



Bat wing biometrics: using collagen–elastin bundles in bat wings as a unique individual identifier

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The ability to recognize individuals within an animal population is fundamental to conservation and management. Identification of individual bats has relied on artificial marking techniques that may negatively affect the survival and alter the behavior of individuals. Biometric systems use biological characteristics to identify individuals. The field of animal biometrics has expanded to include recognition of individuals based upon various morphologies and phenotypic variations including pelage patterns, tail flukes, and whisker arrangement. Biometric systems use 4 biologic measurement criteria: universality, distinctiveness, permanence, and collectability. Additionally, the system should not violate assumptions of capture–recapture methods that include no increased mortality or alterations of behavior. We evaluated whether individual bats could be uniquely identified based upon the collagen–elastin bundles that are visible with gross examination of their wings. We examined little brown bats (*Myotis lucifugus*), northern long-eared bats (*M. septentrionalis*), big brown bats (*Eptesicus fuscus*), and tricolored bats (*Perimyotis subflavus*) to determine whether the “wing prints” from the bundle network would satisfy the biologic measurement criteria. We evaluated 1,212 photographs from 230 individual bats comparing week 0 photos with those taken at weeks 3 or 6 and were able to confirm identity of individuals over time. Two blinded evaluators were able to successfully match 170 individuals in hand to photographs taken at weeks 0, 3, and 6. This study suggests that bats can be successfully re-identified using photographs taken at previous times. We suggest further evaluation of this methodology for use in a standardized system that can be shared among bat conservationists.

Key words: animal identification, bats, biometrics, *E. fuscus*, *M. lucifugus*, *M. septentrionalis*, *P. subflavus*

The ability to recognize individuals within a population is fundamental to many wildlife management and conservation methods. Artificial marking techniques have included ear or toe tags, toe clipping, leg bands, forearm bands, ear notching, and passive integrated transponder (PIT) tags as well as many others (Kunz et al. 2006). Studies on behavior and survival rates have reported conflicting results, indicating that it may be difficult to make broad generalizations about the effects of these techniques on various animal taxa (Perret and Joly 2002; O’Shea et al. 2004; Lacki et al. 2007; Barron et al. 2010; Jepsen et al. 2015). Altered behavior or increased mortality resulting from marking violates an assumption that underlies

most capture–recapture methods, namely that the probability of recapture is not affected by marking (Caughley and Sinclair 1994). As early as the 1970s, wildlife biologists began developing alternatives to invasive marking methods by establishing identification systems for individuals based on phenotypic appearance such as the unique stripe patterns for plains zebras (*Equus burchelli*—Petersen 1972), and tail flukes in humpback whales (*Megaptera novaeangliae*—Glockner and Venus 1983) and southern right whales (*Eubalaena australis*—Payne and Dorsey 1983). Today, the field of animal biometrics has expanded to include recognition and classification of species and individuals based upon variable and unique coat patterns,

whisker arrangements, vocalizations, movement dynamics, and body morphologies (Kühl and Burghardt 2013).

For the 44 bat species, not including subspecies, found in the United States and Canada (Natureserve 2016), identifying individual bats has been almost solely dependent upon marking with bands. The 1st description of identifying and tracking individual bats occurred in 1916, in Ithaca, New York, when bird bands were placed on the legs of 4 female tricolored bats (*Perimyotis subflavus*, formerly eastern pipistrelle—Allen 1921). Since that initial report of banding, the U.S. Bureau of Biological Survey (now U.S. Fish and Wildlife Service, USFWS) began a coordinated Bat Banding Program (BBP) in 1932 in which bands were issued to registered banders and the records kept at the Smithsonian Institution, National Museum of Natural History (Ellison 2008). The program ran from 1932 to 1972, resulting in 36 species being banded with approximately 1.5 million bands (Ellison 2008). The bands initially were placed on the legs, until 1939 when Trapido and Crowe (1946) began placing bands on the forearm, which became the standard. Early banders used standard aluminum bird bands. However, based on multiple reports of both captive and field recoveries of bats, many bats sustained injuries from the sharp metal edges of the bands (Hitchcock 1957). This resulted in the adoption of Dutch-designed “lipped” aluminum bands in the mid-1950s (Hitchcock 1957; Herreid et al. 1960) that were issued along with the bird bands. In 1973, the high number of injuries caused by bat bands and the documented population declines of 22 species that were directly related to banding and banding-related disturbances led the USFWS to discontinue issuing bands to researchers (O’Shea and Bogan 2003; Ellison 2008). In addition to injuries and population declines, less than 1% of bats with bands were recovered (O’Shea and Bogan 2003).

