LETTER

Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia

Susan F. Walker,[1,4] Jaime Bosch,[2] Virgilio Gomez,[3] Trenton W. J. Garner,[4] Andrew A. Cunningham,[4] Dirk S. Schmeller,[5] Miguel Ninyerola,[6] Daniel A. Henk,[1] Cedric Ginestet[7] Christian-Philippe Arthur[8] and Matthew C. Fisher[1]*

[1]*Department of Infectious Disease Epidemiology, Imperial College London W2 1PG, UK*
[2]*Museo Nacional de Ciencias Naturales, CSIC, c/ José Gutiérrez Abascal 2, 28006 Madrid, Spain*
[3]*Departamento de Matemáticas, Escuela de Ingenieros Industriales – Albacete Universidad de Castilla-La Mancha, 02071 Albacete, Spain*
[4]*Institute of Zoology, Regent`s Park, NW1 4RY London, UK*
[5]*La Station d`Ecologie Expérimentale du CNRS, 09200 Moulis, France*
[6]*Unit of Botany, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain*
[7]*Department of Epidemiology and Public Health, Imperial College London W2 1PG, UK*
[8]*Service Scientifique, Parc National des Pyrénées, 59 Route de Pau, 65000, Tarbes, France*
Correspondence: E-mail: matthew.fisher@imperial.ac.uk

Abstract

Amphibian chytridiomycosis is a disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). Whether *Bd* is a new emerging pathogen (the novel pathogen hypothesis; NPH) or whether environmental changes are exacerbating the host-pathogen dynamic (the endemic pathogen hypothesis; EPH) is debated. To disentangle these hypotheses we map the distribution of *Bd* and chytridiomycosis across the Iberian Peninsula centred on the first European outbreak site. We find that the infection-free state is the norm across both sample sites and individuals. To analyse this dataset, we use Bayesian zero-inflated binomial models to test whether environmental variables can account for heterogeneity in both the presence and prevalence of *Bd,* and heterogeneity in the occurrence of the disease, chytridiomycosis. We also search for signatures of *Bd*-spread within Iberia using genotyping. We show (1) no evidence for any relationship between the presence of *Bd* and environmental variables, (2) a weak relationship between environmental variables and the conditional prevalence of infection, (3) stage-dependent heterogeneity in the infection risk, (4) a strong association between altitude and chytridiomycosis, (5) multiple Iberian genotypes and (6) recent introduction and spread of a single genotype of *Bd* in the Pyrenees. We conclude that the NPH is consistent with the emergence of *Bd* in Iberia. However, epizootic forcing of infection is tied to location and shaped by both biotic and abiotic variables. Therefore, the population-level consequences of disease introduction are explained by EPH-like processes. This study demonstrates the power of combining surveillance and molecular data to ascertain the drivers of new emerging infections diseases.

INTRODUCTION

Patterns of infection are informative about the epidemiology and spatio-temporal spread of pathogens, and the role of environmental factors in forcing infection dynamics. Emergence of the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is now widely recognised as a proximate driver of amphibian declines across the globe (Berger *et al.* 1998; Fisher *et al.* 2009b). Following the relatively synchronous discovery of *Bd* in Australia (1993–1994), Panama (1996–1997) and

Europe (1997), the occurrences of global amphibian declines attributed to chytridiomycosis have been accounted for by two non mutually-exclusive hypotheses. The novel/spreading pathogen hypothesis (NPH) states that the timing of disease-driven declines have resulted from the spread of *Bd* subsequent to its introduction into naïve host populations (Rachowicz *et al.* 2005; Skerratt *et al.* 2007). In Central America, for example, the sequential pattern of declines across a NW–SE trajectory are consistent with an introduction of *Bd* in the north of the region (Lips *et al.* 2008). The hypothesis of recent global spread is supported by the low genetic diversity exhibited by *Bd* and the lack of any strong support for phylogeographical clustering of genotypes collected from Africa, the Americas, Australia and Europe (James *et al.* 2009).

In contrast, the endemic pathogen hypothesis (EPH) states that the timing of declines is a consequence of changes in key environmental conditions that drive patterns of disease (Rachowicz *et al.* 2005). In the Neotropics and Europe, an increase in air temperatures has been linked to the timing of amphibian extinctions and declines (Pounds *et al.* 2006; Bosch *et al.* 2007). Climatic conditions can create key constraints on the distributions of pathogens and the epidemic potential of disease. The signature of this influence is typically highest for those infectious organisms with an environmental life history stage (such as the *Bd* zoospore) and a definitive host which is ectothermic; amphibian chytridiomycosis fulfils both these criteria. Akin to other ectotherms, the fitness and immune response of amphibians is particularly sensitive to the ambient temperature (Raffel *et al.* 2006). The survival, growth and reproduction of *Bd* is also temperature-dependent with a growth maximum between 17 and 25 °C (Piotrowski *et al.* 2004).

Disentangling the relative causality of NPH vs. EPH-type processes has proven problematic given the multiple temporal and spatial scales over which chytridiomycosis occurs. Given that *Bd* exhibits extremely low host specificity (http://www.spatialepidemiology.net/bd-maps; Fisher *et al.* 2009b) as well as distinct intra- and interspecific differences in the distribution of infection and disease in amphibians (Briggs *et al.* 2005; Woodhams *et al.* 2007), the understanding of external forcing factors on infection dynamics is best addressed using ecological studies of single species. Our research here focuses on *Alytes obstetricans*. This species of amphibian is ecologically and developmentally plastic and, given a permanence of water, is found across a wide range of ecotypes throughout much of Europe. The species is found from sea-level to altitudes exceeding 2500 m (Vences *et al.* 2003). At high altitudes, larvae undergo a prolonged period of development and may over-winter for at least one season. The potential for *A. obstetricans* to be highly susceptible to chytridiomycosis has been demonstrated by the near extirpation of this species by *Bd* in the Peñalara Natural Park, Spain. This is the first site in Europe where the pathogen and the disease were discovered to be affecting native amphibians (Bosch *et al.* 2001).

In this study, we detail the surveillance and analysis of the distribution of *Bd* and the occurrence of chytridiomycosis in populations of *A. obstetricans* across the Iberian Peninsula. We assess first, whether there is heterogeneity in the distribution of infection and second, whether environmental variables account for heterogeneity in (1) the presence/absence of infection (i.e. whether infection occurs at a site) and (2) the prevalence of infection (i.e. what is the proportion of *Bd*-infected individuals that are present at a site). We then determine whether these factors are linked to outbreaks of chytridiomycosis. Subsequently, we analyse multi-locus genotypes to assess whether there is evidence for single- or multiple- strains of *Bd* occurring in the Iberian Peninsula and whether these can be used to identify local patterns of introduction and spread. To conclude, we consider the implications of these ecological and molecular signatures on the novel and endemic pathogen hypotheses in Iberia.

