Delayed metamorphosis of amphibian larvae facilitates *Batrachochytrium dendrobatidis* transmission and persistence

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ABSTRACT

Highly virulent pathogens that cause host population declines confront the risk of fade-out, but if pathogen transmission dynamics are age-structured, pathogens can persist. Among other features of amphibian biology, variable larval developmental rates generate age structured larval populations, which in theory can facilitate pathogen persistence. We investigated this possibility empirically in a population of *Salamandra salamandra* in Spain affected by *Batrachochytrium dendrobatidis* (*Bd*) at breeding sites that lacked alternative amphibian hosts. None of the adults presented infection by *Bd.* However, for the larvae, while environmental heterogeneity was the most important predictor of infection, the effect on infection dynamics was mediated by transmission from overwintered larvae to new larval recruits, which occurred only in permanent larval habitats. We suggest that interannual *Bd* maintenance in a host population that experiences mass mortality associated with infection can occur without an environmental reservoir or direct involvement of an alternative host in our study system. However the two aquatic habitat types that support intraspecific reservoirs, permanent streams and ponds, are not ideal habitats for long-term *Bd* maintenance, either due to poor transmission probability or low host survival respectively. While intraspecific pathogen maintenance due to larval plasticity might be possible at our study sites, this transmission pattern is not without significant risk to the pathogen. The availability of alternative hosts nearby does indicate that permanent *Bd* fade-out is unlikely.

19 **INTRODUCTION**

20 Emerging infections are increasingly associated with high levels of host mortality followed by
21 persistent host decline or local extirpation (De Castro & Bolker 2005, Fisher et al. 2012). Pathogens
22 causing catastrophic host responses are put at risk by the rapid, large-scale decline in primary host
23 density, which might reduce the likelihood of successful pathogen transmission (Briggs et al. 2010).
24 Pathogens can compensate against this risk of fade-out (i.e. the pathogen is lost from the host
25 population below a density threshold, Bartlett 1960) by exploiting alternative hosts or by
26 environmental persistence outside of a host (De Castro & Bolker 2005, Garner et al. 2006). However,
27 both of these strategies also entail risk for the pathogen. Alternative hosts can exhibit significant
28 resistance to infection (Agrawal 2000) and intraspecific contacts may be significantly more likely
29 than interspecific contacts, reducing the opportunity for interspecific transmission events (Ruiz-
30 González et al. 2012). Similarly, environmental pathogen stages may not survive for extended periods
31 of time outside of a host, or withstand shifts in environmental conditions (Fuller et al. 2012).

32 Pathogens can compensate for these risks by adopting strategies that allow them to exploit the
33 primary host exclusively even when causing it to suffer mass mortality. As long as host mortality
34 remains low enough to allow at least some new infections to occur (i.e., the pathogen's basic
35 reproductive ratio $R_0 > 1$), even highly virulent and generalist pathogens can be maintained in a single
36 host system (Briggs et al. 2010). Within this context, the ecological setting (e.g. different habitats)
37 may offer opportunities for virulent pathogens and highly susceptible hosts to coexist even when
38 hosts experience mass mortalities. Moreover, if host mortality is age-dependent, and survival of the
39 susceptible age class allows for sufficient recruitment into older age classes, high rates of age-specific
40 mortality may be tolerated and infection maintained in the single host species population as has been
41 observed in the infection dynamic between the larvae of tiger salamanders (*Ambystoma tigrinum*
42 *stebbinsi*) and *Ranavirus*, ATV (Brunner et al. 2004).

43 Typically, larval amphibians either accelerate development in response to environmental risk or delay
44 metamorphosis in resource-constrained environments until sufficient resources are accrued to ensure

45 increased post-metamorphic survival exceeding that experienced by those that do not delay
46 metamorphosis (reviewed in Wells 2007). Thus, it is not uncommon, due to either developmental
47 plasticity or multiyear larval period, for different cohorts of larvae to overlap in the same
48 environment, potentially offering pathogens the opportunity to be transmitted amongst age classes.