Alternatives to banding have been investigated. In 1934, Griffin (1934) tattooed numbers on the wing membrane of bats and upon recapture of 2 individuals approximately 1 year later the tattoos were still readable. However, this method was abandoned due to the time required to tattoo, which made it unfeasible for field studies that release captured individuals within an hour. Thirty-eight years later, Bonaccorso and Smythe (1972) reported that punch marking with interchangeable one-quarter inch high numbered punches was superior to banding, but reported 4 years later that the punch marks were only readable for 5 months (Bonaccorso et al. 1976). Barclay and Bell (1988) fashioned collars from bead-clasp ball-chain necklaces threaded through a numbered aluminum band. The materials for this device were difficult to obtain and they could only be used on individuals heavier than 15 g due to weight ratios (Gannon 1994; Kunz and Parsons 2009). Additionally, there was a risk of snagging the necklace on crevices, roost structures, or foliage (O’Shea and Bogan 2003; Kunz and Parsons 2009). Gannon (1994) adapted the collar method to utilize lighter, more easily obtainable materials including plastic zip ties and medical tubing, but there have been no reports of this method being used beyond this study and so evaluation of success is limited. Sherwin et al. (2002) attempted to use freeze

branding to permanently mark bats. However, this technique has limitations as the brand must be distinct, it can take up to 2 months for the brand to appear and requires the hair to be clipped which prohibits its use during or immediately prior to hibernation (Sherwin et al. 2002). Most recently, PIT tags have been used to identify individual bats, but this method requires a reading device that is primarily suitable to bat species with specialized roosting behaviors that allow researchers to locate the device close enough to the tag to read it (Kerth and König 1996, 1999; O’Shea and Bogan 2003).

Due to the lack of success of these alternative marking techniques and the high injury rate caused by banding (Hitchcock 1957; O’Shea and Bogan 2003; Ellison 2008), a noninvasive biometric scheme for identifying individual bats is warranted (Kühl and Burghardt 2013). We investigated whether *Myotis lucifugus*, *Myotis septentrionalis*, *Eptesicus fuscus*, and *P. subflavus* could be individually identified based upon the collagen–elastin bundles that are visible upon gross examination of bat wings (Holbrook and Odland 1978). These bundles are composed of collagen and elastin that help to provide the tensile strength of the wing tissue while allowing it to remain flexible during flight (Holbrook and Odland 1978). We predicted that these collagen–elastin bundles would form a pattern that would satisfy the 4 biologic measurement requirements of all biometric identification systems: 1) universality, 2) distinctiveness, 3) permanence, and 4) collectability (Jain et al. 2004), and that these wing patterns could be captured using transillumination-assisted photography that could serve as a reference file for future identifications.

MATERIALS AND METHODS

All activities conducted in this study were under an approved University of Missouri Institutional Animal Care and Use Committee protocol, a Missouri Department of Conservation (MDC) Wildlife Scientific Collection permit (Permit #15556 and #16409), and adhered to guidelines of the American Society of Mammalogists (Sikes et al. 2016). All bats and photographs were collected as part of a study focused on white-nose syndrome (WNS). Disinfection protocols for bat studies published by the USFWS (U.S. Fish and Wildlife Service 2016) were followed for all collection and sampling activities.

