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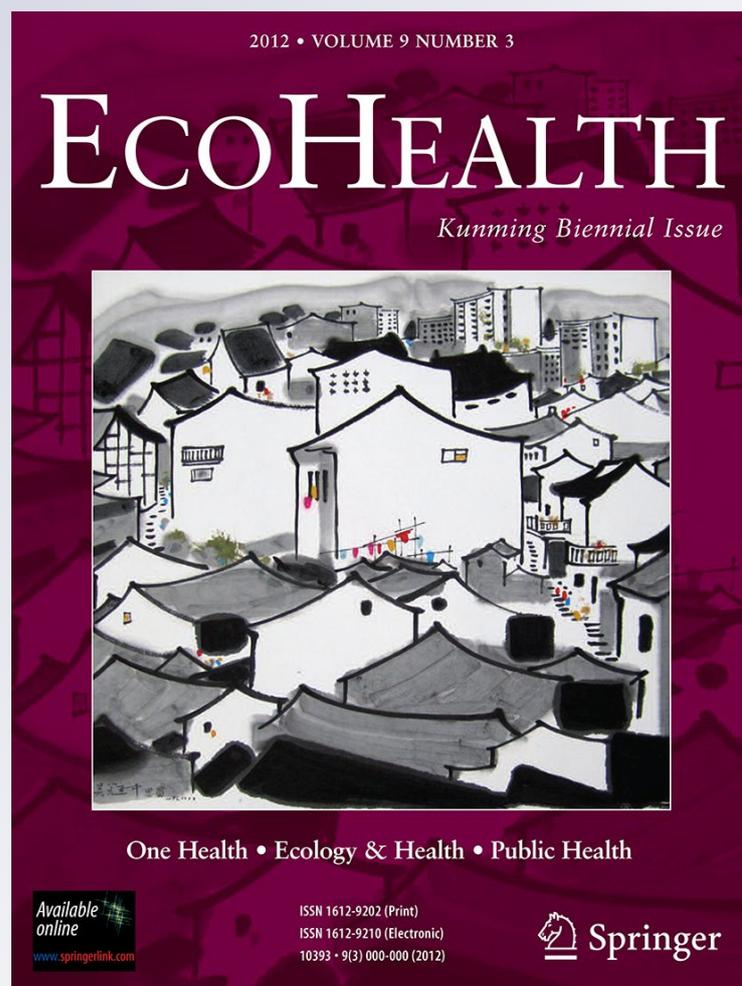
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EcoHealth

Conservation Medicine: Human Health:Ecosystem Sustainability Official journal of International Association for Ecology and Health

ISSN 1612-9202
Volume 9
Number 3

EcoHealth (2012) 9:288-292
DOI 10.1007/s10393-012-0778-2



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Short Communication

Widespread Co-occurrence of Virulent Pathogens Within California Amphibian Communities

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Abstract: The chytrid fungus *Batrachochytrium dendrobatidis*, ranaviruses, and trematodes (*Ribeiroia ondatrae* and echinostomes) are highly virulent pathogens known to infect amphibians, yet the extent to which they co-occur within amphibian communities remains poorly understood. Using field surveillance of 85 wetlands in the East Bay region of California, USA, we found that 68% of wetlands had ≥ 2 pathogens and 36% had ≥ 3 pathogens. Wetlands with high pathogen species richness also tended to cluster spatially. Our results underscore the need for greater integration of multiple pathogens and their interactions into amphibian disease research and conservation efforts.

