DOI: 10.7589/JWD-D-20-00214

Journal of Wildlife Diseases, 57(4), 2021, pp. 942–948

Batrachochytrium salamandrivorans Can Devour More than Salamanders

Anastasia E. Towe,[1,2] Matthew J. Gray,[1] Edward Davis Carter,[1] Mark Q. Wilber,[1] Robert J. Ossiboff,[3] Kurt Ash,[1] Markese Bohanon,[1] Brittany A. Bajo,[1] and Debra L. Miller[1,2,4] [1]Center for Wildlife Health, University of Tennessee Institute of Agriculture, 427 Plant Biotech Building, 2505 E. J. Chapman Drive, Knoxville, Tennessee 37996, USA; [2]Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, Tennessee 37996, USA; [3]Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, 1945 SW 16th Avenue, Gainesville, Florida 32608, USA; [4]Corresponding author (email: dmille42@utk.edu)

ABSTRACT: *Batrachochytrium salamandrivorans* is an emerging fungus that is causing salamander declines in Europe. We evaluated whether an invasive frog species (Cuban treefrog, *Osteopilus septentrionalis*) that is found in international trade could be an asymptomatic carrier when exposed to zoospore doses known to infect salamanders. We discovered that Cuban treefrogs could be infected with *B. salamandrivorans* and, surprisingly, that chytridiomycosis developed in animals at the two highest zoospore doses. To fulfill Koch's postulates, we isolated *B. salamandrivorans* from infected frogs, exposed eastern newts (*Notophthalmus viridescens*) to the isolate, and verified infection and disease by histopathology. This experiment represents the first documentation of *B. salamandrivorans* chytridiomycosis in a frog species and substantially expands the conservation threat and possible mobilization of this pathogen in trade.

Key words: Amphibian, *Batrachochytrium salamandrivorans*, Chytridiomycosis, Cuban treefrog, eastern newt, *Notophthalmus viridescens*, *Osteopilus septentrionalis*.

The chytrid fungi *Batrachochytrium salamandrivorans* (*Bsal*) and *Batrachochytrium dendrobatidis* (*Bd*) cause chytridiomycosis, an often fatal skin disease that has been associated with the decline of more than 500 amphibian species worldwide (Scheele et al. 2019). Though *Bsal* has been isolated from wild anurans in Vietnam, where the fungus is thought to have originated (Nguyen et al. 2017), clinical chytridiomycosis caused by *Bsal* has previously been observed exclusively in urodele amphibians (i.e., salamanders; Martel et al. 2014; Stegen et al. 2017). For example, experimental exposures to *Bsal* showed that a European frog species (*Alytes obstetricans*) could be an asymptomatic carrier but did not develop chytridiomycosis (Stegen et al. 2017). To evaluate whether North American frogs could become infected with *Bsal*, we challenged adult wild-caught *Osteopilus septentrionalis* (Cuban treefrogs) with zoospore doses known to infect salamanders (Carter et al. 2020) and used a multimodal diagnostic approach to determine their susceptibility to infection. This frog is an invasive species in at least four states in the US and 14 countries (Cottrell and Ventosa 2018) and is commonly found in the pet trade (Global Invasive Species Database 2008; Hedges et al. 2019); hence, it could play a role in global dissemination of *Bsal*. All procedures followed approved University of Tennessee Institutional Animal Care and Use Committee protocol no. 2395.

Frogs were captured in the wild in Florida, US, and transported to the University of Tennessee, Knoxville, US. They were housed individually at 30 C for 10 d to clear any preexisting *Bd* infections (Chatfield and Richards-Zawacki 2011) then acclimated over 2 wk to 15 C, the approximate optimal growth temperature for *Bsal* (Martel et al. 2013). We assume that stress associated with heat treatment was negligible, because ambient temperatures of 30 C or greater occur in the native subtropical distribution of Cuban treefrogs and in Florida.

Zoospores used for exposure were harvested from tryptone gelatin hydrolysate agar plates after 6 d of growth. Plates were flooded with 7 mL of autoclaved dechlorinated water and filtered with a 20-μm sieve to isolate zoospores. Zoospores were enumerated with a hemocytometer and verified by flow cytometry. We exposed four animals per treatment

group in 100-mL plastic cylindrical containers with 9 mL of autoclaved dechlorinated water and 1 mL of the randomly assigned *Bsal* zoospore dose (5×10^3, 5×10^4, 5×10^5, or 5×10^6). Two control animals were treated identically but exposed to 10 mL of autoclaved dechlorinated water. After 24 h, we removed frogs from the inoculation tubes and placed them in individual 710-cm^3 vivariums containing a moist paper towel and polyvinyl chloride cover object. We fed animals crickets (2% of their body mass daily) and replaced each animal's container, cover object, and paper towel every 3 d. Animals were housed in environmental chambers on a 12-h light, 12-h dark cycle, at 15 C and >90% humidity.

