Histopathologic Criteria to Confirm White-nose Syndrome in Bats

Carol Uphoff Meteyer, Elizabeth L. Buckles, David S. Blehert, Alan C. Hicks, D. Earl Green, Valerie Shearn-Bochsler, Nancy J. Thomas, Andrea Gargas and Melissa J. Behr

J VET Diagn Invest 2009 21: 411
DOI: 10.1177/104063870902100401

J Vet Diagn Invest 21:411–414 (2009)

Histopathologic criteria to confirm white-nose syndrome in bats

Carol Uphoff Meteyer,[1] Elizabeth L. Buckles, David S. Blehert, Alan C. Hicks, D. Earl Green, Valerie Shearn-Bochsler, Nancy J. Thomas, Andrea Gargas, Melissa J. Behr

Abstract. White-nose syndrome (WNS) is a cutaneous fungal disease of hibernating bats associated with a novel *Geomyces* sp. fungus. Currently, confirmation of WNS requires histopathologic examination. Invasion of living tissue distinguishes this fungal infection from those caused by conventional transmissible dermatophytes. Although fungal hyphae penetrate the connective tissue of glabrous skin and muzzle, there is typically no cellular inflammatory response in hibernating bats. Preferred tissue samples to diagnose this fungal infection are rostral muzzle with nose and wing membrane fixed in 10% neutral buffered formalin. To optimize detection, the muzzle is trimmed longitudinally, the wing membrane is rolled, and multiple cross-sections are embedded to increase the surface area examined. Periodic acid–Schiff stain is essential to discriminate the nonpigmented fungal hyphae and conidia. Fungal hyphae form cup-like epidermal erosions and ulcers in the wing membrane and pinna with involvement of underlying connective tissue. In addition, fungal hyphae are present in hair follicles and in sebaceous and apocrine glands of the muzzle with invasion of tissue surrounding adnexa. Fungal hyphae in tissues are branching and septate, but the diameter and shape of the hyphae may vary from parallel walls measuring 2 μm in diameter to irregular walls measuring 3–5 μm in diameter. When present on short aerial hyphae, curved conidia are approximately 2.5 μm wide and 7.5 μm in curved length. Conidia have a more deeply basophilic center, and one or both ends are usually blunt. Although WNS is a disease of hibernating bats, severe wing damage due to fungal hyphae may be seen in bats that have recently emerged from hibernation. These recently emerged bats also have a robust suppurative inflammatory response.

Key words: Bats; emerging disease; fungus; *Geomyces* sp.; hibernation; *Myotis*; skin erosion.

White-nose syndrome (WNS) has caused mortality in hundreds of thousands of little brown bats (*Myotis lucifugus*) since first reported by biologists in 2007, yet the disease is still poorly understood.[1] Initially detected near Albany, New York, this fungal disease of cave- and mine-hibernating bats has spread rapidly to hibernacula in Vermont, Massachusetts, Connecticut, Pennsylvania, New Jersey, Virginia, West Virginia, and New Hampshire. In addition to little brown bats, other species in which WNS has since been diagnosed include tricolored bats (*Pipistrellus subflavus*), northern long-eared bats (*Myotis septentrionalis*), and endangered Indiana (*Myotis sodalis*) and big brown (*Eptesicus fuscus*) bats.

The classic presentation of WNS in affected bats living in caves and mines includes the delicate, exuberant, white filaments that obscure the muzzle.[1] Fungus on the wings of these bats can appear as an opaque white, tacky film of varying density (Fig. 1A). Grossly visible fungus is not always seen on bats with WNS, and the white facial plume and white fungus on the surface of wings is usually lost when bats are removed from hibernacula and prepared for shipping. Once received in the laboratory, gross signs of fungal infection in bats are subtle and difficult to detect. The small size of little brown bats (5–7 g) make lighted magnifying loops an asset for examining skin. Changes seen in the skin of affected bats are inconsistent and nonspecific, including patches of rough skin on the face, ears, forearms, wing membranes, and feet as well as pinpoint white foci that resemble comedones on the muzzle. Less obvious changes are loss of sheen on glabrous skin and irregular pigmentation with areas of contraction or small tears in wing membranes. Backlighting of extended wings using a light box improves detection of these changes (Fig. 1B).

