SEX-BIASED INFECTIONS SCALE TO POPULATION IMPACTS FOR AN EMERGING WILDLIFE DISEASE

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Keywords: Sex biased infection, emerging infectious disease, white-nose syndrome, bats, Allee effects, fungal disease
ABSTRACT:
Demographic factors are fundamental in shaping infectious disease dynamics. Aspects of populations that create structure, like age and sex, can affect patterns of transmission, infection intensity and population outcomes. However, studies rarely link these processes from individual to population-scale effects. Moreover, the mechanisms underlying demographic differences in disease are frequently unclear. Here, we explore sex-biased infections for a multi-host fungal disease of bats, white-nose syndrome, and link disease-associated mortality between sexes, the distortion of sex ratios, and the potential mechanisms underlying sex differences in infection. We collected data on host traits, infection intensity, and survival of five bat species at 42 sites across seven years. We found females were more infected than males for all five species. Females also had lower apparent survival over winter and accounted for a smaller proportion of populations over time. Notably, female-biased infections were evident by early hibernation and likely driven by sex-based differences in autumn mating behavior. Male bats were more active during autumn, which likely reduced replication of the cool-growing fungus. Higher disease impacts in female bats may have cascading effects on bat populations beyond the hibernation season by limiting recruitment and increasing the risk of Allee effects.

1. INTRODUCTION
Emerging infectious diseases are a serious threat to wildlife health (1, 2). Population structure can shape epidemics by influencing spatial spread, outbreak size, and host impacts (2-4). Elements of populations that create structure such as classes of individuals of specific ages, sexes, or breeding stages, can have profound effects on disease dynamics (3, 5-9). Sex is an especially important factor because sex-biases in infections can contribute to differential transmission through populations due to behavior (10-15), amplify outbreaks due to seasonal changes in susceptibility (16-19), and modify population impacts through disproportionate mortality (20-22). Differences in infection and mortality can also modulate virulence evolution through sex-specific immune responses that affect pathogen replication and growth (23, 24). As such, determining patterns and mechanisms of sex-biases in infections can improve efforts to minimize outbreaks and manage impacts.

Behavioral and physiological traits are two mechanisms that can produce sex-biases in transmission, susceptibility, infection intensity, or disease-induced mortality (25-27). In most systems, males have an elevated risk of disease compared to females due to territory defense and
promiscuous mating, which increases contacts with conspecifics or increase pathogen susceptibility due to physiological stress (9, 25, 28). Sex hormones can also have strong effects on host immunity (29), such that testosterone suppresses immune responses whereas estrogens enhance it, resulting in weaker immune responses in males, which can increase susceptibility to pathogen infection (25, 30, 31). As such, the majority of empirical studies find that infections are typically male-biased (28, 32, 33), although this generalization is sometimes reversed (34-40) and may be linked to seasonal reproductive stress associated with pregnancy, parturition, or parental care (16, 17). Thus, it is likely that the effects of sex-biased infections will be highly pronounced in disease systems where host reproductive strategies differ seasonally among sexes (19).

White-nose syndrome (WNS) is a highly seasonal fungal disease of bats that has caused population collapse, with declines exceeding 95% in many populations of multiple species (41-44). WNS is caused by the fungal pathogen, *Pseudogymnoascus destructans*, which invades the epidermal tissue of bats during hibernation, and disrupts bat homeostasis, causing water and electrolyte imbalances that increase arousals and deplete stored fat (45-52). Transmission of *P. destructans* occurs through host-to-host contacts and contact between hosts and contaminated environmental reservoirs inside winter hibernation sites (53-55). *Pseudogymnoascus destructans* has a thermal growth range of 0-20ºC and optimal growth between 12 and 16 ºC (56). This limits on-host growth to the periods when bats are in torpor and they lower their body temperature to the ambient temperatures of their hibernation sites (57, 58). The restricted growth of *P. destructans* below 20ºC drives the seasonal patterns of WNS, with infection only occurring during winter hibernation and mortality peaking during late winter (59).

