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Partitioning the influence of host specificity in amphibian populations threatened by multiple emerging infectious diseases

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ABSTRACT

Amphibians face many challenges in their conservation, including threats from emerging infectious pathogens/parasites and habitat degradation. In diverse amphibian communities, where multiple emerging pathogens tend to co-occur, we know little about how the structural partitioning of host specificity impacts population maintenance despite disease. Here, we used field data from amphibian communities in north Florida to investigate host-specific traits influencing the prevalence, intensity, and transmission of three emerging pathogens of amphibians: *Batrachochytrium dendrobatidis* (*Bd*), Perkinsea (Pr), and Ranavirus (*Rv*). We found that *Bd* exhibited specificity for later developmental stages, and that overall infection patterns differed between ephemeral and semi-permanent sites and across seasons. For each pathogen, we identified key hosts overwhelmingly contributing to community transmission dynamics and found evidence of pathogen interactions that may facilitate *Bd-Rv* co-infections, and dilution effects of increased host diversity on Pr infection. Our findings confirmed that declining species within the region are routinely infected with emerging pathogens. However, the probability of infection depended on different habitat characteristics and associated host community composition. Thus, our study emphasizes the importance of identifying key and sensitive hosts that drive or succumb to infections in natural communities before reintroducing amphibians into the wild. This approach can help improve conservation efforts in diverse host communities as successful repatriation of sensitive species can benefit from detailed characterization of the established disease dynamics at the release site.

1. Introduction

Emerging infectious diseases increasingly threaten global biodiversity with the rapid expansion of virulent multi-host pathogens across the landscape (Daszak et al., 2000; Fisher et al., 2012; Jones et al., 2008). Under these novel selective pressures, conservationists have a pressing need to examine the outcomes and interactions of pathogen co-occurrence in diverse host communities (Brunner et al., 2015; Rachowicz et al., 2006; Scheele, 2019). For at-risk groups such as amphibians, multi-host pathogens comprise one of the major drivers of global population declines and species extinctions (Fisher and Garner, 2020; Luedtke et al., 2023; Mendelson et al., 2006; Stegen et al., 2017). The main causative agents—amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Scheele, 2019; Stegen et al., 2017), the protist parasite Perkinsea (Pr) (Chambouvet et al., 2020; Isidoro-Ayza et al., 2017), and viruses in the genus *Ranavirus* (*Rv*) (Brunner et al., 2015)—show extensive overlap in hosts and geographic range. Despite the wealth of literature that exists on the ecology of these pathogens, most

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research focuses on a single pathogen, leaving co-infections and multi-pathogen interactions underexamined (Bienentreu and Lesbarrères, 2020; Herczeg et al., 2021). The presence of multiple emerging pathogens in natural amphibian populations can generate a suite of novel host-pathogen-pathogen interactions that can drive infection trends and disease at the individual (Stutz et al., 2018) and community level (Longo et al., 2023). Identifying host and environmental factors associated to infection not only is critical for assessing future decline risk, but also to assign priority when developing conservation strategies in the face of climate change and disease (Grant et al., 2018).

Predicting disease outcomes remains a major challenge in the field of amphibian conservation because the factors influencing infection dynamics are often context dependent (Bienentreu and Lesbarrères, 2020; Blaustein et al., 2012). However, disease outbreaks in amphibian populations typically follow seasonal patterns that provide suitable abiotic conditions for either pathogen proliferation or an abundance of susceptible hosts, or both (Becker et al., 2012; Brunner et al., 2015; Forrest and Schlaepfer, 2011; Karwacki et al., 2018; Longo et al., 2010). In addition, host susceptibility can vary across development, species, and populations, resulting in infection patterns that disproportionately impact some groups (Becker et al., 2012; Blaustein et al., 2005; Hoverman et al., 2010; Langhammer et al., 2014; von Essen et al., 2020). This variable susceptibility drives heterogeneities in pathogen reproduction (Brunner et al., 2017), which are used to quantify host species contributions to the overall transmission and persistence of a pathogen in multi-host communities (Bielby et al., 2021; Fenton et al., 2015; Streicker et al., 2013), highlighting the importance of community composition in modulating infection risk (Longo et al., 2023; Martin et al., 2019).

Interactions among pathogens within co-infected hosts can modify the mechanisms of pathogenicity compared single infections (Johnson and Hoverman, 2012; Pedersen and Fenton, 2007), generating important consequences to host fitness, pathogen reproduction, and disease dynamics. Direct interactions occur when pathogens compete for a shared host resource, while indirect interactions are usually mediated by the host immune system and can be facilitative or inhibitory (Cox, 2001). Some co-infection studies in amphibians have shown evidence of facilitative interactions between Pr-*Rv* (Atkinson and Savage, 2023) and *Bd*-*Rv* (Ramsay and Rohr, 2023; Warne et al., 2016; Whitfield et al., 2013) while others observed no associations between pathogens (Bosch et al., 2020; Olori et al., 2018; Thumsová et al., 2023). However, limited sampling surveys of natural populations may not capture the entire range of phenological or environmental conditions that drive pathogen interactions. The global spread and range overlap of amphibian pathogens (*Bd*, Pr and *Rv*) stresses the need for researchers to adopt multi-pathogen approaches that integrate co-infections to accurately investigate future disease-related declines.

In North America, diseases caused by *Bd*, Pr, and *Rv* have caused mass mortality in both frog and salamander species (Davis et al., 2007; Green et al., 2002; Hall et al., 2018; Hartmann et al., 2022; Isidoro-Ayza et al., 2017; Landsberg et al., 2013; Vredenburg et al., 2010) and pathogen persistence in the environment remains a major obstacle in the conservation of threatened species (Adams et al., 2017; Hossack et al., 2020; Sutton et al., 2014). In the Southeast Coastal Plain of the United States, the decline of many endemic amphibian species (Enge et al., 2014; Farmer et al., 2017; Graham et al., 2010; Jensen and Richter, 2005; Means and Travis, 2007; Semlitsch et al., 2017) has prompted in- and ex-situ conservation efforts to restore depleted populations in the region (Hinkson et al., 2016; IUCN SSC Amphibian Specialist Group, 2022a, 2022b; Means et al., 2011). Some focal species of ongoing conservation efforts are fatally susceptible to Pr (Atkinson, 2016) and *Rv* (Hartmann et al., 2022) infection, and have not met the reintroduction success goals outlined by the IUCN Amphibian Conservation Action Plan (IUCN SSC Amphibian Specialist Group, 2022b). The failure of populations to re-establish and the unassessed impacts of multi-pathogen emergence in Coastal Plain amphibian communities warrants a thorough examination into the drivers of infection and disease in the region. Because transmission of *Bd*, Pr, and *Rv* is primarily waterborne, and Pr and *Rv* are more prevalent in larval stages (Atkinson and Savage, 2023; Duffus et al., 2015; Hall et al., 2018; Karwacki et al., 2018), the potential for pathogen co-occurrence is especially high in aquatic habitats. Additionally, increased prevalence for all three pathogens is associated with cooler months in the Southeastern US (Hall et al., 2018; Horner et al., 2017; Karwacki et al., 2018), posing an acute threat to specialist winter-breeding amphibians. The changes in habits and morphology during amphibian life history and the broad host range of amphibian pathogens present unique considerations when selecting optimal conservation strategies. Understanding the host and environmental traits associated with pathogen proliferation is needed to predict infection dynamics and mitigate disease risk for extant and reintroduced populations.

