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ARTICLE

Disease Ecology

Microbe surveillance in the amphibian pet trade: Results from a pilot study

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Abstract

Regional and global trade of live animals can contribute to the spread and emergence of novel pathogens, including several important pathogens of amphibians. However, understanding the spread or even frequency of infections in large, complex amphibian trade networks has been difficult, in part because businesses tend to be reluctant to participate in surveillance programs. Thus, we developed a novel approach to surveillance in which anonymous participating businesses were sent surveillance kits through a trusted trade advocacy partner, samples were returned to researchers via anonymous prepaid envelopes, and results were provided via a secure website with access regulated by a unique personal identification number (PIN) created by the business. We tested samples for the amphibian pathogens, *Batrachochytrium salamandrivorans* (*Bsal*), *Batrachochytrium dendrobatidis* (*Bd*), and *Ranavirus* spp. (*Rv*), as well as the beneficial microbe, *Janthinobacterium lividum* (*Jliv*), using quantitative real-time polymerase chain reaction (qPCR). Out of 120 businesses invited to complete an anonymous socioeconomic survey, 24 volunteered to participate in pathogen surveillance, of which 14 were sent surveillance kits. Eight of these businesses returned samples consisting of swabs collected from amphibians in 78 terrestrial habitats and water filters from 49 aquatic habitats. Copies of a highly conserved vertebrate gene (EBF3N), quantified using qPCR, were consistently low (<100 copies) in returned samples, but similar to those collected by researchers, indicating comparable sample quality. Three samples (from two facilities) had detectable levels of *Bd* DNA; *Bsal*, *Rv*, and *Jliv* were not detected. This pilot study provides evidence that information about pathogens in pet trade networks can be acquired by developing partnerships with industry, and business participation might be enhanced by ensuring anonymity and inclusion of a trade advocacy partner.

KEYWORDS
amphibians, disease, pathogens, pet trade, surveillance

INTRODUCTION

The global trade of live animals can facilitate the introduction and spread of pathogens (Karesh et al., 2005). For example, the amphibian fungal pathogen *Batrachochytrium salamandrivorans* (*Bsal*)—linked to declines in wild populations of Palearctic salamanders across Germany, Belgium, the Netherlands, and Spain (Lastra González et al., 2020; Spitzen-van der Sluijs et al., 2016; Stegen et al., 2017)—is hypothesized to have been introduced to Europe from Southeast Asia via spillover from the pet trade (Martel et al., 2014; Nguyen et al., 2017). While *Bsal* has not yet been detected outside of Europe and Asia, other important amphibian pathogens, particularly *Batrachochytrium dendrobatidis* (*Bd*) and *Ranavirus* spp. (*Rv*), have both been found in international shipments of pet amphibians around the world (Kolby et al., 2014; Peel et al., 2012; Schloegel et al., 2009; Wombwell et al., 2016). For instance, a random sample of live amphibians entering the United States from Hong Kong revealed 11.7% (31 of 265) of animals carried *Bd* and 56.8% (105 of 185) carried *Rv* (Kolby et al., 2014).

After arriving within a region via imports, a common, if unstated assumption is that pathogens will spread throughout the domestic trade (e.g., for pets) and spillover into wild populations via pathways such as the disposal of untreated waste or released animals (Fisher & Garner, 2007; Ribeiro et al., 2019). For example, introduced populations of American bullfrogs (*Lithobates catesbeianus*), one of the most common species in the global trade of live amphibians (Schloegel et al., 2009), have been implicated in the spread of *Bd* and *Rv* among wild amphibians around the world (Borzée et al., 2017; Garner et al., 2006; Sharifian-Fard et al., 2011; Une et al., 2009; Yap et al., 2018).

Despite this, almost nothing is known about what factors (e.g., common species, husbandry practices, biosecurity) contribute to pathogen spread and amplification within domestic trade networks, or even how common they are. This information is essential for developing evidence-based guidance aimed at preventing pathogens from entering or leaving businesses (Olson et al., 2024). For instance, dozens of species known to carry *Bsal* have been, and continue to be, imported into the United States every year in large numbers (Connelly et al., 2023). Despite *Bsal* being prevalent in the source populations of these commonly traded species (Yuan et al., 2018), *Bsal* was not detected in 639 samples submitted from pet salamanders in the United States (Klocke et al., 2017), nor in wild salamanders in North America more broadly (Basanta et al., 2022; Waddle et al., 2020). What happens to presumed *Bsal* infections in imported animals as they move from borders to pet owners is unknown, but these observations are consistent with the hypothesis that the US domestic trade somehow prevents *Bsal* from spreading. These results stand in stark contrast to the apparent spread of *Bsal* among private collections in the United Kingdom and Western Europe (Fitzpatrick et al., 2018). Clearly, better surveillance within domestic trade networks is needed to improve our understanding of which biosecurity and husbandry practices are most effective in preventing the spread and emergence of amphibian pathogens.

