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Infection and transmission heterogeneity of a multi-host pathogen (*Batrachochytrium dendrobatidis*) within an amphibian community

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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 few 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 a 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*), the causal agent of the disease chytridiomycosis, has caused global amphibian population declines and extinctions. However, not all infected species are affected equally, and thus *Bd* is a good example of a multi-host pathogen that must ultimately be studied with a community approach. To test whether the common midwife toad *Alytes obstetricans* is a reservoir and possible amplifier of infection of other species, we used experimental approaches in captive and wild populations to determine the effect of common midwife toad larvae on infection of other amphibian species found in the Peñalara Massif, Spain. We observed that the most widely and heavily infected species, the common midwife toad, may be amplifying the infection loads in other species, all of which have different degrees 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 · Chytrid fungus · Interspecific transmission · Peñalara Massif · Spain

INTRODUCTION

The majority of parasites are able to infect multiple hosts (Fenton & 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, 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 a species may be of particular importance.

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'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 evidence for the existence of pathogen amplification or dilution by hosts in natural systems is comparatively more limited (but see Searle et al. 2011). By definition, 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 transmission is therefore essential if we are to understand the dynamics of infection and the likelihood of disease emergence within a community.

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is a host-generalist pathogen that infects mainly the keratinized skin of developed amphibians and the mouthparts of amphibian larvae (Berger et al. 1998). Chytridiomycosis is an amphibian-specific emerging infectious disease caused by the fungus, and it has caused severe population declines and species extirpations and extinctions worldwide (Stuart et al. 2004). It is known to have infected over 400 species, including species in all 3 amphibian Orders (Gower et al. 2013; see also www.bd-maps.net), and probably many more species are susceptible to *Bd* infection. However, there is a great deal of variation in the pathogenic effects of *Bd* both among (Lips et al. 2006, Bielby et al. 2008) and within species (Walker et al. 2010). A range of intrinsic (Woodhams et al. 2007, Farrer et al. 2011, Jani & Briggs 2014) and extrinsic factors (Vredenburg et al. 2010, Raffel et al. 2015) have been linked to the variation in the effect of *Bd* on species and communities, but, as yet, relatively little is known about the role of community composition in this context.

The first known chytridiomycosis-related mortalities in Europe occurred in the late 1990s and led the common midwife toad *Alytes obstetricans* (*Ao*) to the brink of local extinction in the Peñalara Massif at the Sierra de Guadarrama National Park (Bosch et al. 2001). The species seems to be the most severely impacted species in Europe (Tobler & Schmidt 2010, Walker et al. 2010), and as a result of the ease with which it becomes infected, the clade Alytidae acts as a reliable sentinel when screening for infection in new regions or populations (Baláž et al. 2014). However, other species in heavily affected assemblages, such as those in Guadarrama, 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 amphibian 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 (www.bd-maps.net). The population-level effects of chytridiomycosis on *B. spinosus* and *S. salamandra* diminished following the near extinction of *Ao* (J. Bosch unpubl. data) and therefore we hypothesize that *Ao* 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 chose to look at the range of tolerance 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 for designing better management strategies, preventing future declines and improving reintroduction programme success. Specifically, we tested the hypotheses that *Ao* acts as an amplification host and that individuals of sympatric species will experience a higher probability and greater intensity of infection than individuals housed only with their own species. Further, we tested the hypothesis that *Bd* can transmit both directly, from *Ao* larvae to sympatric species, and also indirectly through swimming zoospore transmission, rather than relying on direct contact with an infected host. Finally, we investigated whether community members (i.e. individual species) exhibit different levels of infection from one another when housed with *Ao* larvae. Combined, these experiments may help to explain some of the observed community-level impacts of chytridiomycosis in the presence and absence of *Ao*.