Here, we assessed the environmental and host factors associated with infection of three emerging pathogens in diverse Coastal Plain amphibian communities in the Florida panhandle. Sampling focused on communities with extant populations of Striped Newts (*Notophthalmus perstriatus*), a threatened salamander with compelling evidence of pathogen-mediated declines (Farmer et al., 2017; Hartmann et al., 2022). Leveraging survey data from a year-long study period, we sought to (1) identify the environmental factors, host species, and life history traits associated with *Bd*, Pr, and *Rv* infections, (2) quantify host species contributions to community-level infection dynamics, and (3) examine the patterns of coinfections and provide evidence of interactions among pathogens. We hypothesized that, in addition to environmental drivers, host life stage would be an important trait for pathogen transmission (Table 1), because all three pathogens exhibit varying degrees of specificity across amphibian development (Chambouvet et al., 2015; Hoverman et al., 2011; Langhammer et al., 2014). We also expected to see high host taxonomic specificity in Pr because infections overwhelmingly impact Ranid frogs (Atkinson and Savage, 2023; Chambouvet et al., 2015). Taxonomic specificity is generally broader in *Bd* and *Rv* infections (Duffus et al., 2015; Fisher and Garner, 2020), however; many studies report species-specific outcomes during infection outbreaks in the Southeastern US (Green et al., 2002; Hartmann et al., 2022; Horner et al., 2017; Landsberg et al., 2013). We predicted key hosts for a given pathogen type to be those with strong asymmetries from more than one transmission factor (Fig. 1). Finally, if pathogens are interacting within the host, we would expect to observe differences in the rates of co-infection and/or infection intensities in co-infected hosts compared to those infected with a single pathogen. Our findings provide a detailed baseline of the main drivers of infection for three pathogens impacting

Table 1
Shown are the expected development and taxonomic host factors that may predict infection risk by each pathogen type for amphibians in Florida. References supporting each predictor are provided in text.

Host predictor	Pathogen		
	Bd	Pr	*Rv*
Developmental	Post-metamorphs (Basanta et al., 2023; McMahon and Rohr, 2015)	Larvae (Chambouvet et al., 2015)	Larvae (Green et al., 2002; Hoverman et al., 2010) Paedomorphs (Hartmann et al., 2022)
Taxonomic	Hylid frogs (Gervasi et al., 2017) Newts (Hartmann et al., 2022; Rothermel et al., 2016)	Ranid frogs (Atkinson and Savage, 2023; Davis et al., 2007; Isidoro-Ayza et al., 2017)	Newts (Hartmann et al., 2022) Ranid frogs (Hoverman et al., 2011)

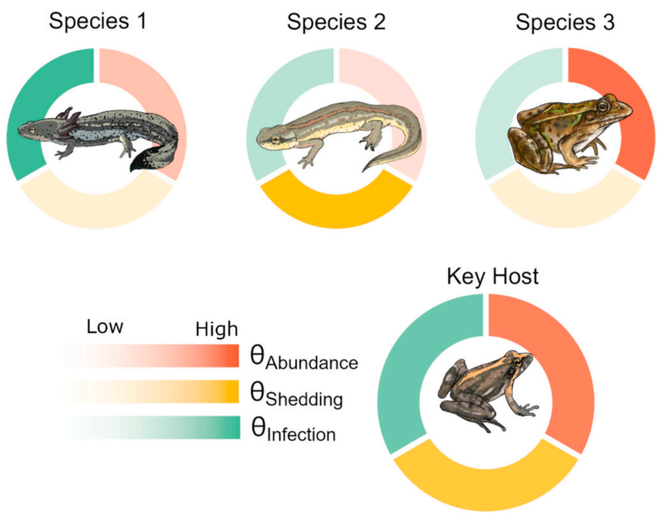

Fig. 1. Conceptual framework adopted from Streicker et al., 2013, illustrating how contributions to pathogen transmission can be quantified from the infection asymmetries among the host community. Mechanisms of transmission are variable, and can rely on super abundant, super-shedding, or super-infected hosts. Key hosts dominate pathogen transmission relative to other members in the host community by having higher values in one or more asymmetries.

amphibian communities in the Florida panhandle, expanding our understanding of the interactions that will need to be targeted to improve the fate of at-risk populations.

2. Materials and methods

2.1. Field site and focal species

We conducted our study at Livingston Place, a 3682-ha conservation property in Jefferson County, Florida, USA (30.626995°, −83.688406°; ~49 m ASL) that is owned and managed by Tall Timbers Research Station. Livingston Place is a working plantation that is heavily managed for bobwhite quail and other game species. It harbors a mosaic of upland pine forest, planted timber stands, and agricultural plots. The understory vegetation community is primarily "old field" due to historical cultivation. It is presently dominated by grasses and forbs and managed with frequent prescribed fire (~2-year return interval). There are approximately 109 freshwater isolated wetlands of various sizes, hydroperiods, and natural communities. Sampling was conducted in nine sites: two semipermanent wetlands consisting of a large (7-ha) semi-permanent depression marsh and a cypress dome, and eight ephemeral sites consisting of smaller depression marshes (Fig. 2). The regional climate is humid and subtropical, with seasonal patterns of precipitation and temperature that alternate between a warm rainy season from May to October, and a cooler dry season from November to April. (Winsberg, 2003). Average maximum temperatures can exceed 29 °C in the summer, and most of the annual precipitation occurs during the peak of the warm rainy season (June to September).