A key problem for conducting surveillance in private businesses is trust. Businesses may view on-site sampling, and even the presence of researchers, as a breach of privacy. Husbandry practices, especially for rare or highly prized species, can be closely guarded trade secrets (Z. Brinks, N. Moherman, personal communication, 2020; Stallins & Kelley, 2013), and participants may worry about reputational damage or government interference in their business if a pathogen is found. Researchers may likewise be wary of the results of self-reported testing from businesses as nonrandom sampling (e.g., sampling in response to apparent disease) and inconsistency in methods of collection make it difficult to estimate prevalence or other important quantities.

In response to these concerns, we designed a novel approach to pathogen surveillance in the US pet amphibian trade network, one that ensures the anonymity of participants and follows a standardized sampling approach (Figure 1). We then conducted a pilot study to determine (1) whether businesses would be willing to invest the time and effort needed to sample their own facilities, and (2) whether business personnel with little or no prior training would collect samples of sufficient quality to detect pathogens.

METHODS

Anonymous surveillance program

Study participants were selected among the respondents of an anonymous, digitally distributed socioeconomic survey that targeted businesses selling pet amphibians (Cavasos et al., 2023). The voluntary survey instruments and protocols were reviewed and approved by the University of Tennessee Knoxville (UTK) Institutional Review Board for human subjects research (approval number: UTK IRB-21-06494-XM). Invitations to participate in the survey were distributed by the Pet Advocacy Network (https://petadvocacy.org/; known as the Pet Industry Joint Advocacy Council [PIJAC] during this study) and industry partners, including Reptiles by Mack (https://reptilesbymack.com/) and Josh's Frogs (https://joshsfrogs.com/). Businesses could also enter the

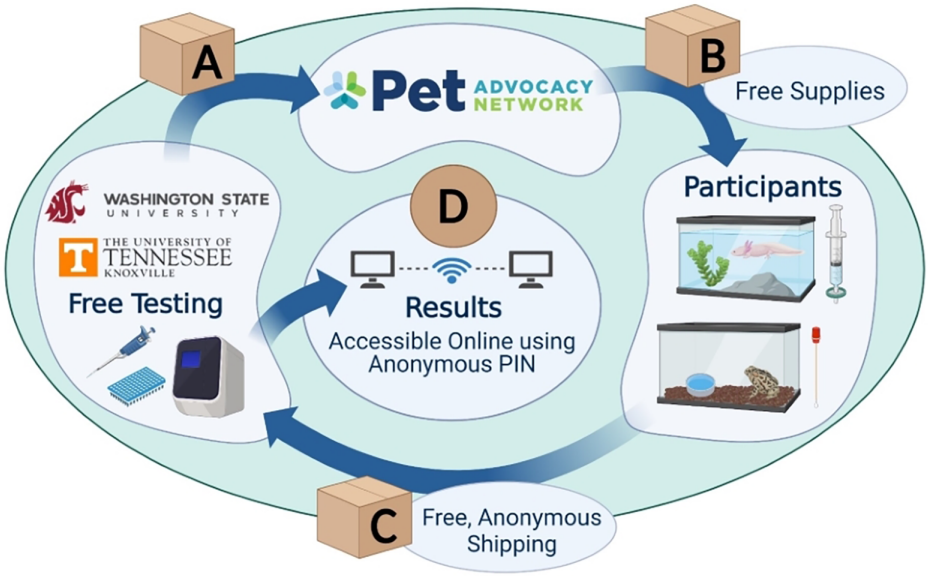

FIGURE 1 Schematic of pathogen testing kit distribution, sampling, return, and processing to maintain participant anonymity. (A) Pathogen testing supplies are given anonymous, numeric ID's and sent from researchers to the Pet Advocacy Network. (B) The Pet Advocacy Network associates anonymous ID's with participants and reships the testing supplies. (C) Participants swab animals or filter water from up to 30 habitats. Samples are returned anonymously to researchers via a free, prepaid shipping label. (D) Samples are processed within 2 weeks of receipt. Results are accessible online, anonymously, with a participant-generated personal identification number (PIN).

survey via a project website: http://tiny.utk.edu/pijac. At the end of taking this survey, respondents were provided with the following prompt: "PIJAC and its partners need help in identifying conditions that result in fewer pathogens and more beneficial microbes in US pet amphibian trade. If you are willing to take advantage of free microbe testing at your business (valued at $30 per sample), please indicate your interest below and provide your business information, which will remain confidential." Only those who responded affirmatively were considered for study participation. Due to limited resources, a subset of volunteering businesses—known to researchers only through their unique, automatically generated, alphanumeric site IDs—were nonrandomly selected to capture the broadest possible diversity of business types (i.e., retailer, breeder, wholesaler, importer) and sizes. Surveillance testing kits were then sent to the Pet Advocacy Network, who associated the anonymized site IDs with participant addresses and reshipped them to selected participants (Figure 1), thereby keeping business identity unknown to the researchers. If participants needed additional supplies during testing, they were advised to contact the Pet Advocacy Network. Participants were also advised to post questions and feedback regarding collection protocols or other study-related activities to an anonymous, online forum operated by the University of Tennessee.

