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Relationship between two pathogens in an amphibian community that experienced mass mortalities

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Article impact statement: Direct negative consequences for amphibians cannot be inferred simply because of the coinfection with the chytrid fungus and *Ranavirus*.

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Abstract

Because host species tend to harbor multiple parasitic species, coinfection in a host is common. The chytrid fungus *Batrachochytrium dendrobatidis* (Bd) and the viruses in the genus *Ranavirus* (Rv) are responsible for the decline of amphibians worldwide. Despite wide geographical co-occurrence and the serious conservation problem that coinfection with these pathogens could represent, little is known about their possible synergistic interactions and effects in a host community. We investigated the occurrence and associations between these two pathogens in an amphibian community after Rv-driven disease outbreaks were detected in four populations of the Iberian ribbed newt (*Pleurodeles waltl*) in northwestern Spain. We collected tissue samples from amphibians and fish and estimated Bd and Rv infection loads by qPCR. A few months after the most recent mass mortality event, Rv infection parameters at the affected sites decreased significantly or were lower than such registered at the sites where no outbreaks were recorded. Both pathogens were simultaneously present in almost all sites, but coinfection in a single host was rare. Our findings suggest that the co-occurrence of Bd and Rv does not predict adverse outcomes (e.g., enhanced susceptibility of hosts to one pathogen due to the presence or infection intensity of the other) following an outbreak. Other variables (such as species identity or site) were more important than infection with a pathogen in predicting the infection status and severity of infection with the other pathogen. Our results highlight the importance of host-specific and environmental characteristics in the dynamics of infections, coinfection patterns, and their impacts.

KEYWORDS
Batrachochytrium dendrobatidis, coinfection, dilution/amplification effects, disease ecology, ranavirosis, *Ranavirus*

Relaciones entre dos patógenos en una comunidad anfibia que experimentó mortalidad masiva

Resumen: La coinfección es común en especies hospederas ya que estas especies tienden a albergar muchas especies parasíticas. El hongo quitridio *Batrachochytrium dendrobatidis* (Bd) y los virus del género *Ranavirus* (Rv) son responsables de la declinación mundial de anfibios. A pesar de la amplia co-ocurrencia geográfica y el problema serio de conservación que podría representar la coinfección con estos patógenos, se conoce muy poco sobre sus posibles interacciones sinérgicas y sus efectos en una comunidad hospedera. Investigamos la incidencia y las asociaciones entre estos dos patógenos en una comunidad anfibia después de que se detectaron brotes de enfermedades causados por Rv en cuatro

poblaciones del tritón estriado ibérico (*Pleurodeles waltl*) en el noroeste de España. Recolectamos muestras de tejido de anfibios y peces y estimamos la carga infecciosa de Bd y Rv con una qPCR. Unos meses después del evento de mortalidad masiva más reciente, los parámetros de infección de Rv en los sitios afectados disminuyeron significativamente o fueron más bajos que los registrados en sitios sin brotes. Ambos patógenos estuvieron presentes de forma simultánea en casi todos los sitios, pero fue raro encontrar la coinfección en un solo hospedero. Nuestros descubrimientos sugieren que la coocurrencia de Bd y Rv no pronostica resultados adversos (aumento en la susceptibilidad de los hospederos a un patógeno debido a la presencia o intensidad de infección del otro patógeno) después de un brote. Otras variables, como la identidad de la especie o el sitio, fueron más importantes que la infección con un patógeno en la predicción del estado de infección y la severidad de la infección con otro patógeno. Nuestros resultados resaltan la importancia de las características ambientales y aquellas específicas del hospedero en las dinámicas de infección, los patrones de coinfección y sus impactos.

PALABRAS CLAVE
coinfección, ecología de enfermedades, efectos de dilución/amplificación, ranavirosis, *Batrachochytrium dendrobatidis*, *Ranavirus*

INTRODUCTION

Organisms, including bacteria and viruses, do no exist in isolation, and they usually form complex ecological relationships (Begon et al., 2006). This factor is important in disease ecology, where a single host is rarely infected by just one parasite or pathogen and most pathogens rarely infect only one host species (e.g., Pedersen & Fenton, 2007; Rigaud et al., 2010). Depending on the characteristics and synergistic interactions between the hosts, the pathogens, and the environment, their interactions can have detrimental, beneficial, or insignificant effects on the involved species (e.g., Stevens, 1960). Even though this fact is almost a truism, its broader ecological context is often ignored. This is especially true for two geographically co-occurring pathogens, the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), the causative agent of chytridiomycosis, and the viruses in the genus *Ranavirus* (Rv), the causative agents of ranavirosis (see reviews by Herczeg et al., 2021 and Thumsová et al., 2023). Both Bd and Rv are generalist waterborne parasites with broad host ranges that seriously affect amphibian populations worldwide (Fisher & Garner, 2020; Price et al., 2017).

Despite hundreds of disease ecology studies on pathogens, multihost–multipathogen interactions in natural systems have been poorly explored (reviewed by Bienentreu & Lesbarrères, 2020). Yet, when Bd and Rv are investigated in natural conditions in an amphibian community, the cumulative nature of these pathogens is often assumed (e.g., Whitfield et al., 2013). However, the presence of these pathogens does not necessarily translate into a negative impact on the host population (Bosch et al., 2020). For example, controlled experiments show a higher intensity of Bd decreases the intensity of Rv (Ramsay & Rohr, 2022).

In addition, most coinfection studies focus only on single-host species interactions without considering the effect of the community (e.g., Bosch et al., 2020; Ramsay & Rohr, 2022; Warne et al., 2016), despite that at the level of the individual, observations do not necessarily match observations at the community level (Stutz et al., 2018). Community structure may represent a key factor in disease dynamics, especially for species whose populations differ in their response to infections due to, for example, pathogen dilution or amplification (e.g., Searle et al., 2011; Snyder et al., 2023; Venesky et al., 2014). The concepts of amplification and dilution rely on the simple relationship between species richness and disease risk. A higher species diversity may dilute the infection and so decrease its impact, but low species richness may facilitate an amplification effect leading to higher disease risk (Halliday et al., 2017; Keesing et al., 2006, 2010). Even though dilution and amplification are strongly context-dependent, they should be considered when investigating the impacts of a multipathogen presence on a particular host community and when implementing disease mitigation strategies (Rohr et al., 2020).

