Heterogeneity in infection and transmission of a multi-host pathogen within a community of amphibians

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Running Head: Infection heterogeneity

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Abstract

The majority of parasites infect multiple hosts. As the outcome of the infection is different in each of them, most studies of wildlife disease focus on the species that suffer the most severe consequences. However, the role that each host plays in the persistence and transmission of infection can be crucial to understanding the spread of that parasite and the risk it poses to the community. Current theory predicts that certain host-species can modulate the infection in other species by amplifying or diluting both infection prevalence and infection intensity, both of which have implications for disease risk within those communities. The fungus *Batrachochytrium dendrobatidis* (*Bd*), causal agent of the disease chytridiomycosis, has caused global amphibian population declines and extinctions. However, not all the species are affected equally, and it is a good example of a multi-host pathogen that must ultimately be studied with a community approach. Using an experimental approach both in captivity and in the field, we focused on the larval amphibian stage to investigate the susceptibility to *Bd* infection of all species found in the Peñalara Massif, Spain, both alone, and in the presence of a proposed reservoir and possible amplifier of infection: the common midwife toad. We observed that the most widely and heavily infected species, the common midwife toad, could be amplifying the infection loads in other species, all of which have different levels of susceptibility to *Bd* infection. Our results have important implications for performing mitigation actions focused on potential "amplifier" hosts and for better understanding the mechanisms of *Bd* transmission.

Key Words: *Alytes obstetricans*, amphibian assemblage, *Batrachochytrium dendrobatidis*, interspecific transmission, Peñalara Massif, Spain

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Introduction

The majority of parasites are able to infect multiple hosts (Fenton and Pedersen 2005). For example, most human pathogens are zoonotic in origin, and the majority of pathogens of livestock and domesticated species originated in wildlife species (Daszak et al. 2000). However, even within the widest host-base there exists a great deal of variation in how frequently and heavily different species become infected (Fenton & Pedersen 2005), as a result of which, host species play different roles in the persistence and transmission of infection within a community.

Just as individual-level transmission is highly skewed towards certain key individuals (Lloyd-Smith et al. 2005), the presence of certain species within a host community can be disproportionately important in the success of parasite invasion and persistence (Rudge et al. 2013). There are a number of ways in which certain species may be of particular importance; vectors, reservoirs, amplifiers and diluters of infection are all terms used to describe species that, in different ways, help maintain, spread or reduce infection within a community. While vectors and reservoirs are widely accepted concepts, empirical evidences for the existence of amplification or dilution hosts in natural systems are comparatively more scarce. The former of these - amplification hosts – are species that make a pathogen more likely to persist and more abundant than it would be in the absence of that species (Begon 2008). By increasing the overall prevalence and infection intensity within sympatric species, amplification hosts may increase the risk of disease emergence within a host assemblage. Quantifying species' differences in host competence and their roles in parasite

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69 transmission is therefore essential if we are to understand the dynamics of infection
70 and the likelihood of disease emergence within a community.
71 The chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), is a host-
72 generalist parasite that feeds mainly on the keratinized skin of developed amphibians
73 and the mouthparts of amphibians larvae (Berger et al. 1998). Chytridiomycosis is an
74 amphibian-specific emerging infectious disease caused by the fungus, and it has
75 caused severe population declines and species extirpations and extinctions worldwide
76 (Stuart et al. 2004). It is known to have infected over 400 species, including species in
77 all three amphibian Orders (Bd-maps; Gower et al. 2013), and probably many more
78 species are susceptible to *Bd* infection. However, there is a great deal of variation in
79 susceptibility to infection and its ill-effects both among (Lips et al. 2006, Bielby et al.
80 2008) and within species (Walker et al. 2010). A range of intrinsic (Woodhams et al.
81 2007, Farrer et al. 2011, Jani & Briggs 2014) and extrinsic factors (Vredenburg et al.
82 2010, Raffel et al. 2015) have been linked to the variation in the impact *Bd* has upon
83 species and communities, but, as yet, relatively little is known about the role of
84 community composition in this context.
85 The first known chytridiomycosis-related mortalities in Europe occurred in the late
86 '90s, and led the common midwife toad (*Alytes obstetricans*, hereafter *Ao*) to the
87 brink of local extinction in the Peñalara Massif at the Sierra de Guadarrama National
88 Park (Bosch et al. 2001). The species seems to be the most severely impacted species
89 in Europe (Tobler & Schmidt 2010, Walker et al. 2010), and as a result of the ease
90 with which it becomes infected, the clade Alytidae acts as a reliable sentinel species
91 when screening for infection in new regions or populations (Balaz et al. 2014).
92 However, other species in heavily affected assemblages, such as Guadarrama NP,
93 exhibit a variety of responses to *Bd* exposure. Following initial *Ao* mass mortalities,

common toads (*Bufo spinosus*) and fire salamanders (*Salamandra salamandra*), also suffered mortality and declines as a result of chytridiomycosis (Martínez- Solano et al. 2003, Bosch & Martínez-Solano 2006, Bosch et al. 2014). In contrast, the rest of the species within the community at Guadarrama seem not to have been seriously affected by the disease, although all of them can be infected by the pathogen (Bd-maps). The population-level effects of chytridiomycosis over *B. spinosus* and *S. salamandra* after the near extinction of *Ao* are lower (Bosch, unpublished data) and, therefore we hypothesize that *Ao* species was driving much of the infection transmission.