Testing kits contained sampling materials (i.e., swabs, eDNA filters and syringes, gloves, drying racks, Whirl-Pak bags, sample labels), instructions (written materials and links to videos), a short questionnaire regarding what kinds of biosecurity practices are employed by participants (e.g., quarantine protocols, glove use, waste disposal), a form to provide written feedback about participation, and a prelabeled, prepaid return envelope. Return labels were generated with the Pet Advocacy Network's address as the point of origin to maintain participant anonymity upon receipt of completed samples. We included sampling materials sufficient to sample up to 30 habitats, or all available habitats in the facility, whichever was less, with swabs and eDNA filters included in rough proportion to the number of terrestrial (>50% enclosure bottom substrate unsubmerged) and aquatic (>50% enclosure bottom substrate submerged) habitats reported, respectively, during the sign-up survey. A habitat, our unit of sampling, was defined as any enclosure in which animals were separated from one another by nonpermeable barriers such as glass, plastic, or metal. Enclosures sharing a common water supply through recirculating pumps were not considered separate habitats. Participants were instructed to randomly select habitats for sampling among all of the habitats in their facility with the aid of an online random number generator (https://brunnerlab.shinyapps.io/RandomNumGenerator/). They were also instructed to sample habitats in the order presented in the randomized list so that sampling would still be random if participants decided not to sample all habitats.

Terrestrial habitats were sampled by swabbing up to five animals per habitat using sterile swabs (Medical Wire 113; Corsham, UK). Participants were instructed to swab each animal five times each along the dorsum (back), ventrum (belly), limbs, and inguinal (cloacal) region and then air-dry swabs prior to storage in a Whirl-Pak bag with other swabs from the same habitat. In order to stabilize swab samples during storage and shipping, three 5-g desiccant pouches were also included in each Whirl-Pak bag. Participants were instructed to sample aquatic habitats by plunging 100 mL of water, or as much as possible prior to clogging, through 0.45 μm pore size, polyvinylidene difluoride membrane filter columns (Millipore SVHV010RS; Burlington, MA, USA) using a sterile 50-mL luer-lok syringe and then dry the filters by plunging air through them. After sealing the outlet of a filter column with a cap containing polymer clay, 2 mL of buffer ATL from the DNeasy Blood and Tissue kit (Qiagen 939011; Hilden, Germany) was added to stabilize the DNA until extraction. Participants were then instructed to seal the filter inlet using a second luer-lok cap. Lastly, sealed filters were to be individually placed in Whirl-Pak bags (one per habitat). The Whirl-Pak bags included labels with additional questions about husbandry practices, including species identity (and total number), life stage of housed animals, substrate type (i.e., wood chips, dirt, paper towel, etc.), and, for aquatic habitats, total water volume, the quantity of water pushed through the eDNA filter column for each habitat sampled, and methods of filtering habitat water (viewable at: https://doi.org/10.6084/m9.figshare.25721169.v3). Collected samples were then returned to researchers using the prepaid, anonymous shipping envelopes (Figure 1).

DNA extraction

Upon receipt by researchers, DNA was extracted using Qiagen DNeasy Blood and Tissue Kits (Qiagen 69506). The tips of all of the swabs from a habitat were cut and pooled in 2-mL round bottom microcentrifuge tubes with 360 μL of buffer ATL and 40 μL of Proteinase K, and then digested at 56°C on a shaking heat block for 24 h. After digestion, 200 μL of lysate was removed and frozen at −20°C to serve as a backup. The remaining liquid and swab tips were placed in a DNeasy column and centrifuged at 8000×*g* for 1 min to ensure lysate trapped within saturated swab tips was not lost. The rest of the extraction proceeded according to the manufacturer's instructions with the exception that samples were incubated at 70°C for 5 min during the elution step to increase DNA yields.

