The Cause and Effect of Bat Wing Tears in Common Pipistrelle Bats *(Pipistrellus pipistrellus)*

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The Cause and Effect of Bat Wing Tears in Common Pipistrelle Bats *(Pipistrellus pipistrellus)*

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Abstract

Bats represent a quarter of all mammalian species and play vital roles in many ecosystems. They are also are the only mammals capable of powered flight and have large, light, thin wings to enable flight. However, bats face many threats, including collisions with man-made structures, fungal infections and predator attacks, all of which can cause severe wing injuries. Hundreds of bats are admitted annually for care to treat torn and injured wings. This thesis aims to investigate the causes and effects of bat wing tears. In a series of studies, this thesis will: i) characterise wing tears in *P. pipistrellus* and other bat species in the UK; ii) explore the anatomy of the wing in *P. pipistrellus*, and see if knowledge of the anatomy is sufficient to understand wing tear placement and healing rates; iii) present a novel method for analysing flight from high-speed video data to assess the effect of tears on flight; and iv) develop a systematic forensic method to identify the presence of cat DNA on wing tears.

Results from Chapter 2 indicate that most tears occurred in the Plagiopatagium wing section (section P), which is closest to the body. Tears in that section also might take longer to heal. Chapter 3 suggested that knowledge of wing anatomy is not sufficient to explain tear position and orientation. Indeed, while material testing did not identify section P as being significantly weaker than the chiropatagium (the distal sections of the wing), section P tended to have the most tears. The position of the tears, close to the body and towards the trailing edge, may suggest that they were caused by predator attacks, such as from a cat, rather than collisions. Consistent with this, 38% of *P. pipistrellus* individuals had confirmed wing tears caused by cats, with an additional 38% identified by rehabilitators as due to suspected cat attacks. Results from high-speed video footage collected in the fourth chapter revealed that tears on both wings significantly affected wing movements, and the body orientation tended to lean towards the healthier wing.

The fifth chapter developed techniques to identify cat DNA from swabs of wing tears, and found cat DNA on 66.67% of the swab samples. Results from this thesis reveal that cat attacks on bats may be far more common than first thought. In addition,

future work should focus on the seasonal timings of wing tear injuries to structure recommendations for cat owners and model the implications of cat attacks on bat populations. In addition, while the tear injuries do heal, post-release monitoring is important to ensure bats survive in the long-term following rehabilitation. Recommendations for bat carers and cat owners formulated in this thesis are the first steps in addressing the prevalent and significant problem of bat wing tears.

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Abbreviations

EDTA: ethylenediaminetetraacetic acid.

CI: The most distal section of the chiropatagium, which is the membrane between digits iii and iv.

CII: The second chiropatagium section, which is the membrane between digits iv and v.

CE: Capillary electrophoresis

P: The plagiopatagium - most proximal section of the wing, which is the membrane between digit v and the body.

PBS: phosphate buffered saline.

PCR: polymerase chain reaction

PFA: paraformaldehyde.

qPCR: quantitative polymerase chain reaction

STR: Short tandem repeat.

VVG: Veroeff-Van Gieson staining.

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Chapter 1 Literature Review

Chapter summary:

This chapter reviews the literature on bat conservation and ecology, as well as aspects of anatomy and behaviour. It begins by considering the role of bats in their ecosystems, their possible threats and the effects on their populations. It goes on to focus on key literature relevant to the thesis, specifically by reviewing wing anatomy, past studies on wing tears, healing and flight.

1.1 Introduction

Bats are mammals and represent 25% of mammalian diversity (Neuweiler, 2000). They belong to the order *Chiroptera*, which includes two suborders; *Yinpterochiroptera* and *Yangochiroptera* (Teeling et al., 2001). So far 1406 bat species have been described worldwide (Simmons and Cirranello, 2019). One of the most important features of bats is that they are the only mammals capable of powered flight (Neuweiler, 2000). Bats are very diverse, and show a lot of variation in their behaviour (Kunz et al., 2011). Roosting behaviours, for instance, are variable, and many bat species inhabit caves, cave-like structures, tree cavities and foliage (Patterson et al., 2003). Bats usually fly at night in order to forage for food, which involves many diverse food types (Kunz et al., 2011), including insects, nectar, fruit and flowers (Patterson et al., 2003). There are even some species that feed on seeds, frogs, fish and small mammals, and also those that can feed on blood (Kunz et al., 2011).

Unfortunately, bats in western cultures have quite negative associations and are often described as rampant disease vectors; as one of the witches' brew ingredients; and are associated with the dark side of some religious traditions (Kunz et al., 2011) and folklore, such as: bats found in women's hair, bats associated with the devil, and that an extract from bat skin can treat baldness (Tuladhar-Douglas, 2008). In contrast, traditionally, bats in China were considered to be icons of fortune: long life, health, wealth, morality and clarity of mind (Asif and Ali, 2019). Indeed, these beliefs

are still preserved in modern Chinese society, although in a marginal form (Kunz et al., 2011).

1.2 Bat Conservation and Ecology

1.2.1 The Role of Bats in the Ecosystem

Bats play an important role in ecosystem services provision (Kunz et al., 2011; Russo et al., 2018). They provide several benefits to humans such as arthropod suppression, seed dispersal and pollination (Kasso and Balakrishnan, 2013). Without bats, these benefits will be reduced and ecosystems will be severely affected (Kunz et al., 2011). Bats can be primary, secondary and tertiary consumers within an ecosystem, and help to support and maintain ecosystem stability (Kunz et al., 2011). Insectivorous bat species feed on airborne insects and other arthropods, which control insect populations. For example, there are many agricultural pest species, which irritate and can transmit particular pathogens to humans and other mammals, which bats feed on (Kunz et al., 2011; Weier et al., 2018). Additionally, some bats contribute to forest diversity by dispersing seeds across a variety of ecosystems, such as frugivorous bats (Wibbelt et al., 2010). Likewise, nectarivorous bats visit flowers and also sow pollen and fertilise plants (Muchhala and Jarrin-V, 2002). Hence, bats redistribute energy and maintain aquatic, terrestrial and cave ecosystems (Kunz et al., 2011). Consequently, these valuable mammals deserve protection and conservation for the services that they provide to the ecosystem.

1.2.2 Threats Faced by Bats

As with many animals, bats face certain issues during their lives, and many specific bat species have become extirpated or extinct (Eiting and Gunnell, 2009). Recently, evidence indicates that many anthropogenic activities have contributed to the mortality of bats (Baerwald et al., 2008; Cryan and Barclay, 2009; Kunz et al., 2011). In particular, this chapter will focus on describing general threats that bats face in the environment, including weather, light pollution, noise and habitat loss. It will also

describe other threats which could lead to wing tears in bats, such as collisions, fungal infections and predation.

Weather

Many studies on bat flight behaviour have been conducted under model conditions; for instance, in wind tunnels and controlled laboratory settings (Hedenström et al., 2007). It is unknown how bat flight is affected under some unsuitable conditions, such as rain or wind. It has been observed that bats avoid flying in the rain (Belwood and Fullard, 1984). Indeed, during heavy rainfall, insectivorous Hoary bats (*Lasiurus cinereus*) discontinued foraging, but continued foraging during light rain (Belwood and Fullard, 1984). There are three possible explanations for this: firstly, the rain drops could interfere with echolocation, which makes detecting insect prey increasingly difficult for bats. In addition, the insect prey themselves might avoid flying during rain (Corten and Veldkamp, 2001), hence there is no motivation for bats to forage. Finally, when the bat body becomes wet, this leads to an increase in the energy exerted in flight, due to additional thermoregulatory costs and decreased lift and thrust production (Voigt et al., 2011).

Several studies report the effect of wind on bat behaviour (Verboom and Spoelstra, 1999; Rydell, 1991). Verboom and Spoelstra (1999) tested the hypotheses that bats fly along tree lines based on food abundance and protection from the wind. They monitored the activity of Common pipistrelle bats (*Pipistrellus pipistrellus*) and found that wind speed did not affect bats activity on the leeward side of the tree line. However, at high wind speed the bats' activities were concentrated near the tree lines (Verboom and Spoelstra, 1999); hence, indicating that bats should avoid facing the wind during flight. Indeed, bats might use the edge of forests to avoid exposure to the wind. Another study in southern Sweden on Northern bats (*Eptesicus nilssoni*) also found a highly significant effect of wind on bat activity. Many bats were observed during the evening during periods of slow or no wind, and fewer were observed during very strong winds (Rydell, 1991). This suggests that bats avoid the wind, possibly because it increases flight cost.

Light Pollution

Anthropogenic light pollution is considered a growing global issue and has a negative effect on wildlife (Rich and Longcore, 2006), impacting animals' survival behaviours, such as in foraging, reproduction and communication (Rich and Longcore, 2006) and flight behaviour (Polak et al., 2011). These effects can lead to changes in animal movement patterns, decreasing breeding success or even increasing predation levels (Rich and Longcore, 2006). The fact that bats are generally active at night makes them a good species to study the effects of light pollution (Stone et al., 2009).

In particular, Lesser horseshoe bats (*Rhinolophus hipposideros*) have been chosen as a study species, due to the decrease in their global population numbers (Stone et al., 2009). In one study, high-pressure sodium lights, which are similar to streetlights in intensity and light spectra, were placed along a bat commuting route. It was found that there was a significant reduction in bat activity and a delay in commuting behaviour when those lights were present (Stone et al., 2009). Furthermore, the effect of light on two bat species, Kuhl's pipistrelle (*Pipistrellus kuhlii*) and Botta's serotine (*Eptesicus bottae*), was also studied in the Negev desert (Polak et al., 2011). It was found that the flight behaviour was affected by artificial light (two strong floodlights) in both species. Both species flew faster under the light condition, than they did in darkness. *P. kuhlii* also flew lower in the light condition, which was regarded as being because they were trying to keep away from the light source. By contrast, the *E. bottae* just foraged during the dark and so their duration of activities decreased significantly with the presence of the light (Polak et al., 2011).

Light pollution has been found to have a considerable negative effect on bat flight routes and activity, especially in nocturnal bats. However, sometimes light can have beneficial effects too. Indeed, light increased bat activity in Lesser noctule (*Nyctalus leisleri)* and *P. pipistrellus* (Mathews et al., 2015). Probably because insects gathered under the light (Mathews et al., 2015). However, in *P. pipistrellus*, increasing activity under light occurred more in habitats with good tree cover, which might be because the cover provided by the trees substituted for the increased predation risk as a result of light conditions (Mathews et al., 2015).

Noise

Ambient noise has an impact on bats and many other animals (Schaub et al., 2008). Many bats depend on echolocation to detect and intercept flying insects (Siemers and Schnitzler, 2000), while others use listening to identify and localise prey-produced sounds (Siemers and Swift, 2006). Thus, it is likely that environmental noise will affect the bat's ability to use acoustic information for finding prey (Schaub et al., 2008). One study was conducted by Schaub et al. (2008) to investigate the effect of anthropogenic and natural noise on bats' foraging behaviour in the Greater mouse-eared bat (*Myotis myotis).* The bats were found to forage more successfully in a 'silent' compartment and also spent more time foraging in a 'silent' compartment than a 'noisy' one, which they generally avoided (Schaub et al., 2008). The findings indicated that noise influences the bats foraging efforts and success, so bats may generally avoid noisy environments.

Loss of habitat

Bat habitat loss occurs in forests, woodlands and caves, and is caused by both natural and anthropogenic factors (Mickleburgh et al., 2002). In the case of the dry forests in Latin America and the Caribbean, harvesting processes and logging have led to a loss of bat habitat, since bats use the forests for roosting and feeding (Mickleburgh et al., 2002). Agriculture has a significant effect on bat habitats. For example, the 'slash and burn' agricultural process, which mainly destroys the vegetation cover, leads to the death of bats that use tree crevices as roosts (Schulze et al., 2000). In addition, removing the dead and decayed trees associated with woodland management practices, can also lead to a loss of bat habitat and reduced availability of bat roosts (Lewis, 1995). Furthermore, during flight, bats use tree lines, hedgerows, canals and other linear elements which play an important role in the connection between roosts and foraging places (Frey-Ehrenbold et al., 2013). Thus, any loss or destruction through agriculture has a negative effect on bat habitat.

Other sites such as caves and mines are also important for bats, since these are used as roosts, or for breeding in the summer, and for hibernation in the winter

(Mickleburgh et al., 2002). Caves can be prone to flooding during unusually heavy rain and can be extremely cold during the winter (O'Shea et al., 2016). In addition, bats in caves compete for space with cave swiftlets (Sankaran, 2001). Caves also attract speleologists and tourists and, when negatively managed, tourism can change the cave ecosystem and affect the bats that roost there (Luo et al., 2013).

Many natural events can also impact habitats, including typhoons, which destroy bat roosts and make the bats more exposed to predation and vulnerable to hunting (O'Shea et al., 2016). Any change to the roosts has an impact on bats, since loss of habitat can lead to greater challenges for survival. These are very specific examples of habitat alteration, but across the world, bats are impacted by drastic habitat fragmentation and loss (Lewis, 1995; Schulze et al, 2000; Sankaran, 2001; Mickleburgh et al., 2002; Frey-Ehrenbold et al., 2013; Luo et al., 2013).

Wind turbines

The number of wind turbines have increased in order to meet the growing demand for energy, and some specific species of bat have faced fatalities (Arnett et al., 2008; Cryan and Barclay, 2009). This issue has been identified in the forests of the Eastern of United Sated of America (USA) (Kunz et al., 2007; Arnett et al., 2008; Korner-Nievergelt et al., 2013). Bat species that tend to use the trees as a place to roost and migrate for long distances, are most affected by the wind turbines (Cryan and Barclay, 2009). This particular phenomenon began in the late 1990s, when bat corpses were found under turbines in the USA (Johnson et al., 2003). Since that time, many studies have concentrated on bat fatalities at wind energy sites in North America (Arnett et al., 2008).

Bat fatalities due to wind turbines are caused by clashing with the turbine tower or rotating blades (Arnett et al., 2008; Baerwald et al., 2008). Bat fatalities may also occur due to barotrauma, which relates to when the bat gets internal injuries due to the rapid pressure change when they come close to the edge or the tips of the turbines' moving blades (Baerwald et al., 2008). There is little evidence of bats

colliding with non-operational turbines (Cryan and Barclay, 2009). If a bat dies as a result of colliding with turbine towers, it is likely that fatalities should also exist at non-operational turbines and other tall structures, such as meteorological towers. However, fatalities are rare at these such structures (Arnett et al., 2008).

If bats die as a result of moving turbine blades, the bat bodies are likely to contain traumatic injuries related to the severity of collision (Cryan and Barclay, 2009). In agreement, bat corpses have been found with broken or amputated wings, crushed skulls, broken vertebral columns, and severe lacerations; although there have also been dead bats found at wind turbines without any external injuries (Baerwald et al., 2008). Bats do often have internal injuries too, which are consistent with rapid decompression, or barotrauma, through the thoracic and abdominal cavities. This can be related to the pressure that is produced around moving turbine blades, which causes damage to small blood vessels in the bats' lungs and bleeding into the thoracic cavity (Baerwald et al., 2008).

In order to explain why bats approach turbines, there are three main causes that have been suggested: random collisions, coincidental collisions and collisions related to the attraction of bats to the turbines (Cryan and Barclay, 2009). Random collisions, which happen just by chance, can happen to any individual near to the wind turbine, regardless of sex, age, reproductive condition or time of the year (Cryan and Barclay, 2009). Separately, coincidental collisions accrue as a result of specific behavioural aspects from the bats, which put them at risk of collision. Those behavioural aspects involve aggregation during migration and seasonal increases in flight activity related to feeding or mating (Cryan and Barclay, 2009). Moreover, attraction collisions suggest that bats are attracted to the wind turbines out of curiosity, misperception, or as potential feeding, roosting and mating opportunities. (Cryan and Barclay, 2009). Attraction collisions are thought to be the most likely cause of many collisions (Cryan and Barclay, 2009). To assess the risk of wind turbines on bats, and find the best methods to avoid or minimize bat fatalities at wind turbines, it is imperative to identify the exact cause of bat fatalities at wind turbine sites (Cryan and Barclay, 2009).

Fungal infection (Bat White-Nose syndrome).

White-Nose Syndrome (WNS) is a disease that occurs in bats as a result of a fungal infection (Reichard and Kunz, 2009; Cryan et al., 2010; Fuller et al., 2011). The first observation of bat WNS infection was in the USA in 2006 (Reichard and Kunz, 2009; Cryan et al., 2010; Fuller et al., 2011). This outbreak killed millions of bats, especially those that hibernate in caves and mines, and threatens some bat species with extirpation and extinction in the USA and Canada (Blehert et al., 2009, Lorch et al., 2016). WNS is caused by a fungus called keratin-digesting fungus (*Pseudogymnoascus destructans*) (Blehert et al., 2009; Reichard and Kunz, 2009; Cryan et al., 2010; Fuller et al., 2011, Lorch et al., 2016), which infects the bat's skin (Blehert et al., 2009; Reichard and Kunz, 2009; Cryan et al., 2010; Fuller et al., 2011). Indeed, this fungus grows rapidly at the low temperatures where the bats hibernate during the winter (Gargas et al., 2009). Furthermore, during hibernation periods, bats face challenges such as a lack of food and water, a decrease in immune function and metabolism, and a reduction in body temperature. Bats often select humid, or heavily populated, areas for hibernation to avoid losing moisture (Blehert et al., 2009; Reichard and Kunz, 2009; Cryan et al., 2010; Fuller et al., 2011). However, all these factors also allow the fungus to grow and successfully spread amongst the bats (Cryan et al., 2010).

Cryan et al. (2010) suggested that the cause of bat mortality, when infected by *P. destructans,* is a disruption in physiological wing function. On examining the wings of WNS-affected Little Brown Bats (*Myotis lucifugus*) that were collected over winter, it was found that they would tear easily since they had lost their elasticity, were no longer strong, and the tissues appeared crumpled (Gargas et al., 2009; Cryan et al., 2010). Furthermore, the microscopic examination of these wings revealed the corrosion of the skin by the fungus, which damaged some glands such as the *apocrine* and *sebaceous* glands, in addition to the hair follicles themselves. As a result of the infection, *P. destructans* digests the wing skin, and also impacts the connective tissues, the elastin and muscle fibres, the blood and lymphatic vessels and the glandular structures (Gargas et al., 2009; Cryan et al., 2010). Although *P. destructans*

only infects the skin, it was proposed that the disruption of physiological homeostasis, caused by the fungus, is what ultimately causes mortality (Cryan et al., 2010).

While many studies focused on bat motility as a result of WNS infection, a study by Fuller et al. (2011) suggested there is a possibility of healing the damaged wings associated with WNS in free-ranging *M. lucifugus*. This study was conducted on two maternity roosts of *M. lucifugus*. The authors found that 78% of the examined bats affected by WNS over the hibernation period were able to heal rapidly in terms of the damaged wings upon arrival at the maternity roosts (Fuller et al., 2011). However, even if the WNS-affected bats survived and the damaged wings had no visible damage, the WNS infection could still have an impact on wing function (Fuller et al., 2011).

Predators

Bats, and many other small mammals and birds are preyed upon by predators such as birds (Speakman, 1991; Jung et al., 2011), monkeys (Tapanes et al., 2016) and domestic cats (Phillips et al., 2001; Woods et al., 2003; Ancillotto et al., 2013; Loss et al., 2013).

A study investigated the effect of predation risk induced by different predator cues, and attempted to identify the anti-predator behaviours of Phyllostomid frugivorous bats, which are Great fruit-eating bat (*Artibeus lituratus)* and Flat-faced fruit-eating bat (*Artibeus planirostris)*, during their foraging, especially focussing on their foraging behaviour and seed dispersal (Breviglieri et al., 2013). This study simulated the presence of predators when bats foraged on calabur trees (*Muntingia calabura*) by using a stuffed animal as a visual cue of different predators, as well as acoustic cues of vocalizations, including Barn owls (*Tyto alba)*, Burrowing owls (*Speotyto cunicularia)* and the Southern Lapwing (*Vanellus chilensis*) as a control. The results indicated that bat foraging behaviour was affected significantly by presenting known predators visually, particularly bat landing behaviour, although flight behaviour was not specifically affected. Moreover, *T. alba* had the most negative impacts on bat

behaviour when compared with the other predators, there was a reduction in bat activity during landing to grab fruits, but not when the bats flew around the tree. The sound and visual display of *S. cunicularia* and *V. chilensis* did not have any effect on bat foraging behaviour. Overall, the presence of the *T. alba* model with its associated sound, led to a decline in the number of the seeds dispersed by bats (Breviglieri et al., 2013). Therefore, foraging behaviour changed as a response to the predator and was also linked to the species of the detected predator (Breviglieri et al., 2013).

Domestic pets kill many wild animals annually (Loss et al., 2013). Furthermore, roaming cats contribute to the extinction of wildlife globally (Loss et al., 2013). Domestic cats (*Felis catus*), are listed in the top 100 of the worst non-native aggressive species (Lowe et al., 2000). Numbers of domestic cats (*Felis silvestris catus*) have increased in Western Europe, and they are now the most abundant carnivore (Pavisse et al., 2019). Between 2000 and 2015, cat-related mortality in garden birds increased by at least 50%, which coincided with an increase in the cat population (Pavisse et al., 2019). However, the mortality rate as a result of predation by domestic cats is still speculative (Loss et al., 2013). Nevertheless, according to the International Union for Conservation of Nature (IUCN), 14% of all birds, mammals and reptiles have become extinct due to the predation of cats (Medina et al., 2011). In addition, it has been estimated that free roaming domestic cats cause the death of 1.3 – 4.0 billion birds and 6.3 – 22.3 billion mammals annually in the USA (Loss et al., 2013). Similarly, domestic cats in the United Kingdom (UK) are the most abundant carnivores and they are found to exist in high densities (typically > 200 cats/km^2) (Beckerman et al., 2007; Baker et al., 2008) with the number of cats increasing annually. Therefore, there is a crucial impact of these predators on wildlife every year (Woods et al., 2003). Several studies have stated that cats have a negative impact on wildlife, (van Heezik et al., 2010; Ancillotto et al., 2013), with their most common prey being birds, followed by rodents (van Heezik et al., 2010). Cats have been estimated to prey on 25–29 million birds annually in Britain (Woods et al., 2003). However, there is no clear scientific evidence that such mortality has reduced bird populations (Parsons et al., 2006; RSPB, 2019). It is likely that most of the birds

killed by cats would have died anyway from other causes before the next breeding season, and cats tend to attack weak or sick birds (Baker et al., 2008).

Many bat species roost in human-made constructions, such as houses, at least for some periods of their life, usually for breeding, hence, increasing the probability of meeting cats (Ancillotto et al., 2013). Although there is only occasional evidence of cats predating bats (Phillips et al., 2001). The Eastern blossom bat (*Syconycteris australis*) was first documented case as being attacked by a cat (Phillips et al., 2001) and are recorded by *the Threatened Species Conservation Act* (1995) as a species that is extremely vulnerable (Phillips et al., 2001). Another study was conducted at four wildlife rescue centres in the Italian peninsular between 2009 and 2011, using 1012 records of admitted bats and came to five main conclusions (Ancillotto et al., 2013). Firstly, according to rehabilitation centre records, 28.7% of the rescued bats had suffered predation by a cat. Secondly, the majority of the bats caught by cats were house-roosting bats. Third, in summer, adult females were more likely to fall prey to repeat predations by cats, which constitutes a threat to the reproductive season. Fourth, there was a strong association between predation by cats and land cover. In short, because cats tend to remain outdoors, the majority of predation occurred in rural and sparse urban areas. Finally, cats are known to be explorative mammals, which means they could be attracted to bat roosts through sensory signals, such as the sound released by bats; the smell of dung located next to the bat roost; or by monitoring the bat when they fly near the roosting entrance (Ancillotto et al., 2013). While it is widely known that cats are responsible for the killing or injuring of bats in these two studies, there appears to be no understanding of how wide-spread this might be, and, as yet, no formal observation of this occurring in the UK at all. There is no evidence for how cat predation impacts bat populations, however, as cat predation does not affect bird abundance overall, this might also be true of bats. Investigating the population-level impacts of cats on bat abundance is outside the scope of this thesis. However, I consider cat predation to primarily be a welfare problem for bats, rather than a conservation one.

As well as cat predation, some studies indicate that bats also experience predation by birds (Speakman, 1991; Jung et al., 2011) and monkeys (Tapanes et al., 2016). Indeed, a study conducted by Speakman (1991) to assess the loss of bats by bird predation found that eleven bird species occasionally fed on bats. In addition, the study found predation by birds accounted for some 11% of bat mortality in Britain annually (Speakman, 1991). Hence, this predation on bats will also have an effect on bat population numbers. In fact, certain scholars have noted the likelihood of owl predation, specifically on the species known as *M. lucifugus*, however there remains no concrete evidence for this (Jung et al., 2011). One study conducted by Jung et al. (2011) was based on the capturing of *M. lucifugus* bats in eight mist nets that were placed over a river and along a narrow forest road in Canada. A Great Horned Owl (*Bubo virginianus*) was observed in the river where one mist net was and where one adult male bat was later found dead. Some puncture wounds were observed on the bat and these were assumed to have been caused by the owl's talons. However, the main cause of the bat's death was not conclusive since part of the mist net was held under the water as a result of the owl's weight, meaning the bat could have also died by drowning (Jung et al., 2011). In fact, it remains unclear as to whether the owl chased the bats into the mist nets or the bats were already there when the owl attacked them. In addition, *B. virginianus* owls are supposed to prey on bats that are locally abundant, while here the bats are already captured so they make easy prey (Jung et al., 2011). Finally, when bats are captured in a mist net they vocalize loudly and that may attract the owl (Jung et al., 2011). Hence it can be argued that this study provides no specific evidence on how *B. virginianus* owls prey on *M. lucifugus* bats in natural circumstances, and therefore, how they may affect bat populations (Jung et al., 2011).

Tapanes et al. (2016) also found that there is predation on bats by *Cercopithecus* monkeys (Tapanes et al., 2016). This study was conducted in Kenya and Tanzania over a period of six and a half years and their observations provided the first behavioural accounts of predation by *Cercopithecus* monkeys on bats. In fact, the authors reported 13 events of predation on bats over the study and thus concluded that monkeys hunt and eat bats. All these predation events were found to have occurred

in forest edges or in plantation habitats (Tapanes et al., 2016). It is worth mentioning that while the *Cercopithecus* monkeys prefer fruits, they are omnivores so they feed on leaves, invertebrates and occasionally on vertebrates. In fact, they consume a wide range of vertebrate species (Tapanes et al., 2016).

All these findings provide new information with regards to the risk of predation for bats that can arise from altering their natural behaviour. This thesis supposes that cats have a negative effect on bats, as they attack them and are likely to cause wing tears.

1.2.3 Bat Population and Ecology in the United Kingdom

This section will first describe general theories of animal population regulation, and then will focus on UK bat species populations, and, specifically, the most commonly occurring UK bats, the Common pipistrelle bat (*P. pipistrellus*).

Population regulation

Factors which affect populations are often grouped into density-dependent and density-independent factors. Density-dependent factors are biological in nature such as predation, competition between individuals, accumulation of waste, and diseases such as those caused by parasites (Kendall, 1990). While density-independent factors are physical or chemical in nature such as weather, natural disasters and pollution in the environment (Kendall, 1990). In addition, there are two further important environmental properties that control whether an individual can live in the population or not; these are conditions and resources. Conditions involve the environment's physiochemical features which make that environment habitable for the individual, such as: temperature, humidity, and the pH of aquatic environments (Townsend et al., 2008). These conditions involve the availability of the animals' shelters or refuge as well. Resources include what the animal consumes and are required for an individual's growth and reproduction (Townsend et al., 2008). These include the availability of food and water in the environment. If these resources are not enough to cover each individual's need in the population, this can lead to

competition between the individuals (Townsend et al., 2008). Competition for limited resources is related to high population density, which not only affects competition, but also reduces birth rate and increases death rate (Townsend et al., 2008). Often food and refuges are the major factors that affect population size overall, as when food and refuges are limited, this leads to competition between individuals and a decline in the population (Stevens, 2010). Therefore, in bats, habitat change and prey availability might have the most effect on population.

United Kingdom bat population

In the UK, bat species make up almost a quarter of all mammalian species, and are affected by insect prey availability, weather conditions, changes in habitats (BCT, 2018a) and predation (Ancillotto et al., 2013). While populations of many bats in the UK are stable or increasing, especially the most abundant pipistrelle species (*P. pipistrellus* and *Pipistrellus pygmaeus*), the number of roost sites are declining (BCT, 2018b). Indeed, urbanisation in the UK is one of the most dramatic forms of land use change, which relatively few species can adapt to (Lintott et al., 2015).

In the UK there are 18 bat species, 17 of which are resident bats. These are: Alcathoe bat (*Myotis alcathoe*), Barbastelle (*Barbastella barbastellus*), Bechstein's bat (*Myotis bechsteinii*), Brandt's bat (*Myotis brandti*), Brown long-eared bat (*Plecotus auritus*), Common pipistrelle (*Pipistrellus pipistrellus*), Daubenton's bat (*Myotis daubentonii*), Greater horseshoe bat (*Rhinolophus ferrumequinum*), Grey long-eared bat (*Plecotus austriacus*), Leisler's bat (*Nyctalus leisleri*), Lesser horseshoe bat (*Rhinolophus hipposideros*), Nathusius' pipistrelle (*Pipistrellus nathusii*), Natterer's bat (*Myotis nattereri*), Noctule (*Nyctalus noctula*), Serotine (*Eptesicus serotinus*), Soprano pipistrelle (*Pipistrellus pygmaeus*) and the Whiskered bat (*Myotis mystacinus*), while one species is vagrant and occasionally visits which is the Greater mouse-eared bat (*Myotis myotis*) (BCT, 2017). According to the IUCN Red List of Threatened Species (2017), almost all of the resident UK bat species are categorised as ‘of least concern’, while two species are categorised as ‘near threatened’, the *B. barbastellus* and *M. bechsteinii* bats (IUCN, 2017).

There was a significant drop in bat populations in the UK during the period of 1980 to 1992 (Harris et al., 1995). However, a more recent bat survey in 2017 indicated a significant increased since the baseline year (1999) for following bat species: *R. ferrumequinum*; *R. hipposideros*; *P. pipistrellus*. *M. nattereri.* Meanwhile, the most species remained stable which are: *M. daubentoniid, M. mystacinus, M. brandti, P. pygmaeus, N. noctula*, *E. serotinus,* and *P. auritus*. For the remainder UK bat species, there are insufficient data available to identify the population trend for those species (BTC, 2018b)

Common pipistrelle populations

The Common pipistrelle (*Pipistrellus pipistrellus*) belongs to the suborder *Yangochiroptera* (Teeling et al., 2001), it is a native species and the most common bat species throughout the UK (Harris et al., 1995). These bats weigh about 5.5g, which makes them the smallest hibernating mammal (Sendor and Simon, 2003). These bats usually start hibernation by mid-August to the end of October (Russ et al., 2003; Gerell and Lundberg, 1985), and finish hibernation by the end of March (Gerell and Lundberg, 1985). The male and female bats start visiting the roost site and by the beginning of the summer the males nominate territories for mating roosts, and the females form maternity roosts. Female bats usually give birth to young bats in June (BCT, 2019), and commonly produce twins (Arlettaz et al., 1999). The young feed on the mother's milk for three-four weeks and are weaned by August (BCT, 2019). They are able to fly and forage by the age of six weeks, and at this time the Summer colony starts to disappear and the bats shift to the mating roost (BCT, 2019). The young female will be reproductive at the age of one year (Arlettaz et al., 1999) and males become sexually mature by the second autumn (Entwistle et al., 1998). After mating, the fertilizations accrue during hibernation, so the female becomes pregnant over May-June and usually they give birth to young bats in June (Davidson-Watts and Jones, 2006; BTC, 2019). When the breeding season starts, by the end of July, the females join a single male in the mating roost and form transient mating harems. This mating system is known as resource defence polygyny (Gerell and Lundberg, 1985).