Although gross lesions can be suggestive of WNS, histopathologic examination is necessary to confirm this disease. To optimize microscopic detection of fungal hyphae, surface area of examined skin was maximized. Muzzle and nose, once dissected from underlying bone and fixed in 10% neutral buffered formalin, were trimmed into multiple longitudinal sections perpendicular to the surface of skin. The skin

From the National Wildlife Health Center, U.S. Geological Survey, Madison, WI (Meteyer, Blehert, Green, Shearn-Bochsler, Thomas), Cornell University, Ithaca, NY (Buckles), the New York Department of Environmental Conservation, Albany, NY (Hicks), the New York State Department of Health, Wadsworth Center, Axelrod Institute, Albany, NY (Behr), and Symbiology LLC, Middleton, WI (Gargas).

[1] Corresponding Author: Carol Uphoff Meteyer, National Wildlife Health Center, 6006 Schroeder Road, Madison WI 53711. cmeteyer@usgs.gov

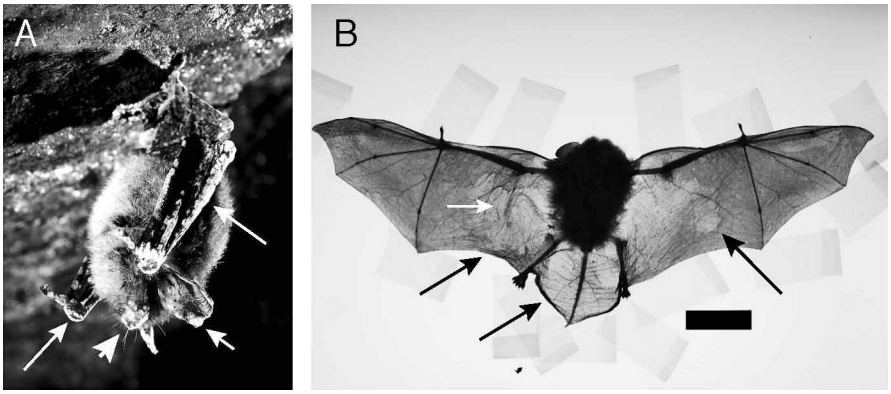

Figure 1. *Myotis lucifugus* with white-nose syndrome, New York. **A**, little brown bat with white foci of fungus on the face (arrowhead) and an irregular pattern of white fungus on the wing (long arrows) and ear (short arrow). **B**, little brown bat submitted to the National Wildlife Health Center, Madison, Wisconsin. The image illustrates the taping method used to extended wings, which are backlit on a light box. Damage to wing with contraction of wing membrane (white arrow) and loss of pigmentation (black arrows). Bar = 2.5 cm.

was embedded with trimmed surfaces presented for sectioning to maintain the orientation of the fungus to dermal structures.

Wing membrane was sampled in multiple rectangular pieces. Each piece of membrane was dipped in formalin, carefully unfolded over a gloved finger, and rolled along the short end onto wooden dowels approximately 0.2 cm in diameter and 3 cm long. After fixation, the dowels were removed from the rolls of wing membrane, and skin rolls were trimmed into multiple cross-sections with the cut surfaces embedded for sectioning and staining. Various fungal stains were initially applied to the tissue sections, but periodic acid–Schiff stain (PAS) proved superior for the microscopic detection of nonpigmented fungal hyphae, appreciation of the pattern of fungal skin invasion (Fig. 2A–2C), and recognition of conidia (Fig. 2D).

