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SANTA CRUZ

LOCAL TO GLOBAL PATHOGEN AND HOST DYNAMICS OF AN EMERIGING FUNGAL DISEASE, WHITE-NOSE SYNDROME

A dissertation submitted in partial satisfaction
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By

Joseph R. Hoyt Jr.

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The Dissertation of Joseph R. Hoyt Jr.
is approved:

_____
Professor A. Marm Kilpatrick, Chair

_____
Professor Bruce Lyon

_____
Professor Winifred F. Frick

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Tyrus Miller
Vice Provost and Dean of Graduate Studies

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Content Pages

Previous published work:

The text of this dissertation includes reprints of the following previously published material:

Hoyt, J.R., K.E. Langwig, K. Sun, G. Lu, K.L. Parise, T. Jiang, S. Yang, W.F. Frick, J.T. Foster, J. Feng, A.M. Kilpatrick. 2016. Host persistence or extinction from emerging infectious disease: insights from white-nose syndrome in endemic and invading regions. Proceedings of the Royal Society: B. 283: 20152861

Hoyt J.R., Sun K., Parise K.L., Lu G., Langwig K.E., Jiang T., Frick W.F., Kilpatrick A.M., Foster J.T., Feng J. 2016 Widespread Bat white-nose syndrome fungus in northeastern China. Emerging Infectious Diseases. 22(1) 140-142

The candidate led the research, performed the analyses, and wrote the papers listed above, with input and from all co-authors.

Abstract:

Joseph R. Hoyt Jr.
Local to global pathogen and host dynamics of an emerging fungal disease, white-nose syndrome

Emerging infectious diseases present a major threat to wildlife populations and have the ability to drive once common species towards extinction. Increasing globalization has resulted in accelerated change in climate, increased anthropogenic movement, and land-use alterations leading to the emergence of infectious diseases in both humans, agriculture and wildlife. Studying disease dynamics at different contexts and scales can provide insight into alternative levers of conservation action. White-nose syndrome, a disease of hibernating bats, was first detected in single tourist cave in northern New York. *Pseudogymnoascus destructans* the fungal pathogen responsible for WNS has since spread across much of eastern North America causing the collapse of hibernating bat populations. *P. destructans* was likely introduced to North America from Eurasia, where it is widely distributed, and has likely been present for thousands of years. The data in this dissertation provide insight into the factors determining temporal variation in mortality from WNS. In addition, we will also provide insight into the mechanisms that contribute to species differences in pathogen transmission. More broadly this research provides a synthesis of data across multiple WNS disease contexts, and highlight the substantial conservation insight that can be gained through this approach.

Illuminating transmission: hidden contacts link networks in populations of hibernating bats

Joseph R. Hoyt[a*], Kate E. Langwig[ab], J. Paul White[c], Heather Kaarakka[c], Jennifer Redell[c], Allen Kurta[d], John DePue[e], William H. Scullon[e], Winifred F. Frick[a], and A. Marm Kilpatrick[a]

[a] *Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, CA 95064, USA*

[b] *Department of Biology, Virginia Polytechnic Institute, Blacksburg, VA, 24060*

[c] *Wisconsin Department of Natural Resources, Bureau of Natural Heritage Conservation, Madison, WI 53707, USA*

[d] *Department of Biology, Eastern Michigan University, Ypsilanti, Michigan, USA*

[e] *Michigan Department of Natural Resources, Iron Mountain, Michigan, USA*

**Corresponding author: hoytjosephr@gmail.com*

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Abstract:

Estimating the rate of infectious contacts among individuals and between species is a fundamental component in understanding, predicting, and controlling epidemics. However, accurately measuring infectious contact rates is extremely difficult for a wide range of directly transmitted pathogens, and most methods miss infrequent contacts that link social groups. We constructed contact networks using direct observations of social behavior in multispecies communities of hibernating bats. We then examined transmission of a surrogate pathogen (different colors of ultraviolet fluorescent dust (UVF-dust)), among bats to elucidate possible transmission networks of a directly transmitted disease, white-nose syndrome. Transmission of UVF-dust uncovered vast numbers of hidden links among social groups of bats that increased the connectedness among individuals and between species by an order of magnitude compared with contact metrics based on direct observations of social groups. These UVF-dust epidemics uncovered the pathways of transmission observed during the invasion of the fungal pathogen causing white-nose syndrome. More broadly, these results illustrate how cryptic social interactions can have dramatic effects on disease dynamics, and emphasize the importance of capturing uncommon or indirect links among social groups.

Introduction:

The rate of infectious contacts among hosts are driven by a complex mixture of host, pathogen and environmental factors [20] and are fundamental components in describing disease systems [21]. Sociality directly influences spread of pathogens, by structuring interactions between individuals within a population [40, 41]. Enormous efforts have been undertaken to characterize contact rates and social networks to understand and predict patterns of pathogen spread [42, 43]. These studies have highlighted the importance of heterogeneity among individuals [44-46], the role of networks in structuring and limiting epidemics [47, 48], and the variation among species in social interactions and pathogen infection [49, 50]. However, accurately predicting pathogen dynamics requires quantification of contact rates and social networks, and measuring infectious contact rates is a fundamentally difficult task due to uncertainties in the types of contact that lead to infection, and challenges in observing and quantifying uncommon or indirect contacts.

Contact rates and networks can be characterized using a variety of techniques, but each of these methods has strengths and weaknesses in terms of easily and accurately capturing infectious contacts within and between social groups in ways that can be used to understand and predict future epidemics. These methods include contact tracing surveys, usually collected over short time periods [43, 51, 52]; surveys of sexual habits [53-55]; household disease surveys [56, 57], observation of social group [58]; GPS or proximity transmitters [59-61]; and the use surrogate pathogens or phylodynamic reconstructions post-outbreak [62, 63]. Short-term contact surveys and social group studies (e.g. family groups, school classrooms) are often relatively easy to conduct, but they may miss important contacts between individuals in different social groups, links between individuals with infrequent, transient, or indirect connections (e.g. taxi drivers and customers, strangers sharing public spaces during transportation, contact with a shared environmental reservoir) [64]. For pathogens with long infectious periods or significant environmental reservoirs short-term survey techniques may do a particularly poor job at characterizing infrequent or

indirect contacts among social groups [65]. Estimation of contact rates based on GPS or proximity collars can sometimes capture these contacts between members of different social groups, but may still underestimate them unless the whole population is marked. In addition, it is difficult to define a potentially infectious contact using location-based methods [66, 67]. Retrospective measurements of pathogen spread using molecular tracing, [68] contact tracing via interviews post-outbreak [51, 64], and model estimation of parameters [46], can be more accurate and complete. However, missing information of all cases and deaths can create difficulties in recreating transmission chains, and circumstantial or epidemic-specific details can limit inference to other settings [20, 69]. Thus, a significant challenge in predicting epidemics is the empirical estimation of infectious contacts among hosts and the environment.

Bat communities provide an ideal system in which to examine how contacts within and between social groups influence subsequent pathogen dynamics[3]. Contact among individuals bats of multiple species can be easily characterized during winter by quantifying clusters of individuals and clustering behavior differs substantially among bat species and individuals [3, 70-72]. Bats spend the great majority of their time (>95%) in these clusters, just as most animals (including humans) primarily interact with a relatively small group of individuals, making these clusters comparable social units [41, 60, 61]. However, hibernating bats occasionally arouse from torpor and contact individuals in other clusters, and may also contact shared environmental spaces. The extent to which infrequent movement among social groups, and indirect contact via the environment influence transmission of pathogens among hibernating bats has not been quantified, and is of substantial interest, due to the recent emergence of the fungal disease white-nose syndrome.

White-nose syndrome (WNS) has decimated hibernating bats [3, 26], as it has spread across North America. The fungus causing WNS, *Pseudogymnoascus destructans*, was first detected in the North America in 2006 [22], and has since spread from New York to over half of North America and recently to the Western

U.S. [23]. White-nose syndrome has killed millions of bats and extirpated species from many sites [3, 73]. Infection with *P. destructans* occurs primarily during the winter when bats hibernate and cool their body temperature to near ambient for several weeks at a time, allowing the fungus to grow on and into their epidermal tissue [34, 74]. Transmission of the fungus can occur through direct contact among bats, or indirectly via the environment (Fig. 1). Infection results in a cascade of physiological effects, disrupting homeostasis, and eventually resulting in death [36, 37].

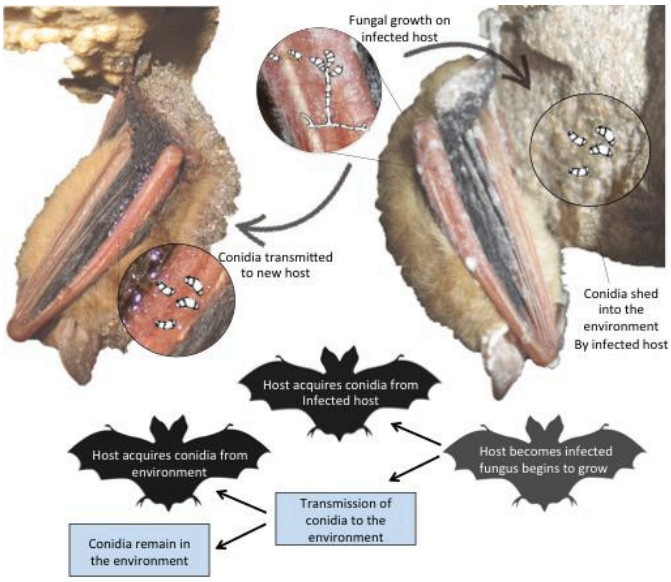

Figure 1: Diagram of *P. destructans* transmission in bats. Hosts acquire infection through direct contact with either other infected bats or the environment. If suitable growth conditions are present, the fungus then grows on and into the epidermal tissue. As the fungus grow, it forms conidia and other structural elements, which can be shed into the environment, reinfect the same host, and/or be transmitted to a new host through direct contact.

Differences in social behavior among species appear to play a role in transmission and impact of the fungus, but rapid mortality and frequent colony extinction in a highly solitary species challenge simple explanations for local within-species pathogen transmission [3], as is the case in other systems [75]. Transmission differs substantially among bat species in the year of pathogen invasion [38], with infection prevalence in two species (*Myotis lucifugus* and *Myotis septentrionalis*) increasing from <5% to 100% over a four month period while prevalence in two others (*Perimyotis subflavus* and *Eptesicus fuscus*) rose from <5% only to 40% [38]. Interestingly, the two species that roost solitarily (*Myotis septentrionalis* and *Perimyotis subflavus*) have very different transmission patterns, suggesting that

contacts among social groups (and possibly between species) plays a key role in transmission dynamics.

Here, we examine the role of within and between social group contacts in influencing pathogen transmission. We quantified social contacts by measuring physical contacts and social group sizes (clusters) of four species of hibernating bats in eight winter bat colonies. We examined between-social group contacts by tracing the spread of seven different colors of a ultraviolet-fluorescent dust (UVF-dust) applied to seven individual bats per site, as repeated surrogate pathogen invasions for the fungus causing WNS. We hypothesized that there would be significant differences between observed and unobserved social behavior, both among species and individuals, potentially explaining the high transmission of *P. destructans* in solitary individuals and species.

Methods:

Study Sites and Sampling for P. destructans

We studied contact rates, networks and fungal infection with *P. destructans* in bats at eight abandoned mines in Wisconsin and Michigan over two winters (Table S1; 2013/2014 and 2014/2015). Mines were previously excavated for minerals including lead, copper, and graphite [76].There were between 18-624 total bats, of 3 or 4 species hibernating at each site (Table S1). Bats in this region begin hibernating in October and leave hibernacula in April-May. We visited each site twice during each winter, in November and March, counted all bats by species, and sampled up to 20 of each species for the fungus, *P. destructans*, using a previously described swab sampling technique [34, 77]. Samples were placed in RNAlater (Thermo Fisher Scientific), and subsequently tested for *P. destructans* by qPCR [34, 78].

Quantifying contact rates and transmission networks

We quantified connections among hibernating bats in three ways, bats physically touching each other, bats hibernating in a cluster, and bats that shared a surrogate pathogen (a unique color of UVF-dust. The first two measures, close

contacts and social groups, are comparable to measures in other systems, because bats spent >99% of the winter hibernating in these conditions. The number of individuals detected with each UVF-dust color provides an estimate of the total epidemic size resulting from a single (dusted) individual and include both direct bat-to-bat and indirect (bat-environment-bat) contacts (Fig. 1).

In early winter, we applied a unique color of UVF-dust to each of 4-7 individual male bats at each site (with one exception; see below). There were seven unique colors: ECO-11 Aurora Pink, ECO-15 Blaze Orange, ECO-16 Arc Yellow, ECO-17 Saturn Yellow, ECO-18 Signal Green, ECO-20 Ultra Violet, ECO-19 Horizon Blue; Risk Reactor: DFSB-C0 Clear Blue (Day-Glo Color Corp., Cleveland, OH, USA; Risk Reactor Inc., Santa Ana, CA, USA). We liberally applied UVF-dust to the entire dorsal and ventral surface of each bat except for the head, and placed a unique band on each bat for identification. We returned to each site during March, and mapped out the location of all bats, including which bats were physically touching each other and which bats were in a cluster (a group of bats touching each other). We visually inspected each bat for UVF-dust using a UV flashlights (395nm; Hayward, CA, USA) and visible light. Each color of UVF-dust observed on each bat was recorded. We also quantified the area of each patch of UVF-dust, by color, in the hibernacula environment to the nearest square centimeter.