Amphibian communities in Iberia are exposed to Bd and Rv, but the number of outbreaks associated with one or the other pathogen has rapidly increased since the late 1980s (Bosch et al., 2001, 2021; Márquez et al., 1995; Price et al., 2014; Rosa et al., 2017; Thumsová et al., 2021, 2022). Although Bd is an introduced and a spreading pathogen in Iberia (O'Hanlon et al., 2018), a more recently described Rv linage (the *Common midwife toad virus* [CMTV]) may be endemic to the region (Thumsová et al., 2022), originating from the area of the most recent mass mortalities (Flechoso et al., 2019). Even though in this region both pathogens are present, these outbreaks were unequivocally attributed to CMTV-Rv (Thumsová et al., 2022).

We explored the interaction of Bd and CMTV-Rv across several amphibian communities in the area recently affected by Rv-driven mass mortality events. We used disease surveillance data to assess within-host and within-site associations between both pathogens. Specifically, we investigated the factors that predict infection at the individual and population levels, correlation between Bd and Rv infection parameters; possible dilution

and amplification effects on infection outcomes; and changes in infection parameters after disease outbreaks.

METHODS

Study system

We conducted our study in the provinces of Zamora and Salamanca, northwestern Spain (Appendix S1), where Bd occurs (Fernández-Beaskoetxea et al., 2015) and ranavirosis outbreaks have been recorded (most recent July 2018). The area has a semi-arid climate with cool winters and hot summers. The landscape harbors many artificial ephemeral and permanent cattle ponds with complex and variable hydroperiods that depend on, for example, location, size, depth, vegetation, and substrate (Alonso & Comelles, 1983; Alarcos et al., 2003). The vast majority of the study ponds were small, ranging from approximately 5 to 25 m in diameter and 1 to 3 m in depth, and dried up in the summertime (Alarcos et al., 2003) (Appendix S1). These water bodies support diverse amphibian assemblages that include spiny common toads (*Bufo spinosus*), natterjack toads (*Epidalea calamita*), Iberian tree frogs (*Hyla molleri*), Iberian spadefoot toads (*Pelobates cultripes*), Iberian green frogs (*Pelophylax perezi*), fire salamanders (*Salamandra salamandra*), Bosca's newts (*Lissotriton boscai*), marbled newts (*Triturus marmoratus*), Iberian ribbed newts (*Pleurodeles waltl*), and amphibian predators, such as the introduced tench (*Tinca tinca*) and invasive western mosquitofish (*Gambusia affinis*).

Sampling procedure

We initiated sampling within a few months following a ranavirosis outbreak recorded in the Zamora province in 2018. This incident led to an estimated mortality of up to 80 *P. waltl* individuals at a single site in 1 day (Flechoso et al., 2019). Mortalities were also identified by park rangers and local herpetologists at an additional site. Mortalities at both sites totaled over 100. Although diseased individuals of other species were detected, mass mortalities appeared restricted to *P. waltl*. In November of 2018 and from March to June of 2019, we surveyed for amphibians and fish across 35 sites in Zamora and Salamanca where Bd and Rv were both present. We collected individuals with dip nets during daylight hours. We took toe clips from metamorphosed amphibians, toe and tail clips from amphibian larvae (417 amphibian individuals total), and pelvic fin clips from 33 fish, up to a maximum of 20 individuals per species and site. From the tissue samples, we estimated Bd and Rv infection loads, which ensures consistency and enhances the accuracy of Bd load estimates (Clare et al., 2016). All sampling sites were frequently checked by local rangers and herpetologists for signs of mass mortality or for individuals presenting typical signs of disease. We followed strict hygiene guidelines to prevent cross-contamination between individuals and sampling sites (Phillott et al., 2010). Tissue samples were fixed in 70% ethanol immediately in the field. Additionally, we took toe and liver samples from ethanol-fixed *P. waltl* individuals collected during previous mortality events at the study area.

Field work was carried out under permission of Consejería de Medio Ambiente of Junta de Castilla y León (reference number EP/CyL/97/2018). The animal research was carried out in accordance with the EU Directive 2010/63/EU for animal experiments and was approved by the animal ethics committee of CSIC (reference number 666/2018).

Laboratory analyses

We extracted DNA from the tissue samples with the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. We conducted real-time Taqman PCR assays on a MyGo Pro machine following the protocols by Blooi et al. (2013) for simultaneous detection and quantification of Bd and *B. salamandrivorans* (Bsal) and Leung et al. (2017) for detection of Rv. Tail clips from anuran larvae and pelvic fin clips from fish were not subjected to Bd and Bsal screening, given their lack of keratin and, therefore, unsuitability for Bd or Bsal colonization (Garner et al., 2009). We ran all samples in duplicate and against negative and positive controls with known concentrations of genomic equivalents (GE) of zoospores or virions (from 0.1 to 1000 for Bd/Bsal and from 3 to 30,000,000 for Rv in $\log_{10}$ increments). A sample was considered positive when both replicates were amplified and infection load was equal to or higher than the lower positive control and the amplification curves presented robust sigmoidal shapes. We reran the samples when obtaining amplification in one replicate only. If that resulted in no amplification, we considered the sample negative for infection.

Statistical analyses

We calculated the prevalence of infections and the 95% Clopper–Pearson confidence intervals for each pathogen, species, and site with Epitools (Sergeant, 2018). We calculated the mean, median, and standard deviation of infection intensities (Bd and Rv loads) expressed in GE of zoospores for Bd or virions for Rv. We conducted statistical analyses at the individual and the site levels. To maximize the power of our analyses, we defined just two life stages: larvae and postmetamorphs (comprising recently metamorphosed individuals, juveniles, and adults).