Keywords: amphibian decline, coinfection, concomitant infection, disease ecology, emerging infectious disease, chytridiomycosis, amphibian malformations

Emerging infectious diseases are believed to be an important cause of amphibian declines (Collins and Storfer 2003; Wake and Vredenburg 2008). Indeed, a diverse assemblage of pathogens including viruses, bacteria, fungi, and helminths are known to infect amphibians (Wright and Whitaker 2001; Densmore and Green 2007). In North America, the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd), viral pathogens in the genus *Ranavirus*, and larval trematodes including *Ribeiroia ondatrae* (hereafter *Ribeiroia*) and several species of echinostomes (e.g., *Echinostoma trivolvis*, *Echinoparyphrium* spp.) have frequently been implicated in

die-offs or severe pathology in amphibian populations (Green et al. 2002; Johnson et al. 2002; Muths et al. 2006). For each of these pathogens, researchers have determined host range, quantified variation in host susceptibility to infection and disease outcomes, and identified environmental correlates of pathogen emergence (Johnson et al. 2007, 2012; Greer et al. 2008, 2009; Johnson and McKenzie 2008; Kilpatrick et al. 2010; Hoverman et al. 2011; Searle et al. 2011). In addition, field studies have demonstrated the widespread occurrence of these pathogens in amphibian populations (Ouellet et al. 2005; Gahl and Calhoun 2008; Padgett-Flohr and Hopkins 2010; Johnson and Buller 2011; Hartson et al. 2012; Hoverman et al. 2012). While these studies have provided insight into individual host–pathogen interactions, the extent to which any of these highly virulent pathogens co-occur within amphibian communities remains poorly understood due to sparse surveillance for

Electronic supplementary material: The online version of this article (doi: 10.1007/s10393-012-0778-2) contains supplementary material, which is available to authorized users.

Published online: July 6, 2012

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multiple pathogens (but see Rohr et al. 2008; Raffel et al. 2009, 2011; Schock et al. 2010; Johnson and Buller 2011; Schotthoefler et al. 2011). Moreover, there have been few attempts to examine the joint occurrence of fungal, viral, and helminth pathogens in “healthy” amphibian populations (Gahl and Calhoun 2008; Greer et al. 2008, 2009).

Here, we conducted field surveys to assess the degree of pathogen co-occurrence in wetland amphibian communities in the East Bay region of California, USA. Between May and August of 2010, >3400 adult and recently metamorphosed amphibian hosts representing six species were sampled from 85 wetlands by a combination of visual encounter surveys, dipnet sweeps, and habitat-stratified seine hauls (for detailed sampling methods see online Appendix). Captured amphibians were non-lethally swabbed for Bd by standardized methods (Annis et al. 2004; Lips et al. 2006). For each species excluding those classified as threatened or endangered, we also randomly necropsied up to 20 metamorphic individuals to evaluate trematode and ranavirus infection. At our collection sites, molecular data support the predominance of *Echinostoma trivolvis* (S. Orlofske et al. unpublished data); however, given their morphological similarity with other echinostomes (e.g., *E. revolutum*, *Echinoparyphrium* spp.), we cannot rule out the possibility that multiple echinostomes were present at our sites (Johnson and McKenzie 2008). For 43 of the 85 field sites, we collected host tissues for ranavirus testing by removing a ~2-mm section of the liver and kidney, which were pooled for all examined individuals for the determination of ranavirus infection status at the species level (Hoverman et al. 2011). Because the detection of Bd and ranavirus were derived from aggregate swabs or tissue samples, our analyses are focused at the site level rather than at the individual host level. We used basic summary statistics to examine patterns in the detection of each pathogen and pathogen species richness across our sites. In addition, we used the checkerboard score (*C* score) to test for patterns of pathogen species co-occurrence, a Mantel test to determine whether similarity in pathogen community composition correlated with distance between wetlands, and Moran's *I* to assess spatial patterns of pathogen species richness (see online Appendix).