The species and number of bats dusted at each site varied depending on species composition and number of bats present, but included three species, *M. lucifugus, M. septentrionalis, and P. subflavus* (Table S1). At six sites, we dusted 3 individuals of one species, and four individuals of another. At one sites site, we dusted four individuals of a single species, *P. subflavus* because the abundance of other species was too low (a single bat). (Table S1). At the eighth site, we dusted five *M. septentrionalis* with five unique colors and five *M. lucifugus* with a single color (Table S1).

Analyses

We quantified the fraction of the bats of each species at a site that were connected to each focal bat either through physical contact, being members of a cluster, or sharing a color of UVF-dust. For the first two measures (physical contact and clusters) every bat in the site served as a focal bat (a single data point). For dust networks, each dusted (focal) bat was a single data point. We compared this number of connections among connection type (physical contact, cluster member, UVF-dust) and species (including within vs. between species) using generalized linear mixed effects models with a binomial distribution (with size equal to the number of bats of that species at the site) and a logit link. We included site and the individual bat ID as random effects to account for repeated observations of the bat with multiple colors of UVF-dust at a site.

We examined whether bats within the same cluster were more likely to share the same UVF-dust color than bats among clusters. For each cluster within a site, we calculated the fraction of individuals within a cluster that had the same color of UVF-dust (within group metric), as well as the fraction of clusters within each site that had a given color (among group metric). We used a generalized linear mixed effects model with a gamma distribution and an inverse link and site as a random effect to determine whether transmission was higher within or among groups.

We also examined differences among species in the probability of becoming infected with *P. destructans* over the winter at the same sites, using a generalized linear mixed model with a binomial distribution and logit link, with species interacting with date as fixed effects, and site as a random effect. UVF-dust and fungal epidemics were compared by regressing the change in *P. destructans* prevalence over the winter during the first year of fungal invasion to the change in UVF-dust prevalence over the winter using a linear mixed model with site as a random effect. Finally, we examined differences among species in the total surface area of the environment covered with UVF-dust, using a linear mixed model with species as a fixed effect, and site as a random effect. All statistical tests were carried out using R 3.3.2 and using package lme4.

Results:

The bat community included three primarily solitary species (*P. subflavus, M. septentrionalis,* and *E. fuscus*, with 93% of bats roosting alone), and one species (*M. lucifugus*) that was more social with cluster sizes averaging 3.2 +/- 5.36 and ranging from 1 to 36 (Fig. 2; Supp. Fig b).

There was on average 20-fold more connections among individuals revealed by transmission of UVF-dust from two species (the more social *M. lucifugus* and the solitary *M. septentrionalis*), than were apparent from physical contact or shared group (cluster) connections (Fig. 2; Table S1, S2). For the third species, (the solitary *P. subflavus*), there were few additional connections among individuals beyond direct contact or membership in a cluster (Table S2d; Fig. 2). Within-species connections were significantly higher for *M. septentrionalis* and *M. lucifugus* than among species connections for all connection types (Table S2d; Fig. 2).

Connections revealed through UVF-dust transmission also differed significantly among species, but not in ways that were apparent from physical contact and cluster membership connections (Fig. 2 & 3). UVF-dust from both *M. lucifugus* (a clustering species) and *M. septentrionalis* (a solitary species) was detected on large fractions of both con- and heterospecifics (and large fractions of the total colony size). In contrast, connections were much lower for *P. subflavus* (a solitary species) than for *M. lucifugus* and *M. septentrionalis* (Fig. 2 & 3, Table S2a). For one species, *M. lucifugus*, within-species transmission of UVF-dust was higher than transmission of UVF-dust to all three other species, *E. fuscus, M. septentrionalis,* and *P. subflavus* (Table S2a; Fig. 3).

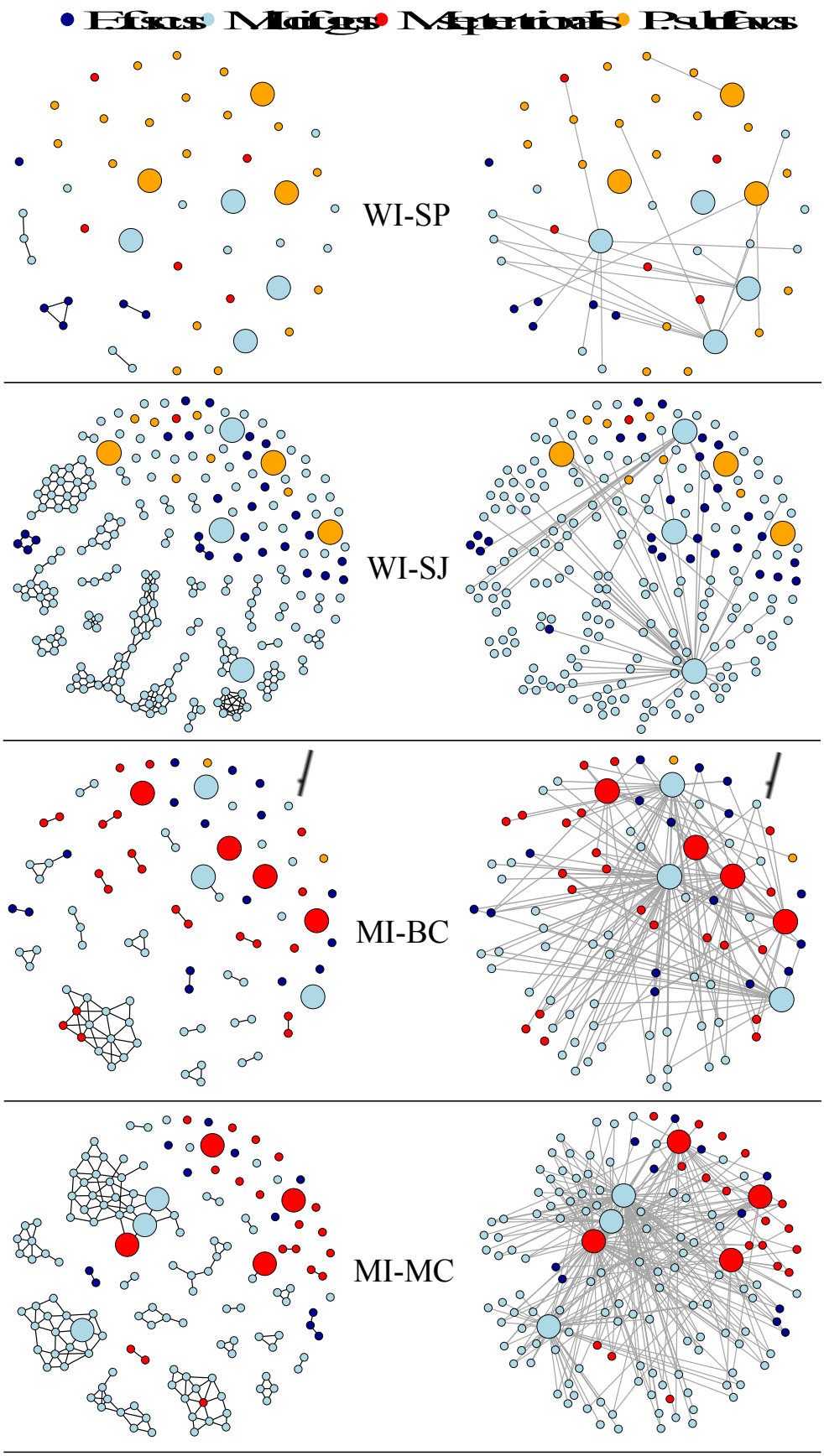
WI-SP
WI-SJ
MI-BC
MI-MC

Figure 2: Cluster and epidemic networks for hibernating bats from 4 mines in Wisconsin and Michigan. Each row shows an individual site. (left) Cluster networks represent observed physical contact networks at the end of winter. All individuals connected represent clusters observed in each site. (right) UVF-dust networks show epidemics created over the winter hibernation period using a surrogate pathogen. Each circle (node) represents a bat in a colony (cave or mine). Larger circles represent bats that were originally dusted in early winter (November). For the cluster networks, smaller circles indicate the bats that were re-sighted in late winter, and edges (black lines) between nodes represent physical contact among bats (March). (right) For the epidemic networks (UVF-dust), circles that are not connected with any other circles indicate bats with no dust, or resighted dusted bats that did not spread dust to any other bats (large circles). (right) Edges (gray lines) represent the transmission from dusted to non-dusted individuals via direct or indirect transmission (Fig. 1). Transmission events originating from non-dusted individuals are not shown. The arrow for the MI_BC cluster networks indicates a bat roosting solitarily in March, and the arrow under the dust epidemics shows the same resighted bat with three colors of dust originating from *M. septentrionalis*.

In contrast, for another species, *M. septentrionalis*, transmission of UVF-dust was similar between other individual *M. septentrionalis* and *E. fuscus* (Table S2a; Fig. 2 & 3). Finally, transmission of UVF-dust from *P. subflavus* was very low with no significant differences within and among species for all comparisons (Table S2a; Fig. 2 & 3). Despite this variation among species, there were no significant differences among species in the total area of the environment with UVF-dust originating from different species (Supp. Fig. 2).

Transmission networks revealed by UVF-dust also showed evidence of group cohesion, transmission increasing with sociality, and differential cross-species contact by cluster size. *M. lucifugus* within the same cluster (group) were more likely to share UVF-dust with each other than with *M. lucifugus* in other clusters in the same site (Fig. 2; coef: 0.180 +/- 0.04, $p<0.0001$). The probability of *M. lucifugus* individuals having UVF-dust originating from another *M. lucifugus* increased with cluster size (cluster size coef: 0.013 +/- 0.005, intercept: -0.549 +/- 0.201, $p = 0.012$; Supp Fig. 1a). At the same time, the probability of *M. lucifugus* having UVF-dust originating from dusted *M. septentrionalis* decreased with cluster size (Fig. 2 (arrow); cluster size coef: -0.014 +/- 0.004, intercept: -1.961 +/- 0.217, $p = 0.0002$; Supp Fig. 1b).

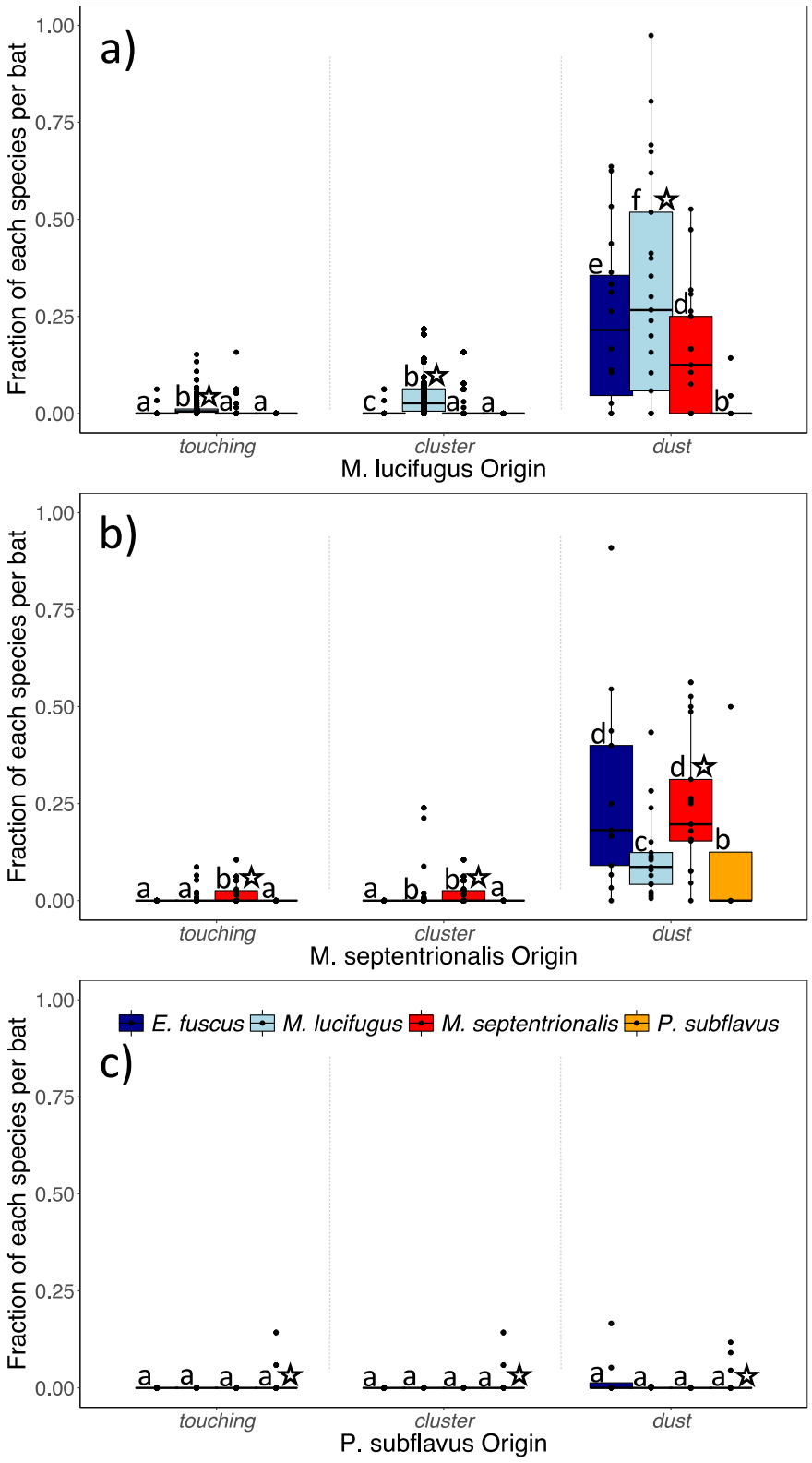
a)
b)
c)
Fraction of each species per bat
1.00
0.75
0.50
0.25
0.00
touching
cluster
dust
M. lucifugus Origin
M. septentrionalis Origin
P. subflavus Origin
E. fuscus
M. lucifugus
M. septentrionalis
P. subflavus

Figure 3: Fraction of individuals of each species at each site observed in physical contact, in a cluster, or observed with dust originating from a single individual of three dusted species, (a) *M. lucifugus, (b) M. septentrionalis, and (c) P. subflavus*. The color of the bar represents the species that was observed in physical contact (touching), in a group of individuals contacting each other (cluster), or with the UVF-dust (dust) from the focal individual. Panels show connections with a) *M. lucifugus, b) M. septentrionalis*, and c) *P. subflavus*. Different letters above bars indicate groups that differ significantly within and among panels (Table S2a-d). The legend indicates the order (left to right) of receiving species when box color is not visible.