Returned filter capsules contained 2 mL of buffer ATL, to which we added 200 μL of proteinase K through the filter inlet. The filters were resealed and vortexed for 30 s, then placed, inlet side down, into 50-mL screw-cap tubes, which were incubated in a 56°C shaking water bath for 24 h. After digestion, the luer-lok cap sealing the filter inlet was replaced with a Qiashredder filter column (Qiagen 79656) and positioned, inlet side down, in a bleach-sterilized (50% bleach solution for 10 min) polyvinyl chloride pipe adapter (Appendix S1: Figure S1) in a sterile 50-mL screw-cap tube and centrifuged at 2000×*g* for 5 min to remove liquids from the Sterivex filter and pass them through the Qiashredder filter column. One milliliter of lysate was then removed and stored at −20°C as a backup. We then added to each sample 1 mL of buffer AL, vortexed for 30 s, incubated it at 70°C for 10 min, and then added 1 mL of 100% ethanol. All 3 mL of the sample volume was centrifuged through the Qiagen DNeasy filter columns, 1 mL at a time, at 8000×*g* for 1 min. The rest of the extraction proceeded according to the manufacturer's instructions with the exception that samples were incubated at 70°C for 5 min during the elution step to increase DNA yields.

Quantitative real-time polymerase chain reaction

Samples were screened with quantitative real-time polymerase chain reaction (qPCR) assays individually for the presence of DNA of the pathogens *Bd* (Boyle et al., 2004), *Bsal* (Blooi et al., 2013), and *Rv* (Stilwell et al., 2018), and the putatively beneficial skin bacterium, *Janthinobacterium lividum* (*Jliv*; Bletz, 2013), that has been associated with reduced risk of infection and disease from *Bd* infections (Becker et al., 2009; Kueneman et al., 2016). Testing for *Jliv* was included in this pilot study to improve our understanding of how common this beneficial microbe is within the pet trade, as well as provide additional incentive for businesses. Each sample was run in duplicate 20 μL reactions with 5 μL of template DNA for 45 cycles on a StepOnePlus thermocycler (Applied Biosystems; Waltham, MA, USA). Exogenous internal positive controls (ExoIPC; Applied Biosystems 4308323) were included in the second of each duplicate well to test for PCR inhibition (2 μL 10× ExoIPC mix per well, 0.4 μL 50× ExoIPC DNA template per well). If a sample showed signs of inhibition—no target amplification and limited or no amplification of the ExoIPC as compared with standards—the sample was diluted 1:10 in buffer AE and rerun. A series of dilutions of gBlock oligonucleotides (10^0, 10^1, 10^2, 10^4, 10^6 copies/μL) with the target sequences were run on each 96-well plate as a standard for quantification. Samples were scored as positive if both wells showed amplification and negative if both did not.

If just one well showed amplification, the sample was rerun, and if at least one well out of two showed amplification, the sample was considered positive.

Sample results were made available to the participants via an encrypted portal that required their participant ID, which was included with their sampling kit, and a user-selected four- to eight-digit personal identification number (PIN), which they returned to researchers with their samples. Thus, researchers remained blinded to participant identities while the Pet Advocacy Network was blinded to the results of testing (Figure 1).

Estimates of vertebrate host DNA quantity

In addition to the microbe data, we estimated the number of copies of the ultra-conserved, noncoding vertebrate gene, EBF3N, in each sample using an additional qPCR assay (Leung et al., 2017). Our hypothesis was that higher quantities of EBF3N would correspond with better sampling technique among participants. We compared these values to (1) swabs we collected from 24 White's tree frogs (*Litoria caerulea*) and (2) eight eDNA filter samples collected from a fully aquatic, 5-L enclosure housing 15 *Xenopus laevis* tadpoles in water that had not been changed in 5 days. Prior to each filtering event, water was thoroughly mixed for 30 s to ensure homogeneity among samples.

RESULTS

Of the 120 businesses that completed the socioeconomic survey (Cavasos et al., 2023), 26 indicated a willingness to receive pathogen surveillance supplies. Due to limited supplies, we selected a total of 14 businesses to participate, 8 of which returned samples. Multiple attempts were made to reach the six businesses (through the Pet Advocacy Network) who did not return samples to better understand what barriers prevented participation, but none responded. Appendix S1: Tables S1–S4 provide additional information self-reported by participants, including responses to categorical questions about the source of their amphibians, who they sell to, sales volume, and adherence to basic biosecurity practices. Feedback from the eight businesses that returned samples was generally positive, with most criticism—reported primarily through the anonymous, online forum operated by the University of Tennessee—centered on the speed at which sample results were posted (average length from receipt to posting results was 30 days).

Returned samples totaled 49 eDNA filters and 78 swab pools collected from 44 amphibian species. The most commonly sampled species were *Ambystoma mexicanum* (12.6%), *Bombina orientalis* (12.6%), *Dendrobates tinctorius* (12.6%), and *Pleurodeles waltl* (7.9%). For a full list of species and their relative abundance among returned samples, see Appendix S1: Table S4.

Bd was detected in two pooled swab samples (48,865 copies and 7.5 copies) from habitats containing *Pyxicephalus adspersus* out of the 14 habitats sampled by participant facility no. 3, and one filter sample (170 copies) from a habitat containing *Tylototriton verrucosus*, out of the 30 sampled habitats in participant facility no. 6. No other microbes were detected.

