Received: 10 August 2020 | Accepted: 18 November 2020

DOI: 10.1111/fwb.13662

ORIGINAL ARTICLE

Freshwater Biology WILEY

Freshwater snails and the green algae *Cladophora* are probably not hosts of *Batrachochytrium dendrobatidis*

Taegan A. McMahon[1,2] | Caitlin L. Nordheim[1] | Devin M. Prokopiak[1]

[1]University of Tampa, Tampa, FL, U.S.A.

[2]Connecticut College, New London, CT, U.S.A.

Correspondence
Taegan A. McMahon, University of Tampa, Tampa, FL, U.S.A.
Email: Taeganmcmahon@gmail.com

Funding information
University of Tampa; National Institutes of Health; National Science Foundation

Abstract

1. *Batrachochytrium dendrobatidis* (Bd) is a pathogenic fungus that has devastated amphibian populations globally by causing the disease chytridiomycosis. *Batrachochytrium dendrobatidis* is capable of infecting non-amphibian hosts, such as crayfish, and has been detected on reptile and bird species. Given the taxonomic heterogeneity in the known hosts and vectors of Bd, it is likely that there is a diversity of undiscovered non-amphibian hosts of the fungus.
2. Here, we investigated whether Bd could survive on freshwater snails (*Physella acuta*) and *Cladophora* algae. We exposed small and large snails (n = 15 snails/size category), *Cladophora* algae (n = 5), and artificial spring water controls (ASW; n = 5) to live Bd. We also maintained Bd-free control snails (n = 5 snails/size category) in ASW. All treatments were maintained for 7 weeks at 18°C. Mortality was checked three times a week, snails were weighed every 2 weeks, and 7 weeks after exposure, the snails, algae, and water were tested for Bd using quantitative polymerase chain reaction.
3. We found that Bd did not grow on live snails, algae, or ASW long term. Additionally, live snails (n = 20) collected from Bd-positive ponds in California were all negative for Bd, as well. Given that we found no Bd on the experimentally exposed or field swabbed snails, snails are probably not a reservoir host of Bd.
4. While negative results are often not published, Bd is one of the deadliest pathogens on earth; it is essential to know what is and is not capable of maintaining Bd for well-designed disease models.

KEYWORDS
alternative host, amphibian decline, chytrid fungus, *Physella*, reservoir host

1 | INTRODUCTION

Batrachochytrium dendrobatidis (Bd) is associated with the decline, extirpation, or extinction of over 500 species of amphibians (Scheele et al., 2019). This pathogenic fungus is widespread and is found on every continent on which amphibians are found (Olson et al., 2013). While Bd was once thought to be an amphibian specialist, we now know Bd has non-amphibian hosts, such as crayfish (Brannelly et al., 2015; McMahon et al., 2013). Additionally, Bd has been found on other organisms in the wild, such as reptiles (Kilburn et al., 2011) and waterfowl (Garmyn et al., 2012; Wimsatt et al., 2014).

The presence of a diversity of reservoir hosts can increase the likelihood that a susceptible host will be extirpated or go extinct (de Castro & Bolker, 2005; Rothermel et al., 2008). There is probably a variety of non-amphibian hosts of Bd; however, most studies examining non-amphibian-Bd interactions have examined Bd growth on tissue in a sterile environment or from wild tissue surface swabs.

While this information is an essential first step, these findings do not account for the species interactions or the hosts' defences, e.g. their microbiome and immune system. More experiments are needed to explore the relationship between Bd and its potential hosts both through field sampling and controlled long-term growth experiments in vivo.