We found that fungal, viral, and helminth infections were widespread within wetland-breeding amphibian communities ($\geq 45\%$ site-level occurrence for each pathogen). Echinostomes were the most commonly detected pathogens (Fig. 1A) followed by ranavirus, Bd, and *Ribeiroia*. Given that

the echinostomes were not identified to species, the high level of occurrence in our wetlands could be due to an increased likelihood of detecting one of the species possible within the region at a local wetland. More importantly, pathogen co-occurrence was extremely common with the majority of sampled wetlands (68%) harboring at least two pathogens (Fig. 1B). These estimates are likely conservative; given that variation in host sample size among sites and the potential for false-negatives (although sample size showed no relationship with detection probability, see online Appendix). In general, there have been few field surveys examining the joint distributions of fungal, viral, and helminth pathogens in amphibian communities, especially in the absence of mortality or morbidity events (Gahl and Calhoun 2008; Greer et al. 2008, 2009). Schock et al. (2010) sampled for Bd and ranavirus in 34 wetlands over the course of two years in the Northwest Territories, Canada, finding just a single site with both pathogens. Recently, positive site-level associations have been detected between echinostomes and *Ribeiroia* (Johnson and Buller 2011; Schotthoefler et al. 2011), likely due to common dispersal mechanisms. While our data only allowed for the exploration of coarse-scale co-occurrence patterns (e.g., among wetlands), the fact that multiple pathogens were commonly found within our sampled amphibian communities suggests that there is ample opportunity for interactions among amphibian pathogens in nature. These results underscore the need for further investigations into the prevalence and patterns of coinfection at the individual host level.

We did not find evidence that the pathogen communities were structured (observed *C* score = 213, simulated mean = 209.6, variance = 13.8, $P = 0.197$) or that similarity in parasite community composition was correlated with distance ($r = -0.04$, $P = 0.468$). However, pathogen species richness exhibited signals of spatial clustering among the sampled wetlands ($I = 0.23$, $P = 0.006$; Fig. 1C). Although these pathogens did not significantly associate with one another within wetlands, regions with a high density of wetlands that supported the pathogens tended to cluster together. Given that wetlands in close proximity were also sampled at relatively similar times due to logistical constraints, temporal variation (i.e., seasonal differences in occurrence) could also be contributing to this spatial pattern. Additional work is necessary to determine whether local biotic and/or abiotic characteristics of these wetlands influence pathogen co-occurrence and individual pathogen success and, thereby, contributed to these non-random spatial distributions.

