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## **Climate forcing of an emerging fungal pathogen across a multihost community**

**Frances C. Clare<sup>1,3\*</sup>, Julia B. Halder<sup>3</sup>, Olivia Daniel<sup>2</sup>, Jon Bielby<sup>1</sup>,  
Mikhail A. Semenov<sup>4</sup>, Thibaut Jombart<sup>3</sup>, Adeline Loyau<sup>5,6,7</sup>, Dirk  
S. Schmeller<sup>5,6</sup>, Andrew A. Cunningham<sup>1</sup>, Marcus Rowcliffe<sup>1</sup>,  
Trenton W. J. Garner<sup>1</sup>, Jaime Bosch<sup>8</sup>, Matthew C. Fisher<sup>3\*</sup>**

1. Institute of Zoology, Zoological Society of London, Regent's Park, London, NW1 4RY, UK

2. Department of Life Sciences, Silwood Park Campus, Imperial College London SL5 9PU, UK

3. Department of Infectious Disease Epidemiology, Imperial College London W2 1PG, UK

4. Computational and Systems Biology, Rothamsted Research, West Common, Harpenden, Hertfordshire,  
AL5 2JQ

5. Université de Toulouse; UPS, INPT; EcoLab (Laboratoire Ecologie Fonctionnelle et Environnement); 118  
route de Narbonne, 31062 Toulouse, France

6. Helmholtz Centre for Environmental Research- UFZ, Department of Conservation Biology, Permoserstr.  
15, 04318 Leipzig, Germany

7. Helmholtz Centre for Environmental Research - UFZ, Department of System Ecotoxicology, Permoserstr.  
15, 04318 Leipzig, Germany

8. Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, Jose  
Gutierrez Abascal, 2 28006 Madrid, Spain

### **Corresponding authors:**

\*Frances C. Clare, Institute of Zoology, Zoological Society of London, Regent's Park, London, NW1 4RY,  
UK; +44 2074496626; [frances.clare@ioz.ac.uk](mailto:frances.clare@ioz.ac.uk)

\*Matthew C. Fisher, Dpt. Infectious Disease Epidemiology, Norfolk Place, London W2 1PG; +44 2073727733  
[matthew.fisher@imperial.ac.uk](mailto:matthew.fisher@imperial.ac.uk)

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## 1 Summary

2 Changes in the timings of seasonality as a result of anthropogenic climate change are  
3 predicted to occur over the coming decades. While this is expected to have widespread  
4 impacts on the dynamics of infectious disease through environmental forcing, empirical  
5 data is lacking. Here, we investigated whether seasonality affected susceptibility to  
6 infection by the emerging fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) across a  
7 montane community of amphibians that are suffering declines and extirpations as a  
8 consequence of this infection. We found a robust temporal association between the  
9 timing of the spring thaw and *Bd* infection in two host species, where we show that an  
10 early onset of spring forced high prevalences of infection. A third highly susceptible  
11 species (the midwife toad *Alytes obstetricans*) maintained a high prevalence of infection  
12 independent of seasonality. Our data show that perennially overwintering midwife toad  
13 larvae act as a year-round reservoir of infection with seasonality determining the extent  
14 to which infection spills over into sympatric species. We used future temperature  
15 projections based on global climate models to demonstrate that the timing of spring  
16 thaw in this region will advance markedly by the 2050's, indicating that climate change  
17 will further force the severity of infection. Our findings of the effect of seasonality on  
18 multi-host infection dynamics show that the community-level impact of fungal  
19 infectious disease on biodiversity will need to be re-evaluated in the face of climate  
20 change.

21

## 22 Introduction

23 Climate change is likely to influence infectious disease dynamics, with many pathogens,  
24 especially those with complex life cycles or those infecting ectothermic hosts, predicted  
25 to increase in severity or range as the earth continues to warm (1-3). *Batrachochytrium*  
26 *dendrobatidis* (*Bd*), one of two fungi known to cause amphibian chytridiomycosis, is  
27 associated with the decline and extinction of amphibians worldwide (4, 5). In common  
28 with other infections, it is widely assumed that climate change has facilitated epizootics  
29 of chytridiomycosis, thus allowing *Bd* to establish in naïve ecosystems (6-8).

