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Pathologic Findings and Liver Elements in Hibernating Bats With White-Nose Syndrome

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Abstract

Two groups of vespertilionid bats were collected from affected hibernacula. In group 1 (n, 14; pathology and microbiology), the average body weights of all species were at the lower limit of published ranges. Twelve bats (86%) had mycotic growth in the epidermis, hair follicles, and sebaceous glands. *Geomyces destructans*, with its characteristic curved conidia, was observed microscopically, cultured, and confirmed by polymerase chain reaction. Dermatitis and mural folliculitis was nil to mild. When focally coinfecting with Gram-negative bacteria, there was necrosis and pustules. Fat stores were little to abundant in 12 bats (86%) and nil in 2. Thirteen bats (93%) had pulmonary congestion and 7 (50%) had bone marrow granulocytosis. In group 2 (n, 24; liver elements), 3 bats (13%) had potentially toxic lead levels and 1 (4%), potentially toxic arsenic level. There was no evidence of major organ failure or consistent element toxicity.

Keywords

bat, fungus, *Geomyces*, metals, minerals, Vespertilionidae, white-nose syndrome

Hibernating bats in the Northeastern United States have been dying in inordinate numbers for unknown reasons. Because a large proportion of these dying bats have a white fungal growth on the face, particularly on the muzzle and ears, this mortality syndrome has been designated *white-nose syndrome* (WNS).¹³ It was first reported from a cave in New York State in February 2006. During the winters of 2007 and 2008, the disease spread throughout the caves of the Northeastern United States. In 2009, it was reported in Pennsylvania and Virginia. A reduction of the bat population at 2 hibernacula in New York was estimated to be at least 75%.²

Affected species included little brown (*Myotis lucifugus*), northern long-eared (*Myotis septentrionalis*), eastern pipistrelle (*Perimyotis subflavus*), and big brown (*Eptesicus fuscus*). Abnormal behavior has been observed in affected colonies, with bats flying out of their caves in the middle of winter and during daylight hours. They have been described as emaciated and dehydrated.¹³ Bats that were examined had minimal fat stores and erosive epidermal lesions on the muzzle and wings, with a white fungus, *Geomyces destructans*. This fungus produces a curved conidia; it is psychrophile; and it grows or can be maintained year-round at the temperatures found in the Northeastern region hibernacula.^{2,10}

In the past, high mortalities of bats have been observed in the summer owing to deliberate organochlorine spraying of nursery colonies.¹⁴ Other reports of similarly scaled massive die-offs are rare and remain unexplained.³ Infectious diseases known to be present in Northeastern bat

populations, such as rabies or histoplasmosis, do not cause such high mortalities.¹⁹

The study was conducted to identify the lesions and any abnormal mineral and metals levels associated with the syndrome.

Sample Collection and Methods

Hibernating bats were collected from hibernacula in New York and Connecticut during February and March of 2008 and 2009. Animals that were collected were dead or moribund and had grossly visible white deposits on the face (Fig. 1). Moribund animals were euthanized with CO₂. The bats collected were randomly separated into 2 groups, one for postmortem and microbiological examination (14 bats) one for mineral and metal determination (24 bats). All specimens were held at 5°C until necropsy.

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Of the 14 animals collected for postmortem analysis, 6 were collected alive and 8 were dead. All were measured (nose to distal perineum) and weighed. Animals were necropsied, and an impression from the brain was submitted for rabies immunofluorescence testing. Tissues were fixed in 10% neutral buffered formalin and processed routinely for histopathology examination. Skin swabs from the faces of 3 animals were cultured for fungus on Sabouraud's dextrose agar at 5° and 30°C, with 80% moisture for a minimum of 2 weeks. Two colonies from different animals had a morphology consistent with the one observed in the histologic skin lesions and were selected for molecular analysis. Fungal DNA was detected by polymerase chain reaction, using primer sets that target the noncoding internal transcribed spacer region 1 and 2 of the 26S ribosomal RNA gene—primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') / ITS2 (5'-GC TGCGTTCTTCATCGATGC-3') and primer set ITS3 (5'-GCATCGATGAAGAACGCAGC-3') / ITS4 (5'-TCCTCCGCTTATTGATATGC-3) respectively—using conditions previously described.⁵ Sequence identities were determined using the National Center for Biotechnology Information software Basic Local Alignment Search Tool.