A total of 28 amphibian species occur in Livingston Place, representing a broad taxonomic diversity that includes five salamander families (Ambystomatidae, Amphiumidae, Plethodontidae, Salamandridae. Sirenidae) and five frog families (Bufonidae, Hylidae, Microhylidae, Ranidae, Scaphiopodidae). The property supports populations of regionally declining species such as the Striped Newt (*Notophthalmus perstriatus*), Ornate Chorus Frog (*Pseudacris ornata)* and Eastern Tiger Salamander (*Ambystoma tigrinum)* (Enge et al., 2014). All these species rely on waterbodies at some or all points in their life histories, and several are obligately or facultatively paedomorphic (*Siren lacertina*, *Pseudobranchus striatus*, *Ambystoma* spp., *Notophthalmus* spp.). Many members of this amphibian community are ephemeral specialists that depend on temporary pools to reproduce (e.g., *Ambystoma* spp., *Hyla gratiosa*, *H. femoralis*, *N. perstriatus*, *P. ornata*).

2.2. Study duration

From March 2021 to March 2022, we surveyed amphibian communities in wetlands if they held water. In 2021 we sampled twice in the cool/dry season (March, April) and twice in the warm-wet season (June and October). In 2022 we sampled twice in the cool/dry season

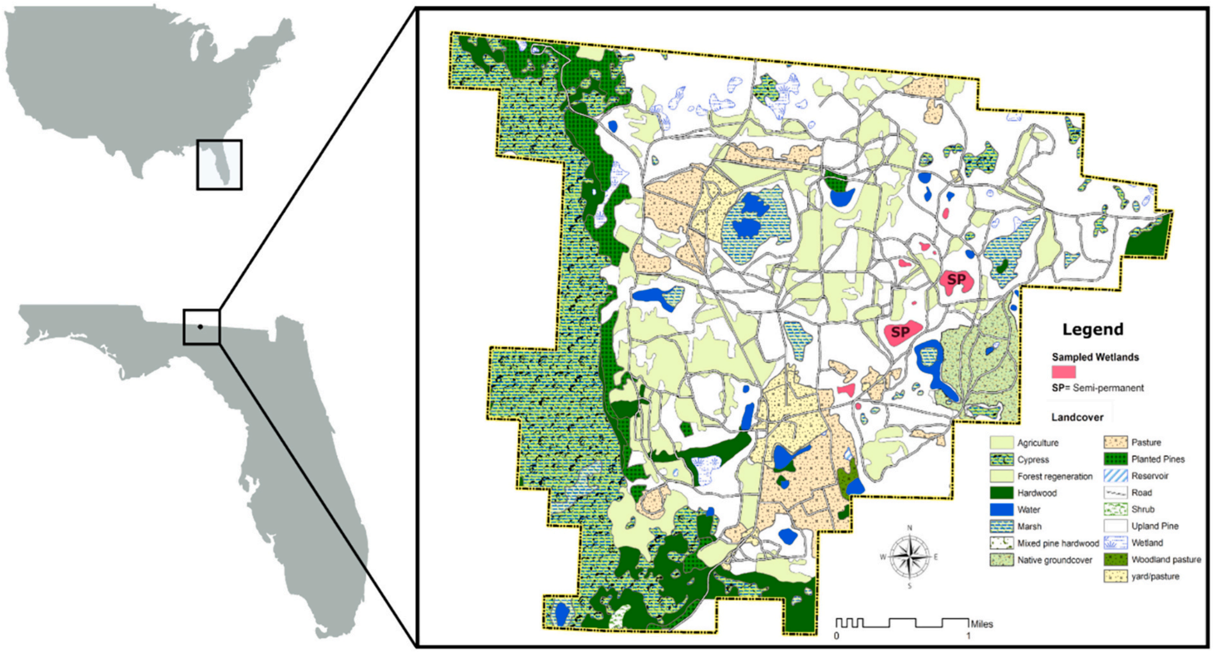

Fig. 2. Location of Livingston Place, Jefferson County, Florida, USA (30.626995°, −83.688406°). Inset map shows the boundary and various landcover types of the site. Surveyed wetlands are depicted in pink and semi-permanent sites indicated with SP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(February, March; Table S1). Due to the sporadic nature of their hydroperiods, not all wetlands could be sampled during each survey, except the semi-permanent marsh (site 48; Table S1).

2.3. Sampling protocol

Amphibians were captured opportunistically by hand or net. We concentrated our sampling efforts in the morning (approximately 09:00) to focus on aquatic stages. We placed each amphibian individually in an unused plastic bag before processing. Because our sampling includes primarily paedomorphic salamanders, we designated individuals into one of three developmental categories (pre-, mid-, or post-metamorphic) based on secondary sexual characteristics and external morphology (e.g., presence of gills, skin granularity) (Duellman and Trueb, 1994; Gosner, 1960). All obligately paedomorphic species were classified as pre-metamorphic, while facultatively paedomorphic species and anurans were categorized as pre-, mid-, or post-metamorphic depending on morphological traits. Pre-metamorphic stages were determined as frogs between Gosner stages 25–41, or presence of gills in salamanders; mid-metamorphic stages were determined as frogs between Gosner stages 42–46, or by the reduction of gills and increased granularity in salamanders; post-metamorphic stages were fully metamorphosed animals of either frogs or salamanders (including juveniles and adults).We swabbed the skin and oral surfaces following standard protocols for pathogen collection (Hyatt et al., 2007; Miller et al., 2015), using a single rayon-tipped sterile swabs (Medical Wire MW-113). Swab tips were placed in individual sterile 2.0 mL screwcap vials and stored at −20 °C until analyzed. Any abnormality, injury, or gross sign of disease was noted. To eliminate cross-contamination between animals, we used new pairs of nitrile gloves in between each animal, and to reduce introduction of pathogens between sites we decontaminated boots and equipment with 10 % bleach.

2.4. Pathogen quantification

We extracted DNA from swabs using Prepman Ultra (Applied Biosciences), following manufacturer protocols. To detect and quantify pathogen infection loads, we performed three individual quantitative PCR (qPCR) reactions for each sample, following the amplification conditions of Boyle et al., 2004 for *Bd*, Allender et al., 2013 for *Rv*, and Karwacki et al., 2018 for Pr. All assays were run against a standard curve of known pathogen quantities generated from serial dilutions of synthetic gene fragments (Integrated DNA Technology), ranging from 10^1 to 10^6 copies for *Bd*, 10^2 to 10^7 for *Rv*, and 10^2 to 10^8 for Pr. Negative controls for all assays consisted of UltraPure DNase/RNase-free water (Invitrogen). All assays were run in duplicate on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) for 50 cycles. For *Bd* and Pr, we considered a sample positive for infection if target gene copies were greater than zero for each reaction. Because *Rv* detection from swabs can be easily confounded by environmental persistence of viral particles (Miller et al., 2015), we only considered samples *Rv* positive if they amplified before the 40th cycle. If only one replicate amplified, we ran a third reaction and used the results to make the final infection determination. Infection loads represent the average quantification values of the replicate reactions.