Collection and housing of bats.—Hibernating adult *M. lucifugus* ($n = 100$) and *M. septentrionalis* ($n = 35$) were collected from 2 privately owned hibernacula, with landowners’ permission, in November 2014. In November 2015, we also collected hibernating adult *E. fuscus* ($n = 35$) from a private residence in central Missouri and a privately owned mine, with landowners’ permission. Each bat was placed in an individual sterile cloth bag, and subsequently placed in an insulated cooler lined with damp towels to maintain high humidity. To reduce thermal stress during transport, bats were maintained at 25–30°C while being transported to a biosecurity level 2 (BSL-2) vivarium room at the University of Missouri, Columbia. All bats were evaluated for sex, mass (MS; Acculab Pocket Pro 150-B, Edgewood, New York), and forearm length (FA). We identified each bat

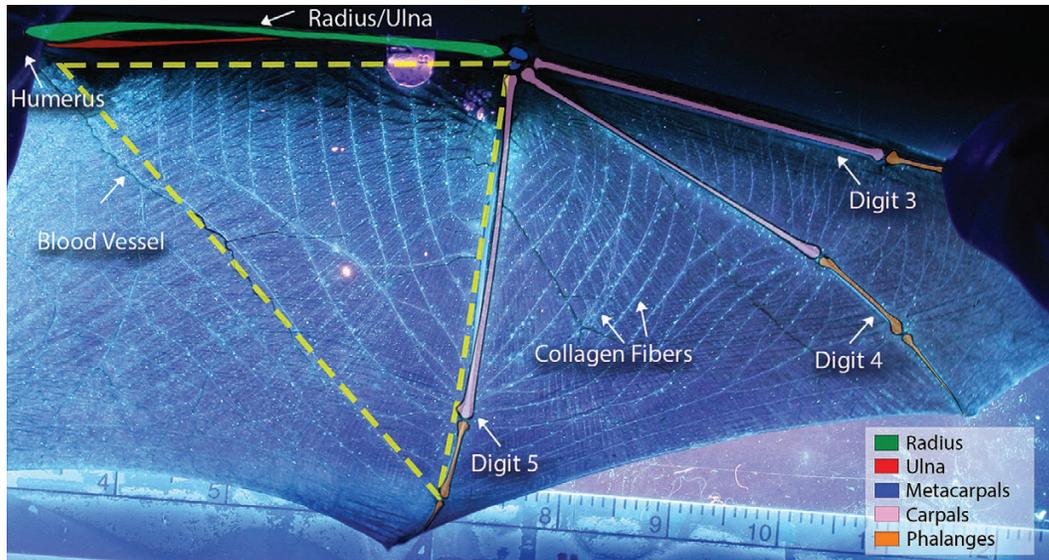


Fig. 1.—Representative photograph of a bat's left wing. Section 1: triangular-shaped section (dashed line) of the plagiopatagium bordered by the radius and ulna, extending the length of the 5th metacarpal and associated phalanges. Section 2: dactylopatagium between digits 4 and 5. Section 3: dactylopatagium between digits 3 and 4. Section 4: plagiopatagium along humerus, extending along the body and along the trailing edge to the distal 5th phalanx.

