Case study: methods and observations of overwintering *Eptesicus fuscus* with White-Nose Syndrome in Ohio, USA

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A healthy large brown bat *(Eptesicus fuscus)*.

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ABSTRACT: Temperate, cave-dwelling bat populations in eastern North America are facing drastic declines due to the emergent disease called White-Nose Syndrome (WNS). In Ohio, USA, wildlife rehabilitators may accept native bats during the winter months when bats are typically hibernating. During the winter months this deadly fungal infection is the most damaging to individual hibernating, temperate bats' physical and physiological condition, because the bats are more vulnerable to disease while their immune response is low during hibernation. Here, we provide observations and methods for successful care and release of overwintering bats with WNS. In the winter of 2016, we administered simple topical treatments and visually investigated patterns during the care of nine *Eptesicus fuscus,* assumed to be infected with *Pseudogymnoascus destructans* through visual confirmation of orange-yellow fluorescence under ultraviolet light and fungal culture. We developed systematic methods for infected-bat husbandry that led to the successful release of seven of the nine big brown bats treated.

KEYWORDS: bats, *Eptesicus fuscus*, *Pseudogymnoascus destructans*, White-Nose Syndrome, wildlife disease, wildlife rehabilitation

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Introduction

White-Nose Syndrome (WNS) has devastated northeast bat populations in the USA, and continues to spread westerly each year.[1] The fungal pathogen *Pseudogymnoascus destructans* (*Geomyces destructans*), which causes WNS, was first detected in Ohio in 2011,[2,3] and state wildlife laws restricted bat rehabilitation during hibernating months in order to monitor populations of infected animals.[4] Currently listed as an endemic state,[5] the Ohio Department of Natural Resources–Division of Wildlife (ODOW) now allows bat rehabilitation during the winter months under newly created decontamination protocols.[6]

Treatments for WNS have become a research focus for cave-dwelling bat research in the USA.[7–10] Simple treatments for individuals, such as apple cider vinegar solutions *in vivo* and orange essential oil concentrations *in vitro*, have resulted in inhibition of *P. destructans*.[7,10] More complex treatments, such as the use of natural microbiota, also result in inhibition of *P. destructans* growth, with potential for applications at a landscape scale.[8,9] In this investigation, we aim to provide methods and simple treatment observations that are helpful to the individual care of *P. destructans*-infected *Eptesicus fuscus* (big brown bats) admitted into wildlife rehabilitation facilities. Excluding the use of apple cider vinegar solution treatments, to our knowledge there are no publications involving other easily accessible, simple treatments for wildlife rehabilitators.[7] We focus on the use of chlorhexidine solution 0.2% and miconazole nitrate 1% topical ointment for treat-

J. Wildlife Rehab. 38(3): 11–16.

ments, which are typically available to wildlife rehabilitators through their veterinarians. Chlorhexidine solution 0.2% is an antimicrobial agent used for common veterinary dermatological fungal and bacterial conditions, and miconazole nitrate 1% topical ointment is a broad-spectrum anti-fungal agent used for yeast and filamentous fungal infections.[11,12] The use of natural microbiota is not investigated here. Additionally, methods of systematic decontamination practices are incorporated with the daily husbandry of individual *E. fuscus* throughout their stay in Brukner Nature Center's Wildlife Rehabilitation Unit in Troy, Ohio.

TABLE 1. Treatment groups and dates for *Eptesicus fuscus* patients at Brukner Nature Center during winter 2016–17.

Patient	Treatment Date	Admit Date	Treatment Start	Treatment End	Disposition	Disposition Date
1465	Miconazol	16 Dec 16	24 Dec 16	4 Feb 17	Released	28 Mar 17
1467	Control	21 Dec 16	4 Feb 17	18 Mar 17	Euthanized	27 Mar 17
1468	Control	23 Dec 16	24 Dec 16	4 Feb 17	Released	28 Mar 17
1469	Chlorhexidine	23 Dec 16	4 Feb 17	18 Mar 17	Released	11 Apr 17
1470	Chlorhexidine	23 Dec 16	24 Dec 16	4 Feb 17	Released	11 Apr 17
2	Control	4 Jan 17	4 Feb 17	18 Mar 17	Euthanized	27 Mar 17
3	Chlorhexidine	7 Jan 17	4 Feb 17	18 Mar 17	Released	28 Mar 17
5	Miconazol	13 Jan 17	4 Feb 17	18 Mar 17	Released	28 Mar 17
11	Miconazol	2 Feb 17	4 Feb 17	18 Mar 17	Released	10 Apr 17

