer: 13 alpine newt 11 smooth newt 6 palmate newt	Summer: 1 alpine newt

LOCATION	alien species present	NJ†	Total N°. amphibians tested	Amphibians tested for Bd	Bd-positive amphibians
P7b - Sowerby Wood, Dalton, Cumbria	No	No	60	Spring: 30 common toad Summer: 5 smooth newt 25 palmate newt	Spring: 1 common toad
WWT Caerlaverock, Dumfriesshire	No	Yes	60	Spring: 26 smooth newt 2 common frog 1 common toad 1 natterjack toad Summer: 26 smooth newt 3 common frog 1 common toad	Spring: 4 smooth newt

† NJ = natterjack toad site