Temperate bat species have sex-based seasonally varying behaviors that may influence their exposure, susceptibility, and mortality from WNS (60-63). Bats mate in autumn swarms, and females store sperm over winter, delaying ovulation, and giving birth at maternity colonies in spring (64). Male reproductive energy expenditures are highest during autumn when they aggregate at hibernacula (subterranean sites where bats spend the winter) and mate indiscriminately with females (65). Since autumn swarm coincides with the seasonal transmission of *P. destructans* (59), breeding stress among males as well as exposure to the environmental reservoirs could increase their susceptibility to infection. Female energy expenditures are greatest during pregnancy and lactation in spring and summer when hosts
typically clear infection (66, 67) and transmission of *P. destructans* is low (59), suggesting females may be the less infected sex. However, female bats may spend more time torpid during winter than males to conserve energy for spring reproduction (62) which could influence the growth of *P. destructans*. Due to the differences in reproductive investment and seasonal energy expenditure, sex-based traits have the potential to affect exposure (e.g., contacting *P. destructans* from other hosts or in the environment) and pathogen growth (e.g., time spent at torpid temperatures that permit fungal growth). Thus, we predict sex-specific behaviors, and the highly seasonal pattern of WNS may drive intersex differences in infection, mortality, and population impacts. Here, we examined differences in *P. destructans* infection between females and males across five bat species in 42 sites across seven years. We then assessed the consequences of intersex differences in infection at both the individual and population-level. Lastly, we explored autumn activity patterns between sexes as a potential mechanism of sex-biased infections.

2. MATERIALS AND METHODS

   a. Study sites and sampling design

   We sampled bats at hibernacula in Illinois, Massachusetts, Michigan, New York, Vermont, Virginia, and Wisconsin between 2011 and 2021 during an annual outbreak at two time points: early winter (November to December) and late winter (March to April) (Supplemental Table 1). During site visits, we walked each section of the hibernacula and counted all the bats of each species and resighted any bats that were previously banded. We sampled up to 20 bats of each species during each visit when possible and stratified sample collection throughout hibernacula to obtain a sample reflective of the spatial distribution of bats at each site. Bat species included little brown (*Myotis lucifugus*), northern long-eared (*Myotis septentrionalis*), tricolored (*Perimyotis subflavus*), big brown (*Eptesicus fuscus*), and Indiana (*Myotis sodalis*) bats. The animal sampling protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee protocol 17-180. We followed the field decontamination procedures outlined by the U.S. Fish and Wildlife Service Decontamination Guidelines as well as the recommendations provided by individual states (68, 69).

   b. *P. destructans* sample collection and quantification

   For every bat we sampled, we determined species and biological sex based on external morphology and attached an aluminum-lipped band (2.4, 2.9, and 4.2mm; Porzana Ltd.,
Icklesham, E. Sussex, U.K.) to the wing for individual identification and resighting. We collected an epidermal swab from individuals to quantify infection prevalence (the fraction of individuals positive for *P. destructans*) and severity (the quantity of *P. destructans* on infected bats) using a standardized swabbing technique (44, 59). We placed swabs in RNAlater® for storage before testing. We extracted DNA and tested for *P. destructans* presence and quantity by quantitative PCR (59, 70).

c. Activity data collection

At three sites in Wisconsin in 2020, we installed radio frequency identification (RFID) systems, consisting of a passive antenna and an automated data logger (IS10001; Biomark, Boise, ID) at the entrances of each hibernaculum (2-3 per site). The systems at each entrance were equipped with a solar or direct power source to run continuously. During autumn swarm, we captured little brown bats swarming near hibernacula and injected 12.5-mm passive integrative transponder (PIT) tags (Biomark APT12; Biomark, Boise, ID). Each RFID system was programmed to record each time a unique individual passed through an entrance with a one-minute delay to avoid duplicate detections of tags. We scored a detection as an active bat and used it to characterize activity of females and males.