MATERIALS AND METHODS

The Peñalara Massif is home to 8 endemic amphibian species: *Ao*, *Bufo calamita*, *B. spinosus*, *Hyla molleri*, *Pelophylax perezi*, *Rana iberica*, *Triturus marmoratus* and *Salamandra salamandra*. Additionally, *Ichthyosaura alpestris* 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 judged to be 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 yr), 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 113) or directly from the corresponding tissue of newly dead individuals or after euthanasia.

Laboratory methods

To quantify infection load in amphibians, we used a quantitative real-time polymerase chain reaction (qPCR) protocol (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 (isolate IA042, Ibón Acherito, Spanish Pyrenees) in a CFX96 machine (BioRad). When only 1 replicate from any sample amplified, we assayed 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 the hypothesis that *Ao* acts as an amplification host by increasing infection prevalence and intensity in sympatric species, and also that *Bd* can initially infect hosts indirectly through waterborne spores, rather than relying on direct contact with an infected host. We conducted a field experiment in the Laguna Grande de Peñalara glacial lake of the Peñalara Massif (2018 m a.s.l.), and *B. spinosus* was chosen as our focal susceptible species as it has been observed to suffer infection and mortality as a result of *Bd* infection in natural surroundings (Bosch & Martínez-Solano 2006) and in experimental settings (Garner et al. 2009). Several hundred *B. spinosus* free-swimming Gosner stage 25 tadpoles (Gosner 1960) were collected from different locations at Laguna Grande to average any possible genetic variation among offspring. Previous studies have shown that at this stage of development, *B. spinosus* tadpoles lack *Bd* infection (Ortiz-Santaliestra et al. 2011). Uninfected *Ao* larvae were obtained from a captive colony located in the studied area that is regularly tested for *Bd* infection by qPCR. Larvae from the stock of our focal species, *B. spinosus*, were assigned to 1 of 4 different treatments in a 2 × 2 experimental design. The 2 factors of interest were density and the presence of *Ao* larvae, and each of these 2 factors had 2 levels: high density (50 *B. spinosus* larvae) or low density (25 *B. spinosus* larvae), and presence (10 larvae) or absence (0 larvae) of *Ao* larvae. The selected densities are within the range typically observed naturally in this system (J. Bosch unpubl. data). Each treatment was replicated 3 times, each group being housed in a separate 4 l container. The containers had ventilated sides and were placed together floating in the lake. Water temperature inside each container was recorded with a thermocouple thermometer in a randomized order and found not to differ between containers. The experimental design removed the possibility that infection was introduced with any of the experimental animals, as they came from uninfected stock, or were placed in the experiment before keratinized mouthparts had developed and had the opportunity to 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 d 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 4 experimental levels resulted in a different probability of infection, we used a chi-squared test, and, in the presence of any significant variation, we used generalized linear models (GLMs) with binomial errors to determine which of the 2 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), 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 analyse whether infection intensity varied with density and presence/absence of *Ao* larvae, we used GLMs with negative binomial errors using the glm.nb function from the R package MASS. The function glht from the multcomp library was used to determine which levels of the 4 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. Plastic containers (2 l capacity, n = 52) were floated together in the Laguna Grande de Peñalara. The containers had holes to allow water exchange with the surrounding lake. 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 13 treatments was replicated 4 times. The 13 treatments were (1) 2 larvae of *Ao* alone, which acted as a control to see how heavy infection was in this species when housed alone; (2–7) 6 treatments consisting of 2 larvae of *Ao* co-housed with 2 larvae of each of *B. spinosus*, *B. calamita*, *H. molleri*, *P. perezi*, *R. iberica* or *S. salamandra*; and (8–13) 2 larvae of each of those 6 species alone (i.e. no *Ao* were added). Larvae of the studied species were collected in the field in several ponds of the Peñalara Massif, and *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 keratinized mouthparts had developed, and their uninfected status was confirmed by qPCR. One overwintered larva of *S. salamandra* from the same lake was introduced into each container for 1 wk. As overwintered larvae, *S. salamandra* have an infection prevalence of 100% in spring in this system (Medina et al. 2015). Therefore 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 13 treatments.