MATERIALS AND METHODS

Field data

A total of 74 populations of *Alytes obstetricans* in the Pyrenees (PY) and a further 52 populations from within the Iberian Peninsula were surveyed for *Batrachochytrium dendrobatidis* (Fig. 1). Site selection was based upon the whereabouts of known populations of *A. obstetricans* (including distribution data from the Asociación Herpetológica Española) and explorations into areas considered to be suitable for *A. obstetricans*. Our studies are centred on two long-term *Bd* study sites: the *Bd* outbreak site at the Peñalara Natural Park (768 ha, 1800–2430 m), an alpine region in the Sierra de Guadarrama mountains of central Spain and Ibon Acherito, a site in the PY (1867 m). Fatal outbreaks of chytridiomycosis were first detected in these localities in 1997 and 2002 respectively.

Each study site was geo-referenced using GPS and sampled at least once during the study period (2003–2008 inclusive). The two long-term sites were visited annually from 2003 to 2008 inclusive. Sampling was conducted at each site during the summer months (June–September). During each site visit up to 30 larvae (L), up to 30 metamorphs (RM) and up to 30 adults (A) of *A. obstetricans* were collected. In localities where *A. obstetricans* was rare, the observed population was often sampled exhaustively. In an infinite population, a sample size of 30 is sufficient to detect at least one infected individual with a 95% probability if the underlying prevalence is 10% (Cannon & Roe 1982).
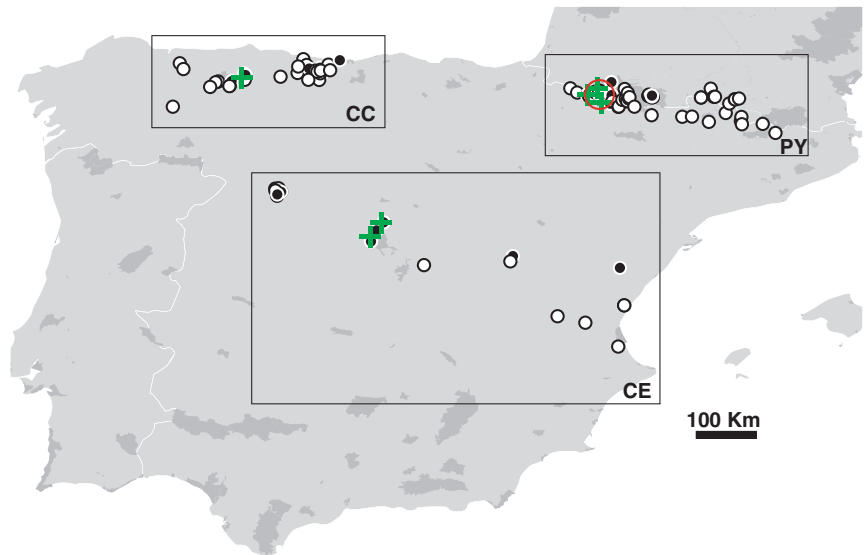
Figure 1 Distribution of surveillance sites. (a) Black circle, *Bd* detected; white circle, *Bd* not detected; green cross, outbreak of fatal chytridiomycosis. In the Pyrenees the spatial cluster detected by SᴀTSᴄᴀɴ v8 software is shown by a red circle. The heavily shaded regions are national parks.

The keratinised mouthparts of larval anuran stages and the skin of post-metamorphic stages were tested for *Bd* infection using the protocols of Hyatt *et al.* (2007). Wearing disposable gloves, the mouthparts of larvae between Gosner stages 26 and 41 were swabbed using sterile cotton-tipped swabs (MW100-100; Medical Wire & Equipment Co, Corsham, UK) (Boyle *et al.* 2004). Post-metamorphic animals were either sampled by toe-clipping (years 2003–2005, predominantly) or by swabbing the lower ventral surface and hind-limbs (years 2006–2008, predominantly). Tissue samples were stored in 70% ethanol and swabs stored dry. Upon return to the laboratory samples were stored at 4 °C until processing (Hyatt *et al.* 2007). The presence/absence of infection was then assessed using the quantitative PCR (qPCR) protocol described by Boyle *et al.* (2004).

Among infected sites, 'outbreak sites' were defined as sites where fatal chytridiomycosis affected over 15% of the total number of metamorphs. At sites where mortality among amphibians was observed, counts of dead and live recent metamorphs (RM) were made by walking the circumference of the target waterbody and searching beneath stones. Any carcasses were collected and stored in either 70% ethanol (for qPCR) or 10% formalin (for histopathology).

Environmental data

Summary climatic statistics (as detailed in Supporting information methods) were calculated by using the modelling framework outlined previously (Ninyerola *et al.* 2007a,b). Climatic grids had a 200 m spatial resolution and covered the period 1950–2000. In addition to the climatic variables, altitude (m), geographical coordinates (lati-tude/longitude) and broad geographical region was speci-fied. Three arbitrary geographical regions were recognised: the Cordillera-Cantabrica (CC) in the north (CC), the Pyrenees in the north-east (PY) and a diffuse NW–SE band running across central Spain including the Sierra de Guadarrama mountain range (CE) (Fig. 1). All of the quantitative covariates were standardised [(*x*-mean)/SD] prior to their use in the statistical models. Environmental correlates of outbreaks were assessed using the univariate non-parametric Wilcoxon test/Mann–Whitney test con-strained to the outbreak period (June, July and August).

Heterogeneity in patterns of infection and detection of disease clusters

In the two main sampled regions where sample sizes were adequate, PY and CC (not CE), the Potthoff Whittinghill test statistic (Potthoff & Whittinghill 1966) was used to assess the null hypothesis of homogeneity among all the relative risks. Under the null hypothesis, the number of cases in each site is Poisson distributed whereas the alternative hypothesis is one of over dispersion, represented by the negative binomial distribution. Subsequently, the spatial scan statistic implemented in SᴀTSᴄᴀɴ v8 software was used to detect spatial clusters with high levels of infection (Kulldorff 1997). For this analysis a Bernoulli model was defined.

Statistical modelling using the Bayesian zero-inflated binomial distribution

Rare diseases where the infection-free condition predomi-nates, are said to be 'zero-inflated'. Zero-inflation results in a

violation of the distributional assumptions of statistical models by, for instance, misinterpreting the variation to mean ratio in the error structure (see Supporting information for further details). As our dataset is characterised by an excess of zeros (i.e. uninfected sites), the number of infected amphibians at each site was modelled using a zero-inflated binomial (ZIB) distribution. Following the framework of Martin *et al.* (2005), this ZIB may be represented as

$$P[K_i = 0|n_i] = p_i + (1 - p_i)(1 - q_i)^{n_i}, i = 1, ..., n; and$$

$$P[K_i = k_i|n_i] = (1 - p_i)\binom{k_i}{n_i} q_i^{k_i}(1 - q_i)^{n_i - k_i},$$

Where k_i represents the number of infected individual in site i and each k_i can only take the following values $k_i \in \{0, 1, ..., n_i\}$, and n_i is the total number of amphibians observed in site i, while n is the total number of sites in the region of interest. The presence of *Bd* at a site is represented by p and the conditional prevalence of infection is represented by q. Here, conditional prevalence is defined as the prevalence of infection given an underlying assumption that the site is infected (i.e. the site is either known to be infected, or is estimated by the bayesian joint-distributions to have an associated probability of being infected). As explained in the supplementary information, we extend this model to incorporate the host's developmental stages [larva (l), recent metamorph (RM) and adult (a)] as a further variable which is nested within site.