d. Statistical analyses

i. Infection

We used generalized (GLMM) and linear (LMM) mixed effect models (71) to compare differences in *P. destructans* prevalence and infection severity between males and females of each of the five species. We defined prevalence as the fraction of bats testing positive for *P. destructans* on qPCR (0|1), and infection severity as the log-transformed quantity of fungal DNA (ng) on positive bats (log10 fungal loads). We used site as a random effect for all infection analyses. We estimated differences in prevalence between sexes using a GLMM with a binomial distribution and sex and species as fixed effects and compared models of additive and interactive versions of these terms. For prevalence, we used data from early hibernation only as every bat was infected at the end of hibernation. To estimate changes in fungal loads between sexes over winter, we used a LMM with species, sex, and date as fixed effects. We also compared changes in over winter fungal loads (the difference between late hibernation and early hibernation loads) using a subset of recaptured individuals that were sampled in early and late hibernation of the same year.
ii. Individual survival

We examined differences between sexes in individual over winter survival using data from a single species, little brown bats, which were abundant enough to obtain reasonable sample sizes. We compared the probability of observing bats in late hibernation (March) that we had observed in early hibernation (November). Bats affected by WNS often emerge prior to spring and die on the landscape (72), enabling the use of recapture as a proxy for individual survival (73, 74). We used a mixed effects model with site as a random effect with a binomial distribution and a logit link to quantify how sex affected the probability of a bat being resighted over winter.

iii. Population-level impacts

We estimated the proportion of female little brown bats sampled at each visit from a core set of sites (N=14) that were sampled in multiple consecutive years to evaluate how female proportions were changing over time. We estimated the probability of sampling a female (vs a male) over time using generalized linear mixed model with a binomial distribution and a logit link with years since P. destructans invasion as a fixed effect, and site as a random effect.

iv. RFID activity

To explore differences in autumn activity of male and female bats, we used a generalized linear mixed model with a binomial distribution and a logit link with sex as a fixed effect and individual bat identification as a random effect. For our response variable, we treated each bat as a series of binomial trials where it could be detected (=1) or not detected (=0) on a given night throughout autumn swarm that any RFID system was operational at an entrance, as determined by the detection of a programmed test tag. We determined the period of autumn swarm to be between the dates in which more than 95% of all individual detections occurred (beginning of swarm; August 19) and all individual detections concluded (end of swarm; October 01). Results were qualitatively similar if we included all dates in which any bat was detected from early August to November. Because bats are nocturnal and daily activity patterns span two calendar days, we treated detections that occurred between hours 0:00 and 07:00 as a detection during the previous night for consistency with bat ecology.

3. RESULTS

We examined the effects of sex on P. destructans infections of five bat species during winter. We sampled a total of 665 females and 1071 males across all species, sites, years, and seasons.
On average, females had higher *P. destructans* prevalence than male bats in early winter (GLMM with site and species as random effects; female coef +/- SE: 0.630 +/- 0.221, P = 0.0043; Figure 1; Appendix 1.1) and suffered from higher fungal loads overall (LMM with site and species as random effects; female coef +/- SE: 0.300 +/- 0.072, P < 0.0001; Figure 2; Appendix 2.1). The best supported model (ΔAIC = -3.11) for predicting early winter *P. destructans* prevalence included species with the additive effect of sex, indicating female infections are generally higher across all bat species in our study (GLMM with site as random effect; female coef +/- SE: 0.637 +/- 0.223, P = 0.0043; Figure 1; Appendix 1.2).