For each pathogen, we ran two generalized linear mixed models (GLMM), the first with infection status (0, 1) as a response and a binomial error distribution and the second with infection intensity as the response and a Poisson error distribution. In both models, we included site as a random effect to account for repeated sampling, and site was nested into a fixed effect to take into account whether mass mortalities were previously recorded at each site. Other fixed effects in the models were species identity, life-history stage, month of sampling, and either infection status or infection intensity of the other pathogen. For the models of infection intensity, we considered infected and uninfected

specimens to increase the sample size. The values of infection intensity were transformed to the $\log_{10}$ after adding 1.

Given that the Rv-driven mass mortalities affected only the *P. waltl* population (Flechoso et al., 2019), we ran an additional GLMM to assess whether the presence of this highly susceptible host species at the site level increased Rv infection in co-occurring species. In this case, and after excluding *P. waltl* individuals from the analysis, we used the same structure as previously described, but site was nested into a new factor that considered the *P. waltl* occurrence (present or not at the time of sampling) at each site instead of the record of mass mortalities.

At the site level, we performed another two GLMMs to determine whether the number of present species was related to the averaged infection intensities for each pathogen. We included the log-transformed number of analyzed individuals per site and month of sampling as a weight variable. Similar to the previous analysis, we included site as a random effect and the log-transformed number of species, month of sampling, and mean infection intensities (again, to test associations between both pathogens) as fixed effects. For all analyses, Tukey tests were run when categorical fixed effects with more than two levels were significant. All analyses were carried out in JMP Pro 17 (SAS Institute).

RESULTS

Infection by at least one of the pathogens was detected in over half of the amphibian individuals sampled for the presence of both pathogens (prevalence = 0.51 [213 out of 417], 95% CI 0.46–0.56; Fig. 1). The Bd-only infections were by far the most prevalent (prevalence = 0.39 [162 of 417], 0.34–0.44), followed by Rv-only infections (prevalence = 0.07 [28 of 417], 0.05–0.10) and infection by both (prevalence = 0.06 [23 of 417], 0.04–0.08). Mean infection load for Bd-only infected individuals was 39.6 GE (SD 132.3) (median = 3.4, range = 0.1–1386.0); for Rv-only individuals was 52.9 GE (80.0) (median = 29.5, range = 11.0–429.0); and for Bd and Rv coinfected individuals was 4.8 GE (8.9) for Bd (median = 1.4, range = 0.2–36.0) and 45.8 GE (34.6) for Rv (median = 33.9, range = 4.0–163.9). We detected Rv infections in another 8 individuals out of a total of 61 individuals tested only for Rv (mean = 30.0 GE [19.0], median = 25.3, range = 10.2–56.2). No individuals were positive for Bsal ($n = 238$), and only one fish tested positive for Rv ($n = 33$).

Thirty-two of 35 sites sampled for the presence of both pathogens were infected by at least one of them (prevalence = 0.91, 95% CI 0.77–0.98; Fig. 1). Nineteen sites harbored both pathogens (prevalence = 0.54, 0.37–0.71), and, of those 19 sites, 14 individuals were coinfected (prevalence = 0.74, 0.49–0.91). Further, Bd-only infected individuals were found in 12 of the 35 sampled sites (prevalence = 0.34, 0.19–0.52), whereas Rv-only infected animals were found in just one site (prevalence = 0.03, 0.001–0.15). A low prevalence of Rv infection was found in the remaining one site that was tested only for Rv (Table 2). Figure 1

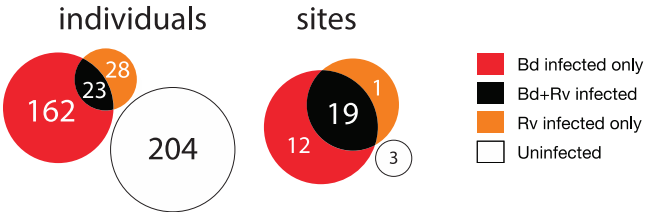

FIGURE 1 Distribution of amphibian individuals and sites tested for *Batrachochytrium dendrobatidis* (Bd) and *Ranavirus* (Rv) across infection categories of uninfected, Bd-only infected, Rv-only infected, and Bd and Rv coinfected (individuals) or Bd and Rv co-occurrence (sites).

Infection patterns at the individual level

Individuals of *P. perezi* and *P. waltl* exhibited the highest prevalence of infection when infected with just one pathogen, independent of their life-history stage (Table 1 & Figure 2). The Bd-only infections were further detected in postmetamorphs of *H. molleri*, *T. marmoratus*, *L. boscai*, and larval individuals of *S. salamandra*. The same pattern was observed for Rv-only infections, except for *H. molleri*, which remained uninfected. Similarly, *P. waltl* and *P. perezi* were the most commonly coinfected (Bd+Rv) species, followed by *T. marmoratus* at a lower prevalence (two postmetamorphs coinfected) (Table 1).

The explanatory models for Bd or Rv infection status of individuals revealed that infection with one pathogen did not predict infection with the other ($F = 2.33$, df = 1, 403, $p = 0.128$; $F = 1.81$, df = 1, 403, $p = 0.180$, respectively). In fact, the Bd infection status of individuals was best predicted by life-history stage ($F = 21.56$, df = 1, 403, $p<0.001$) and species identity ($F = 5.62$, df = 6, 403, $p<0.001$). Postmetamorphs exhibited a higher probability of Bd infection than larvae, and individuals of *P. waltl* were infected at a significantly higher probability than individuals of *H. molleri* ($p = 0.004$), *L. boscai* ($p = 0.010$), and *T. marmoratus* ($p<0.001$). Individuals of *T. marmoratus* also had a significantly lower probability of infection than individuals of *P. perezi* ($p = 0.012$). The Bd infection status was not influenced by site (Wald test $p = 0.066$) or month of sampling ($F = 0.67$, df = 4, 403, $p = 0.669$). In contrast, Rv infection status was marginally predicted by site (Wald test $p = 0.050$) and did not differ across species ($F = 0.95$, df = 6, 403, $p = 0.457$) or life-history stages ($F = 3.09$, df = 1, 403, $p = 0.080$), and, as for Bd, it was not influenced by sampling period ($F = 1.47$, df = 4, 403, $p = 0.210$). An individual's Bd and Rv infection status was not predicted by the occurrence of a previous mass mortality event at a site ($p = 0.722$ and $p = 0.088$, respectively). However, considering the relatively limited number of sites with documented mass mortality events, the level of significance could likely stem from the low statistical power in the case of Rv.