2.5. Statistical analyses and modelling

We built separate binomial generalized linear models GLM (logit link) to assess the factors influencing infection probability for each of the pathogens using infection occurrence as the response. Each probability model included host and environmental covariates that have been shown to affect host pathogen dynamics. Covariates included co-infection by non-focal pathogens (3 levels), species (10 levels) and developmental stage of individual hosts (3 levels: pre, mid, or post-metamorphic), wetland type (2 levels: ephemeral or semipermanent), season (2 levels: cool or warm), and richness (number of species detected during a survey). We included two-way interactions between species and developmental stage, resulting in 79 possible combinations for each model of *Bd*, Pr and *Rv* probability. To assess factors associated with infection severity, we built separate Gaussian GLMs with log10 transformed pathogen copies (infection load) as the response variable. These models consider only infected hosts, and we included the following covariates: developmental stage, wetland type, co-infection by a non-focal pathogen, for a total of 16 possible combinations. Because of the smaller sample sizes, we did not include species or interaction terms to avoid overfitting the models. We generated and ranked models using the R package “MuMIn” (Barton and Barton, 2015), then selected the best model based on the lowest Aikaike information criterion corrected for small sample sizes (AICc; Burnham and Anderson, 2004). If competing models were within ΔAICc ≤2, we retained the model with the fewest explanatory variables. We tested the significance of categorical predictor variables of the top models using ANOVA followed by post-hoc Tukey's HSD tests. To conduct pairwise comparisons of categorical explanatory variables, we used the “pairs” function in the package “emmeans” (Lenth, 2021) to obtain the estimated marginal means and generate Tukey's HSD adjusted *p*-values to control for multiple comparisons.

Differences in habitat characteristics, such as hydroperiods, are important to understanding infection patterns of amphibian pathogens (Atkinson and Savage, 2023). Given that not all individual wetlands could be sampled each survey, we ran an additional GLM to assess whether infection counts were different between ephemeral and semi-permanent wetlands and used pairwise comparison to identify which pathogen counts were different among wetlands. Significance for all analyses was assessed at $\alpha \leq 0.05$. All statistical analyses and visualizations were performed in R version 4.1.1 (R Core Team, 2021).

2.6. Species contributions to overall infection dynamics

We estimated the contribution of each species (i) to community transmission dynamics (π) by quantifying asymmetries of three independent parameters (θ) related to infection: relative abundance (θ^A), infection frequency (θ^I), and pathogen shedding rate (θ^S). Differences in these parameters underlies infection heterogeneity among hosts in a community. The degree of asymmetry for host species *i* is calculated by dividing the observed value by the community average. For example, an asymmetry value of $\theta_i^I = 3$ represents a species with an infection frequency 3× greater than the community average.(Streicker et al., 2013). For each host species in the community, we calculated relative abundance (θ_i^A) using field survey count data, the frequency of infection from pathogen prevalence (θ_i^I) and pathogen shedding rate (θ_i^S) from qPCR values from swabs. We used qPCR-derived infection intensity as a proxy for shedding rates because there are positive associations between infection loads quantified from swabs and pathogen shedding rates for *Bd* (DiRenzo et al., 2014) and Rv (Brunner et al., 2019). We then calculated the relative contribution (π_i) of a host species to community transmission by dividing the product of the three asymmetries by the total number of species in the community: $\pi_i = \frac{\theta_i^A \theta_i^I \theta_i^S}{N}$. Therefore, π_i is proportional to the product of the three asymmetries, and $\pi_{i=}$ 0.6 would indicate a species was responsible for 60 % of transmission. We designated a host species as a “key host” for pathogen transmission if it contributed more to the total transmission than the remaining host community combined (i.e, where $\pi_i > 0.5$)(Streicker et al., 2013).

3. Results

3.1. Sampling results

In total, we sampled 298 amphibians representing 16 species over the study period (Table S2). Pathogen qPCR revealed 38 (12.8 %)

samples were *Bd* positive, 19 (6.4 %) were Pr positive, and 14 (4.7 %) were *Rv* positive (Table S2). Co-infections were rare with *Bd-Rv* being the most common ($n = 5$; Table S2), and we did not detect any sample infected with all three pathogens. Infection prevalence and mean loads were higher during cooler periods for all pathogens (Fig. 3a,b), and ephemeral and semi-permanent wetlands followed similar seasonal infection patterns (Fig. 3c). Of the nine species with sample sizes $\geq$15, *Bd* was not detected in *Ambystoma talpoideum* or *Pseudobranchus striatus*, Pr was not detected in *Acris gryllus* or *P. striatus*, and *Rv* was not detected in *Lithobates grylio* (Fig. 4a). We excluded infrequently encountered species ($n \leq 5$) from analyses that included species as an explanatory variable, retaining 292 samples across 11 species.

3.2. Model results

The top model for *Bd* infection probability included life stage and season as predictors (Table S3). We found a higher probability of *Bd* occurrence in the cool season ($t = 2.00$ $P = 0.046$; Table S4), consistent with our observations of higher *Bd* prevalence during cooler months (Fig. 3a). *Bd* infection occurrence varied significantly among developmental stages ($F = 54.96$, $df = 2$, $P < 0.0001$), where pre-metamorphic hosts were less likely to be infected than other stages (HSD; *Pre-Mid*: $t = -7.84$, $P < 0.0001$; *Pre-Post*: $t = -6.79$, $P < 0.0001$; Table S4, Fig. 4b). The best model for *Bd* load included life stage and wetland type as predictors, and both were significant ($P < 0.05$; Table S5). Post-hoc analyses revealed higher *Bd* loads in post-metamorphic stages (HSD; $t = -3.45$, $P = 0.0043$; Table S6, Fig. 4b), and lower loads in ephemeral wetlands ($t = -2.80$, $P = 0.0084$; Table S6).