30 A link between climatic variables, host phenology (the timing of recurring natural  
31 phenomena) and the population level impact of *Bd* has been investigated by both *in situ*  
32 (6, 7), and *ex situ* (8-10) studies. However, the hypothesis that climate change dictates *Bd*  
33 infection dynamics in nature has not been proven. This is because the key *in situ* studies  
34 to date have confounded two variables, pathogen introduction and environmental  
35 variation (6, 7), and the required longitudinal epidemiological studies in established  
36 diseased ecosystems have not yet been undertaken (11, 12). Further, *Bd* is a generalist  
37 pathogen (13), exhibiting broad variation in its ability to infect and cause disease across  
38 species (14). Within multi-species amphibian assemblages, different host species exhibit  
39 a range of responses to pathogen exposure. These responses include resistance to  
40 infection (diluters of infection), infection tolerance (reservoirs of infection), and variation  
41 in susceptibility to lethal disease (15, 16). Yet, studies to date have focussed on the most  
42 readily infected species assuming homogeneous host response over time, and data that  
43 addresses the medium to long-term temporal impact of *Bd* across all members of a host  
44 community in concert with local climatic data, is absent.

45 Climate variation is pronounced in montane systems, and it is in these environments  
46 that epizootics of chytridiomycosis predominantly occur (17-20) presenting an  
47 opportunity to untangle the complex relationship between disease dynamics and  
48 climate. One such environment, the Pyrenean mountain range, contains many lakes  
49 housing multi-species amphibian assemblages within an expanding epizootic of *Bd*  
50 infection (20). Across the core outbreak region of the Western Pyrenees, the midwife  
51 toad (*Alytes obstetricans*) experiences annual mass mortality due to chytridiomycosis.  
52 Two other anuran species also commonly breed in this area, the common toad (*Bufo*  
53 *spinosus*, previously known as *Bufo bufo* (21)) and the common frog (*Rana temporaria*).

54 Across Europe, the prevalence of *Bd* within affected common toads is usually low (14),  
55 with the majority of individuals tolerating infection (22). In comparison, the common  
56 frog is considered to be resistant to *Bd* infection (14, 23), and until now there has been no  
57 evidence of disease in this species. As a result of the differences that these three species  
58 exhibit in their response to exposure to *Bd*, they constitute an ideal study-system for  
59 exploring the temporal trends in pathogen infection at a community level. To  
60 understand the inter-relationship between infection, community and climate, we  
61 investigated seasonality in this montane ecosystem and studied how this affects  
62 infection dynamics at the present, and when extrapolated into the future using  
63 downscaled outputs of global climate change models.

## 64 Results and Discussion

65 We monitored amphibians at a key remote infected site, Lac Arlet (altitude 1,986 m abs), in  
66 the French Pyrenees over seven years. Across this period we found a robust temporal link  
67 between disease dynamics (Fig. 1a & b) and the timing of spring across this community of  
68 amphibians (Fig. 1c & d). We uncovered a significant effect of the onset of spring on the  
69 prevalence of *Bd* infection in both *B. spinosus* (slope = -0.1000;  $z = -4.715$ , d.f. = 149,  $p < 0.001$ ;  
70 adjusted  $R^2 = 0.19$ ), and *R. temporaria* (slope = -0.039,  $z = -4.356$ , d.f. = 207,  $p < 0.001$ ; adjusted  
71  $R^2 = 0.08$ ), with early spring onset resulting in a higher prevalence of *Bd* infection (Fig. 1d).  
72 The highly susceptible species, *A. obstetricans*, maintained a high prevalence of infection  
73 throughout the study independent of the onset of spring for both overwintering tadpoles  
74 and metamorphs ( $p = 0.09$ ). The timing of the end of season showed little variation between  
75 years (Fig. 1c; Table 1), with the corollary that the length of the amphibian activity period  
76 was increased in years with an early spring onset.