49 The fungus *Batrachochytrium dendrobatidis* (*Bd*), which causes the emergent infectious disease
50 chytridiomycosis, has been implicated in amphibian population declines and extinction worldwide
51 (Fisher et al. 2012). In the *Bd*-amphibian host system, age-specific transmission dynamics amongst
52 dissimilar age classes has been examined mathematically by Briggs et al. (2010) in an American
53 anuran species. Maintenance of *Bd* in an anuran population facilitated by a larval reservoir has also
54 been postulated for another anuran species in Europe (Walker et al. 2010). Although post-
55 metamorphic mortality was and continues to be extremely high in both species, host population
56 persistence is a strong indication that at some locations enough animals survive to adulthood to
57 enable population persistence (Briggs et al. 2010, Walker et al. 2010).

58 In the amphibian-*Bd* system, while a role for delayed maturation of early developmental stages in
59 pathogen maintenance has been justified mathematically (Briggs et al. 2005), empirical data in
60 support of theory are scant. This is because delayed development is exhibited by some larvae in all
61 populations described in Briggs et al. (2010) and at all high-elevation sites studied by Walker et al.
62 (2010), and potential alternative hosts occur in both systems (Reeder et al. 2012). To ascertain if host
63 developmental plasticity is a key factor in pathogen maintenance, it would be necessary to compare
64 infection dynamics across geographically proximate populations with and without delayed
65 metamorphosis and eliminate the potential for pathogen maintenance via alternative hosts.

66 Here we report a comparative study of infection dynamics in larval populations of the fire
67 salamander, *Salamandra salamandra*. At high elevation sites in Western Europe, larvae of this
68 species commonly delay metamorphosis and overwinter in water at rearing sites. However, at some
69 of these locations water may be ephemeral, obliging larvae to complete metamorphosis in the same
70 season that they were deposited into the water. In Guadarrama National Park of Spain, infection with

Bd causes both pre- and post-metamorphic mortality (Bosch & Martínez-Solano 2006). After the local extirpation of *Alytes obstetricans* in the study area (Bosch et al. 2001), fire salamander larvae became the sole occupants of many larval rearing sites throughout the year (Bosch, unpublished data), and the natural history of the species ensures that only female adults make contact with rearing sites, and then only fleetingly (Wake 1993, Schmidt et al. 2007). In our study, we used field data of larval infection to determine if infection in cohorts of new (young of year, YoY) larvae could be the result of cohabitation with infected, overwintered (OW) larvae. We also examined how interactions between YoY and OW larvae were affected by rearing site hydrology (ponds vs. streams) and sampled adults to see if they act as potential pathogen reservoirs in the system. In order to place pathogen dynamics in a broader context, we surveyed *S. salamandra* rearing sites across Guadarrama N.P. for dead larvae and recently metamorphosed juveniles. Although the biology of the *Bd*-amphibian interaction hypothetically allows for the observed infection dynamic, our results provide some of the first empirical evidence that intraspecific infection with *Bd* could be possible without alternative hosts and that interactions between different larval age classes play a pivotal role in *Bd* maintenance.

METHODS

Study design

Salamandra salamandra adults and larvae were sampled in 2011 at 8 larval rearing sites located in Guadarrama N.P., Central Spain (40º50'N, 3º57'W). Sites were located within 800 meters of each other in the same drainage and at nearly identical elevations. Streams (n = 4) and ponds (n = 4) were evenly divided between permanent and temporary (i.e., dried completely each season) sites. Pond surface ranged from 60 to 5452 m^2 and stream lengths ranging from 45.5 to 325 m were included. We visited each site 3 to 5 times between late May and August, weather permitting.