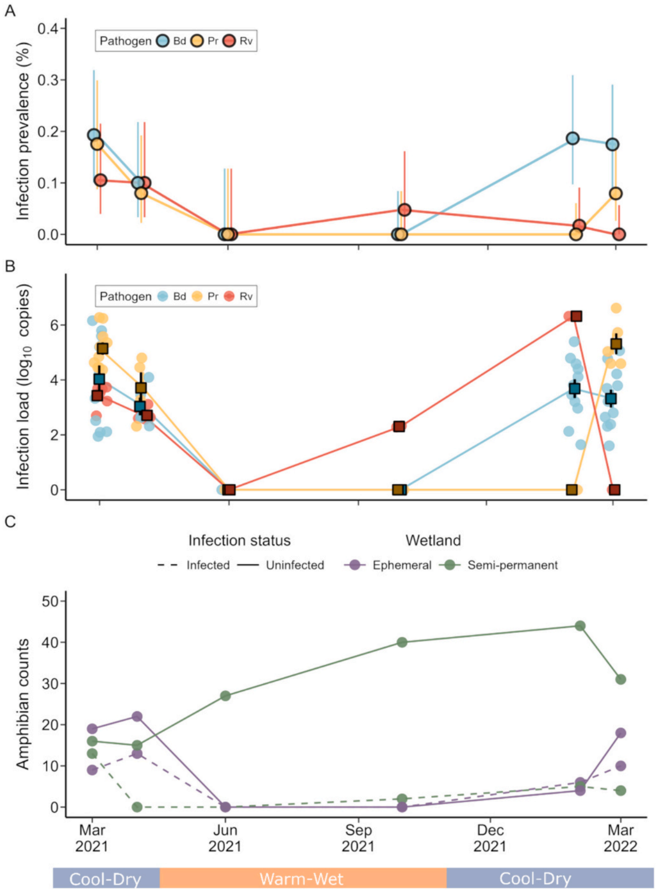

Fig. 3. Infection dynamics over the duration of the study for *Bd*, Pr, and *Rv*. (a) Points represent infection prevalence of each survey, colored by pathogen type, and lines indicate 95 % confidence intervals. (b) Lighter points represent individual infections loads; boxes represent the mean infection load for each survey with vertical lines indicating the 95 % confidence intervals. Points are colored by pathogen type, and all infection loads have been log10-transformed. (c) Total number of sampled amphibians by infection status and wetland type. Point shapes represent the number of infected (by any of the three pathogens) or uninfected amphibians for each sampling event, while colors indicate the wetland type. The bar at the bottom shows the general seasonal trend of the region with the cool-dry (November –April) period in blue and the warm-wet (May – October) in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The top model for Pr infection probability included species richness and season as predictors (Table S3), but only richness was significant and showed a negative association with Pr occurrence ($\beta = -0.2913$, $z = -2.957$, $P = 0.00311$; Table S4). The top model for Pr load did not include any predictors (Table S5).

The top model for *Rv* infection probability included *Bd* co-infection as a positive predictor of *Rv* infection ($\beta = 0.09$, $t = 2.680$, $P = 0.0078$; Table S3). The top model for *Rv* infection loads included only *Bd* co-infection, but this was not significant $P = 0.076$; Table S3).

Irrespective of pathogen type, amphibians sampled in the semi-permanent site were less likely to be infected than those in ephemeral sites ($\beta = 0.364$, $z = -6.865$, $P < 0.0001$, S.Fig, 1a), consistent with our count observations (Fig. 3c). Pairwise comparisons for infection types revealed a significantly higher likelihood of Pr infection in ephemeral sites ($\beta = 4.00$, $z = 2.140$, $P = 0.031$, S.Fig. 1b).

3.3. Host contributions to infection transmission

The highest contributor to *Bd* transmission was *Notophthalmus perstriatus* ($\pi = 0.53$, Fig. 5a), owing to the species' higher rates of zoospore shedding and abundance. *Notophthalmus viridescens* contributed the second most to *Bd* transmission ($\pi = 0.32$), and though their shedding rate was lower than *N. perstriatus*, they were similarly abundant and experienced higher rates of infection. Pr transmission was dominated by L. *sphenocephalus* ($\pi = 0.52$, Fig. 5b), which had high values for all three asymmetries. *Rv* transmission was dominated by *N. viridescens* ($\pi = 0.74$, Fig. 5c) owing to its abundance and high rate of viral shedding, despite a relatively low infection rate. When combining all asymmetries for all pathogens, we did not identify a key host for overall transmission (i.e, none with $\pi > 0.5$, Fig. 5d).

4. Discussion

By examining the infection trends of three emerging pathogens in a diverse amphibian community, we identified host and environmental associations that predict the risk of infection. Using field-collected metrics, we partitioned the contributions of each host species to the overall pathogen transmission of the host community, and identified species that may amplify or dilute infections, or serve as reservoirs that promote pathogen persistence. Understanding infection dynamics in natural systems is essential to inform disease mitigation strategies for species threatened by emerging pathogens, especially when the goal is to reintroduce animals into habitats where pathogens persist. Our study demonstrates the importance of host community on multi-pathogen dynamics by revealing differences in life stage and species specificity among pathogens, positing useful considerations for *N. perstriatus* reintroduction efforts.

4.1. Infections peak in the cool season

Emergence was synchronous across all pathogens, and we found higher probability of *Bd* and *Pr* during cooler months. This finding is consistent with most observations of *Bd* emergence, as the optimal growth of the fungus occurs below 28 °C (Forrest and Schlaepfer, 2011; Piotrowski et al., 2004). We found that *Rv* loads decreased in the warm season, conflicting with previous studies where higher *Rv* prevalence and infection loads are observed in the summer (Hall et al., 2018; Olori