Freshwater snails, for example, might be an ideal reservoir host for Bd, as they are found globally and contain keratin (Hudson et al., 2007), a food resource for Bd (Longcore et al., 1999). Here, we examined *Physella acuta* and *Helisoma trivolvis* because they both co-occur with amphibians (Abbott & Bergey, 2007) and Bd. Freshwater snails are transported from pond to pond by animals (Van Leeuwen et al., 2013) and vehicles (Banha et al., 2014) and, therefore, could function as an effective pathogen vector. Additionally, we examined whether the green algae *Cladophora* sp., used in this study as a food resource for the experimentally exposed snails, could host Bd. This algae is common and widespread in freshwater ponds (Higgins et al., 2008) and is grazed on by snails and tadpoles (Holomuzki & Hemphill, 1996). If *Cladophora* is also a reservoir for Bd, trophic transmission would be an easy mode of transmission to other susceptible hosts, e.g. tadpoles or crayfish. We need to have a better understanding of which organisms are and are not viable hosts in order to adequately manage this pathogen, which is devastating organisms globally.

2 | METHODS

2.1 | *Batrachochytrium dendrobatidis* culture

Batrachochytrium dendrobatidis strain JEL 419 (a virulent strain isolated in Panamá during a mass amphibian die off event) was cultured on 1% tryptone agar plates at 18°C for 10 days. These Bd+ plates were flooded with artificial spring water (ASW; Cohen et al., 1980) to bring the zoospores into suspension, and the liquid from all of the plates was homogenised (Bd+ stock: 1×10^5 zoospores/mL; this concentration has been shown to induce successful infections in both amphibians and crayfish (see McMahon et al., 2013; McMahon and Rohr, 2015). The viability of the Bd+ stock was verified by growing 1 ml on 1% tryptone plates for 8 days ($n = 5$ plates). We had robust growth with this positive control, which verifies that the Bd+ stock used in this experiment was strong and viable. We made control plates by following the same procedure, but the control 1% tryptone agar plates were Bd-free (Bd- stock).

2.2 | Exposure experiment

Physella acuta collected from Tampa, Florida, were housed individually in 50 ml of ASW with 0.165 ± 0.01 g of *Cladophora* sp. algae. All containers were maintained at 18°C (12:12, light:dark cycle) for the duration of the 7-week experiment. We changed the water, container, and the algae for each snail 3 weeks after exposure, to ensure that if Bd was maintained in the system, it was maintained on the snail. We had four experimental groups: (1) small snails (0.01 ± 0.006 g) and *Cladophora* algae; (2) large snails (0.07 ± 0.03 g) and *Cladophora* algae; (3) *Cladophora* algae alone; and (4) an ASW control. Each replicate was dosed with 1 ml of Bd+ stock or Bd− stock ($n = 15$ for each Bd+ snail treatment, $n = 5$ for each Bd- snail treatment (total = 40 snails), and $n = 5$ for all other treatments, all of which were dosed with the Bd+ stock). Mortality was checked three times a week and snails were weighed every 2 weeks.

After 7 weeks, all experimental groups were sampled for Bd. Snails were swabbed (10 times on the entire surface of the shell and 10 times on the foot and operculum), a 0.04 g piece of algae was taken from each *Cladophora* algae alone replicate, and a swab was submerged and moved throughout each ASW replicate for 10 s (covering all surfaces of the container). All samples were analysed for Bd using quantitative polymerase chain reaction (qPCR).

2.3 | Artificial spring water control experiment

We conducted an additional control experiment to verify that Bd did not persist in the ASW treatments alone. We dosed 1,000 μl of ASW with 20 μl of Bd+ ($n = 5$; 1×10^5 zoospores/mL); this is the same ratio of Bd to ASW as used in the Exposure Experiment. We maintained these treatments for two weeks at 18°C (12:12, light:dark cycle); this is long enough that if the Bd added to this system did not survive during this time, the DNA would no longer be detectable (McMahon et al., 2014). We filtered the water through a 1.2 μm filter (GE Whatman Laboratory Products) and qPCR was used to analyse Bd quantity.

