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ORIGINAL RESEARCH

Landscape-scale drivers of spatial dynamics and genetic diversity in an emerging wildlife pathogen

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Abstract

Aquatic pathogens often cannot tolerate drying, and thus their spread, and diversity across a landscape may depend on interactions between hydrological conditions and the movement of infected hosts. The aquatic fungus *Batrachochytrium dendrobatidis* (*Bd*) is a nearly ubiquitous pathogen of amphibians and particular lineages have been associated with host declines. By coupling amphibian surveys with molecular pathogen detection and genotyping techniques, we characterized the spatial dynamics and genetic diversity of *Bd* on a landscape containing both permanent and ephemeral ponds. In doing so, we aimed to clarify how pathogen loads and prevalences vary across seasons and among habitat types, and which host species move the pathogen from place to place. At the start of spring breeding, *Bd* prevalence was lower on amphibians sampled from ephemeral ponds. For the remainder of the amphibian active season, prevalence was similar across both ephemeral and permanent ponds, with variation in prevalence being well-explained by a hump-shaped relationship with host body temperature. The first amphibians to arrive at these ephemeral ponds infected were species that breed in ephemeral ponds and likely emerged infected from terrestrial hibernacula. However, species from permanent ponds, most of which hibernate aquatically, later visited the ephemeral ponds and these animals had a greater prevalence and load of *Bd*, suggesting that migrants among ponds and pond types also move *Bd* across the landscape. The *Bd* we sampled was genetically diverse within ponds but showed little genetic structure among ponds, host species, or seasons. Taken together, our findings suggest that *Bd* can be diverse even at small scales and moves readily across a landscape with help from a wide variety of hosts.

Keywords Amphibian · *Batrachochytrium dendrobatidis* · Drift fence · Ephemeral pond · Genetic diversity · Global panzootic lineage · Permanent pond · Hibernation

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Introduction

As infectious diseases emerge with increasing frequency and impact wildlife populations (Smith et al. 2009), it becomes ever more critical to understand the links between environment, host–pathogen biology, and disease dynamics (Johnson and Paull 2011; Rizzoli et al. 2019). Fungal diseases such as white nose syndrome in bats (Foley et al. 2011), snake fungal disease (*Ophidiomyces ophidiicola*, Guthrie et al. 2016), and chytridiomycosis in amphibians (Berger et al. 1998; Fisher et al. 2009) have been important drivers of recent wildlife declines and extinctions. While host-fungal pathogen systems have received increased research attention in recent years (reviewed in Fisher et al. 2012), important questions remain about how fungal pathogens move across and persist in different landscapes.

To understand how pathogens are transmitted between individuals and across communities, it is critical to understand how infection dynamics differ across habitat types and how pathogens move from place to place (Kozakiewicz et al. 2018; White et al. 2018). For fungal pathogens like *Batrachochytrium dendrobatidis (Bd)*, one of two chytrid fungi that cause chytridiomycosis in amphibians (Longcore et al. 2007; Martel et al. 2013), the presence of a motile infectious zoospore stage means that transmission to new hosts readily occurs in aquatic environments (Greenspan et al. 2012a) and may even be possible through rain or fog (Kolby et al. 2015; Prado et al. 2023). Even in the absence of hosts, some fungal pathogens can persist in aquatic environments for long periods of time. For example, the motile aquatic zoospores of *Bd* can persist for up to 7 weeks in pond water (Johnson and Speare 2003), though they cannot survive desiccation for more than 30 min (Garmyn et al. 2012). Perhaps unsurprisingly then, in many areas, host species associated with permanent ponds (Kriger and Hero 2007) and streams (Hero et al. 2005; Gründler et al. 2012) are more likely to become infected with *Bd* than species inhabiting terrestrial habitats or ephemeral waterbodies. The tadpoles are generally restricted to their natal aquatic environment (Hoff et al. 1999) and as a result, infections on new metamorphs emerging for the first time onto land are a product of the pathogen pool in their natal environment (Wilber et al. 2017). However, the post-metamorphic hosts can move between aquatic habitats (Regosin et al. 2003), potentially bringing new pathogen variants to other permanent water bodies and introducing pathogens into habitats that lack permanent water (and therefore, could not likely sustain pathogens like *Bd* year-round, Garmyn et al. 2012).

The distribution of *Bd* prevalence and load on infected hosts depends on a variety of environmental factors including pH, temperature, canopy cover, and pond depth (Raffel et al. 2010; Sonn et al. 2019; Prahl et al. 2020). *Bd* growth is strongly temperature-dependent both in vitro (Voyles et al. 2017) and in vivo (Raffel et al. 2010). For example, in culture, *Bd* grows well at cool temperatures and can even withstand freezing, but maximal growth of most strains occurs between 17 and 25 °C (Voyles et al. 2017). In the wild, seasonal variation in temperature and humidity and the differences in these environmental variables between sites due to forest cover are known to mediate *Bd* infection prevalence and load (Retallick et al. 2004; Becker et al. 2012; Beyer et al. 2015; Sonn et al. 2019). However, *Bd* dynamics in ponds may also depend on the presence of amphibian (Padgett-Flohr and Hopkins 2009) and/or non-amphibian (McMahon et al. 2013; Brannelly et al. 2015) host species.

Given that *Bd* and many other aquatic pathogens cannot tolerate desiccation (Garmyn et al. 2012), one mechanism for multi-year persistence in ephemeral ponds may be the movement of infected hosts from permanent to ephemeral ponds. The ephemeral ponds are unique habitats often owing to their reduced predator pressure, support reproduction, and early development of many invertebrates and amphibians (Hopey and Petranka 1994). In contrast, permanent ponds are usually larger habitats that hold water throughout the year and often are inhabited by predatory fish, as well as a diverse community of other small-bodied invertebrates and vertebrates that prey on amphibian larvae (Wellborn et al. 1996). Because permanent ponds that are sufficiently deep do not freeze to the bottom, many species, including larval and adult amphibians (McDonald and Alford 1999), overwinter in these environments and can maintain aquatic pathogens like *Bd* on the landscape (Greenspan et al. 2012b; Becker et al. 2019). In eastern North America, some amphibian hosts like northern leopard frogs (*R. pipiens*), green frogs (*R. clamitans*), and bullfrogs (*R. catesbeiana*) usually overwinter and/or reproduce in permanent ponds but migrate to ephemeral ponds in late spring/early summer (Neill 1948), potentially carrying pathogens to ephemeral pond communities. Other species, like wood frogs (*R. sylvatica*) and spotted salamanders (*Ambystoma maculatum*), reproduce either preferentially or exclusively in ephemeral ponds and other semi-permanent water bodies (Karraker and Gibbs 2009). These species, which tend to overwinter terrestrially (Storey and Storey 1984) and reproduce very early in spring, could also bring pathogens to ephemeral ponds if they emerge from hibernation infected or acquire infections during migration to breeding ponds. Understanding how *Bd* and other aquatic pathogens spread to and from ephemeral ponds and how both aquatic communities contribute to the landscape-level dynamics of host–pathogen interactions, will be important for predicting disease risk and developing mitigation strategies.

The genetic diversity and structure of wildlife pathogens can provide important clues about how they move across landscapes to colonize new habitats and hosts (Blanchong et al. 2016; Kozakiewicz et al. 2018). For example, low genetic diversity and structure may indicate that a pathogen has been either recently introduced or that selective sweeps have resulted in the fixation of locally beneficial alleles. In contrast, high genetic diversity is expected when pathogens can colonize new habitats frequently and from a variety of source populations, or when an endemic pathogen is evolving in situ to a variety of different environments (such as host species, e.g., Byrne et al. 2022). When pathogens move readily across a landscape a panmictic pathogen population may result, with little population genetic structure being found among habitat patches. However, finding strong spatial and/or temporal population genetic patterns can help reveal key barriers to dispersal or potential mechanisms of spread (reviewed in Barrett et al. 2008).