To better understand the risk of disease emergence within a host community, it is important to understand how different species within that community differ in their susceptibility to *Bd* infection, and their role in infection transmission. In this study we aim to look at the tolerance range to *Bd* across the amphibian species of the Peñalara Massif and investigate aspects of the transmission of the pathogen within this assemblage. Doing so is important in designing better management strategies, preventing future declines and improving reintroduction programme success. Specifically, we test the hypotheses that *Ao* act as an amplification host and that individuals of sympatric species will experience a higher probability and intensity of infection than individuals housed only with their own species. Further, we test the hypothesis that *Bd* can transmit both directly, from *Ao* larvae to sympatric species, and also indirectly, rather than relying on direct contact with an infected host. Finally, we investigate whether community members exhibit different levels of infection from one another when housed with *Ao* larvae. Combined, these experiments could help to explain some of the observed community-levels impacts of chytridiomycosis in the presence and absence of *Ao*.

Methods

The Peñalara Massif is home to eight endemic amphibian species: *Alytes obstetricans*, *Bufo calamita*, *Bufo spinousus*, *Hyla molleri*, *Pelophylax perezi*, *Rana iberica*, *Triturus marmoratus*, *S. salamandra*. Additionally, *Mesotriton alpestis* was recently introduced in the area (Martínez-Solano et al. 2003).

Bd sampling

To analyse *Bd* infection loads, we took samples when the larvae were close to metamorphosis, from the parts of the body where the concentration of zoospores is generally highest (Garner et al. 2009): the hind limbs of anurans or the complete body of urodeles. As *Ao* tadpoles have a longer developmental time (up to 5 years), samples were taken from the keratinized mouthparts when companion species were sampled, or at the end of the experiment. Samples were taken from live animals with a fine-tipped sterile swab (Medical Wire and Equipment-MW&E 113) or directly from the corresponding tissue when individual had died or been euthanized.

Laboratory methods

To quantify infection load in amphibians we used quantitative real-time polymerase chain reaction protocol, qPCR (Boyle et al. 2004). Extractions were diluted 1:10 before real-time PCR amplification, performed in duplicate, with *Bd* genomic equivalent (GE) standards of 100, 10, 1, and 0.1 GE in a CFX96 machine (BioRad). When only one replicate from any sample amplified, we ran this sample a third time. If the third amplification did not result in an amplification profile, we considered the sample negative for infection.

Experiment 1: This experiment was set-up to test hypothesis that *Ao* acts as an amplification host by increasing infection prevalence and intensity in sympatric

144 species, and also that *Bd* can initially infect hosts indirectly, rather than relying on
145 direct contact with an infected host. We conducted a field-experiment in the Laguna
146 Grande de Peñalara glacial lake of the Peñalara Massif (2018 m.a.s.l.) and *B. spinosus*
147 was chosen as our focal susceptible species as it has been observed to suffer infection
148 and mortality as a result of *Bd* infection in natural surroundings (Bosch & Martinez-
149 Solano 2006), and in experimental settings (Garner et al. 2009). Several hundred *B.*
150 *spinosus* free-swimming Gosner stage 25 tadpoles (Gosner 1960) were collected from
151 different locations at Laguna Grande to average any possible genetic variation among
152 offspring. At this stage of development, *B. spinosus* tadpoles lack *Bd* infection (Ortíz-
153 Santaliestra et al. 2011). Uninfected *Ao* larvae were obtained from a captive colony
154 located in the studied area that is regularly tested for *Bd* infection by qPCR. Larvae
155 from the stock of our focal species, *B. spinosus*, were assigned to one of four different
156 treatments in a 2x2 experimental design. The two factors of interest were density and
157 the presence of *Ao* larvae, and each of these two factors had two-levels: high density
158 (50 *B. spinosus* larvae), low density (25 *B. spinosus* larvae), and presence (10 larvae)
159 or absence (0 larvae) of *Ao* larvae. The selected densities are within the range
160 typically observed naturally in this system (Bosch unpublished data). Each treatment
161 was replicated three times, each being housed in a separate 4 L container. The
162 containers had ventilated sides and were placed together floating in the lake. Water
163 temperature inside each container was recorded with a thermocouple thermometer in a
164 randomized order and found not to differ between containers. The experimental
165 design removed the possibility that infection was introduced with any of the
166 experimental animals, as they came from uninfected stock, or were placed in the
167 experiment before keratinised mouth-parts had developed and had the opportunity to
168 become infected. Instead, experimental animals could only become infected when

exposed to zoospores in the lake water. Once the most advanced *B. spinosus* tadpoles were close to metamorphosis (31 days after the experiment began), the experiment was ended, and we euthanized 20 randomly selected *B. spinosus* tadpoles (Gosner stages 38-42) per container and stored them in 70% ethanol before processing for *Bd* infection. We ended the experiment at this point because we wanted to assess infection in larvae, before they undergo metamorphosis when some individuals lose infection, or infection becomes difficult to detect (Garner et al. 2009).

To see whether the four-experimental levels resulted in different probability of infection we used a chi-square test, and, in the presence of any significant variation, generalised linear models with binomial errors were used to determine which of the two factors best explained variation in infection probability of *B. spinosus*. For the latter analysis backwards stepwise regression of a full model including all terms was implemented, with changes in model fit being measured using analysis of deviance. Because our experimental design does not adequately account for the total density of larvae when considering the presence and absence of *Ao* as a factor (i.e. within each level of the density treatments, *B. spinosus* had different total tadpole densities depending on whether *Ao* was present or not), we used binomial tests to identify whether the proportion of individuals infected significantly varied in the high and low density treatments in the absence of *Ao.* Doing so allowed us to determine whether an increase in the density of the focal host was an important factor in infection levels in the absence of *Ao*.