The wing membrane is composed of 2 single-cell layers of epidermis separated by a thin layer of connective tissue with elastin fibers.[3] Adnexa are only present in wing membrane near the arms and legs. The mildest microscopic changes seen in the wing membranes were cup-like epidermal erosions that were filled with fungal hyphae. Ulceration and fungal invasion of underlying connective tissue was common (Fig. 2B–2D) and could span the full thickness of the wing membrane. When the muzzle was involved, fungal hyphae filled hair follicles, invaded sebaceous and apocrine glands, and extended into the regional connective tissue obscuring epithelial boundaries of the adnexa (Fig. 3A, 3B). Typically, there was an absence of inflammation in the skin of hibernating bats even with extensive fungal invasion (Figs. 2, 3), which was random and nonangiotrophic. Samples of wing membranes from bats euthanized in caves and immediately fixed in formalin had more abundant aerial fungal growth and conidia on the surface of the skin, as well as the characteristic epidermal ulceration and connective tissue invasion (Fig. 2C, 2D).

Lack of inflammation in response to fungal hyphae was surprising. However, tissue invasion noted in samples fixed immediately after euthanasia of bats within hibernacula provided evidence that invasion of living tissue was an antemortem event. When inflammation was present in the skin of hibernating bats, edema and neutrophils were observed microscopically in the regional connective tissue, occasionally with intradermal abscesses. The presence of bacteria was inconsistent but common in bats with inflammation. Typical transmissible dermatophytes of mammals only invade the nonliving structures of skin (keratin, hair, and nails),[4] in contrast to the extensive invasion of skin and underlying connective tissue in bats with WNS. Winter bats with heavy fungal burdens were emaciated, but there were no consistent microscopic lesions in tissues other than skin.

Unlike bats in hibernation, bats with visibly damaged wings that were collected outside hibernacula in May had severe inflammation associated with fungal infection. Histologic changes included suppurative dermatitis with folliculitis, edema, and scattered infiltrates of macrophages. Frequent serocellular inflammatory crusts containing fungal hyphae were present over the intact epidermis (Fig. 3C). Bats collected shortly after hibernation also had small quiescent packets of fungal hyphae within the dermis that were surrounded by a thin layer of acellular material (Fig. 3D).

Fungal hyphae in tissue sections were branching and septate (Fig. 3B) with variable morphology ranging from parallel walls measuring 2 μm in diameter to irregular, bulging, or globose walls measuring 3–5 μm in diameter (Fig. 3B). When present on short aerial hyphae, curved conidia were