Mineral and Toxicologic Testing

The presence of the fungus in the skin lesions was confirmed by direct microscopy of skin scrapings. Animals were necropsied and liver was collected. Wet weight mineral and metal analysis was performed by inductively coupled plasma mass spectroscopy. The dry weight concentration was estimated by multiplying the wet weight result by a factor of 4, a widely used conversion factor.¹⁶

Results

Fourteen bats, 8 males and 6 females, were examined. Table 1 presents the biometry and histopathology findings. At the time of necropsy, 5 bats had one or more 1- to 2-mm delicate white deposits on the fur of the face, and of these, 2 also had deposits on body and wings (Fig. 1). Internal organs of all 14 were free of macroscopic lesions and all were rabies negative. In the skin of 12 bats, particularly on the face but also randomly on the body and wings, there were aggregates of fungal hyphae and keratinic debris adherent to the epidermis, variably mixed with different species of bacteria (Fig. 2). Hyphae extended into the stratum corneum and along hair follicles, colonizing the root sheaths and associated sebaceous glands (Fig. 2). On the non-haired skin (eg, wings), fungal hyphae, when present, were focally pasted at the surface or between thin layers of the stratum corneum. Neutrophilic infiltrates in the hair follicle interface or dermis were usually sparse. In 3 bats, the stratum corneum, as well as the dermal-epidermal interface, was split and formed spaces that were colonized by fungus or Gram-negative bacilli. In these cases, there was necrosis of the epidermis with a marked focal neutrophilic infiltrate (Fig. 3). Careful examination of the fungal morphology in the superficial

epidermis revealed clusters of 2- × 6-μm curved conidia attached to the hyphae (Fig. 4). In tissues, fungal hyphae were slender (2 to 3 μm in diameter), with parallel nonpigmented walls, septation, and right-angle branching (Fig. 5). In dead animals, hyphae often extended deeper from root sheaths and sebaceous glands into the surrounding tissues. In moribund animals, the fungus most often did not traverse the basement membrane of the root sheaths or sebaceous glands. Thirteen bats had marked pulmonary congestion with abundant intravascular neutrophils. Five of these had small foci of neutrophils in alveolar septae or spaces, together with edema material. Seven bats had marked bone marrow granulocytosis. All bats had adipose tissue composed of cells with a single large lipid globule and a marginal compressed nucleus (white fat). The interscapular adipose tissue, composed of adipocytes with multiple lipid droplets and a central nucleus (brown fat), contained fine to large lipid vacuoles in 12 bats and no appreciable lipid vacuoles in 2. Ten of the 14 bats had gastrointestinal flukes; 5 had gastrointestinal nematode larvae; 1 had intestinal cestodes morphometrically consistent with *Hymenolepis christensii*; 2 had arthropod ectoparasites.

Cultures of the skin samples held at 5°C grew white pinpoint colonies in 7 days that expanded and turned smokey green at the center. The microscopic morphology of these colonies (Fig. 6) was consistent with the morphology of the fungus in histologic sections. The molecular analysis of the 2 isolates produced an ITS1 255-base pair (255-bp) amplicon that showed 100% sequence similarity to GenBank accessions of *G destructans* and *Geomyces pannorum*. ITS2 337-bp amplicon (CVMDL ITS2) showed > 99% identity with *Geomyces* spp, including the IFS-2009a isolated from bats with WNS (Fig. 7). Other species that grew at 5° and/or 30°C were *Cladosporium* spp, *Penicillium* spp, *Trichophyton terrestre*, *Aspergillus terreus*, and *Mucor* spp. These fungi were morphologically different from the fungus observed in histologic sections. Table 2 presents liver elements, with corresponding published “normal” values for various bat species.

