CANADIAN BAT WHITE-NOSE SYNDROME NECROPSY PROTOCOL
Canadian Wildlife Health Cooperative
Revised December 18, 2014

PREAMBLE

The purpose of this necropsy protocol is to provide guidance to researchers, laboratories and diagnosticians participating in surveillance for the disease bat white-nose syndrome (WNS). To contribute to Canada's national surveillance program for WNS, **occurrence data must be reported to the Canadian Wildlife Health Cooperative (CWHC) using the diagnostic categories given at the end of this protocol and the criteria for each category of diagnosis must be strictly applied**. This is to ensure uniformity, consistency, accuracy and comparability of results. Also, Canada's national survey for WNS designates each annual surveillance period as the interval from **November 1 of a given year to May 31 of the following year**. During the defined bat WNS surveillance period this detailed necropsy protocol should be followed. This specifically means that all bats submitted will receive a complete necropsy combined with both a histological examination of wing membranes and RT-PCR for the fungus *Pseudogymnoascus* (formerly *Geomyces destructans*). **Best laboratory practices should be followed** to ensure quality assurance and control. As a **biosecurity guideline** for regions where bat WNS is not endemic, it is recommended that bat necropsies and other diagnostic tests involving materials potentially contaminated with *P. destructans* are done in a **biosafety cabinet**. Outside of the surveillance period, the diagnostic tests performed for bat WNS would be optional, and the biosecurity measures to prevent cross contamination of specimens and laboratory contamination could be relaxed.

GROSS EXAMINATION AND SWABBING FOR RT-PCR:

1. Weigh and sex the specimen. (***Note: If examining bats for bat WNS, only handle one bat at a time to prevent cross contamination. DO NOT weigh the bats until the time of necropsy and swab the specimen for RT-PCR for Pseudogymnosascus destructans (Pd) at the time of weighing. Weigh individual bats in a disposable weigh boat [discarded after a single use]. After completion of necropsy, change gloves and decontaminate instruments and working surfaces [see below] between each bat to prevent cross contamination of specimens. If multiple bats are submitted together in one bag, pool the sample by swabbing all of the bats with a single swab and the bats can be necropsied as a group without changing gloves or decontaminating instruments***).

2. RT-PCR for Pd. With a single **polyester** swab, swab the muzzle, ears and dorsal and ventral surfaces of all wing membranes. Place individual swabs in separate labeled sterile whirlpak bags and keep chilled or frozen. ***Change gloves between each bat.*** Samples for RT-PCR can be shipped as diagnostic specimens to Dr. Hugh Cai, Animal Health Laboratory, University of Guelph (hcai@uoguelph.ca; 519-824-4120 ext 54316) or to the Animal Health Centre, Abbotsford, BC (http://www.agf.gov.bc.ca/ahc/; Toll Free 1-800-661-9903 [**BC Only**]; 604-556-3003). Please contact these laboratories prior to

sending specimens. 2013-14 Bat WNS Surveillance Season costs per Pd RT-PCR are $34.00 per sample at the Animal Health Laboratory, University of Guelph and $25.00 + GST per sample (BC **in province** submissions) or $37.50 + GST per sample (BC **out of province** submissions) at the Animal Health Centre, Abbotsford, BC.

If the preference is to do your own RT-PCR, the current RT-PCR protocol is based on:

> Muller, LK, JM Lorch, DL Lindner, M O'Connor, A Gargas and DS Blehert. 2013. Bat white-nose syndrome: a real-time TaqMan polymerase chain reaction test targeting the intergenic spacer region of Geomyces destructans. Mycologia 105:253-259.

