Seasonal & Reproductive Effects on Wound Healing in *E. fuscus*

SEASONAL AND REPRODUCTIVE EFFECTS ON WOUND HEALING IN THE FLIGHT MEMBRANES OF CAPTIVE BIG BROWN BATS (*EPTESICUS FUSCUS*)

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TITLE: Seasonal and Reproductive Effects on Wound Healing in the Flight Membranes of Captive Big Brown Bats (*Eptesicus fuscus*)

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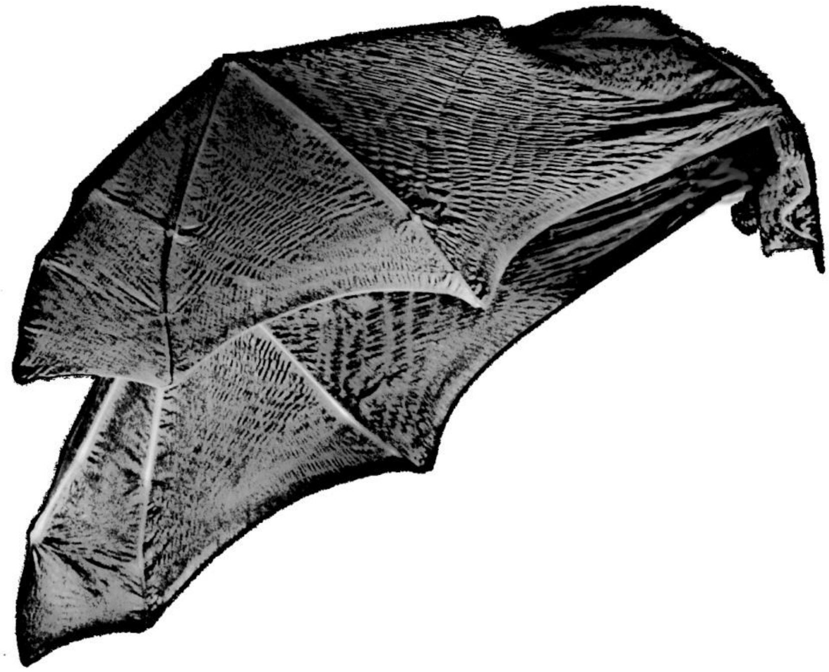
ABSTRACT

Bats (Order Chiroptera) are the only mammals capable of power flight. The flight membranes of bats are not only essential for locomotion, but also play vital roles in homeostasis. Although understanding wound healing in the flight membranes of bats is important because injuries in the wild are common, with the recent emergence of white-nose syndrome, understanding wound healing in bat flight membranes has become even more important.

In order to conduct my studies on wound healing in the flight membranes of bats, it was necessary to manually restrain bats. In this thesis I present a novel bat restrainer that I designed and that reduces stress experienced by restrained bats during experimentation and data collection.

Wound healing is an energy dependent process, as such it is expected that wound healing times will vary during periods of energy constraint (i.e. hibernation) and/or at times of peak demand (i.e. lactation). However, previous studies on wound healing have only looked at healing at times when there are no energy constraints. In thesis I aimed to better understand the effects of seasonality and reproduction on wound healing. Using an 8 mm circular punch, I inflicted biopsy wounds to the chiropatagium of healthy captive big brown bats, *Eptesicus fuscus*. I compared wound healing times between winter and summer seasons, and between reproductive (i.e. lactating) and non-reproductive females. As expected, wound healing times were longer during the winter months when bats are conserving energy. On the other hand, reproductive status did not have an effect on wound healing times. Although

most bats heal, I observed impaired wound healing. This finding is important because it is the first time that impaired wound healing is reported in healthy bats.



To my children Juan Nicolas and Diego-Felipe, you are the engines of my life and I hope one day you will find my desire to never stop learning inspirational.

Los amo inmensamente.

BIOGRAPHICAL SKETCH

Alejandra Ceballos-Vasquez was born in Bogota, Colombia. Although Bogota is a metropolitan city located in the interior of the country, she spent countless vacations, holidays and weekends at her family's cattle farm. It was there that she developed an incredible fascination for nature and animals. It was also there that she had her first encounters with bats. Seeing them fly at dusk was always fascinating, and Alejandra often asked her mom to let her keep a bat as a pet…you can guess what was always the answer. Although Alejandra's fascination with bats began at a very young age, she travelled many routes before joining the McMaster Bat Lab.

Bats were not the only animal that fascinated Alejandra, she has always been very fond of all animals and in particular horses. As a child, she would spend countless hours riding horses at the farm and as she grew older she became more and more involved with horses. At the age of 10 she joined the military equestrian school and after that, her holidays, vacations and weekends began to move from the cattle farm to the equestrian world. Her love for nature, animals and horses in particular led her to enrol in a Veterinary School in Bogota.

In 1999, Alejandra married the love of her life and soon after they welcomed into their lives their first son: Juan Nicolas. At this time, she and her husband made the decision that she would post-pone her veterinary studies to enjoy time with her beloved son. In 2001, the fantastic trio moved to London, ON, and began their journey in Canada; learning a new language, a new culture, and a new everything! In 2004 the family happily welcomed their second son: Diego-Felipe. As things settled in the new country and the family expanded, Alejandra never abandoned the idea of going back to school and to continue her education. Once both children were old enough to attend school, Alejandra went back to University and in 2011 she graduated with a Bachelor of Science degree (Honours in Conservation Biology) from the University of Western Ontario.

It was not until her final year at Western that Alejandra's fascination for bats was rekindled. As part of her electives, she chose to take a course on the "Biology of Bats". It was at this time that she met one of her major "bat" influences: Dr. Brock Fenton. Her fascination for bats and desire to help with the conservation of these fascinating creatures led Alejandra to volunteer on a bat research project. The more she learned about bats, the more she fell in love with them and the more she wanted to learn about them. In 2011, Alejandra had the opportunity to attend the 41st North American Symposium for Bat Research. Attending that conference was a life changing event for her. First, she was surprised at how wonderful all bat researchers were (at least all those she interacted with ☺), and second it was there that she realized that the bat world was a place where she would fit in…after all she had always been batty too. While listening to a talk about the devastating effects of white-nose syndrome, she became aware of the big gap in the knowledge regarding wound healing in the flight membranes of bats. She had been considering going to grad school and coincidentally (or better "bat-cidentally"), her future supervisor prospect Dr. Paul Faure – was also there who had already conducted some wound healing studies on bats in his lab. That was it! The revelation! Alejandra concluded then that it was in the bat world where she could combine all her passions: animals, conservation and animal health.

In 2012 Alejandra joined the McMaster Bat lab, a place where she would have (career wise) the best two years of her life and the place where she would work under the supervision of another major “bat” influence.

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To Dr. Brock Fenton, thank you so much for being a “bat” influence in my life (pun intended) and for introducing me to the fascinating world of bats. For all your words of support and wisdom since I was in my second year of university at Western. Thank you for believing in me and pushing me to keep going.

I do not have sufficient words to express how grateful I am with past and present Bat Lab members. Special thanks to Ryan Caldwell, his incredible hard work made possible all research projects that were completed. To Roberto Valdizon, there are not enough carrots in the world to thank you for the countless trips to pick up material needed in the lab, for helping me troubleshoot computers and cameras, editing, and especially for wanting to keep alive my legacy in the lab (he knows what I’m talking about). A big thank you to Erin Tattersal, Marley Russell and Nabil Khaja for spending their recesses helping me score videos, fly bats and various other tasks. To Imtiaz Karamat for taking on the healing-flight project, your willingness to take on this project made it possible. I will miss the bat-xercise sessions! To Lucas Greville for always being ready to help and for always showing an interest in learning more and doing more for our bats. To Riziq Sayegh for all your wisdom and support when I started in the lab, and for being willing to answer any questions I had (even after you left). To Natalie Cheng, thanks for being the guide on bat care when I joined the lab. To James Morrison for taking over the bat database and for being willing to learn about bat health care. To Brandon Aubie for your help any time the database was giving me a hard time. To all batlabbers, thank you because in one or another way your contributions were essential for the completion this thesis. The Bat Lab was not just that place where I spent countless hours working to obtain a degree, it was also a second home to me. Thanks to all of you.

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I cannot thank enough the members of the Central Animal Care Facilities. Special thanks to Dr. Kathleen Delaney for spending most of her Fridays during the summer time by my side looking after my bats. Special thanks to Dawn Graham for taking such good care of my bats, for truly caring about their wellbeing.

Thank you to the Department of Psychology, Neuroscience & Behaviour, especially to Nancy, Sally, Wendy and Milica for answering my endless questions about everything and anything.

To my bats, I will always be fascinated, curious and ready to learn more about your world.

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Authors: Alejandra Ceballos-Vasquez, John Ryan Caldwell, and Paul Andre Faure.

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The design of this device was conceived and developed by ACV. The design of the experiments was conceived by all authors. The experiment was conducted by ACV and JRC. The manuscript was written by AVC and JRC, and edited by PAF.

Chapter 3 – Seasonal and Reproductive Effects on Wound Healing in the Flight Membranes of Captive Big Brown Bats

Authors: Alejandra Ceballos-Vasquez, John Ryan Caldwell, and Paul Andre Faure.

Publication: In preparation

The design of this study was conceived primarily by ACV, under the supervision of PAF. The experiment was conducted by ACV, and JRC. The manuscript was written by AVC and JRC; and edited by PAF.

Chapter 4 – Future studies

Author: Alejandra Ceballos-Vasquez

Chapter 1

Introduction

1. INTRODUCTION

Perhaps the most striking feature of bats (Order Chiroptera) is their ability to fly. Bats are the only mammal to use flight as a form of locomotion, a trait that possibly enabled them to diversify into the second largest mammalian order (with rodents being the largest). Fossil records from the Eocene, indicate that extinct bats, such as *Icaronycteris index* and *Onychonycteris finneyi* already had wings with similar structure to modern bats and were capable of powered flight (Jepsen, 1966; Simmons et al., 2008).

Powered flight has only evolved 3 times in chordates: the pterodactyla, in birds, and bats. Although these 3 groups all have wings, their morphology differs greatly. Unlike birds, bats don not have feathers nor is the structure of the wing similar in these two animals. In bats the wing is formed mainly by the hand, hence the order's nomenclature – Chiroptera – derived from the Greek words for *chiro* which means hand, and *pteron* which means wing. Bat wings are formed by the elongation of the forearm bones (ulna and radius) and the hand bones (metacarpals and phalanges), covered by a thin layer of skin extending between the body and the digits which forms the flight membranes.

Given all that is known about wing anatomy, physiology and wound healing in the flight membranes of bats, I have hereafter divided the introduction into sections to help the reader navigate the topics necessary for a background on the work described.

1.1 Flight membrane morphology

The flight-membranes of bats, also known as patagia, are composed of skin and are typically subdivided into 3 regions: the plagiopatagium which extends between the trunk of the body and the fifth digit, the chiropatagium which extends between digits V and II, and, in many species, the uropatagium which extends between the hind limbs and the tail. The three flight membrane regions are supported by the forearm, elongated digits and the trunk.

Superficially the skin that extends between the digits to form the wing membrane appears to be similar to typical mammalian skin; however, its morphology differs from that of other mammals (Crowley et al., 1994; Gupta, 1967). In most mammals the skin is formed by three distinct layers: the epidermis, dermis, and hypodermis. In bats there is contention over whether the dermis and hypodermis are well defined in the patagia. Histological studies of the flight membranes in bats present challenges due to the nature of the tissue and its resistance to absorb chemicals needed for staining and imaging (Crowley and Hall, 1994). Nonetheless a variety of visualization techniques have been used to describe the morphology and cytology of bat patagia. Altogether, these studies agree that while there is diversity in the morphology of patagia across genera, the flight membranes are always formed by a bi-layer of epidermis (ventral and dorsal) separated by a central region of connective tissue (Crowley and Hall, 1994; Gupta, 1967; Holbrook and Odland, 1978; Murphy, 1960). Within the central region of connective tissue there are interstitial structures including collagen and elastin fibers, blood and lymphatic vessels, pilosebaceous units, apocrine glands, and sweat glands (Cortese and Nicoll, 1970; Holbrook and Odland, 1978; Murphy, 1960; Yin et al., 2011).

Another morphological feature that is consistent among studies of bat wing is that the flight membrane is significantly thinner than the skin on the rest of the body and thinner than the skin of other mammals. In fact, Swartz *et al.* (1996) suggested that the patagia are 4-10 times thinner than would be predicted by body size alone. Studies using electron microscopy have revealed that the epidermis is highly folded and is thinner on the ventral side than the dorsal side (Crowley and Hall, 1994). Additionally, a recent histological study of the wing of the Common Pipistrelle, divides the patagium into two parts: a thin area where the dermis and hypodermis are not well defined, and a scaffold area where both structures are well defined (Madej et al., 2012).

1.2 Flight membrane functions

The flight-membranes of bats are responsible for generating the necessary lift and thrust to enable forward flight (Swartz et al., 1996). Each region of the wing has a specific role: the chiropatagium aids with thrust and manoeuvrability, the plagiopatagium is the most extensible part of the wing and plays a major role in generating lift, while the uropatagium provides some lift and is used to capture prey (Swartz et al., 1996). The flight membranes also contain tactile mechanoreceptors which provide aerodynamic feedback for flight control (Chadha et al., 2011; Sterbing-D'Angelo et al., 2011). During flight, distortion to the patagia due to changes in air pressure are minimized by elastin bands that maintain skin tension (Crowley and Hall, 1994) and collagen bundles that allow the tissues to stretch (Holbrook and Odland, 1978). These specializations of the flight membrane allow bats to fly while maintaining the integrity of their wings.