We tested whether species differed from one another in their infection probability. Using a Fisher's exact test, we determined 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*, we conducted a Student's *t*-test to compare infection intensity between those individuals co-housed with *Ao* with those housed only with a conspecific for each of the 6 co-housed species.

To investigate whether infection intensity differed among each species when co-housed with *Ao*, we used a GLM 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. GLMs 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 *Bd* infections 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 5 species (*H. molleri*, *P. perezi*, *I. alpestris*, *T. marmoratus* and *S. salamandra*) were captured in the field in several ponds of the Peñalara Massif, and their uninfected status was confirmed by qPCR. Two larvae of each of these species were placed in the presence of a single infected *Ao* larva, resulting in 5 experimental treatments. The sixth treatment was a single infected *Ao* larva, housed alone. Each of the 6 treatments was replicated 10 times in 2011 and 15 times in 2012. All experimental replicates were housed in 1.5 l 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 to compare proportions of infected and uninfected individuals for each species. To test whether species differed from one another in their infection intensity when co-housed with *Ao* larvae, we used a GLM with negative binomial errors and Tukey comparisons between species to determine whether 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 *Bufo 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 4 treatments ($\chi^2 = 38.23$, df = 3, $p < 0.001$; Table 1). In the presence of *Ao* larvae, the prevalence of infection in *B. spinosus* was about 50%, while in the absence of *Ao* larvae, it was less than 7%. The fact that infection occurred suggests that infection can occur and persist via indirect transmission from zoospores in the water and is not initially reliant on direct contact with an infected host.

Our model of infection probability was 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* ($\chi^2 < 0.001$, df = 1, p = 1.000), 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 2 treatments, it was not useful to compare infection burden between treatments.

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 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.

Table 1. Prevalence of infection in *Bufo spinosus* larvae in each of the 4 experimental treatments in Expt 1 ($\chi^2 = 38.23$, df = 3, $p < 0.001$). *Bs* HD/LD indicates high/low density of *B. spinosus* larvae and +/− *Ao* indicates 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

Table 2. Minimal adequate model of infection prevalence in *Bufo spinosus* in Expt 1. +/− *Ao* indicates presence/absence of *Alytes obstetricans* larvae

	Coefficient	Transformed coefficient	SE	*z*	p
−*Ao*	−2.6810	0.06	0.4623	−5.800	<0.001
+*Ao*	2.6810	0.5	0.5081	5.277	<0.001

df = 166, negative log likelihood = 80.956

Table 3. Minimum adequate model of infection intensity in *Bufo spinosus* larvae when housed at different densities with (+) and without (−) *Alytes obstetricans* (*Ao*) larvae in Expt 1. *Bs* HD/LD indicates high/low density of *B. spinosus* larvae

	Coefficient	SE	*z*	p
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

df = 164, negative log likelihood = 242.625

Tukey's HSD 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 6 species showed no significant difference from one another in their probability of becoming infected (Fig. 1; Fisher's exact test: p = 0.072).

Table 4. Tukey's HSD test showing differences in infection intensity among 4 treatment levels in Expt 1. *Bs* HD/LD indicates high/low density of *Bufo spinosus* larvae and +/– *Ao* indicates presence/absence of *Alytes obstetricans* larvae

	Estimate	SE	*z*	p
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

Four of the 6 co-housed species had significantly higher infection intensity in the presence of *Ao* than when housed only with conspecifics (*B. spinosus*: t = 3.097, df = 12, p = 0.009; *B. 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. 1). Species was a significant predictor of infection intensity in our negative binomial glm (F = 9.712, df = 5, p < 0.001), and Tukey's HSD tests highlighted significant differences in the infections between those species (Table 5). *R. iberica* had a significantly lower infection level than *B. spinosus*, *B. calamita* and *H. molleri*. *S. salamandra* had a lower infection intensity than the latter 3 species plus *P. perezi*. *H. 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. 1; Fisher's exact test, p = 0.796). This result is most likely because by the end of the experiment, most *Ao* larvae were fairly heavily infected (Fig. 1).

