University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2013

Adaptation of Sihler's Staining Technique for Visualization of Wing Innervation in Normal and WNS-Positive Bats

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Abstract

Sihler's staining technique is a method for visualizing nerves, while maintaining their positional contexts within organs. The technique stains nervous tissue while rendering other tissues translucent. Initial research assessed the feasibility of this technique on the delicate tissues of bat wings that were preserved by different methods. Specimens of *Myotis lucifugus*, previously frozen, were fixed in solutions of either 10% formalin or 80% ethanol by volume. After modifications, the technique proved successful in showing nerves down to the finest branches in specimens preserved in either solution. Dissection after staining revealed the dactylopatagial membranes (total area 9.87 $cm^2 \pm 0.96$) were supplied by branches of the median nerve, and the plagiopatagium (area 16.97 $cm^2 \pm 1.58$) was supplied primarily by the ulnar nerve, along with segmental spinal nerves and at least one nerve originating from the hindlimb. Sensory cell complexes, observed to be associated with sensory hairs on dorsal and ventral wing surfaces, were regularly patterned along rows through the wing. Three 5x5 mm areas were compared for number of primary rows and density of sensory cell complexes. Density was highest along the trailing edge of the dactylopatagium between digits III-IV (7 primary rows, 63 ± 4 cells), intermediate along the trailing edge of plagiopatagium (4 primary rows, 47 ± 6 cells), and lower near the center of the plagiopatagium (2 primary rows, 21 ± 7 cells). Application of this technique to a sample (n=7) of WNS-positive bats has shown that nerve damage can be seen in some areas where fungal damage was present. This research demonstrated that Sihler's staining is a useful approach for studying the neuroanatomy of bat wings.

1. Introduction

1.1. The Importance of Bats

Bats (Mammalia: Chiroptera) first appeared in the fossil record over 52 million years ago. Bats now live on six continents and are represented by over 1200 species. They have evolved vast diversity in the ecological and dietary niches they occupy[1]. Bats are tremendously important in the maintenance of ecosystems, and they have direct impacts on human health and economy. Bats are critical elements of all terrestrial biotic communities, where they help to control insect populations, reseed cut forests and disturbed habitats, and pollinate a range of plants in desert and tropical ecosystems. Industrially, bat guano is used as a fertilizer and for manufacturing soaps, gasohol, and antibiotics[2]. Because bats are nocturnal, their activities often go unnoticed. Insectivorous species help control insect populations that destroy crops or transmit pathogens to humans and other mammals[1]. Without bats, both we and our food supply would suffer. Due to their nocturnal habitats, the high degree of species variability, and their wide range of unique morphological and physiological adaptations, much remains unknown about these animals.

1.2. Bat Wings

Bats are the only mammalian clade that has evolved true flight, which enabled them to undergo extensive adaptive radiation early in the evolution of placental mammals[3]. Adaptation to flight enables them to inhabit niches that are unavailable to other mammals, and their wings are critically important to them in other ways. Bat wings are very thin, composed of a thin bilayered epidermis separated by a core of connective tissue, with supportive collagen and elastin fibers, fine muscles, nerves, and blood vessels. Due to their extensive vascularization and the fact that they comprise over 85% of body surface area, wings are central to the regulation of body temperature and hydration[4].

1.3. Histology and Sihler's Staining of Bat Wings

Traditionally, histological study is conducted on one of two ways: either by gross dissection of whole specimens, or by fixing, sectioning, and staining of tissue for microscopic analysis. However, both techniques have limitations in the study of neural histology. Terminal nerve endings and sensory complexes are microscopic in scale and difficult to differentiate from surrounding tissues without staining, making them exceedingly difficult to trace by dissection. Slide preparation, by contrast, enables viewing of the smaller structures, but it removes the tissue from its context within an organ.

Sihler's staining is a technique that permits mapping of entire nerve supply patterns, so that all nerves within the stained specimen can be visualized in their three-dimensional contexts[5]. Anatomist Charles Sihler first introduced his method for staining nerve spindles in 1895[6]. Recently, this technique has been rediscovered and applied to structures when conventional approaches of gross anatomical dissection and microscopic sectioning fail to provide adequate details of neuroanatomy. Examples of the use of this technique include studies of skeletal muscle and laryngeal specimens, whose neural distributions are highly complex and further complicated by frequent anastomotic networks[5,6]. This approach has been successfully applied to human, canine, rodent, amphibian, and lagomorph specimens[7-13]. Staining has been performed almost exclusively on fresh specimens that were immediately fixed in unneutralized formalin post mortem. Researchers have made slight adaptations to the process and formulae as required for the specific tissues studied. A research review published in early 2010 listed the known applications of the technique, including the 48 published studies since 1987[5]. None of these studies have explored the innervation of bat wings, nor did they examine the effect of different preservatives on the process.