Thus far, five lineages of *Bd* have been described (O'Hanlon et al. 2018; Byrne et al. 2019). The *Bd* Global Panzootic Lineage (GPL), which has been associated with global declines and extinctions, appears to have diverged from its most recent ancestor and spread worldwide 50 to 120 years ago (Rosenblum et al. 2013; O'Hanlon et al. 2018). In the United States, *Bd* has been detected from amphibian specimens collected as far back as 1888 (Talley et al. 2015). Recent work suggests that many genetic variants belonging to the GPL are infecting amphibians in the United States (Byrne et al. 2019) and that many are shared across ponds and host species (Byrne et al. 2022).

Here, we quantify and genotype *Bd* found on amphibian skin swabs to provide a fine-scaled understanding of the host distribution, genetic diversity, and genetic structure of this pathogen across a landscape of ephemeral and permanent ponds. We aimed to answer three key questions: (1) How does *Bd* load and prevalence change across the amphibian active season in ephemeral versus permanent ponds? (2) Which amphibians play a role in moving *Bd* from place to place? and (3) Are there differences in the genetic diversity and structure of *Bd* across the landscape? To answer these questions, we used a combination of modern molecular tools and traditional ecological techniques. Namely, we used a multi-year amphibian skin swab and environmental DNA (eDNA) surveys at five ephemeral and five permanent ponds in northwestern Pennsylvania to answer question one. To answer questions two and three, we coupled traditional mark-recapture studies at two drift-fenced ephemeral ponds with molecular techniques that enabled both quantification and genotyping of *Bd* from amphibian skin swab samples.

Methods

Unfenced pond surveys

From 2017 to 2019, we sampled amphibians from five ephemeral and five permanent ponds in northwestern Pennsylvania, near the Pymatuning Lab of Ecology (PLE; Fig. 1; Table S1) using visual encounter surveys (see supplemental methods) for post-metamorphic animals and dip netting for larvae. We sampled each pond once per month from March to September in 2017 and 2018. In 2019, we sampled only four of the ten ponds (two ephemeral and two permanent) once in each of April, May, June, July, and October. Prior to amphibian surveys in 2018 and 2019, we also collected 1 L of water from a subset of ponds for *Bd* detection/quantification via environmental DNA (eDNA, see details in supplemental methods). For each captured individual, we recorded species, skin temperature, sex, life stage, mass (using a Pesola spring scale), snout-to-vent length (SVL) and, for salamanders, tail length (using dial calipers), and collected a skin swab sample to test for *Bd* (see supplemental methods) before releasing the animal near the point of capture. After swabbing and taking all measurements, we released each animal near the location where it was captured. We caught most of the amphibians by hand, using a fresh pair of nitrile gloves to prevent pathogen transmission (Phillott et al. 2013). We collected data from up to 20 individuals of each amphibian species that was detected at each pond during each survey (Table S2).

Pitfall-trap surveys at fenced ephemeral ponds

In 2019, we conducted more intensive amphibian surveys at only two ephemeral ponds—Wood Lab pond and Sanctuary Lake pond (1 and 3, respectively in Fig. 1 and Table S1)—around which we installed drift fences and pitfall traps from March to August 2019. Sanctuary Lake pond is in a more developed area, is both smaller and shallower, and generally has a shorter hydroperiod than Wood Lab pond (see Supplemental Methods and Table S5 for additional details about study sites). We installed the drift fences 1–3 m from the water's edge to intercept amphibians moving to and from the pond. A team of two people sampled these ponds, checking traps once or twice per day for amphibians, for 40 sampling days between March 14 and August 8 of 2019 (see supplemental methods). After collecting temperature data and skin swabs as described above, we recorded capture location (outside or inside fence, in pitfall trap or not) and, for animals found in traps, whether the animal was alone or with other captured

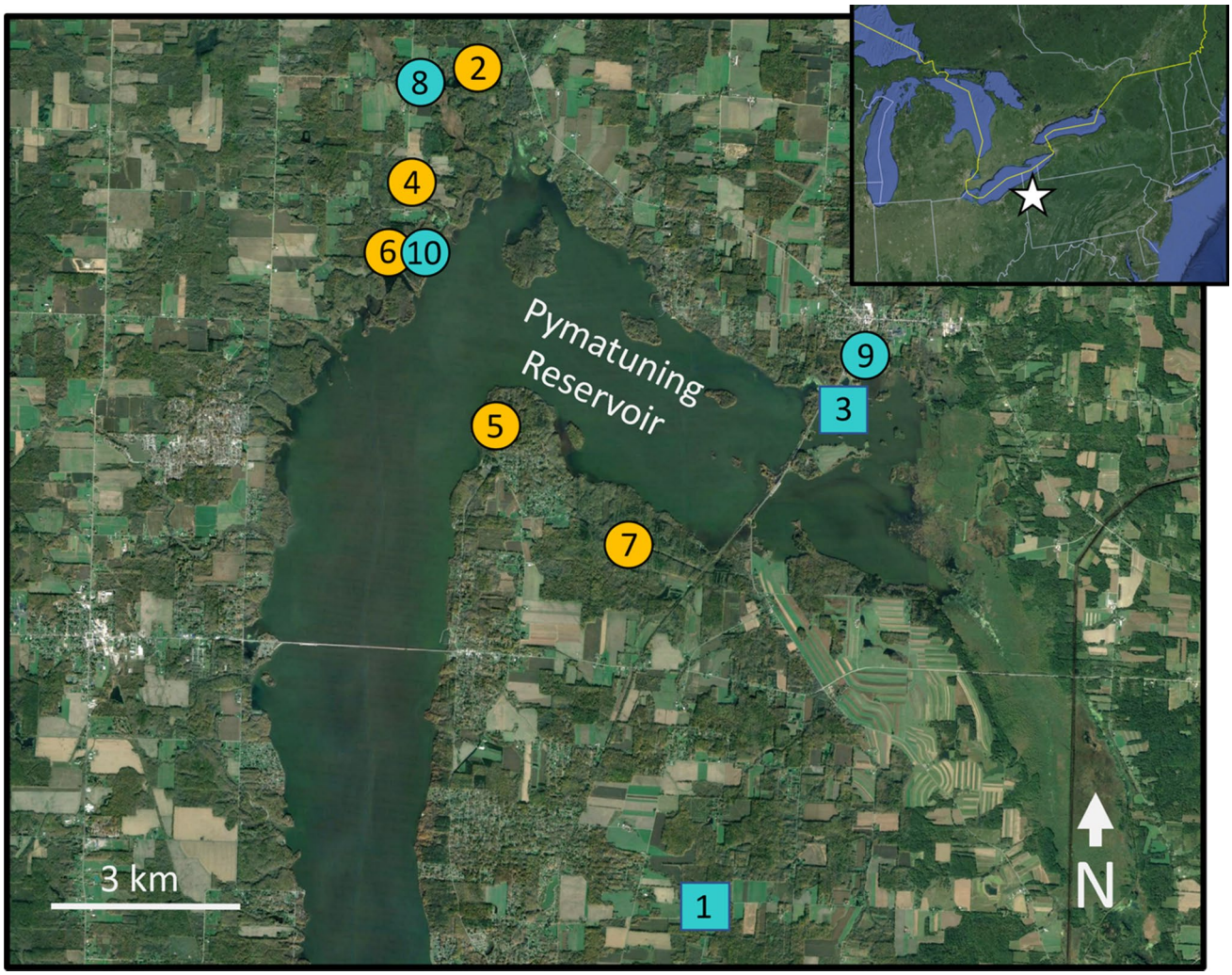

Fig. 1 Maps indicating (upper right) the location of our study region (white star), within the Great Lakes region of northeastern North America and (lower left) the locations of ponds sampled around the Pymatuning Reservoir. Squares indicate fenced ponds and circles unfenced ponds. Ponds 1 (Wood Lab pond) and 3 (Sanctuary Lake pond) were fenced in 2019. Ephemeral ponds are in teal and permanent ponds in orange. Numbers correspond to Table S1. Distances between ponds range from 0.25 to 17 km. Maps are from Google Earth v. 7.3.4.8642 (64-bit), accessed on 25 August 2022. Only ponds 1, 2, 3, and 4 were sampled in 2019 whereas all ponds were sampled in 2017 and 2018

amphibians. We cohort-marked each animal with a toe clip in order to distinguish new arrivals to the ponds from animals we had sampled before and released each animal on the opposite side of the fence from where it was captured.