Methods

Fungal culture

Big brown bat (*Eptesicus fuscus*) patients housed at Brukner Nature Center's Wildlife Rehabilitation Unit were swabbed on 10 February 2017 in areas along the flight membranes and muzzles, selectively chosen through visualization of orange-yellow fluorescence. We swabbed each bat once with a sterile inoculating loop, and once with sterile water and a sterile swab. Each sweep was transferred to an individual Sabouraud Dextrose Agar (SDA) plate (two SDA plates per *E. fuscus*). Plates were transferred to Wright State University in Dayton, Ohio, and kept at 10°C incubation for approximately four months. All United States Geological Survey (USGS) biosafety measures for WNS were followed for transfer, housing, and disposal of contaminated plates in a Biosafety Level-2 laboratory.[5] Culture plates were examined under a dissecting microscope on 13 March 2017 (31 days of incubation) under × 40 magnification for evidence of conidial growth. Slides of culture growth were created with fungal tape on 31 May 2017 (110 days of incubation), examined under a confocal microscope at 60 µm magnification, located at Wright State University's Microscopy Core in Dayton, Ohio. Voucher specimens were taken and stored at 4°C and –80°C.

Animal care and P. destructans treatments

All *E. fuscus* (n = 9) were admitted to Brukner Nature Center's Wildlife Rehabilitation Unit in the winter of 2016 (Table 1), and cared for under permit recommendations (permit #55501).[6] Patients were housed individually in mesh screen 72.8-liter reptariums or ventilated 68.1-liter plastic storage totes with hand towels draped over the sides. Two bats (patients 1467 and 2) were housed together in a 113.6-liter ventilated plastic tote with hand towels draped over the sides, as they were found stranded in the exact same residential home prior to admittance. *Pseudogymnoascus destructans*-infected bat enclosures were quarantined in the same room at 18 to 19°C, with a humidifier, decontaminated every other day with Clorox® wipes, and clean towels provided. All items removed from enclosures were decontaminated following procedures outlined by ODOW Minimum Standards, which requires national decontamination protocols for bats with suspected *P. destructans* through orange-yellow ultraviolet (UV) fluorescence.[6,13–15]

We weighed all bats upon intake, and every other day thereafter. Patients were provided with daily feedings of oral pediatric electrolyte solution within the first week of admittance to account for the dehydration caused by *P. destructans* infection. Bats were roused from torpor daily for hand feedings of 3 g of mealworms, with an additional 3 g of mealworms and water available *ad libitum* until they were consistently gaining weight above 14 g for 3 days. We chose weight consistency above 14 g as a benchmark for a sustainable weight, since it is the low end of the accepted weight range for *E. fuscus* and we did not want to expend more energy during torpor daily by rousing individuals if unnecessary.[16] Hand feedings continued every other day after 14 g, unless the individual regularly free-fed.

Bats were scanned with a 385 nm UV flashlight upon initial exam for bright, orange-yellow fluorescent spots on flight membranes, muzzles, or both. Orange-yellow fluorescent spots and areas were assumed to be cupping erosions formed by *P. destructans* hyphae, and bats were considered infected.[15] Big brown bat patients were not considered for *P. destructans* treatment protocol until additional injuries, conditions, or both were fully resolved (e.g., soft tissue injuries, parasites, etc.). Only those patients presenting emaciation (<14 g), dehydration (skin tugor >3 s), and UV detection of orange-yellow fluorescence were immediately placed into treatment groups.