The GLMM tests for Bd or Rv infection intensities revealed a significant negative association among the intensities of infection of these pathogens ($F = 3.94$, df = 1, 403, $p = 0.048$; $F = 8.26$, df = 1, 403, $p = 0.004$, respectively). Similar to Bd status, Bd infection intensities differed between life-history stages ($F = 19.48$, df = 1, 403, $p < 0.001$); intensity was significantly greater in postmetamorphs than in larvae. Differences were also found

TABLE 1 Mean infection intensities (in genomic equivalents of zoospores or virions) of amphibian individuals infected with either *Batrachochytrium dendrobatidis* (Bd) or *Ranavirus* (Rv) or coinfected with both Bd and Rv and prevalence of infection (with Clopper–Pearson confidence intervals in parentheses) across species and life-history stages.

Species	Life-history stage	Sample size	Bd-only prevalence	Bd-only load	Rv-only prevalence	Rv-only load	Bd+Rv prevalence	Bd+Rv Bd load	Bd+Rv Rv load
Triturus marmoratus	postmetamorphs	71	0.24 (0.15–0.36)	22.2	0.04 (0.01–0.12)	29	0.03 (0.003–0.10)	1.3	61.9
Lissotriton boscai	postmetamorphs	26	0.15 (0.04–0.35)	16.9	0.39 (0.001–0.20)	72.7	0.00 (0.00–0.13)	–	–
Hyla molleri	post metamorphs	22	0.45 (0.24–0.68)	66.5	0.00 (0.00–0.15)	–	0.00 (0.00–0.15)	–	–
Pelobates cultripes	larvae	23	–	–	0.13 (0.03–0.34)	32.7	–	–	–
	postmetamorphs	7	0.00 (0.00–0.41)	–	0.00 (0.00–0.41)	–	0.00 (0.00–0.41)	–	–
Pelophylax perezi	larvae	51	0.22 (0.11–0.35)	5.9	0.20 (0.10–0.33)	44.1	0.04 (0.01–0.13)	0.2	72.2
	postmetamorphs	103	0.63 (0.53–0.72)	73.7	0.07 (0.03–0.14)	79.7	0.06 (0.02–0.12)	7.4	31.9
Pleurodeles waltl	larvae	14	0.36 (0.13–0.65)	0.3	0.14 (0.02–0.43)	70.4	0.07 (0.002–0.34)	1	44.9
	postmetamorphs	75	0.61 (0.49–0.72)	9.4	0.03 (0.003–0.09)	40.9	0.16 (0.09–0.26)	5.2	45.8
Salamandra salamandra	larvae	47	0.09 (0.02–0.20)	1.2	0.06 (0.01–0.18)	33.8	0.00 (0.00–0.08)	–	–
	postmetamorphs	1	0.00 (N/A)	–	0.00 (N/A)	–	0.00 (N/A)	–	–