Discussion

Similar to cases reported previously, the majority of our cases comprised little brown bats,² which are a predominant part of the vespertilionid species in the Northeastern hibernacula of the United States. In the Roxbury mine in Connecticut, for instance, they represented 81% and 70% of the total hibernating bats in 2005 and 2007, respectively, before WNS was observed (unpublished data, Department of Environmental Protection, State of Connecticut).

Body weights of the bats reported here were consistently at the lower end of the normal range, whereas average sizes were mid- to upper-normal range. Previous investigators reported that 65% of their cases had “little or no identifiable” fat stores²; however, the majority of our cases had a significantly higher, appreciable degree of adipose stores. The endoparasitism observed in our cases was consistent with parasite burdens reported previously in little brown bats.⁶

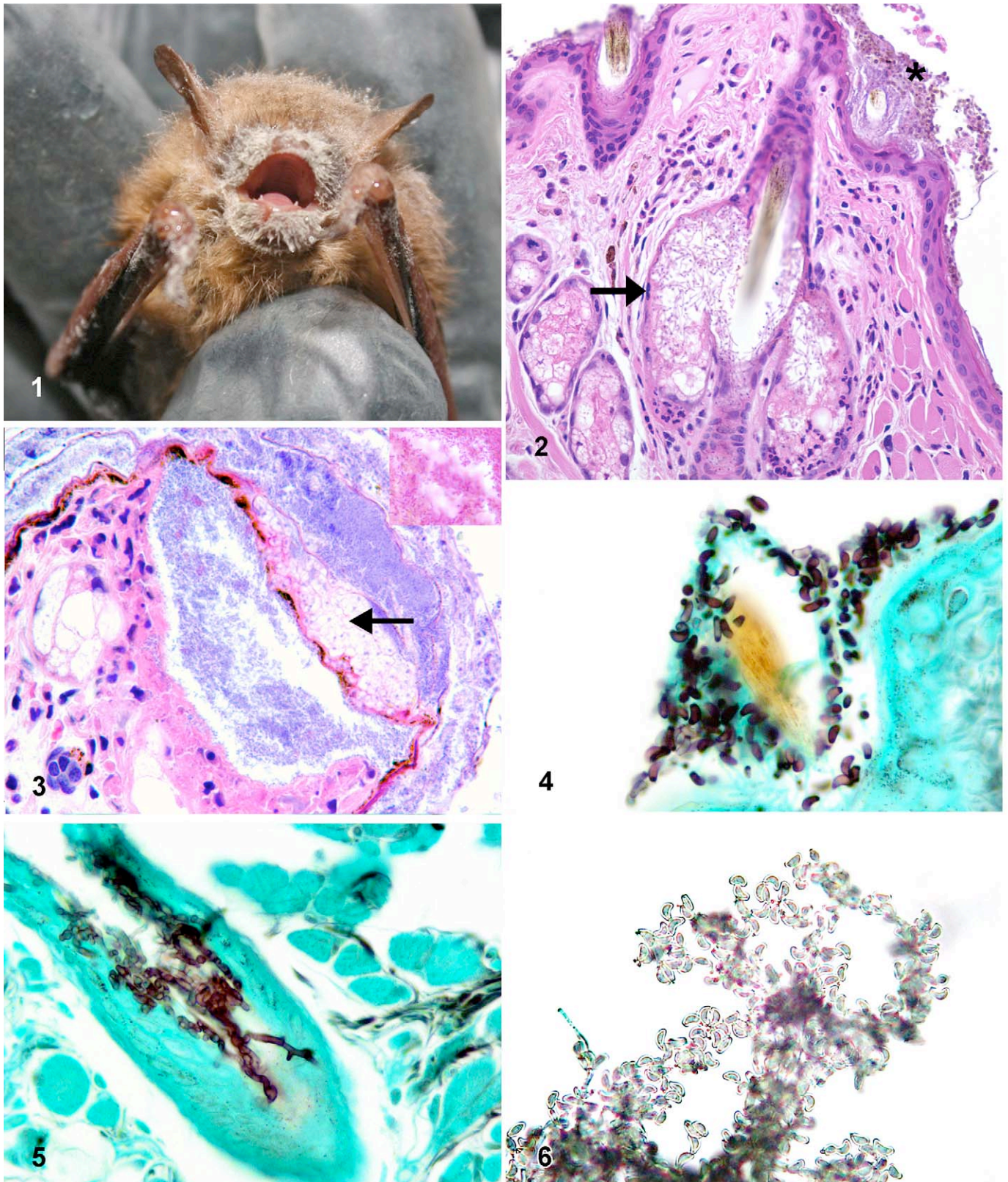


Figure 1. Hibernating eastern pipistrelle bat with white nose syndrome. The fur of the muzzle is coated with white fungus. Numerous small fungal foci are distributed over the head and skin of the forearm, carpus, and digits. Courtesy of Department of Environmental Protection, State of Connecticut. **Figure 2.** Skin of the head; hibernating eastern pipistrelle bat with white nose syndrome. Fungal hyphae and conidia form mats at the surface of the epidermis mixed with keratin debris (*); hyphae stream along the hair shafts and colonize the root sheaths and sebaceous glands