P. pipistrelles forage in many habitats including woodlands, woodland edges, semi-deserts, farmland, rural gardens and urban areas. Like many bat species, they feed on small flies and moths (Hutson et al., 2008). They also tend to roost in buildings and trees over the summer during the maternity period, these maternity colonies generally involve only 25-50 individuals, but some colonies involve up to 200 individuals. In the winter, these bats roost in cracks in buildings and in cliffs and caves, they can also be found in tree holes. During this period, the bats can be found individually or in small groups (Hutson et al., 2008; BCT, 2010). While they can move up to 1 km, they are not migrating bats (Hutson et al., 2008).

Even the last bat field survey in the UK (2017) showed a significant increase for *P. pipistrellus* population, but also, there has been a significant reduction of roost count for *P. pipistrellus* (BTC, 2018b). This could be related to switching roosts, especially maternity colonies, which would significantly affect the counts, thus the roost survey requires more investigation (BCT, 2018b). In fact, the most important issue facing pipistrelles is the major decline in bat roost sites over the last few years as a result of several factors, including current agricultural processes, timber treatment, building renovations and predation by other animals (Hutson et al., 2008; BCT, 2018a).

Common pipistrelle population viability

While there are many threats facing *P. pipistrellus* around the UK, it is important to understand the risks to their populations, which can be achieved using Population Viability Models. A population viability analysis (PVA) is a species-specific tool used in conservation biology to evaluate the risk of population extinction within a given number of years (Boyce, 1992; Sanderson 2006). This analysis requires an understanding of survival rates (Pryde et al., 2005).

The probability of survival, especially adult survival, plays a role in population change in long-lived animals, which bats are considered to be relative to their body size (Sendor and Simon, 2003). Indeed, the probability of survival is thought to have the

largest impact on population dynamics of long-lived species (Sendor and Simon, 2003). An understanding of *P. pipistrellus* survival rates is key to understanding their population dynamics. It is difficult to estimate the survival rates of bats, as most surveys are relatively short-term (Pryde et al., 2005). In addition, bats are nocturnal, and it is difficult to catch and count them (Pryde et al., 2005). Some studies have provided the survival rates of some bats species, but there are limited studies which report a reliable estimation of long-term survival rates, or the difference in survival rates between species or within populations (Sendor and Simon, 2003; Pryde et al., 2005).

A study by Sendor and Simon (2003) investigated the survival rate of *P. pipistrellus* bats over five years (1996-2000), using seasonal (summer/winter) capture-recapture techniques. That study was conducted in a huge bat hibernaculum in Germany. The bats were caught in the summer season between mid-May to mid-September, and the winter season between late November to early March. The bats caught were marked by a coded band, and the bat's sex was recorded. Then the bats were released directly after the capture session. The bats were categorised into four groups: juvenile males, juvenile females, adult males, and adult females. The data was collected over five summers and four winters and analysed to estimate the bat survival probability. The results indicated that there is a persistent difference in survival rates between adults and first-year juveniles, with first-year juveniles having reduced survival probabilities during the first autumn and spring. However, the difference in survival rate between the sexes was small. Over the spring, the survival rates of adults was high, and there was no evidence of mortality increasing during hibernation in the winter, which contradicted the assumption of reduced survival rates over winter due to the reduction in stored fat by the end of hibernation (Sendor and Simon, 2003).

Gerell and Lundberg (1990) estimated higher survival rates in female adult pipistrelles compared to territorial males in southern Sweden (1981-1988). The same results were also found previously by the same authors during a seven years study on a pipistrelle nursery colony in England (Gerell and Lundberg, 1985). Pryde et al

(2005) also showed that adult females had a higher survival rate than males, and adults had higher survival rate than juveniles in New Zealand (Pryde et al., 2005). Also, the adult females were more sensitive to survival and productivity in the population (Pryde et al., 2005). Therefore, these studies suggest that adult females have higher survival rates than males, which means the males might be more at risk. This is because the females spend more time in the maternity roost giving birth and feeding the juveniles, especially between June and July; while the males spend more time out of the roost (BTC, 2019). Although females are less likely to encounter a threat, a decrease in female numbers is likely to have a high impact on the population, as they play a key role in reproduction and productivity. However, the mating system in bats is polygynous, so several females could need just one male to fertilize. Therefore, it is difficult to predict whether threats to males or females might affect a population the most.

1.3 Wing Form and Function

Although habitat loss and prey availability is thought to affect bat populations the most, other threats do significantly impact bat health, welfare and their population numbers. These include weather, collisions, fungal infections and predation. Many of these threats cause wing injuries, especially collisions and predator strikes. Bat wings form about 85% of the body surface (Makanya and Mortola, 2007); indicating the important role they play. They are critical for powering flight, but their thin membranes are prone to damage. This section will present the structure of wings, their anatomy, and the wing material properties; it will then introduce the subject of bat wing tears.

1.3.1 The structure of Flight Membranes

The flight membranes (patagia) comprise 85% of the body surface area of a bat (Makanya and Mortola, 2007). The patagia are made up of 4 sections (Faure et al., 2009; Madej et al., 2012). The skin at the first section that extends from the bat's shoulder to the thumb is called the protopatagium (Pollock et al., 2016). The skin between fingers 1 and 5, is referred to as the chiropatagium (or dactylopatagium).

The section consisting of a skin that extends between the fifth fingers and the body trunk is called the plagiopatagium (Pollock et al., 2016). Various species of bat also have a tail membrane that is situated between the hind limbs, which is knowns as the uropatagium (Madej et al., 2012; Pollock et al., 2016) (Figure 1.1).

Each part of the wing has a particular function in flight. The plagiopatagium supports the bats' body weight during flight (Neuweiler, 2000); it also plays an important role in lifting the bat during flight (Vaughan, 1970; Swartz et al, 1996). Meanwhile, the chiropatagium works to thrust the bat forward (Neuweiler, 2000). The uropatagium is responsible for flight control, and it has a crucial role in the capture of insect prey (Gardiner et al, 2011), and as well as providing lift during flight (Vaughan, 1970; Swartz et al., 1996). Moreover, the hairs along the edge of the wing play a role in a detecting stall (Sterbing-D'Angelo et al., 2011). As well as flight, the patagia plays a vital role in many physiological functions, such as dermal gas exchange (Makanya and Mortola, 2007), thermoregulation (Kluger and Heath, 1970), and maintaining water balance (Cryan et al., 2010).

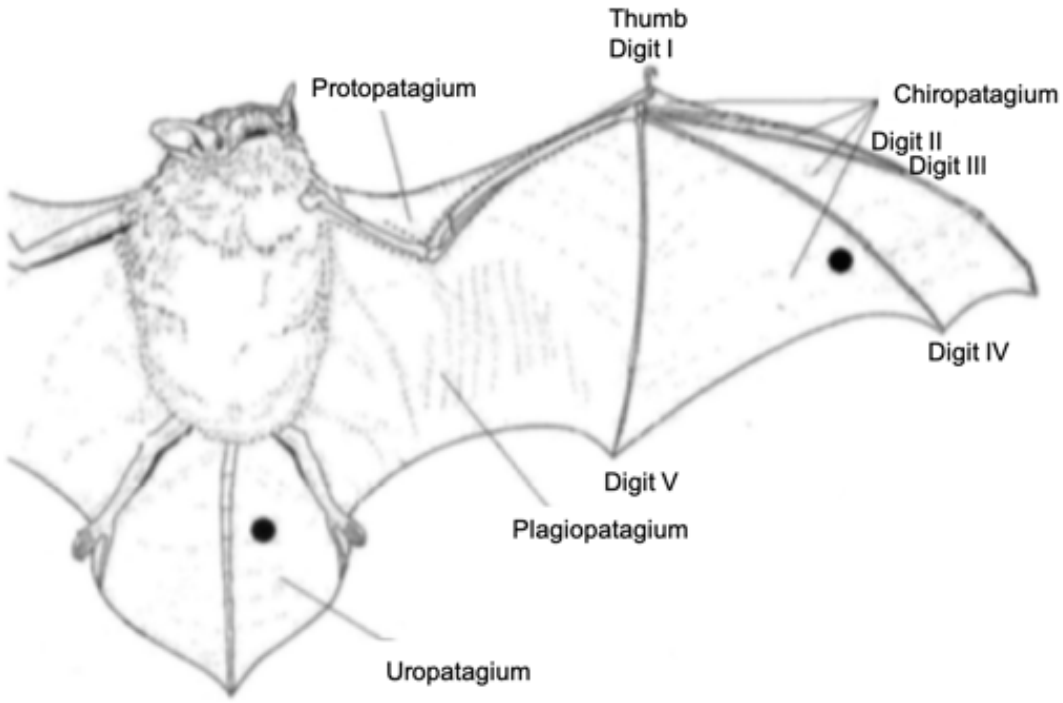


Figure 1.1: Diagram illustrating the 4 parts of the bat flight membranes (patagia), including protopatagium, chiropatagium, plagiopatagium and uropatagium. Image modified from Faure et al., (2009).

1.3.2 The Anatomy of the Bat Wing

Like skin, bat wings are made up of two layers, the epidermal and dermal layers. However, they differ by containing specialist morphological structures and are very thin (Crowley and Hall, 1994; Madej et al., 2012). In bat wings, the thin epidermis covers the dermis, which contains blood vessels, nerves, muscles, elastin bundles and interwoven collagen bundles (Madej et al., 2012; Kovalyova, 2014; Cheney et al., 2017). Additionally, the bones and the skeletal muscles support the wings (Cheney et al., 2017). Gupta (1967) found that the wings consist of a central region of connective tissue that consists of collagen and elastin bundles, sandwiched between a dorsal and ventral layer of epidermis. In contrast, bats' body skin consists of a single-layer, similar to the skin of other non-flying mammals (Sokolov, 1982; Madej et al., 2012).

An early study examined the fibres in the wing of Mexican free-tailed bats *(Tadarida braziliensis).* It used light microscopy and transmission electron microscopy (Holbrook and Odland, 1978), and found that the plagiopataigum consisted of vertical fibre bundles, which were shown in parallel patterns and extended to the trailing edge. These were then also crossed by branched, parallel horizontal fibre bundles, particularly between the joint of the metacarpal and phalanges at the fifth digit (Holbrook and Odland, 1978). In general, this study suggested that the elastin and collagen bundles formed a network of net-like scaffolding over the bat wings (Holbrook and Odland, 1978). A more recent study used polarised light to determine fibres within wing tissue from three male Seba's short-tailed bats (*Carollia perspicillata*) (Cheney et al., 2015). They found that the elastin fibres are arranged in a parallel orientation, along the axis of the unfolded wings, in a 'spanwise' orientation within the membrane (Figure 1.2A, B). The elastin fibres were extremely similar for both wings among the three bats tested (Cheney et al., 2015). However, some tissues were not tested, especially the small sections of the wing next to the bone, and between the fourth and the fifth digits, where the fibres were seen to branch frequently (Cheney et al., 2015). It was also found that the elastin fibres caused wrinkles in the wing in the relaxed position, as removal of elastin fibres reduced the wing wrinkles (Cheney et al., 2015).

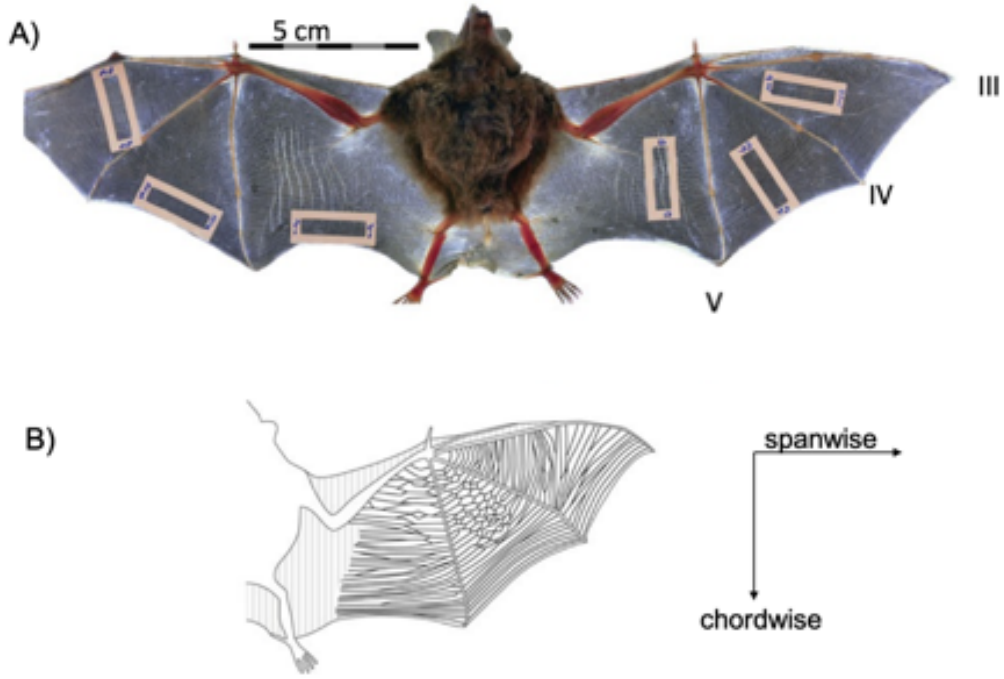


Figure 1.2: The elastin fibres in the wing of the C. perspicillata bat. A) The polarised light image of the bat wing, the rectangles presenting the location of sampling on the wing. B) Diagram of the bat wing presenting the elastin fibres orientation. Image modified from (Cheney et al., 2015).

Another recent study by Cheney et al., (2017) used cross-polarised light to find the diversity of elastin bundles and muscles of bat wings in the plagiopatagium and dactylopatagium (or chiropatagium). The direction of elastin fibres in the wing membrane supports the previous findings by Cheney et al. (2015), and was found in all the bat families tested. However, some variation existed in certain wing sections, which included: 1) the area adjacent to the digit bones, as the elastin bundles often branched and joined to skeletal joints; 2) the area between the fourth and the fifth digits, as in some species the elastin bundles were intersected, which lead to a net-like arrangement of fibres; and 3) the area between the forearm and the fifth digit, as the elastin bundles may section with the distal plagiopatagium (Figure 1.3) (Cheney et al., 2017).

In terms of muscle diversity in the wing membrane, the results of cross-polarised light imaging indicated the muscle architecture was variable, with differences in the length, width and directions of muscle fibres (Figure 1.3) (Cheney et al., 2017). As well as elastin and muscles, collagen bundles are also organised throughout the wing,

forming a cover of both muscles and elastin fibres (Holbrook and Odland, 1978). Histology shows collagen fibres can be found in many different locations and orientations. However, it tended to be distal to the elastin bundles (Cheney et al., 2017), meaning that the collagen bundles occur outside the elastin and cover it (Figure 1.4).

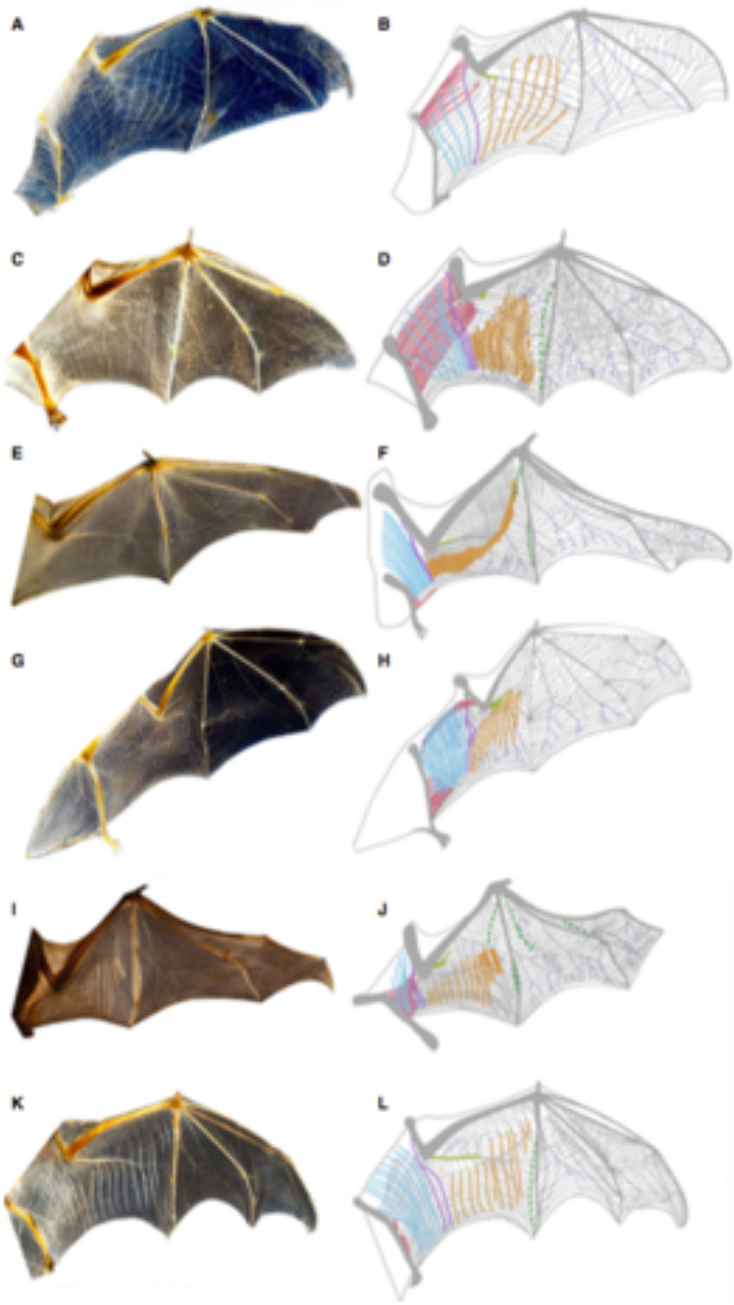


Figure 1.3: The cross-polarised light images of bat wing presenting the elastin bundles (grey lines), muscle (coloured lines), collagen fibre bundles (dashed green lines) and neurovasculature (dashed blue lines). These image sare from different bat families which are: (A, B) Thyropteridae; (C, D) Phyllostomidae; (E, F) Molossidae; (G, H) Natalidae; (I, J) Noctilionidae; (K, L) Mormoopidae (Cheney et al., 2017).

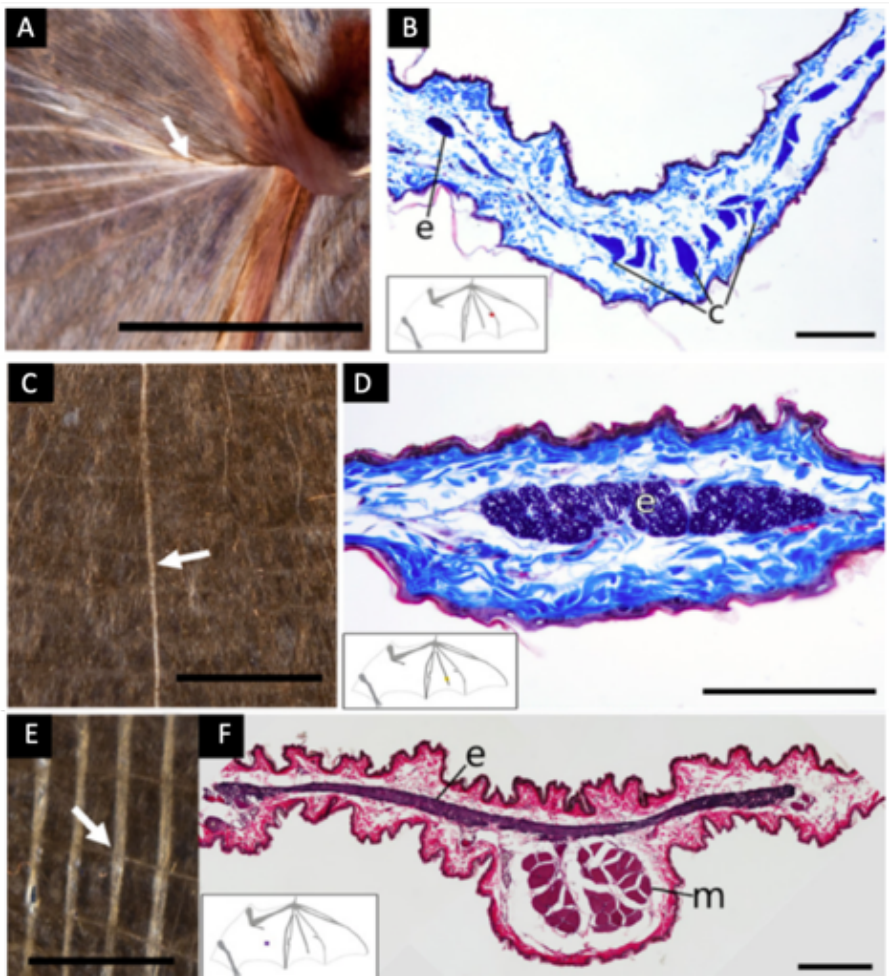


Figure 1.4: A,C,E) The images of wing taken by cross-polarised light, B,D,F) Light micrograph of tissue sample oriented dorsal side up and stained with histological stains. Samples B and D stained with modified Verhoeff's elastin stain and Mallory's triple; elastin bundles in dark blue or navy, collagen in blue. Sample F modified Verhoeff's elastin stain and Van Gieson's stain; collagen, pink; elastin, dark purple; muscle, red. Scale bars; A, C, E) ~ 1 cm, B, D, F) ~ 100 µm. Sample A is from Noctilio leporinus, and samples C and E are from Artibeus lituratus. (Image modified from Cheney et al., 2017).

As well as fibres, there are also blood vessels within the wing. Some studies use the vessel venation on the wing membranes to identify bat species (Amelon et al., 2017; Walker, 2015; Pavlinić et al., 2008; Schofield, 2002). These studies have suggested a specific pattern of wing vessel venation for each bat species, hence it can be used to identify species. In Pipistrelles species, there is a difference in the wing venation pattern between *P. pipistrellus* (Figure 1.5A) and *P. nathusii* (Figure 1.5B), as the middle vein of the plagiopatagium does not branch in *P. pipistrellus*, while it does in the *P. nathusii*. However, in some situations bats cannot be identified by vessel venation, as a result of wing injury and individual variation (Pavlinić et al., 2008).

Other features can be used to identify bat species, such as size, colour, ear shape, and tragus size and shape (Walker, 2002).

One study focused on measuring blood vessels in the wings of the *M. lucifugus* bat (Wiedeman, 1963). It was found that the average length of the main vein was the same as its accompanying artery, even though the diameter of the vein was greater by one-half than the diameter of the artery. Therefore, that main vein had double the number of tributaries than the major artery (Wiedeman, 1963).

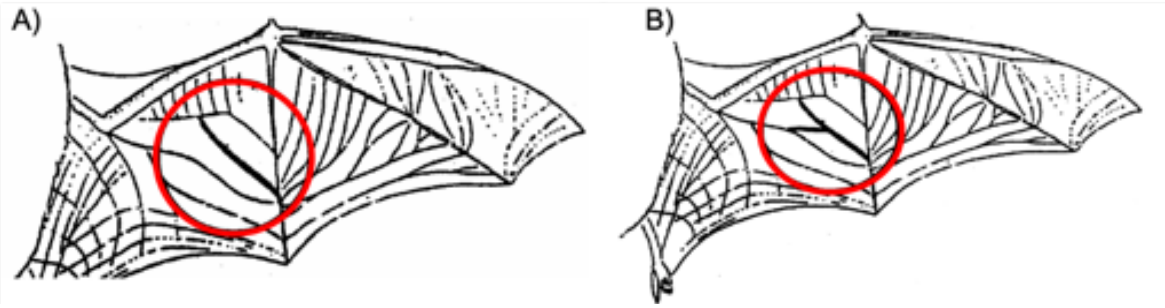


Figure 1.5: The wing venation in: A) P. pipistrellus, B) P. nathusii, the circling area presenting the difference in wing venation to identify bat species (Picture modified from: Walker, 2002).

1.3.3 The Mechanical Properties of the Bat Wing

Bat wing skin is extremely flexible and stretchy, which distinguishes bat wings from the flight membranes of other flying animals (Skulborstad et al., 2015). Swartz et al. (1996) used tensile testing to determine the mechanical properties of bat wings. They compared the wing skin mechanical properties in terms of: stiffness, strength, load at failure, and energy absorption between three particular parts of the wing (chiropatagium, plagiopatagium and uropatagium) in seven bat species: Little brown bat (*Myotis lucifugus),* Cave myotis (*Myotis velifer),* Big brown bat *(Eptesicus fuscus),* Hoary bat (*Lasiurus cinereus),* Parnell's mustached bat *(Pteronotus parnellii),* Big naked-backed bat (*Pteronotus suapurensis),* Jamaica fruit bat (*Artibeus jamaicensis),* Tent-making bat (*Uroderma bilobatum),* Maxican free-tailed bat (*Tadarida brasiliensis)*. It was found that the wing material properties showed robust anisotropy (i.e. different values at different orientations) and had significant variations depending on the orientation of the tested sample, which wing section it

was from and the type of bat species. In particular, the highest stiffness and strength in the wing was found parallel to the wing skeleton, and the highest extension was found parallel to the wing trailing edge. Moreover, there is a significant difference in all mechanical properties, apart from energy absorption, between different sections, especially: 1) the skin is the thickest in the uropatagium and thinnest in the chiropatagium; 2) the highest supportable load is at the uropatagium and it is usually smaller in the other parts; 3) the weakest section is the plagiopatagium; and 4) the least extensible part is the chiropatagium (Swartz et al., 1996). The results also indicated that there was a significant difference between different bat species in all mechanical properties, apart from stiffness (Swartz et al*.,* 1996). This was the first and only study that investigated the mechanical reaction of the bat wing skin to tensile loading under unconstrained uniaxial tension (Skulborstad et al*.,* 2015).

Skulborstad et al*.* (2015) presented the first study using biaxial mechanical characterisation, which applied a mechanical test in two distinct orientations, both chordwise (vertical) and spanwise (horizontal) to investigate local deformation, mechanical properties and fibre kinematics in bat wings. The study was conducted on Pallas' long-tongued bat *(Glossophaga soricina)*, and the results showed a relationship between the wing skin structural morphology, mechanical properties and function (Skulborstad et al., 2015). The study found variation in tissue deformation and fibre strain in the cross-fibre direction for both chordwise and spanwise fibres, which provides evidence for sample orientation affecting material properties. The fibres stretched during deformation, with minimal fibre reorientation at larger stretches. The wing skin was also extremely anisotropic, which affected wing shape under aerodynamic loads. The structural arrangement of the fibres has an important role in providing a low energy mechanism for wing extension and deployment (Skulborstad et al*.,* 2015).

That the wing membranes are thin, compared to body skin, makes them particularly susceptible to injuries, holes and tears (Ceballos-Vasquez et al., 2015). Moreover, the wing or the patagia, beside from its vital role in flight, plays an important role in many other physiological functions, including movement and homeostasis (Ceballos-

Vasquez et al., 2015). Therefore, injury to the wing can be particularly harmful to bats.

1.3.4 Wing Tears

Wing tears are a common wing injury that can be seen in many bat populations (Davis, 1968), and might be caused by collisions (Baerwald et al., 2008; Cryan and Barclay, 2009) or predator attacks (Speakman, 1991; Woods et al., 2003; Jung et al., 2011; Ancillotto et al., 2013; Loss et al., 2013; Tapanes et al., 2016). Even though several studies have investigated bat wing tears in the USA and Canada (Davis, 1968; Powers et al., 2013; Voigt, 2013), there is still little evaluation of bat wing tears in the UK. Davis, over 1966 and 1967, observed the pallid bat (*Antrozous pallidus*) in South Arizona over two summers and found that individual bats from several colonies repeatedly recovered from wing injuries. Significant wing injuries were categorized as either (1) membrane holes, (2) bone abnormalities, (3) missing membrane parts or (4) embedded thorns and cactus spines (Davis, 1968). Holes were the most common injury and ranged from small (1-5 mm in diameter) to large (10-15 mm) (Davis, 1968). Moreover, it was found that wing tears could even lead to losing part of the wing, depending on the breadth of the tear (Davis, 1968). Wing tears and holes can occur as a result of colliding with manufactured objects or plants with spikey branches or thorns (Davis, 1968), fungal infections, such as the *P. destructans* (Blehert et al., 2009; Reichard and Kunz, 2009; Cryan et al., 2010; Fuller et al., 2011, Lorch et al., 2016) or from predator attacks (Speakman 1991; Woods et al., 2003; Ancillotto et al., 2013; Loss et al., 2013), such as cats.

1.4 Wing Wound Healing

Commonly, when bat wings are torn, the bat requires more energy to produce rejuvenated flight membranes, and relatively little is known about membrane healing during times of energy constraint or peak energy demand (Ceballos-Vasquez et al., 2015). Also, the healing procedure requires time, but there is little known about the amount of time needed for healing (Davis and Doster, 1972), even though

it is crucial to understand how injured wings heal and recover the wing function (Fuller et al., 2011). This section will present the mechanisms of wound healing in bat wings and the effect of seasonality on healing.

1.4.1 Healing Process

The healing procedure of wounds in bat wings is somewhat complicated and must pass through numerous steps to be completely successful. A wing tear injury in *A. pallidus* bat needs from 22 to 34 days to heal (for a wound sized 14mm^2) while a larger wound (17mm^2) requires 32 to 43 days to heal. Infection can, of course, cause a delay in healing (Davis and Doster, 1972). There is some evidence that shows that bat wings have the ability to heal and recover from injuries without any significant effect on bat activities (Faure et al., 2009).

Wound healing is an immunological response to an injury, which deactivates the homeostasis of the normal tissue. In relation to mammals, the procedure comprises of a highly organised response that is controlled by several genes, cytokines, and hormones (Gurtner et al*.,* 2008). Many studies on mammalian wound healing have demonstrated that the key element in the complex healing procedure exists in the nearby blood vessels (Faure et al., 2009; Pollock et al., 2016; Davis and Doster, 1972). Generally, there are three main steps for repairing the holes in bats wings: 1) forming red scar tissue around the border of the hole, and after 1-5 days this becomes white; 2) growing of the tissue from the border of the hole inwards to close the wound, which takes place 2-10 days after injury; 3) closing then takes place and any scab will be replaced by scar tissue, which starts 2-3 weeks after the injury and continues for a year or more (Davis and Doster, 1972; Gurtner et al., 2008) (Figure 1.6). The scar tissue is what makes the renewed area usually appear as a pale-colour in comparison to the surrounding area. However, another study has suggested that the pale-colour could be caused by ectoparasites (Davis and Doster, 1972).