DNA extraction and qPCR

We extracted genomic DNA from water samples for *Bd* quantification via eDNA following a modified version of the protocol described in Renshaw et al. (2015) (see details in supplemental methods). We extracted genomic DNA from swabs using the Qiagen DNeasy Extraction Kit (see details in supplemental methods). We then ran a qPCR assay (Blooi et al. 2013, see also supplemental methods) on both sample types to detect and quantify *Bd and B. salamandrivorans* (*Bsal*) DNA. We included negative and positive control samples and a sevenfold dilution series of plasmid standards (2.6 to 2.6×10^6 copies/reaction, Pisces Molecular, CO) in each qPCR run. To generate negative controls, we ran our DNA extraction protocol on a clean swab or, for eDNA, a liter of tap water. To create positive extraction controls we extracted DNA from a swab that had been dipped in a broth culture of *Bd*. We ran samples in singlicate and for swab samples calculated whole-swab *Bd* load by multiplying detected *Bd* DNA copies by 40 to account for the proportion of the full extraction that was aliquoted for qPCR.

Statistical analyses

We performed all statistical analyses involving qPCR data using R Studio 2019 (Racine 2012) with R version 1.1.5019 (R Core team 2019) and produced figures using ggplot2 (Wickham 2009). We used DHARMa (Hartig 2019) and visual assessments of residuals plots to confirm that model assumptions were met. The detailed model descriptions and outputs can be found in the supplemental material (supplemental methods, Tables S5–S16).

To test how *Bd* prevalence (including all sampled animals) and infection load (which in all our analyses included only animals that tested positive for *Bd*) varied over time and with temperature in the unfenced ponds (five ephemeral and five permanent ponds, 2017–2019), we fit generalized additive mixed models (GAMMs) using the *mgcv* package (Wood 2017). We removed two species with very low sample sizes: *Ambystoma jeffersonianum* ($n = 1$) and *Hemidactylium scutatum* ($n = 4$). GAMMs were used to model non-linear patterns in *Bd* prevalence and load across the year and with varying temperature. We used GAMMs because these patterns were clearly non-linear and would not meet assumptions of linear models. The models for prevalence were run with a binomial distribution, with infection status (positive or negative) as the response variable. We included pond type (ephemeral or permanent) as a fixed effect in all models and also included animal body mass as a fixed effect in infection load models, given a possible relationship between load and amphibian size. For each response variable (infection prevalence or load), we ran one model with Julian date by pond type as a smoothed effect (spline (k) = 20 and one with body temperature (˚C) (instead of Julian date) by pond type included as a smoothed effect. For all models, pond (i.e., site code), species (or the species-specific effects of body size for infection load models), and year were included as smoothed random effects. We fit the models with REML, ran diagnostic tests to determine whether the basis dimension choice was adequate and to assess diagnostic plots (library: mgcv, function: gam.check) and checked for concurvity (library: mgcv, function: concurvity). Some models demonstrated significance in the gam.check function, which may indicate the k is too low. However, the effective degrees of freedom (EDF) were not close in value to k', even at high values (e.g., $k = 100$). Following Wood (2017), results were consistent at $k = 20$ and above, so $k = 20$ was used.

Given the unequal distribution of tadpole samples across ponds, seasons, and years, and the relatively low number of tadpole samples overall (Tables S2 and S3), we did not have sufficient power to use GAMMs to compare Bd prevalence across life stages. Therefore, to compare *Bd* infection status between the two life stages (larval vs. post-metamorphic), we used a GLMM (package *lme4*, function 'glmer', Bates 2010) with a binomial distribution including life stage, pond type, species, and their interactions as fixed effects and pond as a random effect. To alleviate violation of the equal variance assumption, we only included the four species with sufficient sampling across ponds and life stages in this model. We did not test for differences in *Bd* load between the two life stages because there were too few (only 15/167) infected tadpoles.

The next set of analyses included subsets of captures of post-metamorphic animals found entering or leaving the two fenced ponds in 2019. For infected animals entering the fenced ponds for the first time during our study period (i.e., animals that had no toe clip), we compared *Bd* prevalence among species hibernating in permanent ponds relative to those hibernating terrestrially. To do this, we analyzed a subset of data that included only amphibians that were known to hibernate either terrestrially (*Ambystoma maculatum*, *A. jeffersonianum*, *Hemidactylium scutatum*, *Plethodon cinereus*, *R. sylvatica*, *P. crucifer*, and *A. americanus*) or in permanent ponds (*R. clamitans*, *R. pipiens*, and *R. catesbeiana*) and only included data from an individual's first capture outside the fence (i.e., on their way into the pond for the first time). We used a GLMM (package lme4, function 'glmer') with a binomial distribution that included hibernation type (terrestrial vs. permanent pond, based on species natural history), pond (because there were different species arriving to the two fenced ponds), and their interaction as fixed effects. We included species as a random effect. To compare *Bd* load between the two hibernation types (permanent pond vs. terrestrial), we used a linear mixed model (function 'lme') that included hibernation type, pond, and their interaction as fixed effects and species as a random effect.

To test for differences in *Bd* load between infected animals entering vs. leaving the fenced ponds, we fit a linear mixed model using generalized least squares (GLS LMM using *nlme* package, function 'gls'). In this case, we used a GLS LMM instead of a GLMM because a GLMM failed the uniformity check. We, therefore, re-fit the model using the *nlme* package in R (Pinheiro et al. 2022) with a variance structure to account for unequal variance across groups, which addressed the violation. The model included hibernation type (terrestrial vs. permanent pond, based on species natural history), migration direction (i.e., whether the animal was captured inside the fence and was leaving or was captured outside the fence and was coming into the pond), pond, and the interaction between hibernation type and migration direction as fixed effects. Species was not included as a random effect in this model because doing so led to violations of model assumptions that we were unable to reconcile. To compare *Bd* infection status between animals entering vs. leaving the fenced ponds, we used an analogous GLMM (package *lme4*, function 'glmer') with a binomial distribution and including all captures (not just those where the animal was infected).

Bd genotyping and analyses of genetic variation

We selected 110 of the 294 *Bd*-positive (by qPCR) swab samples collected from the two ephemeral pond sites in 2019 for *Bd* genotyping. These were selected to balance sample sizes among ponds, seasons, and host species. However, to improve the odds of generating good quality genotype data, we prioritized DNA extracts with higher copy numbers of *Bd* DNA. We used the Fluidigm Access Array platform to perform microfluidic multiplex PCR on 191 regions of the *Bd* genome and one diagnostic locus for the closely related fungus *Bsal* (see supplemental methods and Byrne et al. 2017). Each target locus is 150–200 base pairs long and the targets are distributed across the *Bd* nuclear and mitochondrial genomes.

We used a gene-tree to species-tree approach (see supplemental methods) to construct a phylogeny to explore the relationship of the *Bd* genotypes found in our samples to previously published *Bd* samples representing all known *Bd* lineages ($N = 31$, Byrne et al. 2019; Rosenblum et al. 2013). To further explore the genetic variation of *Bd* sampled from amphibians at the two fenced ponds, we used a principal components analysis (PCA) on variants called from haplotypes (see supplemental methods). To evaluate the relative contribution of season, pond, whether the sampled individual was entering or leaving the pond, and host species, we ran an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using our variant data.

Results

Bd infection and eDNA detections over time in permanent and ephemeral ponds

More than one-third (37.5%) of the 4898 amphibians, we swabbed, tested positive for *Bd* (Tables S2, S4) while none tested positive for *Bsal*. While pond type (ephemeral vs. permanent) was not a significant predictor of *Bd* prevalence overall (GAMM: $\beta = -0.070$, SE = 0.384, $p = 0.855$; Table S5), prevalence in both pond types varied with Julian day (hereafter, JD; GAMM: JD by ephemeral pond [smoothed term]: $\chi^2 = 243.9$, reference degrees of freedom [rf] = 13.75, $p < 0.001$; JD by permanent pond [smoothed term]: $\chi^2 = 295.9$, rf = 17.66, $p < 0.001$). Models predicted that *Bd* prevalence was greater in permanent ponds than ephemeral ponds when animals first emerged from hibernation (JD 60–100; Fig. 2A and Fig. S4A, 24.4% [CI: 10.9–37.8%] on average for ephemeral ponds, and 58.2% [CI: 40.6–75.8% for permanent ponds]. However, by the middle of spring (JD 100–140), the model predicted *Bd* prevalence had peaked to similar levels in both pond types (predicted prevalence was 57% [CI: 47.4–66.6%] for ephemeral and 77.2% [CI: 65.8–88.7%] for permanent ponds). *Bd* prevalence was then predicted to decrease in late spring to early summer (JD 140–180; 32.8% [CI: 21.6–44%] in ephemeral and 41.6% [29.6–53.6%] in permanent) and by mid-summer (JD 180–230) *Bd* prevalence was predicted to be similar in ephemeral ponds and permanent ponds (38.1% [CI: 27.2–49%] in ephemeral and 34.2% [CI: 24.4–44%] in permanent).