The wings are not only important for providing bats with lift and powered locomotion. The patagia are important organs involved in several homeostatic functions essential to bat survival. Previous studies have demonstrated the involvement of wing membranes in thermoregulation, water balance, and possibly in cutaneous gas exchange. Kluger and Heath (1970) studied the response of wing temperatures when bats were subjected to internal heating, and they found that the flight membranes participate in dissipating heat when body temperature rises. This study provides evidence for the role of flight membranes in thermoregulation. There is evidence that the flight membranes are involved in water balance. Measurements of cutaneous evaporative water loss (EWL) have reported values as high as 99% of the total EWL (Thomas and Cloutier, 1992). Additionally, some studies suggest–their involvement in cutaneous gas exchange. Herreid *et al.* (1968) suggested that the thinness and high vascularization of the flight membranes could allow for cutaneous gas exchange; however, they were only able to demonstrate cutaneous CO_2 loss.

1.3 Bats and wound healing

1.3.1 Mammalian wound healing

Injury to tissues disrupts homeostasis. In the case of a cutaneous injury, the barrier formed by the skin becomes compromised and the tissue must be promptly repaired. Wound healing is an immunological response which is achieved in four overlapping phases: hemostasis, inflammation, proliferation and remodelling (Guo and Dipietro, 2010). Each phase is regulated by different genes, cytokines, hormones, etc. (Singer and Clark, 1999), each of which needs to be turned on and off at specific times to achieve proper tissue repair (Gurtner et al., 2008).

1.3.2 Wound healing studies in the flight membranes of bats

Wing injuries are commonly observed in wild bats. In one study on the natural history of the pallid bat (*Antrozous pallidus*), Davis (1968) categorized a number of wing injuries including holes, bone abnormalities, missing membrane parts, and foreign bodies embedded in the membrane. He also noted that bats were capable of healing holes as large as 18 mm in diameter by regenerating large portions of the missing flight membrane.

1.3.3 Relevance of flight membrane healing

An understanding of wound healing in the flight membranes has become more important than ever for two main reasons: tissue biopsy and white-nose syndrome (WNS). Tissue biopsy is an effective and non-lethal technique to collect DNA from the flight membranes of bats. It consists of extending the flight membrane on a flat surface and pressing down to excise the tissue with a sterile biopsy punch tool (Wilmer and Barratt, 1996). Tissue biopsy of bat flight membranes was originally proposed as a technique for temporarily marking individual animals (Bonaccorso and Smythe, 1972); however, with the advent of molecular technologies, membrane biopsy has become widely used as a method to obtain tissue samples for a variety of research purposes. Given the routine use of tissue biopsying from the flight membranes a better understanding of the effect that this procedure can have on bats is needed.

The recent emergence of white-nose syndrome (WNS) and the high mortality associated with infection by the psychrophilic fungus *Pseudogymnoascus destructans* has placed a renewed emphasis on understanding wound healing in bat flight membranes. Structural damage to the wings caused by fungal hyphae invading the hair follicles and sebaceous glands and the subsequent skin erosion (lesion) have been suggested as plausible causes for some of the devastating effects of *P. descructans* infection (Meteyer et al. 2009; Cryan et al. 2010). Fungal damage causes extensive membrane loss resulting in elevated cutaneous water loss and a disturbed water balance that results in severe dehydration for hibernating bats (Cryan et al. 2010; Warnecke et al. 2012). The dehydration causes bats to become more frequently aroused from hibernation resulting in premature depletion of their fat reserves critical for overwinter survival (Cryan et al., 2010; Willis et al., 2011). Furthermore, there is evidence that bats infected with WNS become hyponatremic and hypochloremic (low Na^+ and Cl^- blood concentrations, respectively), and this electrolyte imbalance plays a role in overwinter morbidity (Cryan et al., 2013; Warnecke et al., 2013). These studies highlight the importance of understanding the connection between WNS, wing tissue damage, flight membrane healing, and the combined impacts on winter survival and reproduction (e.g. Cryan et al. 2010; Dobony et al. 2011; Jonasson and Willis 2011).

1.4 Thesis overview

Wound healing would appear to be energetically costly (Archie, 2013; Im and Hoopes, 1970), and while there have been some recent studies on wing wound healing in bats (Faure et al., 2009; Weaver et al., 2009), to my knowledge wound healing has not been studied during times of energy constraints (e.g.. hibernation) or increased energy demand (e.g. gestation and/or lactation). The goal of my thesis research was to compare healing times between summer and winter seasons, and between reproductive (lactating) and non-reproductive adult females. Studying wound healing during the winter was further motivated for two reasons: 1) it is during hibernation that bats experience the most deleterious effects of WNS, and 2) to my knowledge, wound healing in bats has never been studied during the winter months. Altogether, I hope to be able to provide bat researchers with vital baseline information on wound healing rates at times of energy constraint and peak demand.

As previously mentioned, it is common practice to take wing punch biopsies of bats in the field to collect tissue samples, and while studies have concluded that bats are not adversely affected by this procedure, my work will provide new and important information on seasonal and reproductive factors that may interact with tissue biopsy healing.

Despite the high prevalence of wing injuries and the common use of flight membrane biopsies, there have been relatively few experimental studies on wound healing in bats. These studies have been summarized in Table 1.1.

Table 1.1. Summary of experimental studies on wound healing in the flight membranes of bats. Maximum healing time is reported in days.

Species	Wound size	Wound location	Max. healing	N	Source	Citation
Eidolon helvum	2 x 2 cm	Plagiopatagium	24	12	captive	(Church et al., 1968)
Antrozous pallidus	14 mm	Not specified	34	38 [a]	wild	(Davis and Doster, 1972)
	17 mm	Not specified	41	38[a]	wild	
	14 mm	Not specified	33	8	captive	
	17 mm	Not specified	43	7	captive	
Eidolon helvum	12 x 50 mm[b]	Plagiopatagium	11		captive	(Iversen et al., 1974)
Eptesicus fuscus	4 mm	Chiropatagium	52	8	captive	(Faure et al., 2009)
	8 mm	Chiropatagium	193	8	captive	
	4 mm	Uropatagium	24	8	captive	
	8 mm	Uropatagium	125	8	captive	
Myotis lucifugus	3 mm	Plagiopatagium	16	32[c]	wild	(Weaver et al., 2009)
Vesper spp.	3 mm	Plagiopatagium	46[d]	13	wild	(Pierce and Keith, 2011)

[a] Initial number of bats used, but number of bats recaptured was not specified by author.
[b] For this experiment only one of the two layers of epidermis was removed from the flight membrane.
[c] Number of bats that were recaptured, 150 bats were biopsied originally.
[d] Healing might have occurred earlier, but bats were not recaptured until this day.

In chapter 2, I describe a new device for restraining bats. The McMaster restrainer described is an effective alternative to manually restraining bats. The conception and design of this device was the result of my desire to refine my protocol in order to minimize the stress associated with handling. This chapter demonstrates the effectiveness of the McMaster restrainer and also shows that bats are less stressed during experimental procedures compared to manual restraint.

In chapter 3, I describe the effects of season and reproduction status on wound healing times in the wing membranes of big brown bats. This chapter demonstrates that low temperatures have a negative effect on wound healing times in the wing membranes of bats. I also demonstrate that adult females who are rearing young have sufficient energy in captivity to allocate to both wound healing and lactation. This is the first study to describe impaired wound healing in captive healthy bats. This finding is important in the context of WNS related bat mortalities because until now bat biologists believed that impaired wound healing occurred only in bats infected with *Pseudogymnoascus destructans*.

In chapter 4, I offer some suggestions for future studies. These suggestions come from observations made during the course of collecting data for this thesis.

Chapter 2

A Device for Restraining Bats

2. A DEVICE FOR RESTRAINING BATS

2.1 Abstract

Herein we describe a safe, practical, and inexpensive device for restraining bats (Order Chiroptera). With advances in molecular techniques, biopsies from the flight membranes of bats have become routine; however, tissue sampling requires that the bat be restrained and often this work is performed by two people: one to hold the animal and another to conduct the procedure. The McMaster bat restrainer permits a single user to safely, effectively, and comfortably restrain a bat—both in the field and in the laboratory—while still having full access to its forearms, flight membranes, and/or dorsum/ventrum. The restrainer is lightweight, portable, simple to use, easy to modify, and minimizes handling stress on bats. Investigators should take precautions to appropriately decontaminate the restrainer when working in areas where bats could be infected with the psychrophilic fungus that causes white nose syndrome (WNS), *Pseudogymnoascus destructans*.

2.2 Introduction

It is often necessary to manually restrain bats for measurements, marking (e.g. banding), to obtain tissue samples from their flight membranes, and for detailed studies of their wing anatomy and/or sensory physiology (e.g. Chadha et al., 2011; Sterbing-D'Angelo et al., 2011). While most of these procedures could be performed by a single individual, in practice two persons are often required—one to restrain the animal and another to conduct the procedure. For example, during wing membrane biopsy bats are usually held in a supine position against a flat surface, the wing is extended with one hand, and a sterile biopsy tool is pressed against the patagium with the other hand to obtain the tissue sample (Wilmer and Barratt, 1996). Depending on the size of the bat and whether the biopsy is to be collected from the wing (chiropatagium) or tail (uropatagium) membrane, this procedure can require two persons to complete (e.g. Faure et al., 2009). Wing biopsy is commonly used to obtain tissue samples and/or to temporarily mark bats. Although modest-sized holes in the flight membranes do not adversely affect flight or survival (Davis, 1968), bats may suffer from distress during restraint (Ammersdörfer et al., 2012; Heard and Huft, 1998). Previous studies of wound healing in bats have used a clear plexiglass sheet to place bats in a supine position so that the wings can be extended for tissue sampling, measurement, and photography (e.g. Church et al., 1968; Faure et al., 2009; Reichard and Kunz, 2009). In this paper, we describe a device for restraining bats that is light weight, portable, easy to use, inexpensive to construct, safe for both the animal and the researcher, and helps to reduce stress to the bat compared to manual restraint. To the best of our knowledge, this is the first restrainer that has been specifically developed for bats.

2.3 Methods

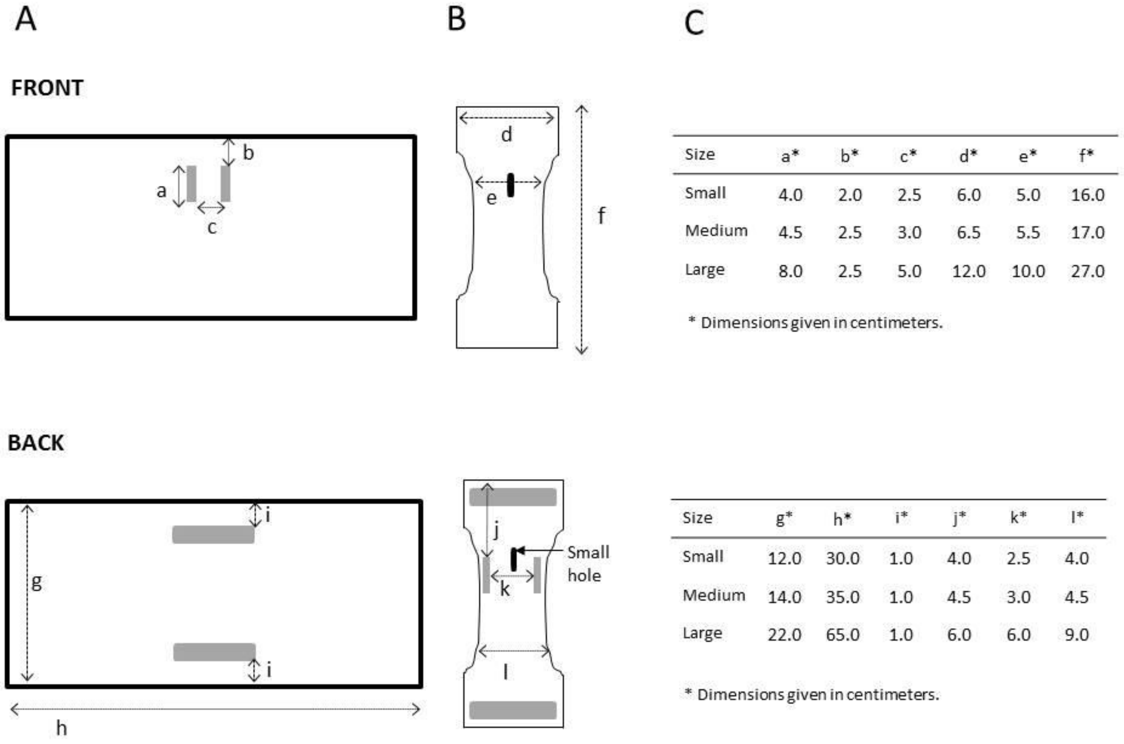
2.3.1 Ethic statement

All procedures adhere to the guidelines for the care and use of wild mammals in research approved by the American Society of Mammalogists (Sikes and Gannon, 2011) and the Canadian Council on Animal Care, and were approved by the Animal Research Ethics Board of McMaster University.