Field methods

Density and infection surveys

97 We counted all visible larvae by walking along transects that covered ~ 90 % of the stream. Ponds
98 were small enough that we were able to walk around the entire perimeter and count all visible larvae
99 within 1-2 m of the shoreline. Due to the small size of the ponds, narrowness of streams (< 1m) and
100 the transparency of the water, this method provides the most accurate larval density estimates in this
101 system (Martínez-Solano et al. 2003). To facilitate comparison between streams and ponds we
102 converted larval counts to densities in animals m^{-1}. We sampled any adult salamanders that we
103 encountered within 1-2 m of the shoreline for evidence of infection with *Bd* by running a fine-tipped
104 swab (MW100; Medical Wire and Equipment Ltd., Wiltshire, England) repeatedly over the epidermis
105 of the abdominal region (10 strokes), all four limbs and digits of each foot (5 strokes/limb). Briefly,
106 all adults and larvae were handled with a pair of powder-free nitrile gloves and although *Bd* is known
107 to occur across the study area, gloves were changed among sites in order to avoid cross-site
108 contamination. Using nets that were sterilized among sites, we randomly captured 20 or 40 larvae at
109 each site, once during the spring surveys (early June) and once during the summer surveys (late
110 August) and sampled each for evidence of infection with *Bd* similarly as described above, with the
111 exception that the whole body of the larvae was swabbed (20 strokes total). Dry swabs were stored at
112 4ºC until being processed in the laboratory. Sample sizes differed between sites based on the presence
113 of OW larvae. Up to 40 larvae were swabbed at sites where both YoY and OW larvae occurred (i.e.
114 permanent sites in May-June), swabbing up to 20 from each category, and up to 20 YoY were
115 sampled at locations where OW larvae were absent (i.e. temporal sites and permanent sites in
116 August). This is because early in the season (May-June), both overwintering larvae from the previous
117 year and new larvae of the current year are found at permanent sites. However, later in the season
118 (August), the overwintering larvae have metamorphosed (or died), and therefore, the sites contained
119 only the current year larvae. OW larvae are distinguished from the small dull grayish-brown YoY by
120 their larger body size, and blackish coloration with the presence of golden-yellowish dorsal spots on
121 both sides of the head. All animals were unharmed and released at point of capture immediately after
122 sampling.

In addition, a parallel survey was conducted with the help of the local park staff to collect and collate dead animals counts for all water bodies located within the park boundaries that have been identified as *S. salamandra* rearing sites. Every site was surveyed for dead larval and recently metamorphosed juveniles 6 times every year, and we report findings for the two years immediately preceding the infection survey and for that same year.

Laboratory analysis

DNA extraction and qPCR amplification was conducted following the protocol of Boyle et al. (2004) using a 96 well CFX machine (BioRad). Each sample was run in duplicate against duplicate standards of 0.1, 1, 10 and 100 zoospore genomic equivalents (GE) and two negative controls. We considered an animal infected if both duplicates amplified with a mean GE of 0.1. We used an internal positive control (IPC) to measure PCR inhibition in randomly selected samples that tested negative for *Bd* infection. Following the methodology of Hyatt et al. (2007), a VICTM labelled synthetic amplicon was used as the IPC (VICTM dye, Applied Biosystems). The IPC was included in one of each duplicate well as 1 µl 10x Exo IPC mix and 0.5 µl 50x Exo IPC DNA.

Statistical analyses

General Linear Mixed Models (GLMM) were applied to analyze *Bd* infection (*Bd*I) dynamics in salamander larvae. The sampling site was considered as a random factor, the habitat, water permanence, larval stage and month as fixed factors, and larval density as the fixed covariate. The random factor "site" was nested within the corresponding levels of the fixed factors. The mean square (MS) and the degrees of freedom (df) of the error terms were estimated following Satterthwaite's method, which finds the linear combinations of sources of random variation that serve as appropriate error terms for testing the significance of the respective effect of interest. We used the unconstrained parameters model to test the significance of the fixed effect of the covariate, where its error term was the interaction of the covariate with the random factor "site" (Quinn & Keough 2002). This analytical procedure is very conservative, because it solves the problem of inflated sample sizes by reducing the degrees of freedom of the error terms and avoids pseudoreplication (i.e. the proper sample unit for the