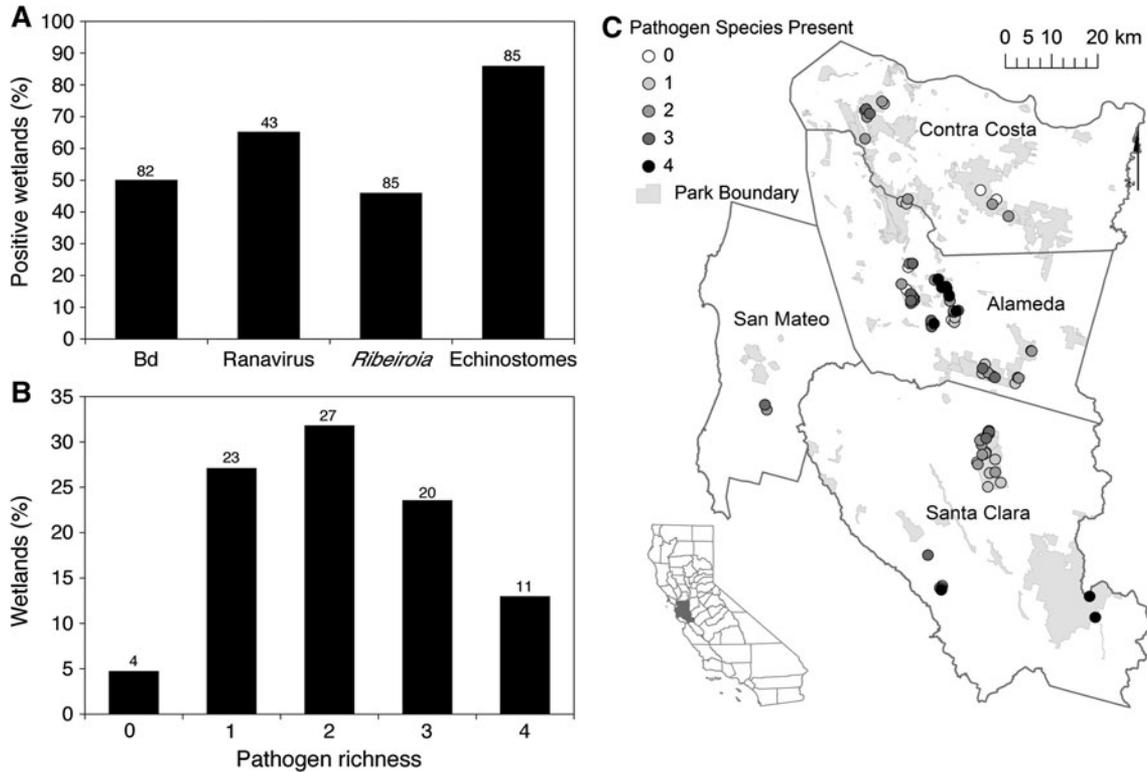


Figure 1. **A** Percentage of wetlands that tested positive for *Batrachochytrium dendrobatidis* (Bd), ranavirus, *Ribeiroia ondatrae*, and echinostomes. *Numbers above each bar* indicate the number of wetlands that were sampled for each pathogen. **B** Percentage of sampled wetlands containing each level of pathogen species richness. *Numbers above each bar* indicate the number of wetlands at each level of pathogen species richness. **C** Map of sampled wetlands in a four county area of the Bay Area of California, USA (Santa Clara, San Mateo, Alameda, and Contra Costa counties). Study sites are depicted as *circles* with the *degree of shading* indicating the number of detected pathogens at the site. *Inset* shows the state of California.

To date, the contributing role of pathogen interactions in amphibian mortality and morbidity events has remained relatively unexplored. The majority of disease studies have focused on Bd due to its sometimes devastating effects on amphibian populations (Bosch et al. 2007; Kilpatrick et al. 2010); however, pathogens such as ranaviruses and *Ribeiroia* have also been implicated in die-off events or severe pathology in amphibians (Green et al. 2002; Johnson et al. 2002; Gray et al. 2009). It is possible that die-offs are more or less likely depending on the presence and prevalence of non-focal pathogens and the outcome of interactions between members of the pathogen community. To understand the population-level impacts of disease and to inform conservation initiatives, studies are needed that discern the combined effects of multiple pathogens on amphibian population dynamics and disease epidemics.

From an applied perspective, intra-host pathogen interactions have been shown to influence disease dynamics in several wildlife and human systems (Bentwich et al. 1995;

Cooney et al. 2002; Druilhe et al. 2005; Ezenwa et al. 2010). Thus, future studies should address how the presence and abundance of one pathogen within a host influences infection by other pathogens and the resulting consequences for individual hosts and population dynamics (Jones et al. 2008; Tompkins et al. 2010). The contributing role of disease in amphibian population declines provides an added urgency to understanding the pathogen community of amphibian populations. Considering that amphibian communities are frequently exposed to multiple pathogens, a broader understanding of coinfection dynamics is essential for the development of effective management strategies.

ACKNOWLEDGMENTS

This project was supported by the grants from NSF (DEB-0553768, MRI-0923419) and a fellowship from the David and Lucile Packard Foundation. We thank S. Paull,

J. McFarland, K. Lunde, and K. Gietzen for assistance in the field surveys and I. Buller, E. Kellermans, and B. LaFonte for conducting amphibian necropsies. J. Rohr and several anonymous reviewers provided helpful comments on the manuscript. We gratefully acknowledge support and property access from D. Bell of East Bay Regional Parks, D. Rocha of Santa Clara County Parks, M. Hamilton of the UC Reserve System, J. Smith of East Bay Municipal Utility District, S. Abbors of Midpeninsula Open Space, and K. Fleming of the California State Parks System.

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