1.4. Bat Wing Membranes

The wing of a bat is comprised of six (6) membranes (Fig. 1). The propataguim, a triangular membrane on the leading edge of the wing, stretches between the anterior surfaces of the humerus and radius, and spans from the shoulder to the first digit. The dactylopatagium consists of three membranes, spanning digits 2-5, and is important to maneuverability in flight. The largest of the flight membranes, the plagiopatagium, runs from the lateral aspect of the hindlimb and body wall to the posterior surfaces of the humerus and ulna, and the medial side of the fifth digit.

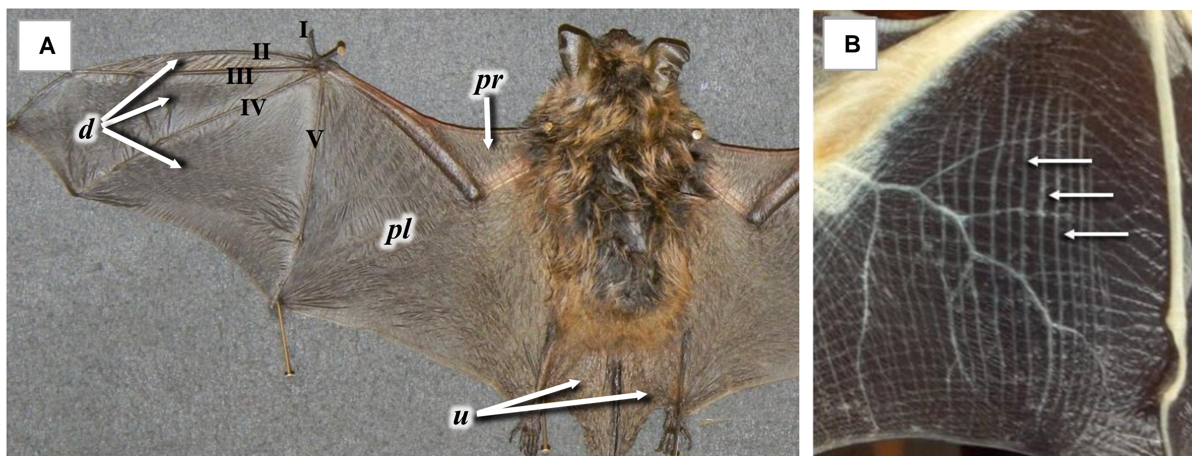

Figure 1. (A) Wing membranes in *Myotis lucifugus*: propataguim (pr), dactylopatagium (d), plagiopatagium (pl), uropatagium (u). Roman numerals (I-V) indicate digits. (B) Plagiopatagiales (arrows) in *Artibeus jamaicensis.*

The plagiopatagium is supplied by several different nerves, and contains the primary intrinsic wing muscles (plagiopatagiales) that run antero-posteriorly within the membrane (Fig. 1). The caudal membrane is the uropatagium, stretching from the medial border of the hindlimb to the tail and, in some species such as *M. lucifugus*, extending to the tail tip.

1.5. White-Nose Syndrome

An emerging disease called white-nose syndrome (WNS) is causing massive mortality in hibernating insectivorous bats in the eastern United States – up to 95% at some sites - placing several species in critical risk of extinction[14]. Current analyses suggest that loss of bats in North America could lead to agricultural losses of more than $3.7 billion per year[15].

White-nose syndrome is named for a white fungus (*Geomyces destructans*) found on the faces and wings of infected bats. The fungus causes bats to arouse more frequently during hibernation, resulting in premature depletion of fat reserves and, ultimately, death by starvation[14]. Since its discovery in eastern New York in early 2006, it has spread to 22 U.S. states and 5 Canadian provinces, killing an estimated 5.7 to 6.7 million bats and increasing the threat to some already-endangered species[16]. The U.S. Fish and Wildlife Service most recently confirmed WNS infections in South Carolina and Georgia[16]. The spread of this disease is exceeding the rate and magnitude of any previously known natural event in bats, and possibly any mammalian group[17]. Such a severe population decline may result in unpredictable changes in ecosystem structure and function[18]. WNS is not a direct human health risk, but it is estimated that with the loss of one million bats, between 660 and 1320 metric tons of insects are no longer being consumed in affected regions, exposing plants and crops to widespread damage; and the downstream impacts on ecosystems from increased use of pesticides could be substantial[15].

The fungus erodes the skin of bat wing membranes, which protect against hypothermia and dehydration during hibernation. The wing membranes represent about 85% of a bat's total surface area and play a critical role in balancing complex physiological processes, such as regulating body temperature, blood pressure, water balance and gas exchange[17,19]. In WNS-affected bats, folded surfaces of severely affected wing membranes adhere to each other, tear easily, and lose tone, tensile strength and elasticity[17]. This disables bats during flight and feeding, which contributes to starvation even when food is available. Irritation as the fungus erodes wing membranes may also play a role in triggering premature arousal from hibernation[19].