2.3.2 Restrainer design

The restrainer base was constructed from a rectangular plexiglass sheet because this allowed for transillumination and observation of the flight membranes. The bat is held against the plexiglass with double-faced fleece fabric that is stretched firmly, yet comfortably, across the animal. The fabric attaches to the back of the base with Velcro strips that have been glued to both the fabric and the plexiglass. A small hole in the fabric permits the researcher to position the bat's head during restraint. Double-faced fleece was selected for the fabric because it is strong enough to immobilize the bat, yet it is stretchable, breathable, and comfortable on the animal. Single-sided fleece fabric was also tested but this material lost its tensibility more quickly than double-faced fleece. Cyanoacrylate adhesive (instant glue) was used to secure the Velcro to both the fabric and the base because it is easy to apply and quick to dry.

All of the materials necessary to construct the restrainer are readily available and inexpensive. To build a single medium-sized restrainer, we used a 35 x 14 cm plexiglass sheet (~ $2.00 CAD per unit), a 17 x 4.5 cm piece of double-faced fleece fabric (~$2.70 CAD per unit), Velcro strips (~$0.50 CAD per unit), and instant glue (~$6.00 CAD per bottle). Suitable dimensions for the base and fabric will vary with the size, species and reproductive condition of the bat to be restrained. We have found that a small and medium-sized device works well to restrain juvenile and adult big brown bats (Eptesicus fuscus), respectively, and we recently built a large version for work with Egyptian fruit bats (Rousettus aegyptiacus). Recommended dimensions to construct the 3 aforementioned restrainer sizes are given in Figure 2.1. In most cases, it is simpler to alter the dimensions of the fleece fabric than the plexiglass base.



Size	a*	b*	c*	d*	e*	f*
Small	4.0	2.0	2.5	6.0	5.0	16.0
Medium	4.5	2.5	3.0	6.5	5.5	17.0
Large	8.0	2.5	5.0	12.0	10.0	27.0

* Dimensions given in centimeters.

Size	g*	h*	i*	j*	k*	l*
Small	12.0	30.0	1.0	4.0	2.5	4.0
Medium	14.0	35.0	1.0	4.5	3.0	4.5
Large	22.0	65.0	1.0	6.0	6.0	9.0

* Dimensions given in centimeters.

Figure 2.1. The McMaster bat restrainer. Dimensions of the front and back of the (A) plexiglass base and (B) double-sided fleece fabric that attaches to it. Shaded areas represent Velcro strips. (C) Suggested dimensions (in cm) for constructing a small, medium, and large restrainer. We have used the small restrainer on juvenile *E. fuscus*, the medium restrainer on adult *E. fuscus*, and the large restrainer on adult female *R. aegyptiacus*.

2.3.3 Procedure for Restraining Bats

To prepare the restrainer for use, attach the top of the fleece to the top of the base by a horizontal strip of Velcro (Fig. 2.1A, back; Fig. 2.2A). Arrange the plexiglass board so that the edge closest to the researcher extends off of the work surface to provide access to the Velcro strip on the reverse side (Fig. 2.1A, back). Using the operator's non-dominant hand, hold the bat in a prone or supine position. The bat's head should be held snug between the middle and index fingers, and the operator's thumb should be extended to support the animal's other side. Place the bat on the base with the long axis of its body aligned between the two parallel strips of Velcro; the bat's head should be level with the top of these strips. Grasp the fabric with the dominant hand and pull toward the operator until the bat's lower jaw (re supine position) or back of its head (re prone position) is visible through the hole in the fleece (Fig. 2.1B). Press the fabric against the midline of the bat's body with the dominant hand; applying sufficient pressure will allow the operator to withdraw their non-dominant hand without providing an opportunity for the bat to escape. Pull the fabric tightly over the bat with the free, non-dominant hand until the horizontal strip of Velcro located at the end of fleece contacts the complimentary strip on the back side of the base. During this step we have observed a tendency for bats to grasp the fabric with their feet, hence users must be careful not to pinch the bat's feet between the fleece and the edge of the base when applying tension to the fabric. Before securing the fabric to the Velcro strips flanking either side of the bat's head, the wings should be partially extended and positioned so they are below the Velcro and remain accessible. Be careful not to pinch the bat's ears between the Velcro strips while securing the head. Active bats will attempt to move in the device, so to help calm them we suggest unfastening the fleece and repositioning the bat. When appropriately positioned with the Velcro fastened, the bat will be comfortably restrained between the fleece fabric and plexiglass base with either the lower jaw (supine position) or back (prone position) visible through the fleece and its forearm and wings fully accessible (Fig. 2.2B). A video demonstrating the procedure is available at http://www.science.mcmaster.ca/pnb/faurelab/videos.

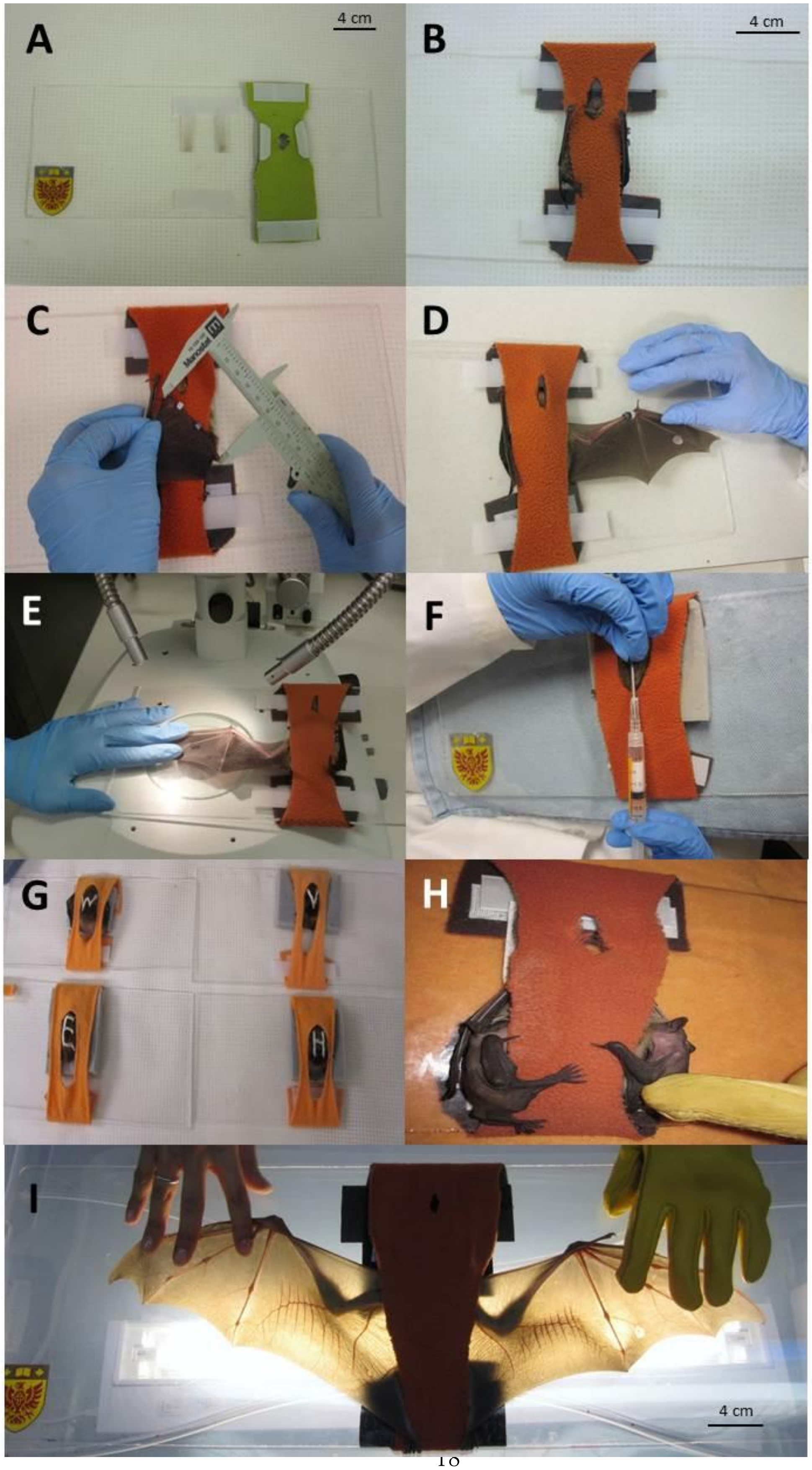

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Figure 2.2. (*Previous page*) Example uses of the McMaster bat restrainer. (A) Restrainer base with unattached double-sided fleece fabric. (B) A big brown bat (*E. fuscus*) properly positioned and successfully restrained, with it's lower jaw visible through the hole in the fabric. (C) Single user measuring forearm length with a vernier caliper. (D) Extending the left wing for tissue biopsy. (E) Photographing the wing with a camera attached to a stereomicroscope. (F) Modified design of fleece fabric for PIT-tagging bats. (G) Four bats restrained with modified fleece fabric to permit bleach marking the fur on the back. (H) A restrained adult female *E. fuscus* with two nursing pups. (I) An Egyptian fruit bat (*R. aegyptiacus*) successfully restrained with wings extended for transillumination and photography. Scale bars in (A), (B) and (I) apply only to those panels.

2.3.4 Restrainer Effectiveness

We used the restrainer once a week for 17 weeks on 17 adult female *E. fuscus* and made observations to improve the device. Success using the restrainer was quantified as follows: a trial was fully successful if the bat was restrained and remained inactive during use of the apparatus (e.g. while measuring the forearm or taking photographs of each wing; Fig. 2.2C-E); partially successful if the bat was restrained but struggled while using the apparatus; and unsuccessful if the bat escaped.

We also tested the effectiveness of the restrainer on 11 naïve users who were pre-vaccinated for rabies virus exposure and had some experience handling bats. Each user was shown once how to properly position and use the device. We then quantified the number of trials required for each participant to successfully restrain a bat.

Finally, we modified the design of the restrainer so a bat could be placed in a prone position with a piece of foam between its body and the plexiglass base, thereby providing access to the back for a routine laboratory procedure. Adult bats were randomly assigned to either the restrainer (n = 25) or manual restraint group (n = 23). Using both the unaided ear and a U30 bat detector (Ultra Sound Advice, UK), we compared the number of vocalizations emitted by bats in each group as an indicator of distress during subcutaneous insertion of a passive integrative transponder tag (PIT-tag) for individual identification (Fig. 2.2F). A Pearson Chi-square test compared the proportion of bats that emitted vocalizations in each group.

2.4 Results

During the initial 3 weeks of testing with a restrainer prototype, about three quarters of restraints were fully successful; however, once we reduced the dimensions of both the base and the fabric, nearly 100% of restraints were fully successful (Table 2.1). The decrease in successful restraints during Week 11 was likely caused by over-stretching the fleece fabric with continuous use because success rates increased after the fabric was replaced (Table 2.1). With the exception of three animals, 14 of 17 bats (82%) were inactive and appeared to be calm in the device. Once restrained, most bats stopped struggling and ceased emitting audible vocalizations, and this was true even while their wings were being manipulated. Two bats ceased vocalizing once restrained but emitted calls during wing manipulation, and one bat vocalized constantly.

Table 2.1. Success (%) in restraining 17 adult female big brown bats over a 17 week period with the medium restrainer.

Week	Fully successful	Partially successful	Unsuccessful
1*	76	0	24
2*	59	24	18
3*	59	12	29
4	94	6	0
5	100	0	0
6	100	0	0
7	100	0	0
8	100	0	0
9	100	0	0
10	100	0	0
11	82	6	12
12	94	6	0
13	94	0	6
14	100	0	0
15	100	0	0
16	100	0	0
17	100	0	0

*Denotes weeks when a restrainer without the appropriate dimensions was used.

We scored the success of using the device with eleven novel users, and all (100%) were fully successful in restraining bats with a mean ± standard deviation of 1.5 ± 0.9 attempts.

When we modified the fleece fabric for insertion of PIT-tags, only five of 25 bats emitted vocalizations during the procedure while in the restrainer compared to 13 of 23 bats that vocalized during manual restraint. The difference in the proportion of bats that vocalized while being PIT-tagged was statistically significant (Pearson Chi-square test: $X^2 = 5.298$, d.f. = 1, P = 0.02).

2.5 Discussion

Our bat restrainer is an easy to use, low-cost, and effective alternative to manual restraint. We are currently conducting a study that requires bats to be restrained once a week to photograph their wings and document flight membrane wound healing. In a previous study by Faure *et al.* (2009), this procedure required two persons to safely complete the work: one to restrain the animal and another to biopsy the bat and operate the stereomicroscope and camera. Using the device described in this paper, a single individual can effectively restrain a bat for a variety of research purposes if the flight membranes or other body areas need to be accessed or examined (e.g. Fig. 2.2). Aside from being helpful to research and increasing safety for both investigators and the bats, our device also reduces handling stress: bats in the restrainer struggled less (i.e. remained calm) and emitted fewer vocalizations during a PIT-tagging procedure compared to bats that were manually restrained. When the fleece fabric of the restrainer is stretched tightly over the body, this may mimic the feeling a bat experiences when it has wedged itself into a crack while roosting. Because the fleece also covers the eyes, this may help to calm the bat and reduce stress (Koprowski, 2002). This assertion is supported by the observation that bats commonly emit distress vocalizations when captured and held in the hand (Fenton et al., 1976).