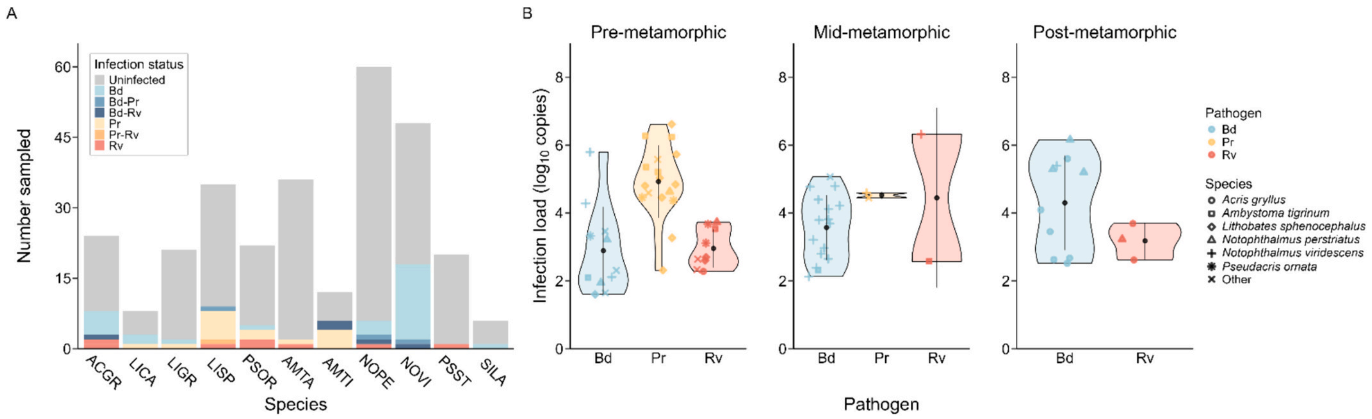

Fig. 4. Species and developmental associations of pathogen infection. (a) Stacked plot represents the infection prevalence for the 11 most abundant species, colored by infection status. Species on the x-axis are abbreviated (ACGR = *Acris gryllus*, LICA = *Lithobates catesbeianus*, LIGR = *Lithobates grylio*, LISP = *Lithobates sphenocephalus*, PSOR = *Pseudacris ornata*, AMTA = *Ambystoma talpoideum*, AMTI = *Ambystoma tigrinum*, NOPE = *Notophthalmus perstriatus*, NOVI=*Notophthalmus viridescens*, PSST = *Pseudobranchus striatus*, SILA = *Siren lacertina*). (b) Scatterplots show the distribution of infection intensities across developmental stages in sampled amphibians. Shapes represent the six most frequently infected species, while the remaining species have been grouped as "Other". Points are colored by pathogen type and shapes represent host species. Infection intensities are log10-transformed, and plot has been horizontally jittered to reduce point overlap.

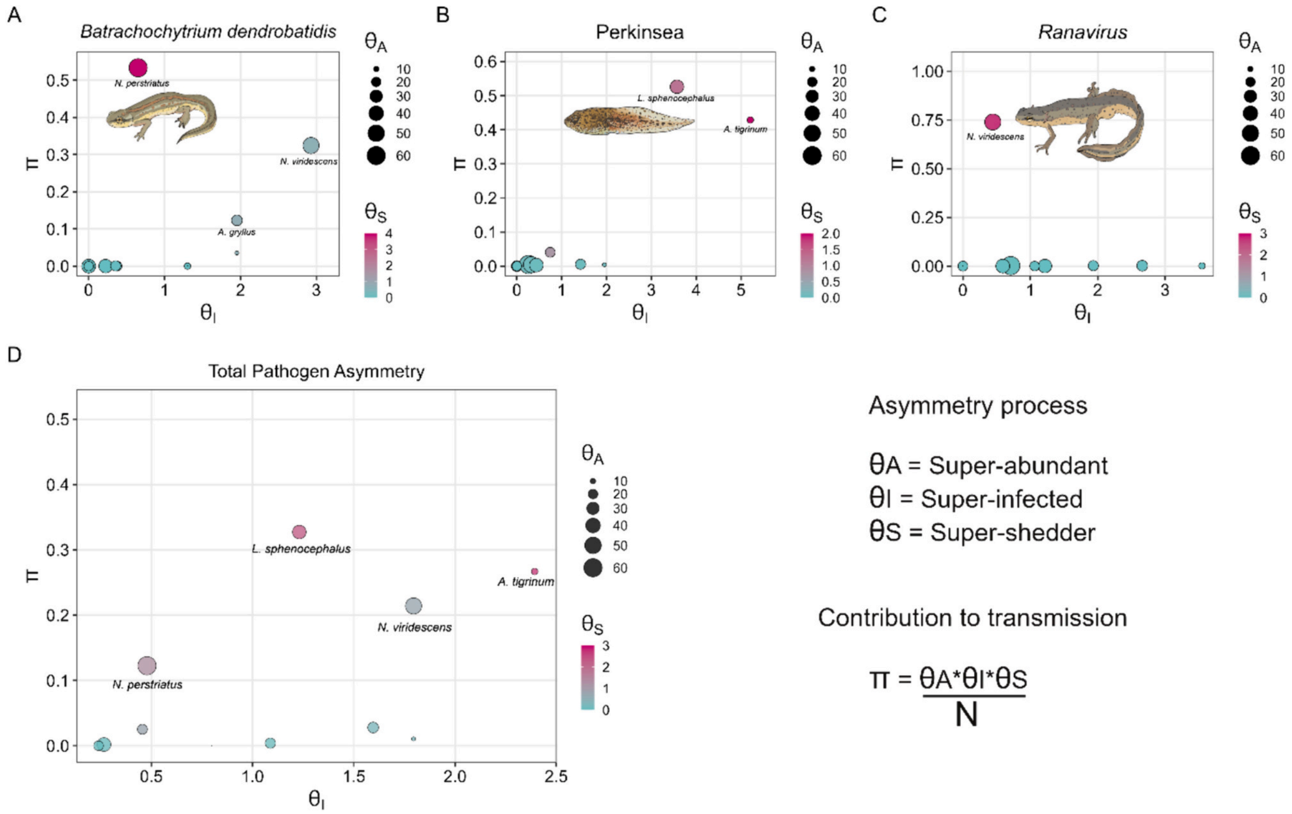

Fig. 5. Species-level asymmetries of abundance, rate of infection, and pathogen shedding determine a species contribution (π) to the overall infection dynamics of the community. Points represent each species contributions to π, point sizes increase with abundance, and colour represents pathogen shedding rate (pink is more intense pathogen shedding, blue is less intense shedding). Points with species names indicates $\pi \geq 0.10$, and illustrations represent the key hosts ($\pi > 0.5$) for each pathogen type. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2018; Rosa et al., 2017; von Essen et al., 2020). Most of these studies come from temperate regions, and the few that examine multi-pathogen dynamics suggest patterns of asynchronous *Bd-Rv* emergence are due to disparities in suitable growth conditions between pathogens. However, as our system is in a hot, subtropical environment, the temperatures of "cooler" months in Florida may be more like those of summer in temperate regions. For example, the average summer (June to August) high temperatures ranged from 23 to 27 °C for a *Bd-Rv* study in Portugal (Rosa et al., 2017), while the average monthly high for our sites ranges from 20 to 27 °C during the cool season (Florida Automated Weather Network). The significant drop in pathogen prevalence we observed during the warm season corroborates recent studies of Pr-*Rv* dynamics in peninsular Florida (Atkinson and Savage, 2023), suggesting that these infection patterns are typical of warm sub-tropical amphibian communities. Future studies should quantify the optimal temperature limits of hosts and pathogens as this information can improve the accuracy of climate change models and predicted disease risk. Warm-adapted hosts, such as those occurring in Florida, can experience higher susceptibility under unusually cooler temperatures as predicted by the thermal mismatch hypothesis (Cohen et al., 2019), which may explain recent disease-induced mortality in the region (Hartmann et al., 2022). Overall, our study stresses the need to increase multi-pathogen surveillance across a wide variety of climatic zones to uncover the suite of context-dependent infection outcomes in natural populations.