Selected covariates were subsequently used in the full nested model. The Bayesian nested versions of the minimum adequate model (MAM) were implemented in the software WinBUGS, version 1.4.3 (Lunn *et al.* 2000). In each model two chains with different initial values for each of the parameters were specified. Given that the covariates and the intercepts act at the logit (Log-Odds Unit) scale, sets of initial values of 0 and 1 were used. In both of the chains for Y_i (the infection status of a site), zero was used if *Bd* had not been detected at the site and NA was used if *Bd* had been detected. Non-informative priors were specified for the beta coefficients (normal priors with mean = 0 and precision = 0.0001) and the alpha intercepts (normal prior with mean = 0 and precision 0.01). Following an initial burn-in of 1000 runs the models were run until acceptable convergence and autocorrelation had been obtained (Gelman *et al.* 2000). In the Bayesian framework, all the parameter estimates are treated as random variables, thus enabling probability statements to be made about them. The Markov Chain Monte Carlo simulation technique used in WinBUGS provides a joint distribution of the parameters in the model given the observed data. This is particularly useful allowing us to provide intervals for the conditional prevalence at a site.

Culturing and multilocus genotypes

Isolates of *Bd* from infected sites were recovered using protocols provided by Joyce Longcore. In some cases, *Bd* was recovered from co-habiting conspecific species. Following culture in mTGhL media, DNA was extracted from cultures following the protocol of Boyle *et al.* (2004). Subsequently, six polymorphic loci (8702 × 2, 8392 × 2, CTSYN, BdC5, BdC18 and BdC24), were amplified by PCR and scored according to the methods and conditions detailed by James *et al.* (2009). Phylogeny was inferred using parsimony with (1) a model of evolution that made loss of heterozygosity three times more likely than either gain of heterozygosity, or change from one homozygous allele to another through relatively rare sexual recombination and (2) a model that weighted movement from any state equally.

RESULTS

Distribution of infection and outbreaks of fatal chytridiomycosis

In total, 3016 individuals from across 126 sites were sampled. Of these sites, 25% ($n = 31$) were shown to be infected with *Bd* (Fig. 1). In the PY, the CC and the diffuse NW–SE band running across central Spain which included the Sierra de Guadarrama (CE), the proportion of infected sites was 23% ($n = 74$), 24% ($n = 34$) and 33% ($n = 18$) respectively (Table S1). There was no significant difference in the prevalence of infection between these regions (Fisher's exact two-sided test, $P = 0.643$). In the PY, all of the infected sites were found occupying a small region in the western half of the mountain range. Here, the prevalence of infection exceeded 0.6 in 35% ($n = 6$) of these sites. At infected sites among all three regions, the prevalence of infection (aggregated across developmental stages) varied from a median of 0.12 in the PY, to 0.72 and 0.90 in the CC, and CE respectively. Across all *Bd*-positive sites in Iberia the median prevalence of infection was 0.63.

Heterogeneity in patterns of infection and detection of disease clusters

In both PY and CC, the Potthoff Whittinghill test statistic (T) was highly significant and the null hypothesis of homogeneity of relative risks was rejected (PY, $T = 22091.77$, $P < 0.0001$; CC, $T = 1385326$, $P < 0.0001$). The spatial scan statistic detected clusters of infection in PY though no clusters were detected in CC. In the most likely spatial cluster [coordinates/radius (42.880200 N, 0.640133 W)/25.17 km] in the PY, 15 of

the 33 sites were actually infected (Fig. 1); this was almost twice that expected (Loglikelihood ratio = 7.79, relative risk = 6.36, P = 0.019). A secondary cluster in the east of the Pyrenees National park was also identified [Coordinates/radius (42.862767 N, 0.143433 E)/1.13 km], though was not statistically significant (P = 0.997). Sample sizes were too small to analyse region CE using these tests. Outbreaks of fatal chytridiomycosis among recently metamorphosed animals (RM) were reported at seven of the 31 positive sites across the whole of Iberia (Fig. 1). The prevalence of *Bd* among RM in these localities consistently exceeded 0.65.

Statistical signature of zero-inflation

The lack of fit of the standard binomial and the presence of zero inflation in our dataset was ascertained by two different means. Firstly, the predicted-vs.-fitted plot from a standard binomial fitted at the site level was shown to produce very poor predictions that were close to zero. Secondly, when no covariates were considered, the P-value from the zero-inflated score test was shown to be highly significant (with Z = 427.68 and P = 5.2×10^{-95}).

Environmental covariates

Summary statistics for the environmental and climatic factors considered are shown in Table 1. The altitude of infected sites ranged from 82 to 2409 m above sea-level and the STempMin and STempMax at these sites ranged from 1.9–11.9 and 14.5–30.1 °C respectively (Table 1). Among the *Bd*-positive sites (n = 31) the localities with known outbreaks (n = 7) of fatal chytridiomycosis were signifi-

cantly associated with Alt [altitude: Wilcoxon Mann–Whitney test, W = 36, P = 0.022 (+)], STempMin [summer temperature minimum: W = 41, P = 0.045 (−)], STempAv [summer temperature mean: W = 0, P = 7.97×10^{-05} (−)] and TempMin [minimum temperature during the coldest month: W = 41, P = 0.045 (−)] (Fig. 2).

In the univariate site-level Bayesian models the following variables were found to have a significant effect on the conditional prevalence of infection (q): StempAv, SradAv (summer radiation average), Seas (seasonality), MDR (mean diurnal range), TempAr (temperature annual range), TempMin and Longitude. By contrast, only a single variable, 'TempMin' was found to have a significant effect on the probability of *Bd* being present at a site (P). From these variables, four pairs of highly correlated variables were identified (STempAv–TempMin 0.91, Seas-TempAr 0.93, MDR–TempAr 0.85 and TempMin–TempAr 0.85) and the following variables with the lower P-value within the pair were dropped: Seas, MDR, TempAr and STempAv. A backwards stepwise regression with TempMin specified as a covariate for P and TempMin, Longitude and SRadAv specified as covariates for q was used to identify the MAM. In this MAM, the conditional prevalence of *Bd* was negatively associated with SRadAv [mean = −0.22, 95% Bayesian credible intervals (BCI) = −0.31 to −0.13], TempMin [mean = −0.39, 95% BCI = −0.57 to −0.22] and Longitude (mean = −0.33, 95% BCI = −0.43 to −0.24). The 95% BCI did not include zero and were considered to be significant. Note that these analyses were conducted for the site-level model, though subsequent cross-checks were made with the nested version of the model which accommodated developmental stage.