We monitored twice daily for signs of *Bsal* chytridiomycosis, including lethargy, focal lesions, ulcerations, increased skin sloughing, hemorrhage, anorexia, and loss of righting response (Martel et al. 2013; Carter et al. 2020). Animals were humanely euthanatized when they lost righting ability and recorded as a mortality event for data analyses. We swabbed each animal every 6 d, starting 4 d postexposure to *Bsal*, using standardized swabbing protocols for *Bd* and *Bsal* (Blooi et al. 2013). We extracted genomic DNA from each swab with Qiagen DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany). To detect the presence and estimate the quantity of *Bsal* DNA on each swab, we performed quantitative PCR with an Applied Biosystems QuantStudio 6 Flex (Thermo Fisher Scientific, Waltham, Massachusetts, USA) quantitative PCR instrument (Blooi et al. 2013). We ran all swab samples in duplicate and considered a sample positive if both replicates reached cycle threshold before 50 cycles. We used a standard curve of synthetic *Bsal* DNA (gBlock) to estimate the number of *Bsal* zoospore copies per microliter in each sample. Swabs collected at the first timepoint after *Bsal* exposure and at postmortem examination were also tested for *Bd* DNA.

We confirmed *Bsal* colonization in histologic cross sections of epidermal tissues stained with H&E. We used RNAscope® (Advanced Cell Diagnostics, Newark, California, USA) in situ hybridization staining to evaluate and highlight the distribution of *Bsal* zoosporangia in infected tissues and verify that frogs were *Bd*-negative (Ossiboff et al. 2019). Although it is possible that the frogs had exposure to *Bd* in the wild, there is no evidence that prior *Bd* exposure affects susceptibility of hosts to *Bsal* infection (Longo et al. 2019; Greener et al. 2020). To demonstrate that *Bsal* was the causative agent of clinical signs in exposed frogs, we reisolated and cultured *Bsal* from infected toe tissue collected from a diseased frog (Martel et al. 2013). After obtaining a pure *Bsal* culture verified by quantitative PCR and microscopically, we grew, harvested, and enumerated *Bsal* zoospores and exposed adult eastern newts (*Notophthalmus viridescens*) to 5×10^6 zoospores from the reisolated culture with the same methods as for the frog experiment. Because adult newts are aquatic, we housed them individually in 2-L plastic containers holding 300 mL of water and a polyvinyl chloride cover object (Malagon et al. 2020). Monitoring, cleaning, and feeding was on the same schedule as described for the frogs.

All statistical analyses were performed in RStudio version 3.5.3 (R Core Team 2020). We analyzed the survival data shown by performing Kaplan-Meier survival analysis and Cox proportional hazard models with the *survival* package (Goel et al. 2010). To compare food consumption among zoospore doses, we fit a binomial generalized linear mixed effects model with a logit link function. We included fixed effects for number of days postexposure, treatment (five levels: control, 5×10^3, 5×10^4, 5×10^5, and 5×10^6), and the interaction between days postexposure and treatment. We included a random effect of individual, and we allowed both the intercept and the effect of days postexposure (i.e., the slope) to vary by individual. We fit the model with the *brms* packages in R with uninformative priors (Bürkner 2018) and used the posterior predictions from the model to test whether percent food consumption differed between the control group and the four dose treatments after 40 d postexposure. We performed an analysis of variance comparing log-transformed *Bsal* zoospore genomic