by a standard aluminum-lipped, numbered forearm band (Porzana, Ltd., East Sussex, United Kingdom) or by a uniquely numbered 3.2-mm fluorescent tag (Fast Signs Inc., Columbia, Missouri), which was attached to the pinna using Osteobond (Zimmer, Inc., Warsaw, Indiana). Animals were housed in 83.3-liter nylon-mesh enclosures (Apogee Reptarium, Dallas, Texas) while in hibernation chambers (Geneva Scientific Model I-36NL, Fontana, Wisconsin and Caron Model 6040, Marietta, Ohio). *M. lucifugus* and *M. septentrionalis* were maintained at a constant temperature of 7°C with 95% relative humidity and *E. fuscus* at 10°C with 95% relative humidity. Animals were maintained in 143.8-liter nylon-mesh cages (Apogee Reptarium) or a flight cage (dimensions 3 × 3 × 3 m) when housed outside the hibernation chambers at ambient temperature (21 ± 1°C) and humidity (30–70%). Enclosures were equipped with infrared cameras (model CM1150H; Nightowl, Walpole, Massachusetts) and video recorders (Apollo, model DVR5; Nightowl) to monitor bat behavior while in hibernation chambers. Bats were visually inspected via camera 2–4 times daily and activity was recorded automatically if movement was sufficient to trigger the camera. At all times animals were provided with ad libitum deionized water and mealworms. As part of a WNS study, hibernating *P. subflavus* ($n = 60$) from a commercially operated cave in Missouri (permission from landowners) were collected and placed individually into sterile cloth bags. These bats were transported to a section of the cave where there were no other bats and where increased activity would not disturb any bats. Each bat was evaluated and identified using the same protocol as for *M. lucifugus*, *M. septentrionalis*, and *E. fuscus*. The *P. subflavus* were housed in 143.8-liter nylon-mesh cages (Apogee Reptarium) suspended from the cave ceiling and enclosed in ¼-inch hardware cloth to ensure protection from potential predators. Bats were subsequently released, as the *P. subflavus* did not meet the diagnostic criteria for WNS.

Transillumination photographs and pattern analysis.—Following methods of Turner et al. (2014), photographs were taken with a digital SLR camera (Canon EOS Rebel XT; Canon U.S.A., Inc., Melville, New York) with a standard zoom lens (Canon EF-S 18-55 mm f/3.5-5.6 IS) mounted on a tripod. UV transillumination photographs (360–385 nm) were taken of all *M. lucifugus* and *M. septentrionalis* wings at weeks 0, 3, and 6. Photographs of *E. fuscus* and *P. subflavus* wings were taken at weeks 0 and 6. Immediately after each photograph, the photograph numbers were recorded for each bat. The initial photograph of each bat wing was labeled with band number, date, and wing (left or right).

Photographs taken at week 3 (*M. lucifugus*, *M. septentrionalis*) and week 6 (*M. lucifugus*, *M. septentrionalis*, *E. fuscus*, and *P. subflavus*) were evaluated by 2 blinded reviewers who used the collagen–elastin bundle patterns grossly visible in the dactylopatagium and plagiopatagium (Holbrook and Odland 1978) to identify each individual. Each bat wing was divided into the following 4 sections: 1) triangular-shaped section of the plagiopatagium with 1 side bordered by the radius and ulna; 1 side extending the length of the 5th metacarpal and associated phalanges; 2) the dactylopatagium between digit 4 and 5; 3) the dactylopatagium between digit 3 and 4; and 4) plagiopatagium along humerus, extending along the body and along the trailing edge to the distal 5th phalanx (Fig. 1). Bats were initially screened by comparing the patterns within section 1. When a bat appeared to be a potential match, other sections were examined in numerical order to ensure an accurate match. Each picture was verified to be correctly labelled based upon the datasheet records that contained the photograph numbers.

Bat identification in hand.—After bats were removed from the hibernation chamber for spring emergence (April 2016) and the numbered tag(s) removed, bats were housed temporarily in labelled 0.3 × 0.3 × 0.3 m nylon-mesh cages (Live Monarch,

Boca Raton, Florida). Subsequently, each bat was presented independently to 2 blinded evaluators who used the previously labelled photographs from week 0 to determine each individual's identification following the procedure described for photographic pattern analysis. After matching the 1st wing, the opposite wing also was examined to further confirm the individual identification of the bat.