3. Identify bat specimens to species. The indigenous bat species vary depending on the geographic location in Canada. Therefore, diagnosticians must become familiar with the bat species in their particular area. Important traits for differentiating among bat species are external morphological measurements including total body, tail, forearm, foot, tragus and ear lengths (***Note: ear and tragus lengths are difficult to measure consistently, resulting in high measuring error***); fur color; and presence or absence of a keel on the calcar. Most characteristics used in bat identification keys are those of adult bats and do not necessarily apply to nursing or immature individuals. Photographing a specimen and consulting a bat biologist or museum curator (eg. Dr. Hugh Broders, Saint Mary's University, Halifax, Nova Scotia; Dr. Don McAlpine, New Brunswick Museum, Saint John, New Brunswick; Dr. Brock Fenton; University of Western Ontario, London, Ontario; Dr. Craig Willis, University of Winnipeg, Winnipeg, Manitoba; and Dr. Cori Lausen, Bats R Us, Birchdale Ecological, British Columbia) may be necessary to obtain an accurate species identification. Nagorsen (2002) gives the following examples of species that are difficult to separate:
    a. Keen's Long-eared Myotis (*Myotis keenii*) and Western Long-eared Myotis (*M. evotis*) cannot be reliably identified from external traits; their identification requires a cleaned skull.
    b. Separation of other species of bats such as the Yuma Myotis (*M. yumanensis*) vs. Little Brown Bat (*M. lucifugus*) is very difficult to accomplish with a 100 percent certainty from their external features alone. Therefore, positive identification requires examination of a voucher specimen by a competent bat taxonomist.

The three species of bats currently most affected by bat white-nose syndrome (WNS) in Canada are the Little Brown Bat (LBB), Northern Long-eared Bat (*M. septentrionalis* or NLE) and Tricolored Bat (*Perimyotis subflavus* or TCB and previously known as the Eastern Pipistrelle). In general, the TCB is smaller than the LBB and NLE, but the overlapping weight and forearm length ranges for these three species make accurate species identification challenging.

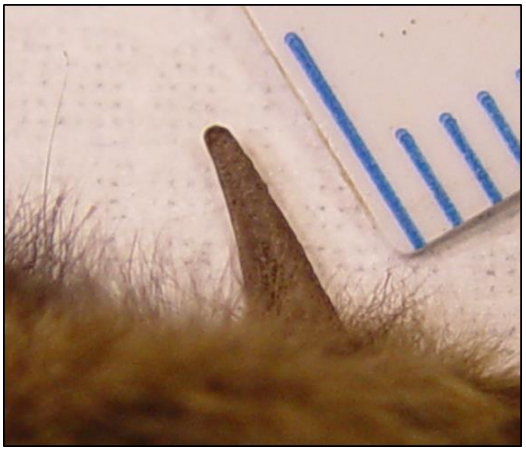
The morphology of the tragus is a distinguishing feature for these species (see images below). However, it can be necessary to use a dissecting scope to carefully and closely examine the tragus in post mortem specimens because dehydration of the individual and/or desiccation of the specimen can affect the size and shape of the tragus.

The LBB tragus is wide with a straight medial surface and bump on lateral edge (note: the bump is often curled inward on partially desiccated specimens so the tragus might have to be manipulated to be accurately visualized); the NLE tragus is conical or triangular in shape with straight lateral surface and slightly curved medial surface; and the TCB tragus is short, blunt and rounded. Placing a piece of white paper behind the tragus helps with visualization of this structure. In addition, the TCB can be recognized by its orange-red forearm and tricolored fur (black base, light brown center and dark tips).

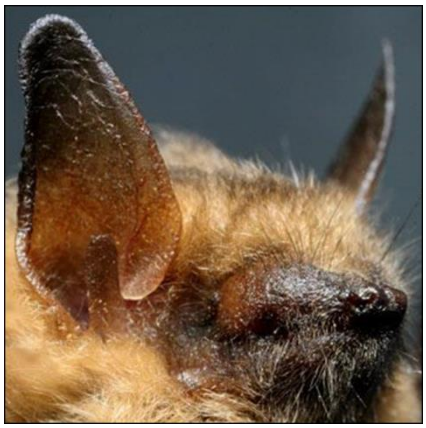
Little Brown Bat
Broders, Saint Mary's University

Little Brown Bat Tragus
McBurney and Needham, CWHC, Atlantic

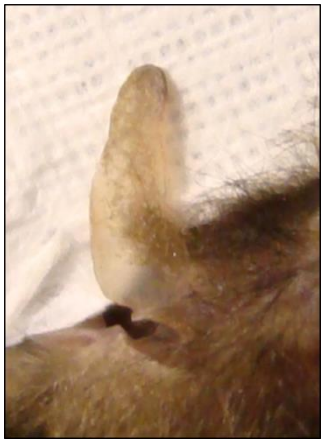
Northern Long-eared Bat
Broders, Saint Mary's University