To analyze whether infection intensity varied with density and presence/absence of *Ao* larvae we used generalised linear models with negative binomial errors using theglm.nb function from the R package MASS, and the function glht from the

multcomp library was used to find which levels of the four treatments varied from one another.

Experiment 2: This experiment tested whether species co-housed with *Ao* larvae differed from one another in probability and intensity of infection. Fifty two 2 L plastic containers were floated together in the Laguna Grande de Peñalara. The containers had holes to allow water exchange with the surrounding lake. Again, water temperature inside the containers was measured with a thermocouple thermometer in random order without mixing the water before sampling began and it did not differ significantly among containers. Each of thirteen treatments was replicated four times. The thirteen treatments were: (1) two larvae of *Ao* alone, which acted as a control to see how heavy infection was in this species when housed alone; (2-7) six treatments consisting of two larvae of *Ao* co-housed with two larvae of each of, *B. spinosus, B. calamita, H. molleri, P. perezi, R. iberica, or S. salamandra*; and (8-13) two larvae of each of those six species alone (i.e. no *Ao* were added). Larvae of studied species we collected at the field in several ponds of Peñalara Massif, yet *Ao* larvae were obtained from the captive colony. All larvae were placed in the experimental set-ups at an early stage of their development before keratinised mouth-parts had developed and their uninfected status were confirmed by qPCR. One overwintered larvae of *S. salamandra* from the same lake was introduced into each container for one week. As over-wintered *S. salamandra* have an infection prevalence of 100% in spring in this system (Medina et al. 2015), this was a guaranteed way to expose experimental animals to infection regardless of whether experimental animals were exposed to zoospores in the lake water. At the end of the experiment we measured the infection intensity of all larvae in each of the thirteen treatments.

We tested whether species differed from one another in their infection probability, first using a Fisher's exact test to see whether the proportion of infected individuals of the different species varied, and in the event of a significantly non-random distribution of infection, we used binomial tests to determine which species varied significantly from the background prevalence of infection in the experiment.

To determine whether infection intensity in co-housed species was higher in the presence of *Ao*, for each of the six co-housed species we conducted a t-test comparing infection intensity between those individuals co-housed with *Ao* to those housed only with a conspecific.

To investigate whether infection intensity differed among each species when co-housed with *Ao* we used a generalised linear model with negative binomial errors and Tukey comparisons. The same statistical tests were used to determine whether *Ao* varied in infection intensity when co-housed with different species. Generalised linear models with negative binomial errors were conducted using the glm.nb function from the MASS library, and the Tukey comparisons on the resulting glm.nb object were made using the glht function from the multcomp library.

Experiment 3: The following experimental set-up in the laboratory was used to test whether *Ao* can transmit directly to other species, whether those species differ from one another in the resulting infection intensity, and whether *Ao* experiences different levels of infection when co-housed with other species. Newly hatched larvae of five species were captured at the field in several ponds of Peñalara Massif and their uninfected status was confirmed by qPCR: *H. molleri*, *P. perezi*, *M. alpestris*, *T. marmoratus* and *S. salamandra*. Two larvae of each of those species were placed in the presence of a single infected *Ao* larva, resulting in five experimental treatments.

The sixth treatment was a single infected *Ao* larvae, housed alone. Each of the six treatments was replicated 10 times in 2011 and 15 times in 2012. All experimental replicates were housed in 1.5L containers maintained at a temperature of 18°C. All *Ao* larvae were collected from a well-studied population (Toro, Zamora, western-central Spain; Fernández-Beaskoetxea et al. 2015) and their infection status was checked by qPCR before the experiment started. To test whether species differed from one another in their probability of infection we used a Fisher's exact test on counts of infected and uninfected for each species. To test whether species differed from one another in their infection intensity when co-housed with *Ao* larvae we used a generalised linear model with negative binomial errors, and Tukey comparisons between species to see where significant differences occurred. The former were conducted using the glm.nb function from the MASS library, and the Tukey comparisons on the resulting glm.nb object were made using the glht function from the multcomp library. All analyses were conducted in the statistical software package, R (R Core Team 2014).

Results

Experiment 1

The prevalence of *Bd* in *B. spinosus* tadpoles at the beginning of the experiment was 0% according to qPCR analyses. The prevalence of infection in *B. spinosus* at the end of the experiment differed significantly among the four treatments ($X^2 = 38.23$, d.f. = 3, $p<0.001$; Table 1). In the presence of *Ao* larvae the prevalence of infection in *B. spinosus* was around 50%, while in the absence of *Ao* larvae it was lower than 7%. The fact that infection occurred suggests that infection can occur and persist via indirect transmission and is not initially reliant on direct contact with an infected host.

Our model of infection probability simplified to leave the presence/absence of *Ao* larvae as the only significant predictor of likelihood of infection (Table 2). Using a binomial test we found no significant difference in the proportion of infection of *B. spinosus* larvae kept at low (2/25) and high density (3/50) in the absence of *Ao* (X^2 = <0.001, df = 1, p = 1), indicating that regardless of the density of *B. spinosus*, infection did not become well established in the absence of *Ao*. Because of the very low number of infected animals in each of these two treatments it was not possible to compare infection burden between the two.

The model of infection intensity contained both density of hosts and the presence/absence of *Ao* as factors affecting infection intensity in *B. spinosus* tadpoles. The model output for this model is presented in Table 3. The model-fit could not be significantly improved by the backwards stepwise regression process, meaning that the best-fitting model was obtained when both terms were left in the model. Tukey's least honest significant differences suggested that *B. spinosus* larvae housed at high-density in the presence of *Ao* larvae had a significantly higher infection burden than those at high density without *Ao* larvae, and that *B. spinosus* larvae held at low density in the presence of *Ao* larvae had a higher infection intensity than *B. spinosus* larvae at high density in the absence of *Ao* (Table 4).