Copy numbers of the vertebrate gene, EBF3N, were consistently low across all participant facilities (Figure 2). The swab samples we collected from *L. caerulea* and eDNA filters from *X. laevis*-bearing water yielded similarly low numbers of EBF3N copies (Figure 2). There was no obvious pattern of higher or lower copy number across sample type, species, or between participants, nor with the number of swabs in a pool or volume of filtered

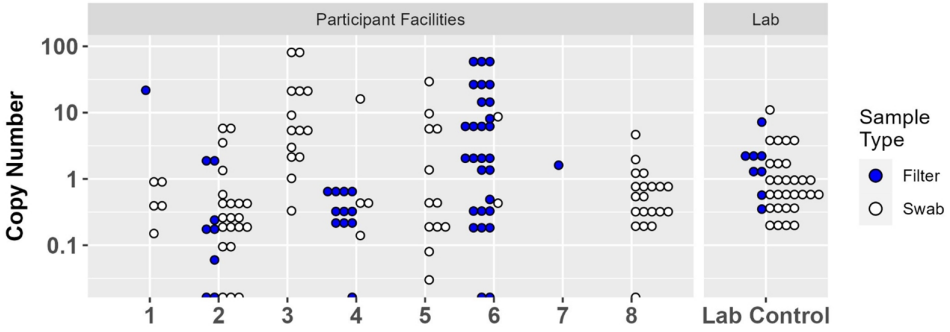

FIGURE 2 Vertebrate DNA content of surveillance samples as measured by a quantitative real-time polymerase chain reaction assay for the ultra-conserved EBF3N gene. Each dot represents a single filter or a pool of between 1 and 5 swabs. Laboratory filter samples were collected from 5-L enclosures containing 15 *Xenopus laevis* tadpoles. Laboratory swab samples were collected from *Litoria caerulea* and extracted in isolation (pool size = 1). Dots along the abscissa were negative.

water (corrected for animal density; Appendix S1: Figures S2–S4).

DISCUSSION

Amphibian pathogens such as *Rv*, *Bd*, and *Bsal* are transported regionally and internationally via the trade of live amphibians (Nguyen et al., 2017; Peel et al., 2012; Picco & Collins, 2008; Schloegel et al., 2009; Wombwell et al., 2016). While the prevalence of these pathogens has been investigated at large, international ports (Kolby et al., 2014; Martel et al., 2014; Wombwell et al., 2016), comparatively little is known about how these pathogens spread and persist upon entering domestic trade networks (dominated by pet amphibian sales in the United States; Mohanty & Measey, 2019). Identifying which pathogens are most common and what factors (e.g., common species, husbandry practices, biosecurity) amplify their prevalence would lay essential groundwork for developing evidence-based guidance aimed at reducing disease-related economic losses in the trade and protecting wild populations from potential pathogen spillover (Olson et al., 2024). We believe that our unique approach to pathogen surveillance, which encourages trust through participant anonymity, could help lay this groundwork.

We found that there was a general willingness to participate in our pathogen surveillance program. Approximately 22% (26 of 120) of businesses responding to a socioeconomic survey indicated a willingness to participate in pathogen surveillance, and of the subset of 14 businesses that were shipped supplies, 57% (8 of 14) returned samples. While our sample of businesses in this pilot study was small, it is noteworthy that two pathogens of concern—*Bsal* and *Rv*—were entirely absent. Even *Bd*, which was detected in two out of eight participant facilities, was of relatively low prevalence among habitats, occurring in 14% (2 of 14) of habitats in one facility and 3% (1 of 30) in the other. Perhaps, the biosecurity practices within these facilities reduce or even eliminate pathogens before they have an opportunity to spread and persist among habitats. Indeed, both participants in whose facilities *Bd* was detected self-reported that they quarantine all new acquisitions and change gloves between habitats containing separate species during handling and cleaning (Appendix S1: Table S3). While this pilot study ultimately lacks the scope to demonstrate strong correlations between specific participant practices and pathogen prevalence in pet trade facilities, it does provide a framework for acquiring such data that can be employed in future work of a larger scale (https://www.healthyamphibiantrade.org).

It is also worth noting that respondents to the recruitment survey generally reported a high degree of prior knowledge regarding amphibian disease as well as an interest in preventing the spread of pathogens within the trade (Cavasos et al., 2023). While participant selection for the pilot study was blind to these aspects of the survey, it is unclear whether this knowledge is representative of businesses in the US pet amphibian trade, and whether this knowledge influenced pathogen detection or prevalence estimates. It is possible that more knowledgeable businesses monitor health more closely or are more likely to incorporate biosecurity practices that reduce the likelihood of pathogen persistence in captivity. Future efforts to estimate pathogen prevalence in the United States and other trade networks should consider expanding the participant pool by advertising more broadly, such as at trade shows or through social media.