Three *E. fuscus* were placed in a control group with no topical treatments applied to the flight membranes. Bats administered with topical chlorhexidine 0.2% solution (n = 3) or topical miconazole nitrate 1% ointment (n = 3) were treated once per day for 14 days, and once per week for 28 days thereafter, at the recommendation of Troy Animal Hospital and Bird Clinic veterinarians. Topical treatments were applied dorsally and ventrally to the wing membranes and uropatagium. Photos of muzzles and all ventral and dorsal flight membranes were taken prior to

initial treatment and then once per week throughout treatment. Treatments opportunistically began on two different start dates, due to an increase in patient intake and recovery of additional aforementioned injuries of *E. fuscus* patients already in our care (Table 1). One replication of each treatment or control group began on 24 December 2016 (n = 3), and two more replications (n = 6) began on 3 February 2017. A total of nine bats were enrolled in this study. Excluding extended time, no changes were made in treatment protocol between replications. Decontamination protocols were followed between each patient.[6]

If considered healthy, patients were released back to the township where originally found in May 2017. Two non-releasable *E. fuscus* candidates were humanely euthanized by cervical dislocation. Both euthanized *E. fuscus* accumulated large wing holes and tears during their care, and concerns for a long-term residence in captivity (a potential stay of 6–8 months, from winter to summer) outweighed continued treatment.

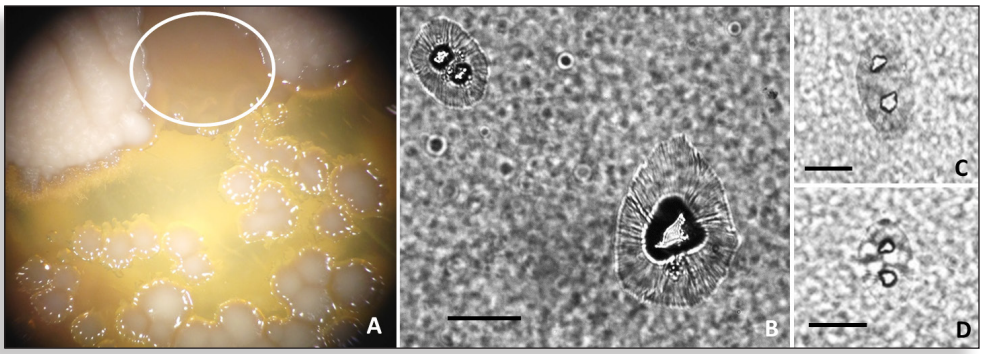

FIGURE 1. Examples of Sabouraud Dextrose Agar (SDA) growth and conidia identification of *Pseudogymnoascus destructans* culture. Scale bars (B to D) are 20 µm. A) Colony formation on SDA plate. The white circle indicates the biofilm associated with *P. destructans* culture growth. Photo taken at 40x magnification under dissecting microscope. B) Two conidia from *E. fuscus* patient 2 (2017 admit). C) Conidia from patient 1470 (2016 admit). D) Conidia from patient 1465 (2016 admit).