77 The fact that species previously resistant to infection and disease alters in different  
78 environmental / climatic contexts illustrates the importance of understanding community-  
79 level dynamics when considering the impacts of infections. Our data show that a species  
80 thought to be highly resistant to infection and disease can suffer mortality (Fig. 2a); prior to  
81 the current study, *R. temporaria* has rarely been found to be infected with *Bd* (14) and has  
82 never been observed to suffer chytridiomycosis despite widespread surveillance across  
83 Europe. However, we detected widespread infection in this species, along with mortality  
84 due to chytridiomycosis, when seasonal onset was early. We also found concurrent temporal  
85 changes in the prevalence of *Bd* infection in the more susceptible species, *B. spinosus*, which

86 became locally extinct in 2013 (Fig. 1a & b). We believe that the abrupt decline of *B. spinosus*  
87 was disease-driven, due to the high prevalence of infection and widespread mortality  
88 detected in metamorphs in the years leading to its disappearance. The number of *A.*  
89 *obstetricans* OW larvae has also declined steadily at Lac Arlet during the seven-year course of  
90 our study alongside high recorded mortality (Fig 2b; Table 2), showing that mortality due to  
91 chytridiomycosis is leading to synchronous multi-species declines across this site.

92 Our data suggest that the susceptible and highly parasitised *A. obstetricans* are acting as the  
93 key biological reservoir of *Bd* in this system, and that seasonality is determining the extent to  
94 which infection spills over into sympatric species. At the altitude of Lac Arlet, *A. obstetricans*  
95 tadpoles over-winter for a number of years before completing development. The tadpole  
96 stage of the majority of amphibians rarely suffer mortality or visible effects of *Bd* infection  
97 (24, 25) and these life-history traits result in over-wintering tadpoles playing a leading a role  
98 in the maintenance of *Bd* across seasons, as has been proposed previously for other systems  
99 (19, 26). The yearly changes in infection prevalence that we detected in hosts that are less  
100 susceptible to infection could imply that the density of one generally heavily infected  
101 species, *A. obstetricans*, drives infection levels within this system. We detected the highest  
102 abundance of *A. obstetricans* metamorphs in 2010 and, in that year we also saw the highest  
103 proportional mortality, with almost half of all *A. obstetricans* individuals encountered being  
104 dead. It is possible that the sharp increase in prevalence of infection in 2010 that we detected  
105 in both *B. spinosus* and *R. temporaria* was a direct result of the observed increase in the  
106 number of infected *A. obstetricans* in that year. However, there was no significant association  
107 between the number of deceased *A. obstetricans* mortalities seen each year and the  
108 prevalence of infection in either species. Further, the prevalence of infection in both *B.*  
109 *spinosus* and *R. temporaria* continued to increase the following year (Fig.1a), despite a  
110 decrease in the abundance of *A. obstetricans*. This contraindicates the hypothesis that the  
111 density of *A. obstetricans* is the sole driver of force of infection upon sympatric species.

112 To assess the impact of climate change on onset of spring, the optimal time period over  
113 which mean air temperature is most closely related to time of spring thaw was estimated  
114 (supplementary Fig. 1). For 2007-2015, mean temperature over days 83-153 (a 70 day period  
115 centred on day 118 of the year) was found to be the best predictor of onset of spring,  
116 explaining 73% of the variance. For every 1 degree increase in the mean temperature over  
117 this time, onset of spring is estimated to be 10 days earlier (Fig. 2c).