In addition to assessing participant engagement, the second objective of this pilot study was to gauge the quality of returned samples by testing for the presence of a highly conserved vertebrate gene (EBF3N) via qPCR. Our assumption was that higher copy numbers of this gene would correspond to better sampling technique among participants. Our results show that the quantity of EBF3N collected from both swabs and eDNA filters was generally low (<100 copies) and highly variable (orders of magnitude within a business), but these values were similar to samples collected in the laboratory, suggesting that participant technique was broadly comparable to that of researchers. The consistently low quantities of EBF3N observed in our results may be due to the fact that EBF3N is a single-copy nuclear gene. If animals were not actively shedding, few epithelial cells may have been deposited on swabs. In aquatic settings, water turnover or filtration may limit the concentration of suspended cells. Future work aimed at sample validation should favor highly conserved vertebrate gene targets with multiple copies in order to improve detection sensitivity. In any case, the detection of *Bd* in three samples from two businesses further suggests participants were able to effectively sample for pathogens.

In conclusion, this pilot study provides compelling evidence that our methods of anonymous pathogen surveillance in the US pet amphibian trade network are an effective, scalable platform for future work. Importantly, our results demonstrate an actionable interest in pathogen surveillance among US businesses, and that DNA samples collected directly by business staff are of an adequate quality to detect pathogens.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Pearhill et al., 2024) are available from Figshare: https://doi.org/10.6084/m9.figshare.25721169.v3.

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REFERENCES

Basanta, M. D., V. Avila-Akerberg, A. Byrne, G. Castellanos-Morales, T. González-Martínez, Y. Maldonado-López, E. Rosenblum, I. Suazo-Ortuño, G. Parra-Olea, and E. Rebollar. 2022. "The Fungal Pathogen *Batrachochytrium salamandrivorans* Is Not Detected in Wild and Captive Amphibians from Mexico." *PeerJ* 10: e14117. https://doi.org/10.7717/peerj.14117.

Becker, M. H., R. M. Brucker, C. R. Schwantes, R. N. Harris, and K. P. C. Minbiole. 2009. "The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus." *Applied and Environmental Microbiology* 75: 6635–38. https://doi.org/10.1128/AEM.01294-09.

Bletz, M. 2013. "Probiotic Bioaugmentation of an Anti-*Bd* Bacteria, *Janthinobacterium lividum*, on the Amphibian, *Notophthalmus viridescens*: Transmission Efficacy and Persistence of the Probiotic on the Host and Non-Target Effects of Probiotic Addition on Ecosystem Components." Masters theses, James Madison University, 153. https://commons.lib.jmu.edu/master201019/153.

Blooi, M., F. Pasmans, J. E. Longcore, A. Spitzen-van Der Sluijs, F. Vercammen, and A. Martel. 2013. "Duplex Real-Time PCR for Rapid Simultaneous Detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in Amphibian Samples." *Journal of Clinical Microbiology* 51: 4173–77. https://doi.org/10.1128/JCM.02313-13.

Borzée, A., T. A. Kosch, M. Kim, and Y. Jang. 2017. "Introduced Bullfrogs Are Associated with Increased *Batrachochytrium dendrobatidis* Prevalence and Reduced Occurrence of Korean Treefrogs." *PLoS One* 12: e0177860. https://doi.org/10.1371/journal.pone.0177860.

Boyle, D. G., D. B. Boyle, V. Olsen, J. Morgan, and A. Hyatt. 2004. "Rapid Quantitative Detection of Chytridiomycosis (*Batrachochytrium dendrobatidis*) in Amphibian Samples Using Real-Time Taqman PCR Assay." *Diseases of Aquatic Organisms* 60: 141–48. https://doi.org/10.3354/dao060141.

Cavasos, K., N. C. Poudyal, J. L. Brunner, A. R. Warwick, J. Jones, N. Moherman, M. George, J. D. Willard, Z. T. Brinks, and M. J. Gray. 2023. "Exploring Business Stakeholder Engagement in Sustainable Business Practices: Evidence from the US Pet Amphibian Industry." *Business Strategy and the Environment* 32: 5909–21. https://doi.org/10.1002/bse.3455.

Connelly, P. J., N. Ross, O. C. Stringham, and E. A. Eskew. 2023. "United States Amphibian Imports Pose a Disease Risk to Salamanders despite Lacey Act Regulations." *Communications Earth & Environment* 4: 1–10. https://doi.org/10.1038/s43247-023-01004-z.

Fisher, M. C., and T. W. J. Garner. 2007. "The Relationship between the Emergence of *Batrachochytrium dendrobatidis*, the International Trade in Amphibians and Introduced Amphibian Species." *Fungal Biology Reviews* 21: 2–9. https://doi.org/10.1016/j.fbr.2007.02.002.