Our model predicted that *Bd* load (in mean ± SE $\log_{10}$ DNA copies per swab) varied over time. The visualization of Fig. 2C suggests that while *Bd* load was similar at the start of the amphibian active season (JD 66–74; predicted load 3.11 ± 0.24), it was predicted to increase in permanent ponds (to 3.5 ± 0.241) in late winter to early spring (JD 84–104) then decline again in late spring to early summer (JD 140–180). In contrast, in ephemeral ponds *Bd* load decreased throughout the year (GAMM: JD by ephemeral pond [smoothed term]: $F = 4.050$, rf = 2.381, $p = 0.013$; JD by permanent pond [smoothed term]: $F = 9.172$, rf = 8.228, $p < 0.001$; Table S6; Fig. 2C, Fig. S4C).

The relationship between amphibian body temperature and *Bd* prevalence was not predicted to differ significantly between pond types (GAMM: $\beta = 0.005$, SE = 0.345, $p = 0.988$; Fig. 2B; Fig. S4B; Table S7). In both pond types, *Bd* prevalence varied with body temperature in a similar fashion (GAMM: temperature by ephemeral pond [smoothed term]: $\chi^2 = 91.26$, rf = 7.333, $p < 0.001$; temperature by permanent pond [smoothed term]: $\chi^2 = 128.71$, rf = 8.313, $p < 0.001$, Table S7); the predicted prevalence increased from 3 to 13 °C and then decreased. There were no differences in the relationship between body temperature and *Bd* infection load between permanent and ephemeral ponds (GAMM: $\beta = 0.137$, SE = 0.150, $p = 0.357$; Fig. 2D; Fig. S4D; Table S8). Overall, *Bd* load decreased with increasing body temperatures.

We did not test statistically for variation in *Bd* eDNA concentration or detection in ephemeral and permanent ponds due to small sample sizes per season and pond type (Table S9). However, some differences were noted upon visual inspection. For example, we detected *Bd* DNA in more than 1/3 (21 of 54) of samples collected in spring or summer but only rarely (in 1 of 14 samples) in winter or fall. When we did detect *Bd* eDNA, its concentration was similar among pond types and seasons (means 3046–3515 DNA copies per liter, Table S9).

Bd load and prevalence in tadpoles and post-metamorphic animals

Bd prevalence was greater for post-metamorphic animals than tadpoles in both pond types (GLMM: $t = -3.718$, $p < 0.001$, Fig. S1B, Table S10); only 15 of 167 tadpoles (9%) tested

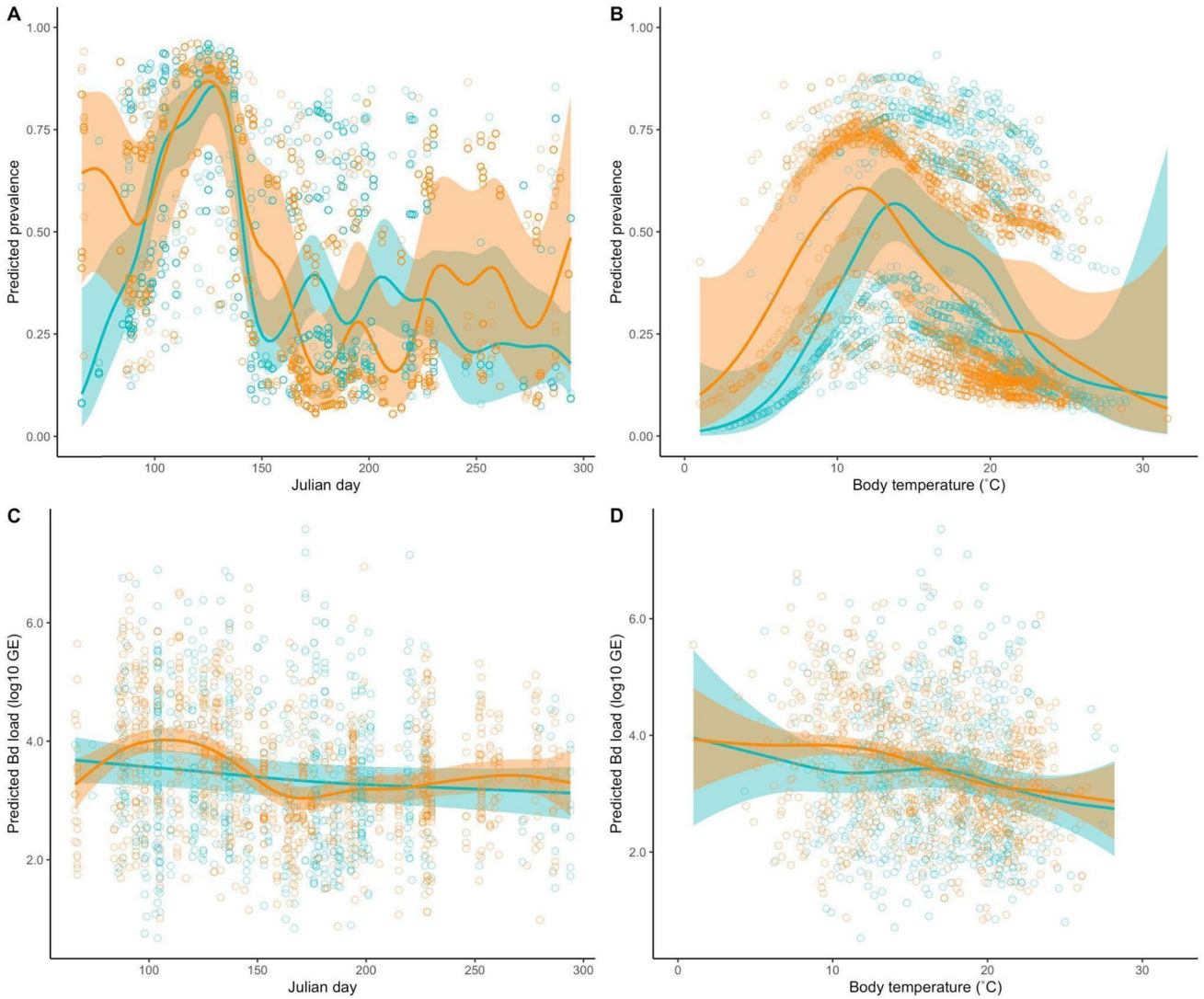

Fig. 2 Generalized additive model (GAMM) predictions of *Batrachochytrium dendrobatidis* (*Bd*) prevalence by **A** Julian day (JD) and **B** amphibian body temperature (in ℃), and $\log_{10}$ *Bd* load by **C** JD and **D** amphibian body temperature in ephemeral (teal color) versus permanent (orange color) ponds (data collected from 2017 to 2019). In these predictions, all additional random effects are fixed at their mean or mode, depending on the factor. Raw data have been superimposed and the shaded areas show 95% pointwise confidence limits. In this region, JD 81–172 is spring, JD 173–264 is summer, JD 265–355 is fall, and JD 356–80 is winter

positive for *Bd* whereas 1812 of 4667, post-metamorphic amphibians (39%) were *Bd*-positive.

We were unable to compare *Bd* load on infected animals across life stages statistically due to low numbers of infected tadpoles. The mean load on infected tadpoles (in $\log_{10}$ DNA copies per swab) was 2.319 (range 1.251–5.438) while for post-metamorphic animals the mean load was 3.382 (range 0.769–7.160; Fig. S1A).