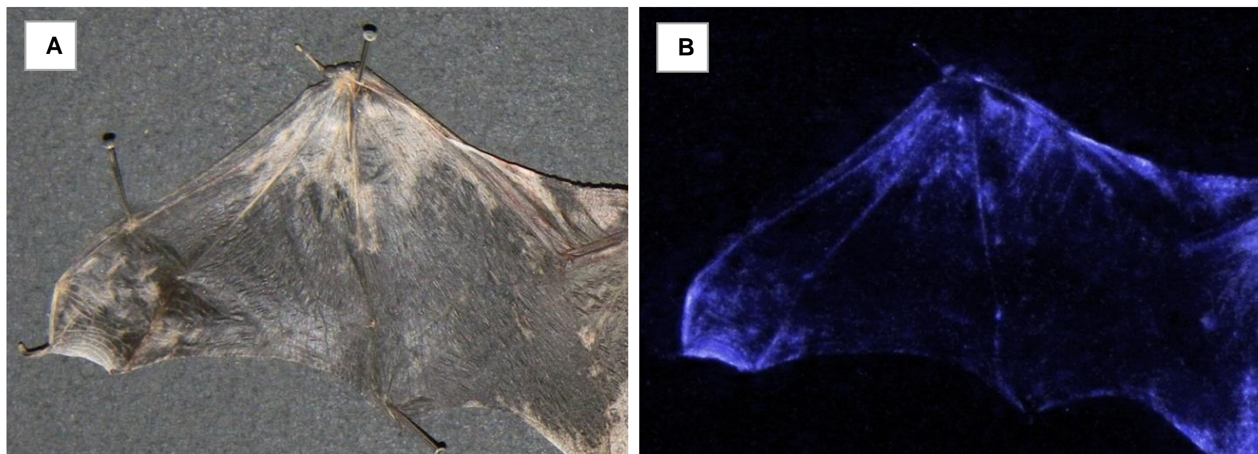

Figure 2. Wing damage in a white-nose positive specimen (A) under white light, and (B) revealing *Geomyces destructans* fungus using UV illumination.

1.6. Objectives of this Study

This project had four primary objectives:

- Compare results of this technique when used on bats preserved in ethanol and formalin. Fixation is required as the first step of this process, which has been commonly used on fresh specimens. Formalin and alcohol are the two most common preservatives used to store species for longer periods of time.

- Modify the published staining protocols to produce the best visualization of nerves in bat wings. These techniques have not been used on bats before, and wing membranes are thinner, more delicate, and have less connective tissue than other structures that have been analyzed, suggesting that several steps may require modification to produce optimal results.

- Examine and document the distribution of nerves and sensory hair complexes in the wings of *Myotis lucifugus,* to assess degree to which this technique improves visualization of normal morphology. To my knowledge, neither the normal pattern of innervation in the wings nor the distributions of sensory hair complexes in different areas of the wing have been previously described for *M. lucifugus*.

- Assess neural damage occurring in WNS-affected bats. This is expected to provide insight into the pathogenesis of WNS damage to wings, and help determine if nerve regeneration occurs in cases where wings have shown healing[20].

2. Methods

2.1. Specimens

White-nose negative specimens used in adaptation of this staining technique were from the collections of the University of North Carolina Asheville, Department of Biology. The sample of *Myotis lucifugus* (n=9) had been stored in a freezer at -20°C for several years. Specimens were intact, with no indications of disease or tissue damage. They had been acquired prior to the discovery of white-nose syndrome in the United States.

White-nose positive specimens were graciously provided by the Conserve Wildlife Foundation, New Jersey (John Gumbs). A sample of (n=7) *Myotis lucifugus* was shipped on ice, individually wrapped, and unfixed. Enclosed documentation indicated that all were WNS-positive and had died from the disease. Upon receipt, specimens were visually inspected, photographed and refrozen. Damage caused by *Geomyces destructans* was visible on the wing membranes of all specimens, each to varying degrees. Presence of the fungus was confirmed using UV illumination, which can be used as an indicator of *G. destructans* infection[21] (Fig. 2).

2.2. Sihler's Staining Technique

2.2.1. initial dissection

It was determined during the initial trials that the fixation of specimen internal organs was compromised by the technique's treatments. Therefore, the wings of all subsequent specimens were removed prior to Sihler's treatment. Though this step could be performed before or after fixation, without affecting the results, wings were easier to remove beforehand, when the tissues were more pliable.

2.2.2. fixation

Frozen specimens of *M. lucifugus* were thawed in cold water to facilitate inspection and, following the initial trial, to ease wing removal. All specimens were fixed in preparations of either 10% unneutralized formalin or 85% ethanol. Fixation time was significantly longer for whole bats, to allow preservative to penetrate the body walls.