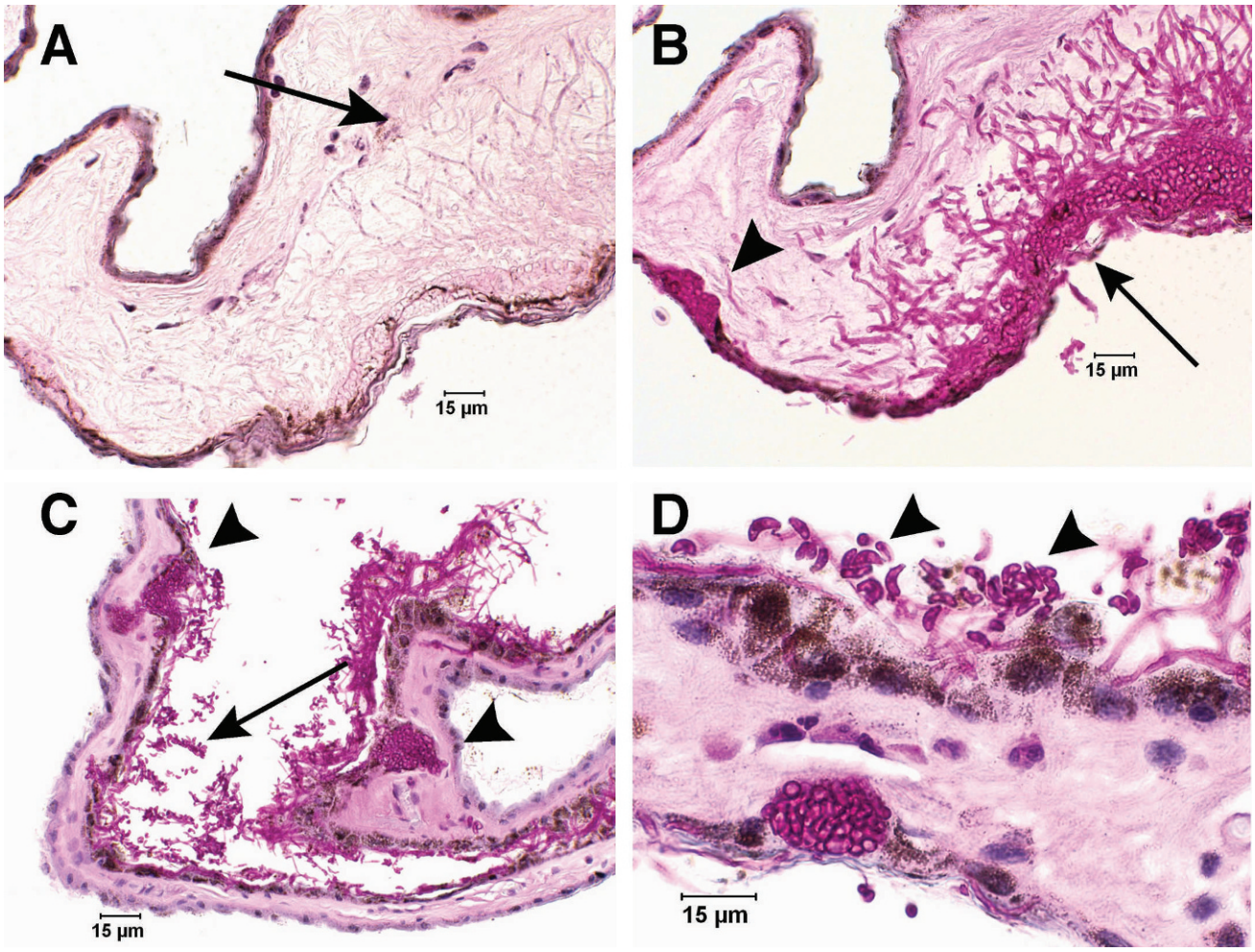

Figure 2. Wing membranes of *Myotis lucifugus* bats infected with white-nose syndrome (WNS). **A**, histologic section of wing membrane from the same bat as in Figure 1B. Invasive fungus (arrow) stains poorly with hematoxylin and eosin stain, and inflammatory infiltrates are not present. Bar = 15 µm. **B**, periodic acid–Schiff (PAS) stain of serial section from same tissue as in panel A. Fungal hyphae stain bright magenta. Hyphae are associated with cup-shaped epidermal erosions (arrowhead) and ulcers (arrow) with invasion of the underlying connective tissue. Bar = 15 µm. **C**, section of wing membrane, collected while inside the cave, from a little brown bat immediately after euthanasia. Exuberant fungal growth is present on the surface of the skin (arrow) and penetrates the wing membrane (arrowheads) without associated inflammation. PAS stain. Bar = 15 µm. **D**, conidia on the surface of the wing membrane of a cave-dwelling little brown bat fixed immediately after euthanasia in the cave. The characteristic curved conidia measure approximately 2.5 µm in diameter and 7.5 µm in curved length, have one or two blunt ends, and have a deeply basophilic central region (arrowheads). These conidia are identical to those of *Geomyces* sp. fungus isolated from bats with WNS.[1] A focal cluster of fungal hyphae is present within the epithelium on opposite wing margin. PAS stain. Bar = 15 µm.

approximately 2.5 µm in diameter and 7.5 µm long measured along the curve of the conidia. The conidia also had a central region of basophilia, and one or both ends were blunt (Fig. 2D). A recently identified *Geomyces* sp. fungus was cultured from the skin of bats with WNS and formed conidia in culture that were identical to those seen in histologic sections (Fig. 2D).[1] The WNS-associated *Geomyces* sp. fungus grows best between 5°C and 14°C, but growth is slow.