2.4 | Field survey

Physa spp. and *H. trivolvis* ($n = 10$ snails/genus) were collected from two small, fishless ponds in East Bay, California (July 2019) that were historically Bd+ (Johnson et al., 2018; Stutz et al., 2018) using seine nets and dipnets in vegetated areas of the ponds (McCaffrey & Johnson, 2017). Each snail was swabbed as described above (see Exposure experiment for swabbing methods). All samples were analysed for Bd using qPCR. Importantly, Bd+ amphibians were also sampled in this region during this collection event; amphibians were swabbed ten times from hip to toe on the hind legs and screened for Bd with qPCR ($n = 79$ frogs swabbed: 45.6% Bd+ prevalence).

2.5 | Quantitative PCR

We followed the qPCR protocol described by Hyatt et al. (Hyatt et al., 2007). In brief, DNA was extracted from the samples using PrepMan Ultra (Applied Biosystems, Foster City, CA, U.S.A.). The *Cladophora* algae samples were processed with one extra step, in which the tissue was disrupted for better extraction efficiency

using 0.035 ± 0.05 g of zirconia/silica beads in a cell disruptor for a total of 2.25 min (Disruptor Genie, Scientific Industries, Bohemia, NY, U.S.A.). To screen for inhibition, we added TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems, Foster City, CA, U.S.A.) to each sample. There was no evidence of inhibition. Additionally, the standard curve in each plate was clean with little variation and we also ran known Bd+ samples to verify that the qPCR methods worked effectively.

2.6 | Data analysis

All statistical analyses were conducted in R statistical software (Development Core Team R 2013). Snail growth rate, snail final mass, and snail size were analysed using a generalised linear model (package: stats; function: glm; family: gaussian). A Cox-proportional hazards regression run to determine effect of treatment on mortality (family: survival, function: coxph).

3 | RESULTS

Bd was not detected in ASW using either the swab or filter methods. Bd was not detected on snails (in the lab or the field), *Cladophora* algae, or in ASW 7 weeks after exposure. Snail growth rate was not affected by Bd exposure nor by snail size (growth rate: $x = 0.91$, $p = 0.34$, snail final mass: $x = 1.06$, $p = 0.30$, snail length: $x = 1.05$, $p = 0.30$). There was no effect of treatment on mortality ($x = 0.56$, $p = 0.46$).

4 | DISCUSSION

Snails are a reasonable candidate for a reservoir host or vector of Bd, given that they co-occur with amphibians (Ebbs et al., 2018), remain fully aquatic throughout their life (ideal for an aquatic pathogen), and contain keratin (Hudson et al., 2007), which is a common food resource for Bd (Longcore et al., 1999). However, we found no evidence that snails can maintain Bd in the field nor that *P. acuta* can be experimentally infected in the laboratory long term. It is important to note that our field sampling methodology may not have found Bd+ snails if they maintain infections at a very low prevalence (we screened 20 snails); however, our laboratory experiments also did not yield any infected animals and so it is our conclusion that snails are not probably a host for Bd. Additionally, we controlled for the presence of the snails' algae food by directly exposing *Cladophora* algae with Bd. While this was originally intended as an experimental control, we can also conclude that *Cladophora* is also not a likely reservoir host of Bd, given that no algae samples were Bd+ at the end of the experiment.

There are several reasons that Bd may not have been able to establish on and infect freshwater snails or *Cladophora* algae. It is possible that Bd was incapable of surviving on the host tissue itself, and that this substrate was not a viable food resource for the pathogen. The snails may have resisted the infection using their innate or adaptive immune responses. Snail immune responses to aquatic fungi are not well understood, but there is evidence that snails can mount a measurable immune response to the presence of other aquatic pathogens, e.g. bacteria (Seppälä & Jokela, 2011). *Cladophora* spp. are also known to produce anti-pathogen chemicals (Lavoie et al., 2019), which may help this species resist Bd infection.