Table 1. Biometry and Histopathologic Findings in 14 Hibernating Bats With White-Nose Syndrome in the Northeastern United States

Species	No. Bats Examined	Average Body Weight, g (normal range)	Average Body Length, cm (normal range)	Skin Mycosis	Lung Congestion	Bone Marrow Granulocytosis
Little brown (<i>Myotis lucifugus</i>)	10	5.8 (5–14) ¹⁷	5.1 (3.5–10.0) ¹⁷	10	10	5
Big brown (<i>Eptesicus fuscus</i>)	1	11.9 (11–23) ¹⁵	7.0 (5.3–8.1) ^{15,a}	0	0	1
Northern long-eared (<i>Myotis septentrionalis</i>)	2	5.0 (5–8) ⁴	4.8 (?–5.0) ⁴	1	2	0
Eastern pipistrelle (<i>Perimyotis subflavus</i>)	1	6.4 (6–10) ²⁰	4.3 (4.2–4.5) ^{20,a}	1	1	1

^a Interpreted by subtracting total length with tail length.

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EU877931 GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC ATTGCGCCCC CTGG-TATTC
CVMDLITS2 .....A.....
FJ977924 .....-.....
AJ608972 .....-.....

EU877931 CGGGGGGCAT GCCTGTCCGA GCGTCATTAC AACCCCTCAAG CTCAGCTTGG TATTGGGCC CCGCCA-CCC GCGGGGCCCT AAAGTCAGTG GCGGTGCCGT
CVMDLITS2 .....-.....
FJ977924 .....-.....
AJ608972 .....-.....

EU877931 CCGGCTCCGA GCCTAGTAAT TCTTCTCGCT CCGGAGSTCC GGTCTGTGTC TTGCCAGCAA CCCCCAAT-T TTTTCAGTT GACCTCGGAT CAGGTAGGGA
CVMDLITS2 .....-.....
FJ977924 .....-.....
AJ608972 .....T.....C.....T.....