The body temperature of bats in torpor drops to within a few degrees of the ambient temperature in their hibernaculum (usually 2–10°C), with a concomitant 96–98% reduction of metabolic rate. Research has shown that mammals in torpor also down-regulate their immune response, which does not return to normal responsiveness until basal metabolic rates and core temperatures return to euthermic levels.[2] The drop in body temperature and depression of the immune response would provide ideal conditions for a psychrophilic fungus, such as *Geomyces* sp., to use hibernating bats as an opportunistic host.

Multiple studies are ongoing to determine if the suppression of the immune response in hibernating bats with WNS is a normal occurrence or compromised beyond what is physiologically expected. Experimental replication of WNS in hibernating bats is having early success in artificial hibernacula, and investigations into a more complete picture of the life

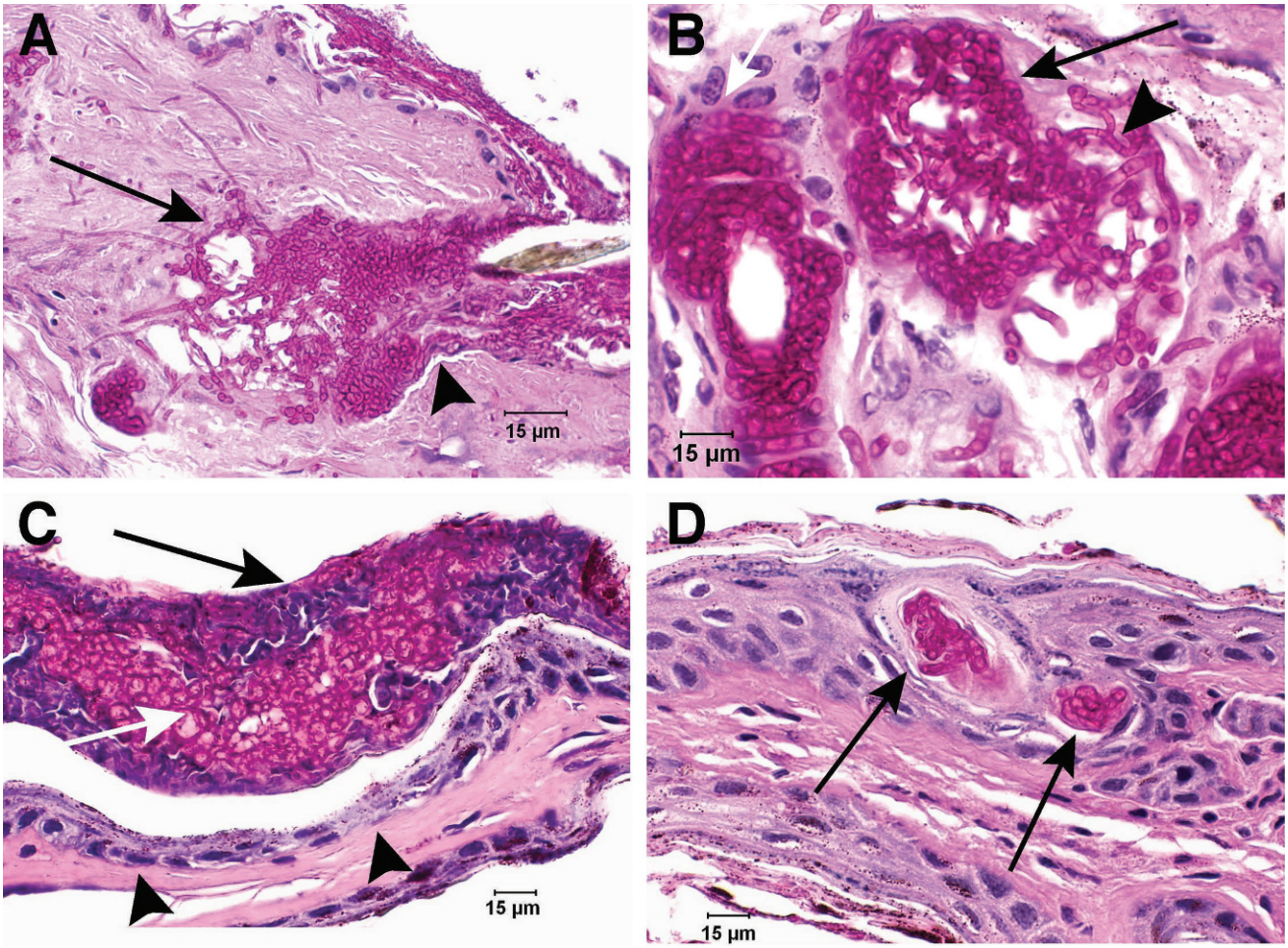

Figure 3. *Myotis lucifugus* with white-nose syndrome (WNS) submitted to the National Wildlife Health Center, Madison, Wisconsin, from Connecticut and Vermont. **A**, histologic section of bat muzzle with fungal hyphae filling the hair follicle (arrowhead), obliterating the epidermal sheath, and invading the regional connective tissue (arrow). No inflammatory response is present and bacteria colonize the surface. Periodic acid–Schiff (PAS) stain. Bar = 15 µm. **B**, fungal hyphae obscure the follicular epithelium (white arrow) and associated sebaceous gland (long arrow) of a bat's muzzle. The fungal hyphae are branching, septate (arrowhead), and of variable morphology ranging from parallel walls measuring 2 µm diameter to bulging or globose walls measuring 3–5 µm in diameter. There is no associated inflammation. PAS stain. Bar = 15 µm. **C**, wing membrane from a bat collected in May after emergence