Transmission of P. destructans

P. destructans was detected at all sites within 0-3 years of the contact rate studies. Prevalence of *P. destructans* in early winter did not differ significantly among species (Fig. 4a). However, prevalence on *M. lucifugus* and *M. septentrionalis* increased rapidly over the winter hibernation period, reaching ~70%-100% at all sites (Fig. 4a). In contrast *P. subflavus* became infected much more slowly, with prevalence reaching only ~40% by the end of the winter (Fig. 4a). Patterns of fungal transmission (the fraction of individuals becoming infected over time) were strongly correlated with the total spread of UVF-dust in a species at a site (Fig. 4b.).

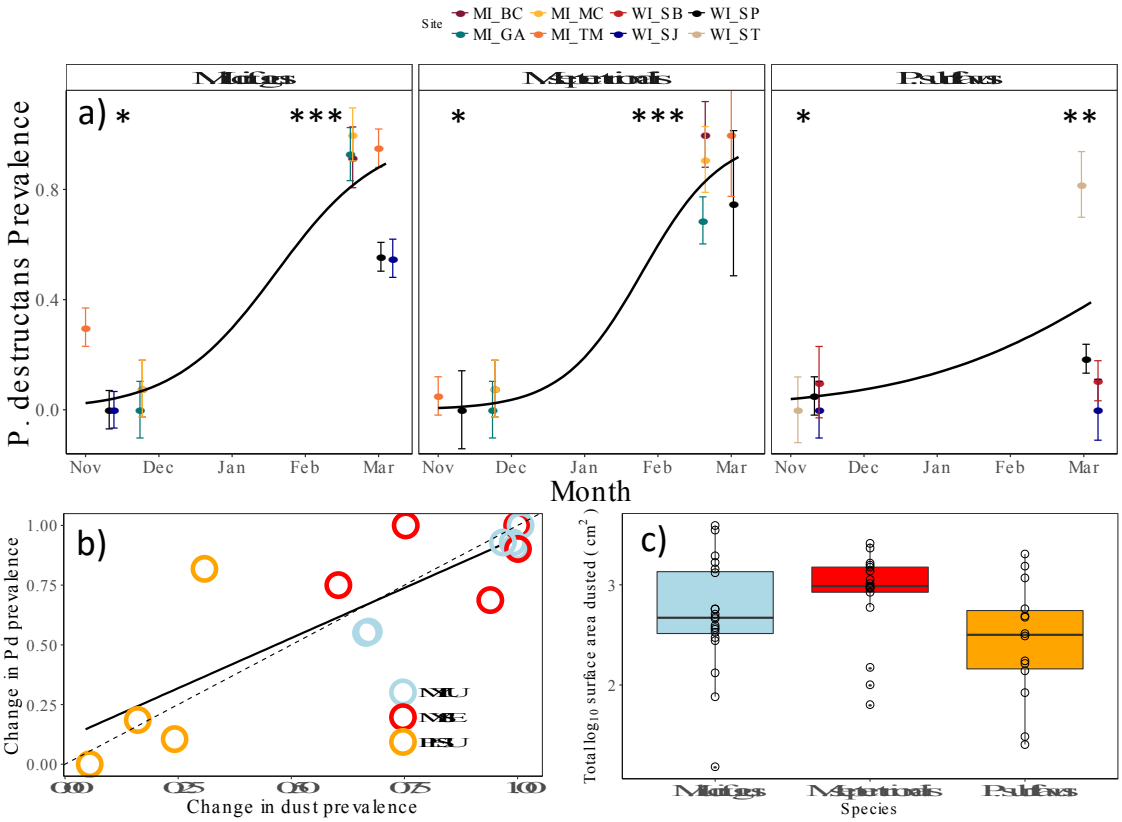

Figure 4. Change in fungal, P. destructans, prevalence and the relationship between the change in UVF-dust prevalence and fungal prevalence. (a) Change in prevalence during the first year of *P. destructans* invasion at eight sites in the Midwestern U.S. Lines show fitted models across the winter hibernation period with points representing site prevalence. Stars above each sampling point indicate significance differences in the probability of infection during early hibernation and the change in probability of infection over the winter (GLMM with site as random effect; *MYLU (*intercept): -3.71 +/- 0.80, *PESU*: 0.50 +/- 1.18, *MYSE*: -1.14 +/-0.20, MYLU date: 1.43 +/- 0.20, PESU date: -0.75 +/- 0.30, MYSE date: 0.41 +/- 0.27) (c) The change in dust and pathogen prevalence (Δ species prevalence at each site divided by 1 – early winter species prevalence). The line shows the fitted linear relationship and 95% confidence intervals of the regression (coef: 0.825 +/- 0.15, t = 5.496, P = 0.0006). Dashed line shows 1:1 line for comparison. **Total surface area of the hibernacula walls and ceilings contaminated with fluorescent dust.** (c) Each point represents the summed and then $\log_{10}$ transformed surface area of an individual color at a site (ref: *MYLU* 2.705 +/- 0.15, *MYSE* coef: 0.141 +/- 0.17, t = 0.788, *PESU* coef: -0.211 +/- 0.18, t = -1.163).

Discussion:

Many studies of contact rates and sociality attempt to quantify connections among individuals that may lead to transmission of pathogens [43, 79, 80], using a variety of methods [43, 58, 61]. These studies provide estimates of contact rates and social connections among cohesive groups that are limited by logistical constraints, including relatively short time periods, data from only a small subset of all hosts, only direct contacts, or a combination of these issues [42, 81]. We have shown that indirect and transient connections among social groups result in an order of magnitude more connections among individuals than are observed from measures of direct contact or shared social groups. These hidden connections revealed by transmission of surrogate pathogens not only link social groups within species, but also create bridges among species, and result in a much more highly connected network across the multi-species community than was apparent from other measures of association.

Discovering hidden connections among social groups can help explain otherwise mysterious transmission patterns. In our system, *M. septentrionalis* were almost always observed as solitary individuals, which one would expect would reduce transmission of a directly transmitted pathogen relative to a more social species such as *M. lucifugus*. However, transmission of UVF-dust showed that this species is, in fact, highly connected to both other *M. septentrionalis* individuals as well as individuals of two other species, *E. fuscus,* and *M. lucifugus* (but not *P. subflavus*). This connectedness was evident in the rapid infection in this species with the fungus *P. destructans*. Although the data cannot determine whether these connections are via direct contact or from indirect contact via the environment, it is clear that they occur during the very short arousal periods from hibernation because *M. septentrionalis* are almost never observed hibernating in clusters with other *M. septentrionalis* or other species (>99.5% of observations are of solitary individuals). The higher transmission of UVF-dust from *M. septentrionalis* to *M. lucifugus* roosting solitary than to *M. lucifugus* roosting in clusters may be a result of mistaken

mate selection during arousal periods, or due to avoidance of areas used by large groups of other bats. Hidden connections outside social groups have been observed in several other systems [82, 83], and are especially important for pathogens with an environmental reservoir, such as cholera, and avian influenza, but they are likely also important for pathogens with more limited survival outside the host, or those with aerosol transmission, including respiratory pathogens and oral fecal pathogens.

The extent of hidden connections outside social groups can also differ greatly for different groups of individuals, and suggests key differences in social behavior beyond differences in space use [84-86]. Transmission of both *P. destructans* and UVF-dust demonstrated that *P. subflavus* had relatively few hidden connections and overall very low contact rates, in sharp contrast to *M. septentrionalis* which was very similar in terms of connections by physical contact and social groups. Interestingly, the low connectance of *P. subflavus* was not simply because individuals of this species were immobile. We found no difference in the amount of the environmental surface with UVF-dust from *P. subflavus* versus the two other dusted species, *M. lucifugus,* and *M. septentrionalis*. Instead, the data suggest that *P. subflavus* individuals have lower rates of contacts resulting in transmission and avoid areas of the environment used by other individuals and species than. Uncovering these asocial space use patterns requires being able to simultaneously map the locations of individuals, and not simply to characterize individual metrics of space use such as home range size.

Studies of surrogate pathogen transmission overcome some challenges with other measures of contact networks, but also have several limitations. Transmission of surrogate pathogens can reveal both direct and frequent physical contact within social groups (which are well-captured by contact surveys, or studies using proximity or GPS transmitters) as well as those that occur through indirect or transient contacts, which are common in many systems [87-89]. Contact surveys or tracking studies also have a key limitation that is shared by some surrogate pathogen studies – accurately differentiating infectious contacts from contacts unlikely to transmit

pathogens. In contact surveys, efforts are usually made to quantify certain types of behaviors that are more likely to result in transmission (e.g. physical contact, conversing; [80]), but these are still approximate at best, since pathogen transmission given contact is often a highly stochastic process [82, 90]. Surrogate pathogens that have similar transmissibility to the pathogens they are models for are obviously best for overcoming this challenge. In our study, the amount of UVF-dust on bats decayed over time due to grooming and general shedding of UVF-dust, whereas pathogens (including *P. destructans*) replicate on hosts and generally increase in load and infectiousness over time. Finally, a limitation of the surrogate pathogen used in our study, UVF-dust, is that we were unable to trace the transmission connections among individual bats if transmission chains occurred that weren't from the primary dusted individual. However, the quantity of UVF-dust observed on individual bats was often very small, suggesting that secondary transmission chains from intermediate individuals were less likely in our study. Molecular studies with surrogate pathogens that mutate at a rate that enables accurate phylogenetic reconstruction of the epidemic overcome this hurdle [62, 63, 68], but suitable surrogate pathogens that approximate the biology of the target pathogen are often hard to find.

Estimating contact rates among individuals is a key element in understanding, predicting, and controlling infectious disease outbreaks. We have shown that hidden connections outside social groups play a key role in transmission dynamics, and increase connectance in multi-host communities by an order of magnitude for within-species transmission and even more for cross species transmission. These results emphasize the importance of contacts that are indirect, transient, or outside social connections for understanding and predicting disease dynamics. Uncovering these links can provide clues and context for disease management to improve health outcomes for affected populations.

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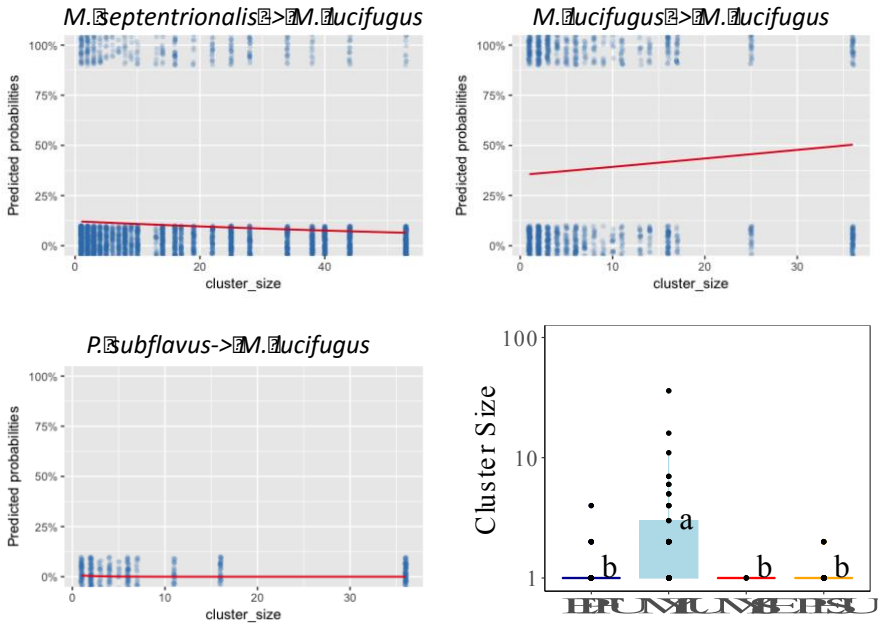
work was performed under protocol FrickW1106 approved by the UCSC IACUC committee.

Supplemental Figures:



Supp Figure 1: Winter clustering behavior of 3 species of hibernating bats and predicted probabilities of having dust from three origin species to different cluster sizes of *M. lucifugus*. Cluster size during winter hibernation for four species of bats. Letters above each bar represent significant differences among species (Likelihood ratio test of models with and without species: X^2 =46.954; df = 3, P < 0.0001) *EPFU – E. fuscus; MYLU – M. lucifugus; MYSE - M. septentrionalis; PESU - P. subflavus.*

Supplemental Tables:

Table S1: Species population counts and number of each species dusted at 8 sites in Wisconsin and Michigan.