Validation of wing print biometrics.—A subset of *M. lucifugus* ($n = 11$) whose wings were severely damaged by WNS and subsequently healed were photographed 16 months after the original week 0 photographs were taken. Twenty-two numerically numbered photographs were provided to 3 blinded bat biologists and 3 blinded naïve evaluators who were asked to match them to the original labelled (band number, date, L or R wing) week 0 photographs. We evaluated the number of images correctly and incorrectly identified by individual observers and all observers combined. We used Cohen's Kappa within SAS 9.3 (SAS 2010) to test for agreement between multiple observers. Any image incorrectly labeled was recorded as an error. The error rate is the proportion of test images misidentified.

$$\text{Success rate}_{(\text{observer})} = \frac{\text{Number of correct matches}}{\text{Total number of images}}$$

$$\text{Error rate}_{(\text{cumulative})} = \frac{\text{Number of incorrect matches all observers combined}}{\text{Total number of images} \times \text{number of observers}}$$

RESULTS

Transillumination photographs and pattern analysis.—A total of 622 *M. lucifugus*, 210 *M. septentrionalis*, 140 *E. fuscus*, and 240 *P. subflavus* photographs were matched successfully to 230 individuals as the wing prints of each animal displayed distinct, unique patterns. Photos taken at weeks 3 or 6 were determined by 2 observers to have unique and identifiable bundle patterns that matched photos taken at week 0.

Bat identification in hand.—Two blinded evaluators were able to successfully match all 100 *M. lucifugus*, 35 *M. septentrionalis*, and 35 *E. fuscus* to their UV wing print photographs in our laboratory. There was some difficulty seeing the collagen–elastin bundles under the vivarium lighting, and so a fluorescent bulb light box was used occasionally to assist in transillumination of wings.

Validation of wing print biometrics.—Cohen's Kappa statistic for agreement between observers evaluating a subset of *M. lucifugus* ($n = 11$) was 0.773 with an asymptotic *SE* of 0.07. Five of 6 evaluators had success rate of 1 while 1 observer had a success rate of 0.77. Cumulatively, 96% of individual bats were correctly identified.

DISCUSSION

This study sought to address the critical need of identifying individual bats (O'Shea and Bogan 2003) without employing marking techniques that could result in injury, disrupt their

behavior, or in any way potentially be detrimental to the species of interest. A total of 230 bats representing 4 North American species, *M. lucifugus*, *M. septentrionalis*, *E. fuscus*, and *P. subflavus*, were found to have universal, yet individually distinct patterns or “wing prints” formed by the collagen–elastin bundle network that is grossly visible. To date, these wing print patterns appear as unique and distinctive as human fingerprints (Herschel 1916) and zebra stripes (Petersen 1972), thereby fulfilling the first 2 requirements of a biometric identification system, universality and distinctiveness (Jain et al. 2004), and allowed the individual identification of all bats within our study. Photographs taken at weeks 3 and 6 were correctly matched with initial (week 0) photographs. A subset of individuals photographed over an additional 16 months confirmed that wing print patterns were permanent and could be collected, thus fulfilling the last 2 requirements of a biometric identification system (Jain et al. 2004).

What is remarkable is the permanence of the collagen–elastin bundle network even for the subset of bats with severe damage to their wings caused by *Pseudogymnoascus destructans* (causative agent of WNS—Bleher et al. 2009). These bats were sampled 16 months after their original photographs were taken, and while they exhibited some scarring with wing damage index scores up to 4 (Reichard and Kunz 2009), the collagen–elastin bundle network within the damaged areas of the plagiopatagium and dactylopatagium maintained the original wing print pattern with only small portions of the bundle network having disappeared due to extensive scarring (Fig. 2). If the scarring was captured with the photographs, it could be used to further identify individuals.

If a bat sustained an injury or loss of the plagiopatagium from WNS, it did not interfere with the analysis unless over 50% of the plagiopatagium was absent (Fig. 3). Bats that sustained injuries were noted to have severe dilation of the blood vessels surrounding the injury (large increase in diameter, prominence and erythema; Fig. 4). We also noted that over the course of 6 weeks, the blood vessels could dilate without any grossly visible signs of injury (Fig. 5), which resulted in the blood vessels appearing to have altered their location. This provides evidence that noncaptive bats whose plagiopatagium or dactylopatagium are damaged could likely still be identified as long as the actual collagen–elastin bundles throughout the wing are used and the observer does not rely on the blood vessels.