In the initial trial, which was used to determine which fixation method resulted in the best staining results, whole specimens (n=3) were soaked in preservative for a month. One was fixed in formalin (Fig. 3-A), another in ethanol, and a third in ethanol for two weeks followed by formalin for two weeks. The remaining WNS-negative bats (n=6) were divided evenly, half of them fixed in ethanol and half in formalin. The wings of each were treated for a minimum of two weeks.

Experience showed that preservation in ethanol produced specimens that required less physical manipulation, and therefore were less likely to be torn during the process. Due to the degree of damage that was present in the wings of the WNS-positive specimens prior to treatment, all were fixed in ethanol to minimize the amount of handling and subsequent risk of tearing the wing membranes.

2.2.3. maceration and de-pigmentation

Specimens were rinsed for 30 minutes and then incubated in a solution containing 3% w/v aqueous potassium hydroxide with three drops of 3% hydrogen peroxide for every 100 mL of solution. Incubation was continued, and the solution changed as necessary, when it darkened, until wing membranes become transparent. Treatment for ethanol-preserved wings required less than 24 hours in most cases (Fig. 3-B). Formalin-preserved wings required a week or longer to clear, and were rubbed gently under cold water periodically to loosen pigmented tissue.

2.2.4. decalcification

Specimens were rinsed for 30 minutes and then treated in Sihler's I solution (prepared from one part glacial acetic acid, glycerin and 1% w/v aqueous chloral hydrate). This step was eliminated for later ethanol-preserved specimens, as the bones and tissues degrade rapidly in this solution. Formalin-preserved wings were treated for up to two days, until wings could be laid flat.

2.2.5. staining

Specimens were rinsed for 30 minutes under cool water, blotted dry, and incubated in Sihler's II solution (prepared from 1 part Ehrlich’s hematoxylin, 1 part glycerin, and 6 parts 1% w/v aqueous chloral hydrate). Wings were stained for three or more days or until the large nerves within the specimens turned dark purple and the terminal nerve branches were observed to be well-stained, when viewed under a dissecting microscope.

2.2.6. destaining

Specimens were rinsed for 30 minutes under cool water, blotted dry, and immersed in Sihler's I solution to remove excess stain. This treatment was conducted on a flat, shallow dish under a dissecting microscope, and gentle agitation was used at times to speed this process. Destaining is complete when nerves appear dark against clear membrane tissues. If nerves become too pale, staining and destaining can be repeated (Fig. 3-C).

2.2.7. clearing

Specimens were rinsed again and then immersed in increasing concentrations of glycerin (40%, 60%, 80% and 100%). The specimen remains in 40% and 60% glycerin for 2 days, and one day each in 80% and 100% glycerin. Specimens can be stored in 100% glycerin, changed every few months or when solution darkens.

2.2.8. visualization and dissection

Ideally, specimens were photographed immediately following destaining, as optimal nerve/membrane contrast fades rapidly. Dissection can be performed at any time, even following storage in glycerin.