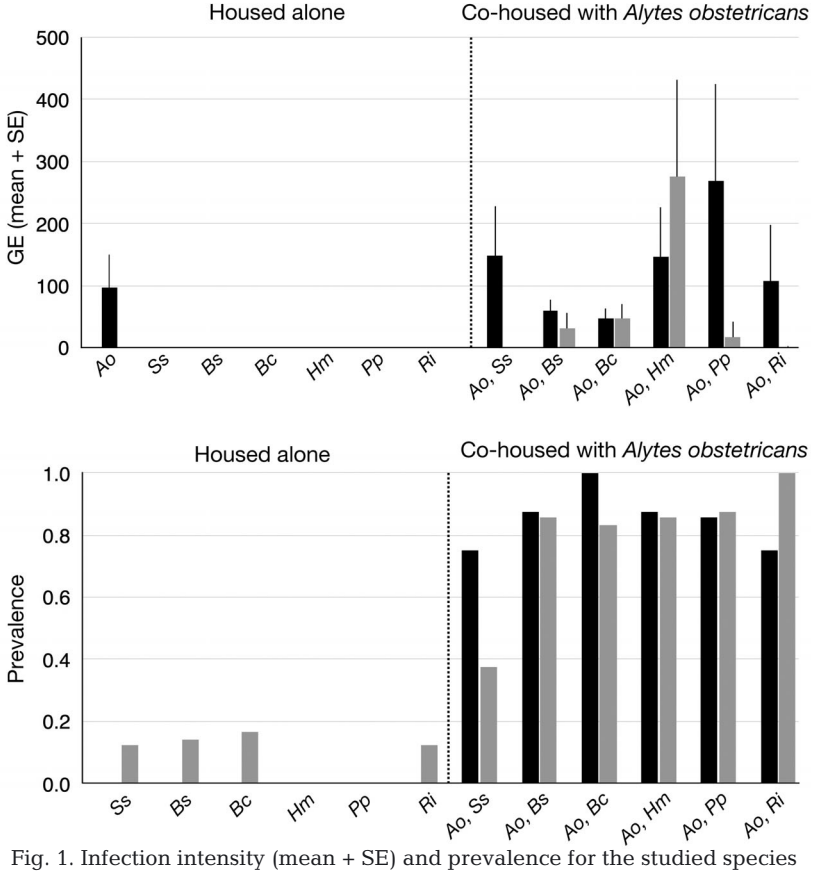

Fig. 1. Infection intensity (mean + SE) and prevalence for the studied species when housed alone (left side) and when co-housed with *Alytes obstetricans* (*Ao*, right side) in Expt 2. Black bars are for *Ao*, gray bars are for the other species: *Salamandra salamandra* (*Ss*), *Bufo spinosus* (*Bs*),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

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 no significant difference among those species in their probability of infection (p = 0.2126; Fig. 2).

Significant differences in infection intensity were present among those species (Fig. 2; F = 4.9807, df = 4, p < 0.001). *P. perezi* had a significantly higher infection intensity than *H. molleri*, *Triturus marmoratus* and *S. salamandra*. *Ichthyosaura 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 test, 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 *I. 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 *Ao* larvae housed with *I. alpestris* (Table 7).