We encountered 3 challenges during the identification process, with the primary challenge being the slow shutter speed required for UV photographs, as this required the bat to remain immobile for several seconds. If the bat moved during the photo, then the photograph would be blurry, usually near the body, forcing the blinded evaluators to rely upon sections 2 and 3 for identification if these were in focus. A 2nd minor challenge was created by poorly restrained bats handled by their metacarpals instead of the distal phalanges resulting in the wings being partially covered. The other minor challenge also was due to improper restraint of the distal phalanges of digits 4 and 5, resulting in curling of the wing, which obscured the lower portion of the wing.

Although this study used UV light photography, a regular light box can be substituted, and in some cases, may be preferential

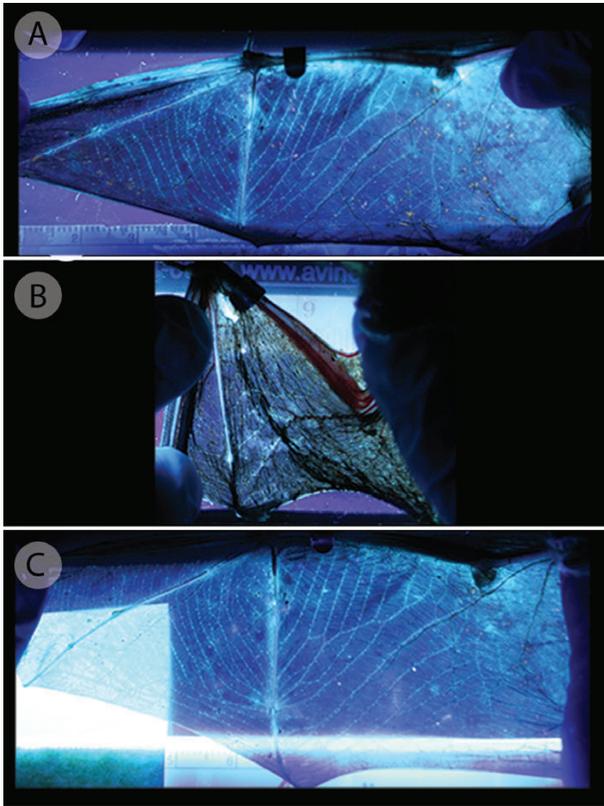


Fig. 2.—A) An example of a bat severely infected with *Pseudogymnoascus destructans*. B) The severe inflammatory reaction that damages the wings. C) If the bat survives, the wing heals, documenting that the collagen-elastin bundles heal unchanged.



Fig. 3.—Bat 10495 lost more than 50% of his plagiopatagium. This loss interfered with the ability to individually identify him based upon wing print patterns.

as the increased exposure time required for UV light images may lead to an increased risk of low quality (blurry) images. These blurry images were difficult and sometimes impossible to use to identify a bat. Additionally, poor handling techniques resulted in the wing print being blocked by a handler's fingers or due to the wing not being fully outstretched. Proper technique can preclude poor-quality photographs if the handler is properly instructed to restrain the bat's wing membrane by gently holding the distal phalanges and ensuring that the wing print

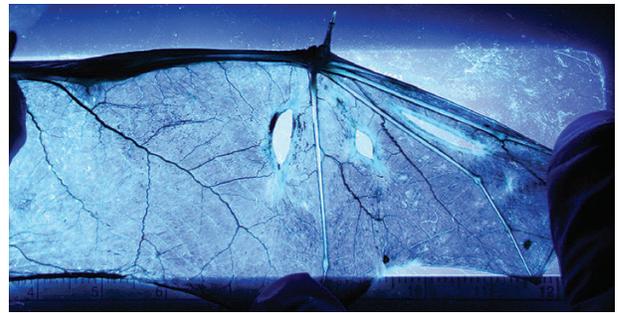


Fig. 4.—Severe venous dilation due to injuries in the plagiopatagium and dactylopatagium.

is as fully visible as possible while also ensuring the wing is completely extended.