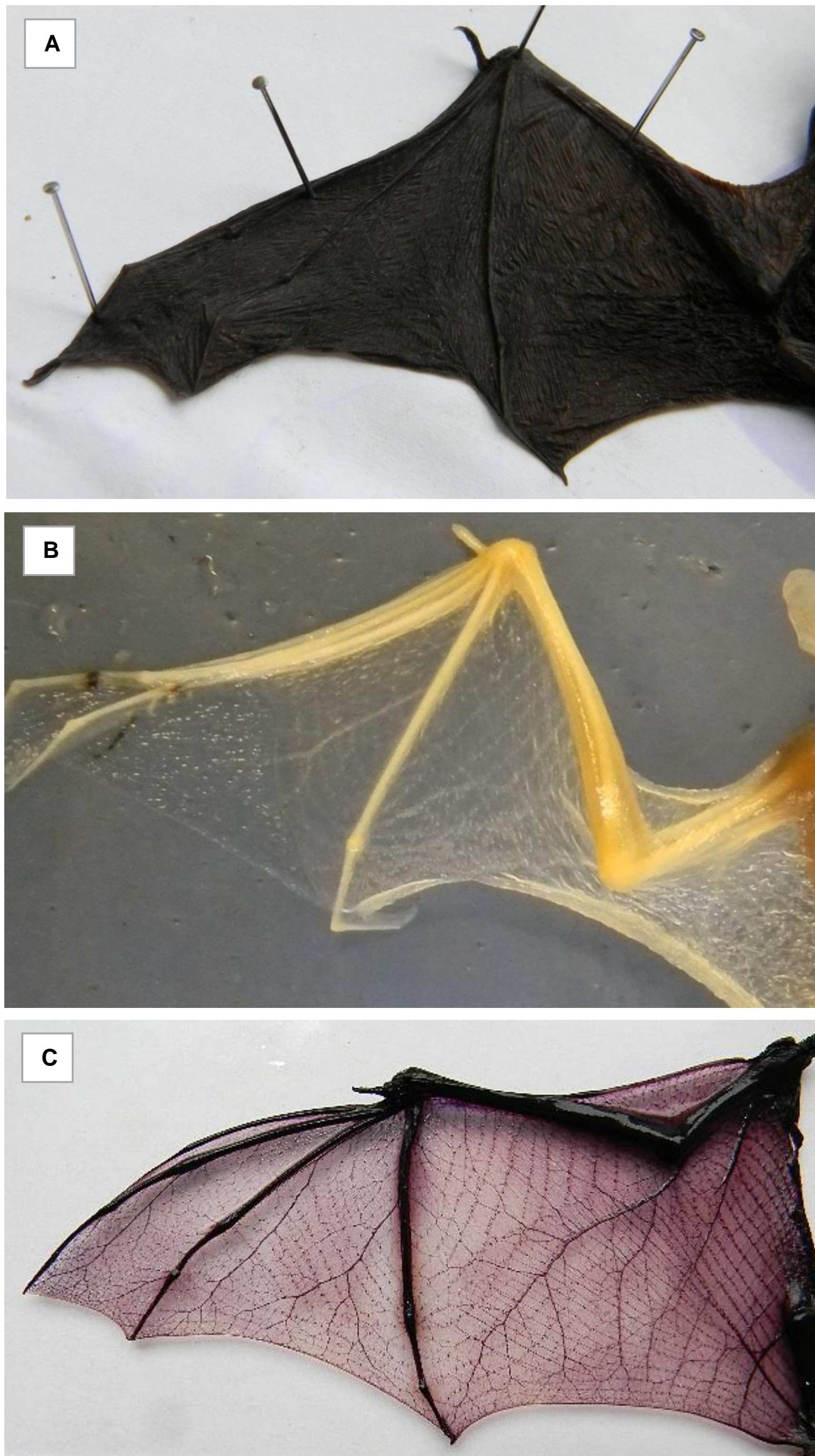

Figure 3. Wing specimens at various treatment stages. (A) following fixation in formalin, (B) following maceration and depigmentation, (C) following destaining.

3. Results

3.1. Staining

Figure 3 shows wings at different stages of preparation. With modifications, the technique worked successfully on bats preserved in both ethanol and formalin. Modifications to the published procedure[5] include initial dissection to remove preservative-resistant tissues, shortening times of the maceration, decalcification, staining and destaining treatments, and elimination of the neutralization step. Specimens fixed in ethanol cleared easily, making them less likely to endure damage from handling. Those fixed in formalin took up to a week longer to clear, and required manual rubbing to remove pigmented tissue and fur. This is a concern because the tissues are easily torn, especially in already compromised wings. However, formalin fixation made wings slightly tougher and often produced better visualization of neural structures in the final product. Wing nerves and tactile hair complexes were clearly visible in specimens following either preservation treatment.

3.2. Analysis of Normal Wing Structure

3.2.1. innervation

This technique enabled visualization and facilitated microdissection of the wing membrane nerves. This analysis focused on cutaneous innervation, rather than the nerve supply to specific muscles (Fig. 4). The median nerve (m) traveled along the ventral surfaces of the arm and forearm, supplying the dactylopatagium. Large branches ran directly along the medial and lateral edges of digits III, IV and V, with numerous smaller branches extending from these into the wing membranes. The ulnar nerve (u) followed the medial surface of the brachium and supplied most of the plagiopatagium. Two large branches of the ulnar nerve diverged distal to the axilla. The plagiopatagium received additional branches from segmental spinal nerves (s) and a nerve emerging along the hindlimb at the knee (h). The radial nerve (r) ran along the cranial edge of the forelimb. Distal branching patterns of all cutaneous nerves were highly variable. Several areas of the wing appeared to show anastomoses between different nerves (Fig. 4, dashed circles), but it was not possible to tell if these represented dual innervation to sensory hairs and section of the wing, or were nerves supplying opposite surfaces of the wing.