Site	*M. lucifugus*		*P. subflavus*		*M. septentrionalis*		*E. fuscus*		*Total Pop.*
	Dusted	Total	Dusted	Total	Dusted	Total	Dusted	Total	
WI_SJ	4	241	3	10	0	1	0	38	290
WI_ST	0	1	4	1	0	1	0	0	18
WI_SP	4	15	3	22	0	6	0	6	49
WI_SB	0	1	4	7	0	1	0	10	19
MI_BE	3	46	0	2	4	19	0	16	83
MI_MC	3	113	0	0	4	16	0	11	140
MI_TM	3	172	0	0	4	66	0	0	238
MI_GA	5 -1col	569	0	0	5	39	0	16	624

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Widespread Bat White-Nose Syndrome Fungus, Northeastern China

Joseph R. Hoyt, Keping Sun, Katy L. Parise, Guanjun Lu, Kate E. Langwig, Tinglei Jiang, Shubao Yang, Winifred F. Frick, A. Marm Kilpatrick, Jeffrey T. Foster,[1] Jiang Feng

Author affiliations: University of California, Santa Cruz, California, USA (J.R. Hoyt, K.E. Langwig, W.F. Frick, A.M. Kilpatrick); Northeast Normal University, Changchun, China (K. Sun, G. Lu, T. Jiang, J. Feng); Northern Arizona University, Flagstaff, Arizona, USA (K.L. Parise, J.T. Foster); Changchun Normal University, Changchun (G. Lu); Jilin Agricultural University, Changchun (S. Yang)

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To the Editor: Emerging infectious diseases have caused catastrophic declines in wildlife populations, and the introductions of many pathogen have been linked to increases in global trade and travel (*1*). Mapping the distribution of pathogens is necessary to identify species and populations at risk and identify sources of pathogen spillover and introduction. Once pathogen distributions are known, management actions can be taken to reduce the risk for future global spread (*2*).

Bats with symptoms of white-nose syndrome (WNS) were first detected in the United States in 2006, and the disease has subsequently caused precipitous declines in temperate bat populations across eastern North America (*3*,*4*). *Pseudogymnoascus destructans*, the causative agent of WNS, is a cold-growing fungus that infects bats' skin during hibernation, leading to more frequent arousals from torpor and death (*3*). *P. destructans* is widespread throughout Europe (*5*), but, to our knowledge, its presence in Asia has not been documented.

We sampled bats and hibernacula surfaces (cave walls and ceilings) across northeastern China during 2 visits (June–July 2014 and March 2015) using a previously described swab-sampling technique (*6*). Bats were captured inside caves and at their entrances. DNA was extracted from samples by using a modified QIAGEN DNeasy blood and tissue kit (QIAGEN, Valencia, CA, USA) and tested in duplicate for the presence of *P. destructans* with a quantitative real-time PCR (qPCR) (*6*,*7*).

In the summer of 2014 and winter of 2015, we collected 385 samples from hibernacula surfaces at 12 sites in 3 provinces and 1 municipality (Figure, panel A) and 215 samples from 9 species of bats at 10 sites (summer: *Rhinolophus ferrumequinum, Rhinolophus pusillus, Myotis adversus, Myotis macrodactylus, Myotis pilosus, Myotis chinensis, Murina usseriensis*; winter: *R. ferrumequinum, Murina leucogaster, Myotis petax*). During the summer, *P. destructans* was widely distributed across the study region with positive samples (determined on the basis of qPCR results) obtained from cave surfaces at 9 of 12 sites and from bats at 2 of the 9 sites where bats were sampled (Figure, panel A).

Prevalence of *P. destructans* was low during summer in the environment (mean prevalence across sites 0.06 ± 0.03) and in bats. Bats of 3 species tested positive for *P. destructans* in the summer: *M. macrodactylus* (1/10), *M. chinensis* (1/1), and *M. ussuriensis* (1/1). *P. destructans* was not detected in bats of 4 other species, of which >20 individual animals of each species were sampled (*R. ferrumequinum*, *R. pusillus*, *M. pilosus*, and *M. adversus*). The low prevalence of *P. destructans* in bats and on hibernacula surfaces in China during the summer was similar to comparable results from studies in North America (*6*).

In winter, prevalence at the 2 sites we revisited was much higher; 75% of 85 samples from 3 species tested positive, including samples from 16/17 *M. petax* bats. We also detected *P. destructans* in bats from 2 additional species (*R. ferrumequinum* [11/19 bats] and *M. leucogaster* [11/16 bats]).

In addition, during March 2015, we observed visual evidence of *P. destructans* in bats (*M. petax*; Figure, panel C) and obtained 2 fungal cultures from swab specimens taken from these bats. To isolate *P. destructans* from these samples, we plated swab specimens from visibly infected bats on Sabouraud dextrose agar at 10°C. We identified potential *P. destructans* isolates on the basis of morphologic characteristics. DNA was then extracted from 2 suspected fungal cultures and tested for *P. destructans* by qPCR, as previously described.

To further confirm the presence of *P. destructans*, we prepared the fungal isolates for Sanger sequencing (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/1/15-1314-Techapp1.pdf). The 600-nt amplification products from these 2 isolates were sequenced and found to be 100% identical to the *P. destructans* rRNA gene region targeted for amplification. In addition, using BLAST (http://www.ncbi.nlm.nih.gov/blast.cgi), we found that sequences were a 100% match with isolates from Europe (GenBank accession no. GQ489024) and North America (GenBank accession no. EU884924). This result confirms that the same species of fungus occurs on all 3 continents. We also obtained wing biopsy punches from these bats and found lesions characteristic of WNS by histopathologic examination (Figure, panel B; online Technical Appendix).

The occurrence of *P. destructans* at most sites sampled indicates that this pathogen is widespread in eastern

[1]Current affiliation: University of New Hampshire, Durham, New Hampshire, USA.

Asia (Figure, panel A). The presence of *P. destructans* in bats from 6 species in China and on bats in 13 species in Europe (*8*) confirms the generalist nature of this fungus and suggests that it may occur throughout Eurasia (Figure, panel D).

Decontamination and restrictions on the use of equipment that has been used in caves in Asia would help reduce the probability of introducing *P. destructans* to uninfected bat populations (e.g., western North America, New Zealand, southern Australia, and temperate areas of South

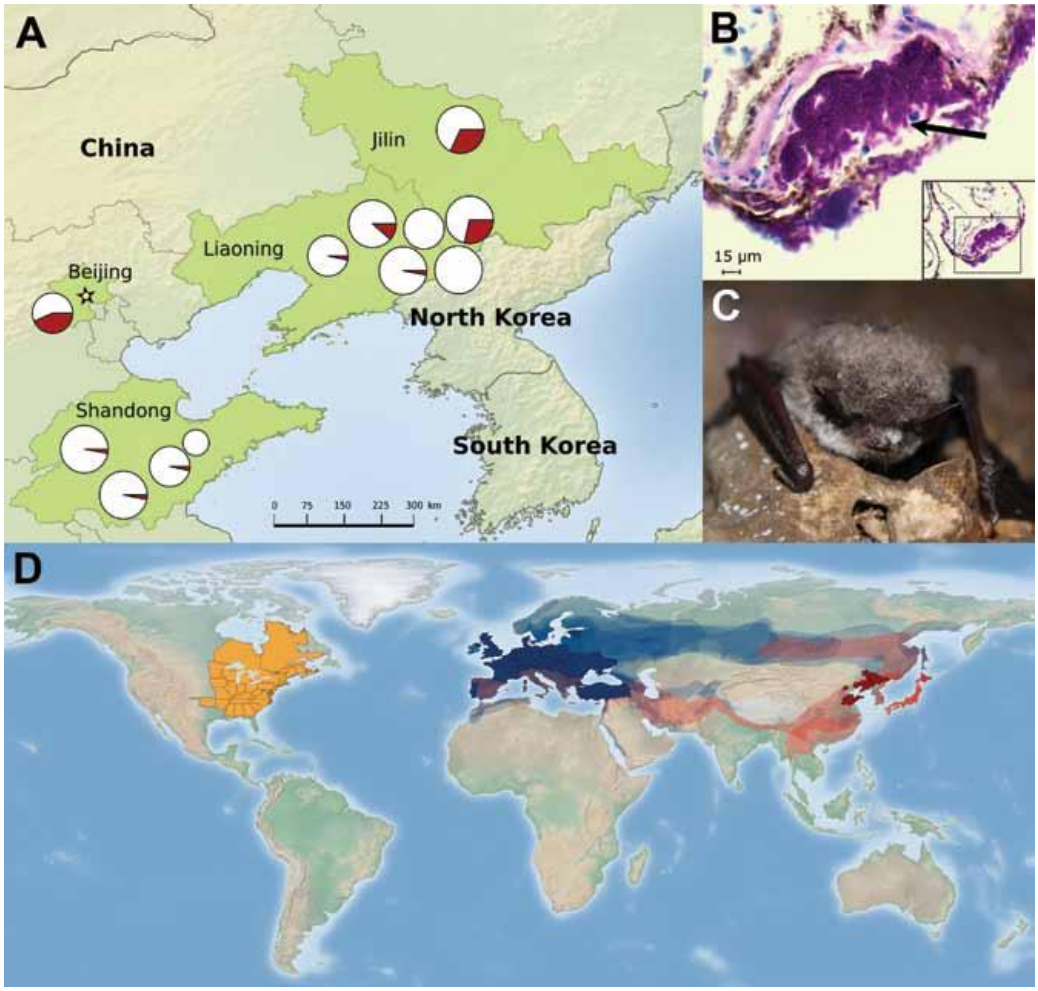

Figure. A) Distribution of *Pseudogymnoascus destructans* in cave environments during summer at 9 sites in northeastern China. Pie charts show the prevalence of *P. destructans,* and the size of pie graphs indicates the number of samples taken at each site (range 10–35). B) Histologic wing cross-section from *Myotis petax* bat collected in March 2015 with cup-like lesion (arrow) diagnostic of white-nose syndrome (periodic acid–Schiff staining). C) *M. petax* bat found in a cave in Jilin, China, showing visible signs of white-nose syndrome, March 2015. D) Documented global distribution of *P. destructans*. Areas in solid black represent the provinces and countries in China and Europe, respectively, where *P. destructans* was detected in this study and from previous research (*5*). Semitransparent regions show the species ranges (range data taken from http://www.iucnredlist.org/) for the bat species detected with *P. destructans* in Asia (n = 6) and Europe (n = 13) (*8*) and possible distribution of *P. destructans*. The solid black region in North America shows the extent of *P. destructans* spread as of May 15, 2015 (https://www.whitenosesyndrome.org/resources/map). A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/22/1/15-1314-F1.htm).

America). These measures would also reduce the risk of introducing new strains of *P. destructans* to regions where bats are already infected (e.g., eastern North America and Europe). These measures are necessary to prevent the devastating effects this pathogen has had on bats in North America and would help maintain the ecosystem services that bats provide (*9,10*).

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Address for correspondence: Joseph R. Hoyt, University of California, Santa Cruz–Ecology and Evolutionary Biology, 1156 High St, Santa Cruz, CA 95064, USA; email: hoytjosephr@gmail.com; and Jiang Feng, Northeast Normal University, Changchun-School of Environment, 5268 Renmin St., Changchun, Jilin, People's Republic of China; email: fengj@nenu.edu.cn

New Clinical Strain of *Neisseria gonorrhoeae* with Decreased Susceptibility to Ceftriaxone, Japan

Takashi Deguchi,[1] Mitsuru Yasuda,[1] Kyoko Hatazaki,[1] Koji Kameyama, Kengo Horie, Taku Kato, Kohsuke Mizutani, Kensaku Seike, Tomohiro Tsuchiya, Shigeaki Yokoi, Masahiro Nakano, Mutsumasa Yoh

Author affiliations: Gifu University, Gifu, Japan (T. Deguchi, M. Yasuda, K. Hatazaki, K. Kameyama, K. Horie, T. Kato, K. Mizutani, K. Seike, T. Tsuchiya, S. Yokoi, M. Nakano); Yoh Clinic, Inazawa, Japan (M. Yoh)

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To the Editor: In 2009, 2010, and 2013, *Neisseria gonorrhoeae* strains H041 (ceftriaxone MIC of 2 mg/L), F89 (ceftriaxone MIC of 1 mg/L), and A8806 (ceftriaxone MIC of 0.5 mg/L) were isolated from samples from patients in Japan (*1*), France (*2*) and Australia (*3*), respectively. In Japan, no other clinical *N. gonorrhoeae* strains with decreased susceptibility to ceftriaxone were reported until 2014, when clinical strain GU140106 (ceftriaxone MIC of 0.5 mg/L) was isolated from a man in in Nagoya, Japan. We report details of this case and sequencing results of the *penA* gene for the strain. The study was approved by the Institutional Review Board of the Graduate School of Medicine, Gifu University, Japan.