fixed effects is the sampling locality "site" and not every salamander larvae captured in the field). *Bd* infection was transformed using the Box-Cox transformation prior to data analyses (lambda=-1.56; $Bd\text{I}' = [(Bd\text{I}+1)^{-1.56}-1]/-1.56$). Homoscedasticity and normality of residuals of the GLMMs were checked and they did not show considerable deviations from the canonical assumptions. Due to the existence of missing cells (i.e., the lack of data at several levels of the interactions among fixed factors; e.g., 'month x larval stage', 'larval stage x permanence', 'month x permanence') three GLMM were carried out: (a) one including the whole sample of salamander larvae and all factors excluding the month, (b) another including the month but restricted to permanent sites of the permanence factor, and finally (c) one examining the *Bd* infection of YoY in August as the response to determine if *Bd* infection of OW in June was a significant predictor. In the second GLMM, we used two planned *a priori* comparisons to attain greater statistical power, testing for the effect of larval stage (YoY vs OW) within the sample of larvae collected in June, and testing for the effect of months (June vs August) using the sample of new YoY larvae. Data were analyzed using StatSoft's Statistica 12 (StatSoft Inc, Tulsa, Oklahoma).

RESULTS

A total of 364 larvae and 116 adults of *S. salamandra* were swabbed. None of the adults tested positive for infection. IPCs showed that there was no evidence of PCR inhibition in any of the samples. Table 1 shows the average figures of prevalence and infection intensity of salamander larvae (original, non-transformed data) according to habitat type, water permanence of water bodies and larval stage. The General Linear Mixed Model with all data ($F_{12,351}= 50.33$, $p << 0.001$, 63.2% of the variance accounted for; Table 2) showed that *Bd* infection intensity in salamander larvae was not affected by larval density, and was significantly influenced by the larval stage, type of breeding habitat and permanence. No significant differences were found across the 'site' factor in infection intensity. There were significant interactions among the fixed factors 'permanence x type of breeding habitat' and 'larval stage x type of breeding habitat'. *Bd* infection intensity was greater in

175 salamanders from permanent ponds, and was absent or weak with little variation in salamanders
176 occupying temporary water bodies and permanent streams, respectively. Also, *Bd* infection intensity
177 of salamander larvae in ponds was greater in OW than in YoY larvae (we could not estimate the
178 remaining interaction terms due to missing cells in the data; see Methods). Habitat type and larval
179 stage were the predictors with the highest magnitude effects (partial η^2) that also explained the largest
180 amount of the variance in *Bd* infection intensity, followed by the interaction term 'type of habitat x
181 larval stage', while the influence of the site and the larval density inside water bodies were negligible.

182 The second General Linear Mixed Model showed that the effects on infection intensity of larval stage
183 (YoY vs OW) within the sample of larvae collected in June ($F_{1,5}$= 57.44, $p < 0.001$), and of months
184 (June vs August) within the sample of new young of the year (YoY) larvae ($F_{1,5}$= 37.94, $p = 0.002$)
185 were highly significant (after controlling for the effect of site, larval density and type of habitat; see
186 Methods for statistical details regarding a design with missing cells). *Bd* infection intensity was
187 greater in August than in June in new YoY salamander larvae inhabiting permanent water bodies,
188 while *Bd* infection intensity was also greater in OW than in new YoY larvae inhabiting permanent
189 water bodies in June.

190 Finally, during parallel field surveys, dead salamanders were found more frequently at locations with
191 permanent water than at temporary ponds across Guadarrama N.P. (Table 3). Taken together, these
192 results suggest that differences in type of breeding habitats and permanence, which foster large,
193 overwintering larvae, were critical in driving *Bd* infection intensities.