Fitzpatrick, L. D., F. Pasmans, A. Martel, and A. A. Cunningham. 2018. "Epidemiological Tracing of *Batrachochytrium salamandrivorans* Identifies Widespread Infection and Associated Mortalities in Private Amphibian Collections." *Scientific Reports* 8: 13845. https://doi.org/10.1038/s41598-018-31800-z.

Garner, T. W. J., M. W. Perkins, P. Govindarajulu, D. Seglie, S. Walker, A. A. Cunningham, and M. C. Fisher. 2006. "The Emerging Amphibian Pathogen *Batrachochytrium dendrobatidis* Globally Infects Introduced Populations of the North American Bullfrog, *Rana catesbeiana*." *Biology Letters* 2: 455–59. https://doi.org/10.1098/rsbl.2006.0494.

Karesh, W. B., R. A. Cook, E. L. Bennett, and J. Newcomb. 2005. "Wildlife Trade and Global Disease Emergence." *Emerging Infectious Diseases* 11: 1000–1002. https://doi.org/10.3201/eid1107.050194.

Klocke, B., M. Becker, J. Lewis, R. C. Fleischer, C. R. Muletz-Wolz, L. Rockwood, A. A. Aguirre, and B. Gratwicke. 2017. "*Batrachochytrium salamandrivorans* Not Detected in U.S. Survey of Pet Salamanders." *Scientific Reports* 7: 13132. https://doi.org/10.1038/s41598-017-13500-2.

Kolby, J. E., K. M. Smith, L. Berger, W. B. Karesh, A. Preston, A. P. Pessier, and L. F. Skerratt. 2014. "First Evidence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and *Ranavirus* in Hong Kong Amphibian Trade." *PLoS One* 9: e90750. https://doi.org/10.1371/journal.pone.0090750.

Kueneman, J. G., D. C. Woodhams, R. Harris, H. M. Archer, R. Knight, and V. J. McKenzie. 2016. "Probiotic Treatment Restores Protection against Lethal Fungal Infection Lost during Amphibian Captivity." *Proceedings of the Royal Society B: Biological Sciences* 283: 20161553. https://doi.org/10.1098/rspb.2016.1553.

Lastra González, D., V. Baláž, P. Chajma, and J. Vojar. 2020. "Surveying for *Batrachochytrium salamandrivorans* Presence in Spanish Captive Collections of Amphibians." *Diseases of Aquatic Organisms* 142: 99–103. https://doi.org/10.3354/dao03535.

Leung, W. T. M., L. Thomas-Walters, T. W. J. Garner, F. Balloux, C. Durrant, and S. J. Price. 2017. "A Quantitative-PCR Based Method to Estimate *Ranavirus* Viral Load Following Normalisation by Reference to an Ultraconserved Vertebrate Target." *Journal of Virological Methods* 249: 147–155. https://doi.org/10.1016/j.jviromet.2017.08.016.

Martel, A., M. Blooi, C. Adriaensen, P. Van Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, et al. 2014. "Recent Introduction of a Chytrid Fungus Endangers Western Palearctic

Salamanders." *Science* 346: 630–31. https://doi.org/10.1126/science.1258268.

Mohanty, N., and J. Measey. 2019. "The Global Pet Trade in Amphibians: Species Traits, Taxonomic Bias, and Future Directions." *Biodiversity and Conservation* 28: 3915–23. https://doi.org/10.1007/s10531-019-01857-x.

Nguyen, T. T., T. V. Nguyen, T. Ziegler, F. Pasmans, and A. Martel. 2017. "Trade in Wild Anurans Vectors the Urodelan Pathogen *Batrachochytrium salamandrivorans* into Europe." *Amphibia-Reptilia* 38: 554–56. https://doi.org/10.1163/15685381-00003125.

Olson, D. H., E. H. C. Grant, M. Bletz, J. Piovia-Scott, D. Lesbarrères, J. L. Kerby, M. J. Adams, et al. 2024. "Preparing for a *Bsal* Invasion into North America Has Improved Multi-Sector Readiness." *Frontiers in Amphibian and Reptile Science* 2: 1347541. https://doi.org/10.3389/famrs.2024.1347541.

Pearhill, R., M. J. Gray, J. Jones, Z. Brinks, and J. L. Brunner. 2024. "Microbe_surveillance_in_the_amphibian_pet_trade_results_from_a_pilot_study.xlsx." Figshare. Dataset. https://doi.org/10.6084/m9.figshare.25721169.v3.

Peel, A. J., M. Hartley, and A. A. Cunningham. 2012. "Qualitative Risk Analysis of Introducing *Batrachochytrium dendrobatidis* to the UK through the Importation of Live Amphibians." *Diseases of Aquatic Organisms* 98: 95–112. https://doi.org/10.3354/dao02424.