Northern Long-eared Bat Tragus
McBurney, CWHC, Atlantic

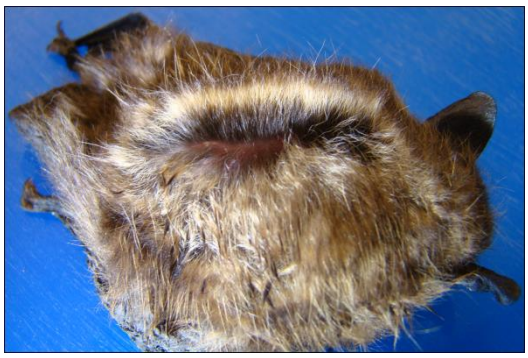


Tricolored Bat
© Hilton Pond Center



Tricolored Bat Tragus
McBurney, CWHC, Atlantic

Tricolored bat (red-orange forearm)
© K. Miles, Tennessee Wildlife Resources Agency



Tricolored bat (black-brown-dark fur)
McBurney, CWHC, Atlantic

There are many references online to help with identification of bat species, and the following are a few examples:

a. Nagorsen, D.W. 2002. An identification manual to the small mammals of British Columbia. Ministry of Sustainable Resource Management, Ministry of Water, Land and Air Protection, Biodiversity Branch, and Royal British Columbia Museum. 165pp. Found at: http://www.env.gov.bc.ca/wld/documents/techpub/id_keys_s.pdf
b. http://www.ontarionature.org/discover/resources/PDFs/atlases/mammal_atlas_bats.pdf
c. http://www.dnr.state.md.us/wildlife/Plants_Wildlife/bats/nhpbatfield.asp
d. http://www.dnr.state.md.us/wildlife/Plants_Wildlife/bats/bat_key.asp - Dichotomous Key

Lastly, “van Zyll de Jong, C.G. 1985. Handbook of Canadian mammals. Volume 2 (Bats). National Museum of Canada and National Museum of Natural History, Ottawa, Ontario. 210pp.” is a good reference to have if you are working with bats in Canada.

5. Examination of Wings. In fresh specimens, examining the spread wings on a light table, backlit by a bright light or using UV light may reveal erosions in the skin of the wing membrane. The lesions may also be photographed at this time. Looking at the wings under a dissecting microscope is also helpful because mites and small skin lesions can be detected using this equipment.

6. Examination of Other Organ Systems. Grossly examine other body organ systems and collect representative samples of organs and any lesions observed for histological examination and/or other ancillary diagnostic tests (eg. bacteriology, virology and parasitology).

7. DNA Sampling. If DNA is needed for genetic studies, we typically collect the majority of the left uropatagium (see wing membrane diagram below to identify this structure) and the entire left ear, including the tragus. These are placed in a DMSO Sodium Solution. However, it is best to use a protocol specified by the individuals who are doing the genetic study.

NOTE: To prevent cross contamination of samples, dissection instruments and working surfaces should be cleaned and decontaminated after each necropsy and before proceeding with the next specimen's necropsy. However, flaming stainless steel dissection instruments or soaking them in 10% bleach solutions can damage them excessively. Therefore, this alternative protocol is provided to avoid flaming and minimize contact with 10% bleach solution:

Cleaning Tools for Processing Bat Tissues for PCR and Culture Analyses
(modified from J. Lorch, E. Bohuski, and D. Blehert, USGS – National Wildlife Health Center, June 2013)

1. Remove dissection tools from storage container, and before use, briefly dip tools in 10% bleach and rinse almost immediately by transferring to a tray containing deionized or distilled water.

2. Transfer rinse tray containing tools to the working surface, remove tools from tray, and place on a clean paper towel.

3. Spray tools on paper towel with a DNA decontamination solution (e.g., DNA-OFF™, Clontech Laboratories, Fisher Scientific Catalogue Number TAK9036; Decon™ ELIMINase™, Decon Laboratories, Fisher Scientific Catalogue Number 04-355-32; or D/RNAse Free™ Decontaminant, Argos Technologies, VWR Catalogue Number 47751-044) and allow tools to sit for approximately 10 minutes or until dry.