EU877931 TACCCGCTGA ACTTAAGCAT ATCAATAAGC GGAGGA
CVMDLITS2 .....
FJ977924 .....
AJ608972 .....

```

7

Figure 7. Alignment of fungal internal transcribed spacer region 2 and adjacent partial sequences of 5.8S and 28S rRNA. CVMDL ITS2 337–base pair DNA was amplified with primer set ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) / ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). GeneBank accession nos. EU877931, *Geomyces pannorum*; FJ977924, *Geomyces* sp IFS 2009a; AJ608972, *Geomyces vinaceus*.

G destructans produces a characteristic conidia that can be easily and reliably identified by direct microscopic examination of skin scrapings of the lesions. Cutaneous mycoses typically produce variable lesions depending on agent properties, host response and bacterial superinfection.¹¹ This fungus has invasive properties that differ from those of dermatophytes in that it extends into the noncorneal layers of the epidermis and sebaceous glands. Hibernation-induced immunodepression may explain a minimal inflammatory host response in many cases. This has been recognized in hibernating red-cheeked ground squirrels (at 3°C to 4°C) that were experimentally exposed to *Trichophyton mentagrophytes*.¹⁸ We observed

significant necrosis and inflammation mainly when there were abundant bacteria. Interestingly, we and others did not observe the fungus in the big brown specimens.² When hibernating, big brown bats rest in drier, more ventilated areas than do little browns.²⁰ Overall, this behavior may have an importance in the exposure to the fungus.

We confirm what others have reported—namely, that there is no evidence of major organ failure.² Pulmonary congestion and mild focal pneumonitis were frequent changes. Pulmonary congestion is often an unspecific necropsy finding. However, in a large number of our cases, it was associated with a large number of circulating neutrophils and with bone marrow

Figure caption continued. (arrow). Few neutrophils are focally aggregated in the sebaceous glands and dispersed in the dermis. HE. **Figure 3.** Skin of wing membrane; hibernating little brown bat with white nose syndrome. Necrosis and splitting of epidermal layers; spaces are filled with fungal hyphae (arrow) or Gram-negative bacilli (inset); the denuded dermis is infiltrated by neutrophils. HE. Inset: Brown and Brenn tissue Gram stain. **Figure 4.** Skin of the head; hibernating little brown bat with white nose syndrome. Fungal curved conidia are aggregated at the surface of the epidermis and hair follicle. Grocott's methenamine silver. **Figure 5.** Skin of the head; hibernating little brown bat with white nose syndrome. Hyphae colonize the hair follicle root sheaths and shaft and are 2 to 3 μm in diameter, with parallel walls, septation, and right-angle branching. Grocott's methenamine silver. **Figure 6.** Microscopic colonial morphology of white nose syndrome *Geomyces* fungus showing profuse numbers of curved conidia attached on either side of hyphae. Distilled water preparation.

Table 2. Metal and Mineral Elements in Livers of Hibernating Bats With White-Nose Syndrome in the Northeastern United States During Winter 2008, and Corresponding Values Reported From Various Bat Species Around the World^{1,9}

Bat Species	n	Average Dry Weight Concentration ($\mu\text{g/g}$)											
		Al	Ar	Cd	Cr	Cu	Fe	Pb	Hg	Mn	Mo	Se	Zn
Little brown (<i>Myotis lucifugus</i>)	17	1.92	0.42	4.31	4.65	37.44	1210	7.04	8.34	26.06	2.23	7.33	158.74
Big brown (<i>Eptesicus fuscus</i>)	4	4.14	1.99	1.15	4.67	43.03	3071	21.42	4.16	14.40	2.93	8.96	181.14
Northern long-eared (<i>Myotis septentrionalis</i>)	3	2.19	5.95 ^a	8.61	5.25	42.02	1718	0.32	8.00	17.03	2.72	6.35	142.74
Total	24	2.33	1.38	4.32	4.73	38.95	1584	8.59	7.60	22.96	2.40	7.47	160.46
Various species ^b				0.21– 6.50	0.33– 4.60	17.0– 29.2	696– 3.435 ^c	0.18– 12.20	0.37– 3.60	23.2– 29.1	2.33– 2.71	2.6– 4.7	134– 174

^a One animal with 15.5 $\mu\text{g/g}$.

^b *Pipistrellus pipistrellus*, *Tadarida brasiliensis*, *Artebeus jamaicensis*, *Myotis vivesis*, *Pteropus poliocephalus*, and *Miniopterus schreibersii*.