Our restrainer can easily be modified to suit other research needs. For example, using the same base we constructed a second piece of fleece fabric that allowed us to restrain bats in a prone position for bleach marking fur on the back (Silva et al., 2007). As with the PIT-tagging procedure, a square piece of foam was placed between the plexiglass base and the bat's ventrum. We also enlarged and changed the position of the hole in the fleece fabric so that the more fur on the bat's back was exposed. With these simple modifications, bats were easily and comfortably restrained for 15-20 min so that we could bleach their fur with unique symbols or numbers (Fig. 2.2G). Again, the majority of bats remained calm and no audible vocalizations were detected during this procedure. We have also used the device to restrain lactating *E. fuscus* females without disturbing their nursing pups (Fig. 2.2H). An additional benefit of using flexible and thin fleece fabric is that it allows for visual monitoring of the bat's breathing movements.

We recently constructed a larger restrainer for a wound healing study on the Egyptian fruit bat, *Rousettus aegyptiacus* (Yinpterochiroptera, Family Pteropodidae), and the dimensions for this larger version are listed in Figure 1. With these changes and the use of a light box for transillumination, *R. aegyptiacus* were easily and comfortably restrained and their wings could be manipulated and photographed (Fig. 2.2I).

The McMaster bat restrainer could be used to assist wildlife investigators, conservation managers, and natural resource personnel in performing standard procedures on bat such as banding, weighing, measuring forearm length, and collecting fur or flight-membrane tissue samples. These procedures are essential for studies estimating the size and genetic diversity of bat populations, determining their habitat fidelity, and for examining the health of individuals.

Our restrainer allows researchers to conduct field and laboratory studies on the flight membranes of bats in a way that reduces animal distress and improves handling efficiency. Bat flight membranes serve a number of physiological functions essential to survival and reproduction. Monitoring the flight membranes during the winter has recently taken on new importance for bat conservation (e.g. Jonasson and Willis, 2011) because it is during hibernation when bats experience the most deleterious effects of infection with the psychrophilic fungus *Pseudogymnoascus destructans*—the causative agent of white nose syndrome (WNS) that has killed millions of bats in North America within the past decade (Cryan et al., 2010). The use of light boxes to visualize bat flight membranes to assess wing damage and monitor for the presence of the WNS fungus has been previously described (Reichard and Kunz, 2009). Our restrainer would permit investigators to more easily evaluate bat flight membranes with transillumination. Although the restrainer can safely be used when working with captive bat colonies, tropical bats, and in areas not afflicted with *P. destructans*, to reduce the risk of fungal transmission to other bats and/or habitats, we recommend that field researchers decontaminate the device when working in areas with WNS. Because the restrainer is inexpensive to construct, we recommend users pre-fabricate many pieces of double-faced fleece fabric (Fig. 2.1B) so that a clean unit can be used with each bat. According to the National WNS Decontamination Protocol published by the U.S. Fish and Wildlife Service (2012), submersing porous clothing or fabric in a 1:10 dilution of bleach:water for 10 minutes is an effective decontamination method. We submersed a medium-sized restrainer in this solution for 30 min and found that the integrity of the device, including the Velcro glued to fabric, was maintained. The plexiglass base can be decontaminated by wiping/spraying with bleach or alcohol. One anonymous reviewer suggested using small magnets (instead of Velcro) to secure the fleece fabric to the plexiglass base because magnets are easy to decontaminate with bleach. We have not tried this. For up-to-date information on WNS decontamination protocols, please visit http://whitenosesyndrome.org/.

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Chapter 3

Seasonal and Reproductive Effects on Wound Healing in the Flight Membranes of Captive Big Brown Bats

3. SEASONAL AND REPRODUCTIVE EFFECTS ON WOUND HEALING IN THE FLIGHT MEMBRANES OF CAPTIVE BIG BROWN BATS

3.1 Abstract

Research on the flight membranes of bats has revealed several physiological functions essential to their survival and conservation. Injuries to the flight membranes are commonly observed in bats captured from the wild and in most cases the damage heals completely. Previous studies on wound healing in bat flight membranes have not taken into consideration energy constraints that could influence healing times. Because wound healing necessitates increased energy demands, we hypothesized that wound healing times would be slower during periods of energy conservation and/or peak energy demand. In this study we used an 8 mm diameter circular tool to biopsy the wing membranes of healthy adult female big brown bats (*Eptesicus fuscus)* from a captive research colony to test the hypothesis that healing times will vary with seasonal temperature changes between the summer and winter seasons, and with reproductive condition between lactating and non-reproductive adult females. As expected, membrane biopsies took significantly longer to heal during the winter compared to the summer months. Surprisingly, there was no difference in flight membrane healing times between lactating and non-reproductive females. Although the wings of most bats in our study fully healed, some bats showed wound expansion demonstrating that impaired healing is occasionally observed in otherwise healthy subjects.

3.2 Introduction

Bats (Order Chiroptera) are the only mammals capable of powered flight. In addition to providing lift and powered locomotion, the flight membranes (patagia) of bats play a vital role in thermoregulation and homeostasis. Patagia are composed of skin extending between the trunk of the body and the fifth digit (plagiopatagium), between digits V and II (chiropatagium), and in many species there is a patagium that extends between the hind limbs (uropatagium). The patagia comprise ca. 85% of the total body surface area of a bat (Makanya and Mortola, 2007). Although the flight membranes of bats are superficially similar to other types of mammalian skin, the morphology and thickness differs from that of other mammals and even from the skin of other body parts in bats (Crowley and Hall, 1994; Gupta, 1967).

A variety of imaging techniques has been used to study the morphology and cytology of bat patagia (e.g., fluorescence, light, transmission and electron microscopy). These studies agree that the membrane is composed of a dorsal and ventral layer of epidermis separated by a central region of connective tissue consisting of collagen and elastin bundles (Gupta, 1967; Holbrook and Odland, 1978; Murphy, 1960); however, there is contention over whether the patagium contains dermal and hypodermal layers. A recent histological study of the wing of the common pipistrelle (*Pipstrellus pipistrellus*) divides the patagium into two components: a thin area where a dermis and hypodermis are not well defined, and a scaffold area where both layers are well defined (Madej et al., 2012). Although there is variation in the composition of the wing, a consistent feature is that it is significantly thinner than the skin covering the rest of the body (Gupta, 1967; Madej et al., 2012) and this makes the patagia prone to injuries. Like other skin, patagia act as a barrier and are involved in several physiological functions essential to survival. For example, the wing membranes have some involvement in cutaneous gas exchange (Makanya and Mortola, 2007), thermoregulation (Kluger and Heath, 1970), and water balance (Cryan et al., 2010; Willis et al. 2011). Given their importance in locomotion and homeostasis, bat patagia have likely been under strong natural selection to heal quickly.

Wing injuries are commonly observed in wild bats. Davis (1968) conducted a study of the natural history of the pallid bat (*Antrozous pallidus*) and categorized a number of wing injuries including holes, bone abnormalities, missing sections, and foreign bodies embedded in the membrane. Despite the prevalence of flight membranes injuries in bats, there have been relatively few studies on the process of wound healing. To our knowledge, the first experimental study of wound healing in bat flight membranes was conducted by Church and Warren (1968) who inflicted full thickness wounds in the plagiopatagium of straw-coloured fruit bats (*Eidolon helvum*), and found that they fully healed in 24 days. Using the same species, Iversen et al. (1974) removed thin layers of the ventral epidermis of the plagiopatagium and reported that the wounds were completely healed in 11 days. Davis and Doster (1972) studied wound healing in captive and free-ranging *A. pallidus* and found that 14 mm holes completely healed in 34 days whereas 17 mm holes required up to 43 days to heal. Although naturally occurring injuries are frequent and presumably this requires bats re-allocate sufficient energy resources to regenerate injured wing tissue, nothing is known about flight membrane healing during times of energy constraint. For example, we hypothesize that wound healing times could be expected to vary across seasons and during times of increased energy expenditure such as pregnancy and lactation (Barclay, 1989; 1991) Because biologists routinely punch bat flight membranes to collect tissue for molecular analyses, genetic studies and/or to mark animals in the field, a better understanding of wound healing is warranted.

Despite the importance of the patagia for survival, experimental studies of wound healing in bats ceased until 2009 when two studies were published. Faure et al. (2009) compared wound healing times in the wing *versus* the tail membranes of 32 captive *E. fuscus* using 4-mm and 8-mm diameter biopsy tools, and demonstrated that 4-mm holes healed faster than the 8-mm holes and that the uropatagium healed faster than the chiropatagium. Faure et al. (2009) attributed the faster healing of the uropatagium to its increased tissue thickness and vasculature. Weaver et al. (2009) examined wound healing in free-ranging lactating little brown bats (*Myotis lucifugus*). Of 150 females that received wing biopsies, only 32 were recaptured and by day 16 all females had completely healed. To our knowledge, there has been no other experimental studies of wound healing in bat flight membranes.

Wound healing is an immunological response to an injury that disrupts normal tissue homeostasis. In mammalian skin, wound healing is a complex process that involves highly organized sets of events regulated by different genes, cytokines and hormones (Singer and Clark, 1999) that are turned on and off at specific stages of healing (Gurtner et al., 2008). Owing to this complexity, wound healing requires a large energy investment (Ennis et al., 2007; Im and Hoopes, 1970). The healing process was originally divided into 3 phases (Kanzler et al., 1986), but has been more recently described as an overlapping process of 4 phases—hemostasis, inflammation, cell proliferation, and remodelling (Guo and Dipietro, 2010)—subdivided according to cellular and physiological events. Hemostasis occurs immediately after tissue injury and consists of vasoconstriction and clotting. In the second phase, an inflammatory response is mounted and neutrophils, macrophages and lymphocytes are recruited to the injury site. During cell proliferation, which overlaps with the inflammatory phase, there is extensive re-epithalization, angiogenesis and collagen synthesis. In the last stage of wound healing, the remodeling phase which overlaps with cell proliferation and can take years to complete, blood vessels formed during angiogenesis regress and there is collagen remodelling. This study focused mainly on the inflammation and proliferative phases of wound healing. The inflammation phase is easy to observe and the proliferative phase is easy to measure as new tissue forms and closes the wound.

In this study we examined the effects of seasonal temperature (i.e., winter *versus* summer) and reproductive status (i.e., lactation *versus* non-reproductive) on wound healing times in the fight membranes of big brown bats (*Eptesicus fuscus*). Because wound healing results in increased energy demands (Im and Hoopes, 1970) it has been used as proxy for studying energy allocation and immune competence (French et al., 2007; Rees et al., 2001). We hypothesized that wound healing times would be slower during periods of energy conservation or peak energy demand. Thus we predicted that wound healing would be slower during the winter compared to the summer months, and also for bats that were lactating compared to non-reproductive females. In line with our predictions, we also measured pup growth (in the lactating condition) to evaluate whether there is a trade-off in energy allocation to healing and lactation. To test these hypotheses, we inflicted biopsy wounds in the flight membranes of captive *E. fuscus*. We were particularly interested in studying wing wound healing during the winter because this is when bats experience the most devastating effects of infection with the fungus *Pseudogymnoascus destructans*, the causative agent of white nose syndrome (WNS) that has killed millions of bats in North America. Additionally, Warnecke et al. (2013) proposes that WNS may compromise a bat's ability to regulate its water balance due to the resultant wing injuries. Therefore this 'drinking hypothesis' suggests that bats with damaged wings may be more susceptible to dehydration. Given that our bats had *ad libitum* access to water, we suspected that bats with biopsied wings would compensate for the water loss by increasing drinking behaviour. Water consumption was monitored in the winter condition to evaluate whether biopsied bats compensate for the loss of wing integrity by increasing water consumption when compared to an un-biopsied control group and the other bats living in the colony. To the best of our knowledge, wound healing in bat flight membranes has not been studied during the winter. Importantly, the results of this study will provide bat biologists with important baseline healing information.

3.3 MATERIALS AND METHODS

3.3.1 Ethics statement

All procedures adhered to the guidelines for the care and use of wild mammals in research approved by the American Society of Mammologists (Sikes and Gannon, 2011), the Canadian Council on Animal Care, and were approved by the Animal Research Ethics Board of McMaster University.

3.3.2 Animals

Seasonal and reproductive effects on wound healing were studied in adult female big brown bats (*Eptesicus fuscus*) collected from colonies in southern Ontario in August 2012 and May 2013. Upon collection, bats were housed together in a free-flight husbandry facility (2.5 x 1.5 x 2.3 m) at McMaster University where the colony temperature and lighting varied with ambient conditions (Faure et al. 2009). Throughout the study bats had *ad libitum* access to mealworms (*Tenebrio molitor*), water, and an outdoor flying area (2.5 x 3.8 x 3.1 m).

3.3.3 Biopsy procedure

Bats included in the winter and summer treatment groups were gas anesthetized during the biopsy procedure, whereas bats included in the lactation and non-reproductive treatment groups were not anesthetized to prevent any interference with lactation., Bats were weighed prior to anesthesia and then placed in an induction chamber where they breathed a 4% isofluorane:oxygen gas mixture (flow rate = 1 L / min). During flight membrane biopsy, bats were held in the McMaster restrainer permitting access to both wings (Ceballos-vasquez et al., 2014), the wing was extended so the fifth digit was parallel to the body and the other digits were fully extended, and tissue was excised by applying pressure with an 8-mm diameter Sklar Tru-Punch® sterile disposable punch tool (Wilmer and Barratt, 1996). Tissue was taken from the chiropatagium of both wings, between the 3rd and 4th digits, using the joint between the metacarpal bone and the phalanges as a landmark to standardize the biopsy location. Week 0 was defined as the week of the biopsy procedure.