Table 5. Pairwise comparisons of infection intensity between the 6 species co-housed with *Alytes obstetricans* larvae in Expt 2. Arrows indicate the relative infection intensity of the species named in the row compared to the species named in the column

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

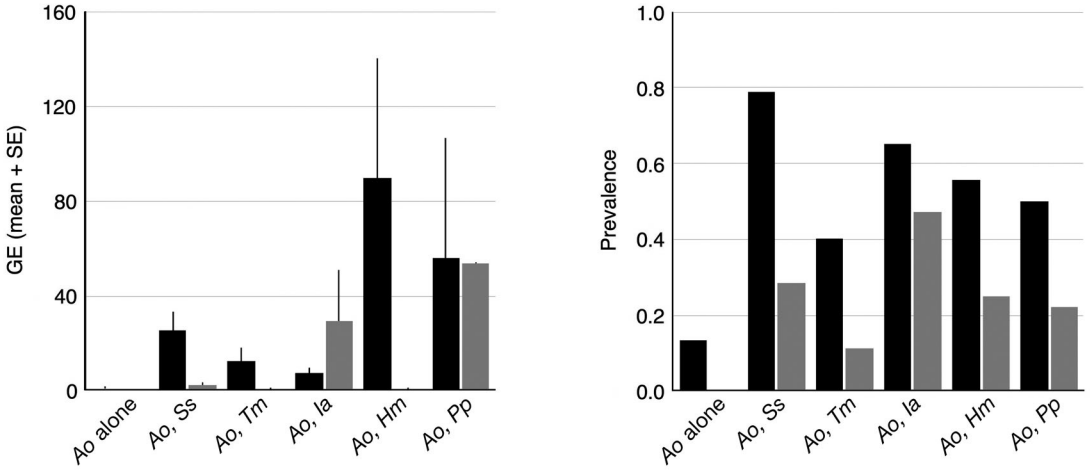

Fig. 2. Infection intensity (GE: *Bd* zoospore genomic equivalent, mean + SE) and prevalence of species co-housed with *Alytes obstetricans* (*Ao*, black bars) larvae in Expt 3. Gray bars represent the other species: *Salamandra salamandra* (*Ss*), *Triturus marmoratus* (*Tm*), *Ichthyosaura alpestris* (*Ia*), *Hyla molleri* (*Hm*) and *Pelophylax perezi* (*Pp*)

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. Our 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, 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 1 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, it may cause 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 overwintering 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 host

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

	I. alpestris		*P. perezi*		*S. salamandra*		*Triturus marmoratus*	
	z	p	*z*	p	*z*	p	z	p
Hyla molleri	↓, 2.890	0.032	↓, 3.445	0.005	0.773	0.940	0.365	0.0987
Ichthyosaura alpestris	–	–	0.500	0.987	2.200	0.180	↑, 3.144	0.015
Pelophylax perezi	–	–	–	–	↑, 2.757	0.046	↑, 3.680	<0.002
Salamandra salamandra	–	–	–	–	–	–	1.115	0.798

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

	H. molleri		*I. alpestris*		*P. perezi*		*S. salamandra*		*Triturus marmoratus*	
	z	p	*z*	p	*z*	p	*z*	p	*z*	p
Control (*Ao* alone)	↓, 5.201	0.001	2.483	0.128	↓,4.774	0.001	↓,3.827	0.001	↓,2.871	0.047
Hyla molleri	–	–	↑, 3.109	0.023	0.594	0.991	1.576	0.613	2.313	0.188
Ichthyosaura alpestris	–	–	–	–	↑,2.585	0.100	1.583	0.638	0.591	0.992
Pelophylax perezi	–	–	–	–	–	–	1.106	0.912	1.804	0.462
Salamandra salamandra	–	–	–	–	–	–	–	–	0.843	0.959

assemblage. Combined, the results of our experiments suggest that *Ao* larvae are able to increase infection prevalence and intensity in a number of co-housed species by directly transmitting infection to them. Further, the ability of *Ao* to act as an amplification host appears to be independent of the overall density of larvae around it, as highlighted in Expt 1. In this experiment, the density of the co-housed focal species *Bufo spinosus* did not affect its likelihood of becoming infected, which remained close to 0 in the absence of *Ao*. In contrast, *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*, suggesting that the presence of a single *Ao* larva resulted in a significant increase in infection probability and intensity regardless of overall host density. The fact that the presence of *Ao* is strongly associated with infection in other species, regardless of the overall density of hosts, suggests that even post-decline, when the overall density of hosts is reduced, infection may still be maintained and spread if *Ao* larvae remain present in the community.