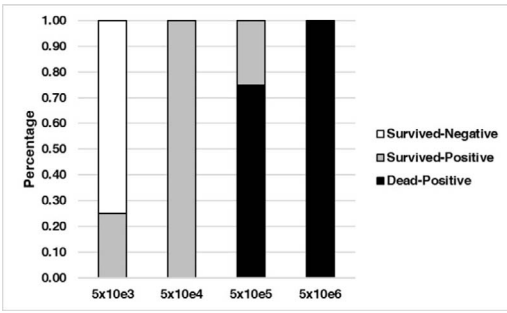

FIGURE 1. Percentage of Cuban treefrogs (*Osteopilus septentrionalis*) in each final mortality-infection category after exposure to five levels of *Batrachochytrium salamandrivorans* (*Bsal*) zoospores. Animals were designated to each category according to whether the animal died or was euthanatized because of clinical signs of disease (i.e., loss of righting ability) before 75 d postexposure and whether their final postmortem swab was positive for *Bsal* DNA by quantitative PCR following methods outlined in Carter et al. (2020). Categories shown include animals that survived and were negative (Survived-Negative, white), those that survived and were positive (Survived-Positive, gray), and animals that died and were positive (Dead-Positive, black). Each treatment group consisted of four animals. The two control frogs, not shown, survived and were negative for *Bsal*.

equivalents per microliter postmortem among exposure doses. When the analysis of variance was significant (α=0.05), post hoc Bonferroni corrected *t*-tests were performed to evaluate pairwise differences in *Bsal* zoospore load.

The skin of all frogs tested positive for *Bsal* DNA after exposure at the three highest doses and contained high genomic copies (Figs. 1, 2). All animals were negative for *Bd* DNA. The frogs experienced dose-dependent survival; individuals exposed to 5×10^5 and 5×10^6 *Bsal* zoospores showed 75% and 100% mortality, respectively (Fig. 3). No frogs in the lowest two doses or controls died by the end of the 75-d experiment. Median survival duration for 5×10^5-exposed frogs was 47 d postexposure (range 46–75 d); median survival duration for 5×10^6-exposed frogs was only 25 d (range 15–45 d). Median survival duration of frogs exposed to 5×10^6 *Bsal* zoospores was similar to frogs exposed to 10^6 *Bd* zoospores at the same temperature (Raffel et al. 2013).

Infected Cuban treefrogs developed erythema and hemorrhage on their feet and ventrums and excessive shedding on their feet (Fig. 4A). On the dorsum, spots of darkened pigmentation developed that progressed to hemorrhages (Fig. 4B). Histologic examination in conjunction with in situ hybridization (Ossiboff et al. 2019) revealed multiple variably sized, necrotizing, crater-like epidermal lesions randomly distributed throughout the body and containing numerous intralesional *Bsal* thalli (Fig. 4C, D). These gross and histologic findings are consistent with *Bsal* chytridiomycosis (Thomas et al. 2018). All animals were confirmed negative for *Bd* chytridiomycosis with in situ hybridization. Infected frogs consumed less invertebrate prey as disease progressed (Fig. 5); median food consumption for animals exposed to 5×10^3, 5×10^4, 5×10^5, and 5×10^6, 40 d after exposure, decreased by 54% (95% confidence interval [CI], 14–81), 51% (95% CI, 8–80), 50% (95% CI, 7–81), and 92% (95% CI, 60–99), respectively, compared with control animals. Anorexia has been associated with amphibian chytridiomycosis caused by both *Bd* and *Bsal* (Martel et al. 2013; Van Rooij et al. 2015).

Zoosporangia of *Bsal* were successfully reisolated from toe tissue collected from a morbid 5×10^5-exposed frog. In cell culture, we observed formation of zoosporangia and motile spores (Supplementary Material Videos S1, S2) diagnostic of chytrid fungi (Van Rooij et al. 2015). The isolate also tested positive for *Bsal* DNA by quantitative PCR, and exposure to 5×10^6 zoospores caused 100% infection and mortality in <10 d in eastern newts, a species susceptible to *Bsal* (Longo et al. 2019). Histopathology and in situ hybridization (Fig. 4E, F) confirmed *Bsal* chytridiomycosis in the newts, fulfilling Koch's postulates.