3.3.4 Experimental design

The study was conducted in two parts. In Part 1 we assessed the effect of seasonality (i.e. winter *versus* summer temperatures) on wound healing times to test the hypothesis that healing is slowed during periods of energy conservation. In Part 2 we assessed the effect of reproductive status (lactating *versus* non-reproductive) on wound healing times to test the hypothesis that healing is slowed during periods of peak energy demand.

3.3.4.1 Part I. Effect of season on wound healing: winter healing (January 2013)

Bats were randomly assigned to either the experimental biopsy (n=12) or control treatment groups (n=5). Experimental bats were biopsied in the same location on the left and right wings using an 8-mm diameter circular punch tool. Control subjects were subjected to identical handling but without wing biopsy. All bats were weighed prior to photographing their wings and a body condition index (BCI=mass/forearm length) score was calculated using the forearm measurement at the time of anaesthesia (Jonasson and Willis, 2011). A BCI score was calculated weekly to determine if experimental bats undergoing wound healing experienced faster depletion of fat reserves, and to monitor to the effects of handling and disturbance during hibernation in control bats. Water drinking behaviour was recorded with an infra-red motion detection camera (8 MP Stealth Game Camera) to determine if there was a difference in drinking frequency between experimental and control bats. Initially, we placed reflective tape on the forearm bands of bats in the experimental group to identify individuals that were drinking; however, some bats removed the tape, so we later bleached marked the fur on the back of the animals to differentiate drinking frequency of experimental and control bats (Silva et al., 2007). Comparisons of water drinking behaviour frequency were drawn between the experimental and control groups, as well as other bats living in the colony. Videos of drinking behaviour were scored by two individuals who were blind to the assignment of bats within each treatment group.

3.3.4.2 Part I. Effect of season on wound healing: summer healing (July 2013)

Wing biopsies were performed on 14 adult female *E. fuscus* using the biopsy procedure described above. Non-reproductive females were specifically chosen for this study to avoid any confounding factors associated with reproduction.

3.3.4.3 Part II. Effect of reproductive status on wound healing (June 2013)

Wing biopsies were performed on 20 lactating (experimental treatment group) and 16 non-reproductive adult female *E. fuscus* (control treatment group) using the biopsy procedure described above. Bats were randomly chosen from the husbandry facility until the appropriate number of animals had been assigned to each treatment group. Lactating females were biopsied when their offspring were between 2–4 days old to ensure that pups and mothers had bonded and established a feeding routine. Wounds measurements were taken once a week starting on the day of the biopsy. We also weighed and measured the forearm length of the pups of lactating females to determine if wound healing influenced pup growth rate.

3.3.5. Wound measurements

Wound healing was monitored using a procedure similar to that of Faure et al. (2009). Biopsied bats were placed in the McMaster bar restrainer (Ceballos-vasquez et al., 2014) and photographs of their wounds were taken once a week with an DP25 CCD camera (5 MPixel resolution; Olympus, Tokyo, Japan) mounted on an Olympus SZX10 stereomicroscope. Wound areas were measured (mm^2) with ImageJ software (National Institute of Health) and an average was calculated for the left and right wings. We calculated wound areas because it was a practical and objective way to measuring healing (Wallenstein and Brem, 2004). The proportion of wound area that had healed was calculated using a modified version of the equation by Baker et al. (1997): fraction healed = ([initial wound area–current wound area]/initial wound area). Theoretically, the area of an 8-mm diameter circle (area = $\pi \cdot r^2$) is 50.27 mm^2; however, due to overstretching of the wing membrane and human measurement errors, the mean ± standard deviation initial wound area was slightly larger at 52.38 ± 2.74 mm^2 ($n = 62$). In four bats we also measured the linear distance between the 4th digit and a large blood vessel below the wound to determine the amount of tissue contraction around the biopsy area.

3.3.6. Statistical analysis

Statistical analyses were performed using SPSS statistical software package for Windows (version 21, SPSS Inc, Chicago). Unless stated otherwise, all data are reported as the mean ± standard error (s.e.m.). Data were first tested for normality (Shapiro and Wilk, 1965) and equality of variances (Bartlett *F*-test), and non-normal or heteroscedastic data were subsequently analyzed with an equivalent nonparametric test. The BCI data collected in Part 1 of the study were not normally distributed, hence a generalized estimated equation (GEE) was used to compare scores between groups. The water consumption data collected in Part 1 was analyzed using a Pearson chi square test to determine whether there were differences in water consumption between experimental and control groups. A Kaplan-Meier survival analysis was used to compare healing times between treatment groups. Healing times are reported for average wound areas of the left and right wings, and the time to complete (100%) healing is reported as the number of weeks post biopsy when both wings no longer had open wounds when viewed under a stereomicroscope. Bats that did not completely heal by week 13 were eliminated from the analysis, and in Part II of the study two bats were removed (P14 control; Blu041 lactating) from the analysis because they had not healed to 50% by week 13.

For Part II of the study the weights and forearm length (FAL) of pups in the study of reproductive effects were used to measure growth rate across the first four weeks post-biopsy of the lactating females. Growth rate was calculated as the slope of the tangent line when the weight or FAL values were plotted against time (weeks). A linear mixed model was applied using R version 2.15.1 (R Development Core Team 2012) to evaluate the influence the number of offspring sired and the growth rate of the pups on the healing.

3.4. RESULTS

3.4.1. Gross morphological observations

During the winter, we did not observe inflammation of the wound area in the first 5 weeks after biopsy and the appearance of the wing was largely unchanged (Fig. 3.1A). We saw no clotting or scabbing but noted that inflammation started to develop around the margins of the wound and the surrounding vasculature became more pronounced by week 6. Once the wound started to heal, changes to the morphology of the wound were quite similar in the winter/summer and lactating/non-reproductive treatment groups (Figs. 3.1 and 3.2), although in general wound inflammation in the winter treatment group seemed less pronounced than in the other groups. Re-epithalization was observed as healing progressed and the wound area decreased in size. Newly formed tissue was noticeably thinner and paler than the surrounding tissue that was not biopsied. As cells divided and new tissue was added there was a contracture (i.e. reduced surface area) in the tissue close to the biopsy location that was observed in all healing bats (see arrowheads in Figs. 3.1 and 3.2). The change in linear distance between the 3rd digit and a major blood vessel posterior to the wound were as follows: Blu002 (winter group) had an initial bone to vessel distance of 9.84 mm in week 4 which decreased to 9.85 mm at week 8, 6.86 mm at week 9, and 5.52 mm by week 11. Blu022 (summer group) had an initial bone to vessel distance of 10.14 mm in week 0 which decreased to 8.98 mm at week 1, 6.10 mm at week 2, and 8.04 mm at week 3. P44 (lactation group) had an initial bone to vessel distance of 9.78 mm at week 0, 8.06 mm at week 1, 4.05 mm at week 2, and 4.17 mm at week 3. P38 (non-reproductive group) had an initial bone to vessel distance of 10.47 mm at week 0, 9.10 mm at week 1, 5.53 mm at week 2 and 7.86 mm at week 3.

Throughout the healing process, the edges of the wound thickened and in some individuals became red and inflamed. In some bats we were able to observe the formation of new blood vessels in the tissue surrounding the wound (e.g. Fig. 3.1B, 3.2AB: weeks 1 and 2). Wounds that had almost completely closed often had a small scab the size of a pin-hole at the center of the wound (e.g. Fig. 3.1A, week 11). When the scab fell off often there was incomplete closure of the wound and a re-expansion of the wound area.

Once wounds were closed and the process of inflammation and cell proliferation had ceased, the remodelling phase commenced. Newly formed tissue began to pigment and slowly it becomes less distinguishable from the surrounding pre-existing tissue (e.g. Fig. 3.1 and 3.2); however, because wrinkles in the chiropatagium did not reform, the newly formed tissue remained smooth and quite distinct when compared to the pre-existing tissue (e.g. Fig. 3.1A, week 13). The "smoothness" in the newly formed tissue was observed in all bats and was evident even after one year post-biopsy.

Bats biopsied in June and July, when temperatures were warmer, reached full wound closure within 3 to 5 weeks (Fig. 3.3B, C, D). Wounds that did not fully close followed the same process of healing during the initial 3 to 5 weeks, but after this point there were no further signs of epithelial proliferation and no additional decrease to the size of the wound area by these bats. In some cases, wounds became larger after week 4 (Fig. 3.3C, D) with no visible signs of inflammation around the wound, blood vessels were less prominent, and there was no swelling around the wound edges. Although our study only reports on wound healing up to 13 weeks, we continued to monitor 14 of 15 bats that had not healed by 26 weeks post-biopsy and observed that in most (n=10) cases there was very little or no change in the wound area after week 6, and in a few cases (n=4) the wounds expanded. Twelve of these bats were re-photographed almost one year after their initial biopsy and in 3 cases there was still no change in the size of the persisting wound hole. Of the remaining 9 bats, 8 had wound areas larger than the last photo taken on week 26 and 1 showed a decrease in wound area. In all 14 bats the tissues surrounding the wound area appeared to be in the remodelling phase (e.g. compare Fig. 3. 4A, week 3 with week 26).

Although not directly related to our study, it is noteworthy to report that during late winter and early spring we observed depigmentation in the flight membranes of all bats. The degree of paleness/translucency in the wings varied greatly between individuals, with some bats showing little depigmentation while others presented wings that were nearly uniformly unpigmented.

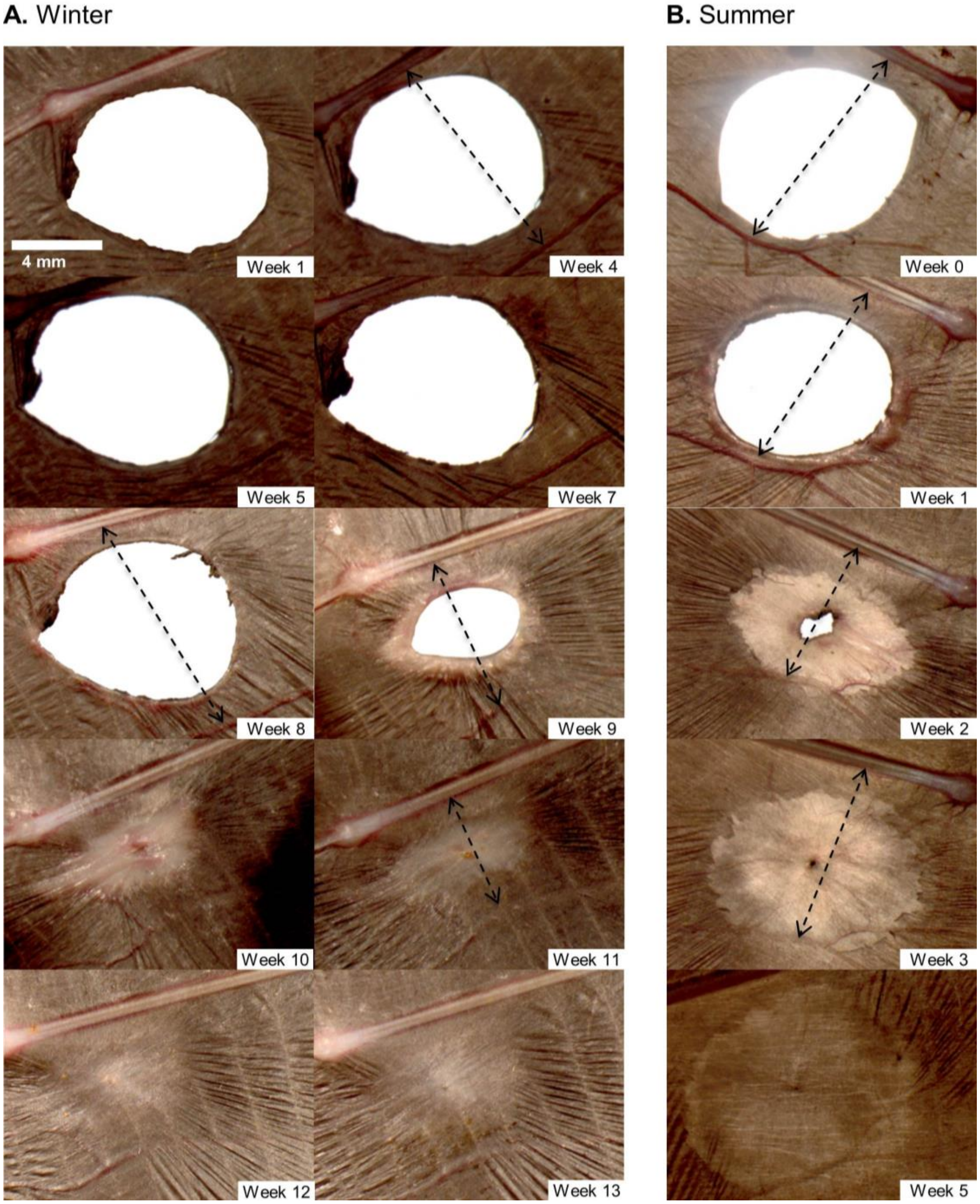

Fig. 3.1. (*Previous page*) **Photographs illustrating the progression of wing wound healing in captive adult female *E. fuscus* for the winter and summer treatment groups**. (A) Female B02 from winter treatment group. (B) Female B22 from summer treatment group. Scale bar in panel A, Week 1 applies to all images. Arrows indicate tissue contracture around the wound (measurements given in text).