118 We then used the LARS-WG weather generator as a downscaling technique to generate  
119 local-scale climate scenarios for the site, based on projections from global climate models  
120 from the CMIP5 multi-model ensemble under the representative concentration pathway  
121 (RCP) 8.5 (greenhouse gas emissions continue rising over 21st<sup>t</sup> Century) (27) and modelled  
122 using local weather data (Fig. 2c). To capture uncertainty in the CMIP5 climate projections  
123 we selected two global climate models, GISS-E2 and HadGEM2, with low and high climate  
124 sensitivities respectively, which thus predict lesser and greater amounts of warming for the  
125 region. The predictions all indicate that an early onset of spring in the western Pyrenees will  
126 become commonplace by the 2050s due to global warming (Fig. 2d). Over days 83-153 of the  
127 year, the mean daily temperature is projected to be around 5.5°C by GISS-E2 and 5.8°C by  
128 HadGEM2 in the 2050s (medians of 100 years plausible weather generated by LARS-WG)  
129 (Fig. 2d). This corresponds, under the current relationship, with an onset of spring around  
130 day 130-133, comparable to that seen in 2011. By the 2090s, the median GISS-E2 scenario for  
131 this time is 6.5°C, potentially indicating an onset of spring around day 122. The median  
132 HadGEM2 scenario is 7.8°C; since this is out of the range of observed temperatures (with the  
133 rest of the year also being substantially warmer) we make no estimated projection of onset of  
134 spring under this scenario. LARS-WG output across the winter months for the 2090s for  
135 HadGEM2 suggests that daily mean temperature will rarely drop below 0°C (daily  
136 minimum temperature may be below freezing for 3 months, compared to currently around 6  
137 months), which may lead to a short, punctuated, or non-existent duration of ice cover  
138 (supplementary Fig. 2). If the trends that we have established here continue, increases in  
139 amphibian activity periods are predicted to correspond to increases in infection levels across  
140 these species, which could have profound effects on populations of hosts we currently  
141 consider to be resistant or tolerant of infection.

142 The mechanisms underpinning the seasonal forcing of infection that we observe are  
143 unknown. A broad range of biotic and abiotic factors are influenced by seasonality, and it is  
144 probable that many factors acting on both the host and the pathogen are driving the  
145 seasonal prevalence of infection in the amphibians studied. On one hand, abiotic drivers  
146 include increased temperature volatility causing a reduction in temperature acclimation of  
147 host resistance to infection (28), and patterns of disease are known to be also exacerbated as  
148 hosts are moved outside of their temperature norms (9). On the other hand, biotic factors  
149 such as the density and species complement of aquatic microfauna present at our research  
150 site are known to modulate the risk of infection (29). Aquatic microfauna have seasonal

151 peaks and dips in abundance throughout the spring and summer corresponding to the local  
152 availability of nutrients (30), therefore, changes in seasonality may affect the abundance of  
153 the microfauna, impacting the removal of *Bd* zoospores and force of infection. When  
154 complex biotic and abiotic drivers such as these interact, nonlinear responses are expected  
155 and the uncertainty associated with predicting future trends in this, and other montane,  
156 systems, will likely be high. However, regardless of the underlying mechanisms, our  
157 findings robustly show that seasonality drives synchronous infection dynamics in *B. spinosus*  
158 and *R. temporaria*, despite previous research indicating a clear difference in susceptibility to  
159 *Bd* infection between these species (14, 31). More broadly, we have shown that failing to  
160 include environmental information may undermine our understanding of how pathogens  
161 spread and persist within host communities. Identifying differences in infection levels across  
162 species under different environmental conditions is therefore central to understanding  
163 disease ecology within multi-host communities, especially where changes in climate are  
164 predicted to exacerbate the impact of emerging infectious disease leading to further losses of  
165 biodiversity.

166

## 167 **Methods**

168 The study was conducted at Lac Arlet (Longitude: 0°36'54.12"W, Latitude: 42°50'24.20"N) in  
169 the Pyrenean National Park between 2007 and 2014 (with dates of thaw up to 2015). This  
170 lake sits at an altitude of 1986 m and encompasses an area of 2.7ha (Fig. 3).