Experiment 2

When co-housed with *Ao* larvae, the six species showed no significant difference from one another in their probability of becoming infected (Fig. 1a; Fisher's exact test: 0.072).

Four of the six co-housed species had significantly higher infection intensity in the presence of *Ao* than when housed only with conspecifics (*Bufo spinosus*: t = 3.097, df

= 12, p = 0.009; *Bufo calamita*: t = 4.705, df = 9, p = 0.001; *Hyla molleri*: t = 3.399, df = 13, p = 0.0475; *Salamandra salamandra*: t = 0.377, df = 14, p = 0.741; *Pelophylax perezi*: t = 4.582 df = 14 p < 0.001; *Rana iberica*: t = 2.037, df = 12, p = 0.064; Fig. 1b). Species was a significant predictor of infection intensity in our negative binomial glm (F = 9.712, df = 5, p<0.001), and Tukey's honest significant tests highlighted significant differences in the infections between those species (see Table 5). *Rana iberica* had a significantly lower infection level than *B. spinosus*, *B. calamita* and *H. molleri*. *S. salamandra* had a lower infection intensity than those latter three species plus *P. perezi*. *Hyla molleri* had a significantly higher infection intensity than *P. perezi*.

There were no significant differences in the proportion of individuals infected or the infection intensity in *Ao* larvae when co-housed with different species (Fig. 1a; Fisher's exact test p-value = 0.796), most likely because by the end of the experiment most *Ao* larvae were fairly heavily infected (Fig. 1b).

Experiment 3

Individuals of other species co-housed with *Ao* did become infected, suggesting that *Ao* can transmit infection to other species. A Fisher's exact test on the species co-housed with *Ao* suggested that there was no significant difference between probability of infection in those species (p = 0.2126; Fig. 2).

Significant differences in infection intensity were present between those species (Fig. 2; F = 4.9807, d.f. = 4, p < 0.001). *Pelophylax perezi* had a significantly higher infection intensity than *H. molleri*, *Triturus marmoratus*, and *S. salamandra*. *Mesotriton alpestris* had heavier infections than *H. molleri* and *T. marmoratus* (Table 6).

The prevalence of infection in *Ao* varied significantly depending on whether they were housed alone or with the larvae of other species (Fig. 2; Fisher's exact, $p = 0.0036$). *Ao* larvae experienced differences in infection intensity depending upon the species with which they were co-housed ($F = 5.068$, $df = 5$, $p < 0.001$). *Ao* housed alone had significantly lower infection burdens than when housed with any species aside from with *M. alpestris*, when the infection intensity in *Ao* did not differ from when housed alone. *Ao* larvae housed with *H. molleri* had significantly higher infections than those *Ao* larvae housed with *M. alpestris* (Table 7).

Discussion

Within an assemblage of hosts it is difficult to predict whether a parasite will become established, will spread, or will cause disease because of heterogeneity in host response within a community. This study shows that all species of the Peñalara Massif are susceptible to *Bd* infection, and that their levels of susceptibility vary greatly from one another, as a result of which different species are likely to play different roles in the infection dynamics within the system. Of particular note, our data suggest that the larvae of one species, *Ao*, could contribute a disproportionate amount to the spread of infection and, in so doing, may act as an amplification host. By carrying severe infections, causing co-housed species to experience elevated levels of infection, and by transmitting directly to other species, overwintering *Ao* larvae may play the role of amplification host within this host community.

The ability of over-wintering amphibian larvae to act as infection reservoirs is well-established (Brunner et al. 2004, Narayan et al. 2014, Medina et al. 2015), yet there is little empirical evidence to suggest that they can increase levels of infection within a

342 host assemblage. Combined, the results of our experiments suggest that *Ao* larvae are
343 able to increase infection prevalence and intensity in a number of co-housed species
344 by directly transmitting infection to them. Further, *Ao*'s ability to act as an
345 amplification host appears to be independent of the overall density of larvae around it,
346 as highlighted in experiment 1. In this experiment the density of cohoused focal
347 species, *B. spinosus*, did not affect its likelihood of becoming infected, which
348 remained close to zero in the absence of *Ao*. In contrast, *B. spinosus* larvae held at low
349 density in the presence of *Ao* larvae had a higher infection intensity than *B. spinosus*
350 larvae at high density in the absence of *Ao*, suggesting that the presence of a single *Ao*
351 larva resulted in a significant increase in infection probability and intensity regardless
352 of overall host density. The fact that the presence of *Ao* is strongly associated with
353 infection in other species, regardless of the overall density of hosts, suggests that even
354 post-decline, when the overall density of hosts is reduced, infection may still be
355 maintained and spread providing *Ao* larvae remain.
356 What characteristics would predispose *Ao* to act as reservoirs or disseminators of
357 infections in the shorter-term? One possible morphological feature that would lend
358 itself to a species harbouring and transmitting high-levels of infection is its large oral
359 disc. In this species this feature is unusually large, and includes numerous rows of
360 large denticles with a high concentration of keratin, and therefore has a greater area to
361 be infected by the pathogen (Berger et al. 1998), but see Searle et al. (2011), in which
362 species with the smaller sizes, such as *Anaxyrus boreas*, presented the highest
363 infection loads. This potential mechanism could be explored further using techniques
364 to track infection prevalence and infection intensity in different body parts, and
365 highlights the importance of understanding a species' biology when considering their
366 roles in transmission of infection within a community of hosts.