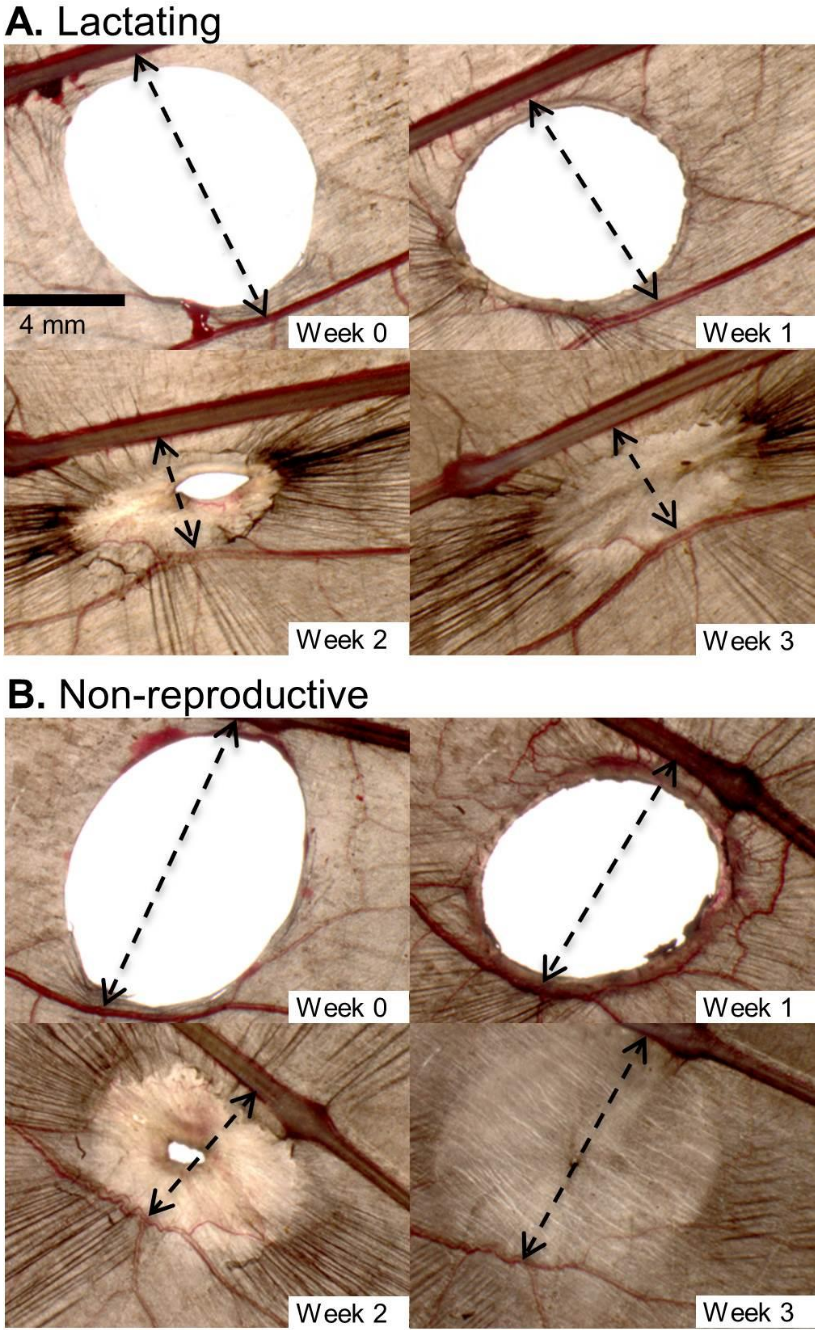

Fig. 3.2. Photographs illustrating the progression of wing wound healing in captive female *E. fuscus* for the lactating and non-reproductive treatment groups. (A) Right wing of female P44 from lactation treatment group. (B) Left wing of female P38 from non-reproductive treatment group. Scale bar in panel A, Week 0 applies to all images. Arrows indicate tissue contracture around the wound (measurements given in text).

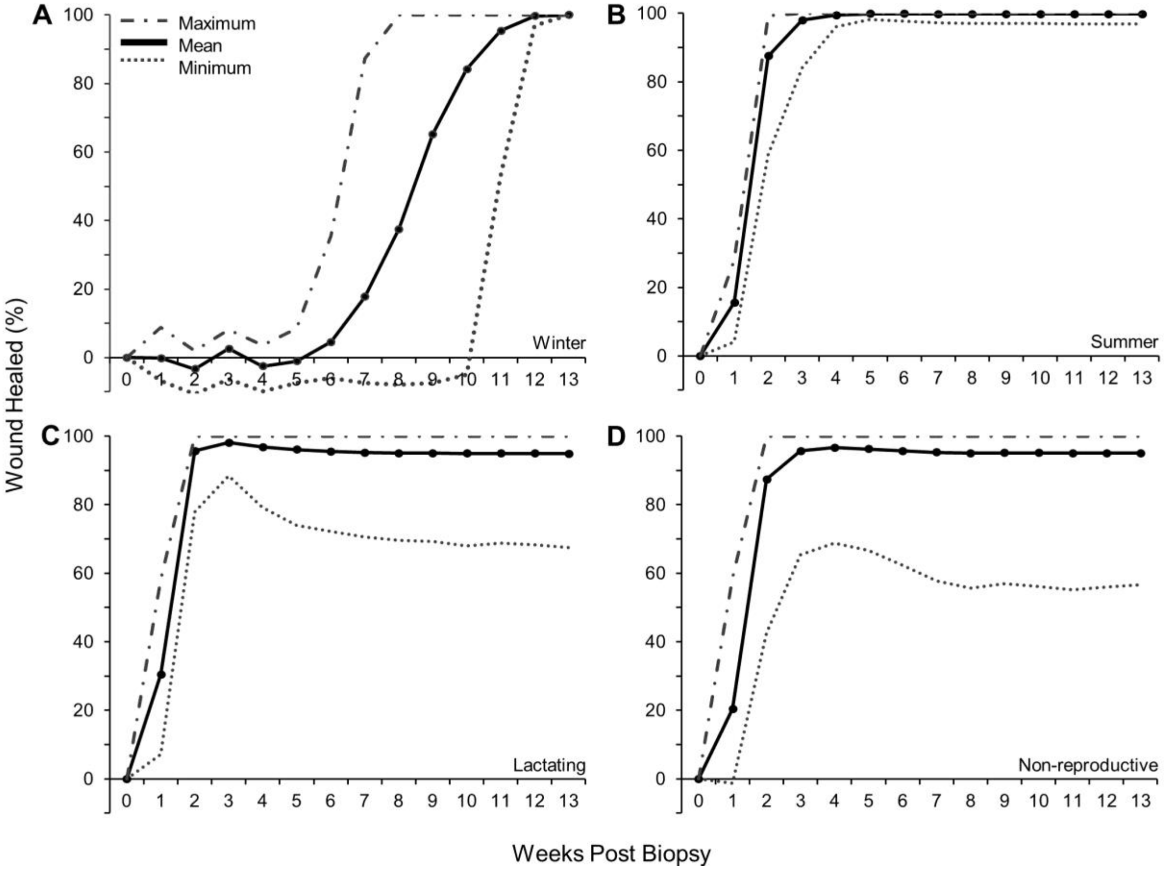

Fig. 3.3. Percent wound healing as a function of the number of weeks post-biopsy in captive big brown bats. Each panel shows the mean (solid line), maximum (dashed line), and minimum (dotted line) percent wound closure calculated as a normalized area (see Materials and Methods), with negative values representing wounds that increased in size re Week 0. (A) Winter treatment group, n=12. (B) Summer treatment group, n=14. (C) Lactating treatment group, n=20. (D) Non-lactating treatment group, n=16. Legend in A applies to all panels.

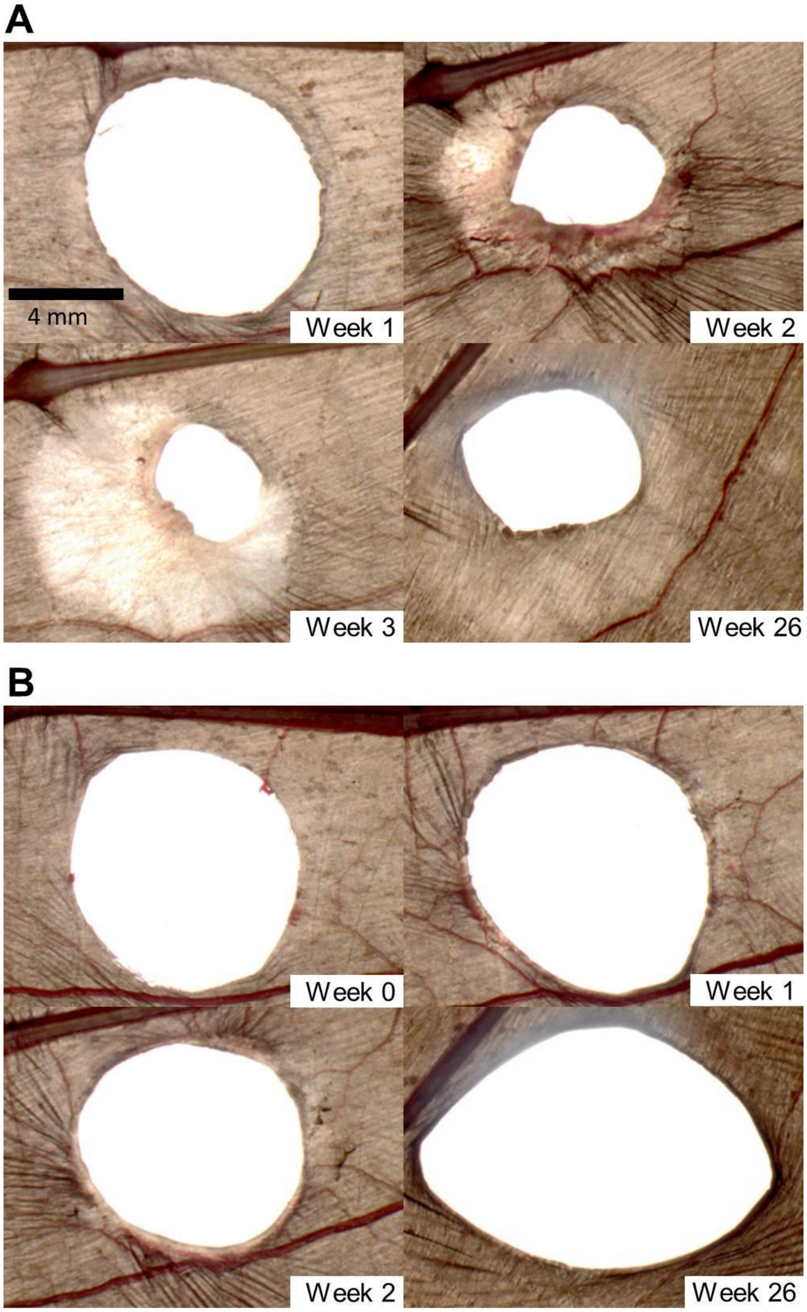

Fig. 3.4. Photographs illustrating examples of impaired wound healing with wound expansion in captive female *E. fuscus*. (A) Right wing of lactating female P08. (B) Right wing of lactating female B41. Scale bar in panel A, Week 1 applies to all images.

3.4.2. Part I. Effect of season on wound healing: winter healing

There was no difference in BCI between the winter biopsy experimental and control groups (Wald X_1^2=1.171, p>0.05). Bats in both groups experienced a decrease in body mass between week 0 (January 17, 2013) and week 5 (February 21, 2013), but then started to gain weight on or after week 6. The increase in body mass was reflected by food consumption as bats in both groups ate on average 0.9 g/day/bat between weeks 0 and 5, and this increased to 3.7 g/day/bat between weeks 6 and 13. Wound healing was not observed in the biopsy group during the initial 5 weeks, but was evident in the latter part of the study after the bats became more active and started gaining mass (Fig. 3.5). The number of observations of experimental bats in the water dish was lower than expected, while the number of observations of the control group was higher than expected. Although the frequency of water consumption between the experimental and control groups did not differ (Pearson Chi-square test: $X^2 = 0.6929$, d.f. = 2, $P > 0.05$).