Bd infections in animals arriving to the fenced ephemeral ponds

Our first survey of the fenced ponds was at Wood Lab pond (pond 1) on March 14 of 2019, 10 days after the ice covering that pond had completely melted. On that day, we found wood frogs (*Rana sylvatica,* $n = 11$), red-backed salamanders (*Plethodon cinereus,* $n = 2$) and spotted

salamanders (*Ambystoma maculatum, n* = 21) along the fence and in the traps. Only two of these animals (both *A. maculatum*) tested positive for *Bd*. The amphibians did not arrive to Sanctuary Lake pond (pond 3) until April and on our first survey there (April 5 of 2019) three species were encountered; three spring peepers *(Pseudacris crucifer, n* = 5) one American bullfrog (*Rana catesbeiana, n* = 1) and one American toad (*Anaxyrus americanus, n* = 1) tested positive for *Bd*.

Bd load on infected post-metamorphic animals at first entry into the fenced ephemeral ponds differed among hibernation types (LMM: $t = 3.103$, $p = 0.003$; Table S11), where the *Bd* load was greater for species that hibernate in permanent ponds than for individuals of species that hibernate terrestrially (Fig. 3A); this pattern was clearer in animals from the Wood Lab pond (pond 1), though the interactive effect of pond site and hibernation type was not significant (LMM: $t = 1.949$, $p = 0.054$; Table S11; Fig. 3A). There was a significant interactive effect of pond and hibernation type on *Bd* status. Terrestrial hibernators had lower prevalence than pond hibernators at Wood Lab (pond 1), whereas *Bd* status was more similar among hibernation types at Sanctuary Lake (pond 3) (GLMM: $z = 4.122$, $p < 0.001$; Fig. 3B; Table S12).

Patterns of infection in post-metamorphic animals entering vs. leaving the fenced ponds

When data were pooled across all infected post-metamorphic animals, including all (i.e., first and subsequent) captures of infected animals moving into (i.e., captured outside the fence) and out of (i.e., captured inside the fence) the ponds, *Bd* load was higher in permanent pond hibernating species than in terrestrially hibernating species (GLS LMM: $t = -4.067$, $p < 0.001$; Fig. 4, Table S13). There was no significant difference between the *Bd* loads of infected animals coming into the fenced ponds and those leaving the fenced ponds and also no significant interaction between hibernation type and animals entering vs. leaving the pond (GLS LMM: $t = 1.296$, $p \geq 0.196$) for *Bd* load. The results for associations between hibernation type, movement direction, and *Bd* status (i.e., infected or not) matched those for *Bd* load (Fig. 4, Table S14).

Genetic diversity and structure in the fenced ponds

Only 28 of the 110 amphibian skin swab samples sent for genotyping produced enough sequence data for downstream analyses. These 28 samples included six of the eight

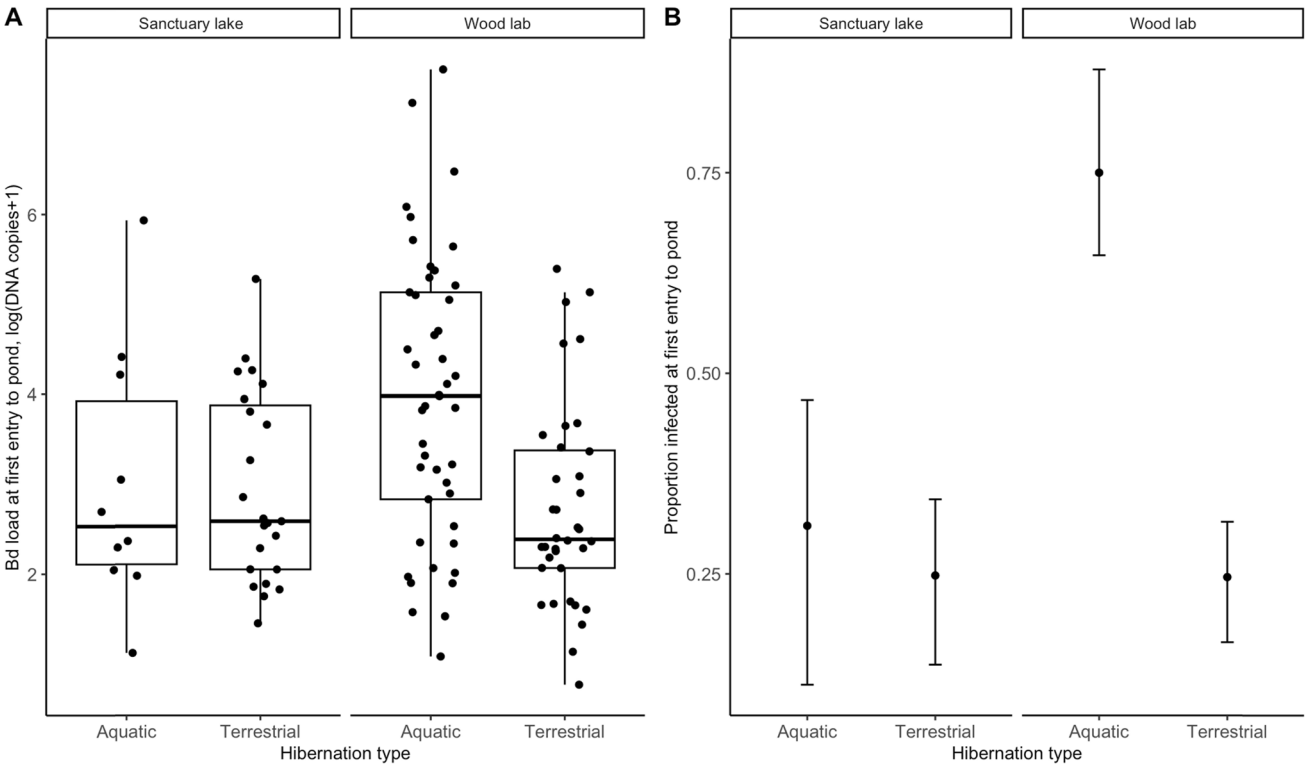

Fig. 3 Box and whisker plots showing the relationship between $\log_{10}$ *Bd* load per swab for infected animals (**A**), and the proportion of post-metamorphic individuals infected with *Bd* (**B**), upon first arrival to the fenced ponds by hibernation type (terrestrial: N = 58 vs. aquatic: *N* = 54). In (**A**), each dot represents one individual. In (**B**), points represent the proportion of individuals infected. The error bars represent 95% confidence intervals calculated using the Clopper–Pearson method (Clopper 1934)