Table 1 Summary statistics [mean, minimum (min.), maximum (max.)] for environmental variables at all sites (all sites), at infected sites (infected) and at infected sites associated with fatal chytridiomycosis (fatal)

Variable	All sites			Infected			Fatal		
	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
Altitude (Alt; m)	1468.4	82.1	2588.0	1552.7	82.1	2409.0	1922.8	1602.0	2196.0
Summer temperature minimum (STempMin; °C)	6.6	1.7	15.7	6.2	1.9	11.9	4.4	4.1	4.9
Summer temperature mean (STempAv; °C)	14.5	8.6	23.7	14.4	8.6	20.5	13.2	11.7	15.3
Summer temperature max (STempMax; °C)	22.2	14.5	32.6	22.3	14.5	30.1	21.9	19.2	26.3
Summer cumulative precipitation (SPrecCum; mm)	231.2	51.4	354.7	225.1	55.8	334.8	232.8	107.1	324.4
Summer radiation average (SRadAv; kJ^{m-2})	2950.5	2504.4	3119.6	2970.5	2640.1	3119.6	2904.7	2640.1	3113.1
Seasonality (Seas; °C)	547.8	431.3	699.5	556.8	435.0	699.5	588.5	518.5	697.9
Mean diurnal range (MDR; °C)	10.5	8.4	14.8	10.5	8.5	13.7	10.9	9.5	12.5
Temperature annual range (TempAr; °C)	25.8	21.7	33.6	26.1	22.4	33.6	27.6	24.8	32.4
Isothermality (Iso; °C)	40.7	35.6	48.7	40.1	36.1	48.7	40.1	37.7	44.6
Minimum temperature during the coldest month (TempMin; °C)	−3.6	−9.1	5.9	−3.9	−8.4	3.1	−5.7	−7.0	−4.1

The acronyms used in the text for each of these variables and the units involved are shown in parentheses.
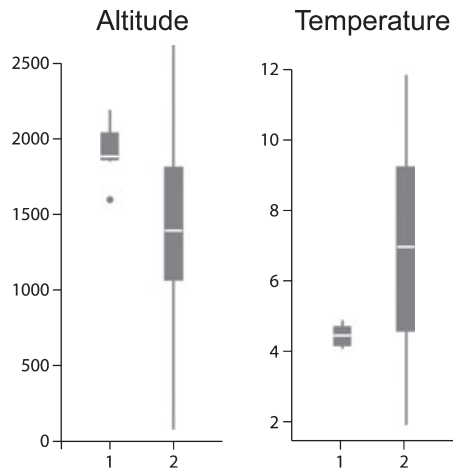
Figure 2 Box-plots displaying the altitude (m) and temperature minima (°C) during the warmest quarter at localities associated with known outbreaks of fatal chytridiomycosis, 1, and at localities from which *Bd*-infected amphibians occur though no outbreaks have been recorded, 2. In both cases, the differences between 1 vs. 2 are significant.

Outputs from Bayesian nested models

In the expanded ZIB with developmental stage nested within site and environmental covariates specified for *q* (model 4TLR; Table S2) the prevalence of infection among larvae (mean = −1.611, 95% BCI = −1.86 to −1.362) and adults (mean = −1.077, 95% BCI = −2.103 to −0.02085) was significantly depressed relative to metamorphic animals (Table S3). As in the non-nested MAM, the conditional prevalence of *Bd* was negatively associated with Longitude (mean = −0.2424, 95% BCI = −0.3496 to −0.1445), SRadAv (mean = −0.2563, 95% BCI = −0.356 to −0.5191) and TempMin (mean = −0.04549, 95% BCI = −0.2391–0.1476), though the significance of the relationship only held for the former two covariates (Table S3). In this model the underlying conditional prevalence and probability of *Bd* presence was 0.7613 (95% BCI = 0.5007–0.7612) and 0.2575 (95% BCI = 0.1850–0.3425) respectively. By contrast, in the nested model where intercepts only were specified, the conditional prevalence was 0.6031 (95% BCI = 0.5784–0.6277).

The infection status and conditional prevalence of *Bd* at sites across the Iberian Peninsula (according to model 4TLR), is shown in Fig. S1, with insets for PY and CC in Figs 3 and S2 respectively. The accompanying caterpillar plots in these latter figures display the 95% BCI associated with each estimate of the conditional prevalence, at infected sites. Across all sites the conditional prevalence of infection was significantly elevated (i.e. lower 95% BCI greater than the underlying value of 0.7612) at 16 of the 31 sites from which *Bd* had been recorded. Beyond these sites, the
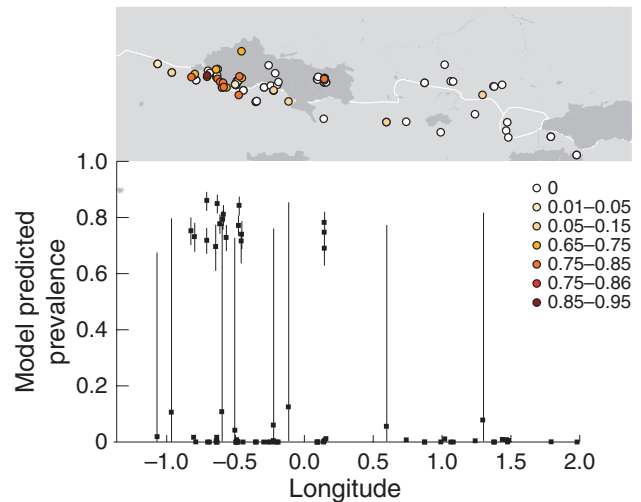
Figure 3 Map and caterpillar plot showing site infection status and conditional prevalence as predicted by the best model 4TLR (Table S2) in the Pyrenees (PY). This model contains environmental covariates (TempMin, Longitude and SradAv) and nested developmental stage effects. 95% Bayesian credible intervals are shown. The equivalent figure for the Cordillera-Cantabrica (CC) is shown in Fig. S2.

estimated mean conditional prevalence of *Bd* was above zero (mean = 0.01912–0.1253) at 19 sites. However the 95% Bayesian credible intervals were large and included zero in all cases.

Model fit

In the nested model with intercepts only (Model 1; Table S2), the values of the AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion) were 1433.174 and 1825.994 respectively. Relative to this null model the greatest reduction in AIC, albeit very marginal (< 1 decimal place) was achieved by the addition of both developmental stage and all three environmental covariates on *q* (model 4TLR, Table S2). By contrast the greatest reduction in BIC was achieved when TempMin was omitted from *q* (model 4RL; Table S2); this omission is consistent with the lack of significance of this variable in the full model (Table S2). In the full version of model 4 (model 4TLR) the predictive check showed overestimation of infection in 26 of the 159 site:stage combinations. This overestimation is likely to be related to the presence of some sites with small sample sizes.