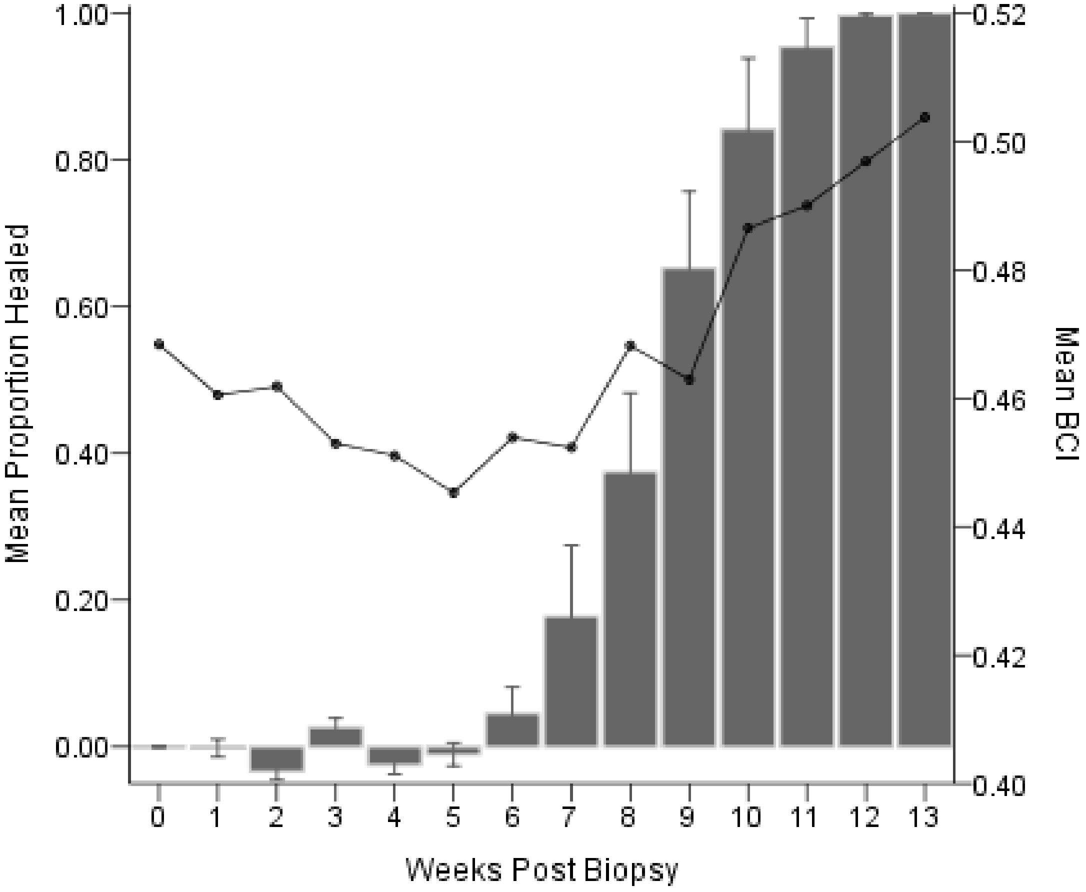

Fig. 3.5. Mean (± s.e.m.) proportion of adult female E. fuscus (n=12) and their Body Condition Index scores as a function of weeks post biopsy. Proportion healed (*solid bars*, left y-axis) was calculated and normalized as ([initial area-current area]/initial area). Experimental group was biopsied on week 0 (January 17 2013) and all bats were 100% healed by week 13 (April 18, 2013). Mean BCI (*solid line*; right axis) for the same group of bats.

3.4.3. Part I. Effect of season on wound healing: summer healing

Unlike bats biopsied in the winter, wound healing was observed within the first week for bats that were biopsied during the summer. Complete wound closure occurred for some bats by week 3, and 13 of 14 bats (92.8%) had fully healed by week 5.

There was a significant effect of season on healing times (Fig. 3.6A) at the beginning (Mantel-Cox = 9.16, $p = 0.002$), during the middle (Generalized Wilcoxon = 15.85, $p< 0.0001$), and at the end of the study period (Tarone-Ware = 13.99, $p< 0.0001$). Throughout the 13 weeks post biopsy in both treatments, weekly average temperatures differed greatly (Fig. 3.6B). The average minimum daily temperature (AMDT) for the duration of the winter study was 14.3 ^{o}C ± 4.8 (s.d.). For the first 5 weeks before healing was observed, the AMDT was 10.8 ^{o}C ± 4.9 and 16.9^{o}C ± 2.3 during the weeks when healing was in progress (weeks 6-13). The AMDT for the duration of the summer study was 24.6 ^{o}C ± 1.8 (s.d.).

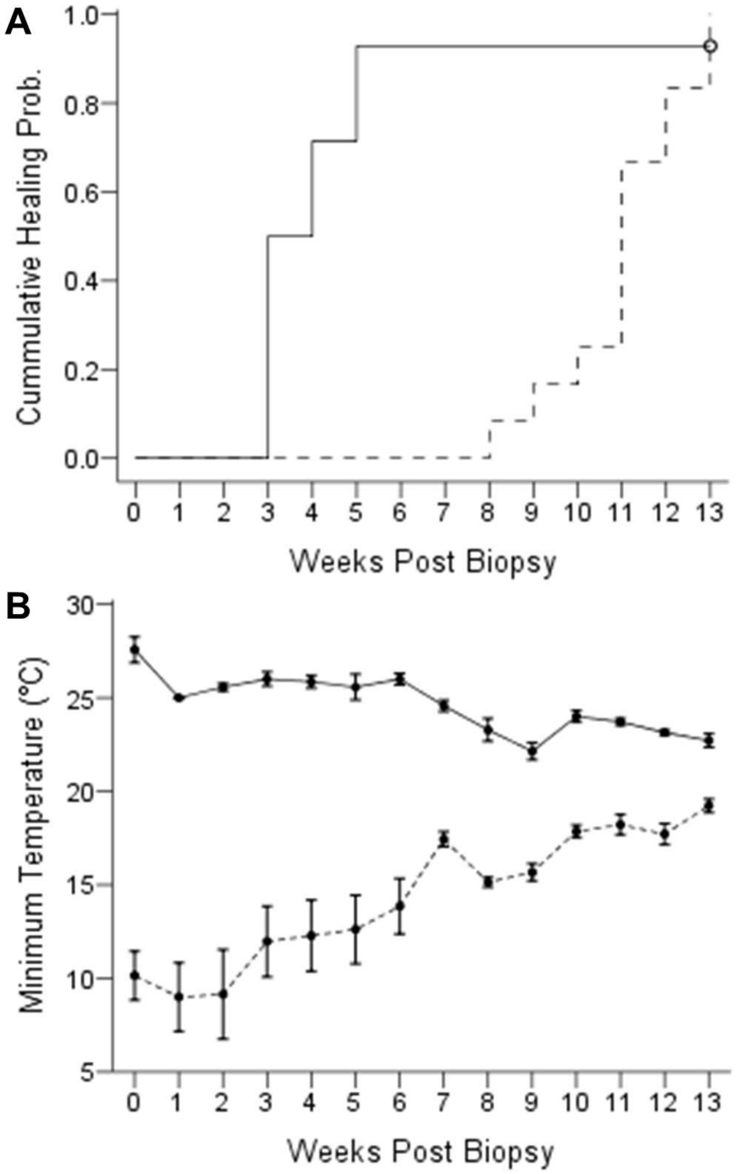

Fig. 3.6. Probability of healing and minimum colony temperature for the winter and summer treatment groups as a function of weeks post biopsy in captive adult female *E. fuscus*. (A) Time-to-event analysis for wing wound closure during the winter (n=12) and summer (n=14) seasons. There was a significant effect of season on wound healing times. (B) Mean (± s.e.m.) weekly minimum colony temperature in the bat husbandry facility. In both panels, *dotted line:* winter treatment group (17 January to 18 April 18 2013); *solid line*: summer treatment group (18 July to 10 October 2013).

3.4.4. Part II: Effect of reproductive status on wound healing

Surprisingly, there was no effect of reproductive status on wound healing times (Fig. 3.7A) at the beginning (Mantel-Cox = 0.24, p = 0.62), during the middle (Generalized Wilcoxon = 15.85, p = 0.84), or at the end of the study period (Tarone-Ware = 0.98, p = 0.75). Data on the mean weekly minimum colony temperature revealed and it was consistently warm during weeks 1 to 5 when wound healing was most active (Fig. 3.7B). Moreover, there was no influence of the mother's healing time (Linear mixed-effects model, p=0.3780) or the number of offspring she reared (Linear mixed-effects model, p=0.7904) on pup growth rate. For the pups, the average rate of mass gain was 2.58 ±0.46 (s.d.) and the average rate of FAL growth was 5.01 ± 0.76 (s.d.).

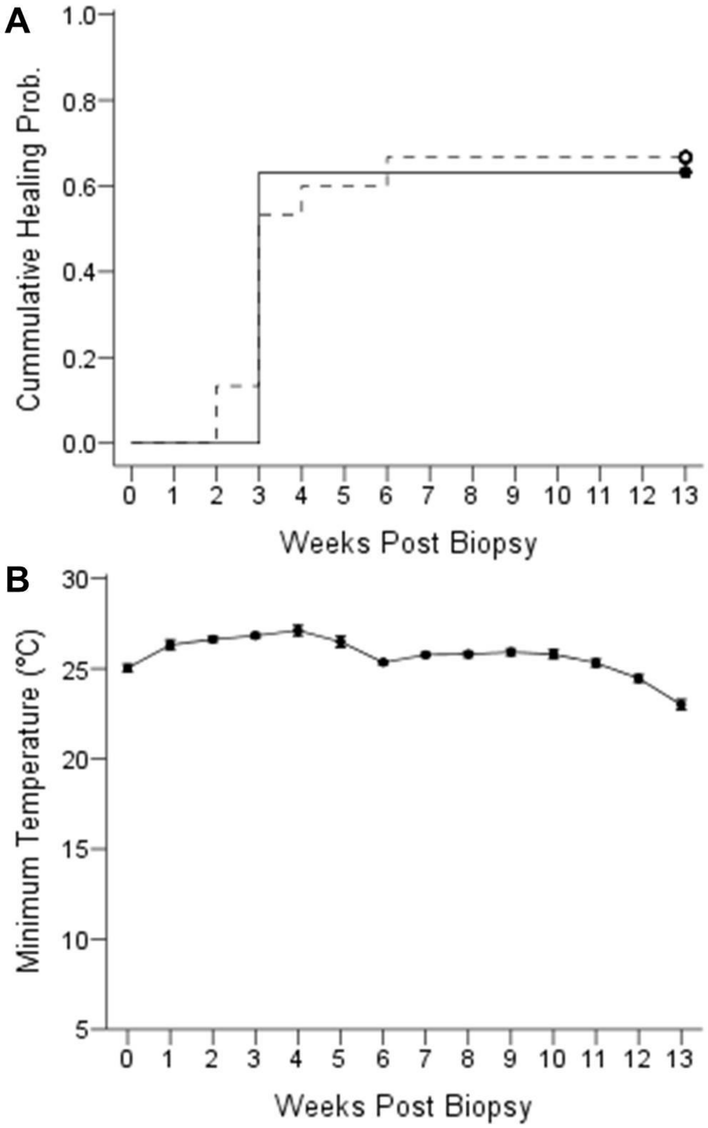

Fig. 3.7. Probability of healing and minimum colony temperature for the lactating and non-reproductive treatment groups as a function of weeks post biopsy in captive adult female *E. fuscus.* (A) Time-to-event analysis for wing wound closure for lactating (n=20) and non-reproductive (n=16) females. There was no effect of reproductive conduction on wound healing times. *Dotted line*: lactation treatment group; *solid line:* non-reproductive treatment group. (B) Mean (± s.e.m.) weekly minimum colony temperature in the bat husbandry facility. In this experiment, bats were housed from 10 June to 19 October 2013.

3.5. DISCUSSION

We hypothesized that wound healing would be slower during periods of energy conservation or peak energy demand, and thus predicted that wound healing by bats would be slower during the winter compared to the summer months. Results from the summer *versus* winter wound healing treatment groups supported our prediction with the data showing slower/delayed wound healing during winter hibernation, a time of energy constraint. We also tested the prediction that wound healing would be slower for lactating *versus* non-reproductive adult females and our results did not support this prediction. Unexpectedly, adult females nursing young did not show delayed healing and were able to successfully partition energy into wound repair and reproduction.

3.5.1 Seasonal effect on wound healing

Whereas the winter treatment group did not show wound healing until week 5, the summer group showed signs of healing within 1 week following the biopsy procedure. This finding agrees with previous suggestions that wound healing should be slower immediately before and during winter hibernation compared to the summer when temperatures are warmer (e.g. Wilmer and Barratt 1996; Weaver et al. 2009). Gross morphological observations indicate that wound healing during the winter is delayed rather than slowed. During the first 5 weeks, no inflammation was observed, the size of the wound area did not decrease, and the edges of the wound showed no signs of swelling, scabbing, or the inflammation typical of healing biopsy wounds during the summer. In a few individuals we saw signs of healing during the winter by week 3; however, by week 4 these bats had reverted to an arrested state and showed no further signals of healing thereafter. The finding of arrested and/or delayed wound healing during winter hibernation is contrary to observation of winter healing in other hibernating mammals. For example, a recent study found that American black bear (*Ursus americanus*) were able to fully heal skin biopsies during the winter and that healing progressed normally throughout all of its phases (Iaizzo et al. 2012). Differences in winter wound healing between *E. fuscus* and *U. americanus* may be accounted for by different body temperatures that are maintained during hibernation; bats allow their core temperature to fall close to 0°C while bears maintain their body temperature between 31–35°C (Nelson et al., 1983).

Wound healing is an immunological response to injury and tissue disruption, and has been proposed as an integrative measure of immune function (Archie, 2013). Immunological suppression during the winter has been described in a number of mammals (for a review, see Bouma et al. 2010).If the bats in our study were immunologically suppressed during hibernation then it may be unsurprising that their wound healing was delayed (Fig. 3.3). If wound healing can serve as an integrative proxy of immune function, then the bats in our winter treatment group with severely delayed healing may have provided indirect evidence of immunolgical suppression. Although we attribute slower healing to winter hibernation, it is important to note that delaying healing most likely resulted because of lower ambient temperatures and/or reduced food consumption by the bats. In our study the time of "arrested healing" occurred during weeks 0-5, which coincided with a period of reduced food intake and lower body mass in our bats. The process of wound healing (e.g. inflammation) occurred mainly after week 6, which coincided with a period of warmer temperatures, increased food consumption and higher daily activity. In contrast to our prediction, wound healing during winter hibernation did not result in faster depletion of fat reserves compared to control bats as there was no difference in BCI scores between the winter biopsy and control treatment groups. The role of nutrition in wound healing is still a matter of controversy. Some studies indicate caloric restriction improves wound healing, while others suggest the opposite (e.g. Albina 1994). In our study it is possible that delayed healing and its associated immune response were arrested due to lack of nutrients and/or to prevent a faster rate of depletion of fat reserves during hibernation.