Our results demonstrate that *Bsal* chytridiomycosis is not limited to urodele host species. Therefore, current amphibian import bans focusing largely on stopping the trade of urodele species may be insufficient to prevent introduction of *Bsal* into the US and elsewhere. Because anurans constitute 99% of global amphibian trade (Can et al. 2019), outright trade bans to prevent possible entry

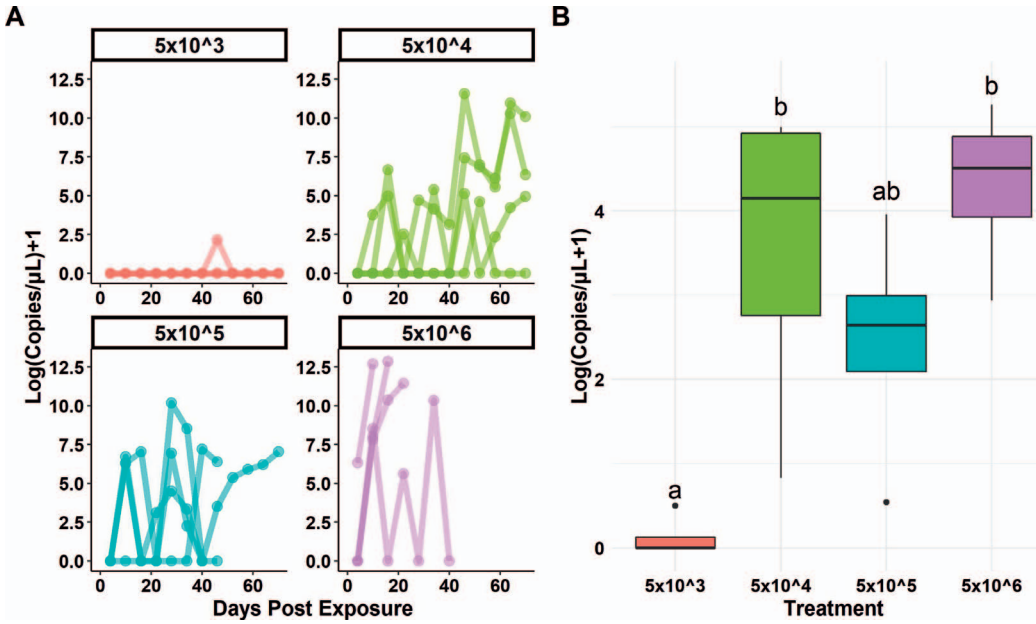

FIGURE 2. (A) Individual *Batrachochytrium salamandrivorans* (*Bsal*) load trajectories during the experiment and (B) mean *Bsal* load (copies/µL) at postmortem examination on adult Cuban treefrogs (*Osteopilus septentrionalis*) skin collected from swabs and estimated with quantitative PCR after exposure to *Bsal* zoospores. Exposure doses of *Bsal* were 5×10^3 (red), 5×10^4 (green), 5×10^5 (blue), and 5×10^6 (purple) zoospores. Unlike letters above box plots indicate significant differences by post hoc Bonferroni-adjusted pairwise *t*-tests; dots indicate outlying data points.

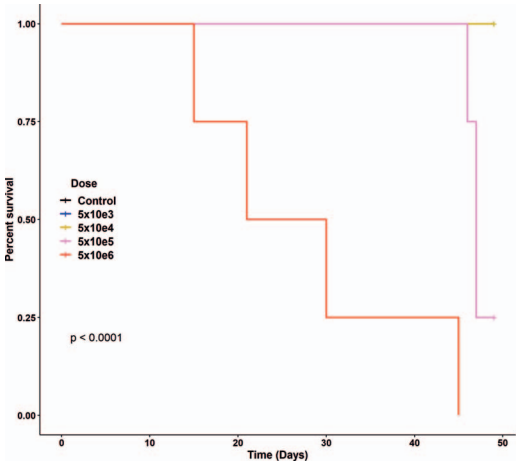

FIGURE 3. Kaplan-Meier survival curves of adult Cuban treefrogs (*Osteopilus septentrionalis*) used as controls (black; 100% survival, not visible) or exposed to 5×10^3 (blue; 100% survival, not visible), 5×10^4 (yellow), 5×10^5 (pink), or 5×10^6 (red) *Batrachochytrium salamandrivorans* zoospores. Rates of mortality differed among exposure doses by a log-rank significance test (n=4 per exposure dose and n=2 controls).

of infected amphibians into *Bsal*-free nations could be met with significant resistance by industry. We suggest as an alternative that animal health certifications or other programs that support sustainable clean trade be considered as intervention strategies. Furthermore, our results show that species in the most diverse anuran family, Hylidae (IUCN 2019), that have experienced multiple species extinctions because of *Bd* (Scheele et al. 2019), may be susceptible to *Bsal*, as well. Additional research into the susceptibility of more anuran species is warranted to formulate a complete picture of the threat *Bsal* poses to amphibian species worldwide.