Results from the analysis of multilocus genotypes

All of the alleles found in Iberia had been previously documented by James *et al.* (2009) and are included here (Supporting information). Three trees were recovered with
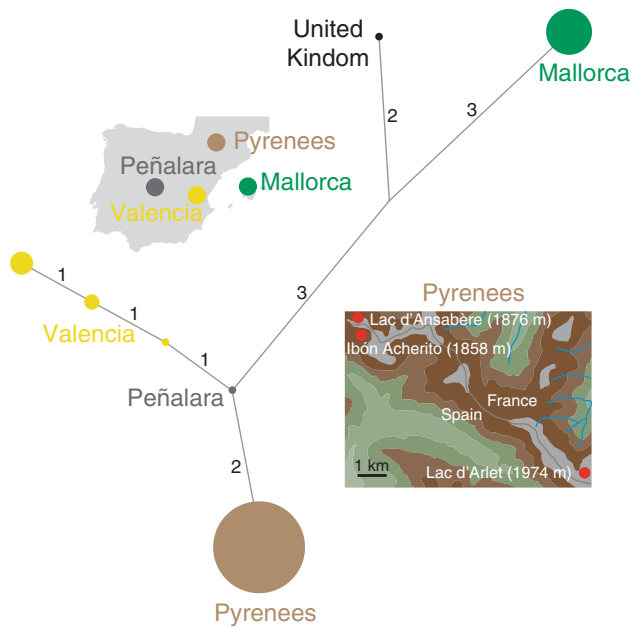
Figure 4 Phylogeny showing relationships among *Bd* strains within Iberia based on six locus genotypes. The tree shown is one of the three generated by a weighted model; these differ only in the placement of the UK isolate relative to Mallorcan isolates. The radius of the circles is relative to the numbers of identical genotypes. Insets show where samples were taken, and the different watersheds that occur in the Pyrenees.

the weighted model and they differed only in the placement of the UK isolate relative to the Mallorca isolates, and in the placement of one Valencia isolate. Twenty-seven unresolved trees were recovered with the unweighted model. For both models, unique multilocus genotypes were found between each of the regions surveyed (Table S4 and Fig. 4). In the cluster of infection in the PY, all isolates were of a single identical genotype. The isolate recovered from the original outbreak site of Peñalara was heterozygous at all six loci in this dataset, and at 12/14 loci in the James *et al.* (2009) dataset; this heterozygosity puts this first European 'outbreak' isolate at the centre of the dendrogram. Maximum genotypic diversity was found in Valencia at a site 400 km distant from Peñalara, where three distinct genotypes were recovered from a single pond and from two species of amphibian, *A. obstetricans* and *Pelophylax perezi*.

DISCUSSION

The widespread yet heterogeneous distribution of *Bd* reported here illustrates the potential risk of chytridiomycosis to populations of *A. obstetricans* across the Iberian Peninsula and perhaps across the host's entire European range. This risk surface is three-tiered, accommodating first, the actual distribution of *Bd*, second, the modelled

conditional prevalence of infection (without necessarily observing infection), and third, the risk of a disease outbreak. In our analyses we find: (1) no evidence for any relationship between the occupancy of *Bd* and environmental variables, (2) a weak relationship between environmental variables (average/minimum temperature of the warmest quarter, minimum temperature of the coldest month, mean solar radiation and longitude) and the conditional prevalence of infection, (3) stage-dependent heterogeneity in the risk of infection, (4) an association between high altitudes and outbreaks of fatal chytridiomycosis, (5) multiple Iberian genotypes and (6) recent introduction and spread of a single genotype of *Bd* in the Pyrenees.

In this study, evidence for weak environmental effects on the establishment of *Bd per se* is consistent with the broad spatial distribution and environmental envelope of *Bd* identified in previous studies (Ron 2005; Fisher *et al.* 2009a). Together, these data are suggestive of *Bd* being an ecological generalist that is able to persist wherever susceptible amphibians occur. The *Bd*-infected localities reported here traverse a broad altitudinal range (82–2588 m a.s.l.). Above 2000 m in our study region, infected sites are typically associated with air temperature minima and maxima below the limits currently recognised for the optimal growth of *Bd*. This disparity is not unprecedented and highlights the need for an improved understanding of the hypervolume of space characterising the fundamental niche of *Bd*. Notably, the highest record of *Bd*, at an altitude of 5400 m, is known from the Peruvian Andes where soil surface temperatures may fall to −13.5 °C and diurnal variability of 30 °C has been recorded (Seimon *et al.* 2007); it is clear from these, and ours, data that the breadth of environments within which *Bd* persists is wider than was previously thought. In addition to not supporting the concept of refugia from infection at high altitudes (Muths *et al.* 2008) our findings motivate the need to: (1) consider any incongruity of scale between the (often coarse) summary climatic statistics used in models and the actual microenvironment occupied by *Bd* and (2) assess whether the physiological thresholds which have been defined for strains of *Bd* from various regions across North America (Piotrowski *et al.* 2004) are applicable to the *Bd* genotypes that are found infecting Europe and elsewhere (Fisher *et al.* 2009a).

Our models present evidence for a negative relationship between prevalence and the minimum temperature during the coldest month (TempMin), radiation (SRadAv) and longitude. However, this relationship is weak and a far greater improvement in model fit is achieved by the specification of developmental stage as an individual-level risk factor (Table S2). This finding is consistent with observations that recently metamorphosed amphibians are predisposed to infection, perhaps owing to transient immunosuppression owing to metamorphosis (Robert *et al.*

1995; Garner *et al.* 2009). The negative relationship between conditional prevalence and increasing (+) longitude is consistent with a signature of spread, with a bias to the west of Iberia; this is weighted to a large degree by the *Bd* hotspot in the western Pyrenees. The negative relationship between conditional prevalence and temperature of the minimum month is somewhat equivocal since, although its significance is lost when developmental stage is not accommodated in the model, when either radiation or longitude is omitted from the full model, this variable's significance is regained. In terms of model fit, the greatest reduction in AIC/BIC in the full model is achieved by the addition of the variable radiation. Elevated levels of UVB are known to be harmful to amphibians (Nagl & Hofer 1997; Blaustein *et al.* 2005) and may increase susceptibility to pathogens such as *Bd*. However, this finding needs to be interpreted with caution as, in addition to the technical shortcomings in the air-based measurements of UVR (Ninyerola *et al.* 2007b), differential attenuation of ultraviolet wavelengths in water (Laurion *et al.* 2000; Blaustein *et al.* 2004) risks distorting the ecological significance of this variable.

The results of our study do not refute the frequently cited hypothesis regarding the predilection of *Bd* for cooler temperatures (Berger *et al.* 2004; Drew *et al.* 2006; Kriger & Hero 2007) however they do emphasize the context-dependent nature of fatal chytridiomycosis as all occurrences of disease were found at altitudes above 1600 m. The environmental forcing of many infectious diseases is well-documented (Rogers *et al.* 2002; Clements *et al.* 2007; Liang *et al.* 2007) and are often related to the prevalence of the infectious organism in the environment. However, in diseases where the outcome of infection is dependent more on the host's ability to tolerate an infection, environmental forcing of pathogenicity rather than prevalence *per se* may dominate the fitness landscape. In addition to the occurrence of fatal outbreaks at high altitudes, the interannual variability in the mortality rate at one of our 'outbreak' sites, at which the prevalence of infection was consistently high (Ibon Acherito), is strongly consistent with the importance of context-dependent host tolerance of infections. Specifically, the association of mean temperature minima during the summer months and mortality is a prediction borne by our knowledge that: (1) the maintenance/clearance of infection and the survival of the host amphibian is temperature dependent (Woodhams *et al.* 2003; Andre *et al.* 2008) and (2) in the field, temperature-dependent immunity may be forced by seasonal patterns (Raffel *et al.* 2006).