Efforts to better understand how and when transmission of infection will take place rely greatly on accurate information on mechanism and modes of transmission. Within this host-pathogen system it is generally assumed that, given the low motility of *Bd* zoospores (Moss et al. 2008, Lam et al. 2011), and the tendency of amphibians to cluster at high densities in suitable conditions (Duellman & Trueb 1994), direct host-contact may be the most common method of infection transmission. The data we obtained from experiment 1 suggests, however, that initial infection can and does occur as a result of exposure to infected lake-water by means of zoospores present in the lake. This finding supports previous research in demonstrating that transmission of infection does not necessarily require a direct contact between the tadpoles (Rachowicz & Briggs 2007), and can help to inform future efforts to understand transmission events within this host-parasite system.

A great deal of variation in host susceptibility to *Bd* infection was observed within our experiments 2 and 3. Although the majority of species had increased probability of infection and infection intensity in the presence of *Ao* larvae, there was a great deal of variation among species as to how prevalent or severe those infections became. These differences reflect how the transmission dynamics within a community may differ depending upon its constituent species, making it difficult to make general recommendations of predictions as to how host communities will respond to the introduction of *Bd*. Additionally, there was little consistency in how the studied species responded to *Bd* introduction levels among performed experiments (for example, *H. molleri* and *P. perezi* on experiments 2 and 3).

Infection levels varied not only in those species co-housed with *Ao*, but also in *Ao* larvae depending upon the species with which they were housed. Co-housed *Ao* generally suffered more frequent and heavier infections than those housed alone, but

392 those with *M. alpestris* did not, having significantly lower infections than *Ao* housed
393 with *H. molleri* larvae. While it is impossible to determine the mechanism behind this
394 difference, the end result is that, even for a host capable of carrying heavy infection
395 burdens, competition with other larvae, or the ability for infection to be transmitted
396 both to and from other species in the assemblage may, at times, be important for the
397 maintenance of infection. These inconsistencies and inter-species differences suggest
398 that the outcome of *Bd* exposure is highly context-dependent, and may differ greatly
399 depending upon the source of infection and the environment in which the larvae
400 develop, illustrating how important it is to consider carefully the generalities of
401 research into the transmission within any host-parasite system.
402 Rachowicz and Briggs (2007) showed that under laboratory or field conditions, there
403 is a clear influence of the density of infected individuals in the rates of *Bd*
404 transmission. The density of both host and pathogen are fundamental parameters in
405 the transmission of infectious disease. In the case of our experiments, although the
406 experimental numbers of tadpoles were similar to those used at the study mentioned
407 above, we did not find a significant effect of density of tadpoles in the variation of *Bd*
408 infection intensity. Our experimental design meant that comparisons of species co-
409 housed with and without *Ao* varied not only in species composition, but also in the
410 density of animals in the experimental treatments. Accounting for both density and
411 species composition would be the ideal approach to take, but the practicalities of these
412 experiments meant that was not possible. Regardless of these different densities,
413 though, the main findings of our experiments remain unchanged, in that *Ao*
414 presence/absence is a greater predictor of infection the overall density of tadpoles
415 (experiment 1), that species co-housed with *Ao* differ in their response to parasite
416 exposure (experiments 2 and 3), that *Ao* varies in its infection levels depending on the

417 species with which it is housed, and that *Ao* can directly infect other species
418 (experiment 3).
419 To add more complexity to the overall findings, competition and stress between two
420 host species may account for some of the observed pattern. Additional experiments
421 with the target host at different densities and addition of non-target and non-*Ao* hosts
422 would be needed to test whether additional host species simply cause competitive
423 stress and thus lead to increased infection.
424 Identifying the roles that different species or life-stages play in the transmission,
425 prevalence and intensity of infection is crucial to better understand the persistence and
426 spread of infection within a host-pathogen system. Knowledge related to which
427 species are more tolerant and more susceptible to infection could allow mitigation
428 design to focus on reducing the levels of infection in a host; in the case of our study
429 system by aiming to reduce the amount of infection in potential "amplifier" hosts.
430 Considering the species composition of a particular host community is therefore
431 essential in efforts to understand the spread of infection, risk of disease emergence,
432 and, ultimately, in managing systems to minimise any negative effects of pathogens
433 on biodiversity.
434
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Literature cited

Balaz V, Voeroes J, Civis P, Vojar J, Hettyey A, Sos E, Dankovics R, Jehle R, Christiansen DG, Clare F, Fisher MC, Garner TWJ, Bielby J (2014) Assessing risk and guidance on monitoring of *Batrachochytrium dendrobatidis* in Europe through identification of taxonomic selectivity of infection. Conser Biol 2:213-223

Bd-maps. http://www.bd-maps.net/ (accessed March 17 2015)

Begon M (2008) Effects of host diversity on disease dynamics. In: Ostfeld RS, Keesing FF, Eviner V (eds) *Infectious disease ecology: effects of ecosystems on disease and of disease on ecosystems*. University Press Princeton, Princeton, p 12-29

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 95:9031-9036

Bielby J, Cooper N, Cunningham AA, Garner TWJ, Purvis A (2008) Predicting susceptibility to future declines in the world's frogs. Conserv Lett 1:82-90

Bosch J, Fernández-Beaskoetxea S, Scherer RD, Amburgey SM, Muths E (2014) Demography of common toads after local extirpation of co-occurring midwife toads. Amphibia-Reptilia 35:293-303