N. gonorrhoeae strain GU140106 was isolated from a urethral swab sample from a man with acute urethritis. The man had received fellatio, without condom use, from a female sex worker in Nagoya in December 2013. He visited our clinic in January 2014 for urethral discharge. Culture of a urethral swab sample was positive for *N. gonorrhoeae*. We used the Cobas 4800 CT/NG Test (Roche Molecular Systems Inc., Pleasanton, CA, USA) to test a first-voided urine sample; results were positive for *N. gonorrhoeae* but negative for *Chlamydia trachomatis*. The infection was treated with a single-dose regimen of ceftriaxone (1 g) administered by intravenous drip infusion. Two weeks later,

[1]These authors contributed equally to this article.

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Authors for correspondence:
Joseph R. Hoyt
e-mail: hoytjosephr@gmail.com
Jiang Feng
e-mail: fengj@nenu.edu.cn

†These authors contributed equally to this study.
‡Present address: Department of Molecular, Cellular and Biomedical Science, University of New Hampshire, Durham NH 03824, USA.

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Host persistence or extinction from emerging infectious disease: insights from white-nose syndrome in endemic and invading regions

Joseph R. Hoyt[1,†], Kate E. Langwig[1,†], Keping Sun[2], Guanjun Lu[2,3], Katy L. Parise[4], Tinglei Jiang[2], Winifred F. Frick[1], Jeffrey T. Foster[4,‡], Jiang Feng[2] and A. Marm Kilpatrick[1]

[1]Department of Ecology and Evolutionary Biology, University of California, EE Biology/EMS, Santa Cruz, CA 95064, USA
[2]Jilin Provincial Key Laboratory of Animal Resource Conservation and Utilization, Northeast Normal University, Changchun 130117, People's Republic of China
[3]Urban and Environmental Science College, Changchun Normal University, Changchun 130032, People's Republic of China
[4]Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, AZ 86011, USA

WFF, 0000-0002-9469-1839

Predicting species' fates following the introduction of a novel pathogen is a significant and growing problem in conservation. Comparing disease dynamics between introduced and endemic regions can offer insight into which naive hosts will persist or go extinct, with disease acting as a filter on host communities. We examined four hypothesized mechanisms for host–pathogen persistence by comparing host infection patterns and environmental reservoirs for *Pseudogymnoascus destructans* (the causative agent of white-nose syndrome) in Asia, an endemic region, and North America, where the pathogen has recently invaded. Although colony sizes of bats and hibernacula temperatures were very similar, both infection prevalence and fungal loads were much lower on bats and in the environment in Asia than North America. These results indicate that transmission intensity and pathogen growth are lower in Asia, likely due to higher host resistance to pathogen growth in this endemic region, and not due to host tolerance, lower transmission due to smaller populations, or lower environmentally driven pathogen growth rate. Disease filtering also appears to be favouring initially resistant species in North America. More broadly, determining the mechanisms allowing species persistence in endemic regions can help identify species at greater risk of extinction in introduced regions, and determine the consequences for disease dynamics and host–pathogen coevolution.

1. Introduction

A major outstanding question in evolution and ecology in the Anthropocene is predicting the state that ecosystems will reach after the introduction of invasive species, and in particular, novel pathogens [1]. The introduction of novel pathogens can have lasting effects on species, communities and ecosystems, but impacts are extremely variable with some species suffering little mortality from disease, while others are driven towards extinction [2–6]. Hosts in endemic regions can coexist with these same pathogens through at least four possible mechanisms: reduced transmission, resistance (host defences that reduce pathogen growth), tolerance (host defences that reduce damage experienced by the host without reducing pathogen growth) and/or demographic compensation [7–10]. Inherent host traits, pathogen evolution and the response of species to the selective pressures of mortality from disease determine the

outcome of pathogen introductions. Understanding host–pathogen interactions in endemic regions can offer insight into both the long-term impacts and potential persistence mechanisms of naive hosts [11]. For example, finding that hosts in disease-endemic areas are resistant to a disease would suggest that phylogenetically and ecologically similar hosts in invading regions might also persist by evolving resistance. However, few, if any, studies exist that have explicitly compared disease dynamics in introduced and endemic regions.

The mechanisms of host persistence with novel pathogens also determine, in large part, the intensity of transmission. Two mechanisms, reduced host density for pathogens in which transmission is density-dependent, and poor environmental conditions for pathogen survival or replication outside hosts, will result in lower pathogen transmission [7,12]. Similarly, if hosts in the introduced region are inherently resistant to the pathogen or evolve to become resistant, then this will limit transmission [9,13]. By contrast, if hosts are tolerant (or evolve tolerance) then transmission of the pathogen will be maintained at a much higher intensity, with correspondingly large impacts on intolerant, non-resistant species. In this way, diseases 'filter' communities [14], allowing resistant or tolerant species to persist while more susceptible species are driven extinct.

White-nose syndrome (WNS), a recently emerged disease of hibernating bats, was first detected in North America in 2006 [15] and has caused precipitous declines in temperate bat populations across eastern and midwestern North America [16–18]. The pathogen that causes WNS, *Pseudogymnoascus destructans*, is a cold growing fungus that infects bats' skin during their hibernation period [19–21], and can persist in the environment for long periods of time in the absence of bats [22,23]. The resulting infections lead to the disruption of homeostatic processes and ultimately mortality [24,25].

Pseudogymnoascus destructans has been documented widely across Europe and Asia on multiple species of bats [26–28]. European isolates of *P. destructans* are at least as virulent as the North American strain for North American bats [20], but the virulence of *P. destructans* from Asia for North American bats is unknown. The widespread declines observed in North America have not been observed in Asia or Europe [29] and genetic data suggest that *P. destructans* has been present in the Old World for millennia [30]. By understanding how bats persist with *P. destructans* infections in endemic regions, we may be able to predict the long-term disease dynamics in North America. Here, we present the first comparison of *P. destructans* infection patterns in introduced and endemic regions and the first data on infection prevalence and intensity using molecular methods from a WNS-endemic region.

2. Material and methods

(a) Sample collection and testing

We sampled bats and hibernacula (caves and mines) substrate for *P. destructans* at five sites across northeastern Asia and five sites in North America between 2012 and 2015. The North American sites were sampled during the first 3 years of pathogen invasion to each site where populations were experiencing severe declines [16,18].

We selected sites in Asia and North America at similar latitudes to control for climate and winter severity. The average daily above-ground temperature at the sites in Asia during winter when sampling was conducted (1 October–30 April 2015) was 0.35°C (s.d. = 9.67°C) and the average at the North American sites was 0.53°C (s.d. = 10.33°C). The number of days during this period when the temperature was below 10°C (an approximate threshold for flying insect and bat foraging activity [31]) was similar between regions, averaging 200 days at Asian sites and 198 at North American sites (an alternate threshold of 5°C produced equivalent results—175 days in Asia versus 172 days in North America).

Samples were collected using identical methods and during the same time period (March) in the two regions to make the comparison as similar as possible. Past studies indicate that prevalence and infection intensities (measured as the amount of fungal DNA detected in a sample) and WNS lesions on bats throughout North America increased over the winter, and were the highest in late winter [19,21], making March an ideal sampling time to examine and compare infection prevalence and fungal growth. In both regions, we estimated fungal prevalence and infection intensity (based on the quantity of fungus on the surface of the skin) by rubbing sterile swabs on bats' wings and muzzles as previously described [19,32,33]. We also swabbed areas of cave/mine surfaces directly under and 10 cm from the swabbed bat to determine the extent of the environmental reservoir. We stored samples in RNAlater prior to testing. We extracted DNA from samples using a modified Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) [19] and tested for the presence of *P. destructans* using quantitative PCR [34]. All samples were run in duplicate, with quantification standards on each plate and 16 negative controls per plate. All quantification standards were within a consistent range and all negative controls had no fungal detection. We also surveyed a subset of bats' wing and tail membranes for orange fluorescence using an ultraviolet (UV) flashlight (395 nm; Hayward, CA, USA). The presence of orange fluorescence on wing and tail membranes under UV light has been shown to be correlated with the presence of lesions in infected bats [35].

We measured the temperature next to the swabbed hibernating bat using a laser temperature thermometer (Fluke 62 MAX Plus Infrared Thermometer) that was calibrated across a range of surfaces and temperatures from 0°C to 20°C using a factory calibrated HOBO temperature logger (model U23–001). We confirmed that these single time point measurements of temperatures next to hibernating bats accurately represent winter hibernacula temperatures; point estimates of temperature (mean per site = 41; range 24–105) at 11 sites in the Midwestern USA were very tightly correlated with winter temperatures measured every 3 h from 1 December–31 March using HOBO temperature loggers ($r = 0.95$; $n = 11$; $p < 0.00001$).

At each site, we estimated colony size by counting all individuals of each species. Hibernating bats roosted on cave and mine surfaces making near complete counts of individuals possible.

(b) Statistical analysis

We used generalized linear mixed-effects models with continent as a fixed effect, and species and site as random effects to make comparisons of *P. destructans* prevalence and loads on bats and in the environment using binomial and Gaussian distributions, respectively. We controlled for temperature and colony size by including these variables as fixed effects in the models. We also examined variation in prevalence and load among species by modelling species as a fixed effect. We used the R package brglm to compare prevalence among species with species, temperature and $\log_{10}$ colony size as fixed effects. We used Tukey's post-hoc HSD test to compare pairs of species. To compare environmental prevalence and loads, we used generalized linear mixed-effects models with sample type (under the bat or

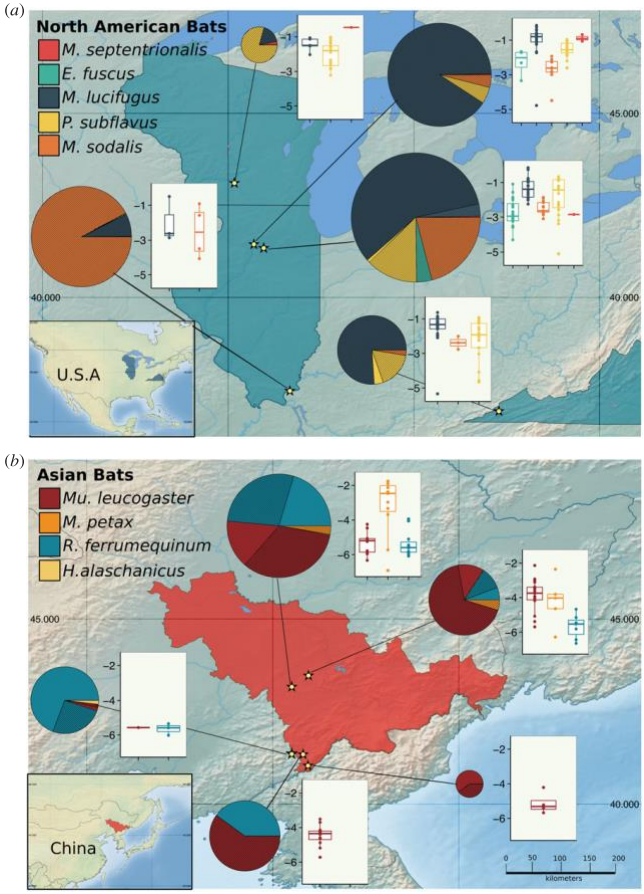

Figure 1. (*a*,*b*) Geographical variation in bat colony size, community composition and *Pseudogymnoascus destructans* prevalence and infection intensity in Asia and North America. Maps show the study regions and study sites (stars) in China and the USA. The pie charts show the total colony size (pie size, on a log scale, range 20–5158) and community composition of bats at each site. The fraction of each species that were tested positive for *P. destructans* is shown by the hatched or darker portion of each pie slice. Boxplots show the infection intensity for each species at the same sites on a log scale (ng) (note different scales for Asia and North America). Pie charts and boxplots use the same colour scheme for species, which is indicated by the legend. (Online version in colour.)

10 cm from the bat) as a fixed effect, and site as a random effect. We standardized roosting temperatures by fitting a mixed-effects model with site as a random effect, and season (early or late hibernation) as a categorical fixed effect because temperatures of hibernation sites were colder as winter progressed and data at all sites was not collected at identical times. We then compared adjusted mean late hibernation site temperatures using a *t*-test. We compared colony sizes among continents using a generalized linear mixed-effects model with Poisson distribution and log link, with site and species as random effects and continent as a fixed effect. All analyses were run in R, v. 3.0.2, and mixed-effects models were fit using the lme4 package.

3. Results

Colony sizes and hibernacula environments were very similar between the two continents (electronic supplementary material, table S1). Colony sizes for individual species ranged from 19 to 3154 bats per hibernaculum and were not significantly different among continents (figure 1; coeff: 0.64 ± 1.54, $Z = 0.42$, $p = 0.68$). Hibernacula temperatures were also very similar in Asia and North America (figure 2*a*; *t*-test of site means: $t = -0.76$, d.f. $= 8$, $p = 0.47$).