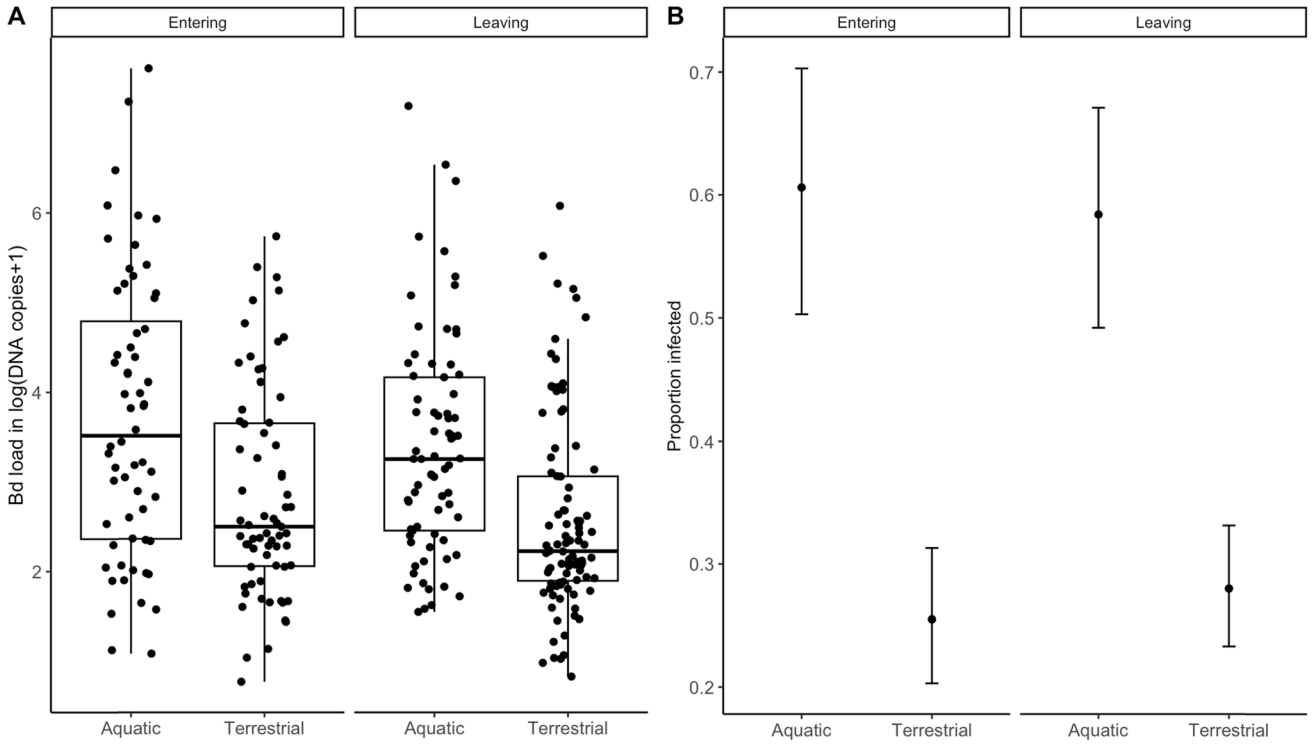

Fig. 4 Box and whisker plots showing the relationship between $\log_{10}$ *Bd* load per swab for infected animals (**A**), and the proportion of post-metamorphic individuals infected with *Bd* (**B**) for each instance where an animal entered ($N = 125$) or left ($N = 149$) the pond by the hibernation type (terrestrial vs. aquatic). In (**A**), each dot represents one individual. In (**B**), dots represent the proportion of individuals infected with *Bd* and *e*rror bars represent 95% confidence intervals calculated using the Clopper–Pearson method

amphibian species sampled at the two fenced ephemeral ponds and were collected between April and August of 2019. *Bd* load, as estimated via qPCR, was not a significant predictor of whether a skin swab sample produced the quality of haplotype data needed for analysis (GLM: $t = 0.603$, $p = 0.547$; Table S15).

The first two principal components (PCs) describing the genetic variation in the 28 genotyped samples explained 33.8% of the total variance in haplotypes. To visualize how the genetic variation was distributed, we plotted the first two PCs by pond (Fig. S2A), by whether animals were sampled coming into or going out of the pond (Fig. S2B), and by season (spring vs. summer, Fig. S2C). In all cases, there was visual overlap in haplotype variation among the sample categories and AMOVAs showed no significant structure among ponds, by direction of animal movement, or by season (999 permutations, $p \geq 0.163$; Fig. S3; Table S16). We were not able to test for the effect of host species on *Bd* genotypes because of small sample sizes for each species.

The phylogeny of *Bd* genotypes from the swab samples revealed a great deal of diversity (i.e., many haplotypes) among samples from each pond and some genetic structure; one haplotype from Sanctuary Lake pond fell within the *Bd* GPL1 clade while many other haplotypes from that pond and from Wood Lab pond fell within *Bd* GPL2. Many of the samples from Wood Lab pond had haplotypes that are highly heterozygous, and therefore could be indicative of animals having been coinfected with both *Bd* GPL clades (GPL1 and GPL2). We could not distinguish coinfection from the possibility of infection by a hybrid *Bd* strain using our dataset. We did not see any obvious phylogenetic structure among samples from the two ponds, among host species, among samples collected during different seasons, or among samples from animals entering vs. leaving the ponds (Fig. 5).

Discussion

The number of reported fungal and fungal-like pathogens responsible for disease outbreaks and declines in freshwater animals, including amphibians (Fisher et al. 2009), fish (Gozlan et al. 2010; Gozlan 2012), and crayfish (Holdich et al. 2009), is ever increasing. Many aquatic fungal pathogens are generalists, causing disease in animals spanning families, or even orders (Poulin and Mouillot 2003; Scheele et al. 2019; Gray et al. 2023). In this case, and in contrast to pathogens with a single host species, the composition of the host community and the susceptibility of each host species can shape many aspects of the epidemiology and overall impact on aquatic communities (Woolhouse et al.

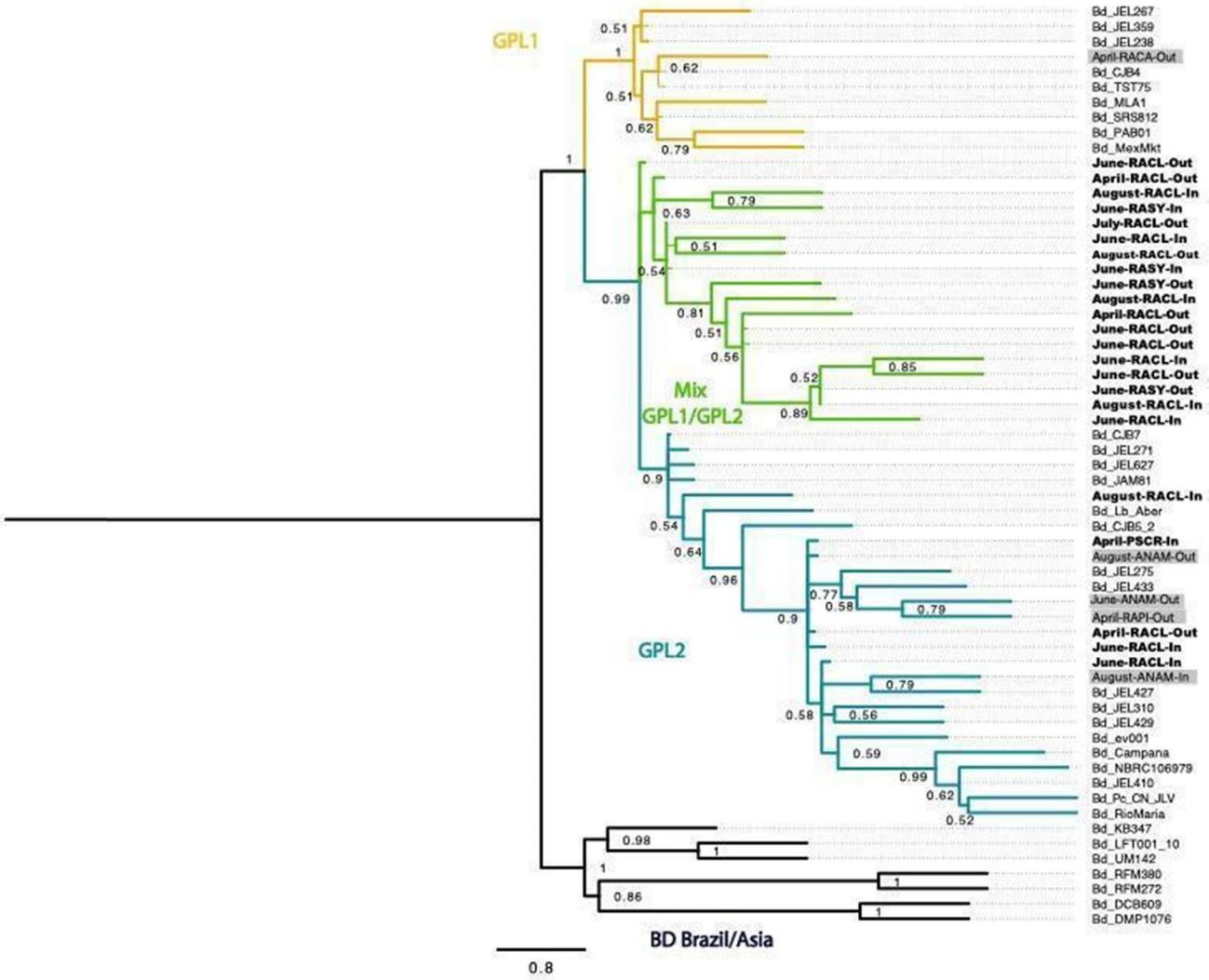

Fig. 5 Phylogeny of *Bd* haplotypes ($N=31$) inferred from ASTRAL and RAxML analyses. The numbers denote ASTRAL local posterior probability values. Names in bold represent the samples collected in Wood Lab (pond 1) pond and names highlighted in grey were collected in Sanctuary Lake pond (pond 3). The rest of names are previously genotyped isolates we used to assign *Bd* lineage designations to the samples from this study (Rosenblum et al. 2013; Byrne et al. 2019). These were from Panama, Brazil, and Asia. Branch coloration indicates clade (yellow for GPL1, blue for GPL2, green for "mixed")

2005). However, in many cases, the lack of external symptoms on hosts (Gozlan 2012) and limitations on methods and support for pathogen detection and monitoring have left us without a complete picture of disease dynamics across host communities. This, coupled with our limited understanding of the ecologies and life cycles of this diverse group of aquatic pathogens, has hampered our ability to understand and appreciate the true risk that the emergence and spread of such pathogens poses to freshwater communities (Gozlan et al. 2014). The amphibian-*Bd* system, where we now have several decades of both experimental and observational studies on both hosts and pathogen, and a diversity of monitoring tools available, stands in stark contrast and presents us with an opportunity to provide a case study, answering questions about drivers of spatial and temporal dynamics that are not yet approachable for many aquatic disease systems.