4. Rinse tools in a tray containing deionized or distilled water.

5. Remove tools from rinse tray, dry with fresh paper towels and proceed with necropsy.

6. Following necropsy, clean tools with hot soapy water and dry them with a clean paper towel. Spray tools with DNA-Off solution and allow tools to sit for approximately 10 minutes or until dry. Lastly, dip them in 10% bleach and rinse immediately by transferring to a tray containing deionized or distilled water before returning tools to the storage container.

7. Douse working surface with 95% EtOH and allow the solution to evaporate. Then wet the working surface with 10% bleach and let stand 2 minutes. Wipe up the bleach solution and rinse working surfaces with deionized or distilled water to remove any residual bleach.

TISSUE SAMPLING:

1. Histology. (***Note: Place all skin samples in one cassette and immediately request a PAS stain at time of submission for histology if bat WNS is the suspect diagnosis.*****)**

    a. Wing membranes: The wing membrane is thin and elastic. The NWHC's proposed method for collecting histology samples from this tissue is as follows: *Collect a large rectangle of wing membrane for histology which includes lesions if present. Roll the skin sample like a cinnamon roll or roll it onto a dowel and place in cassette with a piece of paper towel on it so the tissue doesn't unroll. Immerse the cassette in 10% neutral buffered formalin for fixation (24 hours). After fixation, cut multiple cross sections of the roll for histological processing*.

    In practice, the technique above is difficult to do (especially in partially desiccated or decomposed carcasses), but a sample of wing membrane rolls into a ball quite readily. If you fix and cross section the ball of tissue, a very large surface area is available to examine microscopically. Therefore, collect the entire right and left dactylopatagia major, right and left plagiopatagia and right uropatagium, individually compress the membranes into small balls and place them into the cassette for processing (see diagram below for wing membrane nomenclature). Propatagia and dactylopatagia medius, minus and brevis can also be collected if lesions are observed in them. This methodology is best for simple diagnostic evaluation. However, if histological scoring/grading of wing damage is the goal, a more rigorous methodology is required and is described in Reeder *et al*., 2012, *PloS ONE* 7:e38920, Appendix S2.