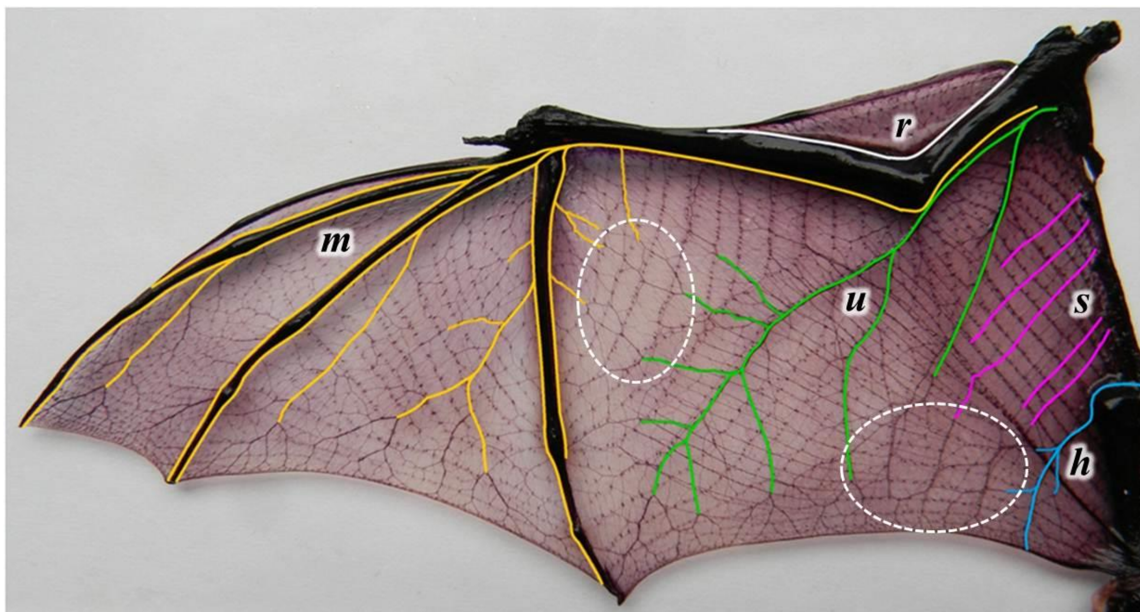

Figure 4. Primary nerve branches traced. (r) radial nerve = white, (m) median nerve = yellow, (u) ulnar nerve = green, (s) segmental spinal nerves = pink, (h) nerve from hindlimb = blue. Dashed circles indicate areas of possible anastomoses.

3.2.2. density of sensory cell complexes

Sensory cell complexes (dome shaped structures containing hairs that help provide aerodynamic feedback)[22-24] occur along regularly patterned rows throughout the wing, and stain darker than surrounding tissues (Fig. 5). The number of primary rows and a total count of sensory cell complexes (domes) were recorded in three 5x5 mm areas: (1) the trailing edge of the dactylopatagium between digits III-IV, (2) the trailing edge of plagiopatagium, (3) center of the plagiopatagium. A one-way ANOVA showed that the dactylopatagium had the highest density of sensory cell complexes, and the plagiopatagium had the lowest density (Table 1).

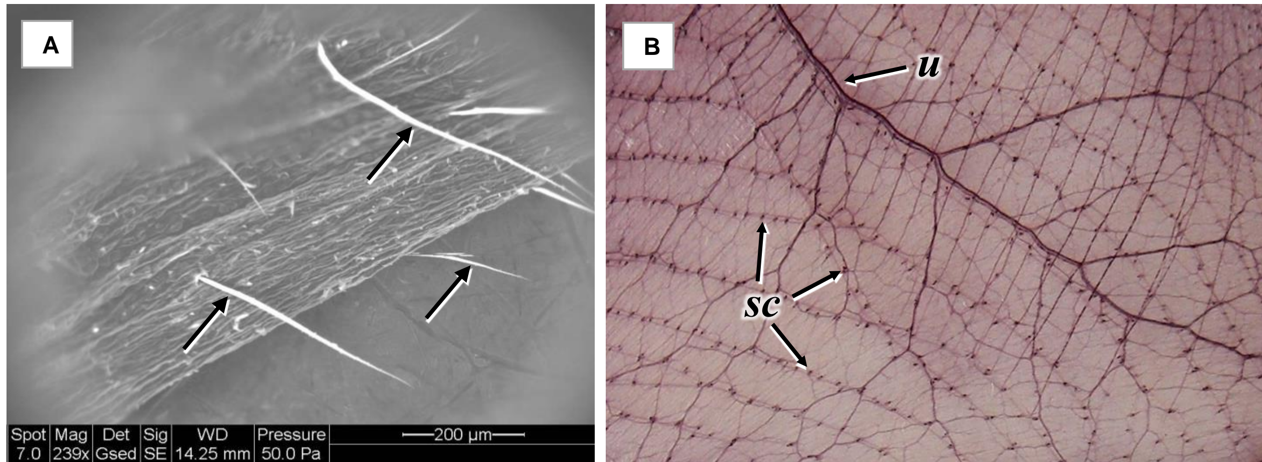

Figure 5. (A) SEM of untreated wing along trailing edge of plagiopatagium shows sensory hairs (arrows). (B) Section of stained wing showing ulnar nerve (u) and sensory cell complexes (sc). The sensory hairs associated with the complexes fell out during preparation.

Table 1. Densities of sensory cell complexes in different areas of the wing. Cells contain mean ± standard deviation.

Area of Wing	#Rows	#Complexes
Dactylopatagium (III-IV), trailing edge	7	63 ± 3.5
Plagiopatagium (trailing edge)	4	47 ± 4.7
Plagiopatagium (center)	2	21 ± 5.4
One-Way ANOVA (n=5)	*** (P< 0.001)	*** (P< 0.001)

3.3. Analysis of WNS-positive Specimens

A sample of (n=7) WNS-positive *M. lucifugus* collected by John Gumbs from Hibernia Mine (Rockaway Township, NJ) was analyzed. Four of the bats had active lesions from *Geomyces destructans,* while the other three bats showed no evidence of active lesions. Wing damage on each bat was scored as category 1-2 on the scale proposed by Reichard[14].