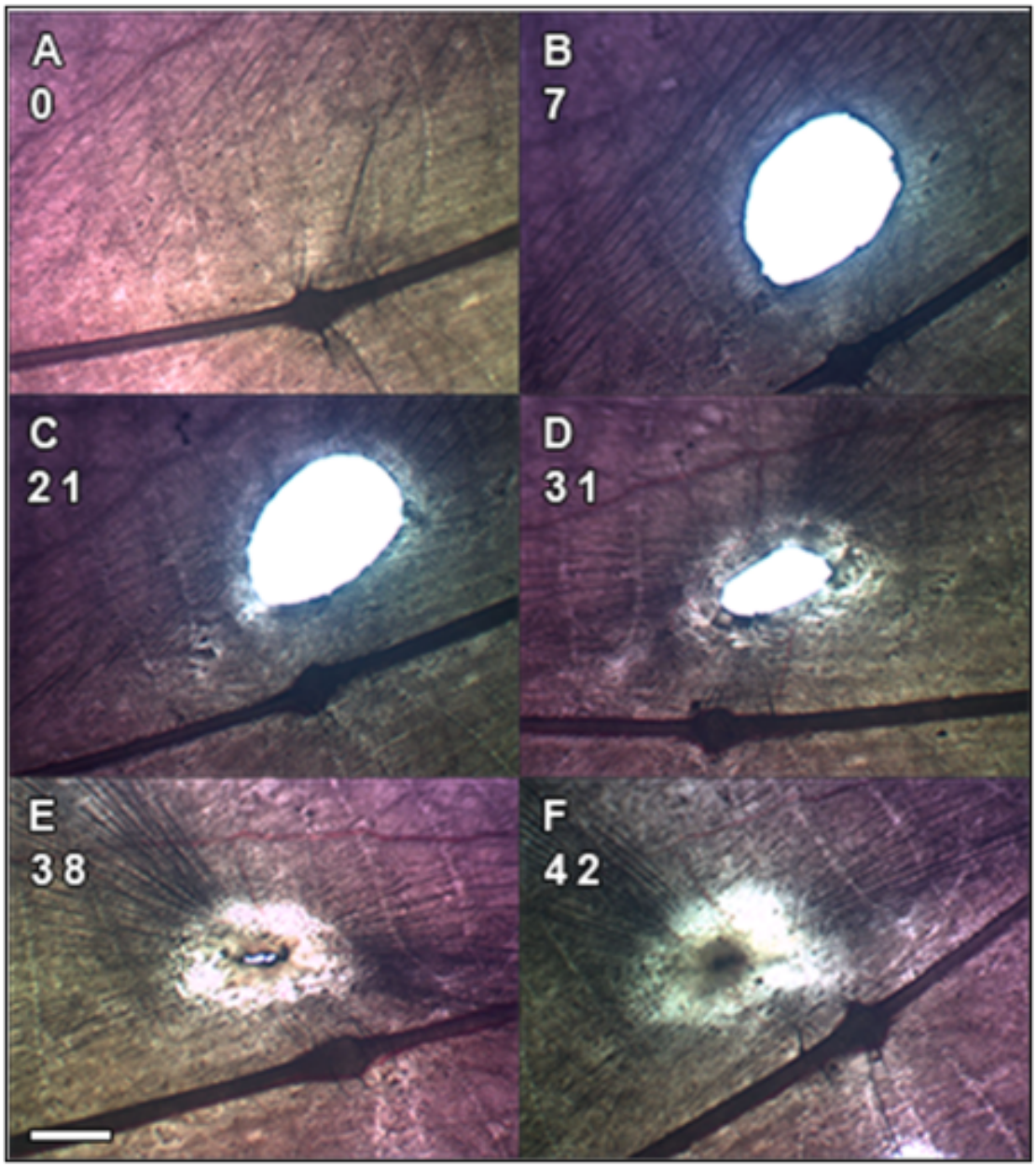


Figure 1.6: Wound healing in the wing membranes of a E. Fuscus bat in Cuba in 2014. (A) Before biopsy (day0). (B) Day 7. (D) Day 31. (E) Day 38. (F) Day 42 (Pollock et al., 2016).

As well as injuries, biologists often collect tissue samples from bat wings for marking in the field, or for molecular analysis (Ceballos-Vasquez et al., 2015). A study by Faure et al. (2009) found that a wound in the tail membrane healed faster than flight membranes as the uropatagium is more highly vascularized than the chiropatagium, thus the tail was the best place to collect tissue as it had the most blood vessels and healed quickest. Moreover, it was found that a wound in the Egyptian fruit bat (*Rousettus aegyptiacus*) in the plagiopatagium section took 50% longer to heal

compared to the same size wound in the chiropatagium (Greville et al., 2018). Indeed, a good blood supply, indicated by the presence of more blood vessels, is associated with quick healing.

1.4.2 Seasonal Wound Healing

In order to heal from a wing tear, there must be a fundamental energy investment (Ceballos-Vasquez et al., 2015). Ceballos-Vasquez et al. (2015), found that wound healing in the flight membranes of Big brown bats (*Eptesicus fuscus*) differed across seasons and during times that depend on energy consumption. Therefore, the healing of the wound will be slower in the Winter compared to that in the Summer, and might also be slower during the periods of energy conservation, such as in hibernation, and during the high-energy demand periods, such as pregnancy and lactation (Ceballos-Vasquez et al*.,* 2015). In the study, an 8 mm biopsy of the wing of adult female E. *fuscus* bats was taken and the time that would be required for healing from the biopsy was compared at different seasonal temperatures between the Winter and the Summer. Consequently, it was found that the Summer group demonstrated healing during the first week, while the winter group did not demonstrate it until the fifth week. Moreover, the results show that there was no difference between lactating and non-reproductive females in healing time, so both groups exhibited full wound healing during the third week (Ceballos-Vasquez et al*.,* 2015).

1.5 Bat Flight Behaviour

Bats are the only mammals capable of flapping flight (Voigt et al., 2012). A bat's wing is highly flexible and stretchable, and is supported by the skeletal structure and musculature, to enable a large change in the wing shape during flight (Skulborstad et al., 2015).

A study by Voigt (2013) investigated how holes and tears in bat wings affect the flight performance and metabolic rate during flight. This investigation was conducted on

two tropical bat species, the Silver-tipped myotis (*Myotis albescens*) and Black myotis *(Myotis nigricans)* (Voigt, 2013). In both study species, injured individuals had torn trailing edges on the left plagiopatagium, which resulted in about a 20% reduction of the wing area when compared to the right wing. The results indicated that flight speed was not altered in the bats with injured wings, compared to their conspecifics with healthy wings. However, the bats with injured wings performed fewer U-turns in a circular flight arena and also had lower flight metabolism (mL CO_2 min^{-1}). The reduction in flight metabolism in the bats with injured wings led to fewer flight manoeuvres, which cost more energy, than in bats with healthy wings. Wing tears reduced the wing area, and impacted the aspect ratio, which is the ratio of wing length and width, and impacted wing loading abilities, which is likely to lead to a reduction in foraging success, and will, in turn, affect body mass and flight metabolism (Voigt, 2013). This study is the first investigation in to the effect of reducing the wing area on flight performance and metabolic rate (Voigt, 2013). In this thesis, the effect of bat wing tears on flight behaviour will be investigated.

1.6 Gaps in knowledge that this thesis will address

There are many studies in the literature on bats, especially in the USA and Canada (Davis, 1968; Powers et al., 2013; Voigt, 2013; Greville et al., 2018). However, there are hardly any studies on bats wing tear in the UK, despite most of the UK bat species roosting in houses (BTC, 2015), and being greatly affected by anthropogenic factors, such as light pollution, habitat loss and fragmentation, and coming in to contact with domestic pets (Mickleburgh et al., 2002; BCT 2018a; BCT 2018b). Urbanisation is increasing in the UK (Lintott et al., 2015), which is also likely to lead to more injuries in bats, especially in terms of collisions with manmade structures (Cryan and Barclay, 2009; Baerwald et al., 2008) and predator attacks (Speakman, 1991; Woods et al., 2003; Jung et al., 2011; Ancillotto et al., 2013; Loss et al., 2013; Tapanes et al., 2016), such as from cats (Phillips et al., 2001). Wing injuries are prevalent in UK bats, causing many hundreds to be admitted to rescue centres every year (Kelly et al., 2008). There are limited studies on wing tears in bats, and so far, no one understands the causes,

healing rates and effects of these injuries. **This thesis, therefore, aims to investigate the causes and effects of bat wing tears, in a series of studies.**

This review has identified that there are no studies that have fully characterised the occurrence of wing tears in bats. While some studies have considered the effect of tears, in terms of healing (Davis and Doster, 1972; Faure et al., 2009; Pollock et al., 2016) and prevalence (Davis 1968), none have systematically recorded their type, shape, position and healing outcomes. Therefore, **chapter 2 of this thesis aims to characterise wing tears in *P. pipistrellus*, and other bat species, in the UK.**

While there is a good understanding of wing anatomy, including fibres, venation and material properties (Wiedeman, 1963; Swartz et al., 1996; Cheney et al., 2015; Skulborstad et al., 2015; Cheney et al., 2017), these tend to vary between species (Swartz et al., 1996). Wing anatomy has never been documented in *P. pipistrellus*, probably due to them being small and difficult to process. Furthermore, how the wing anatomy might predict the positioning of wing tears and their healing capabilities, has yet to be explored. For instance, the plagiopatagium is often the weakest section and, therefore, might have more tears (Swartz et al., 1996). **Chapter 3 aims to explore the anatomy of the wing in *P. pipistrellus*, and see if knowledge of the anatomy is sufficient to understand wing tear placement and healing rates.**

While wing tears have been found to alter turning manoeuvres in bats (Voigt, 2013), a quantitative method has not been developed to measure the effect of wing tears on flight. Therefore, **the aim of chapter 4 is to develop a new method for analysing flight from high-speed video data to assess the effect of wing tear on flight**.

The causes of wing tears have, so far, only been based on circumstantial evidence collected from rescue centres (Ancillotto et al., 2013) and researcher observations (Davis, 1968; Speakman, 1991; Phillips et al., 2001; Jung et al., 2011; Powers et al., 2013). Wing tear causes, reported by bat carers, will be included in Chapter 2. Then **the aim of chapter 5 is to develop a systematic forensic method to identify the presence of cat DNA on wing tears**.

The discussion chapter (Chapter 6) will present the key findings in each chapter and make recommendations for future studies and for bat carers to take on board to improve bat health and welfare.

Chapter 2 Characterising Wing Tears in Common Pipistrelle (*Pipistrellus pipistrellus*) and other UK bat species

This chapter has been accepted for publication in the Journal of Mammalogy:

Khayat, R. O., Shaw, K. J., Dougill, G., Melling, L. M., Ferris, G. R., Cooper, G., and Grant, R. A. 'Characterizing wing tears in common pipistrelles (Pipistrellus pipistrellus): investigating tear distribution, wing strength, and possible causes.' Journal of Mammalogy, pp. 1-13. (Appendix 1)

Chapter summary:

This chapter presents data collected from bat carers around the UK. It provides a quantitative summary of wing tear placement, types and distribution in Common pipistrelles bat and other bat species. Moreover, it introduces the healing outcomes of bats with wing tears, and presents possible causes of the wing tear in bats included in the study. Results indicate that holes were the most common tear types. The most tears occurred in section P, and tended to be oriented rostro-caudally, from the wing membrane to the trailing edge. Tears in section P tended to take longer to heal, and might be associated with predator attacks, such as from cats.

2.1 Introduction

Injuries in bats often occur on the wings, or flight membranes, as they are large, thin and sensitive to damage (Ceballos-Vasquez et al., 2015). Wing tears are a common injury in many bat populations (Davis, 1968). Davis (1968) found over 40% of Pallid bat individuals (*Antrozous pallidus*) in one roost in South Arizona, had wing injuries or abnormalities. Wing tears can even lead to losing parts of the wing, depending on the breadth of the tear (Davis, 1968).

These wing tears often occur as a result of several possible causes such as: colliding with man-made objects or plants with spikes and thorns (Davis, 1968), fungal infections (Reichard and Kunz, 2009; Cryan et al., 2010; Fuller et al., 2011), or

predator attacks (Speakman, 1991; Woods et al., 2003; Ancillotto et al., 2013; Loss et al., 2013; Russo and Ancillotto, 2015). Even though several studies have investigated bat wing tears in the USA and Canada (Davis, 1968; Powers et al., 2013; Voigt, 2013; Greville et al., 2018), there is still little evaluation of bat wing tears in the UK. The UK makes an especially interesting area to study as there is a lot of urbanisation (Lintott et al., 2015), and most of the UK bat species are house roosting (BTC, 2015). This mean that bats in the UK are likely to be affected by many anthropogenic factors, including increased exposure to pets and complex urban landscapes, which may both increase their likelihood of receiving wing tears.

While some studies have considered the effect of tears, in terms of healing (Davis and Doster, 1972; Faure et al., 2009; Pollock et al., 2016; Greville et al., 2018) and prevalence (Davis 1968), none have systematically recorded their type, shape, position and healing outcomes. Therefore, this chapter of this thesis will characterise wing tears in *P. pipistrelles*, and other bat species, in the UK. It will identify: i) which part of the wing is most susceptible to tears; ii) which type of wing tear occurs most commonly in bats; iii) the recovery and rehabilitation outcomes of bats with wing tears; iv) the possible cause of the wing tear in the bats included in the study.

2.2 Methods

This study focuses on the three biggest parts of the wing: The most distal section of the chiropatagium (CI) is the membrane between digits iii and iv. The second chiropatagium section (CII) is the membrane between digits iv and v. The most proximal section of the wing is the plagiopatagium (P), which is the membrane between digit v and the body (Figure 2.1 A). The data was collected from Common pipistrelles bat (*Pipistrellus pipistrellus)* and five other bat species which are: the brown long- eared bat (*Plecotus auritus*), Natterer's bat *(Myotis nattereri*), Serotine (*Eptesicus serotinus*), Soprano pipistrelle *(Pipistrellus pygmaeus*), and the whiskered bat (*Myotis mystacinus*).

2.2.1 Data Collection

Data on bat wing tears was collected over 20 months between March 2016 and October 2017 from live, rehabilitating animals. Bat carers were recruited by advertising the project at the Mammal Society Easter Meetings, the National Bat Conference, National Bat Care Conference and in Bat Care News, as well as from Facebook groups across the UK (UK Bat Workers, Cambridgeshire Bat Group, Kent Bat Group and South Lancashire Bat Group). Bat packs were distributed to bat carers, which involved a small questionnaire about the injured bat, providing details on species, gender and age, as well as a 1 cm gridded card for taking a calibrated image of the wing (Appendix 2). The wing tears were photographed while the bat was awake (not during torpor nor under anaesthetic), and its wing was extended and held against 1 cm gridded card for scale (Appendix 2D). Photographs of torn wings were collected from bat carers across the UK, through the website http://bat-research-mmu.weebly.com. Some pictures of injured bat wings were taken during the filming of bats at Lower Moss Wood Educational Nature Reserve in October 2016 and October 2017.

Soon after admittance, bat carers were also asked, in their own words, to describe how the bat was found and the possible cause of the tear; bat carers emailed free text comments of the possible cause, describing any evidence for their decision. Ethical approval was obtained through the Research Ethics and Governance Committee at Manchester Metropolitan University.

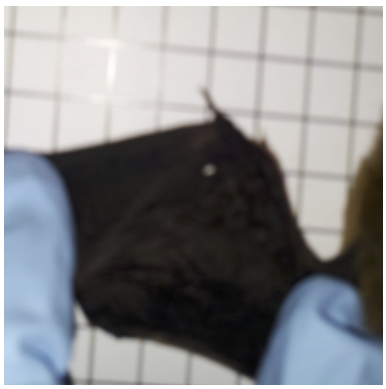
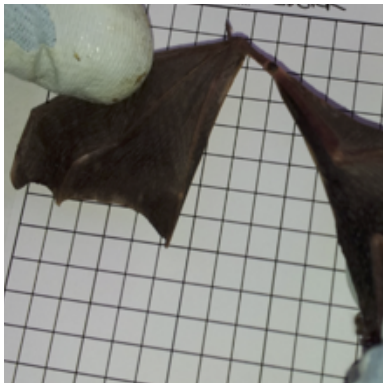
In the study, 55 pictures of *P. pipistrellus* and 21 pictures of other UK bat species were collected, including two *P. auritus*, three *M. nattereri*, one *E. serotinus*, twelve *P. pygmaeus*, and three *M. mystacinus*.

2.2.2 Tear Placement, Categorisation and Description

From each image, every tear was traced as it appeared in the photograph using Inkscape version 0.91 (available from: inkscape.org/en/) onto a wing diagram, and coded by colour for the frequency of its occurrence in that location. The total number

of all tears in each section of the wing was also determined. In addition, the tears were categorised into four major types, based on a criteria of classification that was developed during this study: holes, contained tears, total tears and trailing edge tears and is defined in Table 2.1. Some variation existed in how much the wing was stretched in each photograph; for example, sometimes other injuries prevented the carer from fully extending the wing. This may have influenced some of the classifications of holes and contained tears; however, holes did not have any further ripping, and were puncture wounds (Table 2.1), whereas contained tears tended to be much larger and ragged around the edges, from ripping (Table 2.1). The number of tears in each category were identified in each wing section. In some images more than one tear from the same category was found in the same section, in these instances, the total numbers of tears were counted from each tear category in each wing section.

Table 2.1: The different types of tears in bat wings and the description of each tear type with a picture.

Type of tear	Description	Example
Hole	It is a small puncture, and can be round or oval, it is not more than 2% of a wing segment	
Contained tear	It is larger than a hole. It is a tear, rather than a puncture, that is still entirely contained within the wing. It can also be round or oval, with 5-50% of the membrane missing from a wing segment.	
Total tear	It is a tear that runs from the internal membrane to the trailing edge of the wing, thus not being contained within the wing. It often has a vertical appearance (like a triangle), and the bones are often affected or missing; more than 50% of the membrane tends to be missing from a wing segment	
Trailing edge tear	It is horizontal in appearance and occurs only at the trailing edge of the wing.	

2.2.3 Tear Recovery and Rehabilitation

Bat carers were approached 9-12 months after submitting their photographs and asked what the outcome of the rehabilitation was. No further photographs were collected at this follow-up. Recommendations for bat carers for release, rehabilitation and euthanasia practices are provided by the Bat Conservation Trust and the Department for Environment, Food and Rural Affairs (DEFRA) (Mitchell-Jones and McLeish, 2004; BCT, 2016), but are not quantitative, and rely on the experience and opinions of the individual carers. Bat carers emailed free text comments detailing rehabilitation outcomes. This data was collected and, upon review, fell naturally in to four categories: released after 2 weeks, released within 2- 3 months, still in care after 6 months and euthanized. This follow-up data was collected for thirteen *P. pipistrellus*, and fifteen other species of UK bats, including twelve *P. pygmaeus*, one *M. nattereri*, one *P. auritus*, and one *E. serotinus*.

2.2.4 Statistical Analysis

Statistical analysis was carried out using SPSS Version 24. Total tear numbers, tear types and tear orientation were compared for *P. pipistrellus* between each of the three wing sections using a chi-squared test. In other UK bat species only the total tear numbers were tested with a chi-squared test for each of the wing sections, as there were many zero scores in the tear type data. Sample numbers for the follow-up healing data were too low to run statistics on, but are presented in graphs for visual comparison.

2.3 Results

Across the 76 pictures of bat wings analysed during this study, the tears and injuries on the wings were found in different locations on the wing and took a variety of shapes. In the following sections, the results of the tear placement, categorisation, description, and tear recovery, will be presented for both *P. pipistrellus* and other bat species (Appendix 3). Also, the possible causes of those tear will be presented (Appendix 3).

2.3.1 The Placement, Categorization and Description of Wing Tears

P. pipistrellus Bats

P. pipistrellus had more wing tears in the P section, than in CI and CII (χ^2=18.951, df=2, p<0.001, Figure 2.1A, B, C). The graph in Figure 2.1C shows that CII section generally contains a lower number of wing tears than P section, and all tear types are present but in lower numbers than in P section. Meanwhile, section CI features the lowest number of wing tears, of all types except the total tear. They can be found there but in smaller amounts than in the other sections.

The types of tear did not differ significantly between the wing sections in *P. pipistrellus* (χ^2=3.647, df=2, p=0.161, Figure 2.1 B, C). Holes were the most common tear type in all wing sections and appeared distributed fairly evenly in each wing section. The contained and total tears tended to be rostro-caudally oriented, from the wing membrane towards the trailing edge (Figure 2.1C), and occurred more prevalently in the proximal wing sections (Figure 2.1B, C). Trailing edge tears occurred in a distal-proximal orientation on the trailing edge (Figure 2.1B).

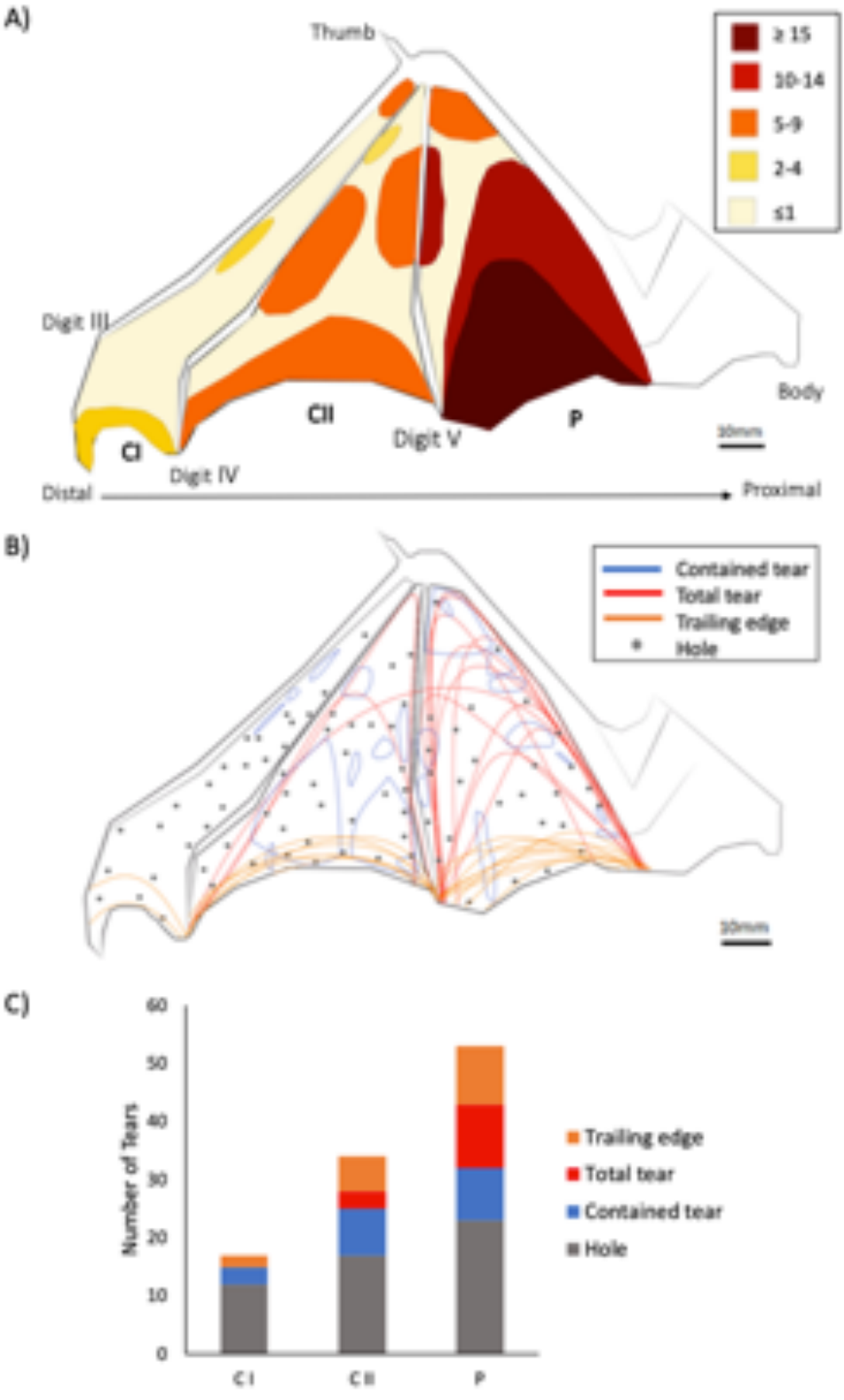


Figure 2.1: Wing tears in P. pipistrellus. A) A diagram of the wing, presenting the number and the location of the tears over the three sections. B) A diagram the wing presenting all tears found in bat wing images, over the three sections of the wing. C) The number of different types of wing tears in each section of the wing. The wing sections are CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section.

Other bat species

Other bat species also had statistically more wing tears in the P section than in CI and CII, (χ^2=8.773, df=2, p=0.012, Figure 2.2A, B, C), even though sample sizes were lower for these species. Holes and contained tears were common tear types, and section P was the only section to exhibit all the possible tear types. Similar to the *P. pipistrellus* results, tear types were commonly oriented in the rostro-caudal direction, from the membrane to the trailing edge, with only section P revealing one trailing edge tear, which was oriented distal-proximally.

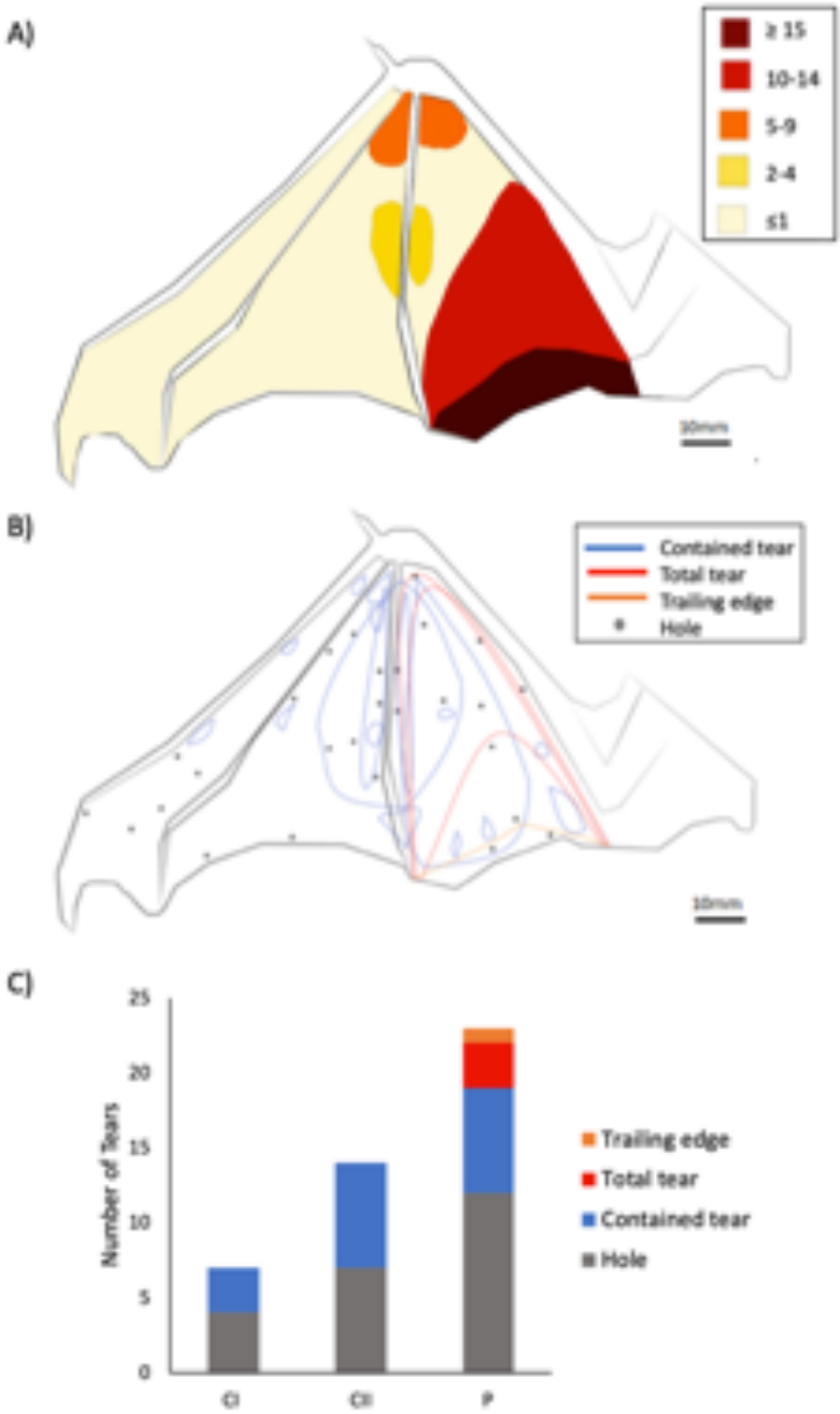


Figure 2.2: Wing tears in other bat species. A) A diagram of the wing, presenting the number and the location of the tears over the three sections. B) A diagram of the wing presenting all tears found in bat wings images, over the three sections of the wing. C) The number of different types of wing tears in each section of the wing. The wing sections are CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section.

2.3.2 Tear Recovery and Rehabilitation

Sample numbers reporting rehabilitation outcomes were fairly low (Appendix 3), so cannot be analysed here, however, images are included for comparison. Figures 2.3A and B show the length of time in rehabilitation of bats with wing tears. Larger tears percentage in CI did not appear to affect the length of time that *P. pipistrellus* spent in care. However, when *P. pipistrellus* had large tears in P, they were still in care after 6 months (Figure 2.3A). In addition, large tears in both CII and P were found in the euthanized *P. pipistrellus* bats (Figure 2.3A).

In other bat species, large tears in section P were found in the two bats that were still in care after 6 months (Figure 2.3 B), but tear size did not vary much between the other wing sections in euthanized bats, or those released after 2 weeks and 2-3 months (Figure 2.3 B).

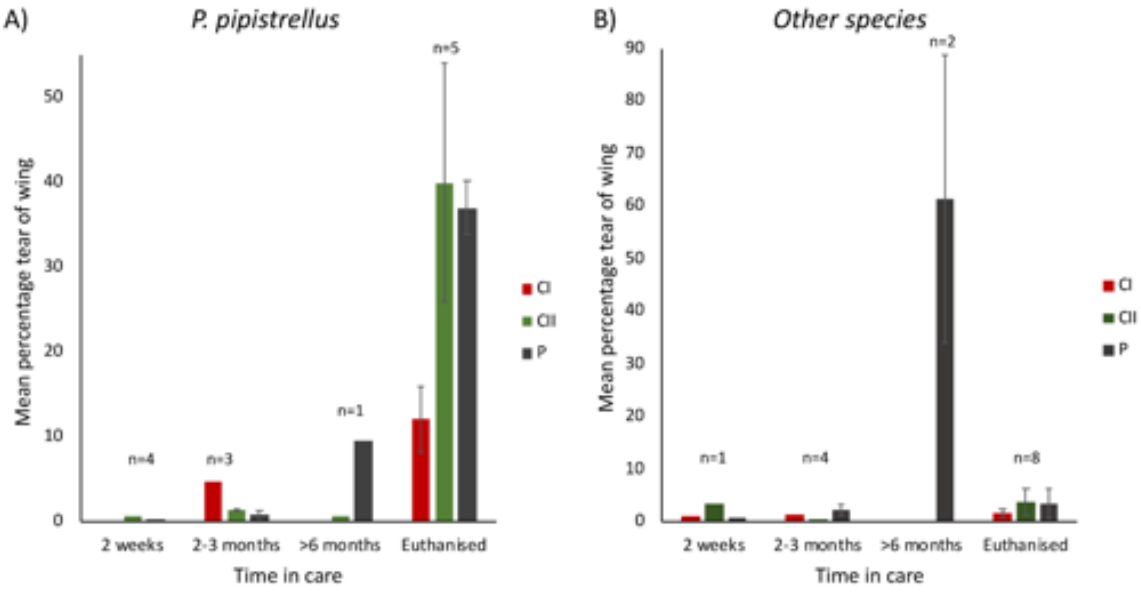


Figure 2.3: The length of time that bats were in care from when they receive a tear in a wing section, A) P. pipistrellus bats, B) Other bat species. The y-axis presents the percentage of tear in the section of the wing with CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section. n represents the total number of bats in that classification. Error bars are standard error.