The authors thank Peter Iacono, Eric Suarez, and Brooke Talley of the Florida Fish and Wildlife Conservation Commission for collecting the Cuban treefrogs used in this study. The University of Tennessee (UT) and University of Florida histology laboratories assisted with slide preparations, and Melissa Brown helped with in situ hybridization. We thank Bobby Simpson and Alex Anderson of

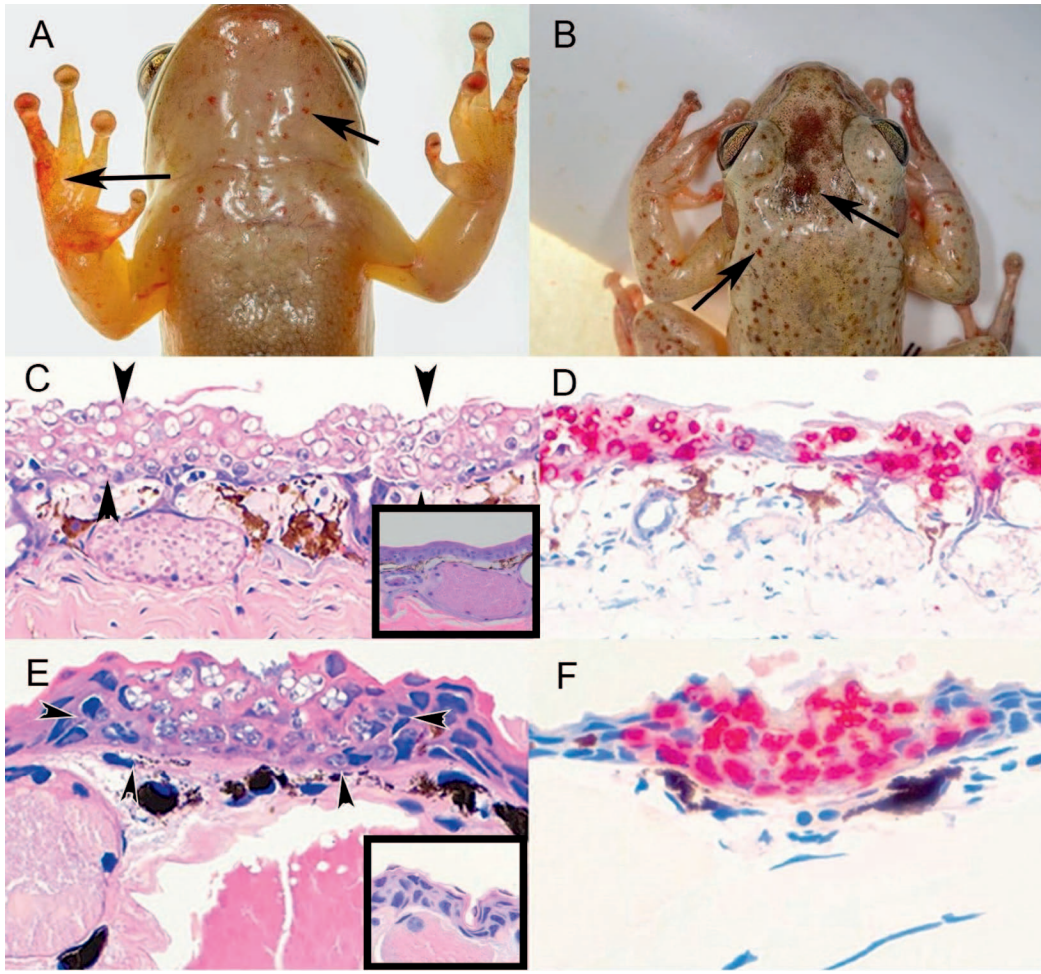

FIGURE 4. (A–D) *Batrachochytrium salamandrivorans* (*Bsal*) chytridiomycosis in adult Cuban treefrogs (*Osteopilus septentrionalis*). Gross lesions included hemorrhages (arrows; A) and dark brown or black pigmentation (arrows; B) within the skin. Microscopically, the gross lesions revealed multifocal to diffuse destruction of the epidermis by *Bsal* organisms (arrowheads; C). A normal section of skin from a control frog is shown for comparison (inset; C). *Bsal* organisms were confirmed by RNAscope (red staining; D) following Ossiboff et al. (2019). To satisfy Koch's postulates, the fungus was isolated from the lesions of the frogs, cultured, and exposed to adult eastern newts, *Notophthalmus viridescens*. Exposed newts developed multifocal craterlike (arrowheads; E) to diffuse *Bsal* chytridiomycosis similar to that seen in the frogs. A normal section of skin from a control newt is shown for comparison (inset; E). *Bsal* chytridiomycosis was confirmed by RNAscope (red staining; F).