### 171 *Prevalence and intensity of Bd infection*

172 All three species of amphibian present at Lac Arlet were sampled: emerging metamorphs of  
173 *B. spinosus* and *R. temporaria* and two life stages of *A. obstetricans*, over-wintered (OW)  
174 tadpoles and recent metamorphs; OW tadpoles are those which have spent at least one  
175 winter in the lake before completing metamorphosis. To sample the animals, sterile rayon-  
176 tipped swabs (MWE medical wire) were gently rotated (10 times per individual) over the  
177 mouthparts of OW tadpoles, and used to swab the hind legs, feet and pelvic patch (five  
178 swipes per area, with the swab rotated between each area) of each recent metamorph. All  
179 swabs were stored in dry tubes at 4°C until processing. Sampling of tadpoles was conducted  
180 each year in July. Metamorphs were sampled in August during the height of metamorphic  
181 emergence. In 2007, toe clips were taken from metamorphic *A. obstetricans* instead of skin

182 swabs; a 2-3mm clip was cut from a single hind toe using a sterile scalpel blade. These toe  
183 clips were fixed in 70% ethanol.

184 We followed the protocol of Boyle et al. (32), to quantify *Bd* prevalence and intensity of  
185 infection, as assessed by quantitative PCR (qPCR). To avoid inhibition, all extractions were  
186 diluted 1:10 prior to qPCR; therefore results were multiplied by 10 in order to determine the  
187 true value. We defined infection intensity as the number of *Bd* zoospore genomic  
188 equivalents (GE) per swab. All samples were run in duplicate, and a sample was assigned a  
189 positive reading if both wells amplified and an average estimate of 0.1GE or above was  
190 produced when comparing the sample to the curve generated by the standards. Samples  
191 were repeated up to three times if only one well amplified, after which time they were  
192 assigned a negative reading if both wells failed to amplify.

### 193 *Population counts*

194 Counts of newly emerged live and dead metamorphic *A. obstetricans* were conducted twice  
195 during each metamorph emergence period each year from 2010 to 2014. Searches were  
196 carried out around the entire circumference of the lake, from the shallows of the lakes (<0.5  
197 metres in depth) to a 1.5m distance from the water's edge onto dry land, including under  
198 movable rocks. Any deceased metamorphs found were removed so as not to be counted a  
199 second time on subsequent visits. We assumed that live animals would either disperse from  
200 the water's edge within a few days of emergence or die, and would therefore not be counted  
201 again in subsequent live estimates. Approximate counts (<100; 100-1000; >1000) were made  
202 for both newly emerged *R. temporaria* and *B. spinous* metamorphs each year, during the  
203 above surveying time. This measure was also applied to *A. obstetricans* OW larvae by  
204 scanning a 2m area of the water, from the water's edge.

### 205 *Diagnosis of chytridiomycosis in R. temporaria*

206 Owing to the high prevalence of *Bd* that we observed infecting *R. temporaria*, we decided to  
207 investigate whether individuals were suffering chytridiomycosis due to *Bd* infection,  
208 something which has not been shown before. Recently deceased, fresh *R. temporaria*  
209 metamorphs found around the edge of the lake were collected and fixed in 10% neutral  
210 buffered formalin. After fixation, the lower half of the each body (including pelvic region)  
211 and the front legs were processed for histopathological examination. Three levels per section  
212 were prepared, using a standard Haematoxylin & Eosin stain. Histological sections were

213 examined microscopically to determine the presence or absence of the disease  
214 chytridiomycosis.

#### 215 *Determining length of season*

216 Lac Arlet water temperature was measured every half an hour throughout the study period  
217 using a data logger (HOBO Water Temperature Pro v2 Data Logger - U22-001). The logger  
218 was positioned two meters from the shore, approximately half a meter below the water  
219 surface, attached to a large rock with non-perishable wire. For each year, the date of the  
220 onset of spring and the date of the presumed end of the amphibian active season was  
221 recorded. The onset of spring was defined as the first day of the year (00:00 – 23:59) with a  
222 mean water temperature above 1°C, which remained so until the winter. The end of the  
223 active season was defined as the first day in the second half of the year when the mean  
224 water temperature dropped below 5°C, as European amphibians will often enter hibernation  
225 around this temperature (33). This allowed the length of the active season (total number of  
226 possible ‘active days’ for amphibians) to be calculated by subtracting the Julian date of the  
227 onset of spring from the Julian date of the end of season.