We considered what characteristics would predispose *Ao* to act as reservoirs or disseminators of infections in the shorter term. One possible morphological feature that would lend itself to a species harbouring and transmitting high levels of infection is its large oral disc. In *Ao*, this feature is unusually large, and includes numerous rows of large denticles with a high concentration of keratin. Therefore, it has a greater area available to be infected by the pathogen (Berger et al. 1998; but see Searle et al. 2011, who reported that smaller species, such as *Anaxyrus boreas*, presented the highest infection loads). This potential mechanism could be explored further using techniques to track infection prevalence and infection intensity in different body parts, and it highlights the importance of understanding species' biology when considering their 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 about mechanisms 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. However, the data from Expt 1 suggest 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 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 Expts 2 and 3. Although the majority of species had an increased probability of infection and infection intensity in the presence of *Ao* larvae, there was a considerable degree 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. This observation makes it difficult to predict 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 the performed experiments (for example, *Hyla molleri* and *Pelophylax perezi* in Expts 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 those with *Ichthyosaura alpestris* did not, having signifi-

cantly lower infections than *Ao* housed with *H. molleri* larvae. While it is currently difficult to determine the mechanism behind these differences, the end result is that, even for a host capable of carrying heavy infection burdens, competition with other larvae, or the ability for infection to be transmitted both to and from other species in the assemblage, may, at times, be important for the maintenance of infection. These inconsistencies and inter-species differences suggest that the outcome of *Bd* exposure is highly context dependent and may differ greatly depending upon the source of infection and the environment in which the larvae develop, illustrating how important it is to carefully consider the generalities of research into the transmission within any host–parasite system.

Rachowicz & Briggs (2007) showed that under laboratory or field conditions, there is a clear influence of the density of infected individuals on the rates of *Bd* transmission. The density of both host and pathogen are fundamental parameters in the transmission of infectious disease. Although the experimental numbers of tadpoles in our study were similar to those used in the study mentioned above, we did not find a significant effect of density of tadpoles in the variation of *Bd* infection intensity. Our experimental design was such that comparisons of species co-housed with and without *Ao* varied not only in species composition, but also in the density of animals in the experimental treatments. Accounting for both density and species composition would be the ideal approach to take, but the practicalities involved with conducting such experiments prevented these dual comparisons. Regardless of these different densities, the main findings of our experiments remain unchanged. We conclude that *Ao* presence/absence is a greater predictor of infection than overall density of tadpoles (Expt 1), that species co-housed with *Ao* differ in their response to parasite exposure (Expts 2 and 3), that *Ao* varies in its infection levels depending on the species with which it is housed and that *Ao* can directly infect other species (Expt 3).

To add more complexity to the overall findings, competition and stress between 2 host species may account for some of the observed patterns. Additional experiments with the target host at different densities and addition of non-target and non-*Ao* hosts would be needed to test whether additional host species simply cause competitive stress and thus lead to increased infection.

Identifying the roles that different species or life stages play in the transmission, prevalence and intensity of infection is crucial to better understand the persistence and spread of infection within a host–pathogen system. Knowledge related to which species are more tolerant and more susceptible to infection could allow designers of mitigation efforts to focus on reducing the levels of infection in a host. In the case of our study system, this might be accomplished by aiming to reduce the amount of infection in potential 'amplifier' hosts. Considering the species composition of a particular host community is essential in efforts to understand the spread of infection, risk of disease emergence and, ultimately, in managing systems to minimize any negative effects of pathogens on biodiversity.