Bosch J, Martínez-Solano I (2006) Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Peñalara Natural Park Spain. Oryx 40:84-89

Bosch J, Martínez-Solano I (2003) Factors influencing occupancy of breeding ponds in a montane amphibian assemblage. J Herpetol 37:410-413

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

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Organ 60:141-148

Brunner JL, Schock DM, Davidson EW, Collins JP (2004) Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. Ecology 85:560-566

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife--threats to biodiversity and human health, Science 287:443-449

Duellman WE, Trueb L (1994) Biology of amphibians. University Press, Baltimore

Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, Bosch J, Cunningham AA, Weldon C, du Preez LH, Anderson L, Pond SLK, Shahar-Golan R, Henk DA, Fisher MC (2011) Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. Proc Natl Acad Sci USA 108:18732-18736

Fenton A, Pedersen AB (2005) Community epidemiology framework for classifying disease threats. Emerg Inf Dis 11:1815-1821

Fernández-Beaskoetxea S, Carrascal LM, Fernández-Loras A, Fisher MC, Bosch J (2015) Short term minimum water temperatures determine levels of infection by the amphibian chytrid fungus in *Alytes obstetricans* tadpoles. PLoS ONE e0120237

492 Garner TWJ, Walker S, Bosch J, Leech S, Rowcliffe JM, Cunningham AA, Fisher
493 MC (2009) Life history tradeoffs influence mortality associated with the
494 amphibian pathogen *Batrachochytrium dendrobatidis*. Oikos 118:783-791
495 Gosner KL (1960) A simplified table for staging anuran embryos and larvae with
496 notes on identification. Herpetologica 16:183-190
497 Gower DJ, Doherty-Bone T, Loader SP, Wilkinson M, Kouete MT, Tapley B, Orton
498 F, Daniel OZ, Wynne F, Flach E (2013) *Batrachochytrium dendrobatidis*
499 infection and lethal chytridiomycosis in caecilian amphibians (Gymnophiona).
500 EcoHealth 10:173-183
501 Jani AJ, Briggs CJ (2014) The pathogen *Batrachochytrium dendrobatidis* disturbs the
502 frog skin microbiome during a natural epidemic and experimental infection.
503 Proc Natl Acad Sci USA 111:E5049-E5058
504 Lam BA, Walton DB, Harris RN (2011) Motile zoospores of *Batrachochytrium*
505 *dendrobatidis* move away from antifungal metabolites produced by amphibian
506 skin bacteria. EcoHealth 8:36-45
507 Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L,
508 Pessier AP, Collins JP (2006) Emerging infectious disease and the loss of
509 biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci USA
510 103:3165-3170
511 Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz W (2005) Superspreading and the
512 effect of individual variation on disease emergence. Nature 438:355-359
513 Martínez-Solano I, Bosch J, García-París M (2003) Demographic trends and
514 community stability in a montane amphibian assemblage. Conserv Biol
515 17:238-244

516 Medina D, Garner TWJ, Carrascal LM, Bosch J (2015) Delayed metamorphosis of
517 amphibian larvae facilitates *Batrachochytrium dendrobatidis* transmission and
518 persistence. Diseas Aquat Organ 00:00-00
519 Moss AS, Reddy NS, Dorta JIM, Francisco MJS (2008) Chemotaxis of the amphibian
520 pathogen *Batrachochytrium dendrobatidis* and its response to a variety of
521 attractants. Mycologia 100:1-5
522 Narayan EJ, Graham C, McCallum H, Hero JM (2014) Over-wintering tadpoles of
523 *Mixophyes fasciolatus* act as reservoir host for *Batrachochytrium*
524 *dendrobatidis*. PLoS ONE 9:e92499
525 Ortiz-Santaliestra ME, Fisher MC, Fernández-Beaskoetxea S, Fernández-Benéitez
526 MJ, Bosch J (2011) Ambient ultraviolet B radiation decreases the prevalence
527 of infection by *Batrachochytrium dendrobatidis* in two amphibian species.
528 Conserv Biol 25:975-982
529 R Core Team (2014) A Language and Environment for Statistical Computing R
530 Foundation for Statistical Computing Vienna Austria, URL http://www.R-
531 project.org/
532 Rachowicz LJ, Briggs CJ (2007) Quantifying the disease transmission function:
533 effects of density on *Batrachochytrium dendrobatidis* transmission in the
534 mountain yellow-legged frog *Rana muscosa*. J Anim Ecol 76:711-721
535 Raffel TR, Halstead NT, McMahon TA, Davis AK, Rohr JR (2015) Temperature
536 variability and moisture synergistically interact to exacerbate an epizootic
537 disease. Proc R Soc Lond [Biol] 282:2014-2039
538 Rudge JW, Webster JP, Lu DB, Wang TP, Fang GR, Basáñez MG (2013) Identifying
539 host species driving transmission of schistosomiasis japonica a multihost
540 parasite system in China. Proc Natl Acad Sci USA 110:11457-11462