the UT Institute of Agriculture East Tennessee Research and Education Center for laboratory and logistic support. We also thank Bailee Augustino for assistance with animal care and data collection. This work was partially supported by the National Science Foundation Division of Environmental Biology (EEID grant 1814520) and US Department of Agriculture National Institute of Food and Agriculture (Hatch Project 1012932) awarded to M.J.G. and D.L.M. The UT College of Veterinary Medicine Center of Excellence program provided salary support to A.E.T. Authors declare no competing interests. All data are available in the main text. Code for statistical analyses is available in the public repository, TRACE (https://trace.tennessee.edu).

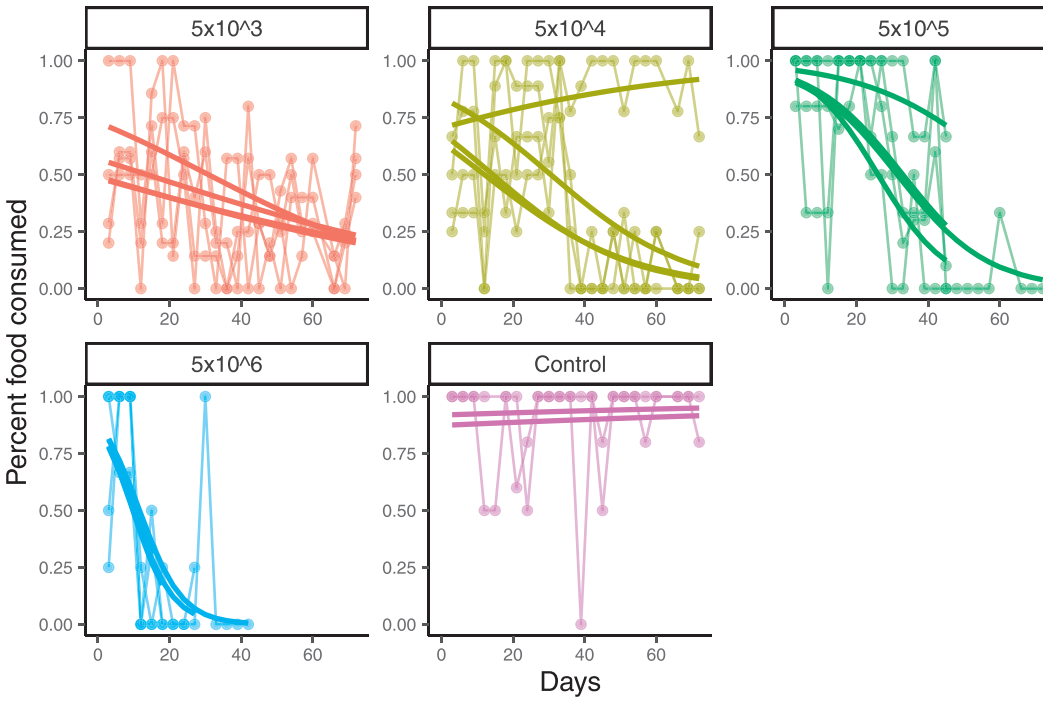

FIGURE 5. Percentage of invertebrates consumed by adult Cuban treefrogs (*Osteopilus septentrionalis*) over 3-d feeding periods after various *Batrachochytrium salamandrivorans* zoospore exposure doses. Points and thin lines represent the observed percentage of food consumed by each individual over time. Thick-colored lines give the mean predictions of the percent invertebrates consumed for each individual frog according to a generalized linear mixed effects model. Confidence bands about individual predictions are not shown for clarity. Colors correspond to zoospore doses. The experiment duration was 75 d.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-20-00214.

LITERATURE CITED

Blooi M, Pasmans F, Longcore JE, Spitzen-van der Sluijs A, Vercammen F, Martel A. 2013. Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. *J Clin Microbiol* 51:4173–4177.

Bürkner PC. 2018. Advanced Bayesian multilevel modeling with the R package brms. *R J* 10:395–411.