2.3.3 Possible Causes of the Wing Tear

Out of 55 *P. pipistrellus* individuals, bat carers gave possible causes of their wing tears for eleven individuals. One was bought in to the house by a cat, four were seen being attacked by a cat (Figure 2.4a), and five were suspected by the carers to be cat attacks. One individual was found on the ground and was likely to have sustained tears from brambles on the floor (Figure 2.4b) and had tears throughout each section of the wing.

In other bat species, out of 21 individuals, bat carers gave possible causes for seven individuals. One bat was caught in flypaper, one was seen being attacked by a cat, and two were suspected by the carers to be cat attacks (Figure 2.4c). Three were seen in a cat's mouth (Figure 2.4d).

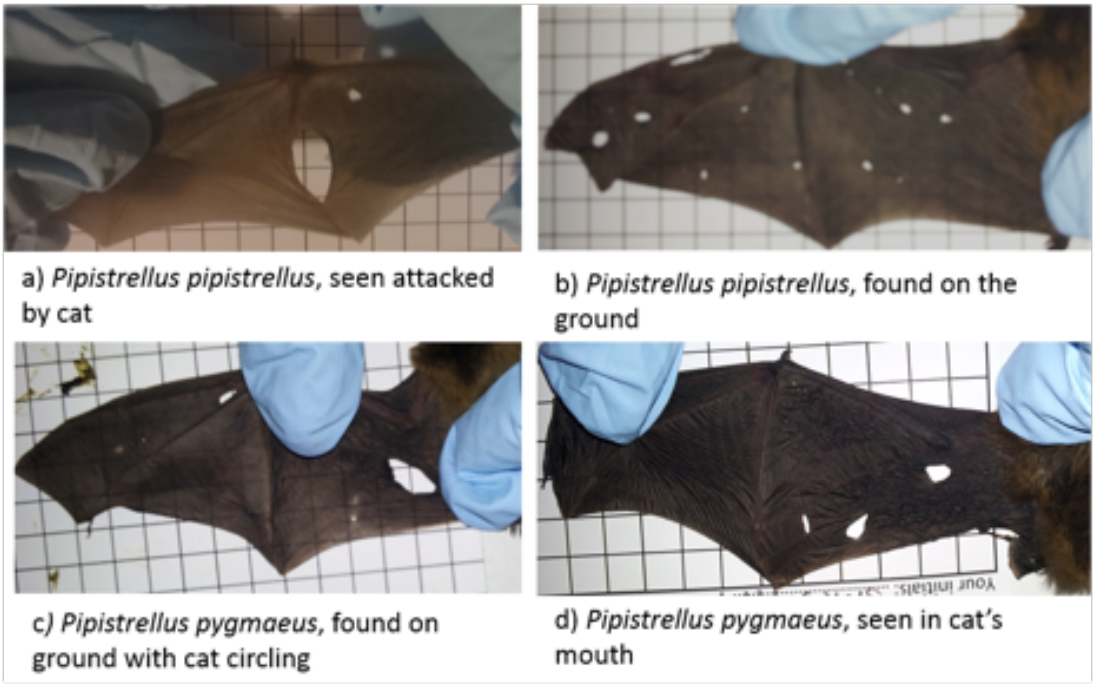


Figure 2.4: Example wing tears, with associated causes. Confirmed cat attacks have damage to the proximal wing sections (Section P) in Pipistrellus pipistrellus (a) and Pipistrellus pygmaeus (c and d). Grounded bats have damage to other areas of the wing in Pipistrellus pipistrellus (b) and Pipistrellus pygmaeus (c).

2.4 Discussion

The plagiopatagium section (P) sustained the most injuries when bats were surveyed in rescue centres. I suggest, through this discussion, that section P, being close to the body, might be targeted by predators, and propose that cat attacks might be contributing to the rostro-caudal tears in the P section. Finally, I discuss the possible causes of the tears.

Our results show that the P section contained the highest number of tears compared with the other sections, and holes were the most common tear types, compared with other tear types; rostro-caudal tears were common. In *P. pipistrellus* all tear types were found in all wing sections, but in the other bat species all tear types only occurred in the P section. Even though sample size is lower in this group, the tears followed the same pattern as seen in in *P. pipistrellus* bats, as the highest number of tears found in P section, compared to the more distal sections.

Across all species I looked at, section P contained the highest number of tears and often had the most varied types of tears too. This has not been specifically documented in the literature before; however, studying the majority of figures in Davis (1968) reveals that torn wings or large holes in Pallid bats (*Antrozous pallidus*) were common in section P, with CI and CII having more trailing edge tears (Davis 1968), consistent with our *P. pipistrellus* results in Figure 2.1B, C. Finding an anatomical explanation for the prevalence of tears in the P section is the aim of the following chapter of this thesis, Chapter 3.

All sections of the wing play key roles during flight. Section P acts to support the bat's body weight during flight and provides lift (Vaughan, 1970; Swartz et al., 1996; Neuweiler, 2000). Meanwhile, the flexible, mobile CI and CII sections provide thrust (Swartz et al., 1996; Neuweiler, 2000). Therefore, damage to different sections of the wing can affect flight in various ways. Tears in the CI or CII sections are more likely to affect manoeuvrability and speed, whereas tears in the P section may inhibit flight. However, bats are capable of flying with large tears in their wings (Davis, 1968). A

study by Voigt (2013) used two species of Myotis bats; Silver-tipped myotis (*Myotis albescens*) and Black myotis (*Myotis nigricans*), with unilateral trailing edge tears (of approximately 20%) in section P. It was found that injured bats made less flight manoeuvres (u-turns) and, as a result, had lower metabolic rates, than healthy individuals (Voigt, 2013). Therefore, foraging success and survival are likely to be strongly impacted due to limited manoeuvrability during flight. Large wing tears, as well as other injuries, can prevent flight altogether. Bats on the ground are likely to sustain even more wing tears from thorns and other ground matter (Davis, 1968) (see also Figure 2.4b, c), and might also explain the increase in tears in all wing sections in the very injured bats that were euthanized. Investigating the effect of wing tears on flight capabilities will help to assess the impact of tears for release and welfare recommendations.

I observed that larger tears in section P, were found in *P. pipistrellus* individuals that spent a long time in care (> 6 months) or were euthanized; although the size of tears in all sections were large in animals that were euthanized. In other UK bat species, the individuals that spent a long time in care (> 6 months) also had large tears in the P section of their wings. However, it must be noted that decisions about release, rehabilitation and euthanasia are highly subjective; they are dependent, not only on the extent of the injury, but on the judgement of the bat carer, and also the season and weather appropriate for release.

Greville et al. (2018) also found that in the Egyptian fruit bat (*Rousettus aegyptiacus*) wounds took around 1.5 days longer to heal to 50% wound closure in section P, compared to the chiropatagium (sections CI and CII), although this was not found in the Big brown bat (*Eptesicus fuscus*). They suggest that understanding more about the fibres and vasculature within the wing, might explain their ability to heal (Greville et al., 2018), which is exactly what I will do in the following chapter.

Possible causes

Wing tears and holes have been found to occur as a result of colliding with manufactured objects or plants with spikes and thorns (Davis, 1968), fungal infections, such as the keratin-digesting fungus (*Pseudogymnoascus destructans*) (Reichard and Kunz 2009; Cryan et al., 2010; Fuller et al., 2011, Lorch et al., 2016), or from predator attacks, including cats (Ancillotto et al., 2013; Loss et al., 2013; Russo and Ancillotto, 2015) and birds of prey (Speakman, 1991). It might be assumed that collisions are more likely to be evidenced by holes or tears on the distal wing sections (i.e. section CI), and may be oriented rostro-caudally, in the direction of flight. I saw significantly more tears in section P; these could be holes and horizontal trailing edge tears, but many were rostro-caudal, starting from the middle of the wing and extending to the trailing edge. I, therefore, suggest that where there are mainly holes or tears in proximal wing sections, these may well be caused by predators, including cat (*Felis silvestris catus*) attacks (Figure 2.4a, c, d) and perhaps failed talon strikes in birds of prey, such as barn owls (*Tyto alba*) (Speakman, 1991).

Some bat carers communicated with us about the cause of the tears. For *P. pipistrellus*, they included being found on the ground (n=1), brought in the house by a cat (n=1), attacked by a cat (n=4), and suspected cat attacks (n=5). For other UK bat species, tear causes included being caught in flypaper (n=1), seen in a cat's mouth (n=3), seen attacked by a cat (n=1), and suspected cat attacks (n=2). Cats have relatively stereotypical predatory behaviour, including hissing, ear flattening, and striking or raking objects with claws (Berntson et al., 1976). Attack behaviour also escalates relatively formulaically, including stalking, cuffing or pushing with the paw, pinning with the paw, and biting (Berntson et al., 1976). These paw-prey interactions may well have caused the tears on the P section that I observed. Perhaps pinning might lead to holes, and cuffing or raking to rostro-caudal tears. One study undertaken in Italy in 2009 – 2011 found that predation by cats accounted for 28.7% of records of adult bats admitted to rehabilitation centres (Ancillotto et al., 2013), which was based on the bat carers subjectively identifying the cause of the tear. As yet, there has been no research to objectively identify the causes of wing tears. Identifying these causes reliably will help us to understand the scale of the problem

and exploring the causes driving these injures will have implications for bat conservation, welfare and rehabilitation recommendations. Moreover, the cause of the wing tear will be investigated further as a part of this thesis in Chapter 5.

Implications

In conclusion the plagiopatagium wing section had the most tears and took the longest time to heal. The position of the tears, close to the body, and their rostro-caudal orientation might imply that this is caused by a predator attack, such as a cat, rather than a collision. This study provides the first quantitative description of wing tears, and further investigations should focus on the cause of the tears, and their effect on flight capabilities. Investigating the extensive problem of wing tear injuries in bats will help to improve rehabilitation plans, and has significant implications for conservation and welfare. Chapter 3 will investigate the anatomy of the wing, and see whether it is sufficient to understand why there are more tears in section P, which take longer to heal.

Chapter 3 Describing the anatomy of Common Pipistrelle (*Pipistrellus pipistrellus*) wing

This chapter has been accepted for publication in the Journal of Mammalogy:

Khayat, R. O., Shaw, K. J., Dougill, G., Melling, L. M., Ferris, G. R., Cooper, G., and Grant, R. A. 'Characterizing wing tears in common pipistrelles (Pipistrellus pipistrellus): investigating tear distribution, wing strength, and possible causes.' Journal of Mammalogy, pp. 1-13. (Appendix 1).

Chapter summary:

This Chapter describes bat wing anatomy, and considers whether it is able to account for the position and orientation of wing tears, as well as their healing rates. I describe the blood vessels, fibres orientations and material properties of each wing sections. The results indicated that while the anatomy might be able to predict healing capabilities, it is not sufficient in accounting for tear position and orientation.

3.1 Introduction

Bat wings cover more than a third of their body surface (Makanya and Mortola, 2007). The wing consists of four sections which are protopatagium, chiropatagium, plagiopatagium, and uropatagium – a tail membrane that can be found in some bat species (Figure 2.1A) (Pollock et al., 2016). The wing skin is very thin and has a distinct anatomy compared to body skin (Madej et al., 2012; Cheney et al., 2017). Due to the large size and thin pattern of the bat wings, the wings are commonly injured (Davis, 1968), and the most frequent injury seen in some bat populations is wing tears (Davis, 1968). However, bat wings have an excellent blood supply that contributes to rapid healing when bat wings are torn (Davis and Doster, 1972; Faure et al., 2009; Weaver et al., 2009). Bat wings have an extensive blood supply to enable wound cleaning, infection prevention and tissue reformation (Faure et al., 2009).

Wing material properties show strong anisotropy and vary between the wing sections and bat species (Swartz et al., 1996; Skulborstad et al., 2015). The wing also has a unique arrangement of fibres in a net-like arrangement, containing collagen and elastin. This supports the wing skin and reinforces it, increasing their resistance to puncture (Studier, 1972; Holbrook and Odland, 1978; Cheney et al., 2017; Madej et al., 2012).

While there is a good understanding of wing anatomy, including fibres, venation and material properties (Wiedeman, 1963; Swartz et al., 1996; Skulborstad et al., 2015; Cheney et al., 2015; 2017), these tend to vary between species (Swartz et al., 1996). Wing anatomy has never been documented in Common pipistrelles *(Pipistrellus pipistrellus)*, probably due to them being small and difficult to process. Furthermore, how the wing anatomy might predict the positioning of wing tears and their healing capabilities, has yet to be explored. This chapter will, therefore, explore the anatomy of the wing in *P. pipistrellus*, and see if knowledge of the anatomy is sufficient to understand wing tear placement and healing rates. The blood vessels in bat wings will be traced to assess the blood vessel number and density over the wing sections. Histological techniques will find fibre orientations and quantify the amounts of collagen and elastin fibres in the wing sections. Finally, material testing will be undertaken in each section of the wing.

3.2 Methods

This chapter refers to the anatomy of *P. pipistrellus* wings over three sections (as per Chapter 2). The most distal section of the chiropatagium (CI) is the membrane between digits iii and iv. The second chiropatagium section (CII) is the membrane between digits iv and v. The most proximal section of the wing is the plagiopatagium (P), which is the membrane between digit v and the body (Figure 2.1A). Ethical approval was obtained through the Research Ethics and Governance Committee at Manchester Metropolitan University, and all tissue was held under a Natural England license (2014-4322-SCI-SCI).

10 adult whole-animal *P. pipistrellus* specimens were donated by bat carers from euthanized animals for the vessel tracing (2 bats, right side wings), histology (2 bats used in Masson's Trichrome staining and 2 bats used in Veroeff-Van Gieson staining, right side wings) and material testing (10 bats in total, left side wings, including the 6 bats from the vessel and histology work). These animals were admitted to care following injury and grounding; although exact details were not known by the carers they likely had many internal injuries and complications. All individuals had intact wings so I could examine their anatomy. All photography was undertaken from live animals during usual husbandry and rehabilitation procedures carried out by bat carers.

3.2.1 Tracing the Vessels

Samples

Ten bat specimens were used to identify all the major blood vessels in *P. pipistrellus* wings. Two whole-bat euthanised specimens were donated by bat carers, and kept in the freezer at -20°C. Their right wings were removed whole and stored in 4% paraformaldehyde (PFA), at 4°C. The wings were stretched out over a bright lightbox (Phlox, SLLUB), and photographed using a digital camera (Nikon, D3200). Another eight photographs were collected from bat carers, who had stretched the wings of live bats, admitted for rehabilitation, over a white piece of 1 cm gridded card. All wings were intact and did not contain any holes or scarring.

Image tracing and data collection

Inkscape software Version 0.91 (available from: inkscape.org/en/) was used to trace the blood vessels in all the photographs. Some vessels were twice as thick as other vessels, and these were traced with thicker lines. Once the vessels had been drawn in all the photographs, they were combined into one figure to give a summary of all the possible blood vessels over the three sections of the wing. This figure was validated against descriptions in the literature (Pavlinić et al., 2008). The number of vessels were counted in each section of the wing (CI, CII, P), and any bifurcations were counted as an additional vessel. The area of each section of the wing was also

measured using Inkscape, to give an approximation of blood vessel density (count/wing area).

Statistical analysis of blood vessels number and density

The statistical analysis was conducted using SPSS Version 24. As the data was not normally distributed, non-parametric tests were used. The Kruskal-Wallis test was run to compare between the three sections of the wing in terms number of blood vessels and density of blood vessels (number/cm^2), pairwise comparisons were undertaken using Mann-Whitney tests, with a Bonferrroni correction applied at the $p<0.01$ level of significance.

3.2.2 Histology

3.2.2.1 Fibre Orientation

Sample sectioning

To identify wing fibre orientation over the three sections of the wing, Masson's Trichrome staining was used. Two wings from euthanised *P. pipistrellus* bats were stored in 4% PFA, at 4°C. A fragment from each section of the wing (approximately 10 mm^2) was dissected and sliced tangential to the wing at 30 µm thickness using a freezing cryostat (Leica CM3050 at -20°C). This thickness was selected to reduce the curling and wrinkling of slices that occurred in thinner sections.

Staining

The slices were transferred to a solution of 10% phosphate buffered saline (PBS) overnight and then mounted on to microscope slides (Thermo scientific, Menzel-Glaser) and left to dry for a further 24 hours. The slices were then stained using Masson's Trichrome (Sigma-Aldrich, Trichrome Stain Kit). Slides were put in a fixative solution (4% paraformaldehyde in 0.1 M PBS) for 1 hr, and introduced to Bouin's Solution (Bouin's solution, Sigma-Aldrich) (which consists of picric acid, acetic acid and formaldehyde in an aqueous solution) for 3 hrs. They were then cleared with xylene (analytical reagent grade, Fisher), rehydrated with descending concentrations

of ethyl alcohol (100, 90, 80, and 70%) and moved through a sequence of solutions for the Masson's Trichrome staining. The slices were stained with Biebrich Scarlet (Biebrich Scarlet, HT151, Sigma-Aldrich) for 5 minutes, then washed three times with double-distilled water for 1 minute each. The fibres were stained by placing the slides for 10 minutes in an acid solution (which consists of 66% double-distilled water to 33% phosphotungstic/ phosphomolybdic acid). The slices were stained with Aniline Blue (Aniline blue, B8563, Sigma-Aldrich) for 5 minutes, then washed three times with double-distilled water for 1 minute each. Next, the slides were transferred to acidified water (1 ml of acetic acid in 200 ml of double-distilled water) for 2 minutes, then washed with double-distilled water for 1 minute, twice. The slices were then dehydrated with ascending concentrations of ethyl alcohol (70, 90, and 100%) and xylene, towel dried and cover-slipped using Distyrene Plasticizer Xylene (DPX) (Sigma-Aldrich).

3.2.2.2 Fibre Quantification

Sample preparation and cutting

To measure relative amounts of collagen and elastin within the sections of the wing, Veroeff-Van Gieson (VVG) staining was used. Two wings from two euthanised *P. pipistrellus* bats were stored in 4% PFA, at 4°C. A sample (approximately 10 mm^2) was removed from each section of the wing and placed in 4% PFA overnight at 4°C (Sears et al., 2006). Each sample was embedded in 2% agar in PBS and transferred in a histology cassette for tissue processing (Shandon - Citadel 200). The samples were placed subsequently in 70% Industrial Methylated Spirits (IMS) (Methanol HPLC, Fisher Chemicals) for 3 hours, 80% IMS for 60 min, 90% IMS for 60 min, 100% IMS for 2 hours twice, 100% IMS for 60 min, then Xylene for 90 min twice, Xylene for 2 hours, and finally in paraffin wax twice for 3 hours. Afterwards, the samples were embedded in paraffin wax for slicing. Each sample was sliced across the wing axis (perpendicular to tangential) at 5 µm thickness (Madej et al., 2012) on an automatic, rotary microtome with water bath (Thermo Scientific microtome HM355S), collected on glass slides (Superfrost Plus, Thermo Scientific) and incubated in an oven at 37°C overnight before staining.

Staining

The slides were cleared with xylene and rehydrated with ethyl alcohol (100%, 90%, 80%, 70% and distilled water) prior to staining. To stain the elastin, the slides were placed for 10 min in working elastic stain solution (Verhoeff's working solution), which consisted of 20 mL of Haematoxylin solution (HT 251, Sigma-Aldrich), 3 mL ferric chloride solution (HT252, Sigma-Aldrich), 8 mL of Weigert's iodine solution (HT253, Sigma-Aldrich), and 5 mL deionised water. The slides were then rinsed in deionised water and differentiated in Ferric Chloride solution, comprising of 3 mL Ferric Chloride Solution (HT252, Sigma-Aldrich) and 37 mL of deionised water. Next, the slides were rinsed in tap water, and placed in 95% ethyl alcohol to remove the iodine and then in deionised water. After that, slides were stained for collagen in Van Gieson's solution (Sigma-Aldrich) for 1-3 min, then rinsed in 95% alcohol. Finally, they were dehydrated (100% ethyl alcohol), placed in xylene and cover-slipped with DPX.

3.2.2.3 Image Capture and Analysis

All slices were visualised using a Zeiss Stereo Lumar V12 light microscope. Figures were captured using Zeiss Axiovision, version 4.8. Additionally, the light intensity was standardised at 3200K for all figures captured. Occasional adjustments to exposure and white balance were made. The fibre orientation was described qualitatively for each section stained with Masson's Trichrome. The relative amounts of collagen and elastin were quantitatively approximated using image processing in MatLab (Mathworks, USA) (available from: uk.mathworks.com/products/matlab.html.) from each section stained with VVG. VVG is a standard histological stain used to identify collagen and elastin fibres (Fullmer and Lillie, 1956; Kazlouskaya et al., 2013; Cheney et al., 2017), and has been previously used to quantify amounts of collagen and elastin in stained tissues (Daamen et al., 2003; Raub et al., 2010; Eberson et al., 2015; Wheeler et al., 2015; Lee et al., 2016). Images were selected for image processing when the section was clear and not folded so that all fibres could be seen in the image. Ten to twelve slices were taken for each wing section and then two to three images captured from each slice, giving a total of 35 images for each section.

Original 8-bit colour images of the slides reveal collagen as a red/pink colour and elastin as a dark, near black colour (Figure 3.1, top panel). Filters were applied to create two black and white images from each original image, one that showed only collagen and a second showing only elastin (Figure 3.1, middle and bottom panels respectively). A range of filter strengths were tested on the sample images and visually inspected for accuracy by three observers, each working independently. The position and presence of collagen fibres was also validated by comparing the Veroeff-Van Gieson slices to a sub-set of slices that were stained with Sirius Red. The settings observed to provide the most accurate separation of collagen and elastin fibres were then applied to the full image set. Elastin images were created by filtering out pixels with a moderate or high 8-bit colour intensity in any RGB channel. Some black colour could be seen at the edges of the slices, consisting of melanin in the bat wing skin. Although some elastin was also likely to occur in this area, the edges of the sample were cropped to focus on measuring the internal elastin fibres only (Figure 3.1, bottom panels). Collagen images were created by filtering out any pixel with a high green or blue intensity or a low red channel intensity; the red channel threshold was determined automatically (graythresh in Matlab using Otsu's method) on an image-by-image basis taking into account the overall colour spectrum of the image. Relative percentages of collagen and elastin were calculated by counting the number of white pixels in each of the generated images. Example images can be seen in Figure 3.1.

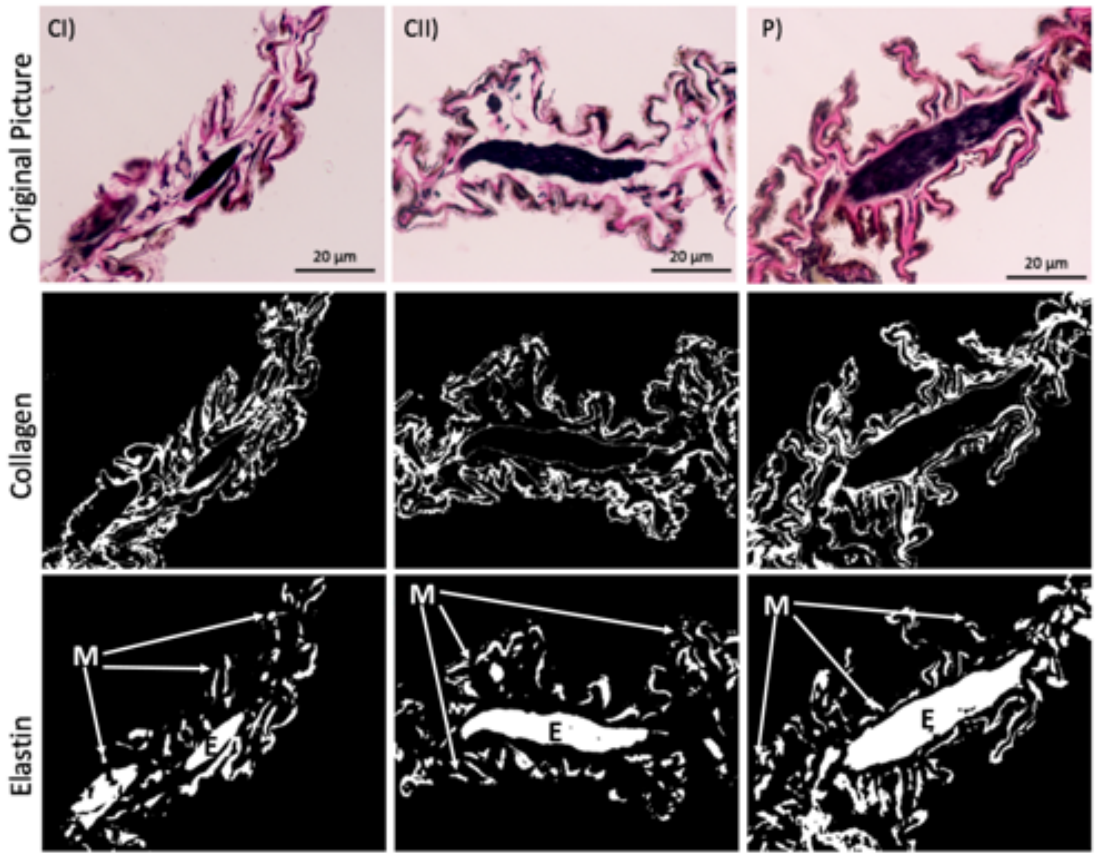


Figure 3.1: Example images demonstrating the processing of elastin and collagen fibres. The top panel shows the original images collected from the microscope following Van Gieson staining. These were processed to find the red/pink collagen colours (middle panels) and the dark elastin colours (bottom panels), for CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section. In the bottom panels, M representing the melanin and E representing the elastin.

Statistical analysis for quantification of collagen and elastin fibres

Statistical analysis was undertaken using SPSS Version 24. The non-parametric Kruskal-Wallis test was performed to compare the percentage of the collagen and elastin between the three sections of the wings, and Pairwise comparisons with Mann-Whitney tests, with a Bonferrroni correction applied at the $p<0.01$ level of significance.

3.2.3 Material Testing

Samples

Ten left wings were used to test the material properties of each wing section; these were all from euthanised bats. Each wing was kept in a freezer at -20°C, and then defrosted in 10% PBS for 10 minutes. Freezing may have affected the mechanical properties of the samples, and previous studies have shown mixed results (Wang et al., 2007; Kaye et al., 2012), with freezing not having an effect in some cases (Foutz et al., 1992; Van Ee et al., 2000; Santago et al., 2009). As all samples were frozen, I was able to compare between and within samples and confidently observe relative differences, but the absolute values may vary from other studies. After defrosting, long strips were cut out from each wing section, from the digit joint to the trailing edge (Figure 3.2). The length and width of each strip sample was measured with a ruler (Table 3.3). The wing thickness was calculated as the mean of three measurements by placing the sample on glass beads and using a microscope (Lumar.V12) with a calibrated camera (AxioCam MRc). Samples were kept hydrated in 10% PBS and tested before drying. The tensile tests were conducted in the engineering laboratories at Manchester Metropolitan University. Each sample was gripped in a tensometer (Hounsfield H10KS) using pneumatic grips (HT pneumatic grip) with an external air supply line, to ensure consistent grip with 5 bar pressure across all tests. A gauge length of 5 mm was used for all samples, with approximately 11 mm in each grip (refer to average length in Table 3.3). Each sample was stretched at 10 mm/min until failure, along its longest axis (from the digit joint to the trailing edge). The load cell used was 100N (Tinius Olsen, DBB MTOL-100N).

Due to the small size and the delicate nature of the wing of *P. pipistrellus* the aspect ratios of the tested samples were fairly small and it was not possible to cut a 'dog-bone' shape which is typically used in materials testing. This shape, which is wider at the ends held between the clamps than in the middle, ensures that test samples fail in the middle due to axial forces and not due to transverse forces applied by the clamps. Therefore, the 3/10 samples which failed at the grips were removed from

further analyses to reduce the effect of incidental bias of transverse stresses on failure stress results.

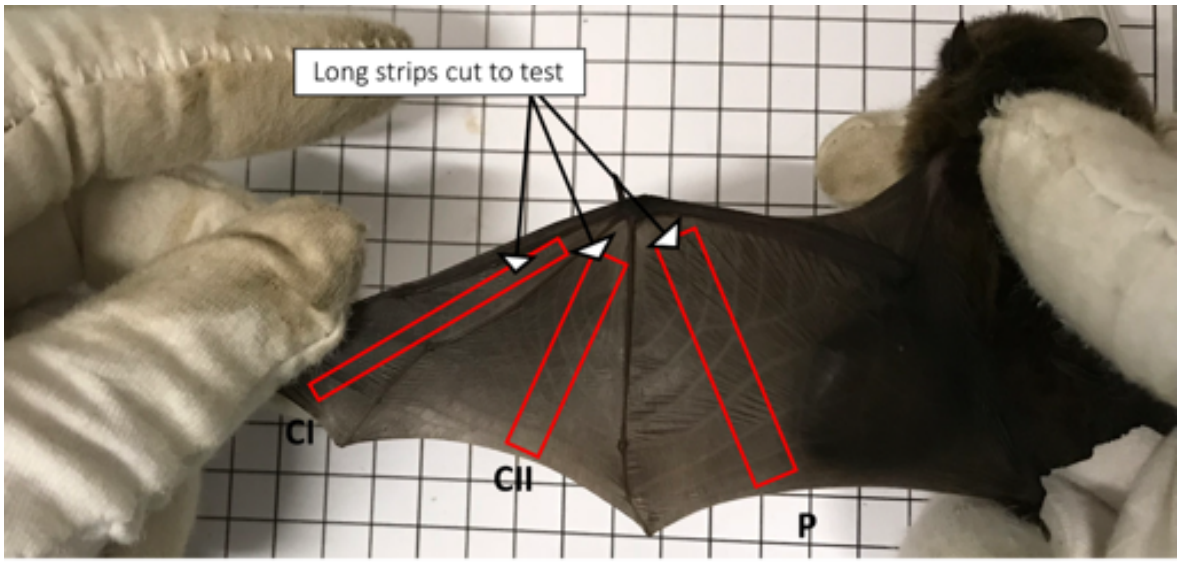


Figure 3.2: Long strips cut from the three sections of the wing CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section, for the material testing.