The mucus produced by snails may also function as a mechanism for pathogen resistance. The mucus produced by other snails (e.g. *Helix aspersa* and *Achatina fulica*) contains antimicrobial peptides (Gunn et al., 2015; Zhonga et al., 2013), which may help fight off pathogens before infection. Antimicrobial peptides produced by *Rana muscosa* have been shown to inhibit Bd growth in vitro (Rollins-Smith et al., 2006), and the antimicrobial peptides produced by *Xenopus laevis* inhibit the growth of zoospores on the skin (Ramsey et al., 2010). Further work is needed to determine the definitive causal mechanism behind the lack of infection in snails.

Batrachochytrium dendrobatidis is potentially responsible for the decline of over 500 species of amphibians (Scheele et al., 2019) and, because of this, we have historically focused on the amphibian–Bd host–parasite system. However, to understand and effectively manage the devastation associated with Bd, we need to expand our research scope to understand how this pathogen fits into the aquatic community. The presence of reservoir hosts increases environmental persistence (de Castro & Bolker, 2005), virulence (Rutrecht & Brown, 2009), and the potential of host extirpation or extinction (Rosà, 2003; Rothermel et al., 2008).

Additionally, we acknowledge that it is rare to publish null results; indeed, there is a bias across the disciplines toward positive findings (Mlinarić et al., 2017). This bias can be particularly detrimental when working with an organism such as Bd, which is arguably one of the deadliest pathogens in the world. Time, money, and research hours are dedicated to asking necessary questions in this field; it would be damaging to our scientific knowledge for this conservation crisis not to publish both positive and null findings. Now that we know that freshwater snails (*Physa* spp. and *H. trivolvis*) and *Cladophora* algae are not reservoir hosts, it would be detrimental and unnecessary for another research group to work through a similar set of questions. We argue that all knowledge regarding which hosts are and are not viable reservoir hosts is crucial to developing a full community-wide view of this pathogen.

ACKNOWLEDGEMENTS

We would like to acknowledge S. Alterio and R. Kurlander for their research assistance in the Exposure experiment, P. Johnson for the field permits, and T. McDevitt-Galles for assistance in the Field survey. Funding was provided by the National Science Foundation (T.A.M.: IOS- 1754862), the National Institutes of Health (T.A.M.: 1R01GM135935-01, subaward KK2022) and the University of Tampa (T.A.M.: Biology Student Research Funds).

CONFLICT OF INTEREST

All authors affirm there are no conflict of interests associated with this submission.

AUTHOR CONTRIBUTIONS

T.A.M. designed experiments, all authors conducted experiments, C.L.N. collected field samples, and T.A.M. conducted PCR and data analyses. All authors wrote the paper and provided editorial advice. T.A.M. provided funding.

DATA AVAILABILITY STATEMENT

The authors will make the data available upon direct request. Please contact the corresponding author, Dr Taegan McMahon.

ORCID

Taegan A. McMahon https://orcid.org/0000-0002-0995-7445
Caitlin L. Nordheim https://orcid.org/0000-0002-9786-060X

REFERENCES

Abbott, L. L., & Bergey, E. A. (2007). Why are there few algae on snail shells? The effects of grazing, nutrients and shell chemistry on the algae on shells of *Helisoma trivolvis*. *Freshwater Biology*, *52*, 2112–2120. https://doi.org/10.1111/j.1365-2427.2007.01832.x

Banha, F., Marques, M., & Anastacio, P. M. (2014). Dispersal of two freshwater invasive macroinvertebrates, *Procambarus clarkii* and *Physella acuta*, by off-road vehicles. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *24*, 582–591.

Brannelly, L. A., Taegan, A., McMahon, M. H., Lenger, D., & Richards-Zawacki, C. L. (2015). *Batrachochytrium dendrobatidis* in natural and farmed Louisiana crayfish populations: Prevalence and implications. *Diseases of Aquatic Organisms*, *112*, 229–235. https://doi.org/10.3354/dao02817

Cohen, L. M., Neimark, H. L., & Everland, L. K. (1980). *Schistosoma mansoni*: Response to cercariae to a thermal gradient. *Journal of Parasitology*, *66*, 362–364.

de Castro, F., & Bolker, B. (2005). Mechanisms of disease-induced extinction. *Ecology Letters*, *8*, 117–126. https://doi.org/10.1111/j.1461-0248.2004.00693.x

Development Core Team R (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing Version 3.1.3.