194

195 **DISCUSSION**

196 Our results show that the type of habitat and larval stage were the most important predictors of *Bd*
197 occurrence (which imply transmission to newly deposited larvae), and these were closely followed by
198 the factor of water permanence (Table 2). A mechanistic link between water availability and the
199 persistence of *Bd* is generally accepted based on the physiological needs of this fungal pathogen
200 (Kriger & Hero 2007). Thus, although *Bd* can persist in moist sand for extended periods of time

(Johnson & Speare 2005), drying at four of our eight study locations might have been lethal to any *Bd* that might be present in the environment given its sensitivity to desiccation (Johnson et al. 2003, Garmyn et al. 2012). At the three sites where infection persisted (i.e., two permanent ponds and one permanent stream), infection intensity in larval cohorts was significantly reduced when water was flowing rather than standing. Environmental conditions restricted to the aquatic environment can have a direct effect on transmission rate and infection dynamics by altering the density of viable zoospores (Schmeller et al. 2014). Increased water flow rate should also reduce the density of infectious particles that are available for transmission and reduce the likelihood that successful transmission will occur. In contrast, Kriger & Hero (2007) also described a significant difference in *Bd* prevalence when comparing amphibian infection data generated from sites with standing versus flowing water, but instead found that frogs at streams were more likely to be infected than those at ponds. The difference between these two studies is likely attributable to susceptibility differences across species: post-metamorphic stages of our focal species are not carrying the fungus, whereas in Kriger & Hero (2007) adult frogs were heavily infected. Therefore, our system excludes any influence of the terrestrial contact rates on transmission dynamics.

Despite the consistent effect of water dynamics on infection in our system, the necessity of an aquatic environment for the survival and persistence of *Bd* has been challenged by detection of infection in strictly terrestrial amphibian species (e.g. Weinstein 2009). Permanent water is also not a guarantee for zoospore survival (Schmeller et al. 2014). Although recent evidence suggested that *Bd* can persist in the water through the year in pond habitats, the detection probability of *Bd* increased as density of amphibians increased at the sampling sites (Chestnut et al. 2014). Thus, efforts to detect environmentally persistent forms of the fungus in aquatic habitats (including water bodies in Guadarrama N.P.) suggest that persistence outside the host in water is limited and that detection of *Bd* in aquatic environments is predicated on the presence of infected animals at the time of sampling (Kirshtein et al. 2007, Walker et al. 2007, Hossack et al. 2009). Certainly, the lack of transmission to YoY at ephemeral sites cannot be directly attributed to pond or stream drying over the course of our field season, as water was present at all four sites during our August surveys. We can also eliminate

228 the potential for vertical transmission, as none of the adults we sampled in the area exhibited
229 detectable infections. Instead, our data suggest that OW larvae -which did not occur at ephemeral
230 sites as drying either forced larvae to complete metamorphosis or die, leaving these sites unoccupied
231 for the incoming cohort of larvae in the subsequent year- drive infection dynamics in this system. We
232 conclude that the effect of water permanence on infection dynamics was potentially driven through its
233 direct effect on the presence of the intraspecific reservoir OW larvae. As argued in Fisher et al.
234 (2009), the amphibian host is the primary environment for *Bd*, thus maintenance of infection in a host
235 species and its ability to transmit infection to susceptible hosts should be the most important
236 predictors of pathogen persistence.

237 Field experiments investigating transmission probabilities of *Bd* in larval populations have shown that
238 site effects are interactive with host density (Rachowicz & Briggs 2007). This may go some way to
239 explaining why larval abundance was not an important factor affecting infections in our system,
240 which should be the case if transmission was density-dependent (Table 2). The few attempts that have
241 been made to determine the transmission function of *Bd* are inconsistent in their support of wholly
242 density-dependent transmission (Rachowicz & Briggs 2007, Briggs et al. 2010). The probability of
243 transmitting *Bd* resulting in sustained infections may instead be more strongly affected by factors that
244 influence the frequency of individual transmission events, such as temperature (Fernández-
245 Beaskoetxea et al. 2015), rather than the frequency of individual transmission events alone. Infections
246 in even highly susceptible host species are often lost then reacquired through subsequent re-exposure,
247 and threshold burdens are associated with clearance and maintenance of infection, as well as host
248 mortality; burdens that are realized through re-exposure and re-infection (Briggs et al. 2010,
249 Vredenburg et al. 2010).