Picco, A. M., and J. P. Collins. 2008. "Amphibian Commerce as a Likely Source of Pathogen Pollution." *Conservation Biology* 22: 1582–89. https://doi.org/10.1111/j.1523-1739.2008.01025.x.

Ribeiro, L. P., T. Carvalho, C. G. Becker, T. S. Jenkinson, D. da Silva Leite, T. Y. James, S. E. Greenspan, and L. F. Toledo. 2019. "Bullfrog Farms Release Virulent Zoospores of the Frog-Killing Fungus into the Natural Environment." *Scientific Reports* 9: 13422. https://doi.org/10.1038/s41598-019-49674-0.

Schloegel, L. M., A. M. Picco, A. M. Kilpatrick, A. J. Davies, A. D. Hyatt, and P. Daszak. 2009. "Magnitude of the US Trade in Amphibians and Presence of *Batrachochytrium dendrobatidis* and *Ranavirus* Infection in Imported North American Bullfrogs (*Rana catesbeiana*)." *Biological Conservation* 142: 1420–26. https://doi.org/10.1016/j.biocon.2009.02.007.

Sharifian-Fard, M., F. Pasmans, C. Adriaensen, S. Devisscher, T. Adriaens, G. Louette, and A. Martel. 2011. "Ranavirosis in Invasive Bullfrogs, Belgium." *Emerging Infectious Diseases* 17: 2371–72. https://doi.org/10.3201/eid1712.110236.

Spitzen-van der Sluijs, A., A. Martel, J. Asselberghs, E. K. Bales, W. Beukema, M. C. Bletz, L. Dalbeck, et al. 2016. "Expanding Distribution of Lethal Amphibian Fungus *Batrachochytrium salamandrivorans* in Europe." *Emerging Infectious Diseases* 22: 1286–88. https://doi.org/10.3201/eid2207.160109.

Stallins, J. A., and L. Kelley. 2013. "The Embeddedness of a North American Snake in the Wildlife Pet Trade and the Production of Assemblage Biogeographies." *Annals of the Association of American Geographers* 103: 417–436. https://doi.org/10.1080/00045608.2013.765770.

Stegen, G., F. Pasmans, B. R. Schmidt, L. O. Rouffaer, S. Van Praet, M. Schaub, S. Canessa, et al. 2017. "Drivers of Salamander Extirpation Mediated by *Batrachochytrium salamandrivorans*." *Nature* 544: 353–56. https://doi.org/10.1038/nature22059.

Stilwell, N. K., R. J. Whittington, P. M. Hick, J. A. Becker, E. Ariel, S. Van Beurden, N. Vendramin, N. J. Olesen, and T. B. Waltzek. 2018. "Partial Validation of a TaqMan Real-Time Quantitative PCR for the Detection of *Ranaviruses*." *Diseases of Aquatic Organisms* 128: 105–116. https://doi.org/10.3354/dao03214.

Une, Y., A. Sakuma, H. Matsueda, K. Nakai, and M. Murakami. 2009. "*Ranavirus* Outbreak in North American Bullfrogs (*Rana catesbeiana*), Japan, 2008." *Emerging Infectious Diseases* 15: 1146–47. https://doi.org/10.3201/eid1507.081636.

Waddle, J. H., D. A. Grear, B. A. Mosher, E. H. Campbell Grant, M. J. Adams, A. R. Backlin, W. J. Barichivich, et al. 2020. "*Batrachochytrium salamandrivorans* (*Bsal*) Not Detected in an Intensive Survey of Wild North American Amphibians." *Scientific Reports* 10: 13012. https://doi.org/10.1038/s41598-020-69486-x.

Wombwell, E. L., T. W. J. Garner, A. A. Cunningham, R. Quest, S. Pritchard, J. M. Rowcliffe, and R. A. Griffiths. 2016. "Detection of *Batrachochytrium dendrobatidis* in Amphibians Imported into the UK for the Pet Trade." *EcoHealth* 13: 456–466. https://doi.org/10.1007/s10393-016-1138-4.

Yap, T. A., M. S. Koo, R. F. Ambrose, and V. T. Vredenburg. 2018. "Introduced Bullfrog Facilitates Pathogen Invasion in the Western United States." *PLoS One* 13: e0188384. https://doi.org/10.1371/journal.pone.0188384.

Yuan, Z., A. Martel, J. Wu, S. Van Praet, S. Canessa, and F. Pasmans. 2018. "Widespread Occurrence of an Emerging Fungal Pathogen in Heavily Traded Chinese Urodelan Species." *Conservation Letters* 11: e12436. https://doi.org/10.1111/conl.12436.

SUPPORTING INFORMATION

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

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