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LITERATURE CITED

Baláž V, Voeroes J, Civis P, Vojar J and others (2014) Assessing risk and guidance on monitoring of *Batrachochytrium dendrobatidis* in Europe through identification of taxonomic selectivity of infection. Conserv Biol 28: 213–223

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. Princeton University Press, Princeton, NJ, p 12–29

Berger L, Speare R, Daszak P, Green DE and others (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, 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, García-París M (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biol Conserv 97:331–337

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

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 Org 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. Johns Hopkins University Press, Baltimore, MD

Farrer RA, Weinert LA, Bielby J, Garner TWJ and others (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 Infect 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 10:e0120237

Garner TWJ, Walker S, Bosch J, Leech S, Rowcliffe JM, Cunningham AA, Fisher MC (2009) Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis.* Oikos 118:783–791

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190

Gower DJ, Doherty-Bone T, Loader SP, Wilkinson M and others (2013) *Batrachochytrium dendrobatidis* infection and lethal chytridiomycosis in caecilian amphibians (Gymnophiona). EcoHealth 10:173–183

Jani AJ, Briggs CJ (2014) The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. Proc Natl Acad Sci USA 111:E5049–E5058

Lam BA, Walton DB, Harris RN (2011) Motile zoospores of *Batrachochytrium dendrobatidis* move away from antifungal metabolites produced by amphibian skin bacteria. EcoHealth 8:36–45

Lips KR, Brem F, Brenes R, Reeve JD and others (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci USA 103:3165–3170

Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz W (2005) Superspreading and the effect of individual variation on disease emergence. Nature 438:355–359

Martínez-Solano I, Bosch J, García-París M (2003) Demographic trends and community stability in a montane amphibian assemblage. Conserv Biol 17:238–244

Medina D, Garner TWJ, Carrascal LM, Bosch J (2015) Delayed metamorphosis of amphibian larvae facilitates *Batrachochytrium dendrobatidis* transmission and persistence. Dis Aquat Org 117:85–92

Moss AS, Reddy NS, Dorta JIM, Francisco MJS (2008) Chemotaxis of the amphibian pathogen *Batrachochytrium dendrobatidis* and its response to a variety of attractants. Mycologia 100:1–5

Narayan EJ, Graham C, McCallum H, Hero JM (2014) Overwintering tadpoles of *Mixophyes fasciolatus* act as reservoir host for *Batrachochytrium dendrobatidis.* PLoS ONE 9:e92499

Ortiz-Santaliestra ME, Fisher MC, Fernández-Beaskoetxea S, Fernández-Benéitez MJ, Bosch J (2011) Ambient ultraviolet B radiation decreases the prevalence of infection by *Batrachochytrium dendrobatidis* in two amphibian species. Conserv Biol 25:975–982

R Core Team (2014) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. www.R-project.org/

Rachowicz LJ, Briggs CJ (2007) Quantifying the disease transmission function: effects of density on *Batrachochytrium dendrobatidis* transmission in the mountain yellow-legged frog *Rana muscosa.* J Anim Ecol 76: 711–721

Raffel TR, Halstead NT, McMahon TA, Davis AK, Rohr JR (2015) Temperature variability and moisture synergistically interact to exacerbate an epizootic disease. Proc R Soc Lond B Biol Sci 282:20142039

Rudge JW, Webster JP, Lu DB, Wang TP, Fang GR, Basáñez MG (2013) Identifying host species driving transmission of schistosomiasis japonica, a multihost parasite system, in China. Proc Natl Acad Sci USA 110:11457–11462

Searle CL, Biga LM, Spatafora JW, Blaustein AR (2011) A dilution effect in the emerging amphibian pathogen *Batrachochytrium dendrobatidis.* Proc Natl Acad Sci USA 108:16322–16326

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, Waller RW (2004) Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786

Tobler U, Schmidt BR (2010) Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans.* PLoS ONE 5:e10927

Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proc Natl Acad Sci USA 107:9689–9694

Walker SF, Bosch J, Gomez V, Garner TWJ and others (2010) Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecol Lett 13:372–382

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

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