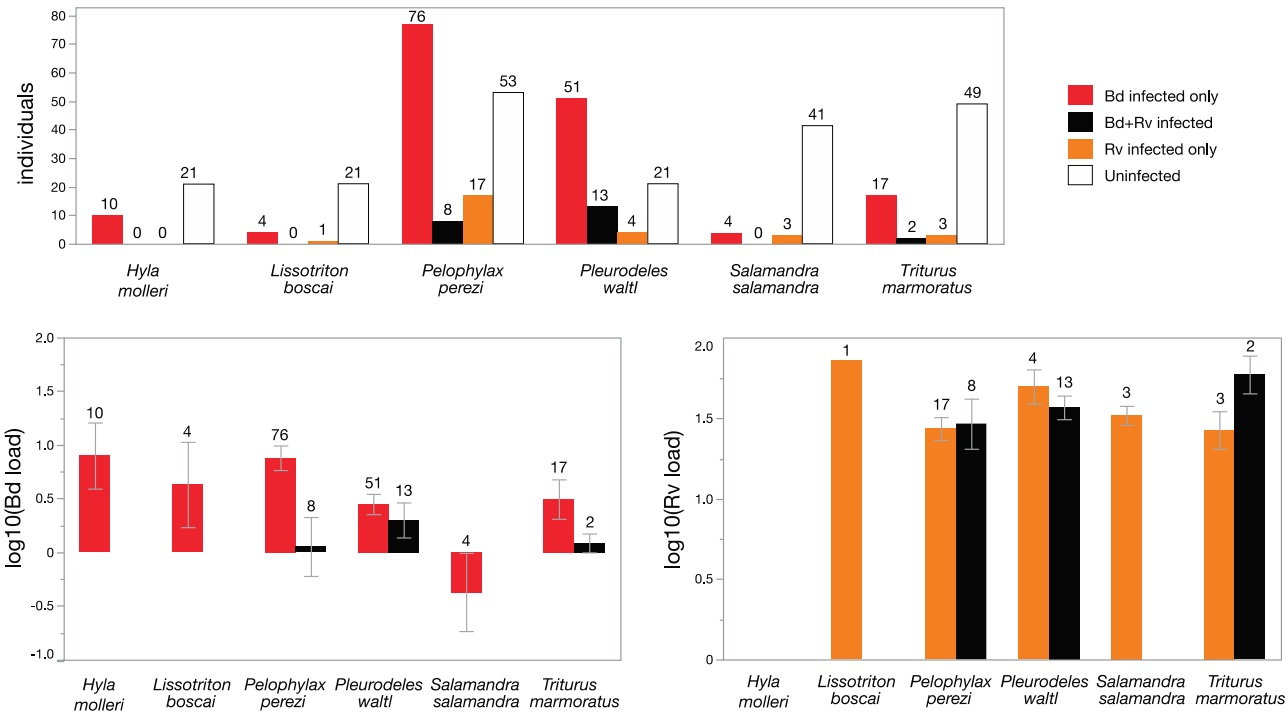

FIGURE 2 Counts of amphibian individuals tested for *Batrachochytrium dendrobatidis* (Bd) and *Ranavirus* (Rv) and Bd and Rv infection intensities (mean [SE]) across species and coinfection categories (Bd-only infected, Rv-only infected, Bd and Rv coinfected, and uninfected).

across species ($F = 4.19$, df = 6, 403, $p < 0.001$). In particular, individuals of *P. perezi* and *P. waltl* presented greater intensities of infection than individuals of *T. marmoratus* ($p < 0.001$ and $p = 0.038$, respectively). Nevertheless, consistent with the results above, Bd intensities of infection did not differ across sites (Wald test $p = 0.205$) or among sampling months ($F = 0.67$, df = 4, 403, $p = 0.615$). The same pattern was not found for Rv intensity of infection, for which infection loads differed among sites (Wald test $p = 0.024$), but not between life-history stages ($F = 2.30$, df = 1, 403, $p = 0.130$), across species ($F = 1.31$, df = 6, 403, $p = 0.252$), or across sampling months ($F = 1.78$, df = 1, 403, $p = 0.132$). The occurrence of a previous mass mortality event at a site was related to Rv infection intensities of the individuals ($F = 4.26$, df = 1, 30, $p = 0.048$), but not to Bd infections ($F = 0.13$, df = 1, 17, $p = 0.722$). Individuals from mass mortality sites had lower Rv infection levels than individuals at sites without mass mortality records. Finally, the presence of *P. waltl* did not amplify Rv infections in co-occurring species ($F = 0.36$, df = 1, 23, $p = 0.553$).

Infection patterns at the site level

The averaged infection intensity of one pathogen in a particular site was not predicted by the averaged infection intensity of the other pathogen ($F = 0.00$, df = 1, 41, $p = 1.000$; $F = 0.06$, df = 1, 15, $p = 0.806$, for Bd and Rv, respectively) (Table 2). However, species richness, month of sampling, and site contributed significantly to differences in Rv infection ($F = 16.54$, df = 1, 17, $p<0.001$; $F = 24.57$, df = 3, 15, $p<0.001$, respectively; Wald test $p<0.001$) but not to the differences in Bd infection intensities ($p>0.116$ in all cases). The relationship between Rv intensity of infection and species richness was negative (t ratio = −4.07).

All available carcasses ($n = 13$) collected at the study area during two independent mass mortality events that occurred in the regions of Zamora and Salamanca belonged to *P. waltl* and tested positive for Rv but not for Bd or Bsal. Overall infection loads were extremely high: mean (SD) = 10,184,861 GE (13,167,324) of virions (median = 4,870,390; range = 11,602−41,601,884).

DISCUSSION

The ranavirosis outbreaks recorded in the study area resulted in dramatic mortalities of over a hundred individuals of the largest European newt species, *P. waltl*, endemic to Iberia and Morocco (Flechoso et al., 2019; G.A., personal observations). Although those carcasses reached 100% prevalence of Rv and infection intensities of millions of GE of virions, our postoutbreak survey, conducted shortly after the most recent ranavirosis outbreak, showed a significant decrease in infection levels in live animals. Overall, in the sites where dead animals had never been recorded, at least one pathogen, Bd or Rv, was detected in almost all places and the prevalence of infection was close to 50%. Most individuals were infected with only Bd. Individuals infected only by Rv or simultaneously by both pathogens represented only a small proportion. Despite the very low proportion of coinfected individuals, the co-occurrence of the pathogens at a single site was detected in most cases. Throughout the sampling area, postoutbreak infection loads were lower and never reached the values recorded during the mass mortality events. Our findings thus indicate that the probability and intensity of infection with one of the studied pathogens did not, on its own, increase the probability or infection intensity of the other pathogen. Similarly, at the site level, we did not find an association between the averaged infection intensities of one pathogen and the infection intensities of the other. Finally, we found evidence of a dilution effect of Rv infection at our sites but no evidence of an amplification effect, even in the presence of *P. waltl*.

Despite the low number of field studies in which the potential interaction and effects of Bd and Rv were examined directly, the lack of a strong association between these two pathogens under natural conditions is increasingly supported (e.g., Bosch et al., 2020; Olori et al., 2018; Warne et al., 2016). For example, cumulative or amplified effects caused by the asynchronous emergence of these two lethal pathogens were not found in a study conducted in the Serra da Estrela in Portugal (Rosa et al., 2017). In fact, Rv drove the declines of host assemblages and changed host community composition and structure, even at sites where Bd was not having deleterious impacts (Bosch et al., 2021; Rosa et al., 2013, 2017). Moreover, infection with one pathogen did not increase the probability of co-infection in two susceptible species across two sites in Picos de Europa National Park, Spain (Bosch et al., 2020). Results of that study showed a negative association between both pathogens when the presence of one was linked to lower infections by the other. We found the same patterns, but they encompassed a larger number of species and sites.