Data collection

Qmat 5.52 software was used to collect the data from each sample. The maximum force at failure (N) and maximum extension (mm) was recorded. From these values, failure stress (force at failure divided by the cross-sectional area) (Equation 3.1), failure strain (maximum extension divided by the original sample length of 5 mm) (Equation 3.2) and Young's modulus (change in stress divided by the change in strain) (Equation 3.3) (Vable, 2012), were all calculated for each sample from each wing section. As the P section's strips tended to be wider than the other sections (Table 3.3), component stiffness (force at failure divided by sample width, divided by failure strain) (Equation 3.4) was also calculated to control for sample width but not thickness. Results did not exhibit the extreme variation between "toe" – an initial period of low, non-linear stiffness in polymers – and "upturn" – the major linear region of a polymer stress-strain curve – that was observed in Skullborstad et al., 2015. Therefore, a single gradient was calculated from the major linear region of each curve.

$$Stress = force/(the\ thickness\ of\ the\ sample \times the\ width\ of\ the\ sample) \quad (3.1)$$

$$Strain = extension / length \quad (3.2)$$

$$Young's\ modulus = \frac{\Delta\ stress}{\Delta\ strain} \quad (3.3)$$

$$Component\ stiffness = (force/sampl\ width)/ failure\ strain \quad (3.4)$$

Statistical analysis of the wing material testing

The three sections of the wing were compared in the following variables: section thickness (mm), stress (N/mm^2), strain (mm/mm), Young's modulus (N/mm^2), and Component Stiffness (N/mm) using a Kruskal-Wallis test in SPSS Version24. Pairwise comparisons were carried out using Mann-Whitney tests, with a Bonferroni correction applied at the $p<0.01$ level of significance.

3.3 Results

3.3.1 Tracing the Vessels

The 10 traced bat wing images can be seen in Figure 3.3. While there is some variation, section P contained the fewest vessels, and also the thickest vessels. All these images were combined into one 'model' bat wing, containing all the vessels present in the 10 images (Figure 3.4B). The results indicated that section P had significantly fewer blood vessels than sections CI and CII (Table 3.1, $p<0.01$, Figure 3.5A). From the observation it also appeared to have the thickest blood vessels, as indicated by the thicker lines (Figure 3.4B). However, section P was also the largest section. When the number of blood vessels was normalised to the area of each wing section, there was no significant difference in blood vessel density between sections P and CII (Table 3.1), but section CI had the densest arrangement of blood vessels (Table 3.1, $p<0.01$, Figure 3.5B).

Table 3.1: *Comparing the blood vessels number and density at the three wing sections for CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section. Values are mean ±sd, n= 10. The statistics present the results of Kruskal-Wallis tests between the three sections of the wing, with a Bonferroni correction applied at the p<0.01 level of significance.*

Wing Section:	**CI**	**CII**	**P**	**Statistics**
Number	14.00±1.70	12.60±1.51	9.00±1.56	X^2=18.686, df=2, p<0.001 (CI, CII > P[i])*
Density (no/cm^2)	2.16±0.99	0.98±0.54	0.63±0.48	X^2=13.628, df=2, p=0.01 (CI > CII, P[ii])*

** Pairwise comparisons values:*

i) Number: CI vs CII: W=5.5, p=0.463, CI vs. P: W=16.4, p<0.001 and CII vs. P: W=10.9, p=0.005.

ii) Density: CI vs CII: W=9.4, p=0.51, CI vs. P: W=14.3, p=0.001 and CII vs. P: W=4.9, p=0.64.

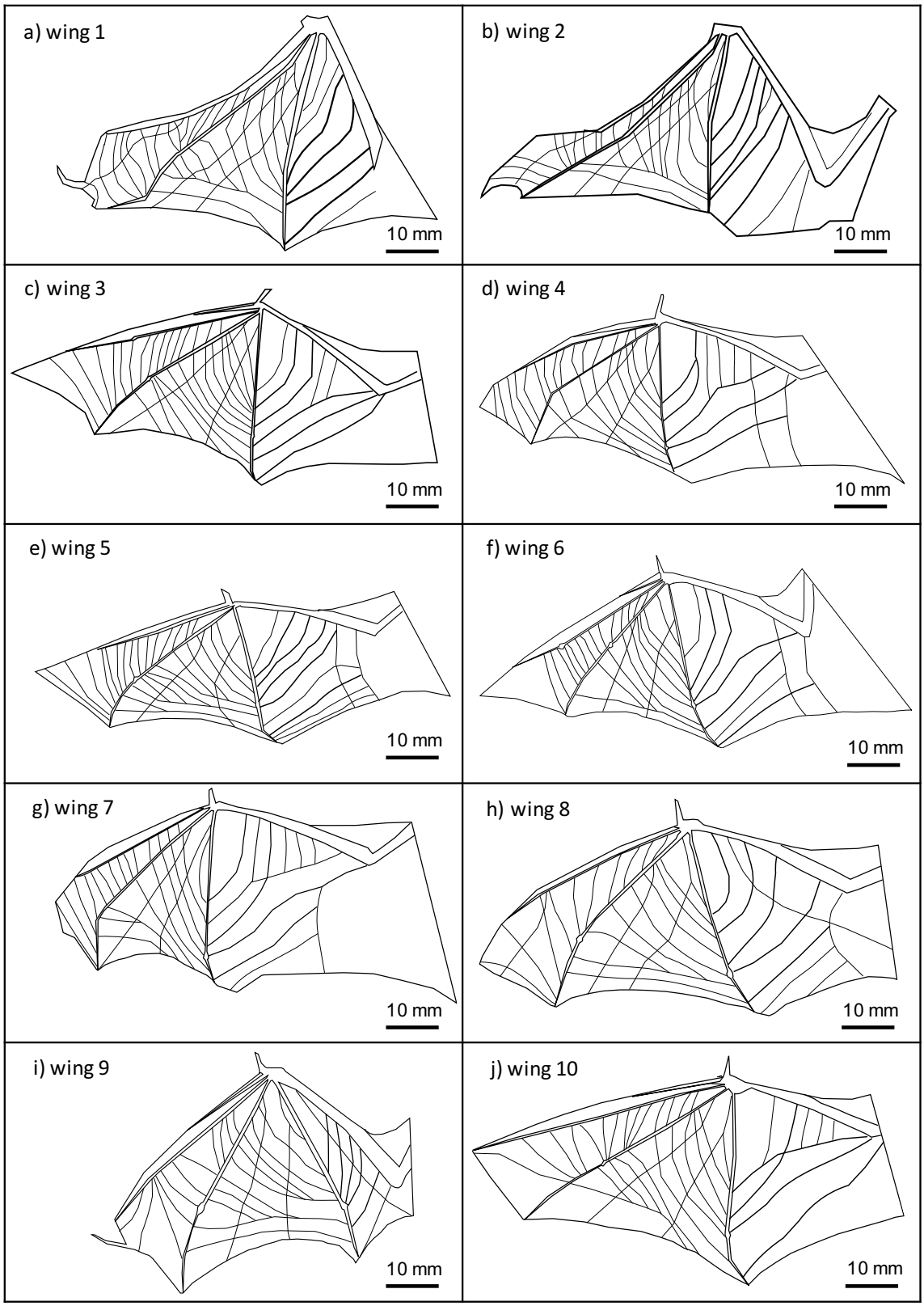


Figure 3.3: Tracing the vessels on 10 bat wing images at the three wing sections.

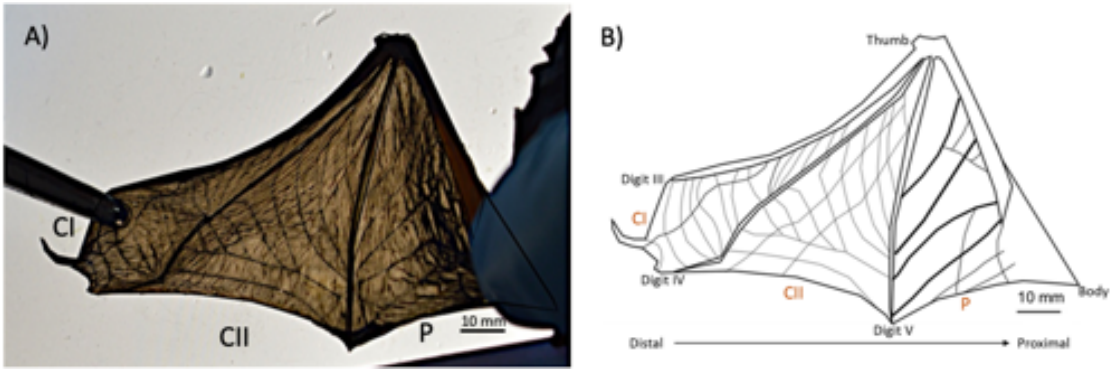


Figure 3.4: Tracing the vessels on the bat wing sections, the first chiropatagium section (CI); the second chiropatagium section (CII); and the plagiopatagium section (P). Panel A shows the three sections of the wing that were used to trace the vessels. Panel B shows tracing the vessels at those three sections of the wing (CI, CII, P). Digits III, IV and V are also indicated on the figure, digit II is not visible as it is folded against digit III, and digit I is the thumb.

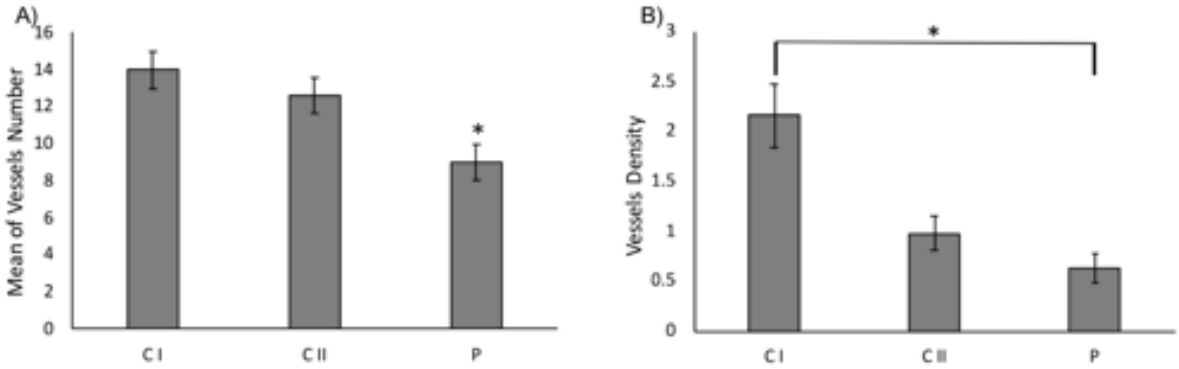


Figure 3.5: Panel A, shows the average vessel number in each section of the wing. Panel B, showing the blood vessels density in the three sections of the wing. Those sections are the first chiropatagium section (CI); the second chiropatagium section (CII); and the plagiopatagium section (P) (in panel A, significant difference between P section and CI, CII sections, in panel B, significant difference between P section and CI section). Error bars are standard error.*

3.3.2 Histology

3.3.2.1 Fibre Orientation

The orientation of fibres within the wing tended to be multi-directional and distributed in a net-like fashion throughout the membrane, with a similar appearance in each wing section (Figure 3.6). The appearance of the fibres at many orientations indicates that the material is visually isotropic.

Figure 3.6: Fibre orientations over the three section of the wing, the first chiropatagium section (CI); the second chiropatagium section (CII); and the plagiopatagium section (P).

3.3.2.2 Fibre Quantification

By analysing the histological images and quantifying the amounts of collagen and elastin in each section and by calculating the percentage of collagen and elastin in each section, the amount of collagen (%) was significantly higher in section CI and the amount of elastin (%) was significantly lower in section CI, compared to section CII and P (Table 3.2, $p<0.01$; Figure 3.7)

Table 3.2: Comparing the percentage of fibres in the three wing sections CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section. Values are mean ±sd, n= 2 bats, 35 histological images per section. The statistics present the results of Kruskal-Wallis tests between wing sections, with a Bonferroni correction applied at the p<0.01 level of significance.

Wing Section:	**CI**	**CII**	**P**	**Statistics**
% Collagen	79.92±13.73	43.09±21.78	52.47±26.95	X^2=35.922, df=2, p<0.001 (CI > CII, P^{i})*
% Elastin	20.08±13.73	56.91±21.78	47.53±26.95	X^2=35.922, df=2, p<0.001 (CI < CII, P^{ii})*

** Pairwise comparisons values:*

i) % collagen: CI vs CII: W=41.97, p<0.01, CI vs. P: W=31.41, p<0.001 and CII vs. P: W=-10.66, p=0.44.

ii) % Elastin: CI vs CII: W=-41.97, p<0.01, CI vs. P: W=-31.314, p<0.01 and CII vs. P: W=10.65, p=0.43.

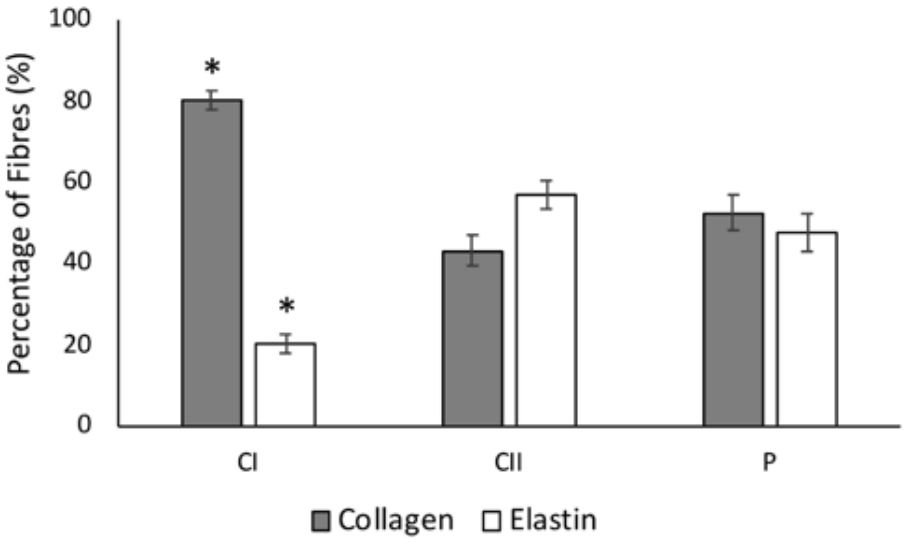


*Figure 3.7: The percentage of collagen and elastin fibres in the three sections of the wing, the first chiropatagium section (CI); the second chiropatagium section (CII); and the plagiopatagium section (P). (*Significant difference in collagen and elastin fibre percentage between CI section and CII, P sections). Error bars are the standard error.*

3.3.3 Material Testing

The results of comparing the material properties between the three sections of the wing showed that, section P was the thickest section (Table 3.3, $p<0.05$, Figure 3.8A). No significant differences were observed between the wing sections for any of the material testing measurements (Table 3.3). Although, CI tended to have the smallest deformation (failure strain) and highest Young's modulus (compared to section P) (Table 3.3, Figure 3.8C, D); and section P also tend to have the lowest failure stress (Table 3.3, Figure 3.8B) and Component Stiffness (Table 3.3, Figure 3.8E).

Table 3.3: Comparing material properties of the three wing sections, the first chiropatagium section (CI); the second chiropatagium section (CII); and the plagiopatagium section (P). Values are mean ±sd, and n.a refers to when it was not applicable to run statistical tests. n=7. The statistics present the results of Kruskal-Wallis tests between wing sections, with a Bonferroni correction applied. p was considered significant at the <0.01 level.

Wing Section:	**CI**	**CII**	**P**	**Statistics**
Section Length (mm)	33.6±7.97	26.66±7.45	26.03±5.79	n.a
Section Width (mm)	2.83±1.18	4.59±0.96	5.6±1.36	n.a
Section thickness (mm)	0.22±0.04	0.24±0.02	0.33±0.04	X^2=13.569, df=2, p=0.001 (CI, CII < P[i])*
Failure Stress (N/mm^2)	2.32±0.81	2.48±0.94	1.58±0.61	X^2=4.364, df=2, p=0.113
Failure Strain (mm/mm)	0.37±0.110	0.61±0.18	0.63±0.20	X^2=9.062, df=2, p=0.011 (CI < CII, P)
Young's Modulus (N/mm^2)	8.07±2.19	6.22±3.12	4.45±1.97	X^2=6.033, df=2, p=0.049 (CI>P)
Component Stiffness (N/mm)	1.37±0.29	1.09±0.61	0.93±0.55	X^2=2.879, df=2, p=0.237

** Pairwise comparisons values:*

i) Section thickness: CI vs CII: W=-3.57, p=0.83, CI vs. P: W=-11.85, p<0.01 and CII vs. P: W=-8.27, p<0.01.

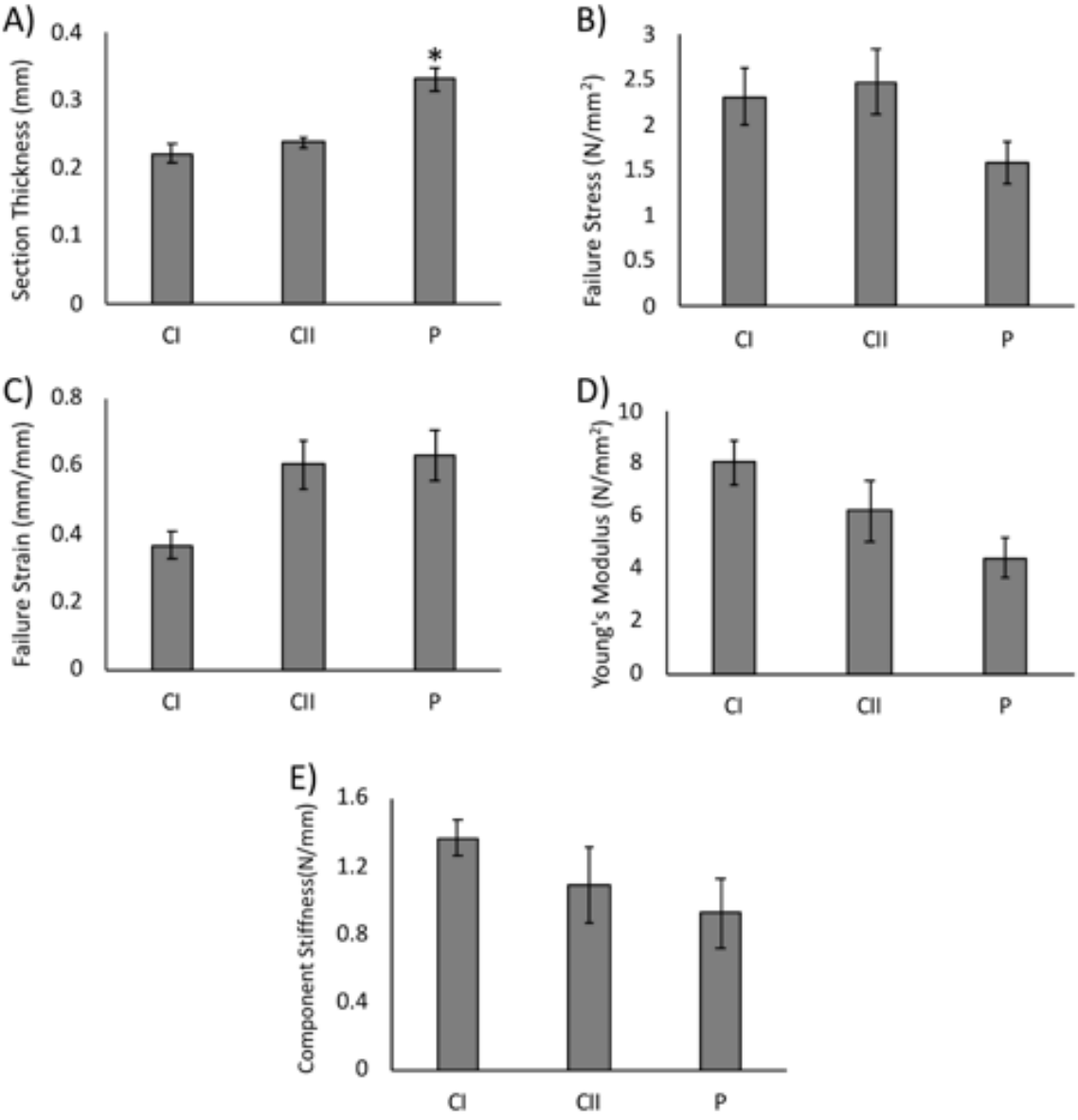


*Figure 3.8: Material testing of bat wing samples. A) section thickness, and B) failure stress, C) failure strain, D) Young's modulus, E) Component stiffness. Wing sections correspond to: the first chiropatagium section (CI), the second chiropatagium section (CII) and the plagiopatagium section (P). (*Significant difference in section thickness between P section and CI, CII sections). Error bars are the standard error.*

3.4 Discussion

CI section has the highest percentage of collagen fibres and the lowest percentage of elastin fibres compared to CII and P sections. However, there are no significant differences in material testing measurements between the three wing sections, which means that each is as likely to tear. Coupling these findings, with those from Chapter 2, I consider tearing capacity, and suggest that, according to the anatomy, section P should not be more prone to tearing than any other section. Therefore, the position of the plagiopatagium, rather than its anatomy, is an important factor in determining the number, location and orientation of wing tears. Moreover, while rostro-caudal tears might take longer to heal, owing to the orientation of elastin fibres, they should not be more commonplace in the first instance. Section P has the fewest blood vessels and lowest vessel density. I suggest that low blood vessel density and high amounts of elastin fibres might lead to slow healing in this section.

3.4.1 Tear Positioning

In Chapter 2, across all species I looked at, section P contained the highest number of tears and often had the most varied types of tears too. Within a bat wing, elastin fibres run perpendicular to the wing bones and collagen fibres create a network parallel and perpendicular to the elastin, which has been described in a number of studies (Holbrook and Odland 1978; Madej et al., 2012; Cheney et al., 2015; 2017). This net-like fibre array provides tensile strength and limits extension of the wing membranes, which is important for flight (Holbrook and Odland 1978). Qualitatively, I have also observed a fibrous net, which can be seen with a similar appearance in each section of the wing (Figure 3.6). This net might act to limit tears from extending, and its equal distribution across all the wing sections reinforces the whole wing surface (Holbrook and Odland 1978). It probably contributes to maintaining the holes as small holes, rather than tearing any further. Indeed, holes are the most common tear type (Chapter 2). That this net does not visually appear to differ between the three wing sections, indicates that they should each be similarly resistant to tearing.

Quasi-static material testing and analysis of collagen-elastin percentages were carried out to compare the three wing sections further. Section CI had significantly higher relative collagen percentages than the other wing sections (% collagen, Table 3.2) and tended to exhibit the least extension (failure strain, Table 3.3, Figure 3.8C, although not significant $p>0.01$). Sections CII and P exhibited more equal collagen-elastin ratios and consequently failed at lower stress values and underwent greater extension. However, when accounting for the increased thickness of the CII and P sections, no significant difference was found in the component stiffness of any of the three sections. Component stiffness normalises for the dissected width of tested samples but allows for the natural variation in thickness of the wing sections. The similarity of values across CI, CII and P suggests that no one section is inherently 'easier' to induce failure in than any other. The P section, whilst weak as a pure material, requires a similar force to break when viewed as a component. The higher relative percentages of elastin in the CII and P sections may be an adaptation to improve wing folding. As the largest and most proximal sections they are required to unfurl (stretch out) the most during flight and higher levels of elastin should benefit this function (Cheney et al., 2015).

There were some differences between the sections, in terms of fibre composition and material properties. The association between the material properties and the anatomy of the wing is relatively complex. The CI section contained the greatest collagen percentage, and the lowest elastin percentage, which might account for it being less compliant and deforming less. Furthermore, collagen provides strength to the tissue (Grover et al., 2012), while the elastin is a highly elastic protein (Holbrook and Odland, 1978) which provides the elasticity to the tissue (Grover et al., 2012). Qualitatively, in CI section the collagen percentage was significantly higher compared to the other sections (Figure 3.7, Table 3.2). This could lead to more strength in the CI section. Section CI also contains the lowest percentage of elastin (Figure 3.7, Table 3.2), which could lead to an increase in tears, as elastin helps the wing skin to resist tears (Holbrook and Odland, 1978).

The variation between wing sections in terms of thickness, could relate to their different functions (Swartz et al., 1996). As P section plays an important role in lifting bats during flight (Vaughan, 1970), this section needs to be thick. However, the three sections of the bat's wing had similar material properties and there was no significant difference between these sections in term of Component Stiffness, which is related to the force that is applied on the section. If each section of the wing is equally disposed to being torn, it is puzzling that the majority of tears are on the P section.

A study on seven different species of bats, not including *Pipistrellus sp.*, by Swartz et al. (1996), found that the plagiopatagium was the weakest wing section overall, it also tended to be thicker, had the lowest Young's modulus and stretched the most before breaking. This does fit the general trend in our data, however, I do not observe statistically significant differences in these parameters. There is a lot of variation between the genera, which might explain why my results differ from Swartz et al. (1996), as they did not measure *Pipistrellus sp.*. I also observed a lot of variation within individuals too (note high standard deviation values in material property data in Table 3.3).

There may be a number of reasons for there being more tears in section P. It is the largest section so it might just be more likely to be torn (Figure 3.2), so it might just be more likely to tear. It also contains the fewest bones (Figure 3.2), which may act to stop tearing. Section P also gets extended first before flight, therefore, it might get caught or snagged during flight preparation (Figure 3.9) (Gardiner and Nudds, 2011). Overall, consider whether any anatomical properties within section P makes it more likely to tear, and conclude that, based on fibre type and material testing data, it should not be more prone to tearing than sections CI and CII.

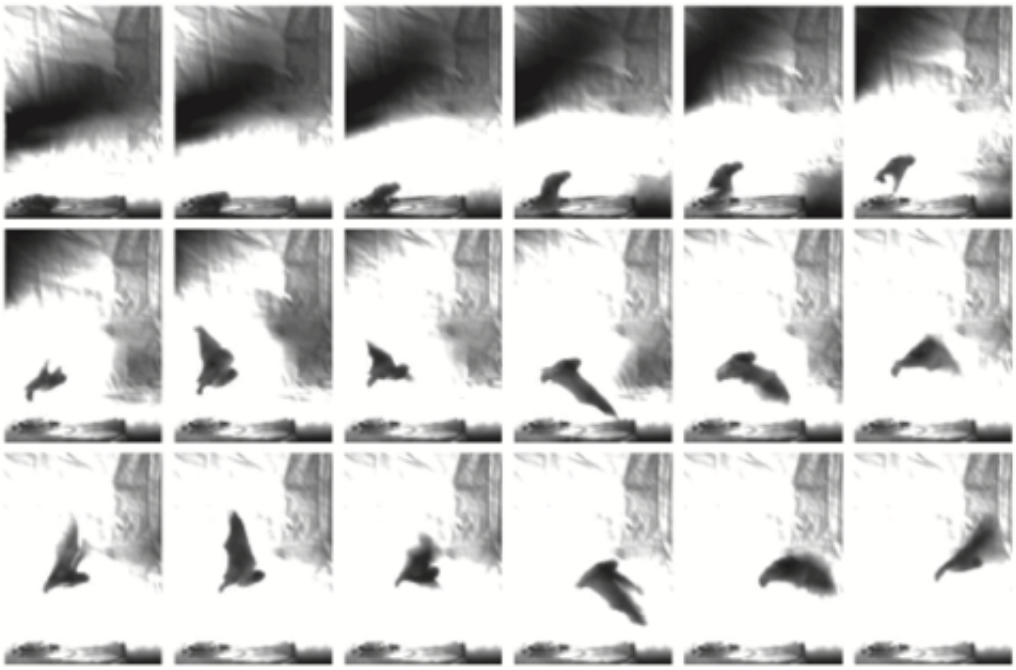


Figure 3.9: Selected frames from high-speed video footage of Miniopterus schreibersii jumping from a platform to initiate flight, showing that the P section extends first. (Gardiner and Nudds, 2011).

3.4.2 Tear Orientation

If the wing is isotropic when it is stretched out, it should be equally susceptible to tearing in every orientation. Bat wings were thought to be highly anisotropic (Swartz et al., 1996), however, the elastin accounts for much of this difference and once the elastin has 'unwrinkled' the wing is isotropic (Cheney et al., 2015). Therefore, our force results are likely to be representative of the wing as a whole, regardless of the orientation of the sample; although displacement will be significantly higher in samples perpendicular with ours, as the elastin stretches out ('unwrinkles').

Despite the presence of a fibrous net in all wing sections, many tears occurred in a rostro-caudal orientation (Chapter 2, Figure 2.1C, 2.2C), especially in wing section P. This coincides with the direction of travel, and might be indicative of the bat wing being snagged while moving forward. Proximal-distal tears tended to occur only around the trailing edges of the wings (Chapter 2, Figure 2.1C, 2.2C), despite them being reinforced here by bundles of skeletal muscle fibres (Holbrook and Odland 1978). While the wing anatomy indicates that it is just as likely to tear in any

orientation, I see many rostro-caudal tears (Chapter 2); therefore, the wing anatomy does not appear to be sufficient in explaining tear orientation.

3.4.3 Tear Healing

The results indicate that Section CI has the highest density of blood vessels, while section P has the lowest number of blood vessels (Figure 3.4, Table 3.1), which occurs as the vessels naturally bifurcate from proximal to distal. Extensive vasculature is associated with increased healing capabilities in bat wings and tails (Faure et al., 2009), with both the wound and scarring healing quicker; however, previous studies have not quantified the vasculature as presented here (Faure et al., 2009; Weaver et al., 2009). It is the blood which carries important factors to the wound site; to clean the wound, prevent infection, and begin the process of reforming the tissue matrix, therefore I propose that being close to a vessel is important for quick healing responses, which has also been suggested by Faure et al. (2009) and Pollock et al. (2016). As CI had the most extensive vasculature, I would expect it to heal quicker than section P. In addition, section P, which had the lowest numbers of blood vessels, also had the thickest vessels, which supply blood to the thinner, highly branched, densely-distributed vessels in the other sections of the wing. Following a tear, these thicker vessels also might bleed more, and lead to additional complications. The majority of tears occurred on section P of the wing, which I suggest is the section that is likely to be the slowest to heal.

Greville et al. (2018) found that in the Egyptian fruit bat (*Rousettus aegyptiacus*) wounds took 50% longer to heal in section P, compared to the chiropatagium (sections CI and CII), although this was not found in the Big brown bat (*Eptesicus fuscus*). They suggest that, not just blood vessels, but also the collagen and elastin fibres are likely to play a role in healing. Indeed, Greville et al. (2018) suggest that over-stretching of the collagen or elastin fibres during healing can cause the tear to even get bigger before healing (Greville et al., 2018). I suggest that the proximal-distal orientation of the elastin fibres may be holding the common rostro-caudal tears apart, thus increasing healing times. This will be especially true in the P section,

which has a lot of elastin and is the largest wing section with the most movement, so tears may well be stretched and extended during the healing process.

Implications

Anatomical evidence suggests that each wing section is just as likely to tear in *P. pipistrellus*. The entire wing is protected somewhat from tearing by a fibrous net, which maintains the majority of tears as small holes. However, when tears do occur, I see significantly more of them in section P, and many are rostro-caudal, starting from the middle of the wing and extending to the trailing edge (Chapter 2). Therefore, tear positioning and orientation cannot be accounted for by the anatomy of the wing, when material properties, and fibre types and orientations are considered. I suggest that where there are mainly tears in proximal wing sections, these may well be caused by predators, including cat (*Felis silvestris catus*) attacks (Figure 2.4a, c, d) and perhaps failed talon strikes in birds of prey, such as barn owls (*Tyto alba*) (Speakman, 1991), that are targeting the body of the bat, and tearing the proximal P section.

While anatomical evidence cannot explain tear placement and orientation, it may well be associated with healing. Tears in the P section tend to take longer to heal, and this section contains the lowest number of blood vessels and the most elastin. Indeed, results indicate that large tears at the P section might cause bats to stay in rescue centres for longer than 6 months (Chapter 2).