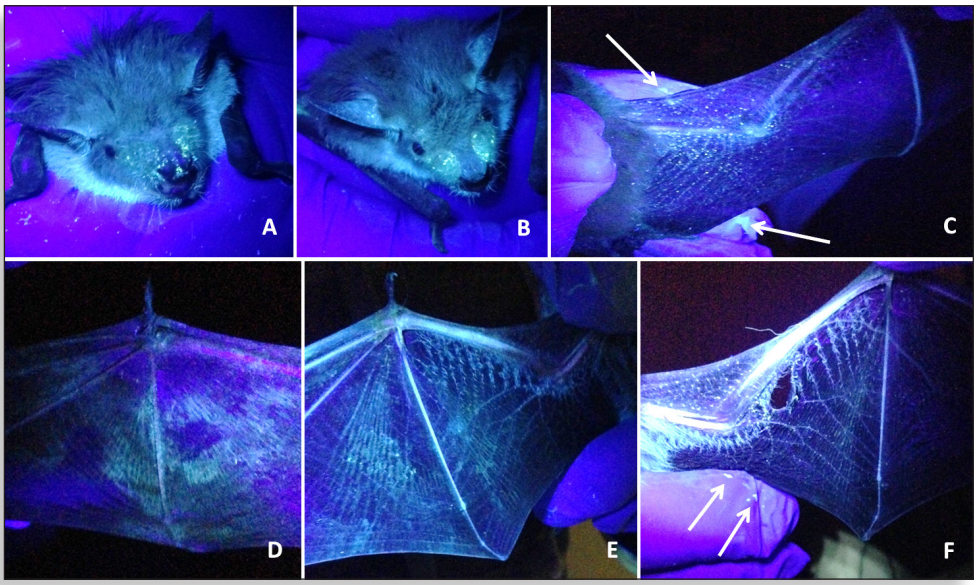

FIGURE 2. Examples of orange-yellow ultraviolet (UV) fluorescence and damage visualized on *E. fuscus* during treatment. Arrows indicate highly fluorescent urine stains on gloves, which only occurred while handling patients 2 and 1467. A) Patient 11 with fluorescence on muzzle. B) Patient 5 with fluorescence on muzzle and ears. C) Patient 2 with fluorescence on right dorsal wing. D) Patient 11 with fluorescent smudging on left dorsal wing. E) Patient 1467 with fluorescent smudging on right ventral wing. F) Patient 1467 with hole and necrotic tissue obtained during its stay.

Results

Pseudogymnoascus destructans culture

Incubation of culture swabs began on 10 February 2017, and growth was first seen on 24 February 2017. By 13 March 2017 (31 days of incubation), five culture plates had fungal growths distinctive of *P. destructans*. Colonies were cream-colored, with a mucoid biofilm surrounding elevated colonies (Fig. 1A). Observed slides indicated conidia typically associated with *P. destructans* (Fig. 1B to 1D), and were used to confirm our initial determinations of bats infected with assumed *P. destructans* cupping erosions detected by UV light.[17]

Patient observations

Eptesicus fuscus (big brown bat) patients within this study were admitted to Brukner Nature Center's Wildlife Rehabilitation Unit in the winter of 2016. Five *E. fuscus* were admitted in 2016 (patients 1465, 1467, 1468, 1469, and 1470), and four *E. fuscus* in 2017 (patients 2, 3, 5, and 11). We considered all patients admitted infected with *P. destructans* through visual confirmation of orange-yellow spots found on their flight membranes, muzzles, or both (Fig. 2A to 2C).

Upon initial exams, it was noted patients 1467 and 2 appeared to have greater fluorescent burden assumed to be *P. destructans*. Patients 1467 and 2 were also the most independent *specimens*, needing the least amount of individual husbandry. These specific patients were both treated as controls (receiving no additional treatments), and their observed fluorescent burden increased throughout the winter. Much of their orange-yellow fluorescent spots became orange-yellow fluorescent smudging or scarring (Fig. 2E). Both patients 1467 and 2 also began to form necrotic holes and tears in their flight membranes (Fig. 2F). Although in the same enclosure, patients 1467 and 2 were never observed roosting together. Both patients additionally expelled highly fluorescent