A comparison of the frequency with which bats engaged in drinking behaviour revealed no differences between the biopsy and control groups. This suggests that bats with compromised wing membranes do not drink water more often than bats with intact wing membranes. Our results contradict some recent studies that suggest that bats with damaged wings would arouse more often to drink water during hibernation (e.g. Cryan et al., 2013; Warnecke et al., 2013; Willis et al., 2011). Though we cannot be sure that bats with damaged flight membranes do not drink more water volume or for longer periods.

Wound healing by bats in the summer progressed quite rapidly. Except for one female that did not completely heal, all bats in the summer treatment group reached 100% wound closure in both wings by week 5. This result is similar to the findings of Davis and Doster (1970) who reported that 14-mm wounds in the wings of *A. pallidus* had healed by 34 days. In a separate study, Faure et al. (2009) reported that 4-mm diameter holes in the wings of *E. fuscus* required, on average, 27 days to heal, whereas 8-mm diameter wounds required an average of 127 days. Although the biopsy size and location in the present study were identical to that of Part II in Faure et al. (2009), a difference in healing times may have resulted because of animal handling and seasonal influences. The bats in Faure et al. (2009) were handled and photographed every second day, whereas bats in the current study bats were measured weekly. Moreover, Part II of the Faure et al. (2009) study was conducted between September and March, and although their experimental design included maintaining the colony temperature (mean = 21.5 °C), undocumented additional seasonal factors may have influenced wound healing times. The difference in healing time between Parts I and II of Faure et al. (2009), together with our study, support the inference that there are strong temperature and possibly other seasonal effects on wound healing.

3.5.2. Reproductive effects on wound healing

Contrary to our prediction, there was no difference in healing times between lactating and non-reproductive female *E. fuscus*. On average, females in both groups were fully healed by week 3. Despite being an energy dependent process with lactating females being in a period of peak energy output, wound healing progressed rather quickly and did not differ between the lactation and non-reproductive treatment groups. This demonstrates that captive bats were capable of quickly healing their wings even when faced with the energetic challenge of nursing pups. Two previous field studies observed wound healing in lactating female bats (Weaver et al. 2009; Dobony et al. 2011), although neither reported on the fate of the offspring. While it is certainly possible that lactating females have had less energy available to allocate to milk production and the rearing of their pups, most lactating females in our study healed completely and successfully reared one or both offspring. One must be cautious in applying this result to wild populations because the bats in our experiment had access to *ad libitum* food. Thus, it is entirely possible that lactating females in our study experienced higher energy demands but compensated by increasing their food intake to accommodate both lactation and healing. McLean and Speakman (1999) suggested that brown long-eared bats (*Plecotus auritus*) compensate for the expenditure of lactation by increasing food consumption. A trade-off between reproduction and healing has been documented in tree lizards (*Urosaurus ornatus*). For example, during reproduction lizards forego wound healing when food resources are limited (French et al., 2007). Future studies controlling food intake are needed to determine whether a trade-off exists between reproduction and wound healing in insectivorous bats when resources are limited and to evaluate whether bats face additional costs detrimental to their locomotion and survival by delaying wing wound healing when resources are limited during the breeding season.

3.5.3. Flight membrane wound healing implications

The flight membranes of bats serve both locomotory and physiological roles, but the importance of the integrity of the patagia for these functions is not understood. During healing the skin around the edges of the wound contracts but it is unclear if this influences bat locomotory performance. We know bats can fly even with very large holes in their wings (e.g. Davis 1968 and personal observations), but it is unknown if flight performance is affected by the healing process. Billingham and Russell (1956) discussed limitations in movement caused by the contracture of healing skin in humans, so it seems possible that wound contractures may limit wing movements and/or cause flight impairments as bat wings are delicate organs highly innervated with somatosensory receptors (Chadha et al., 2011; Sterbing-D'Angelo et al., 2011). During proliferation of the wing epithelium, tissue contracture resulted in a reduced area between the bone of the 3rd digit and the first major blood vessel below the wound (e.g. Fig. 3.1 and 3.2). It is unknown if this contracture in the wing affected the bat's flight performance. It has been suggested that flight membrane wounds have negative effects on the physiology of the wings and their functional performance in flight, resulting in decreased foraging success (Reichard and Kunz, 2009). For example, Voigt (2013) studied flight performance in two species of wild *Myotis* with wing membrane defects and found that injured animals performed fewer U-turns, suggesting functional consequences impacting foraging and the ability to evade aerial predators. Bats sustaining severe wing damage following infection with the WNS fungus have lower BCI scores, but it is unclear if the compromised integrity of their flight membranes alters their foraging ability (Fuller et al., 2011).

It is noteworthy to highlight that newly formed wing tissue is smooth and does not regain the wrinkled appearance of older tissue surrounding the wound site. This wrinkling is caused by microscopic collagen and elastin bundles located within the flight membrane (Holbrook and Odland, 1978). That newly healed tissue does not regain the same gross morphology as uninjured tissue demonstrates that injuries to bats flight membranes do not result in full tissue regeneration, as has been previously suggested (Bekkum, 2004; Goss, 1987; Maginnis, 2006). At present, the functional consequences of having smooth *versus* wrinkled wing membranes are unknown.

3.5.4. Impaired wound healing

While most of wounds we inflicted in our study healed completely, some wounds did not fully close and/or did not heal at all (e.g. Fig. 3.4). In Part I of our study (summer treatment group) one adult female had a wound that was 97% closed before healing arrested. In Part II of our study, impaired healing was observed in 8 of 20 (40%) experimental and 6 of 16 (37%) control bats. We closely monitored healing for up to 13 weeks, although we continued to monitor bats that did not fully heal for up to 26 weeks and for these individuals we found that their wounds were largely unchanged. We have observed impaired wound healing while conducting other (e.g. neurophysiological) studies on bats but have been unable to attribute a cause for the impairment. Impaired wound healing was also noted by Dobony et al. (2011) who reported that not all recaptured bats had wing wounds that healed and attributed this to infection with the WNS fungus. Their observations were on lactating females, hence Dobony et al. (2011) argued that the bats with impaired healing were unable to allocate sufficient energy to both reproduction and tissue repair; however, this cannot explain the impaired healing observed in the non-reproductive female *E. fuscus* in our captive study. Wing damage has also been noted in bats prior to the emergence of WNS (Powers et al., 2013). Because impaired healing has been observed in both healthy lactating and non-reproductive females, we caution researchers to avoid concluding that impaired healing in wild bats is a result of a previous WNS infection. Interestingly, all bats in Part II of our study (winter treatment group) healed completely. As wound healing is a dynamic process involving many overlapping steps, precision and timing of the different events is also likely to be important for successful healing (Guo and Dipietro, 2010). For example, perfect healing is typically observed in the embryonic stage of development, and lower levels of circulating macrophages and less inflammation have been suggested as being responsible for this near perfect repair (Redd et al., 2004). Perhaps the lack of inflammation that was observed during the first few weeks of healing following wing biopsy can explain why all bats in the winter treatment grouped healed completely.

Our observations on bats with wounds that did not fully heal by week 6 indicates that healing continued to progress through each stage even though the wound area may have been unchanged for up to 51 weeks. Perhaps these bats progressed to the third (re-epithalization) stage of healing but then arrested before wound closure was achieved. In other bats we noticed that the wound holes were very small and could easily be missed with the naked eye. In our study this was not a problem because stereomicroscopy was used to image all wounds. Many bats healed to the point where only a pin-sized hole was present, and in a few cases this hole grew larger over time. Although such holes are unlikely to cause adverse effects, they have the potential to enlarge and compromise the integrity of the flight membrane. Fluorescence imaging on the wings of *Myotis* spp. have shown that even small injuries (e.g. minute needle stabs) activate the lymphatic system (Murphy, 1960). It seems prudent not to underestimate the physiological effects of such small holes in the flight membranes. For example, many bats have sharp claws and roost together in large colonies, and once we saw the entire plagiopatagium tear after an Egyptian fruit bat (*Rousettus aegyptiacus*) caught its thumb nail in a small hole of the wing of a conspecific.

With the emergence of WNS, there has been renewed interest in understanding the process of wing wound healing in bats. Field studies have shown that bats can heal wing injuries caused by infection with *P. destructans,* the psychrophilic fungus that causes WNS and that has killed millions of hibernating bats in temperate North America. Fuller et al. (2011) and Dobony et al. (2011) reported finding bats with more severe wing damage early in the summer, with damage scores lower later in the season presumably because the bats had healed. Although both studies suggest a positive outlook for wing damage associated with WNS, it is important to note that these conclusions were drawn from recapture data. Fuller et al. (2011) reported a 10% recapture rate but recognized that the fate of bats with wing damage that were not recaptured was unknown. It is also possible that bats with severe wing damage die because of starvation or predation (Reichard and Kunz 2009). Although some bats are capable of fully recovering from the wing damaged associated with *P. destructans* infection, the fate of bats with moderate to severe damage remains unknown (see Francl et al., 2011; Powers et al. 2013). In this study, about 40% of the *E. fuscus* we biopsied had not achieved full wound closure after one year. Additional field and lab studies are needed to assess the factors that influence how wing injuries heal and to better assess the functional consequences to individuals that do not achieve full wound closure.

3.6. Acknowledgments

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3.7. Competing interests

The authors declare no competing financial interests.

3.8. Author contributions

All authors participated in the conception and design of the study. A. C. V. and J. R. C. collected all data, J. R. C. completed the image analysis, A. C. V. and J. R. C. completed the statistical analysis, and all authors contributed to the writing of the manuscript.

3.9. Funding

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3.10. List of abbreviations

AMDT = average minimum daily temperature

BCI = Body Condition Index

FAL = Forearm length

GEE = General estimated equation

S.D. = Standard deviation

S.E.M. = Standard error of the mean

WNS = White nose-syndrome

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Chapter 4

Future directions

4. FUTURE RESEARCH

In this thesis I documented that bats have an incredible ability to heal injuries and regenerate missing tissue in the flight membranes. I was also able to demonstrate that not all bats heal (see Chapter 3). This latter result is important because, to my knowledge, there has only been one other report on impaired wound healing in bats not related to WNS (see Powers et al., 2013). Most observations on wound healing in the field are conducted using mark recapture techniques; therefore, it is important to consider the possibility that bats that there is a recapture bias towards bats that heal their wounds. A recent study of wound healing associated with WNS presented data with a recapture rate of ~10% (Fuller et al., 2011), leaving a large proportion of the original sample as unknown data. Future studies should further investigate impaired wound healing in bats. Molecular, histological and physiological studies comparing bats that are able to fully heal their wings *versus* bats that show impaired wound healing could help elucidate the causes behind failures to heal. This information could be very useful for bat researchers, but also for translational studies.

Another possible project that stems from my research is studying how limited resources can impact wound healing in the flight membranes of bats. For example, in humans, poor nutrition has been proposed as a systemic factor having a negative effect on wound healing (Guo and Dipietro, 2010), and in tree lizard (*Urosaurus ornatus*) it has been reported that during times of increased energy demands (e.g. reproduction) wound healing is negatively affected if resources are limited (French et al., 2007). The studies presented in Chapter 3 were conducted while bats had access to *ad libitum* food, therefore it is possible that bats may have compensated for the increased energy demand necessary for wound healing during lactation by increasing food consumption. Studying wound healing under conditions of limited food intake could help to resolve this question and would have important for conservation implications.

Another avenue for future studies is understanding if there are differences in healing times between different regions of the wing flight membrane. The uro- and plagiopatagium depend on the process of morphogenesis to grow and extend from the distal part of the trunk to the 5th digit, and between the tail (Giannini et al., 2006; Weatherbee et al., 2006). On the other hand, the chiropatagium depends on the retention of early developmental characteristics in the interdigital tissue. Apoptosis and regression does not occur giving rise to the wing tissue that forms the chiropatagium (Adams, 2008; Weatherbee et al., 2006). To my knowledge, it is not known if such differences during patagia development impact wound healing in different regions of the wing membrane. For example Faure *et al.* (2009) determined that wound healing in the uropatagium was faster than in the chiropatagium and attributed this difference to a higher vascularization of the tail membrane; however, the difference could also be the result of differences in patagia development. To help elucidate if vascularization if the best explanation for the difference in healing time reported by Faure *et al.* (2009), future research on wound healing should look at the difference between high vascularization *vs.* low vascularization in the same wing region. Uncovering possible differences in wound healing across the flight membrane regions and/or high *vs.* low vascularization could provide bat researchers with improved techniques for wing biopsies in the field.

Another area of future work that could prove fruitful is a comparative study between males and females. Differential wound healing between males and females in other mammalian species has been linked to different levels of circulating sex hormone levels

(Gilliver et al., 2008). Furthermore, it has been discussed that males and females expend energy differently throughout the year (Jonasson and Willis, 2011). Such a study would help to further characterize physiological differences between males and females in bats. Furthermore, it could provide bat researchers with information on whether or not it is preferable to biopsy one sex over the other.

Finally, although I have mentioned some future directions for research, these suggestions represent only a fraction of the work that could be continued in this area.It is important to remember that, although very important ecologically, field studies result in low recapture rates and thus missing data points. This highlights an important difference that research with bats in captivity does not suffer from. Therefore, additional research on wound healing in captive bats should be conducted. Because of the importance that flight membranes have on the survival of the second largest group of mammals, further research in wound healing is necessary.

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