While an understanding of the anatomy of the wing, and occurrence of tears, is important to investigate the mechanism of tearing and healing, it is not sufficient to measure the effect of the wing tears on the animal. The following chapter, therefore, will investigate the effect of wing tears on flight.

Chapter 4 Determining the Effect of Bat Wing Tears on Flight in Common Pipistrelles (*Pipistrellus pipistrellus*)

Results from this chapter were peer-reviewed in an abstract and presentation at the Measuring Behaviour conference, 2018:

Khayat, R. O., Shaw, K. J., L. M., and Grant, R. A. (2018). The Effect of Wing Tears on the Flight Behaviour of Common Pipistrelles Bats (Pipistrellus pipistrellus). Abstract in Measuring Behavior Conference Proceedings: 11th International Conference on Methods and Techniques in Behavioral Research. (Appendix 4)

Chapter summary:

This chapter aims to compare the body orientation and wing movements of *P. pipestrellus* during flight, in animals with and without wing tears. Bats were filmed in high-speed and videos analysed to quantify the effect of the wing tears on flight. Results suggest that tears on both wings affected wing movements the most, and body orientation tended to orient towards the healthier wing. There was no significant association between wing movements and body orientation with tear size and bilateral tear asymmetry.

4.1 Introduction

Bats are the only mammals capable of flapping flight (Neuweiler, 2000; Voigt et al., 2012; Kovalyova, 2014). As the bats' wings are flexible and stretchable, their shape can be modified during flight (Skulborstad et al., 2015). However, these large wings are also thin and delicate, which makes them prone to tearing (Ceballos-Vasquez et al., 2015). While bats can fly with large wing tears (Davis 1968; Voigt 2013); many have to be taken to rescue centres for rehabilitation, which affects hundreds of bats annually in the UK, especially the most abundant *P. pipestrellus* (Kelly et al., 2008). There is no clear recommendation for rehabilitated bats to be released and usually bat carers subjectively judge when bats are ready for release.

Bat wing sections play different roles in flight. It is thought that the P section acts to support the bat's body weight during flight and provides lift (Vaughan, 1970; Swartz et al., 1996; Neuweiler, 2000) and the CI and CII sections provide thrust (Swartz et al., 1996; Neuweiler, 2000). This is also true for bird flight. In bird wings there are two types of flight feathers, primary feathers and secondary feathers. Primary feathers are located at the tip of the wing and provide thrust. Secondary feathers are located closer to the body and provide lift (Videler, 2006). In addition, gaps occur in bird feather during moulting. Specifically, gaps can vary in terms of size and position (Hedenström and Sunada, 1999), and large gaps in the proximal sections of the wing impact flight in most in birds (Hedenström and Sunada, 1999; Kiat et al., 2016). If bat wings are affected in a similar way, we might predict bats with large, proximal (P section) tears to have the most impacted flight.

This current study developed a method to investigate the effect of bat wing tears on the flight of *P. pipistrellus*. This chapter will initially compare between bats in three conditions: i) without wing tears; ii) with tears on one wing; and iii) with tears on both wings. It will measure their wing movements and body orientation. Secondly, it will identify any associations between tear size and bilateral tear asymmetry on wing movements and body orientation.

4.2 Methods

4.2.1 Samples

Over a period of three years, 36 *P. pipestrellus* bats were filmed in several locations around the United Kingdom. Three of these samples were female and one male, the others were all unknown. The bats were filmed at rescue centres and carer's houses, in collaboration with Lower Moss Wood Educational Nature Reserve at Knutsford in Cheshire, Wildwood Trust in Kent, Sussex bat group in Sussex, and the Yorkshire bat group in Otley. The filmed bats were categorised as: i) bats without wing tears (No tears) (10 bats); ii) bats with tear(s) on only one wing (Unilateral tear(s)) (16 bats); and iii) bats with tear(s) on both wings (Bilateral tear(s)) (10 bats).

Before filming, pictures of the injured wing/wings were also captured. The tears of those images were traced, counted and classified according to the methods described in Chapter 2. Tear area was measured to approximate tear percentage size (tear size (%)). The difference in percentage tear size between each wing was also calculated to give bilateral tear asymmetry (%). Each bat was also categorised as having no tears, unilateral tear(s) only on one wing, or bilateral tear(s) on both wings. The more injured wing was also identified in bats with bilateral tears, since one wing tended to have a much larger tear (with mean bilateral tear asymmetry values of 77.9%).

4.2.2 Video Data Collection

Bats were encouraged to fly around the space where they were usually assessed for flight. Filming was conducted during the day time in a flight cage (with approximate size of 5x5x3 m), indoor room (with approximate size of 4.5x2.5x2.4 m) or corridor (with approximate size of 6x3x3 m). The bats were filmed using a digital high-speed camera (Phantom Camera, MIRO_M110), at 200 frames per second, with a resolution of 1280 x 800 pixels. A manual trigger was used to capture 3-12 video clips with at least 5-seconds for each clip per bat, when the bat flew in to the field of view of the camera. All the clips that were recorded were saved as cine-files, giving a total of 375 clips. Subsequently, the clips were reviewed using the Phantom Camera Control Application (PCC 2.7) and converted to avi. files. The videos and video portions were selected for conversion and analysis when the bat was i) clearly in view, ii) was either directly front-on, or back-on to the camera, and iii) flying straight, so not making any turns. A total of 99 clips were excluded when the bats were in a side-on position or turning round. During this review process, the bats filmed in the corridor flew straight more often than the bats filmed in the flight cage or indoor room as they turned around more during flight. Hence, 276 clips were used for tracking, with 51.4% from the corridor, 48.5% from the flight cage and 6.02% from the indoor room. All clips were a minimum of 1-2 seconds in length, and included at least 1 wing beat.

4.2.3 Video Tracking

The bat wing movements were tracked using the Manual Whisker Annotator software (MWA) (Hewitt et al., 2016). For tracking, 2 points (a and b) were placed on the right and left side of the body (Figure 4.1), which were used to measure body orientation. Three points (1, 2, 3) were placed on each wing: the first one was at the wing tip; the second was in the middle of the wing; and the last on the base of the wing, closest to the body (Figure 4.1). The wing tracking points were always placed at the upper edge of the wing. These eight points were manually tracked through every frame of the video. The x-y coordinates of the two body points were extracted to calculate body orientation (Figure 4.2A, defined in Table 4.1) such that a horizontally-oriented bat would be at zero degrees, a bat leaning towards the left would have a positive body orientation, and a bat leaning towards the right would have a negative body orientation. The wing coordinates were used to calculate wing angle (Figure 4.2B and C), which is the angle the wings make with the body. A wing positioned straight up above the head would give an angle of 180˚, and one positioned straight down would give an angle of 0˚. The wing angles were then used to calculate the following variables: maximum angle, minimum angle, mean angle and amplitude, which are defined in Table 4.1 (Hewitt et al., 2016). Wingbeat frequency was also calculated by counting the wing beat cycles visually in each clip and dividing by the total time of the clip to give a value in Hertz.

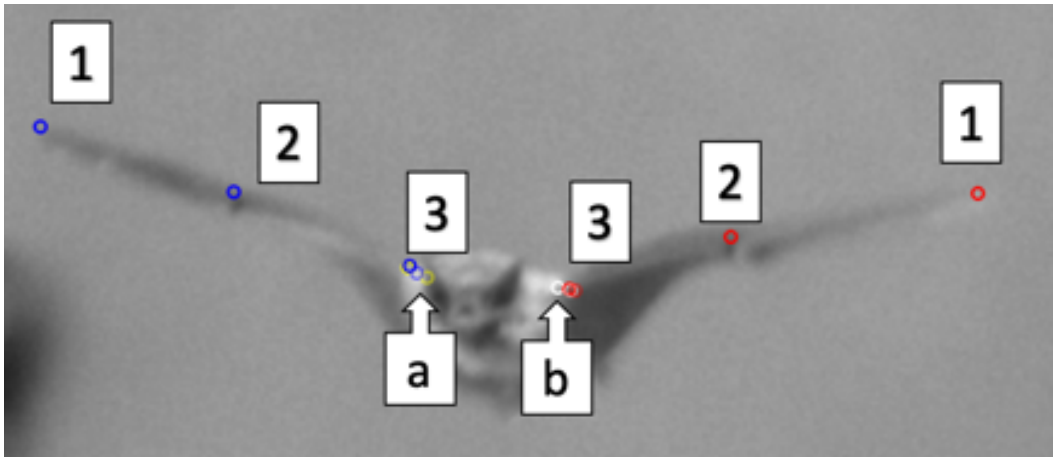


Figure 4.1: The Tracking points on the bat wing. The tracking points were used with the Manual Whisker Annotator (MWA) software to track the bat wing movement and to calculate the variables. The two points on the middle of the bat (a, b) are for the body orientation. The three points on each the wing (1,2,3) are for tracking wing movements.

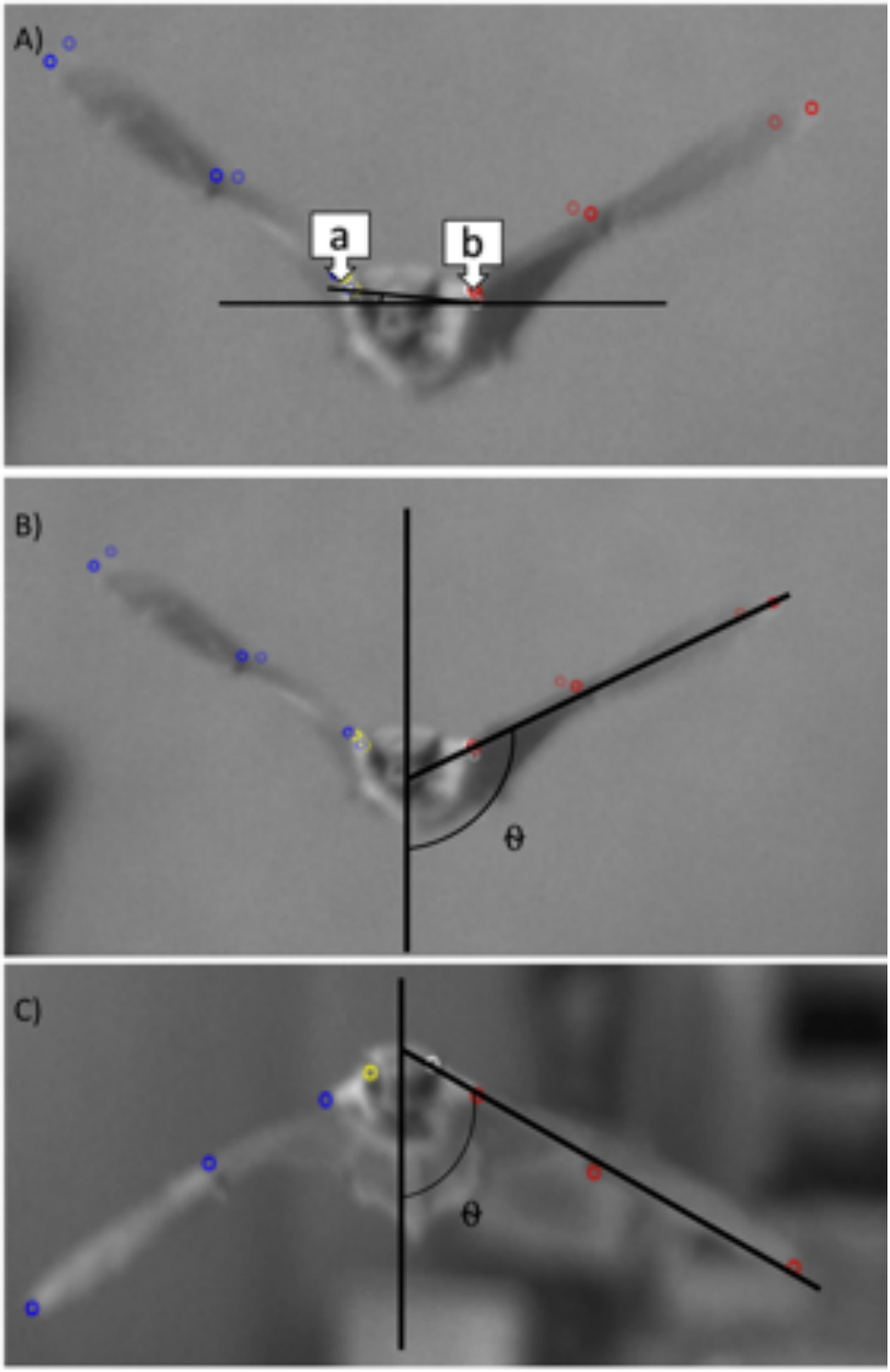


Figure 4.2: A) The angle to measure the body orientation, B) The maximum angle of the bat wing movement. C) The minimum angle of the bat wing movement.

Table 4.1: The wing movement and body orientation variables calculated from the footage.

Characteristic	Description
Maximum angle	The largest wing angle, when the wings were at their highest, calculated individually for left and right wings. Measured in degrees (Figure 4.2B).
Minimum angle	The smallest wing angle, when the wings were at their lowest, calculated individually for left and right wings. Measured in degrees (Figure 4.2C).
Mean angle	A mean of all wing angles, calculated individually for left and right wings. Measured in degrees.
Amplitude	It is the root mean squared error, which is the deviance of wing movements from the mean angle. Measured in degrees.
Wing frequency	The number of wing beats per second. Measured in Hertz.
Body orientation	The mean angle of the body orientation, to a horizontal line (Figure 4.2A). A horizontal body position would give 0˚. Measured in degrees.

4.2.4 Statistical Considerations

Wing movement and body orientation data was classified into three categories: No tears, Unilateral tear(s) and Bilateral tear(s). All the statistical analysis was completed using SPSS Version 24. The normality test for the data showed that the data was not normally distributed, so non-parametric tests were used.

To compare the wing movement data, firstly a Wilcoxon test was conducted to ascertain the differences between each side of the wing in all bat categories: no tears,

unilateral, bilateral. The data for the bats with no tears was compared between the left and right wings. In bats with unilateral wing tears, data was compared between the intact and torn wings. For bats with bilateral tears, the most injured and least injured wings were compared. The Wilcoxon test showed no significant difference between the wing sides in wing movement, over the three groups of bats. Therefore, for graphing, data from the two sides were combined for the bats with no tears and bilateral tears, and kept separate for those with unilateral tears.

To further compare wing movements between bat categories (no tears, unilateral, bilateral) a Kruskal-Wallis Test was conducted on all the wing measurements. Data were averaged (mean) between each wing in bats with no tears and bilateral tears, and in bats with unilateral tears, data from the injured wing was used in the analysis. Pairwise tests were run to identify significant differences, and a Bonferroni correction was used to make adjustments for multiple testing ($p<0.01$).

In order to explore whether body orientation was angled towards or away from a wing tear, the body orientation data was divided depending on whether the tear location was in the left wing or the right wing, in bats with unilateral tears. For bats with bilateral tears, the data was divided depending on the location of the biggest tear(s), in left wings or in the right wings. Therefore, the mean body orientation values were calculated for the bats with tears on the left wings and for the bats with tears on the right wings, to identify the direction of the body orientation. For ease of analysis clips were chosen only when the bat was flying front-on. A Mann-Whitney U Test was run to find the difference in the body orientation between the bats with tears on left wings and bats with tears on the right wings.

For each bat with a wing tear, 'Inkscape' software was used to measure the tear size as well as the wing size. This calculates the percentage size of a tear, compared to the size of the wing. In addition, the difference in percentage tear size between the two wings was calculated and termed bilateral tear asymmetry. A bivariate Spearman's rank correlation was conducted to correlate percentage tear size and

bilateral tear asymmetry with wing movement and body orientation data (mean values of maximum angle, minimum angle, mean angle, amplitude, frequency and body orientation for each bat).

4.3 Results

4.3.1 Wing Tear Classification and Placement

The distribution of the wing tears for bats with unilateral wing tears and bats with bilateral wing tears is presented in Figure 4.3, and shows that the most tears were found in section P, which matches the results found previously in chapter 2. Also, bats with unilateral wing tears had significantly more tears in P section than CII and CI sections (χ^2= 14.362, df=2, p=0.001), however there was no significant difference in tear numbers between the wing sections (χ^2=1.750, df=2, p=0.417) in bats with bilateral wing tears. Bats with bilateral wing tears had more tears in all wing sections when compared to bats with unilateral wing tears. However, the number of tears did not significantly differ between bats with unilateral wing tears and bilateral wing tears (χ^2=0.581, df=1, p=0.446).

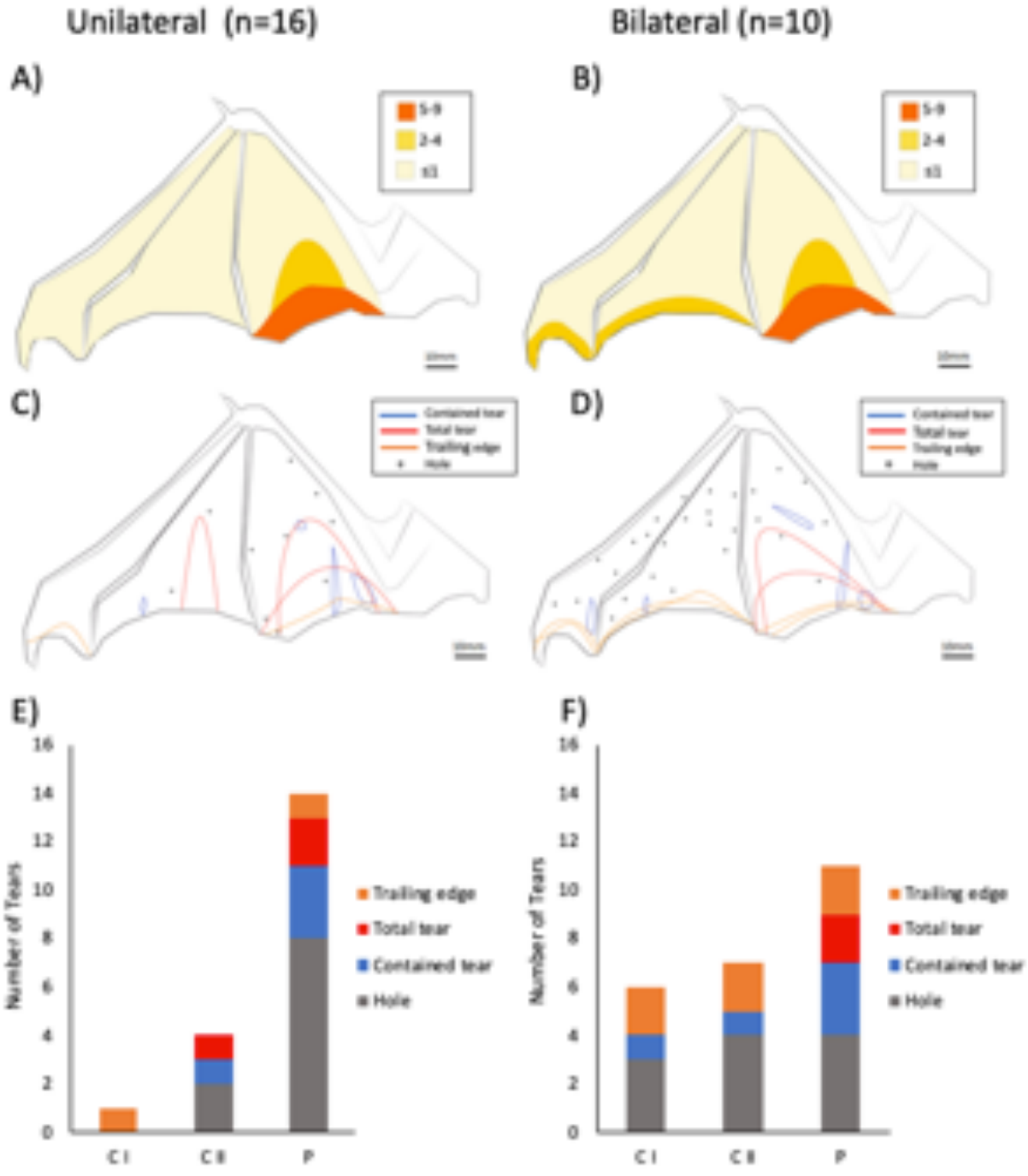


Figure 4.3: Wing tear classification, A) and B) showing the wing tear distributions over the wing sections; C) and D) showing the distribution of the different types of tear over the wing sections; E) and F) presenting the count of each tear type in each section of the wing. The first chiropatagium section (CI), the second chiropatagium section (CII) and the plagiopatagium section (P).

4.3.2 Wing Movements and Body Orientation

The Wilcoxon signed-ranks test showed that there were no significant differences between left and right sides of the wing for the movement variables: maximum angle, minimum angle, mean angle, amplitude, and wing frequency in all three bat categories (all p's > 0.05) (Table 4.2). Therefore, this data was combined for the no tear and bilateral tear groups.

Table 4.2: The Wilcoxon results to compare between the left and right wing in the in bats with no tears, bats with unilateral wing tears and bats with bilateral wing tears.

	No tears	**Unilateral**	**Bilateral**
Maximum angle	*Z*=-0.018, p=0.985	*Z*=-0.819, p=0.413	*Z*=-0.808, p=0.419
Minimum angle	*Z*=-0.135, p=0.892	*Z*=-0.434, p=0.665	*Z*=-0.104, p=0.917
Mean angle	*Z*=-0.263, p=0.792	*Z*=-1.188, p=0.235	*Z*=-0.450, p=0.653
Amplitude	*Z*=-0.691, p=0.490	*Z*=-0.387, p=0.699	*Z*=-1.444, p=0.149
Wing Frequency	*Z*=0.000, p=1.000	*Z*=-1.802, p=0.072	*Z*=0.000, p=1.000

Wing Movements

Bats with bilateral tears had significantly lower maximum angles than bats with no tears and bats with unilateral tears (Table 4.3, Figure 4.4A). Bats with bilateral tears also had significantly higher minimum angles compared to the other groups (Table 4.3, Figure 4.4B). The mean angle and amplitude were not significantly different between the three groups of bats (Table 4.3, Figure 4.4C, D). Bats with bilateral wing tears also had significantly higher wing beat frequencies, so beat their wings more often than bats with no wing tears and bats with unilateral wing tears (Table 4.3, Figure 4.4E).

Body Orientation

Body orientation for bats with no tears was around horizontal at 0 degrees (Table 4.3, Figure 4.4F). Although not significant, bats with unilateral wing tears had a more tilted body orientation towards the left hand wings, and the bats with bilateral wing tears had a slightly lower body orientation, toward the right hand wings, compared to bats with no tears (Table 4.3, Figure 4.4F).

To examine body orientation in more detail, bats were split in to those with the highest percentage of tears on the left wing and those with the highest percentage of tears on the right wing. When the tear, or the largest tear, was found on the left wing, the body orientation was significantly lower and negative in the bats with unilateral wing tears (-1.08 ± 14.31) ($p=0.014$) (Figure 4.5B), and also in the bats with bilateral wing tears (-5.12 ± 12.84) ($p=0.006$) (Figure 4.5B). This means that the body orientation was more angled towards the right side when the left side was more injured (Figure 4.5A). In contrast, when the tears, or the largest tear, was located on the right wing, the body orientation was significantly higher and positive in the bats with unilateral wing tears (4.67 ± 12.37) ($Z=1.515$, $p=0.014$) (Figure 4.5B), and also in the bats with bilateral wing tears (3.96 ± 9.86) ($Z=780$, $p=0.006$) (Figure 4.5B). Hence, the body orientation tended to lean towards the left side in bats with more injured right wings (Figure 4.5A). This means that when the bats have wing tears the bat's body is oriented towards the most intact wing (Figure 4.5A).

Association with tear size or bilateral tear symmetry

Despite the tears significantly affecting wing movements and body orientation, these changes did not significantly correlate with percentage tear size or bilateral tear asymmetry. Indeed, there was no significant correlation between percentage tear size or bilateral tear asymmetry between the wing movement variables (maximum angle, minimum angle, mean angle, amplitude, frequency), nor body orientation (Table 4.3).

Table 4.3: Mean, standard deviation and statistics of wing angles, wing frequency and body orientation in bats with no tears, bats with unilateral wing tears and bats with bilateral wing tears. Bat groups were compared using Kruskal-Wallis Tests with a Bonferroni correction applied at the p<0.01 level of significance, and all correlations were Spearman's Ranked.

	No tears[i]	**Unilateral[ii]**	**Bilateral[iii]**	**Comparing between bat groups**	**Correlation to tear size (%)**	**Correlation to bilateral tear asymmetry (%)**
Maximum angle	158.97± 25.14	159.22± 21.83	147.19± 29.06	χ^2=16.287, df=2, p<0.001*	r_s=-0.03, p=0.885	r_s=0.018, p=0.930
Minimum angle	2.73± 10.78	2.61± 4.01	4.26± 7.83	χ^2=14.732, df=2, p=0.001*	r_s=0.264, p=0.192	r_s=0.186, p=0.364
Mean angle	45.05± 13.95	44.47± 10.80	48.15± 9.11	χ^2=6.543, df=2, p=0.038	r_s=-0.100, p=0.626	r_s=-0.050, p=0.807
Amplitude	41.72± 7.55	40.99± 6.81	38.70± 9.11	χ^2=8.563, df=2, p=0.014	r_s=0.147, p=0.473	r_s=0.169, p=0.410
Wing frequency	12.35± 2.67	12.31± 2.46	13.30± 9.11	χ^2=10.580, df=2, p=0.005*	r_s=0.175, p=0.393	r_s=0.150, p=0.464
Body orientation	0.03± 14.49	3.78± 11.46	-1.00± 13.63	χ^2=6.111, df=2, p=0.047	r_s=-0.071, p=0.731	r_s=0.011, p=0.959

** Pairwise comparisons values:*

Maximum angle: i vs ii: W=3.13, p=0.78, i vs. iii: W=43.76, p<0.001 and ii vs. iii: W=40.62, p=0.001.

Minimum angle: i vs ii: W=2.04, p=0.85, i vs. iii: W=-39.14, p=0.001 and ii vs. iii: W=-41.17, p=0.001.

Wing Frequency: i vs ii: W=-1.22, p=0.91, i vs. iii: W=34.67, p=0.004 and ii vs. iii: W=-33.45, p=0.005.

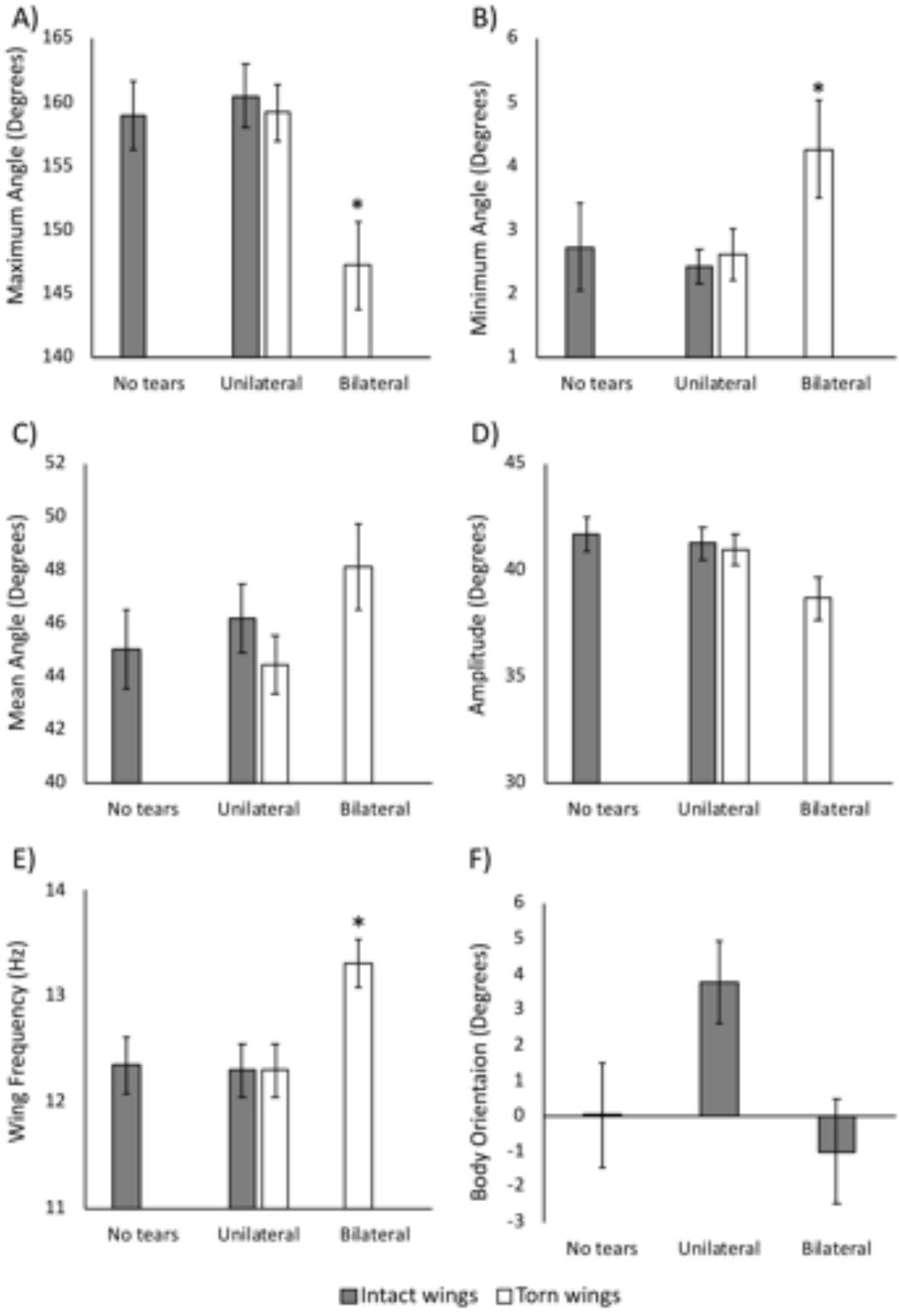


*Figure 4.4: The graphs of: A) Maximum angle, B) Minimum angle, C) Mean angle, D) Amplitude, E) Wing frequency, and F) Body orientation, of bats with no tears, bats with unilateral wing tears and bats with bilateral wing tears. (*Significant difference in maximum angle, minimum angle, and wing frequency at bilateral wing tear bats compared with no tear and unilateral wing tear bats). Error bars represent the standard error means.*

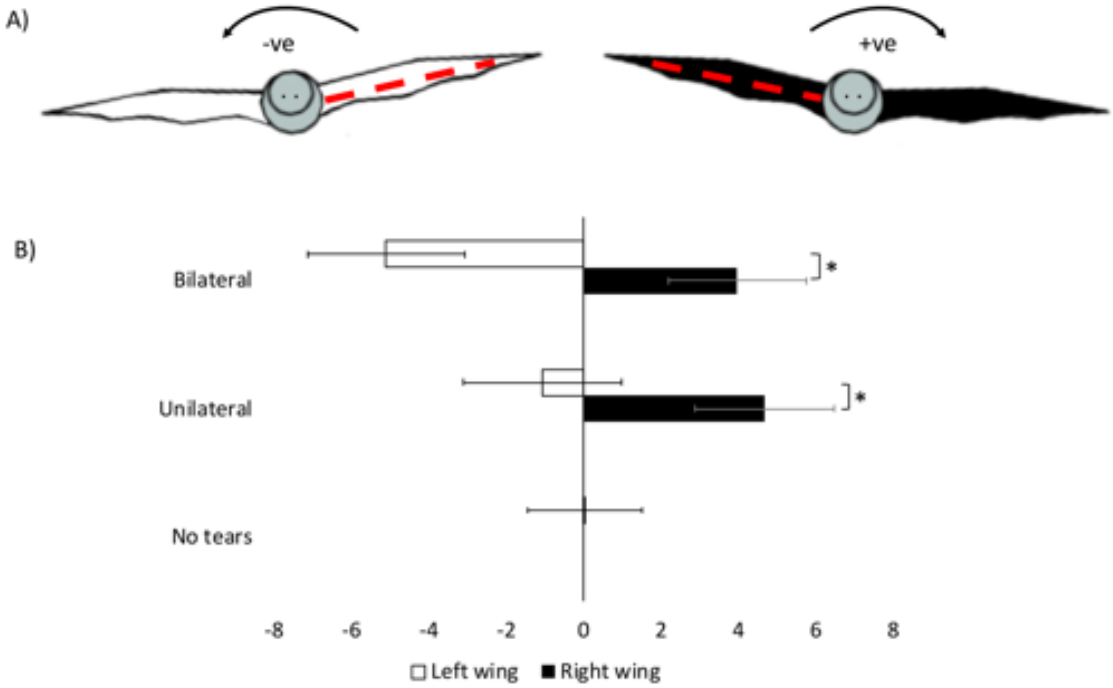


*Figure 4.5: A) The diagram presents the body orientation when the tear/ largest tear is in the left wing (white colour), and when the tear/ largest tear is in the right wing (black colour), the red dashed line represents the torn wing (Image adapted from Iriarte-Díaz and Swartz, 2008), B) Presents the body orientation degree in: bats with no tears, bats with unilateral wing tears, and bats with bilateral wing tears, when the tear/ largest tear is found in the left or right wing. All body orientation was measured when the bat was flying front-on to the camera. (*Significant difference in body orientation degree between bats with tear/ tears on left wings and bats with tear/tears on right wings). Error bars are the standard error.*

4.3.3 Effect of Tear Placement on Wing Movements and Body Orientation

Further investigation of the data compared the wing movements and body orientations in bats with tears in different wing sections; including: either chiropatagium sections (CI, CII), chiropatagium and plagiopatagium sections (CII, P), the plagiopatagium section (P), or in all sections (CI, CII and P), for bats with unilateral wing tears and bilateral wing tears (Figure 4.6). Samples were not large enough to conduct statistics, but visually these groupings followed a similar pattern to the full dataset. Bats with bilateral wing tears had lower maximum wing angles (except the group with the tear in CII, P sections), and higher minimum angles than the bats with no tears and bats with unilateral wing tears (Figure 4.6A, B). However, bats with unilateral wing tears had lower mean angles and higher wing amplitudes than the

bats with bilateral wing tears, except the bat with tears on all wing sections (CI, CII, P) (Figure 4.6C, D). Comparing wing frequency and body orientation between the groups was not clear (Figure 4.6E, F). However, body orientation was more tilted (i.e. more deviation from horizontal) when there were tears in just the P section. Overall, it is difficult to spot any strong visual associations between the position of the tear and the resulting wing movements and body orientation.