Ebbs, E. T., Loker, E. S., & Brant, S. V. (2018). Phylogeography and genetics of the globally invasive snail *Physa acuta* Draparnaud 1805, and its potential to serve as an intermediate host to larval digenetic trematodes. *BMC Evolutionary Biology*, *18*, 103. https://doi.org/10.1186/s12862-018-1208-z

Garmyn, A. N., Van Rooij, P., Pasmans, F., Hellebuyck, T., Van Den Broeck, W., Haesebrouck, F., & Martel, A. N. (2012). Waterfowl: Potential environmental reservoirs of the chytrid fungus *Batrachochytrium dendrobatidis*. *PLoS One*, *7*, e35038. https://doi.org/10.1371/journal.pone.0035038

Gunn, A., Pitt, S. J., Graham, M. A., Dedi, C. G., & Taylor-Harris, P. M. (2015). Antimicrobial properties of mucus from the brown garden snail *Helix aspersa*. *British Journal of Biomedical Science*, *72*, 174–181.

Higgins, S. N., Malkin, S. Y., Howell, T. E., Guildford, S. J., Campbell, L., Hiriart-Baer, V. et al (2008). An ecological review of *Cladophora glomerata* (Chlorophyta) in the Laurentian Great Lakes. *Journal of Phycology*, *44*, 839–854.

Holomuzki, J. R., & Hemphill, N. (1996). Snail-tadpole interactions in streamside pools. *The American Midland Naturalist*, *136*, 315–327. https://doi.org/10.2307/2426735

Hudson, L. G., Choi, C., Newkirk, K. M., Parkhani, J., Cooper, K. L., Lu, P., & Kusewitt, D. F. (2007). Ultraviolet radiation stimulates expression of snail family transcription factors in keratinocytes. *Molecular Carcinogenesis*, *46*, 257–268. https://doi.org/10.1002/mc.20257

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., … Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, *73*, 175–192. https://doi.org/10.3354/dao073175

Johnson, P. T. J., Calhoun, D. M., Stokes, A. N., Susbilla, C. B., McDevitt-Galles, T., Briggs, C. J., … de Roode, J. C. (2018). Of poisons and parasites—the defensive role of tetrodotoxin against infections in newts. *Journal of Animal Ecology*, *87*, 1192–1204. https://doi.org/10.1111/1365-2656.12816

Kilburn, V. L., Ibáñez, R., & Green, D. M. (2011). Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama. *Diseases of Aquatic Organisms*, *97*, 127–134. https://doi.org/10.3354/dao02409

Lavoie, S., Sweeney-Jones, A. M., Mojib, N., Dale, B., Gagaring, K., McNamara, C. W. et al (2019). Antibacterial oligomeric polyphenols from the green alga *Cladophora socialis*. *Journal of Organic Chemistry*, *84*, 5035–5045.

Longcore, J. E., Pessier, A. P., & Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. *Mycologia*, *91*, 219–227.

McCaffrey, K., & Johnson, P. T. J. (2017). Drivers of symbiont diversity in freshwater snails: A comparative analysis of resource availability, community heterogeneity, and colonization opportunities. *Oecologia*, *183*, 927–938. https://doi.org/10.1007/s00442-016-3795-y

McMahon, T. A., Brannelly, L. A., Chatfield, M. W. H., Johnson, P. T. J., Joseph, M. B., McKenzie, V. J., … Rohr, J. R. (2013). Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. *Proceedings of the National Academy of Science of the United States of America*, *110*, 210–215. https://doi.org/10.1073/pnas.1200592110

McMahon, T. A., & Rohr, J. R. (2015). Transition of chytrid fungus infection from mouthparts to hind limbs during amphibian metamorphosis. *EcoHealth*, *12*, 188–193. https://doi.org/10.1007/s10393-014-0989-9

McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., … Rohr, J. R. (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature*, *511*, 224–227. https://doi.org/10.1038/nature13491

Mlinarić, A., Horvat, M., & Smolčić, Š. (2017). Dealing with the positive publication bias: Why you should really publish your negative results. *Biochemical Medicine*, *27*.