The photographs in this study were labelled and reviewed manually. However, this approach would be extremely time-consuming with larger data sets comprised of thousands of individuals. Further research should be conducted to determine whether pattern recognition algorithms can be generated to locate, identify, and classify the wing bundle patterns to allow an automated identification system. There are multiple examples of successful automated animal biometric systems for other taxa including zebras (Lahiri et al. 2011) and whale sharks (*Rhincodon typus*—Graham and Roberts 2007), where the user interface allows a photograph to be uploaded and an animal identified. It is important to recognize that when an individual animal is identified by means of these animal biometric systems, the results are reported as the most probable match or the percent probability that the match is correct (Kühl and Burghardt 2013). A human reviewer must still determine if the match is correct, and not simply accept the most probable match as correct, as identification error is one of the common problems associated with automated species identification systems (Gaston and O'Neill 2004).

If biometric identification could be widely applied using an automated system to identify individual bats, this would be the 1st easily employable identification system for bats that does not require physical alteration of the animal that could negatively affect it. Currently, all other bat-marking techniques alter the physical appearance of the bats and result in increased risk of injury and behavioral disruption. The most commonly used method for marking bats is forearm banding, known to cause minor injuries such as abrasions or swelling as well as more severe injuries to the skeleton and patagium (Baker et al. 2001) that could lead to death (Pierson and Fellers 1993). Additionally, bands may cause damage to the dentition (Baker et al. 2001) of bats that chew on them. Chewing on the band can cause the bands to become unreadable (Bonaccorso and Smythe 1972; Humphrey and Kunz 1976; Baker et al. 2001). Necklaces have the risk of becoming snagged on foliage or rocky projections (O'Shea and Bogan 2003). Dyes and microtaggants could be potentially toxic (O'Shea and Bogan 2003). Wing punches and freeze branding are short-term marking solutions that have the potential to result in long-term negative effects to the bat's health (Sherwin et al. 2002; O'Shea and Bogan 2003).