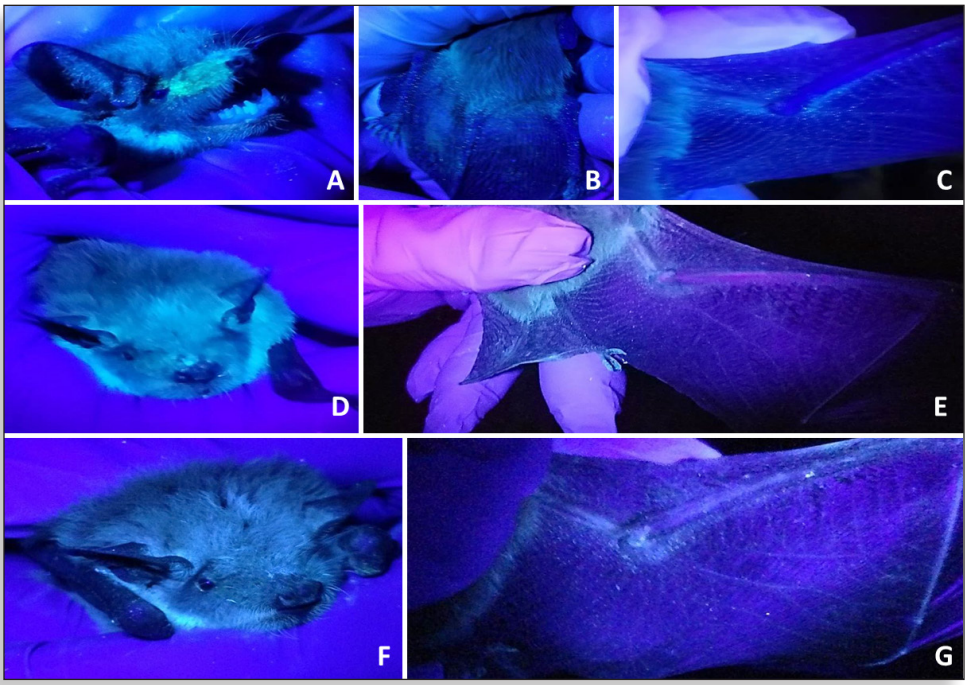

FIGURE 3. Photos of *E. fuscus* patient 1468 under UV light throughout its stay at Brukner Nature Center's Wildlife Rehabilitation Unit. A to C) Photos taken 23 Dec 2016 (0 d). D and E) Photos taken 3 Feb 2017 (42 d). F and G) Photos taken 24 Mar 2017 (91 d). B, C, E, and G) All are dorsal views and correctly oriented.

urine while handled (Fig. 2C and 2E), and this behavior was not observed in any other patients. All observed areas of orange-yellow fluorescence on all control bats (n = 3) intermittently lost its fluorescent color during the treatment period. Areas appeared under UV light as white, dry spots in the same areas where once fluorescing orange-yellow. Big brown bat patients 1467 and 2 were euthanized on 27 March 2017, due to progressive wing damage and an increased observed fluorescent burden. The third control *E. fuscus* (patient 1468) showed improvement with supportive care and fluoresced minimally upon release (Fig. 3).

Bats treated with topical chlorhexidine 0.2% solution (n = 3) visually had the least fluorescent smudging and scarring over time. These *E. fuscus* patients additionally had minimal amounts of observed orange-yellow fluorescent areas by the end of the treatment period. Visual orange-yellow fluorescence of assumed *P. destructans* cupping erosions disappeared and reappeared throughout the treatment period. When orange-yellow color was not visible, the same areas appeared as mentioned above in the control patients. *Eptesicus fuscus* in the chlorhexidine 0.2% solution treatment group were additionally observed to have a decrease in muzzle fluorescence, although treatment was not applied facially. All *E. fuscus* in the chlorhexidine 0.2% solution treatment group (n = 3) were released.

Bats treated with topical miconazole nitrate 1% ointment visually had the most fluorescent smudging over time (patient 11 in Fig. 1D). Within the first two weeks of treatment, *P. destructans* cupping erosions appeared larger, and then dissipated to original size with the included blotchy fluorescence on the wing membranes. All *E. fuscus* treated with miconazole nitrate 1% ointment also displayed a decrease in fluorescence on muzzles, contrary to where treatment was applied. Visual fluorescence additionally increased and decreased throughout the treatment period. All *E. fuscus* in the miconazole nitrate 1% ointment treatment group (n = 3) were released.

Discussion

Fungal culture and slide examination indicated that at least five of the nine *E. fuscus* patients were most likely infected with *P. destructans*. For the four patients without fungal growth in culture, it is likely there was no transfer of *P. destructans* during swabbing and plating, even though we swabbed bats opportunistically in fluoresced areas. Multiple culture plates were contaminated with other fungal growth (mainly yeast, *Aspergillus*), which we expected to happen since the Rehabilitation Unit at Brukner Nature Center is not a sterile environment. Although histology is used for confirming WNS and qPCR analysis indicates *P. destructans* fungal load best across all stages of disease severity, as a nonprofit organization, we used the resources that were readily available to us at the time to confirm *P. destructans* presence on our big brown bat patients.[18,19]

Seven of the nine bats in this case study were successfully released back into the wild. Although patients 1467 and 2 were euthanized, it is not to be assumed because they did not receive treatment in a control group. They are to be considered as individuals with infections that appeared more virulent than others. What should be more encouraging is patient 1468. Big brown bat patient 1468 only received supportive care throughout its stay and showed improvements during the treatment period, and more visual improvements upon release (Fig. 3). Although statistics are not shown here (due to blurred photos refraining post-hoc analysis in Image-J®), it is possible that the health of patient 1468 benefited from a warmer environment, feeding, and supplemented pediatric electrolyte solution. Big brown bats have varying thermoregulatory patterns across geographic locations and have longer bouts of torpor during hibernation in comparison to *Myotis lucifugus* (little brown bat) under *P. destructans* infection.[20,21] Since all *E. fuscus* in our study were local (within ~100 km of the Rehabilitation Unit), and we regularly disrupted torpor to ensure caloric intake under the warmer temperatures, it is likely *E. fuscus* 1468 and all other released bats benefited from the same supportive care, regardless of topical treatment application. Further analysis with more detailed, measurable metrics is needed to determine if our methods of supportive care alone do provide the proper means

of rehabilitative aid to individual bats during the winter months.