Temporal data documenting the rapid spread of fatal chytridiomycosis and the subsequent collapse of *Rana muscosa* populations in the Sierra Nevada, as well as the decline of *A. obstetricans* in Peñalara, serve as a cautionary note for the potential fate of *Alytes* populations in the Pyrenees (Bosch *et al.* 2001; Briggs *et al.* 2005). Akin to *A. obstetricans*, the larval stage of *R. mucosa* is typically prolonged in high altitude montane lakes. The prolonged period of exposure to *Bd* and the associated window of time for the accumulation of fitness costs may be pivotal to explaining the susceptibility of these montane species and populations to fatal chytridiomycosis. In our study, although the configuration of infected sites containing identical genotypes in the Pyrenees is strongly suggestive of a pathogen in the early stages of its emergence [in contrast with that observed for *R. muscosa* (Morgan *et al.* 2007)], the widespread distribution of *Bd* across the Iberian Peninsula suggests that *Bd* has been introduced into this wider region some time ago. Such a conclusion is consistent with the broad distribution of *Bd* across Europe (Garner *et al.* 2005). That *Bd* is in relative equilibrium with its environment and not undergoing a period of range expansion is a critical assumption of landscape epidemiology (Pavlovsky 1966) since spatial heterogeneity in the environmental extent of disease is attributed directly to the underlying variation in the ecological conditions that support the pathogen, its vectors, its reservoirs and its hosts (Ostfeld *et al.* 2005). Given that the relative endemicity of infection in our study region is debated, the ZIB model is particularly suited as it models separately occupancy and conditional prevalence. The signature of zero-inflation in our data has not been reported from previous *Bd*-surveillance in other systems and it is plausible that, if *Bd* is not at equilibrium within Iberia, this signature may be lost with time. Notably, the proportion of sites occupied by *Bd* in each of our regions (23–33%) is considerably lower than that reported by Muths *et al.* (Muths *et al.* 2008) in their study on *Bufo boreas* in the Rockies (north 84% clusters occupied $n = 49$; south 42%, $n = 48$) and, more generally, across the United States. This observation suggests that *Bd* is at an earlier phase of it's emergence within Iberia relative to North America.

Our finding of distinct genotypes within each of the sampled regions is consistent with either a history of multiple introductions or of a single relatively ancient introduction of *Bd* into Iberia (Table S4). James *et al.* (2009) using 14 marker loci show that Iberian genotypes from Peñalara (C2A) and the Pyrenees (IA042/043) co-occur within a clade of genotypes that are exclusively found in North America, raising the hypothesis that a transatlantic introduction of *Bd* into mainland Iberia has ocurred. The high levels of heterozygosity found in Peñalara C2A (Fig. 4) are concordant with this hypothesis as this site is arguably the first European outbreak site, and the most heterozygous isolate yet found comes from a North American bullfrog in Maine (JEL404). However, owing to low genetic diversity and global sampling, the loci utilised by James *et al.* (2009) lack the power to accurately describe potential global sources of infection and a definitive identification of the

original source of the Iberian infections will likely require a next-generation sequencing approach.

On the other hand, the Iberian genotypes clearly show local patterns of introduction and spread. Principally, in the Pyrenees the identical nature of each of the isolates from three lakes in three different drainage basins (Fig. 4) is strongly supportive of a single introduction and subsequent spread, or multiple introductions of an identical clone from an unknown source population. We believe that the former is the most parsimonious interpretation of our data, as evidenced by the discovery of a recent single introduction of *Bd* into an endemic species of *Alytes* in Mallorca (Walker *et al.* 2008).

Our findings have shown that the expression of an NPH-like process (the introduction of *Bd* into Iberian biomes) is dependent on its EPH-like environmental context (the expression of chytridiomycosis). We have shown that these processes have likely not reached their spatial equilibria within Iberia, and that further spread (both of the pathogen and the disease) across this region is likely. It is also possible that future changes in these environments will further modify the *Alytes*/Bd host/pathogen relationship. Montane systems are particularly vulnerable to climate change (Catalan *et al.* 2006; Nogues-Bravo *et al.* 2007) and the global trends in changes between minima and maxima temperatures reported by Pounds *et al.* (2006), are supported by temperature trends in the Pyrenees (Dessens & Bucher 1995). For this region, an increase of between 2.8 and 4 °C in the mean annual temperature and a decrease of between 10.7% and 14.8% in the annual precipitation is predicted for the next century (Lopez-Moreno *et al.* 2008). However, beyond the need to assess the impact of climate change on the physiology of *Bd* and the host amphibian *per se*, there is a need also to consider changes in: hydrological patterns, phenology, community composition and changes in the amphibian epidermal microfauna. All of these variables are likely to impact on the epidemiology of *Bd* and chytridiomycosis, greatly increasing the challenge of developing a predictive understanding of the emergence and occurrence of this increasingly devastating infectious disease.

REFERENCES

Andre, S.E., Parker, J. & Briggs, C.J. (2008). Effect of temperature on host response to *Batrachochytrium dendrobatidis* infection in the mountain yellow-legged frog (*Rana muscosa*). *J. Wildl. Dis.*, 44, 716–720.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L. *et al.* (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl Acad. Sci. U.S.A.*, 95, 9031–9036.

Berger, L., Speare, R., Hines, H., Marantelli, G., Hyatt, A.D., McDonald, K.R. *et al.* (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust. Vet. J.*, 82, 31–36.

Blaustein, A.R., Han, B., Fasy, B., Romansic, J., Scheessele, E.A., Anthony, R.G. *et al.* (2004). Variable breeding phenology affects the exposure of amphibian embryos to ultraviolet radiation and optical characteristics of natural waters protect amphibians from UV-B in the US Pacific Northwest: comment. *Ecology*, 85, 1747–1754.

Blaustein, A.R., Romansic, J.M. & Scheessele, E.A. (2005). Ambient levels of ultraviolet-B radiation cause mortality in juvenile western toads, *Bufo boreas*. *Am. Midl. Nat.*, 154, 375–382.

Bosch, J., Martínez-Solano, I. & García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biol. Conserv.*, 97, 331–337.

Bosch, J., Carrascal, L.M., Duran, L., Walker, S. & Fisher, M.C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proc. R. Soc. B Biol. Sci.*, 274, 253–260.

Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. & Hyatt, A.D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Org.*, 60, 141–148.

Briggs, C.J., Vredenburg, V.T., Knapp, R.A. & Rachowicz, L.J. (2005). Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology*, 86, 3149–3159.

Cannon, R.M. & Roe, R.T. (1982). *Livestock Disease Surveys – A Field Manual for Veterinarians*. Australian bureau of animal health, Canberra, Australia.

Catalan, J., Camarero, L., Felip, M., Pla, S., Ventura, M., Buchaca, T. *et al.* (2006). High mountain lakes: extreme habitats and witnesses of environmental changes. *Limnetica*, 25, 551–584.

Clements, A.C.A., Pfeiffer, D.U., Martin, V., Pittliglio, C., Best, N. & Thiongane, Y. (2007). Spatial risk assessment of Rift Valley fever in Senegal. *Vector Borne Zoonotic Dis.*, 7, 203–216.

Dessens, J. & Bucher, A. (1995). Changes in minimum and maximum temperatures at the Pic-Du-Midi in relation with humidity and cloudiness, 1882–1984. *Atmos. Res.*, 37, 147–162.