The strong differences in infection prevalence during early winter paralleled higher fungal loads on female bats during the hibernation season. Across species, females began hibernation with higher fungal loads than males (LMM with site as random effect; female coef +/- SE: 0.490 +/- 0.093, P < 0.0001; Figure 2; Appendix 2.2). Fungal loads appeared to become more similar by the end of winter (Figure 2); however, this is likely a result of a mortality bias (Figure 3; Appendix 3.1) such that individuals with higher fungal loads are less likely to survive to late winter (Supplemental Figure 1A).

We did not detect a clear effect of early hibernation temperatures or body mass that explained the sex differences in prevalence, infection intensity, or mortality consistently across the species for which we had sufficient data to analyze (Supplemental Figure 2). Data from individual little brown bats sampled in both early and late winter indicated that the change in fungal loads over winter did not differ between sexes (LMM with site as a random effect; female coef +/- SE: -0.141 +/- 0.263, P = 0.593; Supplemental Figure 1B; Appendix 5.2).
Figure 1. Early winter *Pseudogymnoascus destructans* prevalence by sex for five bat species. The colored points show the proportion of individuals that were infected at a given site and year. The size of colored points is scaled to the number of bats sampled during the site visit. The black circles show model-predicted prevalence with 95% confidence intervals denoted with error bars. Horizontal blue and orange lines show the grand mean prevalence for all females and males, respectively.
Figure 2. (a-e) Change in *Pseudogymnoascus destructans* infection severity (fungal loads) throughout winter by sex for each of the five host species. A point represents an individual bat, and females and males are shown in blue and orange, respectively. Lines represent model predicted fungal loads over winter. (f) Fungal loads in early winter by sex for each host species. Black points show model predicted fungal loads. Error bars show 95% confidence intervals. Blue and orange horizontal lines show grand mean fungal loads for all females and males, respectively.
At an individual-scale, using data from little brown bats that were banded in early hibernation (Supplemental Table 2), we found clear support that female little brown bats were less likely to be recaptured in late winter than males (GLMM with site as random effect; female coef +/- SE: -0.855 +/- 0.380, P = 0.0240; Figure 3A; Appendix 3.1). At a population-level, the proportion of females sampled in the same populations at the end of winter (Supplemental Table 3) decreased with time since WNS invasion (GLMM with site as random effect; years since invasion coef +/- SE: -0.363 +/- 0.173, P = 0.0365; Figure 3B; Appendix 3.2). Collectively, these data indicate that females have higher, more severe infections, and correspondingly experience higher mortality during winter from WNS.

Females begin hibernation with more severe infections than male bats and differential activity between sexes may influence infections. We found that the probability of a female being active on a given night was five-fold lower than males during autumn. (GLMM with individual bat as random effect; female coef +/- SE: -1.643 +/- 0.182, P < 0.0001; Figure 4; Supplemental Table 4; Appendix 4.1).
Figure 3. (A) Recapture probability of little brown bats (*Myotis lucifugus*) by sex. Blue and orange points show the mean probability of recapture from early (November) and late (March) winter from a given site and year for females and males, respectively. Colored horizontal bars show the grand mean recapture probabilities for each sex across all sites. (B) Proportional change in females with time since *Pseudogymnoascus destructans* invasion. Points show the proportion of sampled individuals that were female at the same sites sampled over time where *P. destructans* had been present for 1 & 2 years compared to years 3 & 4. Black points in both
panels show the model predicted means and error bars show 95% confidence intervals. The size
of points in both panels is scaled to the number of bats sampled during the site visit.

Figure 4. Differences in autumn activity of female and male little brown bats (*M. lucifugus*) at
three hibernacula. Each data point represents the proportion of nights a bat was active at a site.
The proportion of nights active was calculated as the number of nights an individual was
detected at least once on the RFID systems divided by the total number of nights the RFID
systems at each site were operating throughout autumn swarm. Points at 0 represent tagged bats
that were never detected on a reader. Black points show model predicted activity by sex. Error bars show 95% upper and lower confidence intervals.