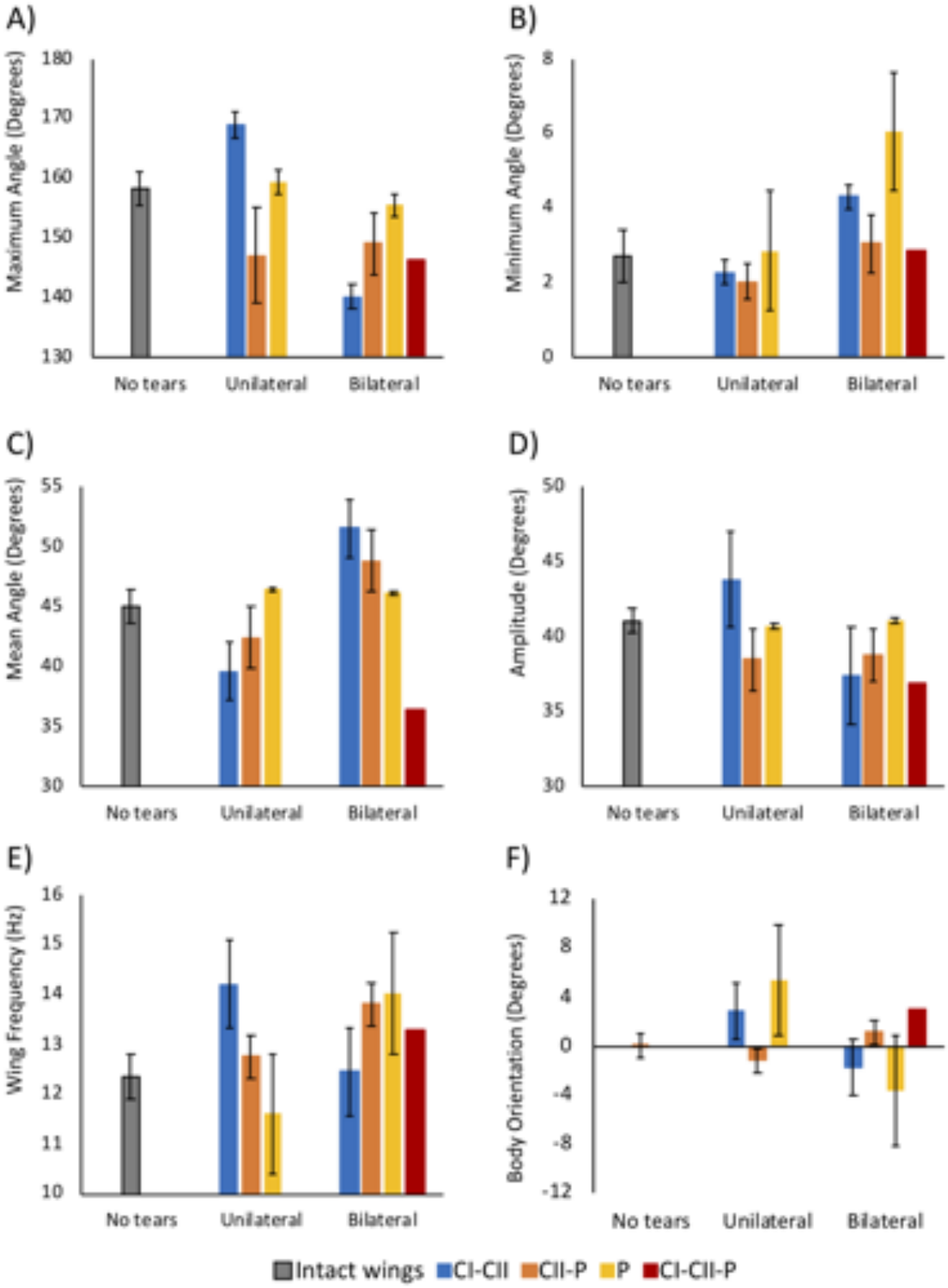


Figure 4.6: The graphs of: A) Maximum angle, B) Minimum angle, C) Mean angle, D) Amplitude, E) Wing frequency, and F) Body orientation, for bats with No tears, bats with Unilateral and Bilateral wing tears. In term of tear placement over the wing sections: the first chiropatagium section (CI), the second chiropatagium section (CII) and the plagiopatagium section (P). Error bars are the standard error.

4.4 Discussion

Flight analysis indicated that the maximum wing angle, the minimum wing angle and wing frequency were all affected in bats with bilateral tears. Moreover, body orientation tended to orient towards the healthier wing. Flight has been found to be affected in bats with wing tears in previous studies, with injured bats found to make fewer turns than healthy bats (Voigt, 2013). Although I only look at straight flight without turning, flight manoeuvres are likely to be even more affected by tears (Pollock et al., 2016) due to a reduction in wing area (Voigt, 2013); although I did not see any correlation in wing movements and tear size here.

Results showed that bats with bilateral wing tears had overall reductions in wing movements with lower maximum angles and higher minimum angles (see Figure 4.4A, B). Although not significant, this contributed to a reduction in wing amplitude. While the wing moved through less of an angle, it also moved more times per second with a higher frequency (>13Hz) (Figure 4.4E) in bats with bilateral wing tears. This might be to compensate for the smaller wing movements, and ensure appropriate lift and thrust for flight (Norberg and Norberg, 2012). The wing frequency values in this study for the bats with no tears, unilateral tears and bilateral tears were 12.35 ± 2.67, 12.31 ± 2.46, and 13.30 ± 9.11 respectively. Therefore, despite wing beat frequency elevating in bilaterally injured bats, these values were within the range of wing frequency values ascertained in the literature, of around 11-14 Hz (Thomas et al., 1990; Berg and Rayner, 1995).

There is a complex interplay between wing amplitude and wing beat frequency, which affects aerodynamics and flight energy expenditure (Taylor et al., 2003). These values are all combined with forward velocity to give an animal's Strouhal Number. Such that:

$$\text{Strouhal Number} = (\text{Frequency} * \text{Amplitude}) / \text{Forward Velocity} \quad (4.1)$$

The Strouhal Number describes kinematics in swimming and flying animals, it is dimensionless and captures vortex behaviour during flapping motion (Taylor et al., 2003; Nudds et al., 2004; Tian et al., 2006). Animals have the highest propulsive efficiency at Strouhal Numbers of 0.2-0.4 during cruising flight or swimming, and most bats will have Strouhal Numbers of around 0.3 (Taylor et al., 2003) (Figure 4.7A). A study by Bullen and McKenzie (2002) measured the aerodynamics of bat flight in 23 species of Australian bat. They found that frequency tended to be independent of forward velocity, especially at higher speeds (Figure 4.7B), while amplitude and velocity were positively correlated (Figure 4.7C) (Bullen and McKenzie, 2002). In bats with bilateral wing tears, the frequency was higher and amplitude lower than in bats with unilateral and no wing tears. If I look at the numerator part of the equation, the changes in amplitude and frequency might cancel each other out and not affect the Strouhal Number, and hence flight efficiency. However, if amplitude and velocity are positively correlated, then the denominator term will also be lower in the equation and the Strouhal Number will, therefore, be larger in bats with bilateral wing tears. Any deviation of the Strouhal Number from 0.3 is likely to be less efficient (Taylor et al., 2003). In addition, any change from a preferential frequency is also likely to be less efficient (Taylor et al., 2003; Norberg and Norberg, 2012). Therefore, these changes in amplitude and frequency suggest a reduction in flight efficiency in bats with bilateral wing tears. Indeed, wingbeat frequencies can also increase in birds with reduced wing areas and this is also thought to increase flight costs (Hambly et al., 2004).

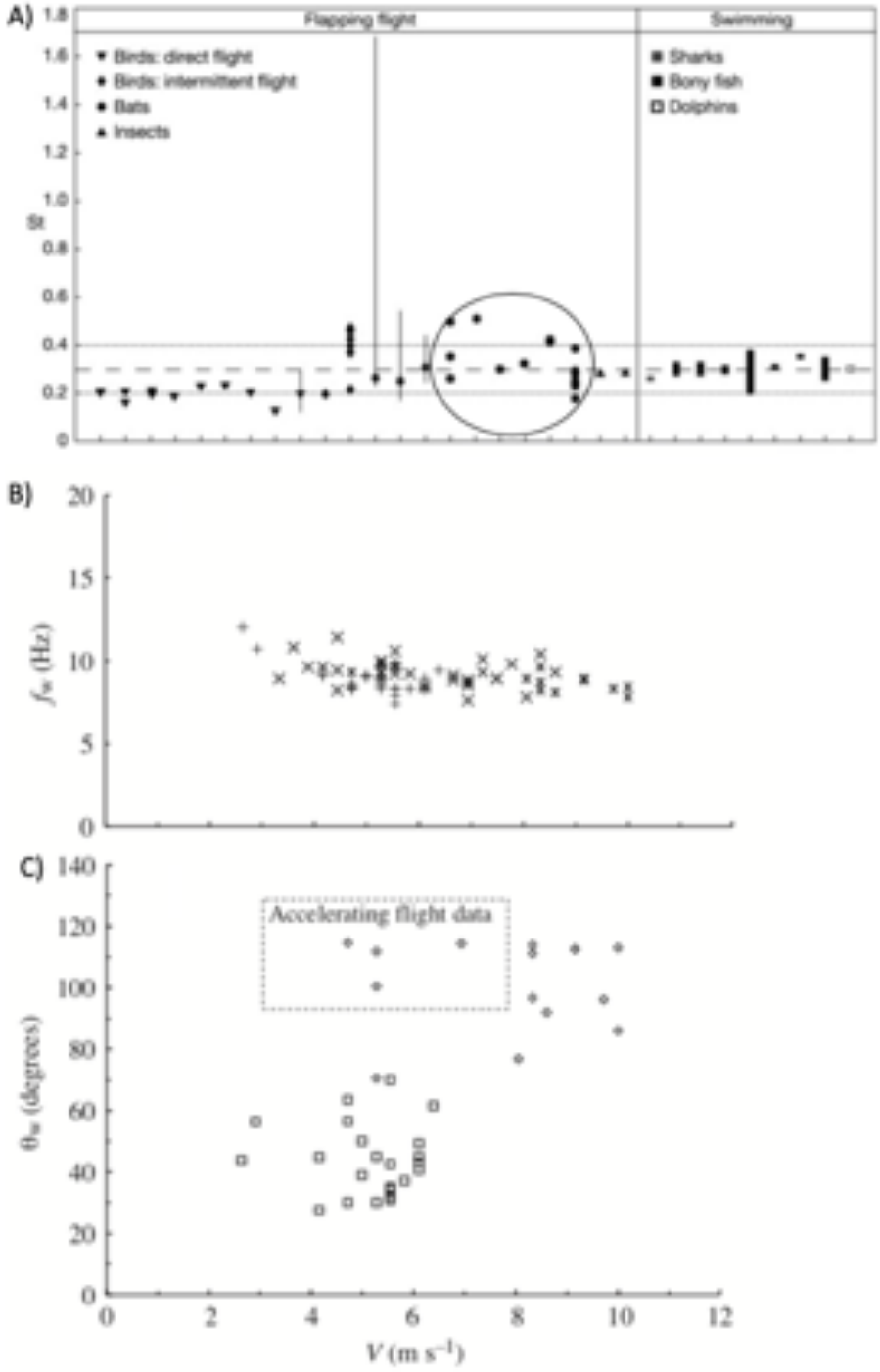


Figure 4.7: A) Strouhal Numbers for cruising flight and swimming, which range between 0.2-0.4. The circling data showing the bats' Strouhal Numbers (image modified from Taylor et al., 2003). Example of the wing beat frequency (f_W) (B), and the wing amplitude (θ_W) (C) versus the flight speed (V (ms^{-1})) for one of the bat species included in the study by Bullen and McKenzie, (2002). It shows increasing amplitude with flight speed (images modified from Bullen and McKenzie, 2002).

As well as wing movements, body orientation was also affected. Bats with wing tears had less horizontal body positions. In particular, bats with both unilateral and bilateral wing tears tended to lean towards the healthier side. Similar results have been found in quadrupedal mammals, where they will shift weight off an injured limb towards the healthy side; this has been documented in both horses (Duberstein, 2012) and rats (Ängeby Möller et al., 2012). It might serve to take some of the weight or pressure off the more injured wing. Birds with asymmetric flight feathers also have reduced manoeuvrability (Swaddle et al., 1996), due to differential lift on each wing (Thomas, 1997).

There was no clear association between the size of the tears, bilateral asymmetry of the tears and the position of the tears with any changes in wing movements and body orientation. All sections of the wing play key roles during flight. It is thought that the P section acts to support the bat's body weight during flight and provides lift (Vaughan, 1970; Swartz et al., 1996; Neuweiler, 2000), while the flexible, mobile CI and CII sections provide thrust (Swartz et al., 1996; Neuweiler, 2000). Therefore, damage to different sections of the wing might affect flight in various ways. One might imagine that tears in the CI or CII sections are more likely to affect manoeuvrability and speed, whereas tears in the P section may inhibit flight. In birds, moult gaps in the more proximal part of the wing have a greater effect on flight than those close to the wing tip (Hedenström and Sunada, 1999). Specifically, aerial manoeuvrability and take-off speed are affected (Swaddle et al., 1996; Hedenström and Sunada, 1999). Perhaps these aspects could be looked at during bat flight to identify the effect of tear position on flight.

However, bats are capable of flying with large tears in their wings (Davis, 1968), and all our bats could fly successfully with no clear differences in wing movements or body orientation depending on tear size or placement. In birds, it has been found that larger moult gaps affect flight the most (Kiat et al., 2016), and I would predict that larger tears would affect flight more in bats. It may be that the sample sizes in this study are just too small to view these associations. Or it may be that internal injuries, that I cannot see, might also be affecting flight. Moreover, it is likely that

tear size and position will affect flight in rather complex ways. Indeed, a complicated interaction of skin, bones, muscles, wing area and sensory innervation all influence wing function during flight (Swartz and Konow, 2015). Hence, any change in those structures will potentially affect bat flight. This association could only be investigated by conducting a larger-scale, more-detailed experimental project, or developing detailed mathematical models.

Indeed, other studies have developed detailed, complex experimental set-ups for measuring many aspects of bat flight; these include measuring precise 3D wing kinematics in multi-camera rigs (Riskin et al., 2008; Schunk et al., 2017), wing vortices (Hedenström et al., 2007; Muijres et al., 2008; Hubel et al., 2016) and even electromyograms (EMG), which measure muscle activity (Konow et al., 2017). These set-ups can measure the precise biomechanics of bat flight, although none have ever been used to study the effect of wing tears. Applying one of these methods would be beneficial to furthering our understanding of how the tears impact flight. However, for bat carers, studies of this nature are only useful if results can be distilled down into key messages for the carer to judge healthy flight for release. I suggest that counting the wing beats and looking at the orientation of the body, would be a good place for bat carers to focus their attention. In particular, wing beats of over 13 per second and body orientations of more than four degrees from horizontal might suggest flight being affected in *P. pipistrellus*.

Body weight also significantly affects aerodynamics during flight (Norburg and Norburg, 2012). Wing amplitude and frequency are both associated with body mass (Bullen and McKenzie, 2002). While not measured here, injured bats are probably lighter as they are likely to be less successful at foraging (Voigt, 2013). Lighter bat individuals tend to have smaller amplitude wing movements and faster frequency movements than heavier individuals (Bullen and McKenzie, 2002). Therefore, the patterns I see in wing movements in bats with bilateral wing tears could be caused by these bats simply being lighter, rather than being a direct result of the wing tear itself.

Implications

Wing movements and body orientation were affected in bats flying straight-on to the camera. Turning and manoeuvrability are likely to be even more affected by tears in these animals, than the straight flight investigated here (Voigt, 2013; Pollock et al., 2016). This has serious implications for the ability of the animals to forage successfully and survive. Indeed, I have developed a novel filming and tracking method to objectively assess wing movements and body orientation during rehabilitating flights of bats in care. However, it is necessary to find relationships between these wing movement and body orientation variables with survival outcomes in order to validate this approach, and make it useful for rehabilitators to adopt. Indeed, it is important to move towards objective guidelines for release, so bat carers can appropriately judge which injuries are serious for bats, and when they are ready for release. I suggest that looking at the number of wing beats and the orientation of the body during straight-on flat flight might be a good place to start.

Chapter 5 Genetic Analysis to Investigate the Potential Cause of Bat Wing Tears

Chapter summary:

This chapter investigates whether cats are interacting with bats to cause bat wing tears. Specifically, cat DNA was identified from swabs collected from injured bat wings sent in by bat carers. The samples with cat DNA present were also subjected to DNA profiling to identify individual cats. The results found that cat DNA was present in 66.67% of all the bat swabs samples.

5.1 Introduction

Many bat species roost in man-made structures, at least for breeding periods, in rural and semi-urban areas. The species *P. pipistrellus* is able to exploit urban environments for water and foraging under lights, and for roosting (Russo and Ancillotto, 2015). Therefore, this leads to an increase in the likelihood of the bats colliding with buildings and other man-made structures, as well as having more encounters with domestic cats (*Felis catus*) (Davis, 1968; Woods et al., 2003; Ancillotto et al., 2013), which may increase the occurrence of wing tears. In the UK, domestic cats are the most abundant carnivores (Woods et al., 2003) and are listed in the top 100 of the worst non-native aggressive species (Lowe et al., 2000). However, the amount of mortality in bat species due to predation by domestic cats are based on speculative observations, and mainly by householders and bat carers (Ancillotto et al., 2013; Woods et al., 2003; Loss et al., 2013). An objective measure of identifying cat predation on bats is important in order to understand the scale of the problem.

It has been reported that cats attack bats by catching the bat in the air - either with their paws in a swift movement whilst standing, or by jumping and catching the bat (Rodríguez-Durán et al., 2010). Thus, the cat's DNA is likely to be transferred to the bat's wing, from saliva in the cat's mouth or on the paws from grooming. Genetic

analysis could confirm whether cat DNA is present on injured bat wings and would provide strong evidence of cat attacks as a cause of wing tears. The utilisation of DNA analysis in forensic investigations can identify the source of human biological samples; however, the same principles can also be applied to non-human samples (Menotti-Raymond et al., 2012) to identify species, gender and individuals (Menotti-Raymond et al., 2005; Menotti-Raymond et al., 2012).

The most common method for genetic identification uses short tandem repeats (STR) profiling, due to its high power of discrimination between individuals and rapid analysis speed (Butler, 2001; Butler, 2012). STR markers are short repeated sequences of DNA, usually between 2 and 7 base pairs (bp) in length, which vary in the number of repeats present in different alleles (Butler, 2001; Butler, 2012). In addition, multiple STRs can be examined simultaneously, increasing the power of discrimination of the analysis and produce a complete DNA profile of an individual (Butler, 2001). This type of STR analysis is used in forensic investigations to compare the DNA profile of individuals (suspects) with samples collected from a crime scene. The same basic principles have also been applied to DNA profiling of cats (Butler et al., 2002; Menotti-Raymond et al., 2005; Menotti-Raymond et al., 2012). A panel of 11 STR markers and a gender marker (SRY) have previously been selected and developed for the genetic identification of individual cats, termed the 'MeowPlex' (Butler et al., 2002; Menotti-Raymond et al., 2005; Menotti-Raymond et al., 2012). The MeowPlex has been used successfully in forensic cases, with a similar power of discrimination as human STR kits (Butler et al., 2002; Menotti-Raymond et al., 2005). The most famous example might be that of Snowball the cat (documented in Coyle 2007). White hairs were found at a murder scene in Canada in 1994. DNA from the hairs were extracted, amplified and compared to the suspect's cat, Snowball. The DNA profile of the hairs at the murder scene matched that of Snowball's and were presented in court as part of the evidence used to convict the defendant in court (with extremely strong support likelihood ratios of: 2.2×10^{-8} and 6.9×10^{-7}, depending on the exact US cat population databases).

This chapter will evaluate the use of forensic genetic techniques as a means to identify the presence of cat DNA on bat wing tears. Specifically, this chapter will present the results of using quantitative polymer chine reaction (qPCR) to find cat DNA on bat wing swabs that were sent by bat carers from bats in the UK. The genetic data will be compared to information collected from bat carers on i) bat characteristics i.e. species, gender, and age, ii) where the bats were found; and iii) wing tear type and placement.

5.2 Methods

5.2.1 Sample Collection

Ethical approval was obtained through the Research Ethics and Governance Committee at Manchester Metropolitan University. Cat blood samples, used as positive controls for all genetic experiments, were collected as part of routine clinical testing at the Institute of Veterinary Science, University of Liverpool. The blood samples were stored in a fridge at 4°C, containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant to prevent blood clots formation in the tubes (Banfi et al., 2007).

Bat wing swab samples were obtained from bat carers in the UK. Bat carers were all trained individuals and registered with the Bat Conservation Trust (BCT). Carers were recruited by advertising the project at the Mammal Society Easter Meetings (2016, 2017, and 2018), the National Bat Conference (2016), National Bat Care Conference (2017) and in Bat Care News (2017), as well as from Facebook groups across the UK (UK Bat Workers, Cambridgeshire Bat Group, Kent Bat Group and South Lancashire Bat Group). Samples were collected between March 2016 and September 2018 from live, rehabilitating animals. Bat packs were issued to collect swabs from injured bat wings. Each pack included: two swabs (TS/8-A, woodshaft with cotton tip, Technical Service Consultants, UK), an Eppendorf tube for dry swabs (D), another Eppendorf tube containing 100 µL of molecular grade water (Microbial Cell Culture Grade Water, Fisher BioReagents) for wet swabs (W), gloves, bubble bag, instruction card, a 1 cm gridded card to take a picture of the injured wing against and send it in by

email, a questionnaire card (Appendix 2), and a pre-paid envelope to return the swabs and the questionnaire card to the university.

Bat carers were asked to swab the site of any bats wing injuries on bats that had arrived in their care. This was done using both a wet and dry double swabbing technique to maximise DNA recovery (Butler, 2012). A sterile swab, wetted with 100 µL of molecular biology grade water immediately before use, was gently rubbed over the bat wing (Butler, 2012) and then the swab tip was snapped off and placed in a 1.5 mL tube. This was followed by swabbing the same bat wing area with a dry sterile swab to collect the remainder of the material (Butler, 2012) and placing it in a separate 1.5 mL tube.

By September 2018, a total of 72 pairs of bat swabs were provided by bat carers. This included 40 swabs from injured *P. pipistrellus* and 32 swabs from other UK bat species, which were: 4 swabs from the brown long-eared bat (*Plecotus auritus*), 2 swabs from Natterer's bat *(Myotis nattereri*), a swab from Serotine (*Eptesicus serotinus*), 18 swabs from Soprano pipistrelle *(Pipistrellus pygmaeus*), 4 swabs from the whiskered bat (*Myotis mystacinus*) and 3 swabs from unknown bat species. Once received, all swabs were stored at -20°C prior to genetic analysis. Before analysis it was observed that 25 samples (34% of all paired swabs) had a suspected fungal growth on the swabs and therefore extra analysis was conducted on those samples, to identify if the fungal growth significantly impacted the detection of cat DNA (see section 5.2.6 on Fungal DNA).

5.2.2 Other Data Collected

Bat carers were asked to complete a short questionnaire for each sample providing information on species, age and gender (if known). Also, photographs of wing tears were taken and causes of the wing tears were collected from bat carers (see section 2.2.1), then the tears were classified in the same protocol that is described in chapter 2 (see section 2.2.2). From the 72 samples that were submitted, 52.7% were received

with images of the injured wings, and 14 samples (19.4%) provided a possible cause of the injury.

5.2.3 DNA Extraction and Quantification

To extract any DNA from the swabs and the control cat blood samples, an ISOLATE II Genomic DNA kit (Bioline, UK) was used, together with the Purifying Genomic DNA protocol provided. First, 500 µL of phosphate-buffered saline (PBS) (Sigma-Aldrich, UK) was added to each swab (D, W) and vortexed for 2 minutes to extract all cells from the swab, and then the swab was removed. Next, 180 µL lysis buffer GL and 25 µL proteinase K solution were added to each swab, vortexed, and then incubated at 56°C for 1 hour (until completely lysed). Vortexing for 10 seconds was performed 4 times, at regular intervals, during the incubation. Subsequently, the samples were vortexed for 10 seconds and 200 µL lysis buffer G3 added, then vortexed for 30 seconds and incubated at 70°C for 10 minutes. After this, all samples were vortexed for 10 seconds and 210 µL molecular grade ethanol (96-100%) added and vortexed for 30 seconds. Subsequently, an ISOLATE II Genomic DNA Spin Column (green) was placed in a 2 mL collection tube and loaded with both the wet and dry swab samples from each bat and centrifuged for 1 minute at 11,000 xg (Thermo Scientific Heraeus, Thermo Fisher Scientific).

Samples were then washed using 500 µL wash buffer GW1, centrifuged for 1 minute at 11,000 xg, and then 600 µL wash buffer GW2, centrifuged for 1 minute at 11,000 xg. The silica membrane was dried by centrifugation for 1 minute at 11,000 xg, to remove any residual ethanol. The DNA was then eluted by adding 100 µL preheated elution buffer G (incubated at 70°C) onto the centre of the silica membrane, incubating at room temperature for 1 minute, then centrifuging for 1 minute at 11,000 xg. DNA samples were stored at -20°C prior to subsequent analysis.

The same protocol was followed to extract the DNA from the cat blood samples, starting from the addition of lysis buffer GL and proteinase K solution.

Extracted DNA was analysed using a NanoDrop™ 2000 Spectrophotometer (ThermoFisher, UK) to determine both nucleic acid concentration and purity (Appendix 3).

5.2.4 Cat DNA Presence / Absence

Primers and probes

Quantitative polymerase chain reaction (qPCR) was used to determine the presence or absence of cat DNA. Primers and probes were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Eurofins qPCR Primer and Probe Design Tool software (https://www.eurofinsgenomics.eu/en/ecom/tools/qpcr-assay-design/) for amplification of the target sequence FCA749 locus of *Felis catus* (Genbank accession number AY988149.1). All primer and probes were obtained from Eurofins MWG Operon, Germany. Specificity of the developed primer and probe sequences was confirmed by a BLAST search on the NCBI website (http://www/ncbi.nlm.nih.gov/BLAST/) and showed 100% identity and coverage of cat DNA (*Felis catus).*

Method optimisation

The following parameters were evaluated to optimise the qPCR method: i) annealing temperature (52°C, 54°C, 56°C, 58°C and 60°C); ii) Magnesium chloride ($MgCl_2$) concentration (1 mM, 1.5 mM, 2 mM and 2.5 mM); and iii) probe concentration (0.2 µM 0.25 µM and 0.3 µM). Optimisation experiments were run only once to select the values for the $MgCl_2$ and probe concentrations, and twice for the temperature values.

Experimental

Following optimisation, samples were run in a total volume of 50 µL in a 96 well plate using the following reagents: 5U/µL DNA polymerase (BIOTAQ DNA Polymerase, Bioline, UK), 1x reaction buffer (10x NH_4 Reaction Buffer, Bioline, UK), 25 mM dNTPs (dNTPs mix, Bioline, UK), 1.5 mM $MgCl_2$ (Bioline, UK), 0.5 µM forward primer (5'-ATGCGTTCTCTGTCTCTC-3'), 0.5 µM reverse primer (5'-CATCTCACCGACCTAAAC-3'),

0.25 µM probe (5'-[HEX]-TCACTGCTGGCCTCTTTCAAATCAC-3') and 5 µL of extracted DNA. Positive controls were prepared using DNA extracted from cat blood samples, along with negative controls containing no template DNA. Samples were run on a MX3005P Real-Time qPCR System (Stratagene, UK) with a thermal cycling profile of 94°C for 10 minutes, followed by 45 cycles of: 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds. The experiments were repeated 3 times.

Agarose gel electrophoresis

qPCR results were corroborated by running the amplified qPCR products on a 2% agarose gel (Bioline, UK) made up using 1x TBE buffer (containing of 0.1 M Tris Base (Fisher Scientific, UK), 0.1 M boric acid (Fisher BioReagents, UK), and 0.02 M EDTA sodium salt (diaminoethanetetra-acetic acid, Fisher Scientific, UK) in distilled water), with 0.1% of DNA stain (Midori Green, Nippon Genetics).