Drew, A., Allen, E.J. & Allen, L.J.S. (2006). Analysis of climatic and geographic factors affecting the presence of chytridiomycosis in Australia. *Dis. Aquat. Org.*, 68, 245–250.

Fisher, M.C., Bosch, J., Yin, Z., Stead, D.A., Walker, J., Selway, L. *et al.* (2009a). Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. *Mol. Ecol.*, 18, 415–429.

Fisher, M.C., T.W.J, G. & Walker, S.F. (2009b). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annu. Rev. Microbiol.*, 63, 291–310.

Garner, T.W.J., Walker, S., Bosch, J., Hyatt, A.D., Cunningham, A.A. & Fisher, M.C. (2005). Chytrid fungus in Europe. *Emerg. Infect. Dis.*, 11, 1639–1641.

Garner, T.W.J., Walker, S., Bosch, J., Leech, S., Rowcliffe, J.M., Cunningham, A.A. *et al.* (2009). Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos*, 118, 783–791.

Gelman, A., Carlin, J.B., Stern, H.S. & Rubin, D.R. (2000). *Bayesian Data Analysis*. Chapman and Hall/CRC, Boca Raton, London, New York, Washington, D.C.

Hyatt, A.D., Boyle, D.G., Olsen, V., Boyle, D.B., Berger, L., Obendorf, D. *et al.* (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Org.*, 73, 175–192.

James, T.Y., Litvintseva, A.P., Vilgalys, R., Morgan, J.A.T., Taylor, J.W., Fisher, M.C. *et al.* (2009). Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. *PLoS Pathog.*, 5, e1000458.

Kriger, K.M. & Hero, J.M. (2007). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *J. Zool.*, 271, 352–359.

Kulldorff, M. (1997). A spatial scan statistic. *Commun. Stat. Theory Methods*, 26, 1481–1496.

Laurion, I., Ventura, M., Catalan, J., Psenner, R. & Sommaruga, R. (2000). Attenuation of ultraviolet radiation in mountain lakes: factors controlling the among- and within-lake variability. *Limnol. Oceanogr.*, 45, 1274–1288.

Liang, S., Seto, E.Y.M., Remais, J.V., Zhong, B., Yang, C., Hubbard, A. *et al.* (2007). Environmental effects on parasitic disease transmission exemplified by schistosomiasis in western China. *Proc. Natl Acad. Sci. U.S.A.*, 104, 7110–7115.

Lips, K.R., Diffendorfer, J., Mendelson, J.R. III & Sears, M.W. (2008). Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biol.*, 6, 441–454.

Lopez-Moreno, J.I., Goyette, S. & Beniston, M. (2008). Climate change prediction over complex areas: spatial variability of uncertainties and predictions over the Pyrenees from a set of regional climate models. *Int. J. Climatol.*, 28, 1535–1550.

Lunn, D.J., Thomas, A., Best, N. & Spiegelhalter, D. (2000). WinBUGS – a Bayesian modelling framework: concepts, structure, and extensibility. *Stat. Comput.*, 10, 325–337.

Martin, T.G., Wintle, B.A., Rhodes, J.R., Kuhnert, P.M., Field, S.A., Low-Choy, S.J. *et al.* (2005). Zero tolerance ecology: improving ecological inference by modelling the source of zero observations. *Ecol. Lett.*, 8, 1235–1246.

Morgan, J.A., Vredenburg, V., Rachowicz, L., Knapp, R., Stice, M., Tunstall, T. *et al.* (2007). Enigmatic amphibian declines and emerging infectious disease: population genetics of the frog killing fungus *Batrachochytrium dendrobatidis*. *Proc. Natl Acad. Sci. U.S.A.*, 104, 1385–1390.

Muths, E., Pilliod, D.S. & Livo, L.J. (2008). Distribution and environmental limitations of an amphibian pathogen in the Rocky Mountains, USA. *Biol. Conserv.*, 141, 1484–1492.

Nagl, A.M. & Hofer, R. (1997). Effects of ultraviolet radiation on early larval stages of the Alpine newt, *Triturus alpestris*, under natural and laboratory conditions. *Oecologia*, 110, 514–519.

Ninyerola, M., Pons, X. & Roure, J.M. (2007a). Monthly precipitation mapping of the Iberian Peninsula using spatial interpolation tools implemented in a Geographic Information System. *Theor. Appl. Climatol.*, 89, 195–209.

Ninyerola, M., Pons, X. & Roure, J.M. (2007b). Objective air temperature mapping for the Iberian Peninsula using spatial interpolation and GIS. *Int. J. Climatol.*, 27, 1231–1242.

Nogues-Bravo, D., Araújo, M.B., Errea, M.P. & Martínez-Rica, J.P. (2007). Exposure of global mountain systems to climate warming during the 21st century. *Glob. Environ. Change*, 17, 420–428.

Ostfeld, R.S., Glass, G.E. & Keesing, F. (2005). Spatial epidemiology: an emerging (or re-emerging) discipline. *Trends Ecol. Evol.*, 20, 328–336.

Pavlovsky, E.N. (1966). *The Natural Nidality of Transmissible Diseases*. University of Illinois Press, Urbana, IL.

Piotrowski, J.S., Annis, S.L. & Longcore, J.F. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, 96, 9–15.

Potthoff, R.F. & Whittinghill, M. (1966). Testing for homogeneity. II. The Poisson distribution. *Biometrika*, 53, 183–190.

Pounds, A.J., Bustamante, M.R., Coloma, L.A., Consuegra, J.A., Fogden, M.P.L., Foster, P.N. *et al.* (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, 439, 161–167.

Rachowicz, L.J., Hero, J.M., Alford, R.A., Taylor, J.W., Morgan, J.A.T., Vredenburg, V.T. *et al.* (2005). The novel and endemic pathogen hypothesis: competing explanations for the origin of emerging infectious diseases of wildlife. *Conserv. Biol.*, 19, 1441–1448.

Raffel, T.R., Rohr, J.R., Kiesecker, J.M. & Hudson, P.J. (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Funct. Ecol.*, 20, 819–828.

Robert, J., Guiet, C. & Dupasquier, L. (1995). Ontogeny of the alloimmune response against a transplanted tumor in *Xenopus laevis*. *Differentiation*, 59, 135–144.

Rogers, D.J., Randolph, S.E., Snow, R.W. & Hay, S.I. (2002). Satellite imagery in the study and forecast of malaria. *Nature*, 415, 710–715.

Ron, S. (2005). Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the New World. *Biotropica*, 37, 209–221.

Seimon, T.A., Seimon, A., Daszak, P., Halloy, S.R.P., Schloegel, L.M., Aguilar, C.A. *et al.* (2007). Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. *Glob. Chang. Biol.*, 13, 288–299.

Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., Phillott, A.D. *et al.* (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, 4, 125–134.

Vences, M., Grossenbacher, K., Puente, M., Palanca, A. & Vieites, D.R. (2003). The Cambalès fairy tale: elevational limits of *Rana temporaria* (Amphibia: Ranidae) and other European amphibians revisited. *Folia Zool.*, 52, 189–202.