from hibernation but unable to fly. Inflammatory cells (long arrow) surround fungal hyphae (white arrow) forming a cellular crust overlying intact epidermis (arrowheads). PAS stain. Bar = 15 µm. **D**, different bat with similar history to that in panel C. Quiescent nests of fungus are surrounded by a thin layer of amorphous material within the epidermis of the wing (arrows). PAS stain. Bar = 15 µm.

cycle of the fungus and its associated host response through multiple seasons are planned.

Histopathologic confirmation of WNS is efficient, cost effective, and reliable. Awareness, surveillance, and early diagnosis of this emerging infectious disease of hibernating bats will be essential for subsequent management efforts to contain the spread of WNS and its impact on populations of cave-hibernating bats.

Acknowledgements

The authors thank Drs. Anne Ballmann and Krysten Schuler for coordinating the field aspects of the cases submitted to the National Wildlife Health Center, Madison, WI. The anatomic pathology residents and Ms. Krisi Hulme provided slide review of bats submitted to Cornell University. For carcass submissions, information on field observations, and bat biology, the authors thank the U.S. Fish and Wildlife Service, biologists from multiples states where WNS has been diagnosed, and those biologists performing disease surveillance in states where WNS has not yet been detected.

References

1. Blehert DS, Hicks AC, Behr MJ, et al.: 2009, Bat white-nose syndrome: an emerging fungal pathogen? Science 232:227.
2. Carey HV, Andrews MT, Martin SL: 2003, Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev 83:1153–1181.
3. Makanya AN, Mortola JP: 2007, The structural design of the bat wing web and its possible role in gas exchange. J Anat 211:687–697.
4. Scott DW, Miller WH, Griffin CE: 2001, Muller and Kirk's small animal dermatology, 6th ed. WB Saunders, Philadelphia, PA.