Olson, D. H., Aanensen, D. M., Ronnenberg, K. L., Powell, C. I., Walker, S. F., Bielby, J., … Fisher, M. C. (2013). Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PlosOne*, *8*, e56802. https://doi.org/10.1371/journal.pone.0056802

Ramsey, J. P., Reinert, L. K., Harper, L. K., Woodhams, D. C., & Rollins-Smith, L. A. (2010). Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infection and Immunity*, *78*, 3981–3992. https://doi.org/10.1128/iai.00402-10

Rollins-Smith, L. A., Woodhams, D. C., Reinert, L. K., Vredenburg, V. T., Briggs, C. J., Nielsen, P. F., & Conlon, J. M. (2006). Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Developmental and Comparative Immunology*, *30*, 831–842. https://doi.org/10.1016/j.dci.2005.10.005

Rosà, R. (2003). *The Importance of aggregation in the dynamics of host-parasite interaction in wildlife: A mathematical approach*. University of Stirling, Institute of Biological Sciences.

Rothermel, B. B., Walls, S. C., Mitchell, J. C., Dodd, C. K., Irwin, L. K., Green, D. E., … Stevenson, D. J. (2008). Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Diseases of Aquatic Organisms*, *82*, 3–18. https://doi.org/10.3354/dao01974

Rutrecht, S. T., & Brown, M. J. F. (2009). Differential virulence in a multiple-host parasite of bumble bees: Resolving the paradox of parasite survival? *Oikos*, *118*, 941–949. https://doi.org/10.1111/j.1600-0706.2009.17392.x

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Martel, A. N., Beukema, W., … Canessa, S. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, *363*, 1459–1463. https://doi.org/10.1126/science.aav0379

Seppälä, O., & Jokela, J. (2011). Immune defence under extreme ambient temperature. *Biology Letters*, *7*, 119–122. https://doi.org/10.1098/rsbl.2010.0459

Stutz, W. E., Blaustein, A. R., Briggs, C. J., Hoverman, J. T., Rohr, J. R., & Johnson, P. T. J. (2018). Using multi-response models to investigate pathogen coinfections across scales: Insights from emerging diseases of amphibians. *Methods in Ecology and Evolution*, *9*, 1109–1120. https://doi.org/10.1111/2041-210X.12938

Van Leeuwen, C. H. A., Huig, N., Van der Velde, G., Van Alen, T. A., Wagemaker, C. A. M., Sherman, C. D. H., … Figuerola, J. (2013). How did this snail get here? Several dispersal vectors inferred for an aquatic invasive species. *Freshwater Biology*, *58*(1), 88–99. https://doi.org/10.1111/fwb.12041

Wimsatt, J., Sanford, H., Feldman, M. H., Hammond, M., Ruehling, M. P. R., Grayson, K. L., & Mitchell, J. C. (2014). Detection of pathogenic *Batrachochytrium dendrobatidis* using water filtration, animal and bait testing. *Zoo Biology*, *33*, 577–585.

Zhonga, J., Wang, W., Yang, X., Yan, X., & Liu, R. (2013). A novel cysteine-rich antimicrobial peptide from the mucus of the snail of *Achatina fulica*. *Peptides*, *39*, 1–5. https://doi.org/10.1016/j.peptides.2012.09.001

How to cite this article: McMahon TA, Nordheim CL, Prokopiak DM. Freshwater snails and the green algae *Cladophora* are probably not hosts of *Batrachochytrium dendrobatidis*. *Freshwater Biology*. 2021;66:582–586. https://doi.org/10.1111/fwb.13662