The lack of a cumulative pattern between both infections is probably related to the distinct temperature requirements of each; Bd does best at low temperatures (e.g., Fernández-Beaskoetxea et al., 2015) and Rv at much higher temperatures (Herczeg et al., 2021; Price et al., 2019). Based on this, we hypothesize that coinfection can only occur when temperatures reach values that enable the successful proliferation of Rv without completely clearing Bd infections. This could be the reason coinfected individuals are usually less abundant than individuals infected with only one pathogen (this study, Bosch et al., 2020). Similarly, the low proportion of *Rv*-infected individuals, low intensities of infection, and absence of mass mortality events we found could be related to the lack of extremely high-temperature events during the study period (Appendix S1). The mean, minimum, and maximum air temperatures recorded during the study period were slightly lower than temperatures recorded during the same months across the 30 years preceding the sampling year (Appendix S1). As Thumsová et al. (2022) demonstrated, Rv emergence and the associated mass mortalities detected in the study area were clearly related to a significant increase in temperature (around 10°C). Due to the low elevation of sampled sites (629–874 m above sea level), the proportion of Bd-infected individuals and the outcome of the infection were, as expected, not very expressive; strong associations between elevation and fatal chytridiomycosis have been demonstrated for

TABLE 2 Mean infection intensities (in genomic equivalents of zoospores or virions) and prevalence of infection (with Clopper–Pearson confidence intervals in parentheses) of amphibian individuals infected with just one pathogen (*Batrachochytrium dendrobatidis* [Bd] or *Ranavirus* [Rv]) or coinfected with Bd and Rv per site and month of sampling.

Site	Latitude, longitude	Sampling month	Sample size	Bd-only prevalence	Bd-only load	Rv-only prevalence	Rv-only load	Bd+Rv prevalence	Bd+Rv Bd load	Bd+Rv Rv load
Cubo del Vino 0[a]	41.28, −5.71	March	4	0.25 (0.01–0.81)	0.4	0.00 (0.00–0.60)	–	0.00 (0.00–0.60)	–	–
		June	5	–	–	0.00 (0.00–0.52)	–	–	–	–
		November	4	0.25 (0.01–0.82)	0.2	0.00 (0.00–0.60)	–	0.00 (0.00–0.60)	–	–
Cubo del Vino 1	41.29, −5.71	March	2	1.00 (0.16–1.00)	2	0.00 (0.00–0.84)	–	0.00 (0.00–0.84)	–	–
		April	1	–	–	0.00 (N/A)	–	–	–	–
		June	3	–	–	0.00 (0.00–0.71)	–	–	–	–
		November	1	1.00 (N/A)	0.2	0.00 (N/A)	–	0.00 (N/A)	–	–
Cubo del Vino 2	41.28, −5.84	April	10	0.40 (0.12–0.74)	14.8	0.10 (0.003–0.45)	43.7	0.50 (0.19–0.81)	2.4	66
		November	2	0.50 (0.01–0.99)	7.8	0.50 (0.01–0.99)	429	0.00 (0.00–0.84)	–	–
Cubo del Vino 3	41.28, −5.72	March	10	0.90 (0.56–1.00)	232.1	0.00 (0.00–0.31)	–	0.00 (0.00–0.31)	–	–
		April	3	1.00 (0.29–1.00)	2.7	0.00 (0.00–0.71)	–	0.00 (0.00–0.71)	–	–
Cubo del Vino 4	41.27, −5.7	April	1	1.00 (N/A)	2.7	0.00 (N/A)	–	0.00 (N/A)	–	–
Cubo del Vino 5	41.28, −5.68	April	1	0.00 (N/A)	–	0.00 (N/A)	–	0.00 (N/A)	–	–
Cubo del Vino 8	41.29, −5.74	April	1	0.00 (N/A)	–	1.00 (N/A)	29.9	0.00 (N/A)	–	–
Cubo del Vino 10	41.26, −5.69	April	16	0.38 (0.15–0.65)	1.8	0.00 (0.00–0.21)	–	0.06 (0.002–0.30)	1	44.9
Cubo del Vino 11	41.27, −5.69	June	1	1.00 (N/A)	0.3	0.00 (N/A)	–	0.00 (N/A)	–	–
Muga 0[a]	41.38, −6.18	March	19	0.32 (0.13–0.57)	13.7	0.05 (0.001–0.26)	27.6	0.11 (0.01–0.33)	1.9	16.8
		May	4	0.50 (0.07–0.93)	0.9	0.00 (0.00–0.60)	–	0.00 (0.00–0.60)	–	–
Muga 1	41.38, −6.17	March	19	0.52 (0.29–0.76)	25.3	0.16 (0.03–0.40)	14.8	0.05 (0.001–0.26)	0.8	21.4
		April	8	0.13 (0.003–0.53)	192.6	0.00 (0.00–0.37)	–	0.00 (0.00–0.37)	–	–
Muga 2	41.38, −6.18	March	20	0.30 (0.12–0.54)	9.9	0.10 (0.01–0.32)	21	0.05 (0.001–0.25)	1	78.2
		April	6	0.50 (0.12–0.88)	7.1	0.00 (0.00–0.46)	–	0.00 (0.00–0.46)	–	–
		May	3	0.67 (0.09–0.99)	5.5	0.00 (0.00–0.71)	–	0.33 (0.01–0.91)	21.4	48.2
Muga 3	41.38, −6.19	March	17	0.18 (0.04–0.43)	0.7	0.24 (0.07–0.50)	36.6	0.00 (0.00–0.20)	–	–
		April	12	0.67 (0.35–0.90)	20.7	0.00 (0.00–0.26)	–	0.00 (0.00–0.26)	–	–

(Continues)

TABLE 2 (Continued)