Prevalence of *P. destructans* on bats across all sites and species was significantly lower in Asia than on the invasion front in North America (figures 2*a* and 3; $N = 337$, continent coeff.: -4.70 ± 1.44, $t = 3.3$, $p < 0.001$) and neither temperature nor colony size were significant predictors of prevalence in this analysis (temperature coeff.: 0.13 ± 0.14; $t = 0.93$; $p = 0.36$; $\log_{10}$ colony size coeff.: -0.17 ± 0.51, $t = 0.33$, $p = 0.74$). Prevalence in late winter on all five species of bats in North America was more than 95%, whereas prevalence in late winter on four species of bats in Asia averaged 51% (figure 3*a*). Infection intensity, measured as fungal loads on bats' wings and muzzles, was significantly lower

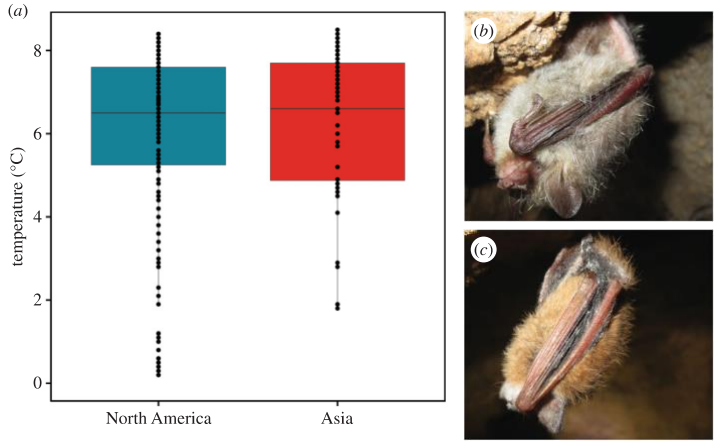

Figure 2. Hibernacula temperatures and visual evidence of WNS in Asia and North America. (*a*) Late winter (March) hibernacula temperatures for individual bats at five sites in each region. Analyses were performed on site average temperatures as described in the methods. (*b*) A bat (*Mu. leucogaster*) in Asia that tested positive for *P. destructans*, but shows no signs of visual infection. (*c*) A bat in North America (*P. subflavus*) that also tested positive, but shows intense visual evidence of fungal growth. Lower visual evidence has been correlated with lower fungal loads [36] among bats. (Online version in colour.)

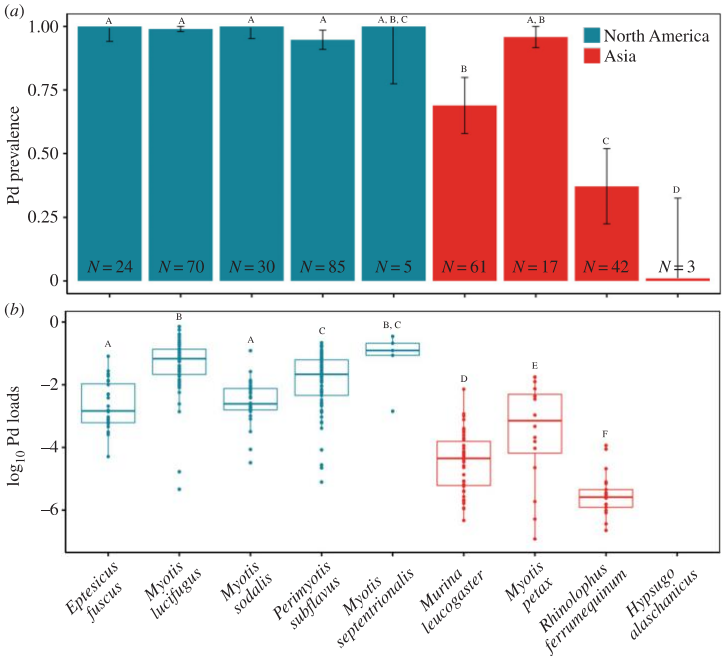

Figure 3. Prevalence and infection intensity of *P. destructans* (Pd) on five species of bats in North America and four species of bats in Asia. (*a*) Fraction of bats testing positive for *P. destructans* at five sites in each region (site random effect s.d.: 1.02, species random effect s.d.: 0.92, intercept: 4.52 ± 0.9). (*b*) Infection intensity, measured as fungal load, on a $\log_{10}$ scale in nanograms at the same sites as in (*a*) for *P. destructans* positive bats only (site random effect s.d.: 0.15, species random effect s.d.: 0.76, intercept: -1.98 ± 0.36). In both panels shared letters indicate species that do not differ significantly by Tukey's HSD tests. (Online version in colour.)

on Asian bats than North American bats (figure 3*b*; $N = 291$, continent coeff: -2.57 ± 0.62, $t = -4.12$, $p < 0.00001$). As with prevalence, neither temperature nor colony size were significant predictors of infection intensity in this analysis (temperature coeff.: -0.026 ± 0.03, $t = -0.76$, $p = 0.30$; $\log_{10}$ colony size coeff.: 0.03 ± 0.12, $t = 0.24$, $p = 0.39$).

There were significant differences in fungal loads among species, but all species in Asia were statistically significantly lower than all North American species (figure 3*b*). The most heavily infected species in Asia, *Myotis petax*, had loads that were twofold lower than the species with the lowest infection intensity in North America, *Eptesicus fuscus* (figure 3*b*). At the extreme, the species with the lowest infection intensity, *Rhinolophus ferrumequinum*, had loads that were 1000-fold lower than the average load of North American species.

We found that, on average, 28% of 124 bats in China (range among species: 2–73%), showed orange UV fluorescence consistent with the presence of skin lesions due to *P. destructans* infection, whereas the prevalence of UV orange fluorescence in North America was higher (75% of 127 bats; range 38–100%; species random effect s.d.: 2.2; continent coeff: -4.5 ± 1.8; $Z = -2.5$; $p = 0.01$). This difference was likely partly related to the differences in fungal load (figure 3*b*), because the probability of UV orange fluorescence increased with fungal infection intensity (generalized linear model with binomial distribution and logit link: coeff: 0.93 ± 0.28, d.f. = 262; $p = 0.01$), and the difference in orange UV fluorescence among continents was not significant after accounting for differences in fungal loads (coeff: -0.79 ± 0.51, d.f. = 262; $p = 0.13$).

Spatial variation in prevalence within each continent was much higher in Asia than North America (figure 1; Kruskal–Wallis $p = 0.001$), despite sampling from a narrower geographical region. For all five species sampled in North America, prevalence was uniformly high (95–100% for all species at all sites). By contrast, prevalence varied from 40 to 100% in *Murina leucogaster*, and 0–67% in *R. ferrumequinum* across sites (figure 1). Similarly, median fungal loads were almost uniformly high across sites in North American bats, including *Myotis lucifugus* ($10^{-1.5}$–$10^{-0.5}$ ng), and *P. subflavus* (10^{-2}–$10^{-1.5}$ ng), whereas loads were highly variable for bats in Asia, with median loads for both *Mu. leucogaster*, and *R. ferrumequinum* spanning two orders of magnitude (figure 3).

Finally, environmental prevalence, while highly variable among sites in Asia, was significantly lower in Asia than North America (figure 4*a*; $N = 198$, coeff: -1.58 ± 0.79, $Z = -2.0$, $p = 0.045$). *Pseudogymnoascus destructans* fungal loads on substrates were also significantly lower in Asia (figure 4*b*; $N = 131$, coeff: -1.4 ± 0.62, $t = -2.24$, $p = 0.01$).

4. Discussion

Disease can drive population cycles, cause extinctions that re-structure communities, and create continental-scale differences in abundance [17,37,38]. WNS has had devastating effects on bat populations across eastern and midwestern North America, with dozens of populations being extirpated and several species predicted to be driven extinct [16–18]. By contrast, although *P. destructans* is widespread in Europe and Asia, bat species have likely persisted with this pathogen for millennia [26,28,30].

We found that prevalence, the presence of UV orange fluorescence, and infection intensity of *P. destructans* was much lower on bats in Asia than on bats at invasion sites in North America, and that the environmental reservoir was less extensive in Asia as well. These data are consistent with Asian bats having higher resistance to pathogen growth, and are inconsistent with three other mechanisms that can lead to coexistence of hosts with an initially virulent pathogen: tolerance, reduced transmission because of smaller population size and lower pathogen growth rate because of lower temperatures. If tolerance, alone, was the mechanism allowing persistence of Asian bat populations with *P. destructans*, then prevalence and infection intensity would be similar on bats in both continents [9]. Colony sizes in Asia and North America were broadly overlapping, and differences among continents in prevalence and infection intensity were still highly significant in analyses that included colony size. Thus, the data are inconsistent with reduced transmission in Asia being due to smaller colony sizes and reduced density-dependent transmission. Similarly, hibernacula temperatures (as well as winter severity) were very similar between sites in Asia and North America and, as analyses indicated that the lower fungal loads of *P. destructans* on bats and hibernacula substrate in Asia cannot be attributed to cooler environmental conditions.

We found that the environmental reservoir was lower in Asia than North America, which is consistent with overall lower transmission intensity and reduced shedding of *P. destructans* by resistant bats in Asia. Lower transmission intensity, as well as differences in hibernation behaviour and phenology may have contributed to the higher variability in prevalence and infection intensity in Asian bats compared with North American bats. In addition, the higher prevalence and infection intensity in North American bats is not simply a transient phenomenon associated with the spreading wavefront of *P. destructans*, because both prevalence and infection intensity has been shown to remain high

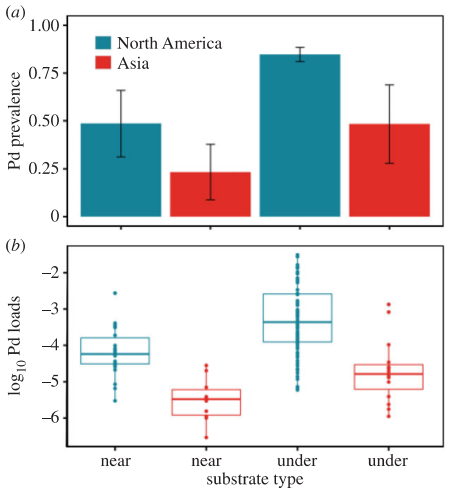

Figure 4. Extent of environmental reservoir for *P. destructans* (Pd) in hibernacula in North America and Asia. (*a*) Fraction of hibernacula substrate samples from under and near (10 cm from bats) testing positive for *P. destructans* at five sites in each region (site random effect s.d.: 0.44, substrate type random effect s.d.: 0.18, intercept: 0.32 ± 0.56). (*b*) Fungal loads on a $\log_{10}$ scale in nanograms at the same sites as in (*a*) for *P. destructans* positive samples only (site random effect s.d.: 0.1, substrate type random effect s.d.: 0.52, intercept: -3.96 ± 0.38). (Online version in colour.)

at sites several years after populations had declined [19]. Fungal loads were much lower on Asian bats, resulting in a lower probability of WNS lesions, measured by UV florescence. For some bat species in Asia with especially low fungal loads (e.g. *R. ferrumequinum*), the fungus may be present only on the surface of their skin and only rarely causing invasion and pathology. However, the absence of *P. destructans* detection on non-hibernating *R. ferrumequinum* bats during the summer at some of these same sites [28] suggests that our winter estimates of prevalence and infection intensity do not simply represent surface contamination, and the presence of detectable amounts of fungus suggests that these bats are likely infectious to other individuals and the environment.

Our data show that while there are significant differences in infection intensity between North America and Asia, there is also substantial variation in infection intensity among individuals within most species in North America, and these data may offer insight into which species are at greater risk of extinction than others. *Eptesicus fuscus* and *Myotis sodalis* have a relatively large fraction of individuals with fungal loads similar to Asian bats, and thus are unlikely to be driven extinct by WNS. By contrast, loads on *Myotis septentrionalis* were high, with most individuals having loads higher than bats in Asia. Two other species, *My. lucifugus* and *P. subflavus*, have populations that include many individuals with high loads, but a small fraction of these species at some sites have lower infection intensities, similar to those of bats in Asia. Thus, these species would be predicted to suffer large declines from WNS, but some individuals should be able to persist with the fungus.

These predictions are consistent with patterns of initial declines of these species reported previously [17,18]. Specifically, *My. septentrionalis* populations have been extirpated from most sites where WNS has been present for 3 or more years [17,18] whereas, *My. lucifugus* and *P. subflavus* populations have declined more than 90%, but have stabilized at much smaller sizes in some colonies [18]. Finally, as would be predicted based on their low infection intensity, colonies of *My. sodalis* and *E. fuscus* suffered much lower declines from WNS.

The traits conferring resistance in Asian bats and some species and individuals of North American bats are yet to be identified. They may include differences in microbial communities on bats [39], or increased immune function, although differences in antibodies do not appear to be important [40]. The smaller environmental reservoir in Asia could be due to the presence of natural enemies (e.g. mycoviruses or nematodes) that are not present or less abundant in North America.

The extent of declines in multiple species and the presence of both environmental and biological reservoirs of *P. destructans* [16,19], result in intense and persistent selective pressure on North American bats. It remains to be seen if the speed of evolution will be rapid enough to prevent extinctions of the most heavily impacted species [2,6,8,38,41,42]. Management actions to conserve these species face the challenge of reducing disease impacts to prevent extinction without compromising species' evolutionary response to this and other diseases.

Ethics. All sampling was conducted under UCSC IACUC protocol FrickW1106 and National Animal Research Authority in Northeast Normal University, China, approval number: NENU-20080416.

Authors' contributions. J.R.H., K.E.L., K.S., J.T.F., W.F.F., J.F. and A.M.K. designed the study. J.R.H., K.E.L., K.S., G.L., K.L.P., T.J. and A.M.K. collected the data. J.R.H., K.E.L. and A.M.K. analysed the data. J.F. and J.T.F. provided materials, and J.R.H., K.E.L. and A.M.K. wrote the manuscript with input from all authors.

Competing interests. We declare we have no competing interests.