Lesions and scars from WNS often stained differently than surrounding tissues. In 5 of 7 WNS-positive wings examined, areas of apparent nerve breakage were found (Fig. 6). These breaks always occurred in parts of the wing where scarring and lesions were present (Fig. 7). However, nerve breakage was rarely observed, even in areas with heavy fungal infection and significant damage to membranes. This damage was only observed in WNS-affected bats. No similar breakage was observed in any of the non-WNS bats that were examined.

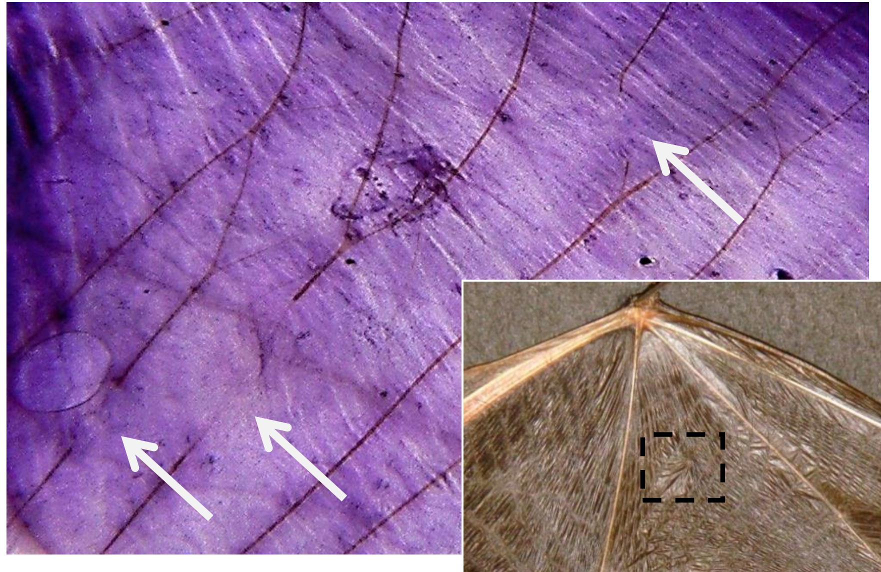

Figure 6. Staining revealed areas of likely nerve tissue damage (arrows) on a wing membrane scarred by WNS. The specimen had active *G. destructans* lesions.

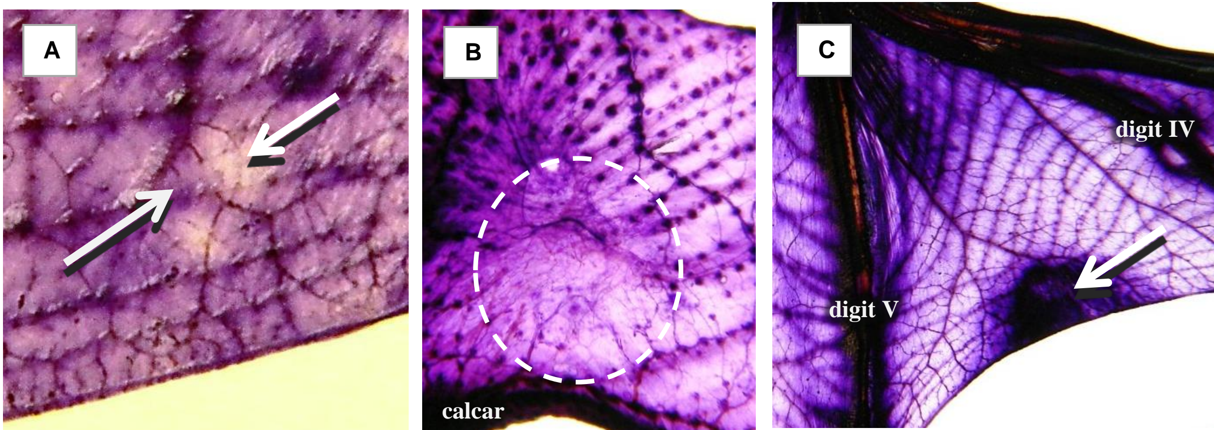

Figure 7. WNS-associated damage to (A) nerves in plagiopatagium (arrows), (B) uropatagium with scarring and nerve deterioration (encircled), (C) dactylopatagium, showing large lesion (arrow). Images not to scale.

4. Discussion

With modifications, Sihler's staining technique can be successfully used on bats preserved in either ethanol or formalin. Freshly collected specimens are not required for this technique -- it can be used on specimens that have been stored for long times in either preservative. As applied to *M. lucifugus*, Sihler's staining takes from 3 to 5 weeks. The optimal duration of certain steps varies depending upon preservative used and the individual character of specimens. Both preservatives have benefits and drawbacks to their application.