250 Although the presence of OW larvae strongly influences transmission to YoY larvae at our study
251 sites, transmission to and reinfection of YoY is likely a process that continues after OW larvae have
252 completed metamorphosis. Infection intensity of larval cohorts was weak at permanent stream sites
253 and remains so across the season, which we attribute to the diluting effect of running water. In
254 comparison, significant amplification of infection intensity in YoY larvae, between June and August

255 in permanent ponds, indicates that the processes of transmission and intensification of infections are
256 better supported in permanent ponds and ongoing across the sampling period. Permanent stream sites
257 therefore represent a riskier environment for age-specific, single host species maintenance of *Bd* in
258 Guadarrama N.P. Nevertheless, YoY sampled at the end of the season in ponds with permanent water
259 exhibited weaker infections than their OW reservoir counterparts at the beginning of the sampling
260 period. Thus, although our study did not look at interannual patterns of infections, we would expect
261 that in the following year the remaining larvae will exhibit higher infection levels (i.e., similar to
262 those of the OW we sampled).

263 Although we conclude that *Bd* infection can be maintained in *S. salamandra* populations through
264 intraspecific transmission among larval age classes, it remains unresolved whether infection can be
265 sustained over the long term exclusively in this species without the benefit of an interspecific or
266 environmental reservoir. Our study indicates that intraspecific dynamics at permanent streams are
267 more likely to favour host persistence due to decreased intensity of infections compared to permanent
268 ponds, and in the long run might also favour pathogen fade out. Transmission dynamics at ponds
269 instead favour pathogen persistence over the short term. Importantly, declines of *S. salamandra* were
270 documented predominantly at pond locations and local extirpation associated with chytridiomycosis
271 was recorded at many of these ponds (Bosch & Martínez-Solano 2006). Salamanders are still dying
272 due to chytridiomycosis, and at much higher rates at locations with permanent water (Table 3).
273 Extirpation of OW larvae from a pond site could presumably be compensated for through
274 recruitment, but without the reservoir, the persistence of *Bd* at such a location seems highly unlikely.
275 Overall, while the maintenance of *Bd* infection in *S. salamandra* without an exogenous source of
276 infection in sites with OW larvae appears possible from our study, for the long term, the strategy is
277 risky for the pathogen.

278 Persistence of *S. salamandra* at Guadarrama N.P. is also uncertain. Our survey data shows that the
279 mortality rate that likely contributed to previously recorded extirpations is ongoing (Table 3).
280 *Salamandra salamandra* existence at higher elevations is closely linked to the ability of larvae to
281 delay metamorphosis and as we have shown this strategy requires that water be available throughout

282 the year. Ponds are a more reliable permanent water body than streams, as they are far more common
283 in the park, and far less likely to dry out than streams. If *Bd* does render these locations unavailable to
284 *S. salamandra* larvae, the overwintering strategy will be far less suitable. Notwithstanding, there is
285 reason to be more optimistic than when *Bd* was first detected in the park (Bosch et al. 2001).
286 Salamanders were thought to be in decline in Guadarrama N.P. and infection with *Bd* was considered
287 to be the culprit (Bosch & Martínez-Solano 2006), but more recent surveys suggest that the park
288 population may be at a relatively stable equilibrium (J. Bosch, unpublished data). Although adult
289 salamanders are susceptible to lethal disease under high *Bd* intensity (Bosch & Martínez-Solano
290 2006), after the near extirpation of *A. obstetricans* from our study sites they appear not to contract
291 infections. Irrespective, persistence of *Bd* and *S. salamandra* at Guadarrama N.P. cannot be predicted
292 based on intraspecific infection and transmission dynamics. Within this context, the recently
293 described amphibian pathogen, *Batrachochytrium salamandrivorans* (*Bsal*), although not present at
294 this point at the study site (Martel et al. 2014), would potentially affect the interaction between *Bd*
295 and *S. salamandra* due to its high virulence to *S. salamandra* and lower thermal range for optimal
296 growth compared to *Bd* (Martel et al. 2013).