Seasonal patterns of infection in ephemeral and permanent ponds

Observing patterns of variation in infection prevalence and load across seasons and habitat types can provide clues as to the factors that influence the spread of aquatic pathogens. In early spring in our northwest Pennsylvania study system, just after amphibians in emerge from hibernation and begin migrating to breeding ponds, *Bd* prevalence was lower in ephemeral ponds than in permanent ponds. This difference, however, was gone by mid-spring when *Bd* prevalence

peaked at ~ 70% then declined in both pond types (Fig. 2A). The *Bd* load also peaked in spring and was lower in summer, though it rose again in fall in both pond types (Fig. 2C). These seasonal patterns, which are similar to those seen in other sub-tropical and temperate zone amphibian communities (Brannelly et al. 2018; Sonn et al. 2019; Wilber et al. 2022) are perhaps easiest to understand in terms of relationships between temperature and *Bd* prevalence and load, and our models showed strong support for such relationships (Fig. 2B, D).

Body temperature was a significant predictor of *Bd* infection in the amphibians inhabiting both the ephemeral and permanent ponds in our study area, which is similar to findings of previous studies (Phillott et al. 2013; Sonn et al. 2019). In both pond types, we found that *Bd* load decreased with amphibian body temperature (Fig. 2D). *Bd* prevalence, on the other hand, was lowest at the extremes of amphibian body temperature and reached a peak in intermediate temperature ranges (Fig. 2B), mirroring the thermal performance limits for *Bd* growth in culture (Voyles et al. 2017). *Bd* infections peaked at a lower temperature (~ 11 ºC) in permanent than in ephemeral ponds (~ 15 ºC). The explanations for this difference between pond types might include (1) differences in the thermal environments available, (2) differences in the thermal physiology of distinct *Bd* lineages (as seen in Voyles et al. 2017; Sheets et al. 2021, though our data do not suggest structuring of *Bd* lineages by pond type), or (3) differences in the thermal sensitivity of the defenses of hosts (as seen in Cohen et al. 2017; Moretti et al. 2019) that inhabit these two pond types. Previous studies have attributed high *Bd* prevalence in the early spring to the fact that *Bd* survives well at cool temperatures (Voyles et al. 2012) whereas the immune defenses of amphibians tend to be reduced (Robak et al. 2019; Rollins-Smith 2020). Thus, in the case of *Bd* and possibly other aquatic pathogens, a clearer understanding of the thermal physiology of both hosts and pathogens seems likely to shed light on the mechanisms generating variation in infection across habitat types and seasons.

Contributions to a *Bd* reservoir in permanent ponds

Amphibians exhibit a diverse range of breeding (Duellman and Trueb 1986) and overwintering (Neill 1948) strategies that can influence their susceptibility to pathogens as well as their potential to transport pathogens to and between aquatic habitats (Wilber et al. 2017). For example, tadpoles and/or post-metamorphic amphibians that overwinter in permanent ponds may act as reservoirs (Wilber et al. 2020) maintaining pathogen presence in a pond year-round. In contrast, because *Bd* and many other aquatic pathogens are intolerant of desiccation, their presence in ephemeral ponds year after year likely depends upon a different set of infected hosts (those that overwinter terrestrially and enter ephemeral ponds to breed each spring), bringing the pathogen anew each year. We used our sampling from permanent and ephemeral ponds to ask how differences in host life histories, including overwintering and breeding habitats, contribute to patterns of *Bd* infection dynamics and spread. In early spring, when amphibians in our study area first become active, the prevalence of *Bd* was greater in animals sampled from permanent ponds. While we did not sample overwintering amphibians themselves, and therefore, could not test this directly, we suspected that this pattern reflects the fact that infected overwintering animals shed zoospores throughout the winter in permanent ponds creating a pathogen reservoir. Our eDNA data, however, fail to support the idea that permanent ponds represent environmental reservoir for *Bd* over winter. While we detected *Bd* eDNA in pond water sampled during spring and summer in both pond types, during the cooler months of the year, we did not detect *Bd* DNA in water sampled from permanent ponds. However, our small sample size for this time period, coupled with the knowledge that eDNA sampling for *Bd* has a much higher detection threshold than amphibian swab samples (Brannelly et al. 2020), means that we cannot rule out the possibility that *Bd* was present in winter and we did not detect it.

Some of the frogs in our study area overwinter as larvae in permanent ponds (e.g., *Rana catesbeiana*, *Rana clamitans*) and thus we suspected that infected overwintering larvae might be an important pathogen reservoir in permanent ponds. However, our comparisons of infection prevalence and load among life stages suggest that tadpoles play a more minor role in pathogen maintenance and transmission in our study area. We found larvae to have similar *Bd* load but lower *Bd* prevalence than post-metamorphic life stages in both ephemeral and permanent ponds. In permanent ponds the difference in prevalence between life stages was dramatic; prevalence in the post-metamorphic animals was close to 40% but only 10% of the permanent pond larvae we sampled were found to be infected with *Bd.* Other studies in the northeastern United States have shown a wide range of *Bd* prevalences in tadpoles that overwinter aquatically. For example, Richards-Hrdlicka et al. (2013) found no evidence of infection in *R. catesbeiana* tadpoles in Julian et al. (2016) found a wide range of prevalences in *R. clamitans* tadpoles in Pennsylvania.

Another potential cause for the high prevalence of aquatic pathogen infections in permanent ponds in early spring could be infected post-metamorphic animals that hibernate in those ponds. The data we collected from the two fenced ephemeral ponds support this idea for *Bd*. We found that for animals arriving at the fenced ponds in spring, *Bd* prevalence and load were higher in permanent pond hibernators (*Rana catesbeiana*, *R. clamitans*, and *R. pipiens*) than terrestrial hibernators (all sampled salamander species, *R. sylvatica*,

Pseudacris crucifer, *Anaxyrus americanus*, and *Hyla versicolor*). This suggests that post-metamorphic anurans that hibernate in permanent ponds may be important contributors to the pool of infectious zoospores not only in permanent ponds (where they also breed) but also in ephemeral ponds, which they often visit (but do not breed in). A survey like ours, which sampled amphibians throughout their active season in Maine, USA, also found that infection prevalence was lower in species that hibernate in terrestrial habitats than that in species that hibernate in aquatic habitats (Longcore et al. 2007). The permanent pond hibernators entering Sanctuary Lake pond had a much lower *Bd* prevalence than those entering Wood Lab pond. There are many variables that could contribute to this pattern, but differences in the most abundant species (*R. pipiens* for Sanctuary Lake vs. *R. clamitans* for Wood Lab, see Table S2) and surrounding landscape (heavily disturbed by fish farming for Sanctuary Lake vs. a matrix of agricultural and forested land for Wood Lab) seem likely to be important ones. More work is needed to understand whether the patterns we found linking hibernation location to pathogen dynamics are common in other aquatic host–pathogen systems as well.

Sources of *Bd* in ephemeral ponds

Little is known about how aquatic fungal pathogens, many of which cannot persist during extended dry periods (Garmyn et al. 2012), reach habitats that are terrestrial or only episodically aquatic, like ephemeral ponds.