^c *Rousettus aegyptiacus*. Interpreted from wet weight data by multiplying by a factor of 4.

granulocytosis. One interpretation is that these represent early inflammation.

Mercury and selenium were consistently higher than levels found in other species of bats around the world.¹ These are not excessive considering that mercury is toxic above 20 $\mu\text{g/g}$ and that selenium levels between 4 and 10 $\mu\text{g/g}$ were measured in healthy birds.⁸ Three bats had elevated lead, and 1 elevated arsenic, which are potentially toxic levels when compared to those of fruit bats, birds, and domestic animals.^{7,12} However, these elevated levels were sporadic, and we did not observe microscopic lesions compatible with toxicity in our postmortem study group.

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Declaration of Conflict of Interest

The authors declared that they had no conflicts of interests with respect to their authorship or the publication of this article.

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References

- Allinson G, Mispagel C, Kajiwara N, Yasumi A, Hashimoto J, Laurenson L, Allinson M, Tanabe S: Organochlorine and trace metal residues in adult southern bent-wing bat (*Miniopterus schreibersii bassanii*) in southeastern Australia. *Chemosphere* **64**:1464–1471, 2006.
- Bleher DS, Hicks AC, Behr M, Meteyer CU, Berlowski-Zier BM, Buckles EL, Coleman JTH, Darling SR, Gargas A, Niver R, Okoniewski JC, Rudd RJ, Stone WB: Bat white-nose syndrome: An emerging fungal pathogen? *Science* **323**:227, 2009.
- Booth ES: A bat die off in Mexico. *J Mammal* **46**:333–334, 1965.
- Caceres C, Barclay RMR: *Myotis septentrionalis*. *Mammal Species* **634**:1–4, 2000.
- Chen YC, Eisner JD, Kattar MM, Rassoulina-Barret SL, Lefe K, Yarfitz SL, Limaye AP, AND Cookson BT: Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J Clin Microbiol* **36**:2302–2310, 2000.
- Coggins JR, Tedesco JL & Rupprecht CE: Seasonal changes and overwintering of parasites in the bat, *Myotis lucifugus* (Le Conte), in a Wisconsin hibernaculum. *Am Midl Nat* **107**: 305–315, 1982.
- Eisler R: Arsenic hazards to humans, plants, and animals from gold mining. *Rev Environ Contam Toxicol* **180**:133–165, 2004.
- Fairbrother A, Locke LN, Hoff GL: *Noninfectious Diseases of Wildlife*, 2nd ed., pp. 113, 114, 124, 133. Manson Publishing Ltd, London, UK, 1996.
- Farina LL, Heard DJ, LeBlanc DM, Hall JO, Stevens G, Wellehan JFX, Detrisac CJ: Iron storage disease in captive egyptian fruit bats (*Rousettus aegyptiacus*): relationship of blood iron parameters to hepatic iron concentrations and hepatic histopathology. *J Zoo Wildl Med* **36**:212–221, 2005.
- Gargas A, Trest MT, Christensen M, Volk TJ, Bleher DS: *Geomyces destructans* sp nov associated with bat white-nose syndrome. *Mycotaxon* **108**:147–154, 2009.
- Ginn PE, Mansell JEKL, Rakich PM: Skin and appendages. *In: Pathology of Domestic Animals*, ed. Maxie MG, 5th ed., pp. 696–700. Elsevier-Saunders, Philadelphia, PA, 2007.
- Hariano B, Sutton RH: Lead concentrations in tissues of fruit bats (*Pteropus* sp.) in urban and non-urban locations. *Wildl Res* **20**:315–20, 1993.
- Kelley T: Bats perish, and no one knows why. *The New York Times*, March 25, 2008.
- Kunz TH, Anthony ELP, Ramage WT: Mortality of little brown bats following pesticide applications. *J Wildl Manage* **41**: 476–483, 1977.

15. Kurta A, Baker RH: *Eptesicus fuscus*. *Mammal Species* **356**:1–10, 1990.
16. Mochizuki M, Mori M, Hondo R, Ueda F: A new index for evaluation of cadmium pollution in birds and mammals. *Environ Monit Assess* **137**:35–49, 2008.
17. Nowak RN: *Mammals of the World*, 6th ed., vol. 1, pp. 420, 440. The Johns Hopkins University Press, Baltimore, MD, 1999.
18. Sharapov VM: Influence of animal hibernation on the development of mycoses. *Mycopathologia* **84**:77–80, 1984.
19. Williams ES, Barker IK: *Infectious Diseases of Wild Mammals*, 3rd ed., pp. 15–16, 519. Blackwell Publishing, Ames, IA, 2001.
20. Wilson DE, Ruff S: *The Smithsonian Book of North American Mammals*, pp. 94–96, 114–116. Smithsonian Institution Press, Washington, DC, 1999.