297 Considering the infection dynamic we described and the threat posed by *Bsal*, mitigation strategies at
298 the Guadarrama N.P. to allow future amphibian reintroductions should focus on the life-history stages
299 that undergo extended periods of exposure to the pathogen (Scheele et al. 2014). Therefore, reducing
300 transmission events between larval stages of *S. salamandra* at permanent ponds could be an effective
301 starting point, while also assuring the conservation of habitats (with established *S. salamandra* larvae
302 populations) that are unsuitable for *Bd* transmission, such as permanent streams. On the other hand,
303 Guadarrama N.P. is host to several other amphibian species, including hosts that can harbour
304 infections. Whatever the fate of *Bd* in single host species populations, it is unlikely to be extirpated
305 from the area and the pattern of amphibian host decline still continues.

306

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Table 1. The effect of habitat type (pond / stream), water permanence (temporary / permanent) of water bodies and larval stage (young of year, YoY / overwintered, OW) on prevalence and *Bd* infection intensity of salamander larvae (mean and SD of zoospore genomic equivalents). N: number of salamander larvae.

Permanence	Habitat	Larval stage	N	Prevalence	Infection intensity	
					mean	SD
Permanent	Pond	OW	40	0.975	38.47	34.02
Permanent	Pond	YoY	80	0.438	5.17	18.09
Permanent	Stream	OW	40	0.075	0.41	1.46
Permanent	Stream	YoY	80	0.063	0.05	0.35
Temporary	Pond	OW		not applicable		
Temporary	Pond	YoY	60	0.000	0.00	0.00
Temporary	Stream	OW		not applicable		
Temporary	Stream	YoY	64	0.000	0.00	0.00

428 Table 2. Results of a general linear mixed-model of *Bd* infection in salamander larvae (Box-Cox
429 transformed) in relation to larval density (fixed covariate), type of habitat (pond vs stream),
430 permanence (permanent vs temporary), and larval stage (young of year vs overwintered larvae) as
431 fixed factors, and site as a random factor (nested within the levels of the previous fixed factors). The
432 effect sizes are also given as % of variance explained (according to sum of squares) and partial η2.
433 The interaction terms 'permanence x type of habitat' and 'larval stage x type of habitat' were only
434 estimated considering the missing cells design. Denominator degrees of freedom have been rounded
435 to the nearest unit. For more details on error term definitions, see Methods.

436

	Sum of squares	% variance	partial η^2	df	F	p
Larval density	0.001	0.00	0.000	1, 6	0.03	0.864
Permanence (P)	0.712	3.41	0.784	1, 6	23.38	0.002
Type of habitat (TH)	2.847	13.63	0.882	1, 14	105.50	<0.001
Larval stage (LS)	2.261	10.82	0.921	1, 6	73.94	<0.001
P * TH	0.587	2.81	0.756	1, 6	19.09	0.004
LS * TH	1.670	7.99	0.896	1, 6	54.63	<0.001
Site	0.185	0.89	0.024	6, 351	1.41	0.209

Table 3. Counts of ponds located in Guadarrama National Park occupied by *S. salamandra* larvae of any age class. Counts are split by water permanence and data are summed across 3 year's sampling (2009-2011). Numbers in parenthesis are the number of carcasses counted across all ponds in a given water permanence category.

	Number of ponds	Ponds with larvae of any age class	Ponds with only overwintered larvae	Ponds with observed mortality
Permanent	29	29	21	7 (58)
Temporary	213	153	0	1 (1)