4.2. Pathogens show different specificities

Specificity for certain hosts can reduce competitive interactions between pathogens and can promote pathogen coexistence (Seabloom et al., 2015). We found heterogeneity in pathogen infection across species and life stages, supporting the hypothesis that infection associations are host specific. In temperate systems *Bd* infections are more commonly detected in post-metamorphic stages than in larval aquatic stages (Bosch et al., 2001, 2023). Similarly, we found that *Bd* was more prevalent during cooler months and mid- and post-metamorphic stages experienced more frequent and intense infections, as we had expected. Furthermore, the vast majority of *Bd* infections occurred in only three species (*N. perstriatus*, *N. viridescens*, and *A. gryllus*), coinciding with the breeding and metamorphosing period for *Notophthalmus* species. The predominantly aquatic lifestyle and periods of high activity in winter and spring, when temperatures favor *Bd* growth, may explain why *N. viridescens* in this and other regions, is frequently infected with *Bd* (Raffel et al., 2010; Rothermel et al., 2008). Conversely, *A. gryllus* are a widespread generalist species that can be found year-round, and are known to harbor both *Bd* and *Rv* infections (Hartmann et al., 2022), potentially serving as a reservoir in this system.

Among salamanders, the facultatively paedomorphic *Notophthalmus* species were frequently infected with *Bd*, while we detected only a single infection in obligately paedomorphic salamanders in the family Sirenidae, supporting developmental and phylogenetic signals of *Bd* specificity. Obligately paedomorphic species do not metamorphose and retain larval traits throughout their lives, including larval-type skin with mucus-secreting Leydig cells and low keratin (Brown and Cai, 2007; Jarial, 1989), which may limit the establishment of keratophilic pathogens like *Bd* in the epidermis.

Our results corroborate other studies identifying larval Ranid frogs as highly susceptible to Pr infection (Atkinson and Savage, 2023; Chambouvet et al., 2015), with most infections detected in *Lithobates sphenocephalus* tadpoles. While anuran susceptibility to Pr has been examined across several families (Chambouvet et al., 2015), the susceptibility of salamanders is not well established, and our findings of Pr infected *A. tigrinum* indicates a much broader host range for Pr than previously known. Interestingly, *A. tigrinum* were more often infected with Pr than other salamanders, including the closely related Mole Salamander, *A. talpoideum*, suggesting a difference in host traits or habitat that may increase or modulate susceptibility in salamanders. Throughout their ranges, *A. tigrinum* and *A. talpoideum* exhibit facultative paedomorphosis and are major predators of larval amphibians, including conspecifics (Holomuzki and Collins, 1987), providing a route of Pr infection via ingestion of infected prey (Chambouvet et al., 2015). However, in this system *A. tigrinum* are restricted to ephemeral sites and do not develop paedomorphic stages, while *A. talpoideum* are found in both semi-permanent and ephemeral sites and frequently develop as paedomorphic adults. We hypothesize that these findings may suggest increased disease infection risk in ephemeral sites, or that paedomorphs may have more developed immune functions compared to larvae, and thus are better equipped to fend off potential parasite infections. Future studies should examine the susceptibility of salamanders to Pr compared to anurans, specially taking into consideration the alternate developmental routes of facultative and obligate paedomorphosis.

4.3. Co-infection interactions are likely context-dependent

Despite similar seasonal infection trends for all three pathogens, we found few instances of co-infection, which may further underscore the importance of host specificity in diverse amphibian systems. Contrary to other studies (Bosch et al., 2020; Olori et al., 2018; Warne et al., 2016; Whitfield et al., 2013), our findings indicate that *Rv* occurrence positively associates with *Bd* infection (Table S4), but these co-infection associations are likely dependent on host factors, such as life stage (Fig. 4a,b). Hosts are known to exhibit different infection patterns across life stages, disproportionately contributing to the maintenance of *Bd* (Bielby et al., 2021; Fernández-Beaskoetxea et al., 2016; Narayan et al., 2014) and *Rv* (Brunner et al., 2004, 2017). *Bd*-*Rv* co-infections were detected in only four species and primarily in metamorphosing individuals, probably due to the different life stage affinities of the pathogens. While some studies found no synergistic link between *Bd* and *Rv* infection dynamics, these studies occurred in either tropical (Warne et al., 2016; Whitfield et al., 2013) or temperate regions (Bosch et al., 2020; Olori et al., 2018). Our study is the first to examine subtropical amphibian communities with year-round amphibian activity and across different life stages and developmental modes (e.g., metamorphic, obligate and facultatively paedomorphic). Lethal and sublethal effects of infection with *Bd* (Bielby et al., 2015; Hanlon et al., 2015) or *Rv* (Brunner et al., 2004; Kirschman et al., 2018; Langwig et al., 2015) in amphibian larvae may occur and carry over into post-metamorphic stages, but the effects are less clear for developmental modes where metamorphosis does not occur or is delayed. The synergism between *Bd* and *Rv* suggests that these effects may be exacerbated by the presence of co-infections, or sequential infections.

We did not observe cumulative or amplified patterns of infection load between any of the three pathogens, such as those reported for Pr-*Rv* dynamics (Atkinson and Savage, 2023). However, our study period extended just over one year, and continued surveys may reveal different patterns over longer time scales. Altogether these findings highlight that despite possible synergistic interactions between pathogens, negative host outcomes do not always occur when multiple pathogens are enzootic in a system. Data from these natural populations showed that host traits (species, development) and environmental (temperature) covariates may be more important when predicting infection status or load than co-infection for most pathogens, demonstrating the importance of ontogeny and environment in modulating host-pathogen dynamics. The mechanisms driving infection proliferation across life stages may rely on the shifts in immunological function (Rollins-Smith, 1998) or microbial composition across amphibian development (Hartmann et al., 2023). Acquired immunity responses to pathogens have been examined primarily in the context of *Bd* infection. Thus, future immunological studies should expand to other pathogens and co-infections to uncover mechanisms that drive pathogen interactions and the resulting outcomes at the host level (Smith and Holt, 1996).