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Global pathogen dynamics of white-nose syndrome

Joseph R. Hoyt[1,2]*, Keping Sun[3], Kate E. Langwig[1,2], Katy L. Parise[14], Yujuan Wang[3], Xiaobin Huang[3], Lisa Worledge[4], Helen Miller[4], Tamas Gorfol[5], Sandor Boldogh[6], Dai Fukui[7], Muneki Sakuyama[8], Munkhnast Dalanast[9], Ariunbold Jargalsaiken[10], Nyambayar Batbayar[11], Yossi Yovel[12], Eran Michael[12], Isoseb Natradze[13], Winifred F. Frick[1, 15], Jeffrey T. Foster[14], Jiang Feng[3], A. Marm Kilpatrick[1]

[1]*Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, CA 95064 USA*
[2] *Department of Biology, Virginia Polytechnic Institute, Blacksburg, VA, 24060 USA*
[3]*Jilin Provincial Key Laboratory of Animal Resource Conservation and Utilization, Northeast Normal University, Changchun 130117, China*
[4] *Bat Conservation Trust, London, United Kingdom*
[5] *Department of Zoology, Hungarian Natural History Museum, Budapest, Hungary*
[6]*Department of Nature Conservation, Aggtelek National Park, Josvafo, Hungary*
[7] *The University of Tokyo Hokkaido Forest, The University of Tokyo, Hokkaido, Japan*
[8] *Association of Bat Conservation, Morioka, Japan*
[9] *Bat Research Center of Mongolia, Ulaanbaatar, Mongolia*
[10] *Department of Biology, School of Mathematics and Natural Science, The Mongolian University of Education, Ulaanbaatar, Mongolia*
[11] *Wildlife Science and Conservation Center, Ulaanbaatar, Mongolia*
[12] *Department of Zoology, Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel*
[13]*Institute of Zoology, Ilia State University, Tbilisi, Republic of Georgia*
[14]*Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, AZ 86011, USA*
[15]*Bat Conservation International, Austin, TX, USA*
**Corresponding author*: hoytjosephr@gmail.com

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Abstract:

The introduction of novel pathogens to naïve host populations can cause catastrophic impacts and species extinctions. However, the mechanisms allowing hosts to persist with these same pathogens in disease endemic regions are poorly known. We examined the global dynamics of white-nose syndrome (WNS) on bats and in the environment to determine how species and populations in endemic regions persist with this disease. WNS, a devastating disease of North American hibernating bats, was recently introduced to North America from Eurasia, where it has been present for millennia. In Eurasia, we found that infection dynamics during winter in species with the highest prevalences were similar to the first year of pathogen invasion in sites in North America (when bat mortality is low), with prevalence increasing from near zero to high levels (50-100%). These dynamics differed substantially from infection patterns during the decline-phase of WNS in North America, when most North American bats have sustained high infection prevalence (100%) over winter. Although *P. destructans* has been present in hibernacula in Europe and Asia for thousands of years, we found that *P. destructans* in the environment, which is the source for bat reinfection each winter, started at low levels at the beginning of winter, and decreased over the summer. In contrast, in North America, *P. destructans* increased in the environment with each year of WNS detection, reaching much higher levels than endemic regions with no decline between winters. The reduction in *P. destructans* each summer in endemic regions could explain the limited mortality in bat populations across Eurasia. These results suggest that factors that regulate the

fungal populations in endemic regions (e.g. natural enemies) are absent in North America, leading to the ecological release and high population impact in invading regions.

Introduction:

Introduced pathogens have caused widespread population declines and extinctions and limit the distributions of many host populations [1-5, 91]. Although disease dynamics of emerging pathogens are frequently intensively studied in introduced regions, far less is known about how species persist with emerging pathogens in endemic regions [92, 93]. Many emerging pathogens are generalists that infect a diverse range of host species, with substantial variation in disease dynamics and impact in both introduced and endemic ranges. In some cases, hosts in endemic regions have evolved physiological traits (e.g. resistance or tolerance [94] that reduce disease impacts [95]. Myriad other factors could also allow for long term host persistence with pathogens including behavioral avoidance [41, 96], differences in pathogen strains [97], or environmental conditions that limit the impact of the pathogen [98, 99]. One factor that often differs between invasive species in their native and introduced regions is the presence of natural competitors that regulate their populations [100-102], which may be particularly important for pathogens with environmental reservoirs.

Abiotic reservoirs can play a key role in the dynamics of seasonal host-pathogen systems [103-105], and can increase the likelihood of host extinction from disease [4, 65]. Likelihood of host extinction is increased by the maintenance of

transmission even after host populations have decreased to very low levels and host-to-host transmission has become inefficient. Abiotic reservoirs can also serve as sources of infection (or reinfection) for immigrating hosts, or for hosts that recover with non-sterilizing immunity [106]. Controlling abiotic reservoirs is the cornerstone of many disease management programs, including treatment of water to reduce cholera transmission [107], as well as household and hospital sanitation practices [108].

White-nose syndrome (WNS) is a recently emerged fungal disease of hibernating bats in which the environmental reservoir appears to play a key role in population impacts [3, 22, 109]. WNS, caused by the fungus *Pseudogymnoascus destructans*, has caused severe declines in multiple species of bats in North America, and threatens several species with extinction [3, 25, 26]. Declines due to WNS are low in the first year *P. destructans* is detected in a site and increase with time since arrival [38, 39]. There is also significant variation in species impacts from WNS. Following WNS detection, four species of bats declined significantly (*Myotis lucifugus, Myotis septentrionalis, Myotis sodalis, and Perimyotis subflavus*) while two others (*Eptesicus fuscus* and *Myotis leibii*) had only small reductions in population growth rate [3].

Host and pathogen ecology are both important in determining seasonal dynamics and impacts of WNS [34]. Bats become exposed to *P. destructans* when they enter hibernacula each fall. Fungal loads on bat skin increase over winter when bats reduce their body temperature to a range suitable for *P. destructans* growth [34,

110]. Infection with *P. destructans* disrupts homeostasis, and leads to increased arousals that can result in death approximately 70-120 days after infection [25, 37]. Bats clear infection each summer when they become euthermic and leave hibernacula [34]. However, the fungus persists in hibernacula over the summer and can survive for long periods of time in the absence of bats [32, 109].

Pseudogymnoascus destructans is endemic to Eurasia, and genetic analyses suggest it has been present for thousands of years [9, 31]. *P. destructans* has been detected throughout Europe and in east Asia, where it has not been associated with significant declines [28, 29], although bat colony sizes are an order of magnitude lower in Europe than they were in in North America before WNS emerged [111]. Late winter infection prevalence and fungal loads on bats in Asia were lower than in the North America, suggesting that bats in Asia were more likely to be resistant than tolerant [93]. The mechanisms responsible for the lower infection prevalence and fungal loads on bats in Asia remain unknown, but could not be explained by lower colony sizes of bats, lower winter severity, or lower hibernacula temperatures in Asia [93].

Here, we examine the global dynamics of *P. destructans* to understand how bat populations throughout endemic regions persist with WNS. To explore mechanisms influencing infection, we sampled bats and hibernacula environments for *P. destructans* across seven countries on three continents over five winters. This data will help elucidate the interplay between bat infections and contamination in the

environment across a global scale and provide potential insight for developing effective disease management strategies in invading regions.

Methods:

Sample collection, species identification and testing

We examined dynamics *P. destructans* on bats and in the environment in hibernacula across the Northern Hemisphere. We collected swab samples from both hibernating bats and hibernacula substrate surface (walls and ceilings) in caves and mines in North America, Europe and Asia (Fig. a). We visited sites twice per year over a 5-year period (2012 – 2017) to measure changes in infection prevalence and fungal loads in the United States, the United Kingdom, Hungary, and China. In addition, we collected samples from a single time point in mid- to late-winter from Israel, the Republic of Georgia, and Japan.

Epidermal and environmental swab samples were collected using standardized techniques across all regions using a previously described swabbing protocol [34, 93]. For bat sampling, we dipped polyester swabs (Puritan, Guilford, ME) in sterile water, and swabbed the wing and muzzle five times back and forth [112]. We collected environmental samples by swabbing a section of substrate that was similar to the length of a bats' forearm (36-40mm) using the same methods, but without dipping the swab in sterile water. Three types of environmental samples were collected: under hibernating bats ("under"), approximately 10 cm from hibernating bats ("near"), and >2 m from hibernating bats ("far"). Bat and environmental samples were stored in salt buffer (RNAlater; Thermo Fisher Scientific) immediately following collection to

preserve DNA. We extracted DNA from samples using a modified Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) [34] and tested for the presence of *P. destructans* using quantitative PCR [78]. All samples were run in duplicate, with quantification standards on each plate and 16 negative controls per plate. All quantification standards were within a consistent range and all negative controls had no fungal detection.

We counted all bats present at each site by species. For bats that could not be identified based on morphological criteria, we collected a wing biopsy (3mm; Miltex, Plainsboro, NJ) for molecular identification. The DNA from wing biopsies was extracted using a Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and we used Sanger sequencing to amplify the Cytochrome c oxidase I gene (CO1) [113]. The CO1 gene sequences were compared with known sequences in the EMBL database [114] using MEGA BLAST (BLASTN 2.1.1, [115]) to identify the most similar sequence alignment. All sampling was conducted under UCSC IACUC protocol FrickW1106 and National Animal Research Authority in Northeast Normal University, China, approval number: NENU-20080416.

Analyses

We analyzed changes in *P. destructans* prevalence on bats or the environment in the North America using generalized linear mixed models with a binomial distribution, and species or substrate type (under bats, near bats, or far from bats), date, and years since *P. destructans* detection as fixed effects, and site as a random effect. To show changes in *P. destructans* prevalence over the winter in endemic

regions, we subsetted to an individual country and ran four candidate models: date interacting with species, date with an additive effect of species, date alone, and species alone as fixed effects with site as a random effect. We selected the best model based on AIC. For countries where data was collected during a single time period during the winter, we excluded date from the analyses. We examined the relationship between early winter infection prevalence in bats and the prevalence of *P. destructans* in the environment at each site using linear mixed models with site as a random effect. We also examined the relationship between fungal loads on bats in late winter (which are highly correlated with WNS impacts among species [116], and with lesions [117]), with early winter infection prevalence in bats using a linear mixed effects model with site as a random effect.

Results:

We collected an average of 86 samples (range 5-496) from 35 species from over 75 sites with a total of 6,054 samples from bats and the environment (Table S1). In North America, prevalence on bats and in the environment increased sharply in the first year of *P. destructans* detection, from very low levels in early winter to moderate to high levels by winter's end (Fig. 1a; Table S2). In the second and subsequent years after *P. destructans* detection, prevalence started at much higher levels in early winter in all but one species, and increased to 100% by late winter (Fig. 1a; Table S2). There was some variation among species, with *M. lucifugus and M. septentrionalis* becoming infected the fastest, followed by *P. subflavus* and *Eptesicus fuscus* (Fig. 1a, Table S2). Prevalence in the environment showed a similar pattern of increasing over time within each winter and with each subsequent year, but the increase was

substantially slower than infection prevalence in bats. Prevalence in the environment did not begin to saturate until year three years after *P. destructans* detection (Fig. 1b; Table S3).

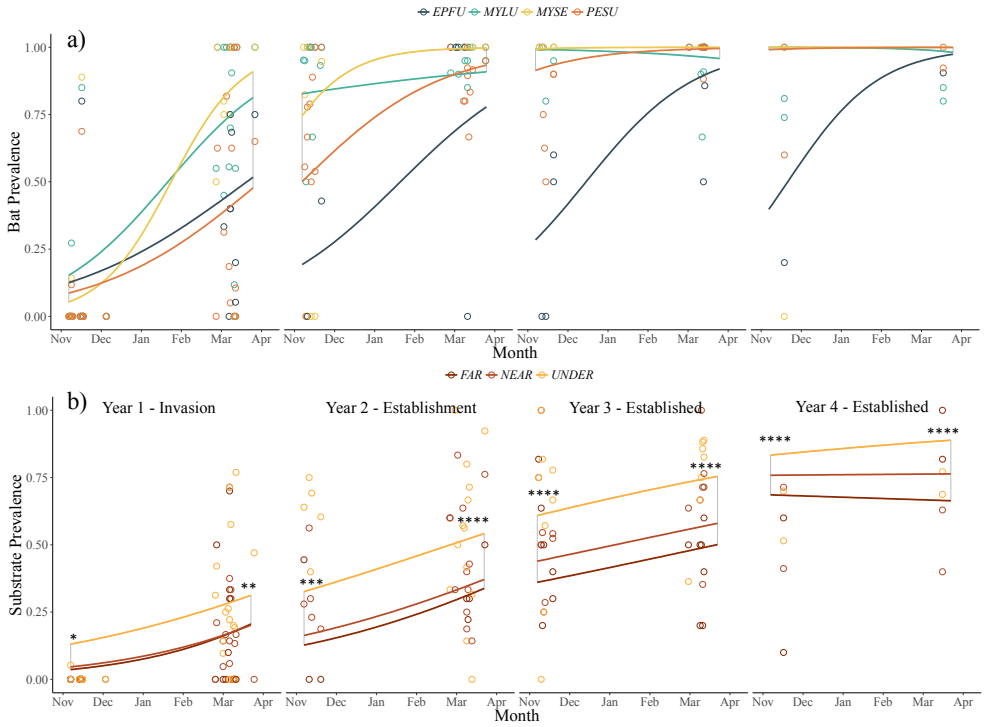

Figure 1: Changes in *P. destructans* prevalence on bats (a) and in the environment (b) during the invasion and establishment in North America. Invasion of *P. destructans* is shown from left to right, over a total of four years of WNS. The best supported model for both bats and the environment include a triple interaction between winter date, years since WNS (continuous), and species or substrate type (Table S2,3). Gray bars show the difference between the upper and lower limits of the predictions for all species and sample types, excluding *E. fuscus*. * indicates different levels of significance both within and among winters ($P<0.05$, Table S3).