Fixation in ethanol produces final specimens more quickly, and is more suitable for delicate specimens. However, the maceration step should be shortened to minimize tissue degradation. In many cases, ethanol-preserved wings were cleared within 24 hours, having sloughed their epidermal layers in sheets without abrasion. The decalcification step may be omitted to avoid excessive softening of the supporting bones. Decalcification of these specimens for longer than a day caused membrane tissues to separate from the bones. Despite rendering wing membranes very delicate, ethanol is recommended for severely damaged tissues, such as those with WNS lesions and tears.

The maceration and clearing of formalin-preserved wings can take up to two weeks, and requires rubbing of tissues to remove all epidermal tissue. Abrasion in this process can tear the delicate remaining tissue, even in normal (WNS-negative) specimens, so it is not recommended for wings with existing damage. Decalcification of formalin-preserved wings, softens the bones enough to be easily laid flat for photographing, after the staining process. Formalin-preserved specimens retain more rigid membrane tissues and enable better final visualization of neural structures.

Only the initial specimens were treated as prescribed in the published protocol, and gradual adaptations were made during subsequent treatments. The initial trial revealed that a prescribed "neutralization" treatment in lithium carbonate solution, performed after destaining, bleached the purple stain from the nerves, leaving them a faint brown color. This step was eliminated from use on subsequent bats. The durations of treatments were gradually modified to reduce damage and improve overall results.

Following initial trials, all wings were always handled with non-textured gloves to prevent inadvertent tissue tearing. To preserve some structural support to the plagiopatagium, a small margin of the body wall was removed along with the wings. Additionally, all steps were performed in shallow glass dishes, to facilitate handling between treatments, after it was observed that some treated wings can tear under their own weight. Destaining of wings requires care and vigilance, as 5-10 additional minutes can be too long. For this reason, these treatments were performed in their flat dishes under a dissecting scope.

Successful staining of whole bats followed by microdissection enabled identification of the major nerves visible in the wings. The radial nerve ran along the margin of the propataguim adjacent to the humerus and radius. The dactylopatagium was innervated by the median nerve, whose branches followed digits 3-5 into the membranes. The plagiopatagium was supplied by the median nerve, ulnar nerve, segmental spinal nerves from the body wall, and a nerve emerging along the hindlimb at the knee. Complete dissection of the lumbosacral plexus was not conducted, so it was not possible to reliably confirm if the nerve coming off the hindlimb was homologous to the lateral femoral cutaneous nerve or a branch from the femoral nerve, or was a nerve that may be unique to bats.

Nerve branching patterns were highly variable within wing membranes, and appear to hold little potential value as a reliable indicator for future phylogenetic or functional studies. Possible anastomoses between distal branches of different nerves were also observed in several locations on the wings. However, even when viewing under high magnification, it was difficult to determine whether these branches were actually connected at those points or were overlapping branches that supply opposite faces of the wing.

In addition to facilitating visualization of nerves, this staining technique was useful for observing sensory hair complexes. The greatest densities of sensory cell complexes were observed in the dactylopatagium and along the trailing edge of the wing. Densities were lower in the plagiopatagium, which is more proximal to the body. The density of sensory hair complexes on the more mobile parts of the wing is consistent with their use in sensation of air currents in flight[22-24].

In bats with wing damage indices of 1-2[14], nerves generally persist in WNS-affected areas with moderate scarring and lesions. In several cases (5 of 7 specimens), some nerves appeared broken in areas where there had been significant WNS involvement. Apparent breakage of nerves was usually limited to fairly small areas. No similar morphology was seen in any WNS-negative wings, nor in WNS-postive bats in areas of the wing that were free of fungal damage, suggesting that this damage is due to destruction of nerve fibers by the fungus. In some of the affected areas that were conspicuously lacking in large nerve branches, it appeared that possible re-growth of new fibers was taking place. Healing of wing membranes in bats that survive WNS infection has been documented[20]. However, due to the small sample size, and high variability of innervation patterns in specimens, we could not verify that new growth of nerves into WNS-damaged areas had occurred.

5. Acknowledgements

The author thanks John Gumbs of the Conserve Wildlife Foundation of New Jersey and Kevin Keel of the UGA College of Veterinary Medicine for loan of WNS-positive specimens, and Sharon Swartz and Jorn Cheney of Brown University for loan of *Artibeus jamaicensis* specimens. The author thanks Dr. Eric Pyeritz for assistance in obtaining reagents. Special thanks go to Chris Nicolay at the University of North Carolina Asheville for the loan of WNS-negative specimens, and for his insight and guidance during this study. The author also thanks the Biology Department at the University of North Carolina Asheville for assistance with laboratory equipment and photography. This work was supported by grants from the UNCA Undergraduate Research Program.

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