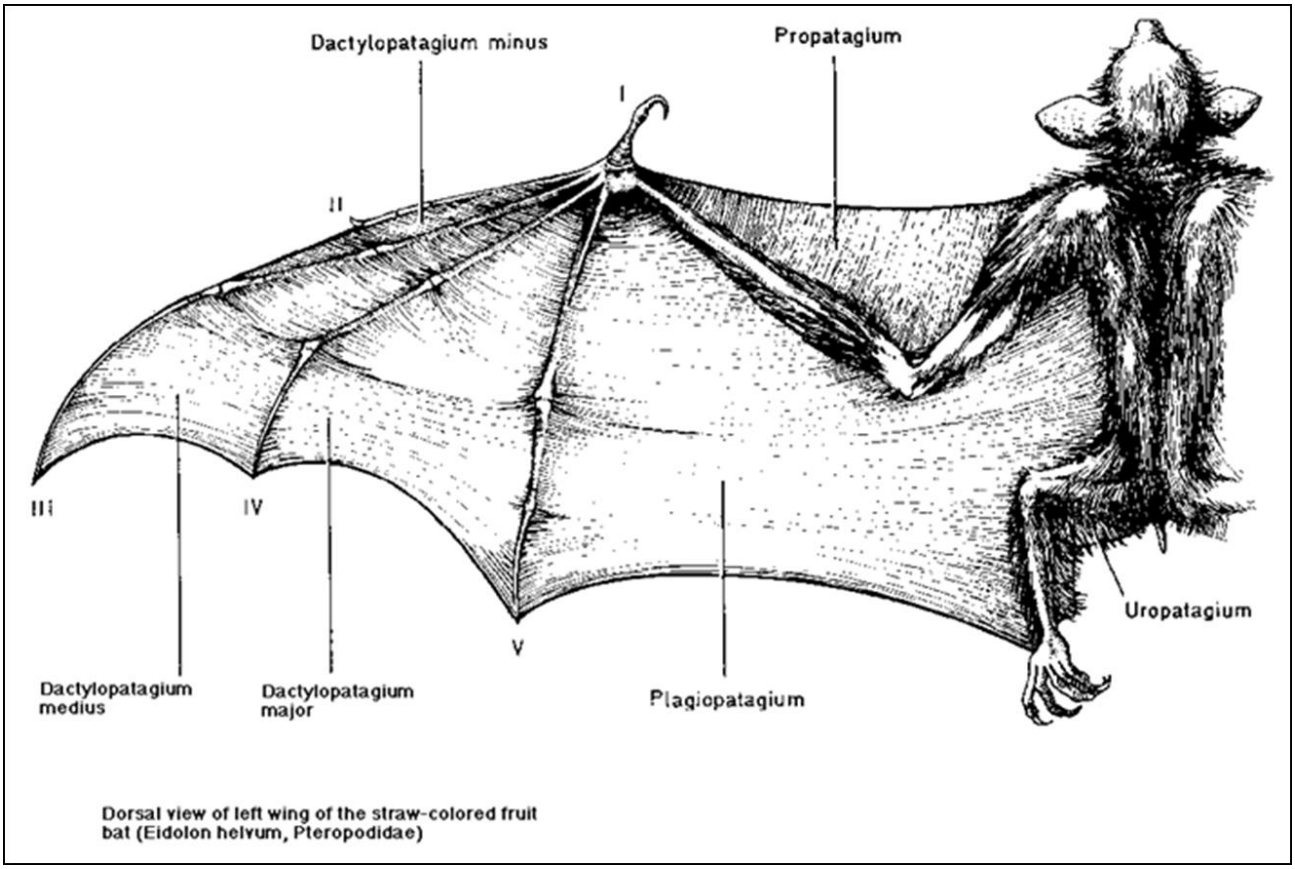


Diagram from: Halstead, L.B., and A.O. Segun, 1975. Dissection guides of common tropical animals, 3 Fruit bat (Eidolon helvum). Publication No. 6. Ethiope Publishing House, Benin City. (***Note: Uropatagium of Myotis and Perimyotis spp. forms a wide membrane that connects the leg to the tail***)

b. Skin of the head and face: Take two transverse sections across the muzzle using a scalpel. Histology technicians can decalcify the surface of the block at the time of sectioning. Another option is to remove the skin from the nose and submandibular region for sectioning, but this is more time consuming. Include these sections in the same cassette containing wing membrane sections.

c. Other tissue samples for histology: One half of the brain, one entire lung, heart (entire), liver, spleen and stomach (together), intestine, kidney (often with adrenal) and other tissues with lesions as identified. Place in a second cassette and immerse it in 10% neutral buffered formalin for fixation (24 hours) and standard histological processing.

2. Screening for Rabies at CWHC, Atlantic Region: All bats are screened for rabies using the Direct Rapid Immunohistochemical Test (dRIT). Half of the fresh brain is given to a wildlife technician for dRIT. After making the dRIT smears, the wildlife technician archives the brain tissue @ -80C for CFIA confirmation by FAT if dRIT positive.

3. Frozen Samples**.** (***-80 C is best***) The remainder of the bat carcass and tissues not utilized for diagnostic purposes can be placed in a labeled whirlpak bag and frozen for further diagnostic work or other research initiatives as deemed necessary.

DIAGNOSTIC CATEGORIES FOR REPORTING CASES OF BAT WNS:

1. **Positive for WNS** – Histologic lesions of bat WNS are present **AND** *Pseudogymnoascus* (formerly *Geomyces*) *destructans* (*Pd*) is detected (either by the Muller et al. PCR or by fungal culture).