4. DISCUSSION

Our results demonstrate that intersex differences in infection are an important driver of disease dynamics. Across all species, females experienced higher infection prevalence and fungal loads by the beginning of winter hibernation. In little brown bats, females were also less likely to be recaptured over winter, indicating that more severe infections result in higher mortality among females. Finally, female-biased mortality has likely resulted in a population-level reduction in females over time. Higher impacts to female bats may mask even more severe long-term impacts from WNS (75, 76) as a disproportionate loss of recruiting females will likely have more severe population-level consequences than male-biased declines.

We consistently observed higher pathogen prevalence and intensity in females than males across bat species. In other vertebrates, males maximize fitness at the cost of greater exposure and susceptibility to pathogens (33, 77-79), in part through higher testosterone concentrations which correlate negatively with immune defense (80-82). Given male bats undergo their most substantial investment in reproduction during autumn swarm compared to females that invest relatively little towards reproduction throughout autumn and over winter (64), we expected infection to be higher in males. However, we found the opposite pattern with females having more severe disease. Drivers of higher female infections likely include sex-based physiological differences (25). Temperate female bats use fat stores acquired in autumn more slowly than males suggesting a more substantial constraint on energy expenditure compared to males throughout the autumn to spring period (62, 63). Therefore, different physiological strategies (e.g., torpor patterns) between female and male bats could be shaping sex-biased infections.

Differences in sex-based physiology and behavior were supported by our data showing that the proportion of nights males were active during autumn was substantially higher than females. Differences in autumn activity patterns are likely motivated by differences in reproductive energy allocation between sexes. For males, their fitness is enhanced by remaining active so they can mate with as many females as possible. However, female bats, which store sperm and delay ovulation until spring, may prefer to conserve energetic resources during fall and use torpor more extensively (63). As bats arrive to contaminated hibernacula for autumn swarm and are exposed
to the pathogen, torpor use in autumn after exposure may permit pathogen growth when hosts reduce their body temperatures to be within the thermal range of *P. destructans* growth. The relationship between sex-based activity and fungal intensity may arise through two potential pathways associated with torpor expression. First, less active females may provide favorable conditions for pathogen growth for a longer period prior to hibernation compared to males, thus resulting in the more severe female infections. Second, more active males may be able to inhibit pathogen growth through euthermia compared to females, as euthermic mammals mount more robust immune defenses than torpid or hibernating mammals (83, 84). Our activity estimates, which are consistent with other studies (61, 85), suggest that vast differences between male and female bats likely contribute to differences in infection.

We were less likely to recapture female little brown bats over winter than males, indicating greater mortality in females affected by WNS. Generally, infections increase on bats overwinter before reaching a threshold at which bats die from their infections (42). Female bats begin hibernation with higher fungal loads than males but have similar fungal growth patterns over winter (Supplemental Figure 1), indicating that females begin dying in mid-winter as they reach high infection levels. Ultimately, the death of females with the highest infections early in winter results in similar infections between males and the remaining females at the end of hibernation (Figure 2). Our report of higher mortality in females with higher fungal loads is supported by previous work demonstrating that WNS severity is positively correlated with mortality, and mechanistically linked by more frequent arousals (42, 45, 51, 73). Several lines of evidence also suggest that females did not leave hibernation sites early and survive elsewhere. In our study region, emergence from hibernation in healthy populations does not begin until four to six weeks after our sampling (86). Given that the proportion of females showed consistent declines each year following WNS invasion, our results indicate that reductions in overwinter recaptures of females are due to WNS mortality. Previous studies on intersex differences in survival associated with WNS have found contrasting results. One study observed lower female survival in naturally infected bats (87) whereas another reported higher female survival in experimental infections where each sex was inoculated with the same dose of *P. destructans* at identical times (88). Our results suggest that differences in disease outcomes are due to differences in pathogen growth during autumn. Therefore, inoculating both sexes with the same dose simultaneously in the
experimental infection may have eliminated the underlying difference in early winter infections between female and males.