There was no evidence of a decline over the summer in fungal prevalence in the environment in any of the four years after *P. destructans* detection (Figure 1, Table S3).

Across Eurasia, prevalence on bats in early winter was very low for most species, and increased at variable rates among species and regions (Figure 2, Table S4). The increase in prevalence over winter for the most infected species was similar to the rise in infection in North America during the first year of *P. destructans* invasion (Fig. 2, Table S2, Table S3). For example, increases in *P. destructans* on *Myotis petax* in China over the winter (slope coef: 0.93 +/- 0.09) were similar to increases in *P. destructans* on *M. lucifugus* in North America during the first year of WNS detection (coef: 1.20 +/-0.12).

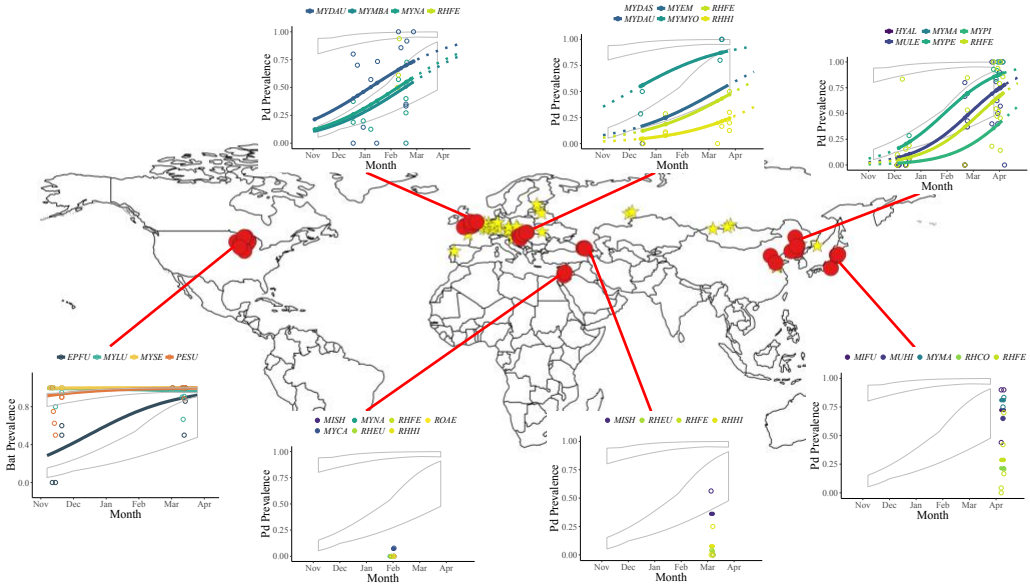

Figure 2: Global *P. destructans* prevalence dynamics on bats. Red dots indicate sites where samples were collected for this study and the yellow stars represent all known locations where *P. destructans* has been detected across Eurasia. Transparent overlays on each graph represent the upper and lower prevalence limits of highly impacted species for both the invasion (lower) and establishment (upper; mean for years 1, 2, and 3) of *P. destructans* in the North America, excluding the less-affected *E. fuscus*. Open circles represent prevalence for a given species at a site and the lines indicate best fit model for each species in each region (Table S4). Dashed lines indicate predictions beyond the date range of the data. Four letter species codes correspond to the first two letters of the genus and species names (Table S1). The

North American data show the changes in prevalence for year 3 of WNS for comparison of dynamics across all established regions.

In contrast, several species across Eurasia (*Rhinolophus ferrumequinum* and *Rhinolophus hipposideros*) showed only moderate increases in prevalence (25-30%), and one species, despite being well-sampled (*Hypsugo alaschanicus*, N=47) was never detected with *P. destructans* (Table S4).

P. destructans prevalence in the environment in early winter was lower in Eurasia than in the North America (years 2-4 after *P. destructans* detection). Prevalence of *P. destructans* in the environment increased significantly over the winter across Eurasia (Table S4). However, in China, where we collected multiple years of data in early and late winter, the prevalence of *P. destructans* significantly decreased over the summer (Figure 3). We found that early winter infection prevalence on bats across Europe, Asia, and North America increased significantly with *P. destructans* in the environment (Figure 4, left). In turn, early winter infection prevalence on bats predicted late winter fungal loads (Figure 4, right).

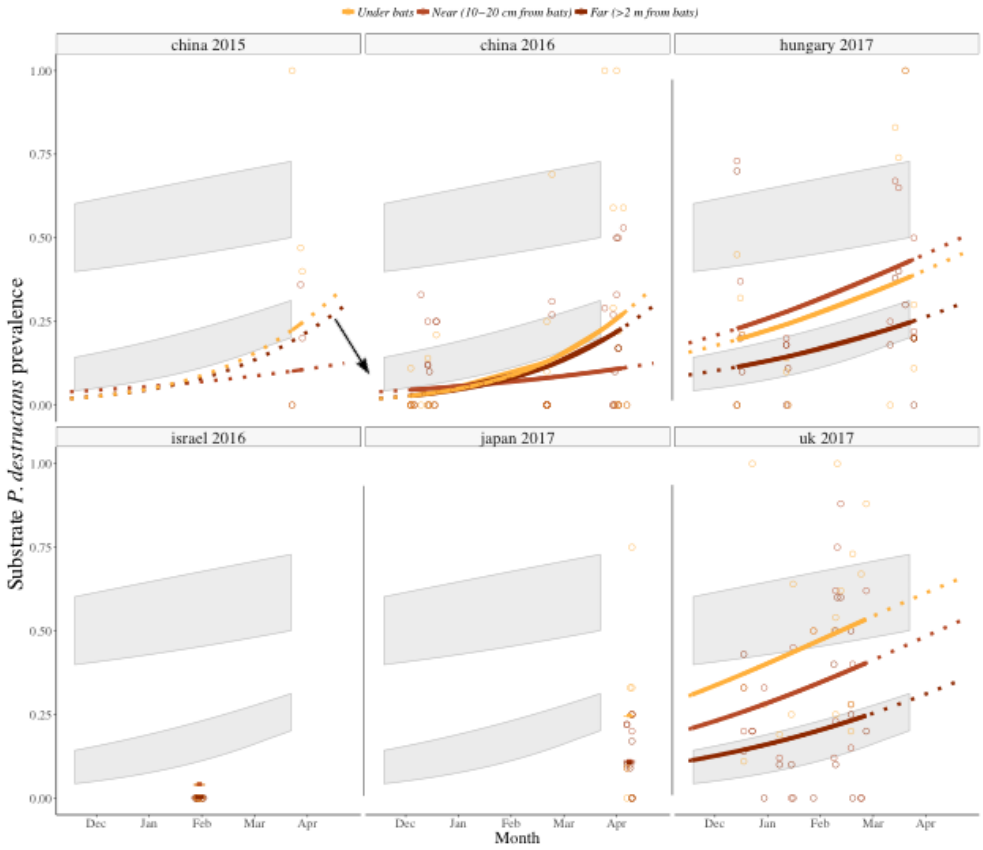

Figure 3: Global *P. destructans* prevalence dynamics in the environment. Transparent overlays on each graph represent the upper and lower prevalence limits of samples collected from under, near (10cm), and far (>2m) from bats for both the invasion (lower) and establishment (upper; mean for years 1, 2, and 3) of *P. destructans* in the North America. Open circles represent prevalence for a given substrate sample type at a site. Lines show model fits to the data and dashed lines indicate predictions beyond the date range of the data. Arrow shows decrease in substrate prevalence over the summer in China (GLMM: intercept (China early hibernation 2016): -2.819 +/- 0.420, coef (China late hibernation 2015): 1.729 +/- 0.463, $P < 0.0001$).

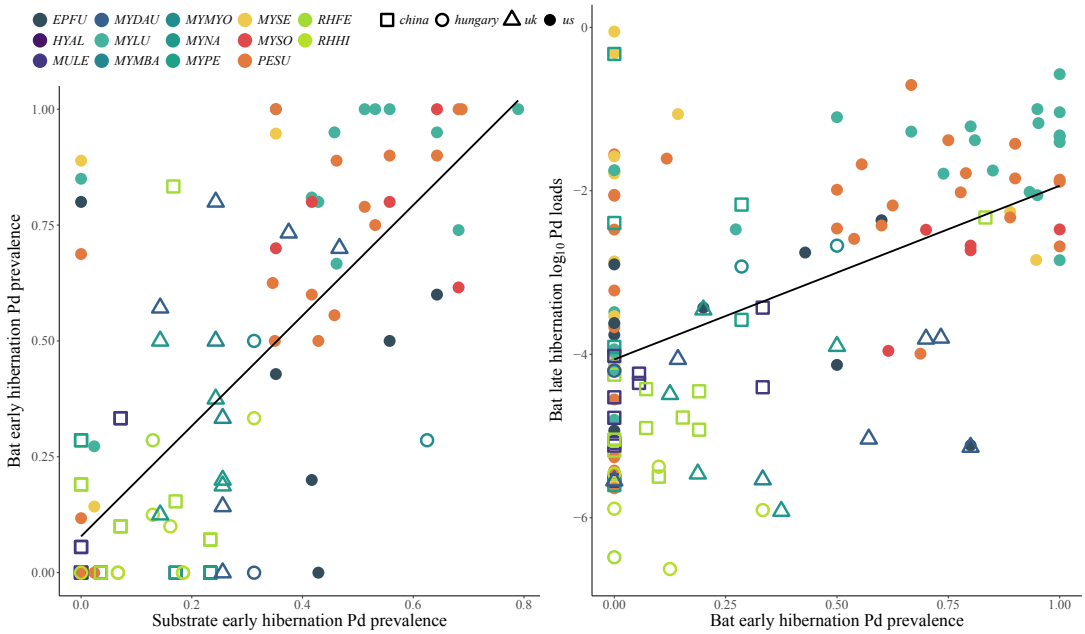

Figure 4: Relationship between early winter *P. destructans* substrate prevalence, early winter bat prevalence, and late winter bat fungal loads. (Left) Each point shows the relationship between substrate prevalence in early winter for a site, and the early hibernation prevalence of bats of different species in each site (intercept: 0.053 coef: 1.267 +/- 0.115, t = 11.045, P<0.001). (Right) Points show the relationship between *P. destructans* prevalence on bat species in early winter and average late winter fungal loads (log_{10}) on that same species in a given site (intercept: -4.118 coef: 1.743 +/- 0.312, t = 5.597, P<0.001).

Discussion:

Introductions of pathogens over the last several decades have resulted in substantial impacts to wildlife and human health [3, 5, 118]. Despite the often-devastating effects of introduced disease, the dynamics of emerging pathogens in areas where host impact is reduced are often poorly understood. Our results indicate that bats in Eurasia likely persist with *P. destructans* through multiple mechanisms. Several bat species appeared to be inherently resistant or resilient to infection, showing no evidence of *P. destructans* infection, or relatively small increases in prevalence over the winter. Several other bat species did not demonstrate inherent resistance with *P. destructans*. After becoming infected, these species showed

increases in *P. destructans* comparable to North American species. A key difference allowing these susceptible species to persist with *P. destructans* in Eurasia appears to be a reduction in early hibernation prevalence, determined by decreases in *P. destructans* in the environmental reservoir when bats are absent from hibernacula over the summer. Mortality from WNS in North America occurs at least 70-120 days after initial infection [25], and the substantially later infection of most bats in Eurasia may explain the persistence of these species with this disease.

In contrast, in North America, there was no evidence of decreases in *P. destructans* in the environmental reservoir over the summer. Instead, the environmental reservoir showed a steady increase from initial invasion through year three of establishment. The difference between North America and Eurasia in the persistence of the environmental reservoir are consistent with the enemy or ecological release hypothesis observed in other invasive species [119-122]. Although the natural enemies causing the decrease in *P. destructans* over the summer in Eurasia are unknown, substrate dwelling bacteria and nematodes can have profound impacts and regulate populations of saprophytic fungi [123-126]. It appears that North American hibernacula may lack the natural enemies that compete with or consume *P. destructans*.

While the *P. destructans* in the environment across Europe and Asia provides a plausible explanation for reduced population impacts, there are numerous other factors that may also contribute to differences in impacts between North America and Eurasia, as well as the differences in dynamics among endemic countries. These

include differences in hibernation phenology related to climate or behavioral factors, innate resistance or resilience limiting fungal growth, density-dependent or species composition related transmission patterns, or differences in pathogen strains. Future work should aim to explore these additional sources of variation in both Eurasia and North America.

Our data suggest that reductions in *P. destructans* in the environment could be an effective tool for managing WNS. An important next step is to identify agents that are effective in reducing *P. destructans* in the environment and can be applied during the summer when bats are absent from hibernacula. Biological controls that regulate fungal populations have been suggested [123], but substantial hurdles exist in demonstrating safety and non-target effects.

More broadly, our results underscore the importance of understanding disease dynamics in invading, epidemic, and endemic contexts. Although links between the environmental reservoir and bat infection dynamics were apparent during *P. destructans* invasion into North American sites, the environmental decay in Asia coupled with lower *P. destructans* environmental reservoirs in endemic sites across the globe, drew attention to the management of environmental reservoir through biological control as a potential conservation strategy to preserve bat populations. Our results also highlight the potential importance of ecological release of pathogens in driving disease dynamics and determining species extinction.

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