4.4. Key hosts and community-level dynamics can inform conservation efforts

Understanding phylogenetic and developmental signals that predict infection consequences can be applied to conservation efforts. Our results can be expanded to predict which species, life stages, or habitats, might be more susceptible to a given pathogen depending on their developmental routes, habitat, or phylogenetic placement. These predictions can guide species suitability assessments for reintroduction efforts before disease screenings of wild populations occur, or at times when funds are not available for molecular diagnostics. Additionally, our findings show that amphibian pathogens in the Southeastern US follow seasonal peaks in prevalence, disproportionately affecting ephemeral communities. Our approach can be used to inform the release schedule of captive-reared individuals by targeting periods of the year where infection risk is low, or to prioritize in-situ conservation efforts by habitat type. Ideally, community-level disease analyses should be integrated during reintroduction planning to predict possible outcomes for the repatriated species and the recipient community. For example, a highly susceptible species released into a site where a pathogen is prevalent will not only fail to establish, but it may also amplify transmission to other species within the community. Furthermore, the community composition should be considered when selecting for release sites, specifically avoiding sites with high abundances of infection-key hosts, since a single host species can maintain annual pathogen persistence (Bielby et al., 2021; Wilber et al., 2020).

Determining the key species that drive infection transmission can also help to understand past population declines and current distributions of hosts and pathogens. For example, *Bd* epizootics impacting amphibian communities in Panama were initially amplified by the presence of a super-abundant and super-shedding key host species, *Atelopus varius*, then declined after *A. varius* was extirpated (Longo et al., 2023). We found that *Bd* transmission was dominated by *Notophthalmus perstriatus* via high shedding loads, which is useful for understanding how ongoing captive releases of this species might impact other amphibians in the release sites. Ranavirosis is likely a driver of *N. perstriatus* declines (Hartmann et al., 2022), and syntopic species that amplified infection transmission may have hastened the extirpation of some populations. The sites in this study represent one of the few extant populations of the western genetic unit of *N. perstriatus* (Farmer et al., 2017), and we observed the closely related *Notophthalmus viridescens* was a major contributor to *Rv* infection dynamics in this system. *Notophthalmus viridescens* is known to be a highly competent host of *Bd* and *Rv* throughout its range (Duffus et al., 2008; Rothermel et al., 2016), providing support for its role as a disease amplifier. Interestingly, eastern populations of *N. perstriatus* do not overlap in habitat with *N. viridescens*, and *Rv* outbreaks in these populations also affect locally abundant Gopher Frogs, *Lithobates capito*, which may serve as amplifiers of *Rv* infection (Hartmann et al., 2022, 2024).

Our examination of host contributions to transmission is by no means exhaustive, but it does offer a way to compare the mechanisms that generate patterns infection across diverse communities of hosts and pathogens. By identifying key hosts of a pathogen within a particular site or host community, we can better understand infection risk to guide disease mitigation strategies. Considering the vast evidence indicating that the composition of species assemblages alters infection dynamics, pathogen surveys should quantify how changes in host abundance and susceptibility are causing new outbreaks and contributing to species declines. Quantifying infection asymmetries in a community would require little effort as these models are parameterized with data that is routinely collected during pathogen surveys (abundance, infection load, and prevalence), and can help to uncover trends and key players in host-parasite dynamics. Despite evidence that these pathogens have co-occurred for >100 years (Karwacki et al., 2021), the Southeastern US has many examples of enigmatic amphibian declines that are consistent with range-wide disease outbreaks (Graham et al., 2010; Maerz et al., 2015). Conservation efforts must transition from individuals and populations to improve the likelihood of persistence of the entire amphibian community, whether species are considered vulnerable, threatened, or of least concern.

4.5. Wetland characteristics and host diversity

The physical characteristics of habitats largely determine the composition of the associated amphibian community, which in turn drives spatial patterns of infection. Semi-permanent sites supported higher species diversity than ephemeral sites, while also experiencing far fewer infections, possibly due to dilution effects. Dilution effects resulting from high host diversity can dampen pathogen transmission by increasing variability in the infection process (Keesing and Ostfeld, 2021). Similar to determining key hosts, the magnitude of dilution relies on variable infection competence and abundances of host species within a community (Keesing et al., 2010), and shifts in the composition of the community can reduce infection risk for even the most competent hosts (Johnson et al., 2013). In our system, Pr occurrence decreased as host diversity increased. The key host of Pr transmission, *L. sphenocephalus*, was present at all sites, yet Pr infections were more frequent in the relatively species-poor ephemeral wetlands (Table S2). This suggests that wetland qualities can influence infection dynamics by structuring the community composition of amphibian hosts, which can modulate the pathogen-amplification of competent hosts depending on the other hosts in the community. However, the presence/abundance of a key host may not be the only factor that helps predict Pr outbreaks. Perkinsea zoospores can persist in harsh environments and remain viable in the soil long after ponds have dried (Cook, 2008), which may explain the more frequent Pr infections we observed in ephemeral wetlands (S. Fig. 1b). This underscores the need of future work to disentangle the combined environmental and host factors that drive spatially and temporally heterogeneous patterns of infection.

5. Conclusion

Amphibians face great challenges to their survival and the emergence and persistence of different pathogens can halt any hope of reintroduction. Repatriation of extirpated species is a costly and often last resort of conservation that should be informed by detailed quantification of potential threats, including pathogens such as *Bd*, *Rv*, and Pr. Although there are no easy solutions to solving the threats posed by amphibian pathogens, assessing communities and environments through time can identify traits and associations that best predict infection risk, providing a comprehensive and dynamic framework to determine the best strategy for a species. We must consider the *which*, *when*, and *where*: which life stages are suitable candidates for release, when is the optimal time to release them, and where will they have the best chances of survival? Our community-level approach highlights the infection metrics that are most important to understand disease dynamics and can be easily implemented in any system.

Ethics statement

Animal sampling was carried out under the permission of the Florida Fish and Wildlife Conservation Commission (FWC-LSSC-17-00031B; LSSC-21-00056A) and the University of Florida's Institutional Animal Care and Use Committee (#201810502; #202100000012).

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CRediT authorship contribution statement

Arik M. Hartmann: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kimberly Sash:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation. **E. Pierson Hill:** Writing – review & editing, Investigation, Conceptualization. **Natalie Claunch:** Writing – review & editing, Investigation. **Max L. Maddox:** Writing – review & editing, Investigation, Formal analysis. **Sarah McGrath-Blaser:** Writing – review & editing, Investigation, Formal analysis. **Cory C. McKinstry:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Robert J. Ossiboff:** Writing – review & editing, Methodology, Visualization, Investigation, Formal analysis. **Ana V. Longo:** Writing – review & editing, Supervision, Resources, Project administration, Methodology,

Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data will be deposited into a repository, and a Github link to the scripts for analyses will be made available upon submission.

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Appendix A. Supplementary data

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