We provide the first evidence that sex-based survival of bats affected by WNS likely scales to population-level impacts. In the first year after *P. destructans* invasion, the mean proportion of females in late hibernation was 45%, but this percentage declined to 21% after four years of WNS. The reduction in the percent of females with time since pathogen invasion differs from previous studies of healthy bat populations that show relatively consistent interannual proportions of females (89-92). This suggests that that a comparable decline in females would not be typical in non-diseased populations. Losing females at higher rates than males will affect the ability of bat populations to respond to WNS. First, a reduction in the number of females will limit the recruitment potential of bat populations and is likely to be especially critical for imperiled populations that remain small and vulnerable to Allee effects. Many temperate bat species, including little brown bats, form maternity colonies during summer when they cooperatively rear offspring and larger colony sizes afford females lower energy expenditure (93). If birth and offspring survival rates decline with density, as shown in other temperate bat species (94), the disproportionate loss of females could further impact recruitment. Second, distorted sex-ratios may negatively affect reproductive success during autumn. In other taxa, a significant increase in male to female sex-ratios resulted in increased stress to females from exacerbated pressure for males to mate with limited females, negatively affecting female fecundity (95, 96).

Our results include impacts to female bats up to four years after the arrival of WNS, which includes the epidemic phase of the disease, when the majority of mortality occurs (75). Females are likely under strong selection pressure to evolve mechanisms of survival given their increased mortality and will need to adapt in for populations to rebound. Future work focusing on the effects of female infection and mortality biases on bat population persistence and recovery could benefit conservation efforts, especially as the negative effects are likely to compound over time as sex ratios become increasingly distorted.

We find a novel example in which female-biased infections are shaping population-level impacts with an emerging disease. Our results provide a clear instance of an emerging pathogen that consistently affects females more than males regardless of host species. We describe a new mechanistic explanation to female-biased infections that links temperature-dependent fungal
growth to sex-specific seasonal physiology. Ultimately, disparate infections among demographic
classes of hosts are fundamental for understanding and managing emerging infectious diseases,
and cross-scale analyses can provide insights into the important consequences of demographic
biases on disease systems.

**ACKNOWLEDGEMENTS**

We thank Steffany Yamada for data curation support, Skylar Hopkins for analysis support, Rick
Reynolds and Carl Herzog for logistical support, and the many landowners for site access.

**FUNDING**

The research was funded by National Science Foundation grants DEB-1115895 to A.M.K. &
J.T.F., DEB-1911853 to K.E.L., J.R.H., A.M.K. & J.T.F., the USFWS (F17AP00591) to K.E.L.,
and by Virginia Tech Institute for Critical Technology and Applied Science to M.J.K.

**DISCLAIMER**

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endorsement by the U.S. Government.

**AUTHOR CONTRIBUTIONS**

M.J.K.: conceptualization, investigation, methodology, visualization, data curation, writing-
original draft, writing-review and editing, formal analysis; J.R.H.: conceptualization,
investigation, methodology, visualization, funding acquisition, resources, project administration,
data curation, writing-review and editing; J.P.W.: investigation, writing-review and editing;
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investigation, writing-review and editing; W.H.S.: investigation; K.L.P.: investigation, writing-
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A.M.K.: investigation, funding acquisition, writing-review and editing; K.E.L.: conceptualization, investigation, visualization, methodology, funding acquisition, resources,
project administration, data curation, writing-original draft, writing-review and editing,
supervision.
All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

**DATA, CODE, AND MATERIALS**

The datasets and code generated in this study will be deposited in Dryad Digital Repository upon final submission. Exact site locations are not disclosed to protect endangered species and landowners.

**CONFLICT OF INTEREST DECLARATION**

The authors declare no competing interests.

**ETHICS**

All bat handling procedures were reviewed and approved by Virginia Tech Institute for Animal Care and Use Committee protocol 17-180.

**LITERATURE CITED**


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