#### 228 *Establishing the relationship between air temperature and lake thaw (onset of spring)*

229 To project the impact of rising temperatures, we first quantified a linear relationship  
230 between air temperature and time of thaw, which corresponds to onset of spring for this  
231 ecological system. This is consistent with findings from other studies showing air  
232 temperature to be highly informative for thawing of many lakes (34), even if temperatures  
233 are measured some distance from the lake. To establish this relationship for this site, daily  
234 weather data (maximum and minimum temperatures, precipitation) were obtained from the  
235 nearest meteorological station to Lac Arlet, Canfranc Los Arañones, 13km south-east of Lac  
236 Arlet. This is situated at 1160 metres above sea level, 826 metres below Lac Arlet. Hence, a  
237 correction of -5.29 °C was applied to the air temperatures to account for tropospheric  
238 temperature decrease with altitude, in accordance with previous studies (35). These adjusted  
239 temperature data along with daily precipitation for 1995-2015 were also used as baseline  
240 input for LARS-WG.

241 To find the optimal time period over which these air temperatures are most predictive for  
242 ice thawing time, and to quantify that relationship, linear regressions were performed  
243 between air temperature and thaw dates for 2007-2015 inclusive. Mean daily air temperature  
244 was calculated over time periods of lengths varying from 21-81 days in 10-day increments,

245 centred on days 30-170 of the year. The time period with the best predictive power for onset  
246 of spring, as judged by R-squared value and standard model checking plots of the linear  
247 regression, was chosen. Analysis was performed in R version 3.2.3.

248 We used the LARS-WG weather generator as a downscaling technique (36) to generate local-  
249 scale climate scenarios, based on climate projections from global climate models (GCMs)  
250 from the CMIP5 multi-model ensemble used in the latest IPCC Assessment Report 5 (AR5)  
251 (27). To capture uncertainty in the CMIP5 climate projections we selected two GCMs with  
252 low, (GISS-E2), and high, (HadGEM2), climate sensitivities (37), which thus predict lesser  
253 and greater degrees of warming for this region. This allowed us to quantify uncertainty in  
254 predictions of the onset of spring under climate change. We generated 100 years of daily  
255 plausible weather for the periods 2050s and 2090s under Representative Concentration  
256 Pathway (RCP) 8.5. The mean temperatures across the time period ascertained as most  
257 predictive for lake thaw were calculated from each of the 100 years of synthetic daily  
258 weather, for each GCM and time period combination.

### 259 *Statistical analysis*

260 All statistical analyses were carried out using the statistical software package 'R', version  
261 3.2.3. All *Bd* DNA values (GE) were rounded to the nearest whole number and treated as  
262 count data. Any value of 0.1 to 0.9 was assigned a value of 1. Negative binomial regression  
263 models (function `glm.nb` from the R-package MASS) were used to look for any differences in  
264 the intensity of infection (GE values) between years and species. Likelihood ratio tests were  
265 used to assess the significance of predictor variables and of differences between factor levels  
266 within predictors. Where more than three factor levels remained significant, Tukey post-hoc  
267 tests (function `glht` from the package multcomp) were applied to allow pairwise  
268 comparisons. Fisher's Exact Test was used to compare differences in the prevalence of  
269 infection in each of *A. obstetricans* metamorphs and tadpoles, over the years 2007 (2008 for  
270 tadpoles) to 2014; in *B. spinosus* compared to *R. temporaria* over the years 2008 to 2012 and  
271 between each of *B. spinosus* and *R. temporaria* compared to *A. obstetricans* metamorphs over  
272 the years 2008-2014 (2008 -2012 for *B. spinosus*). Pearson Correlation tests were used to  
273 determine whether there was a correlation between the prevalence of infection in both *B.*  
274 *spinosus* and *R. temporaria* and, 1) the mean infection intensity (GE) in *A. obstetricans*  
275 metamorphs, 2) the number of dead *A. obstetricans* metamorphs encountered each year.