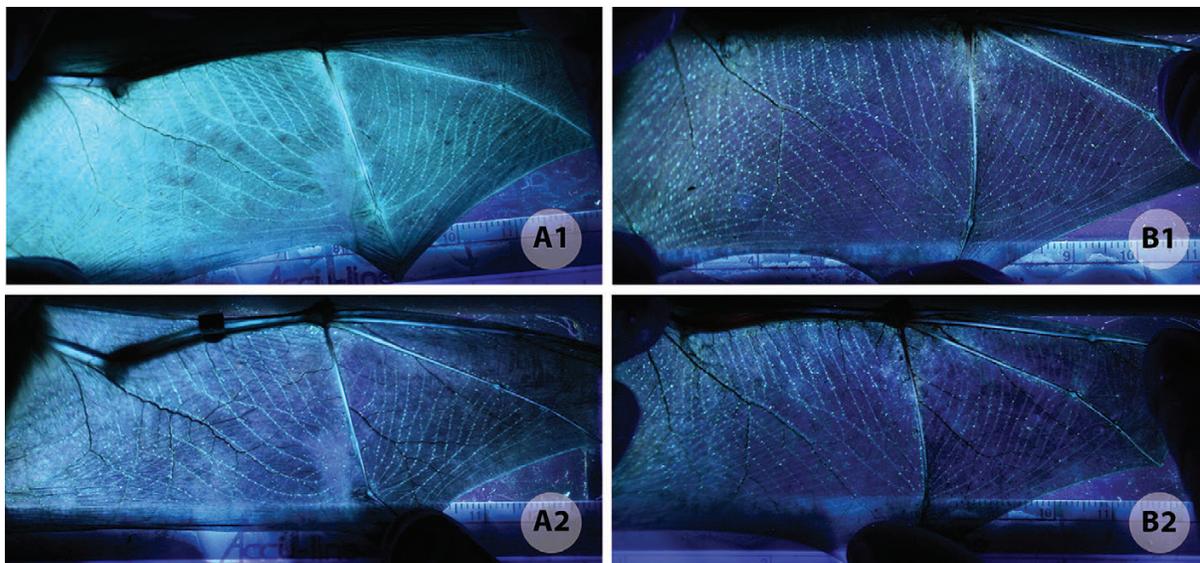


Fig. 5.—Week 0 images (A1 and B1). Week 6 images (A2 and B2) that show venous dilation of blood vessels within the plagiopatagium and dactylopatagium without any signs of injury.

Radiotransmitters are not only short-lived, but force the bat to carry additional weight that could alter its behavior (Lacki et al. 2007; Barron et al. 2010). The long-term effects of PIT tags are unknown, and only a limited number of studies have investigated the impact of PIT tags on survival, reproductive condition, and body mass indexes in 2 bat species, *E. fuscus* and *M. daubentonii* (Neubauer et al. 2005; Rigby et al. 2012). Although PIT tags appear to be the most promising marking method, they are plagued with a fairly high failure rate, up to 16%, within a 3-year study period (Rigby et al. 2012).

Any marking techniques linked to alteration of behavior, injury, or death raise not only ethical issues, but also lead to the introduction of biases into the data (O’Shea et al. 2004). The effect of these biases can vary. However, in certain studies where outcomes such as survival are measured, the bias can be significant. For example, mark and recapture studies are most commonly used to estimate survival of bats (Thompson 2004) and the majority of those studies used banding (Mohr 1952; O’Shea et al. 2004). Banding injuries vary greatly among species, with reports of up to 41.2% of a study population suffering severe banding injuries from bat bands (Baker et al. 2001), thereby reducing survival in marked individuals and leading to biased survival estimates. Therefore, studies investigating survival only serve to estimate the survival of the banded individuals, which is not representative of the overall study population. Other biases introduced by commonly accepted marking methods include physical loss of tags (Hoyle et al. 2001; O’Shea et al. 2004), failure of electronic marking methods (Rigby et al. 2012), and marked but unidentifiable individuals such as when bats chew on the bands rendering them unreadable (Mohr 1952; Bonaccorso and Smythe 1972; Humphrey and Kunz 1976; Baker et al. 2001).

Although biometrics would help eliminate these biases resulting in more accurate survival analysis, the potential disadvantage of a wing biometric identification system is that bats cannot be identified during hibernation without disturbance.

However, this disadvantage may be falsely perceived. Banding efforts often occurred during winter hibernation resulting in increased disturbance-induced arousals that lead to significant declines of bat populations (O’Shea and Bogan 2003; Ellison 2008). We expect that if scanning for PIT tags occurred within a hibernaculum, then the same disturbance-induced arousals would occur and lead to an increase in overwinter mortality. Therefore, we recommend that any biometric identification should be conducted within or surrounding hibernacula only prior to hibernation (i.e., fall swarming activities) or immediately after (i.e., spring emergence), and otherwise employed in the field during the summer.

Yet, despite this potential disadvantage, using the patterns formed by the collagen–elastin bundles throughout the wing, researchers and biologists can eliminate the severe injuries and death that can affect their study population. With the population declines of some North American bat species caused by WNS, wind energy, human activity, and loss of habitat (O’Shea et al. 2016), the need exists to adopt a biometric identification system that does not further contribute to the decline. Our study supports the idea that wing prints can be used to identify individuals as the wing print patterns satisfy the 4 biologic measurement requirements of all biometric identification systems. They are universal, distinct, permanent, and can be collected (Jain et al. 2004) through transillumination-assisted photography.

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