Our observations are in agreement with the 2011 findings of Meteyer and his colleagues, where treatments of apple cider vinegar were used to treat infected bats.[7] They found that both treated and untreated bats benefited at the end of the experimental procedure, due to supportive care and warmer body temperatures.[7] We cannot confirm without histology or PCR that all bats released were negative for *P. destructans,* as shown at the end of the apple cider vinegar treatments conducted by Meteyer and others, nor do we believe they were ever negative for *P. destructans* due to the waxing and waning of fluorescence during their stay.[7] However, we believe quality of life was greatly increased.

We observed orange-yellow UV fluorescence on the wings and muzzles of all nine *E. fuscus* patients. Spots of orange-yellow muzzle fluorescence are documented, but images are not commonly represented across the literature.[15] We do not find our observation to be outside the realm of possibility, since fungal swabbing protocols require sweeps across the wings and muzzles of bats.[5] It is possible that the fluorescence on *E. fuscus* patients' muzzles could be attributed to other microbes or ocular, nasal, or both types of secretions. However, these areas of the muzzles that glowed orange-yellow in color also went in and out of fluorescence in the same fashion as the assumed fluoresced cupping erosions on the flight membranes.

We can confirm observations of drastic changes to the fluorescent appearance of fungal cupping erosions week to week. The fluorescence of assumed cupping erosions along the wing membranes of experimental patients would not always appear with the typical bright, orange-yellow color associated with *P. destructans* infection and WNS. When not fluorescing under UV light, the wing membranes appeared to have flaky, white flecks of skin in the same areas once fluoresced. Conversations between other Ohio-based wildlife rehabilitators and RA Crow revealed that no other organization had detected UV fluorescence on admitted bats' flight membranes and muzzles throughout the winter of 2016 (2017 phone conversations with Ohio Wildlife Rehabilitators Association bat rehabilitation members and RA Crow; unreferenced). The alternating appearance of the wing membranes thought to be infected by *P. destructans* in combination with other wildlife rehabilitators' observations suggests two opposing ideas: 1) we were indicating false positives of infection by UV detection during the initial intake exams, or 2) other rehabilitators were indicating false negatives of infection by UV detection within a WNS endemic state. Cupping erosions can sometimes be microscopic. Original testing from infected New York bats indicated about 30% of negatively fluoresced biopsies had single, microscopic cupping erosions upon further investigation.[15] Additionally, UV fluorescence for *P. destructans*-infected bats is greatly increased in late hibernation.[19] Although active fluorescent properties of *P. destructans* are not always present under UV light, we overwhelmingly agree that detecting orange-yellow fluorescence is a highly resourceful, noninvasive solution for accurately detecting *P. destructans* with high confidence. This convenient method of detection is simple and available with minimal resources to any wildlife rehabilitator. Since we observed periods of changing individual fluorescence, we would highly recommend full decontamination of all patient materials, regardless of UV fluorescent status upon intake. This recommendation is extended beyond our current state guidelines, where full decontamination is required only for those individual bats that present active UV fluorescence of *P. destructans*.[6] Recent reports provide support for the spread of *P. destructans* during the summer months, when fluorescence is not typically detected during this time.[22] We would recommend systematic decontamination practices in North America during any bat's residency within a rehabilitation unit, regardless of geographic location, life history stage, or time of year, to prevent further exposure, disease severity, or both, similar to what we practiced during the experimental time period of this case study.

About the Authors

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Acknowledgements

We thank the staff at Brukner Nature Center for their extended time dedicated to the care of the bats in this study and the donors who contributed husbandry resources for this project. We also thank Shannon Romer, PhD, for her time and imaging assistance at Wright State University's Microscopy Core. Finally, we thank bat rehabilitation members of the Ohio Wildlife Rehabilitators Association for their input and time discussing their wintering bat observations with us.

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