Can ÖE, D'Cruze N, Macdonald DW. 2019. Dealing in deadly pathogens: Taking stock of the legal trade in live wildlife and potential risks to human health. *Glob Ecol Conserv* 17:e00515.

Carter ED, Miller DL, Peterson AC, Sutton WB, Cusaac JPW, Spatz JA, Rollins-Smith L, Reinert L, Bohanon M, Williams LA, et al. 2020. Conservation risk of *Batrachochytrium salamandrivorans* to endemic lungless salamanders. *Conserv Lett* 13:e12675.

Chatfield MWH, Richards-Zawacki CL. 2011. Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Dis Aquat Organ* 94:235–238.

Cottrell V, Ventosa E. 2018. Osteopilus septentrionalis *(Cuban treefrog)*. https://www.cabi.org/isc/datasheet/71203. Accessed October 2019.

Global Invasive Species Database. 2008. *Species profile:* Osteopilus septentrionalis. http://www.iucngisd.org/gisd/species.php?sc=1261. Accessed September 2020.

Goel M, Khanna P, Kishore J. 2010. Understanding survival analysis: Kaplan-Meier estimate. *Int J Ayurveda Res* 1:212–216.

Greener MS, Verbrugghe E, Kelly M, Blooi M, Beukema W, Canessa S, Carranza S, Croubels S, De Troyer N, Fernandez-Giberteau D, et al. 2020. Presence of low virulence chytrid fungi could protect European amphibians from more deadly strains. *Nat Commun* 11:5393.

Hedges B, Díaz L, Ibéné B, Joglar R, Powell R, Bolaños F, Chaves G. 2019. Osteopilus septentrionalis *(Cuban treefrog)*. https://www.iucnredlist.org/fr/species/55811/11368202. Accessed September 2020.

IUCN (International Union for Conservation of Nature). 2019. *The IUCN red list of threatened species*. 2019-1. https://www.iucnredlist.org. Accessed October 2019.

Longo AV, Fleischer RC, Lips KR. 2019. Double trouble: Co-infections of chytrid fungi will severely impact widely distributed newts. *Biol Invasions* 21:2233–2245.

Malagon DA, Melara LA, Prosper OF, Lenhart S, Carter ED, Fordyce JA, Peterson AC, Miller DL, Gray MJ. 2020. Host density and habitat structure influence host contact rates and *Batrachochytrium salamandrivorans* transmission. *Sci Rep* 10:5584.

Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, Farrer RA, Schmidt BR, Tobler U, Goka K, et al. 2014. Recent introduction of a chytrid fungus endangers western Palearctic salamanders. *Science* 346:630–631.

Martel A, Spitzen-van der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, Woeltjes A, Bosman W, Chiers K, Bossuyt F, et al. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proc Natl Acad Sci U S A* 110: 15325–15329.

Nguyen TT, Nguyen TV, Ziegler T, Pasmans F, Martel A. 2017. Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. *Amphib Reptil* 38:554–556.

Ossiboff RJ, Towe AE, Brown MA, Longo AV, Lips KR, Miller DL, Carter ED, Gray MJ, Frasca S Jr. 2019. Differentiating *Batrachochytrium dendrobatidis* and *B. salamandrivorans* in amphibian chytridiomycosis using RNAScope® in situ hybridization. *Front Vet Sci* 6:304.

R Core Team. 2020. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. Accessed September 2020.

Raffel TR, Romansic JM, Halstead NT, McMahon TA, Venesky MD, Rohr JR. 2013. Disease and thermal acclimation in a more variable and unpredictable climate. *Nat Clim Change* 3:146–151.

Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukema W, Acevedo AA, Burrowes PA, Carvalho T, Catenazzi A, et al. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science* 363:1459–1463.

Stegen G, Pasmans F, Schmidt BR, Rouffaer LO, Van Praet S, Schaub M, Canessa S, Laudelout A, Kinet T, Adriaensen C, et al. 2017. Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. *Nature* 544:353–356.

Thomas V, Blooi M, Van Rooij P, Van Praet S, Verbrugghe E, Grasselli E, Lukac M, Smith S, Pasmans F, Martel A. 2018. Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*. *Transbound Emerg Dis* 65:e478–e488.

Van Rooij P, Martel A, Haesebrouck F, Pasmans F. 2015. Amphibian chytridiomycosis: A review with focus on fungus-host interactions. *Vet Res* 46:137.

Submitted for publication 29 November 2020.
Accepted 12 March 2021.