541 Searle CL, Biga LM, Spatafora JW, Blaustein AR (2011) A dilution effect in the
542 emerging amphibian pathogen *Batrachochytrium dendrobatidis*. Proc Natl
543 Acad Sci USA 108:16322-16326
544 Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, Waller
545 RW (2004) Status and trends of amphibian declines and extinctions
546 worldwide. Science 306:1783-1786
547 Tobler U, Schmidt BR (2010) Within-and among-population variation in
548 chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. PLoS ONE
549 5:e10927
550 Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging
551 disease drive large-scale amphibian population extinctions. Proc Natl Acad Sci
552 USA 107:9689-9694
553 Walker SF, Bosch J, Gomez V, Garner TWJ, Cunningham AA, Schmeller DS,
554 Ninyerola M, Henk D, Ginestet C, Arthur CP, Fisher MC (2010) Factors
555 driving pathogenicity vs, prevalence of amphibian panzootic chytridiomycosis
556 in Iberia. Ecol Lett 3:372-382
557 Woodhams D, Ardipradja K, Alford R, Marantelli G, Reinert L, Rollins-Smith L
558 (2007) Resistance to chytridiomycosis varies among amphibian species and is
559 correlated with skin peptide defenses. Anim Conserv 10:409-417
560

Table 1: Prevalence of infection in *Bufo spinosus* larvae in each of the four experimental treatments in experiment 1 (X^2 = 38.23 d.f, = 3 $p<0.001$). Bs HD/LD indicates high density/low density of *B. spinosus* larvae and +/- Ao presence/absence of *Alytes obstetricans* larvae.

	Infected	Uninfected
Bs HD, - Ao	3	49
Bs HD, + Ao	28	26
Bs LD, - Ao	2	24
Bs LD, + Ao	17	19

573 Table 2: Minimal adequate model of infection prevalence in *Bufo spinosus* in
574 experiment 1, +/- Ao indicates presence/absence of *Alytes obstetricans* larvae.
575

	Coefficient	Transformed coefficient	SE	Z-stat	p-value
- Ao	-2.6810	0.06	0.4623	-5.800	<0.001
+ Ao	2.6810	0.5	0.5081	5.277	<0.001

580
581 d.f. = 166, negative logLikelihood = 80.956
582

Table 3: Minimum adequate model of infection intensity in *Bufo spinosus* larvae when housed at different densities with and without *Alytes obstetricans* larvae in experiment 1. Bs HD/LD indicates high density/low density of *B. spinosus* larvae and +/- Ao presence/absence of *A. obstetricans* larvae.

	Coefficient	SE	Z-stat	p-value
Bs HD, - Ao	1.727	0.665	1.763	0.078
Bs HD, + Ao	2.303	0.930	2.478	0.012
Bs LD, - Ao	-21.475	3048.011	-0.007	0.994
Bs LD, + Ao	3.555	1.036	3.430	0.001

d.f. = 164, negative logLikelihood = 242.625

Table 4: Tukey's honest significant difference test showing differences in infection intensity between in four treatment levels in experiment 1. Bs HD/LD indicates high density/low density of *Bufo spinosus* larvae and +/- Ao presence/absence of *Alytes obstetricans* larvae.

	Estimate	SE	z-value	p-value
Bs HD + Ao / Bs HD - Ao	2.303	0.925	2.478	0.048
Bs HD + Ao / Bs LD + Ao	1.251	1.026	1.220	0.565
Bs HD + Ao / Bs LD - Ao	23.779	3048.011	0.008	1.000
Bs HD - Ao / Bs LD + Ao	-3.555	1.036	-3.430	0.002
Bs HD - Ao / Bs LD - Ao	21.475	3048.01	0.007	1.000
Bs LD + Ao / Bs LD - Ao	25.030	3048.011	0.008	1.000

Table 5: Pairwise comparisons of infection intensity between the six species co-housed with *Alytes obstetricans* larvae in experiment 2. Arrows indicate the relative infection intensity of the species named in the row compared to the species named in the column.

	Bufo calamita	*Hyla molleri*	*Pelophylax perezi*	*Rana iberica*	*Salamandra salamandra*
Bufo spinosus	z=0.463, p=0.997	z=2.538, p=0.112	z=0.624, p=0.989	↑, z=3.077, p=0.025	↑, z=4.250, p<0.001
Bufo calamita	-	z= 1.855, p=0.428	z=1.042, p=0.903	↑, z=3.280, p=0.013	↑, z=4.368, p<0.001
Hyla molleri	-	-	↑, z=3.243, p=0.014	↑, z=4.368, p<0.001	↑, z=3.243, p<0.001
Pelophylax perezi	-	-	-	z=2.590, p=0.099	↑, z=3.796, p=0.002
Rana iberica	-	-	-	-	z=1.147, p=0.860

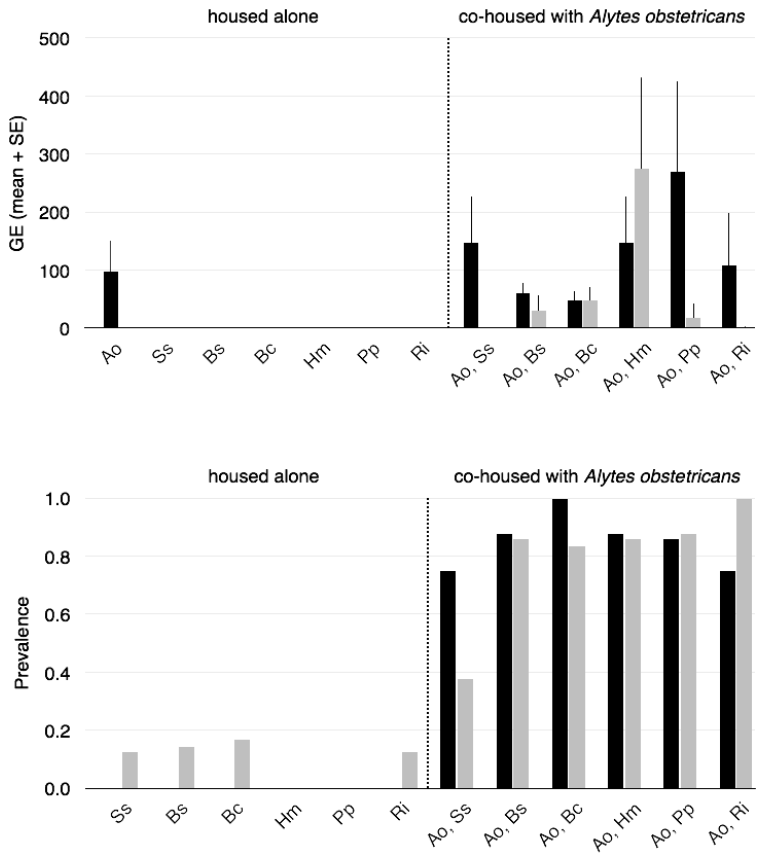
Table 6: Tukey's honest significant difference tests of infection intensity in species co-housed with *Alytes obstetricans* larvae in experiment 3. Arrows indicate the relative infection intensity of the species named in the row compared to the species named in the column.