Walker, S.F., Bosch, J., James, T., Litvintseva, A., Oliver Valls, J., Piña, S. *et al.* (2008). Invasive pathogens threaten species recovery programs. *Curr. Biol.*, 18, R853–R854.

Woodhams, D.C., Alford, R.A. & Marantelli, G. (2003). Emerging disease of amphibians cured by elevated body temperature. *Dis. Aquat. Org.*, 55, 65–67.

Woodhams, D.C., Ardipradja, K., Alford, R.A., Marantelli, G., Reinert, L.K. & Rollins- Smith, L.A. (2007). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Anim. Conserv.*, 10, 409–417.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Map of the Iberian Peninsula showing site infection status and the conditional prevalence as predicted by model 4TLR (Table S2). Green cross = outbreak of fatal chytridiomycosis. The heavily shaded regions are national parks.

Figure S2 Map and caterpillar plot showing site infection status and the conditional prevalence as predicted by model 4TLR in the Cordillera-Cantabrica (CC). 95% Bayesian credible intervals are shown. The heavily shaded regions are national parks.

Table S1 Summary statistics for the infection status of sites within and across the three regions (Pyrenees (PY), Cordillera Cantabrica (CC), a diffuse NW-SE band running across central Spain including the Sierra de Guadarrama mountain range (CE)). When both infected and non-infected sites are considered together (total) the means, minima and maxima are provided for the observed cases, the samples sizes and the prevalence of infection (Prev.). The median is not provided because it is zero in most cases. Similar statistics are provided for the infected sites only. Given the highly skewed distribution of the observed number of positives, the median (instead of the mean) is reported.

Table S2 Model fit for the nested models as determined by the BIC and AIC. Any environmental covariates are indicated in parentheses. (T=TempMin, E=Easting, R=SRadAv). Model 1 contains intercepts only, model 2 contains nested developmental-stage effects with no environmental covariates, model 3 contains environmental covariates in the absence of developmental-stage effects, model 4 contains environmental covariates and nested developmental stage effects.

Table S3 Parameter estimates from model 4TLR. As detailed in Table 3, T=TempMin, E=Easting, R=SRadAv. In the assessment of developmental stage, the risk of infection for larvae (L) and adults (A) is considered relative to recent metamorphs (the outgroup). Covariates shown in bold are, based on 95% Bayesian credible intervals, significant. In this model the underlying conditional prevalence and probability of *Bd* presence is given by the expit of alpha[1] and alpha[2], respectively.

Table S4 Identity of *Bd*-isolates used for multi-locus sequence typing. Multiple isolates from a single specimen are indicated by underscores. The developmental stage (DS) of specimens is listed as larva (L) or recent metamorph (RM). With the exception of isolates VRP1 and VRP2, which were from *Pelophylax perezi*, all isolates were from *Alytes obstetricans*.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Supplementary Methods:

Nested Extension of Zero-inflated Binomial Model.

In this supplementary document, we give further details about the development of the nested model and the treatment of the covariates within that framework.

Nested Model

To fix notation, we review the original zero-inflated binomial (ZIB) model, as proposed by Martin et al. (2005). It has been summarised as follows in the main article:

$$\mathbb{P}[K_i = 0|n_i] = p_i + (1 - p_i)(1 - q_i)^{n_i}, \qquad i = 1, \ldots, n; \tag{1}$$

and

$$\mathbb{P}[K_i = k_i|n_i] = (1 - p_i)\binom{k_i}{n_i}q_i^{k_i}(1 - q_i)^{n_i - k_i}, \tag{2}$$

where k_i represents the number of infected individual in site i and each k_i can only take the following values $k_i \in \{0, 1, \ldots, n_i\}$, and n_i is the total number of amphibians observed in site i, while n is the total number of sites in the region of interest.

We extended this model in order to incorporate the amphibians' developmental stages as another variable. In this version of the model, the three developmental stages (from larvae to adult) are nested within each site. We denote by n the total number of sites in the region under scrutiny, and by N the total number of observations of infected individuals in one developmental stage in a particular region. Naturally, $n < N$. However, since we did not observe amphibians in all developmental stages in all regions, it follows that $N < n \times 3$, where 3 is the number of developmental stages.

Our proposed nested model is therefore a slight departure from Martin et al.'s (2005) ZIB model, described in equation (1) and (2). It was formulated as follows,

$$\mathbb{P}[K_j = 0|n_j] = p_{z_j} + (1 - p_{z_j})(1 - q_j)^{n_j}, \qquad i = 1, \ldots, n, j = 1, \ldots, N; \tag{3}$$

and

$$\mathbb{P}[K_j = k_j|n_j] = (1 - p_{z_i})\binom{k_j}{n_j}q_j^{k_j}(1 - q_j)^{n_j - k_j}, \tag{4}$$

where z_j is an allocation variable, which returns for each j the index i of the corresponding

site, within which the observation for that developmental stage has been conducted. The n_j's, here, represent the number of observations conducted for a particular developmental stage, for a particular site, as opposed to n_i in equations (1) and (2), which represented the total number of observations, for all developmental stages, included in a site i. Note that the N quantities q_j correspond to the probabilities of an amphibian in the age category of K_j in site z_j to be infected. That is, $q_j = \mathbb{P}[K_j = k_j | n_j, Y_{z_j} = 1]$.

Variable Selection

Environmental covariates can enter the model at two different points: (i) either by affecting the probability of a site to be infected, which we have denoted p_i, or (ii) by influencing the probability of each individual in a particular developmental stage, in a specific site to be infected, given that that site is infected, which is denoted by q_j. Thus, we have the following formulations,

$$q_j = \frac{\exp\left\{\alpha_1 + \sum_{l=1}^{L_1} \beta_{1l} X_{lj} + \beta_A(I(q_j))\right\}}{1 + \exp\left\{\alpha_1 + \sum_{l=1}^{L_1} \beta_{1l} X_{lj} + \beta_A(I(q_j))\right\}}, \qquad j = 1, \ldots, N; \tag{5}$$

and

$$p_i = \frac{\exp\left\{\alpha_2 + \sum_{l=1}^{L_2} \beta_{2l} X_{li}\right\}}{1 + \exp\left\{\alpha_2 + \sum_{l=1}^{L_2} \beta_{2l} X_{li}\right\}}, \qquad i = 1, \ldots, n; \tag{6}$$

where L_1, and L_2 are the number of covariates affecting the q_j's and p_i's, respectively, and the β_A are the coefficients for controlling the influence of the developmental stages, through a labelling function $I(\cdot)$, which stipulates which developmental stage the jth observation belongs to.

The L_2 covariates in equation (6) are independent of the developmental stages and only affect the final observations k_j, through the Y_i's. Thus, we tested for the statistical significance of these covariates in a frequentist setting, using the standard model, proposed by Martin et al. (2005). These analyses provided no support for the action of any covariates at that level of the model. Subsequent analyses were conducted in a Bayesian framework, using the nested model for verification purposes. Similarly, the L_1 covariates in equation (5) were first screened using the standard model, as proposed by Martin, without nesting, in a frequentist setting. The selected covariates were then evaluated in the full nested model.