276 Using the temperature data we were able to determine the date of the spring onset for all  
277 years apart from 2010, due to a failure of the datalogger resulting in missing data. However,  
278 a strong association was seen between the onset of spring and the date at which the first *A.*  
279 *obstetricans* metamorph was seen in the years excluding 2010 ( $t= 7.724$ ;  $p = 0.005$ , adjusted  $R^2$   
280  $= 0.94$ ). We used this strong association to predict the onset of spring in 2010 and included  
281 the predicted value in all further analyses. The onset of season (mean Julian days=150, SD  
282 =20) varied more than the end of season (mean Julian days=302, SD =4), therefore the onset  
283 of spring is the measure which primarily dictates season length. For this reason, we used  
284 spring onset as a proxy to assess changes in season length. Generalized Linear Models  
285 (GLM) using a binomial response (logistic regression) were used to determine if there was a  
286 relationship between the prevalence of infection in all three species and the onset of spring  
287 each year. Likelihood ratio tests were used to assess the significance of these effects. A  
288 generalized adjusted  $R^2$  was calculated to assess the predictive power of each model (38).

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399

400 **Supplementary Information:** see appended supplement

401

402 **Table 1:** The timing, in Julian days, of the start of spring and the end of season

403

<b>Year</b>	<b>Start of spring</b>	<b>End of season</b>	<b>Days of activity</b>
<b>2008</b>	162	302	140
<b>2009</b>	150	NA	NA
<b>2010</b>	137	298	161
<b>2011</b>	120	298	178
<b>2012</b>	145	302	157
<b>2013</b>	181	308	127
<b>2014</b>	160	309	149

404

405 **Table 2:** Visual estimates of amphibian abundance + <100; ++100-1000; +++ >1000

406

<b>Year</b>	<b><i>Ao OW</i> tadpoles</b>	<b><i>Rt Mets</i></b>	<b><i>Bb Mets</i></b>
<b>2008</b>	+++	+++	+++
<b>2009</b>	+++	+++	+++
<b>2010</b>	+++	+++	++
<b>2011</b>	+++	+++	++
<b>2012</b>	++	+++	+
<b>2013</b>	+	+++	0
<b>2014</b>	+	+++	0

407

408

409 **Figure Legends**

410 **Figure 1 a.** Temporal change in prevalence of infection for *Alytes obstetricans* (Ao), *Bufo*  
411 *spinosus* (Bs) and *Rana temporaria* (Rt); **b.** Temporal change in intensity of infection; **c.**  
412 Seasonal changes in water temperature and timing of spring onset in Lac Arlet; **d.**  
413 Relationship between spring onset and the prevalence of *Bd* infection across all species

414 **Figure 2 a.** Histology slide showing a section from the hind legs of a deceased *R. temporaria*  
415 metamorph demonstrating clear evidence of the disease chytridiomycosis. The two long  
416 arrows point to two of many sporangia full of zoospores, and the two short arrows point to  
417 empty sporangia cases (once zoospores have burst out), both embedded with the upper skin  
418 layers; **b.** Counts of live and dead *A. obstetricans* metamorphs over time with the number of  
419 dead (black) and alive (white) *A. obstetricans* metamorphs encountered. No 'alive' counts  
420 were made for years 2008 - 2009; **c.** Relationship between air temperature and the onset of  
421 spring at Lac Arlet; **d.** Future predictions of air temperatures at Lac Arlet

422 **Figure 3 a.** Lac Arlet showing the position of temperature datalogger (red arrow). **b.** Mass  
423 mortalities of midwife toads *Alytes obstetricans* caused by *Batrachochytrium dendrobatidis*  
424 lineage *BdGPL* at Lac Arlet