	Mesotriton alpestris	*Pelophylax perezi*	*Salamandra salamandra*	*Triturus marmoratus*
Hyla molleri	↓, z=2.890, p=0.032	↓, z=3.445, p=0.005	z=0.773, p=0.940	z=0.365, p=0.987
Mesotriton alpestris	-	z=0.500, p=0.987	z=2.200, p=0.180	↑, z=3.144, p=0.015
Pelophylax perezi	-	-	↑, z=2.757, p=0.046	↑, z=3.680, p=0.002
Salamandra salamandra	-	-	-	z=1.115, p=0.798

Table 7: Tukey's honest significant difference tests between *Alytes obstetricans* larvae (Ao) co-housed with different species in experiment 3. Arrows indicate the relative infection intensity of the species named in the row compared to the species named in the column.

	Hyla molleri	*Mesotriton alpestris*	*Pelophylax perezi*	*Salamandra salamandra*	*Triturus marmoratus*
Control (Ao alone)	↓, z=5.201, p=0.001	z=2.483, p=0.128	↓, z=4.774, p=0.001	↓, z=3.827, p=0.001	↓, z=2.871, p=0.047
Hyla molleri	-	↑, z=3.109, p=0.023	z=0.594, p=0.991	z=1.576, p=0.613	z=2.313, p=0.188
Mesotriton alpestris	-	-	z=2.585, p=0.100	z=1.583, p=0.638	z=0.591, p=0.992
Pelophylax perezi	-	-	-	z=1.106, p=0.912	z=1.804, p=0.462
Salamandra salamandra	-	-	-	-	z=.0843, p=0.959

Fig. 1: Infection intensity (mean + SE) and prevalence for the studied species when housed alone (left side) and when co-housed with *Alytes obstetricans* (right side) in experiment 2. Black bars are for *Alytes obstetricans* (Ao), gray bars for the other species: *Salamandra salamandra* (Ss), *Bufo spinosus* (Bs), *Bufo calamita* (Bc), *Hyla molleri* (Hm), *Pelophylax perezi* (Pp) and *Rana iberica* (Ri).



649 Fig. 2: Infection intensity (mean + SE) and prevalence of species co-housed with
650 *Alytes obstericans* larvae in experiment 3. Black bars are for *Alytes obstetricans* (Ao),
651 gray bars for the other species: *Salamandra salamandra* (Ss), *Triturus marmoratus*
652 (Tm), *Mesotriton alpestris* (Ma), *Hyla molleri* (Hm) and *Pelophylax perezi* (Pp).
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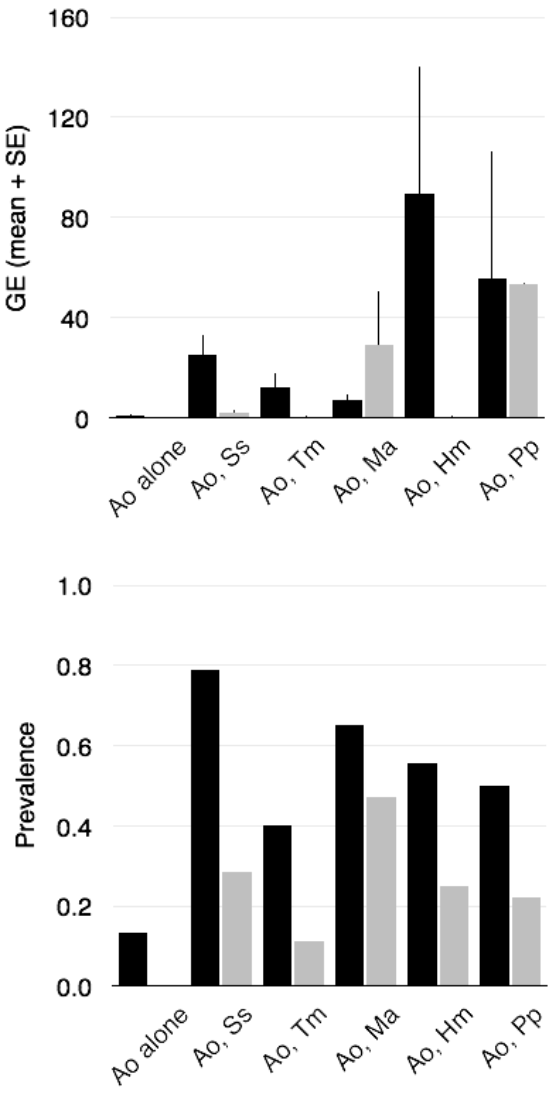

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