2. **Suspect for WNS** – one of the following criteria must be met:
    a. Histologic lesions of WNS are present but *Pd* is **NOT** detected (either by the Muller et al. PCR or by fungal culture).
    b. One or more clinical/field signs (see below) are observed, but gross and/or histological lesions are **NOT** identified **AND** *Pd* is detected (either by the Muller et al. PCR, fungal culture, or a tape lift performed directly on visible fungal growth on bat skin).
    c. **MULTIPLE** clinical/field signs (see below) are observed within the currently recognized range of WNS but no samples are collected for diagnostic workup.
    d. Individual bats that are part of a confirmed WNS morbidity/mortality event are submitted to, but not tested by, a laboratory. This criterion is for instances in which multiple samples from the same site are submitted, but only a subset of those samples is tested. The untested samples may be classified as suspect for WNS if the subset of tested samples is positive and consists of the same species as the untested samples. Representatives of all species involved in the disease event should be tested.

3. **Negative for WNS** – Histologic lesions are not present **AND** *Pd* is not detected (either by the Muller et al. PCR or fungal culture).

CATEGORIES FOR REPORTING DETECTION OF *Pd*:

1. ***Pd* Present** – *Pd* detected (either by Muller et al. PCR or fungal culture - ******when screening for the presence of *Pd*, PCR is preferred over fungal culture due to the greater sensitivity of PCR) in an environmental sample or on an individual bat with no other clinical or field signs of WNS (see below) observed in the population at the hibernaculum. Bat carcasses submitted for diagnostic testing may be placed in this category if *Pd* is detected on the carcass but there were no clinical/field signs of WNS (see below) observed at the collection site and histopathology is negative.

2. ***Pd* not detected** – *Pd* not detected (either by Muller et al. PCR or fungal culture – see ** in previous section) in an environmental sample or on an individual bat [**NOTE**: A negative PCR result indicates that the amount of *Pd* is

below the level of detection for the test but cannot guarantee that the hibernaculum or bat population is free of *Pd*. A lack of observed field signs in the resident bat population is also not sufficient for assuming that a hibernaculum is *Pd*-free. All negative results from a statistically robust sample size can, however, increase confidence that *Pd* is absent from the sampled population or environment.] .

For management purposes, hibernacula should be considered contaminated with *Pd* if they contain at least one sample (bat or environmental) that tests positive for the fungus by the Muller et al. PCR or fungal culture regardless of whether field signs of the disease were observed within the hibernaculum. A contaminated hibernaculum retains this designation indefinitely. The ability of *Pd* to persist long-term outside of hibernacula is not currently well understood.

CLINICAL/FIELD SIGNS ASSOCIATED WITH WNS IN BATS:

1. **Winter/Spring** – excessive or unexplained mortality at or near a hibernaculum; visible fungus on flight membranes, muzzle, or ears of live or fresh dead bats; yellow-orange fluorescent pattern of non-haired skin under UVA light [Turner et al.]; abnormal behaviors including daytime activity, premature egression from the hibernaculum or population shift to entrance of the hibernaculum; moderate to severe wing damage in nontorpid bats [Reichard et al.]; and thin body condition (**NOTE**: Moderate to severe wing damage in nontorpid bats or thin body condition are considered nonspecific field signs when they are observed alone).

2. **Summer/Fall** – There are not consistent field signs associated with WNS during summer/fall.

CITATIONS FOR BAT WNS DIAGNOSTIC AND Pd DETECTION CATEGORIES:

1. Muller, L.K., J.M. Lorch, D.L. Lindner, M. O'Connor, A. Gargas, and D.S. Blehert. 2013. Bat white-nose syndrome: a real-time TaqMan polymerase chain reaction test targeting the intergenic spacer region of *Geomyces destructans*. *Mycologia* 105: 253-259.
2. Reichard, J.D. and T.H. Kunz. 2009. White-nose syndrome inflicts lasting injuries to the wings of little brown myotis (*Myotis lucifugus*). *Acta Chriopterologica* 11: 457-464.
3. Turner, G.G., C.U. Meteyer, H. Barton, J.F. Gumbs, D.M. Reeder, B. Overton, H. Bandouchova, T. Bartonička, N. Martínková, J. Pikula, J. Zukal, and D.S. Blehert. 2014. Non-lethal screening of bat-wing skin with the use of UV fluorescence to detect lesions indicative of white-nose syndrome. *Journal of Wildlife Diseases* 50: 566-573.

Questions regarding this protocol should be directed to:

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