Site	Latitude, longitude	Sampling month	Sample size	Bd-only prevalence	Bd-only load	Rv-only prevalence	Rv-only load	Bd+Rv prevalence	Bd+Rv Bd load	Bd+Rv Rv load
Muga 4	41.39, −6.2	April	5	0.80 (0.28–0.99)	5.3	0.00 (0.00–0.52)	–	0.20 (0.01–0.72)	3.5	92.4
Muga 5	41.39, −6.2	April	4	0.00 (0.00–0.60)	–	0.00 (0.00–0.60)	–	0.00 (0.00–0.60)	–	–
		May	1	0.00 (N/A)	–	0.00 (N/A)	–	0.00 (N/A)	–	–
Muga 6	41.39, −6.17	April	10	0.40 (0.12–0.74)	21.5	0.20 (0.03–0.56)	92.1	0.00 (0.00–0.31)	–	–
Muga 7	41.37, −6.21	April	10	–	–	0.30 (0.07–0.65)	32.7	–	–	–
Muga 7B	41.38, −6.21	May	6	0.50 (0.12–0.88)	2.8	0.17 (0.004–0.64)	38.1	0.33 (0.04–0.78)	12.3	21.8
Muga 8	41.37, −6.19	April	13	0.54 (0.25–0.81)	92.9	0.00 (0.00–0.25)	–	0.00 (0.00–0.25)	–	–
		May	3	0.33 (0.01–0.91)	6.1	0.00 (0.00–0.71)	–	0.33 (0.01–0.91)	1.5	33.9
Muga 9	41.38, −6.2	April	32	0.69 (0.50–0.84)	73.5	0.06 (0.01–0.21)	36.6	0.06 (0.01–0.21)	18.1	44.5
		May	5	0.40 (0.05–0.85)	4.9	0.40 (0.05–0.85)	70.4	0.20 (0.01–0.72)	0.3	53.6
Peñausende 0[a]	41.28, −5.91	–	–	–	–	–	–	–	–	–
Peñausende 1	41.29, −5.89	April	4	0.25 (0.01–0.81)	12.8	0.25 (0.01–0.81)	18.5	0.25 (0.01–0.81)	0.5	18.7
Peñausende 2	41.3, −5.91	April	3	0.67 (0.09–0.99)	8.4	0.33 (0.01–0.91)	20	0.00 (0.00–0.71)	–	–
		May	1	0.00 (N/A)	–	0.00 (N/A)	–	0.00 (N/A)	–	–
Peñausende 3	41.27, −5.93	April	4	0.25 (0.01–0.81)	1.6	0.00 (0.00–0.60)	–	0.00 (0.00–0.60)	–	–
		May	1	1.00 (N/A)	40.2	0.00 (N/A)	–	0.00 (N/A)	–	–
Peñausende 4	41.27, −5.92	April	4	0.50 (0.07–0.93)	14.7	0.00 (0.00–0.60)	–	0.00 (0.00–0.60)	–	–
		May	5	0.80 (0.28–0.99)	10.1	0.00 (0.00–0.52)	–	0.20 (0.01–0.72)	0.4	41.7
Peñausende 5	41.28, −5.92	April	3	0.00 (0.00–0.71)	–	0.00 (0.00–0.71)	–	0.00 (0.00–0.71)	–	–
Peñausende 6	41.28, −5.92	April	4	0.25 (0.01–0.81)	3.1	0.00 (0.00–0.60)	–	0.25 (0.01–0.81)	2.8	23.2
Peñausende 7	41.28, −5.9	April	2	0.00 (0.00–0.84)	–	0.00 (0.00–0.84)	–	0.00 (0.00–0.84)	–	–
		May	2	1.00 (0.16–1.00)	2.5	0.00 (0.00–0.84)	–	0.00 (0.00–0.84)	–	–
Peñausende 9	41.27, −5.89	May	6	0.50 (0.12–0.88)	1.9	0.00 (0.00–0.46)	–	0.17 (0.004–0.64)	0.8	20.5
Puerto Seguro 0[a]	40.79, −6.75	March	22	0.05 (0.001–0.23)	3.4	0.00 (0.00–0.15)	–	0.00 (0.00–0.15)	–	–
		April	12	0.17 (0.02–0.48)	15.3	0.00 (0.00–0.26)	–	0.00 (0.00–0.26)	–	–

(Continues)

TABLE 2 (Continued)

Site	Latitude, longitude	Sampling month	Sample size	Bd-only prevalence	Bd-only load	Rv-only prevalence	Rv-only load	Bd+Rv prevalence	Bd+Rv Bd load	Bd+Rv Rv load
Puerto Seguro 1	40.8, −6.75	March	6	0.33 (0.04–0.78)	1	0.00 (0.00–0.46)	–	0.00 (0.00–0.46)	–	–
		April	6	0.17 (0.004–0.64)	1.3	0.00 (0.00–0.46)	–	0.00 (0.00–0.46)	–	–
Puerto Seguro 2	40.78, −6.74	March	6	0.17 (0.004–0.64)	4.1	0.50 (0.12–0.88)	33.8	0.00 (0.00–0.46)	–	–
		April	2	0.00 (0.00–0.84)	–	0.00 (0.00–0.84)	–	0.00 (0.00–0.84)	–	–
Puerto Seguro 3	40.79, −6.72	March	18	0.00 (0.00–0.19)	–	0.00 (0.00–0.19)	–	0.00 (0.00–0.19)	–	–
		April	20	0.35 (0.15–0.59)	61.5	0.00 (0.00–0.17)	–	0.00 (0.00–0.17)	–	–
Puerto Seguro 4	40.79, −6.74	March	9	0.56 (0.21–0.86)	67.9	0.00 (0.00–0.34)	–	0.00 (0.00–0.34)	–	–
		April	11	0.36 (0.11–0.69)	4.9	0.00 (0.00–0.28)	–	0.00 (0.00–0.28)	–	–
Puerto Seguro 4b	40.79, −6.75	March	10	0.10 (0.003–0.45)	1.2	0.20 (0.03–0.56)	58.8	0.00 (0.00–0.31)	–	–
Puerto Seguro 4C	40.79, −6.75	April	10	0.40 (0.12–0.74)	4.8	0.10 (0.003–0.45)	25.6	0.10 (0.003–0.45)	0.2	80.6
Puerto Seguro 5	40.79, −6.75	April	3	1.00 (0.29–1.00)	10.9	0.00 (0.00–0.71)	–	0.00 (0.00–0.71)	–	–

[a]Sites where mass mortalities of *Pleurodeles waltl* were recorded.