In our study system, *Bd* was frequently detected on animals sampled from ephemeral ponds and we also detected it via eDNA sampling from ephemeral pond water, suggesting that *Bd* is able to colonize these temporary water bodies consistently once they refill. *Ambystoma maculatum*, *A. americanus*, and *P. crucifer*, all of which hibernate terrestrially, were the first amphibians to enter our fenced ephemeral ponds infected with *Bd*. Approximately 20% (28 of 137) of the ephemeral pond breeders (8 *A. maculatum*, 12 *R. sylvatica*, 5 *P. crucifer*, and 2 *A. americanus*) entering the fenced ponds in the first month after these species became active were infected with *Bd*. This suggests that animals entering the pond to breed after overwintering in terrestrial hibernacula may be bringing *Bd* to ephemeral ponds each spring. Thus, it appears that transmission from permanent to ephemeral ponds via infected hosts or environmental sources (e.g., rain or fog, Kolby et al. 2015; Prado et al. 2023) is not required to sustain *Bd* in these ephemeral pond communities over time.

Despite its potential importance for understanding the dynamics and spread of *Bd*, little is known about how hibernation affects the course or outcome of *Bd* infections in amphibian hosts. The only study we are aware of that has addressed this directly is Rumschlag and Boone (2018), which found that experimental *Bd* exposure prior to hibernation reduced overwinter survival in the northern leopard frog. While it is also possible that they acquired infections during migration to the fenced ponds, it appears that some of the terrestrially hibernating animals in our study may have emerged infected from hibernation. If they emerged infected, this means they were able to survive with *Bd* over the long winter, though how infection was acquired and how infection loads may have changed over the winter remain unknown. It may be possible for species hibernating underground to contract *Bd* infections while hibernating, as *Bd* has been shown to persist in moist sand for extended periods (12 weeks, Johnson and Speare 2003). Alternatively, given that *Bd* was detected on many terrestrial hibernators leaving our ephemeral ponds in fall (47 of 134 infected, including 15 *A. maculatum,* 29 *R. sylvatica*, 1 *P. crucifer,* and 2 *A. americanus*), animals may have entered hibernacula infected and maintained those infections over winter. The predicted *Bd* prevalence and load just after emergence from hibernation were similar to those just prior to hibernation for animals sampled in our ephemeral ponds. Though we do not have individual-level data to test this explicitly, this pattern suggests that infections are not commonly gained or lost and that loads do not increase dramatically during the cold months of hibernation in our study area.

While they breed, develop, and hibernate in more permanent water, some of the permanent pond associated amphibians in our study area (e.g., *R. catesbeiana*, *R. clamitans*, and *R. pipiens*) were often encountered in and around our sampled ephemeral ponds. While they were not the first to bring *Bd* to the ephemeral ponds, we found that individuals of species that hibernate in (and in all cases also breed in) permanent ponds had higher *Bd* prevalence and load when entering the fenced ephemeral ponds than did those that hibernate terrestrially. As early as April 5th, we began to encounter *R. catesbeiana*, *R. pipiens*, and *R. clamitans* along our drift fences and 60.6% (60 of 99) of these permanent pond associated species were infected with *Bd* upon first entry into a fenced pond. Whereas, this number was much lower, only 25.4% (66 of 259), for the terrestrially hibernating animals. Most of the permanent pond associated animals found entering the fenced ephemeral ponds with *Bd* infections were *R. clamitans* (50/64 infected). This supports the idea that species associated with permanent ponds play an important role in the pathogen dynamics of ephemeral ponds in addition to permanent ones. It also suggests that permanent ponds, and the species associated with them, might have a large influence on the distribution of aquatic pathogens across the landscape.

Genetic diversity of *Bd* in ephemeral ponds

Despite finding a surprising amount of genetic diversity in *Bd* across the two fenced ephemeral ponds, there was no appreciable structure to that diversity between ponds or among the amphibian species. The two ephemeral ponds are separated by ~ 9 km, a distance greater than amphibians in this region are thought to disperse (Smith and Green 2005), suggesting that movement of infected individuals between the two ponds would be quite unlikely. The lack of genetic differentiation could be explained by these pond communities being part of a metapopulation, connected by occasional dispersal (Hamer and McDonnell 2008) or by movement of *Bd* by more vagile animals (e.g., birds, reptiles, or humans; Burrowes and De la Riva 2017; Prahl et al. 2020) or through environmental sources like rain or fog (Kolby et al. 2015; Prado et al. 2023). There was a large amount of overlap in the *Bd* genetic variants found on animals entering vs. leaving the fenced ponds and also across the spring and summer seasons. The nearly complete lack of genetic structure among *Bd* from different ponds in our study area stands in stark contrast to the pattern seen among high-elevation lakes in the Sierra Nevada of California (where *Bd* haplotypes show strong genetic structure and a pattern of isolation by distance among high elevation lakes) but resembles the pattern of *Bd* genetic diversity seen in western Panama (where *Bd* shows little genetic diversity across geographically distant stream systems) (Rothstein et al. 2021).

In both of our fenced ponds, we found a diversity of *Bd* haplotypes belonging to the globally invasive panzootic lineage (GPL). The GPL comprises two genetic clades: *Bd*GPL-1, which is primarily found in North America and Europe, and *Bd*GPL-2, which is distributed worldwide (James et al. 2015; O'Hanlon et al. 2018). GPL-1 is often thought of as a North American lineage and is thought to be ancestral to GPL-2, which is globally distributed and is the lineage responsible for amphibian declines in Central America (James et al. 2015). While GPL-1 and 2 are both known to be present in the United States (Schloegel et al. 2012), in this study and in a study of a larger sample of ponds from this region (Byrne et al. 2022), we found evidence for a clade with less than 0.7 posterior support that appears to be a mix of *Bd* GPL1 and GPL2. This finding could be explained by coinfected animals harboring *Bd* strains belonging to both sublineages. The lab experiments have demonstrated the potential for such coinfection (Jenkinson et al. 2018). Alternatively, it could indicate that recombination has occurred between the two sublineages. Hybrids between clades of *Bd* have been reported previously (e.g., in Brazil; Greenspan et al. 2018). The ongoing work aimed at clarifying the history of the host–pathogen relationship in this region, as well as mechanisms by which these populations have managed to avoid the catastrophic declines that *Bd* caused in many other amphibian communities, may also shed light on the cause of the large genetic diversity and 'mixed' *Bd* genotypes we detected in this study.

Conclusions

By combining host surveys with molecular pathogen detection and genotyping, we were able to gain new insights into the landscape-scale spatial dynamics and diversity of an emerging wildlife pathogen. Some ephemeral pond breeding amphibians emerged from terrestrial hibernation sites infected with *Bd,* suggesting that transmission by animals visiting from permanent ponds is not required to bring aquatic pathogens like *Bd* to ephemeral ponds each year. However, the amphibians that hibernate in permanent ponds and later visit ephemeral ponds tend to have greater pathogen loads than those that hibernate terrestrially. Unlike other systems where anuran larvae are important reservoirs (e.g., Narayan et al. 2014; Hagman and Alford 2015), tadpoles seem to be a less important reservoir for *Bd* than later life stages in our study area. We found a large amount of genetic variation and little to no genetic structure in the *Bd* sampled from amphibian hosts, suggesting that the pathogen has been present for some time and has little difficulty moving across the mosaic landscape of rural northwestern Pennsylvania. Studies using a similar combination of approaches in other systems hold promise for understanding whether the patterns we have documented in our study area hold for other aquatic communities and other fungal disease systems more generally.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00442-024-05642-8.

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Author contribution statement V.S and C.L.R.-Z. conceived and designed the study. V.S, M.E.B.O., A.Q.B and T.T.H. analyzed the data; C.L.R.-Z. provided material and financial support; V.S., L.A.B. M.E.B.O, T.T.H. M.E.B.O. and K.A.A. collected the data in the field and V.S and C.L.R-Z wrote the manuscript. V.S., M.K. and C.N. performed molecular work on eDNA samples. All authors edited and gave final approval of the submitted manuscript. The authors declare no conflicts of interest.

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Declarations

Competing interests The authors have not disclosed any competing interests.

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