Iberia (e.g., Doddington et al., 2013; Rosa et al., 2013; Walker et al., 2010).

Both Bd and Rv can infect multiple species, which exhibit great variation in their susceptibility to infections (e.g., Duffus et al., 2015; Price et al., 2017; Van Rooij et al., 2015). Some hosts can develop signs of disease and experience high mortality (susceptible species), whereas others may be tolerant, sustain sublethal infections, and act as reservoirs of the pathogen (Van Rooij et al., 2015). Discrimination between susceptible species and those able to tolerate and act as pathogen reservoirs is crucial for understanding infection dynamics in the host community (e.g., Martel et al., 2014). Even though Bd can infect over 1000 species (Castro Monzon et al., 2020), severe impacts have been more frequently associated with anurans than urodeles. Infections of Bd are particularly severe in early metamorphosed individuals, whereas amphibian larvae are usually associated with mild symptoms and very rare mortality (Blaustein et al., 2005; Garner et al., 2009). Contrastingly, Rv has an extremely broad host range, affecting equally urodeles and anurans of all life-history stages (Price et al., 2017). This is also corroborated by our findings that Bd intensity was significantly higher in metamorphosed individuals than larvae, but there were no differences in Rv infection rates among species or life-history stages.

P. waltl and *P. perezi* had the highest prevalence of infection for both pathogens. That all dead *P. waltl* exhibited extreme Rv infection loads during these and other ranavirosis outbreaks (Thumsová et al., 2022) led us to hypothesize that the species could serve as an amplification host for Rv, increasing infection risk and altering disease dynamic in co-occurring species (Snyder et al., 2023). Yet, we did not confirm the hypothesis because the presence of *P. waltl* individuals did not lead to an amplification of infection in co-occurring species. This outcome could be attributed to, as described above, the lack of significantly elevated temperature events during our sampling period (Appendix S1). Alternatively, it is plausible that only individuals resistant to both Bd and Rv endured at the sites following the episodes of mass mortality. The death of the most susceptible individuals bearing high Rv loads during the ranavirosis outbreaks might have contributed to the low prevalence and intensity of Rv infection detected here. Furthermore, this occurrence could potentially account for the absence of a correlation between the presence and intensity of Rv infection and the likelihood and severity of Bd infection. Although all deceased *P. waltl* individuals tested positive only for Rv, the possibility of a prior infection with *Bd* cannot be ruled out. However, most of our sampling effort took from March to June 2019 ($n = 410$), when new larvae have already hatched from the eggs and spend enough time in the water to become infected with both pathogens. Therefore, the likelihood that only resistant individuals were sampled in this instance seems rather weak. In any case, for a comprehensive validation or dismissal of our hypotheses, obtaining samples from live animals prior to and during outbreaks would be essential.

The assumption that the susceptibility of individuals to pathogens is taxonomically dependent raises the question of why the mortalities we found were detected only in *P. waltl*. Especially when other species that experienced Rv-associated mortalities in other parts of Iberia were present. Bayesian phylogeography findings from Thumsová et al. (2022) suggest that CMTV-like Rv has likely been present in the study area for over 500 years. At the same time, the CMTV-like Rv we detected appeared to be genetically different from other CMTV-like Rv detected in other areas of Iberia, where the viruses appear to have been for a shorter period. These factors could account for the pronounced variability in host response and impacts associated with endemic Rv, resulting in some populations facing ongoing substantial mortality (Price et al., 2014; Thumsová et al., 2022) and others remaining stable and displaying resistance. In contrast, the high susceptibility of *P. waltl* to Rv remains consistent in different populations because the species is one of the primary causes of detected Rv incidences in Iberia (Thumsová et al., 2022). This suggests that hosts may possess some components specific to a given group of species, such as genetic makeup, ecology, life history, and so on (reviewed in Thumsová et al., 2023).

Contrary to Bienentreu et al. (2022), averaged intensities of Rv infection in a site decreased as species richness increased. However, Tornabene et al. (2018) found a positive correlation between taxonomic richness and the probability of Rv presence at the site level but a negative relationship between vertebrate richness and individual-level Rv infection prevalence. These outcomes indicate that both effects are strongly context-dependent and that whether dilution or amplification of the pathogens occurs, will most likely depend more on host community composition, transmission-competence of hosts, and densities than on simple species richness (Bienentreu et al., 2022; Luis et al., 2018; Randolph & Dobson, 2012; Tornabene et al., 2018; Venesky et al., 2014).

Understanding the factors affecting infection risk and disease progression is fundamental to developing an effective strategy to maintain amphibian diversity. A combination of intensive management guided by close collaboration between policy and science is currently a priority to prevent the invasion of new pathogens and to mitigate the impact of pathogens that are already present. The threat of introduction of novel pathogens or strains is still acute, especially when the non-native FV3-like Rv is already present not so far away from the study area (Price et al., 2014; Rosa et al., 2017). This threat is enhanced by the introduction or spread of non-native amphibian and fish species (Martel et al., 2020; Price et al., 2017; Rosa et al., 2022). However, that the emergence of the present CMTV-like Rv strain is clearly associated with a significant increase in temperature will likely prompt decision-makers to act rapidly to mitigate the consequences of ongoing climate warming. Because the study area consisted of open grassland ponds exposed to strong solar radiation, an increase in canopy cover could create a habitat for the hosts while reducing the impact of the pathogens (Thumsová et al., 2023) (Appendix S1). Even though the severity of Rv decreases as canopy cover increases, caution must be exercised when implementing this strategy because the reverse trend has been observed for Bd (e.g., Becker et al., 2012; Gahl & Calhoun, 2010).

Although direct negative consequences are frequently inferred when both pathogens are detected in the same host, our results strengthen the evidence that host susceptibility to one pathogen is not always enhanced by the presence or infection intensity of the other. Thus, given the potential endemic nature of CMTV-like Rv in Iberia, further field studies are critical to understanding the interactions and epidemiological consequences of coinfections in the context of Rv-triggering scenarios.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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