To run the gel, 2 µL of loading dye (Loading Buffer Blue, Bioline, UK) was added to 10 µL of each qPCR product, and then loaded into the gel alongside 5 µL of DNA size ladder (HyperLadder ^{TM} 25bp, BIOLINE, UK). Gels were run with in 1x TBE buffer for 45 minutes at 75 volts and the results documented with a UV transilluminator (Geneflash Gel Documentation Darkroom, Syngene, UK).

5.2.5 Cat DNA STR Profiling

Any positive samples containing cat DNA were also subjected to DNA profiling to identify if the same cat was responsible for the bats wing tears. STR profiling was trialled on cat blood samples and those swabs which tested positive for cat DNA using the qPCR assay. Reactions was prepared in a 96 well plate in a total volume of 20 µL using the following reagents: 5 U/µL DNA polymerase (BIOTAQ DNA Polymerase, Bioline, UK), 1X reaction buffer (10X NH_4 Reaction Buffer, Bioline, UK), 25 mM dNTPs (dNTPs mix, Bioline, UK), 1.5 mM $MgCl_2$ (Bioline, UK), 12 µL cat STR primer mix (see Table 6.1 for details), and 4 µl extracted DNA. Positive controls were prepared using DNA extracted from cat blood samples, along with negative controls containing no template DNA. Samples were then subjected to thermal cycling on a Prime thermal

cycler (Techne, UK) using the following parameters: initial denaturation at 90°C for 10 minutes; 28 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute; and a final extension at 60°C for 45 minutes (Menotti-Raymond et al., 2005).

Capillary electrophoresis and data analysis

PCR products from STR amplification were sized using fragment analysis. Samples were prepared by adding 0.5 µL of the PCR reaction product to 0.15 µL of GeneScan™ LIZ 500 Size Standard (ThermoFisher, Scientific, UK) and 9.85 µL of Hi-Di Formamide (ThermoFisher Scientific, UK). The samples were run on a 3737 Genetic Analyzer (Applied Biosystem, UK) at the DNA Sequencing Facility at the University of Manchester.

Analysis of the STR profiles was carried out using GeneMapper® software (version 3.7) (ThermoFisher Scientific, UK), to facilitate sizing of the observed alleles at each locus (Butler, 2012). Complete DNA profiles were obtained from some of the positive control samples extracted from cat blood (an example is shown in Figure 5.1) and the negative controls were clear of contamination. However, the DNA from swab samples failed to present any allele at any locus, with no peak appearing significantly larger than the noise observed in the profile, which typically the minimum peak should be larger, between 5-10 times higher than the noise (Wells et al., 2011; Butler, 2015) (an example is shown in Figure 5.2). Failure of this method could be due to i) low quality of DNA from the swabs; ii) poor optimisation of the procedure; or iii) combining all the markers in one test. As profiling was not a key focus of the thesis and was not integral to identifying the presence of cat DNA on the swab samples, this method was not further investigated here.

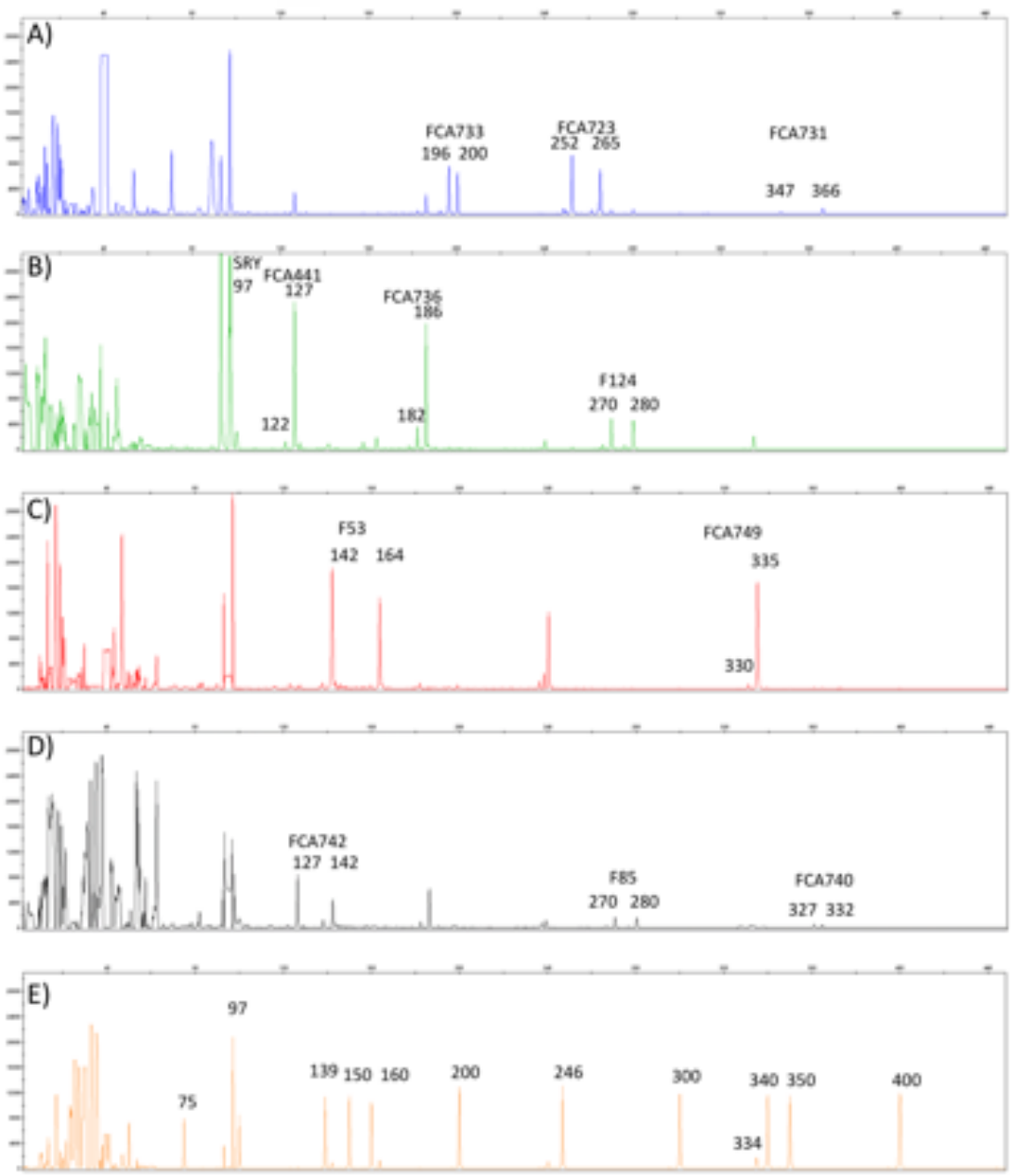


Figure 5.1: An example of a complete genetic profile from cat blood (DNA from cat number 01606). The panels present the observed alleles at each of the following loci: A) FCA733, FCA723, FCA731, B) SRY, FCA736, F124, C) F53, FCA749, D) FCA742, F85, FCA740. Panel E) DNA size ladder.

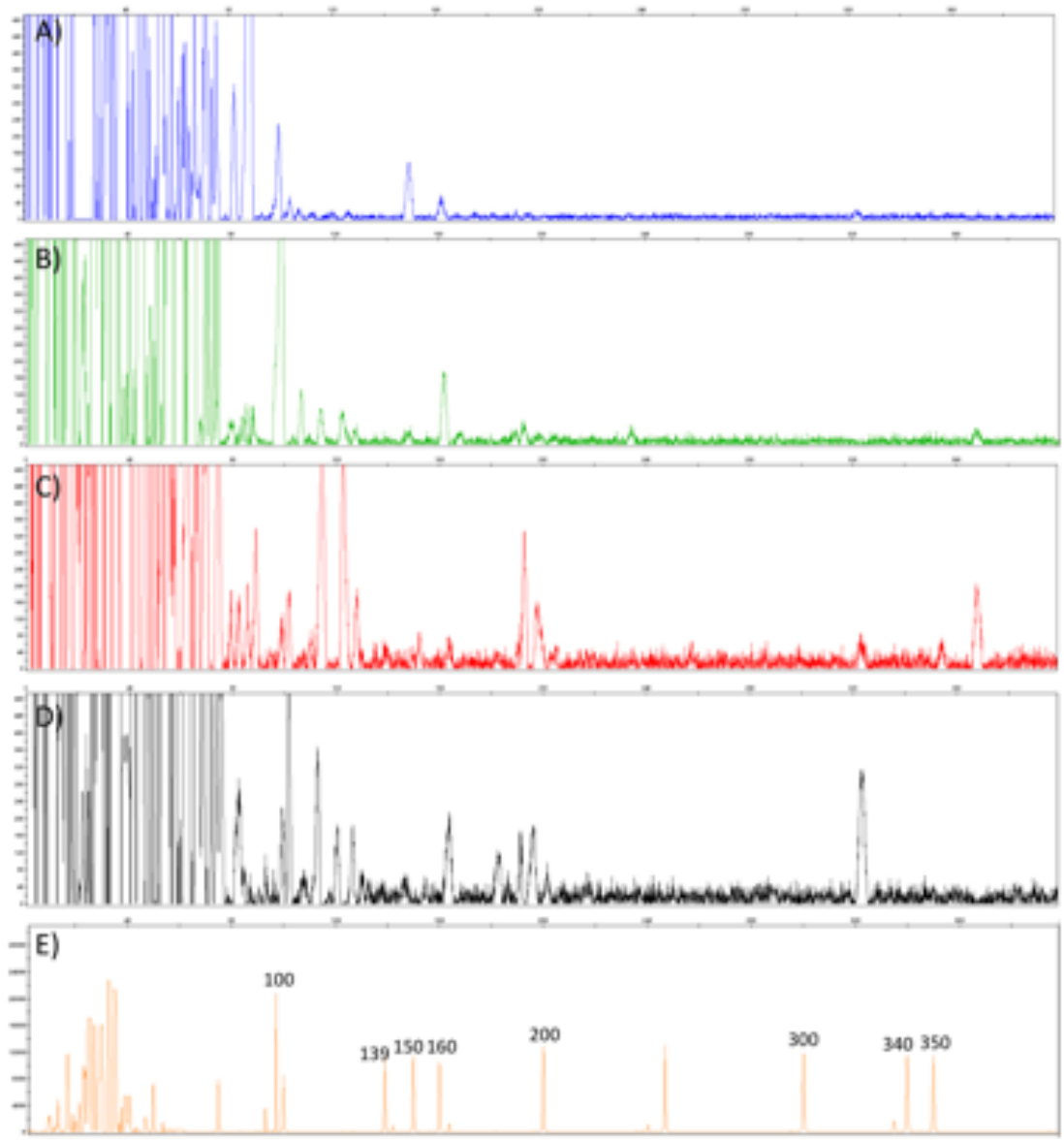


Figure 5.2: An example of a DNA profile from a swab sample (DNA from swab sample 45). The panels show no observed alleles at any loci: A) FCA733, FCA723, FCA731, B) SRY, FCA736, F124, C) F53, FCA749, D) FCA742, F85, FCA740. Panel E) DNA size ladder.

Table 5.1: Cat multiplex primer sequences, final concentrations of each pair of the primers, the fluorescent dyes used for each markers and the expected product size ranges.

STR marker	Final concentration (µM)	Dye	Primer	Primer sequences (5'-3')	Size range (bp)
FCA733	0.8	6-FAM	F	GATCCATCAATAGGTAAATGGATAAAGAAGATG	128–226
			R	6FAM-TGGCTGAGTAATATTCCACTGTCTCTC	
FCA723	0.8	6-FAM	F	6FAM-TGAAGGCTAAGGCACGATAGATAGTC	243–317
			R	GCCACCCAGGTGTCCTGCTTC	
FCA731	1.6	6-FAM	F	6FAM-ATCCATCTGTCCATCCATCTATT	337–401
			R	GGTCAGCATCTCCACTTGAGG	
SRY	0.04	VIC	F	VIC-TGCGAACTTTGCACGGAGAG	96–97
			R	GCGTTCATGGGTCGTTTGACG	
FCA441	0.3	VIC	F	GTGTCTTGATCGGTAGGTAGGTAGATATAG	113–137
			R	VIC-ATATGGCATAAGCCTTGAAGCAAA	
FCA736	0.1	VIC	F	VIC-CCGAGCTCTGTTCTGGGTATGAA	164–222
			R	GTGTCTTTCTAGTTGGTCGGTCTGTCTATCTG	
F124	1.1	VIC	F	VIC-TGTGCTGGGTATGAAGCCTACTG	255–367
			R	GTGTCTTCCATGCCCATAAAGGCTCTGA	
F53	0.8	PET	F	PET-CCTATGTTGGGAGTAGAGATCACCT	115–272
			R	GTGTCTTGAGTGGCTGTGGCATTTCC	
FCA749	1.1	PET	F	PET-GAGGAGCTTACTTAAGAGCATGCGTTC	276–416
			R	GTGTCTTAAACCTATATTCGGATTGTGCCTGCT	
FCA742	1.1	ROX	F	NED-AAATTTCAATGTCTTGACAACGCATAAG	122–175
			R	GCCAGGAACACCATGTTGGGCTA	
F85	1.3	ROX	F	NED-TAAATCTGGTCCTCACGTTTTC	183–301
			R	GCCTGAAAATGTATCCATCACTTCAGAT	
FCA740	1.1	ROX	F	NED-CCAAGGAGCTCTGTGATGCAAA	308–336
			R	GTTCCCACAGGTAAACATCAACCAA	

5.2.6 Fungal DNA

Samples with suspected fungal growth were also subjected to conventional PCR using fungal specific primers aimed at amplifying the internal transcribed spacer (ITS) region of ribosomal DNA from fungi (Toju et al., 2012; Romanelli et al., 2014). DNA amplification reactions contained the following reagents in a total reaction volume of 20 µL: 1x MyTaq Red Mix (Bioline, UK), 0.5 µM ITS1 forward primer (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Invitrogen, Thermo Fisher Scientific), 0.5 µM ITS2 reverse primer (5'-GCTGCGTTCTTCATCGATGC-3') (Invitrogen, Thermo Fisher Scientific) and 2 µL of extracted DNA. Samples were amplified on a Prime thermal cycler (Techne, UK) using the following parameters: initial denaturation at 95°C for 10 minutes; 30 cycles of 95°C for 60 seconds, 55°C for 60 seconds and 72°C for 90 seconds; followed by a final extension at 72°C for 10 minutes. DNA for the positive control was extracted from *Saccharomyces cerevisiae* (Purchased strain, NCYC 87) that had been grown on agar plates. A single colony was picked using a sterile loop, added to 500 µL of molecular biology grade water (Fisher Scientific, UK) in a 1.5 mL sterile Eppendorf tube, vortexed, incubated at 100°C for 5 minutes to break the fungal cell wall and release the DNA, and centrifuged at 6000 xg for 5 minutes before transferring the supernatant to a fresh sterile Eppendorf tube. Negative controls were also run which contained no template DNA.

Agarose gel electrophoresis

The PCR results were analysed by running the amplified PCR products on a 1% agarose gel in 1x TBE. A 10 µL aliquot of the PCR products were loaded onto the gel along with 5 µL of DNA size ladder (HyperLadder™ 100 bp, Bionline, UK). Gels was prepared and run using the same protocol described in section 5.2.4.

Sanger sequencing

Samples which were found to be positive for fungal DNA, were subjected to Sanger sequencing. The PCR products were cleaned up by adding 4 µL of ExoSAP-IT™ reagent (Express PCR Product Clean-up, Applied Biosystems, UK) to 10 µL of the PCR products, vortexing gently and briefly spinning down. Samples were incubated at 37°C for 4

minutes, then at 80°C for 1 minute, before being snap-cooled on ice. Cleaned samples were then prepared for sequencing by combining 6 µL of PCR product with either 0.5 µM of ITS1 forward or ITS2 reverse primer and made up to a 10 µL total volume with molecular biology grade water. Samples were then sent to the DNA Sequencing Facility at the University of Manchester where Sanger sequencing was performed, using BigDye v3.1 terminator and run on an ABI 3730 48-well capillary DNA Analyser (Applied Biosystems, California, USA). Sequence data was aligned and compared to the sequence chromatogram using the BioEdit Sequence Alignment Editor software (version 7.2.5) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 2004), then analysed using BLAST. Fungal identifications were made based on maximum identities and coverages.

5.2.7 Geographical Distribution

QGIS3 software (version 3.2.2) (https://www.qgis.org/en/site/) was used to compare the presence and absence of cat DNA, from the genetic results, with the location of the bat wing swab samples. A 20 mile radius was drawn around each rehabilitation centre based on communication with bat carers about their typical working area and the areas where injured bats are brought in from (personal communication, Sally Humphreys, East Dorset Bat Rescue and Rehabilitation). These 20 mile areas were: i) identified using 'The Web Mapping Application (Rural-Urban Classification for Output Areas Locator V2)', from the Office for National Statistics geography data (Contains OS data Crown copyright and database right [2019], available at https://ons.maps.arcgis.com/apps/webappviewer/index.html?id=20467878cc2041 0d961a3f71db356b6d) and ii) classified in terms of rural-urban classification by using 'The 2011 Rural-Urban Classification For Output Areas in England' by the Office for National Statistics geography data (contains National Statistics data Crown copyright and database right [2017]). The rural-urban percentage of each area was calculated using Inkscape software to measure areas. For locations with sea (such as in Kent), the sea area was excluded from the measurement.

5.2.8 Statistical Consideration

Statistical analysis was performed using SPSS Version 24. A chi-square test was used to compare: i) the percentage of bats showing the presence of cat DNA in terms of species, gender and age; ii) the percentage of the rural area and the samples with presence cat DNA between the locations; iii) the percentage of tears between the samples that showed presence and absence of cat DNA; iv) the number of tears in each section of the wing; v) the percentage of each type of tear (holes, contained tears, total tears, and trailing edge tears) between the sample with cat DNA and sample without; vi) the percentage of samples with the presence of cat DNA between the sample with and without fungal growth. The Mann-Whitney U test was used to compare the average number of DNA loci that amplified in the samples with and without fungal growth.

5.3 Results

5.3.1 Cat DNA Presence/ Absence Analysis

5.3.1.1 qPCR Method Optimisation

Optimisation of the annealing temperature

PCR products were produced using annealing temperatures of 52°C, 54°C and 56°C, but not at the higher temperatures of 58°C and 60°C (Figure 5.3). The PCR product sizes were calculated from the gel photograph, and found to be 289 bp, which was within the expected size range for the DNA fragment for this locus, which is 276-416 bp (Butler, 2012). The negative control was clear of any contamination. The optimum annealing temperature was selected as 56°C as the PCR product had the brightest band intensity.

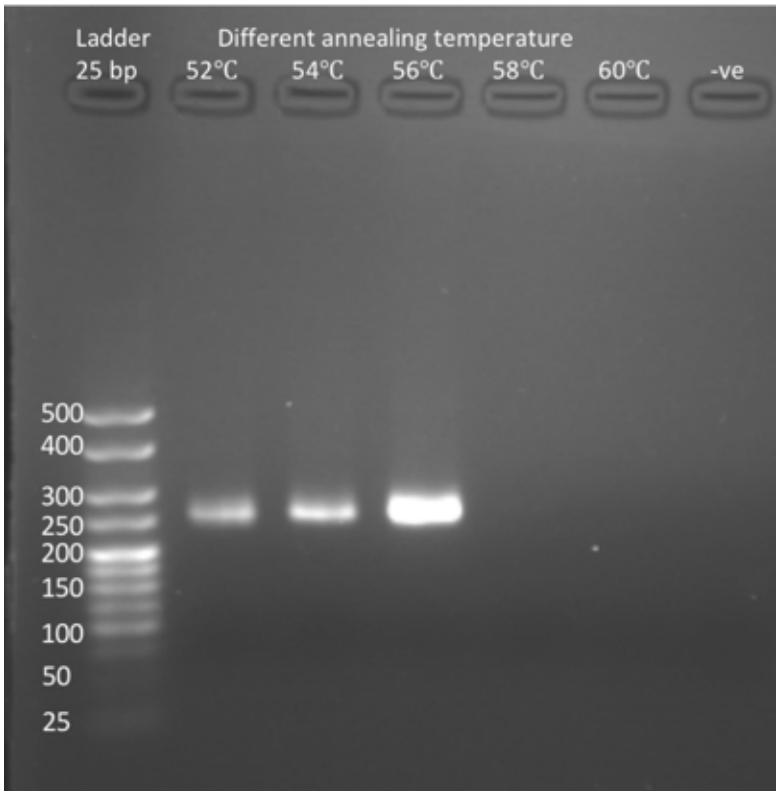


Figure 5.3: Gel photograph of amplified control cat DNA using specific cat DNA primers, with different annealing temperature, PCR products showing bands in multiple lanes and the brightest band at the annealing temperature of 56°C.

Magnesium chloride ($MgCl_2$) concentration optimisation

PCR products were obtained at all $MgCl_2$ concentrations tested (Figure 5.4A). In the amplification with qPCR, the lowest number of the cycles required for the fluorescent signal to cross a threshold (CT) value was the preferred concentration. The CT values at the $MgCl_2$ concentrations of 2 mM and 2.5 mM showed the lowest CT values at 28.76 and 28.66 cycles (Figure 5.4A), respectively, but non-specific amplification was also observed, indicated by additional bands on Figure 5.2B. Therefore, the optimum $MgCl_2$ concentration was chosen to be 1.5 mM. At 1.5 mM, the CT value was lower (28.97 cycles) than for 1mM (29.78 cycles) (Figure 5.4A), and on the gel photograph, the1.5 mM showed a single, bright band the with a product size of 282bp, which is within the expected size range of the locus (Figure 5.4B). The negative control was clear from contamination. (Figure 5.4B).

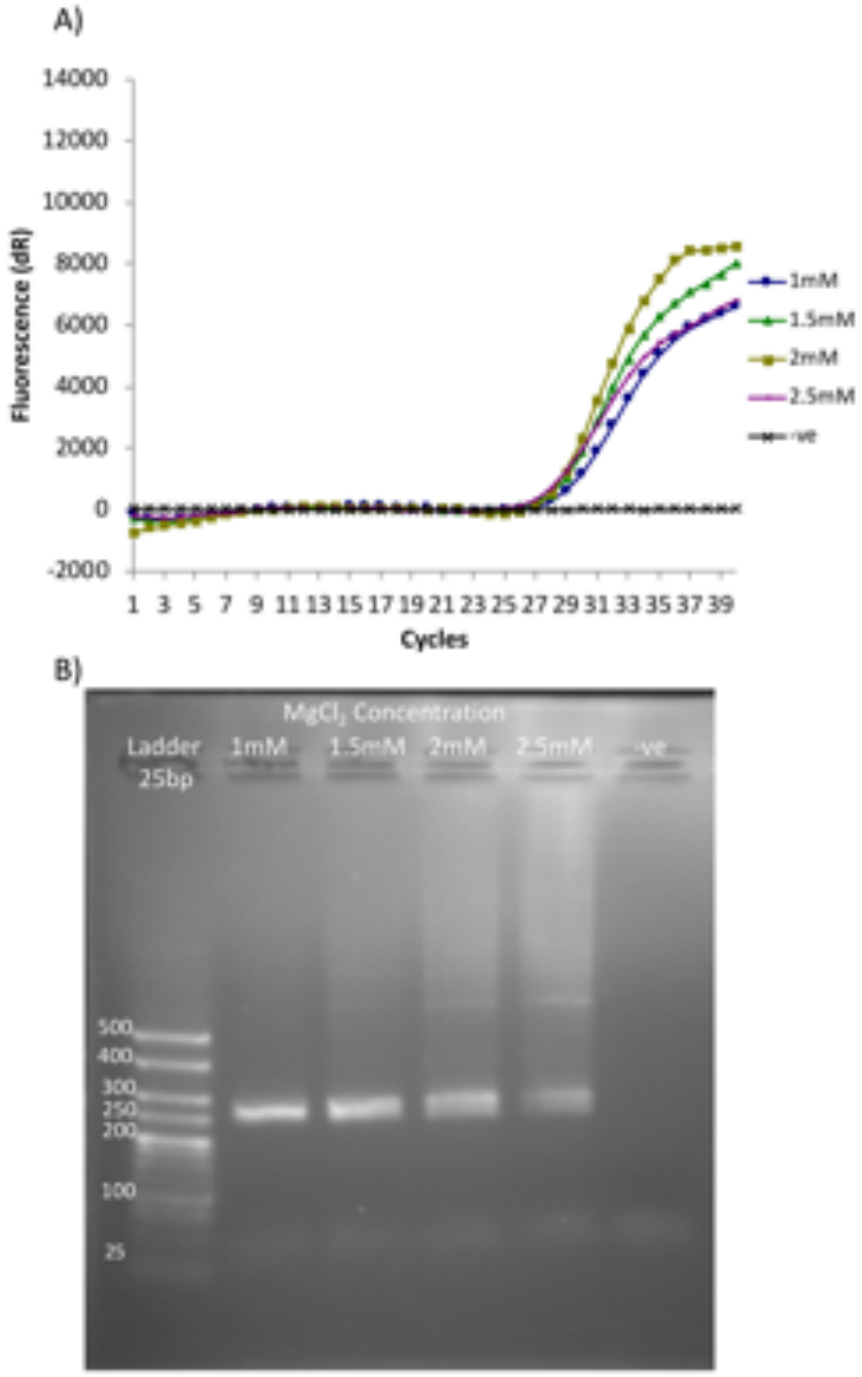


Figure 5.4: A) Amplification plot from qPCR of cat blood DNA (+ve) and the negative control (-ve) with different $MgCl_2$ concentrations. B) Gel photograph of the qPCR products. (n=1)

Probe concentration optimisation

PCR products were produced from all probe concentrations tested (Figure 5.5A). The optimum probe concentration was found to be 0.25 µM due to the lowest CT value (28.91 cycles) at that concentration comparing to the CT values at probe concentrations of 0.2 µM and 0.3 µM, which were 30.44 cycles and 29.03 cycles,

respectively (Figure 5.5A). Also, 0.25 µM concentration showed a bright band on the gel, with a product size of 283bp which is within the expect size of the locus. The negative control was clear from contamination (Figure 5.5B).

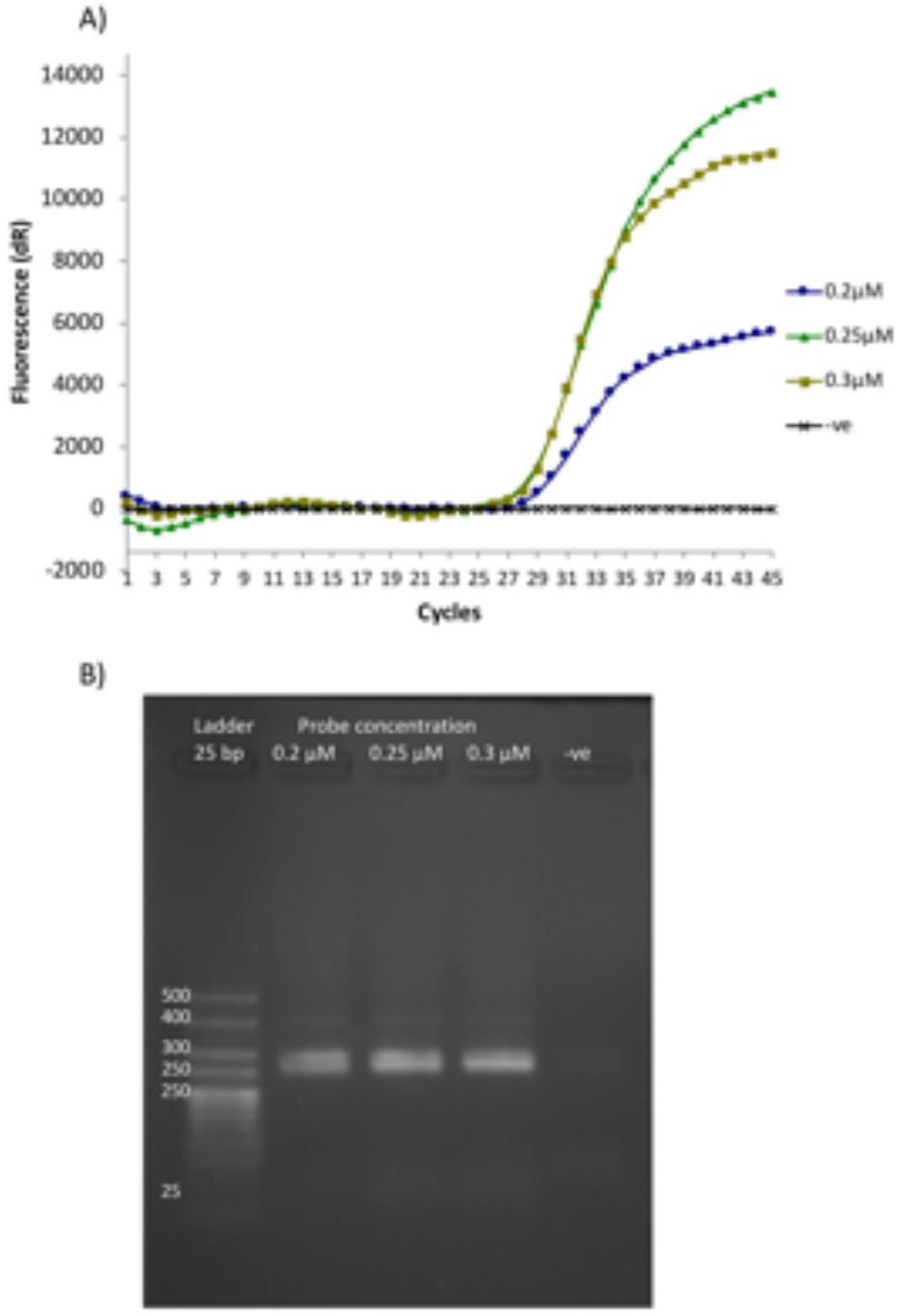


Figure 5.5: A) Amplification plot from qPCR of cat blood DNA (+ve) and the negative control (-ve) with different probe concentrations. B) Gel photograph of the qPCR products. (n=1)

5.3.1.2 Presence of cat DNA

All samples received from bat carers were analysed for the presence of cat DNA using qPCR, and confirmed using agarose gel electrophoresis (Appendix 5). The results showed that 48 out of 72 (66.7%) samples were positive for the presence of cat DNA (Figure 5.6A). The genetic data was also compared to the free-text comments received from bat carers, where they were asked to suggest any possible causes of the bat's wing injuries. When bat carers suspected cat attacks (in 15 cases), cat DNA was found to be present in all but one case. Therefore, there was 92.9% agreement between the bat carer observations and DNA analysis (Table 5.2).

Regarding the different bat species, 70% of *P. pipistrellus* samples, 65.5% of other UK bat and 33.3% of the samples from unknown species had cat DNA present (Figure 5.6B). There was no effect of species on the presence of cat DNA ($\chi 2$=0.118, df=1, p=0.732). Also, there was no difference in presence of cat DNA between males (64.2%) and females (72.7%) ($\chi 2$=0.591, df=1, p=0.442) (Figure 5.6C). The samples with unknown gender had cat DNA in 62.5% of the samples (Figure 5.6C). There was also no significant difference in the percentage of samples with cat DNA present in juveniles and adults ($\chi 2$=7.424, df=1, p=0.06) (Figure 5.6D).

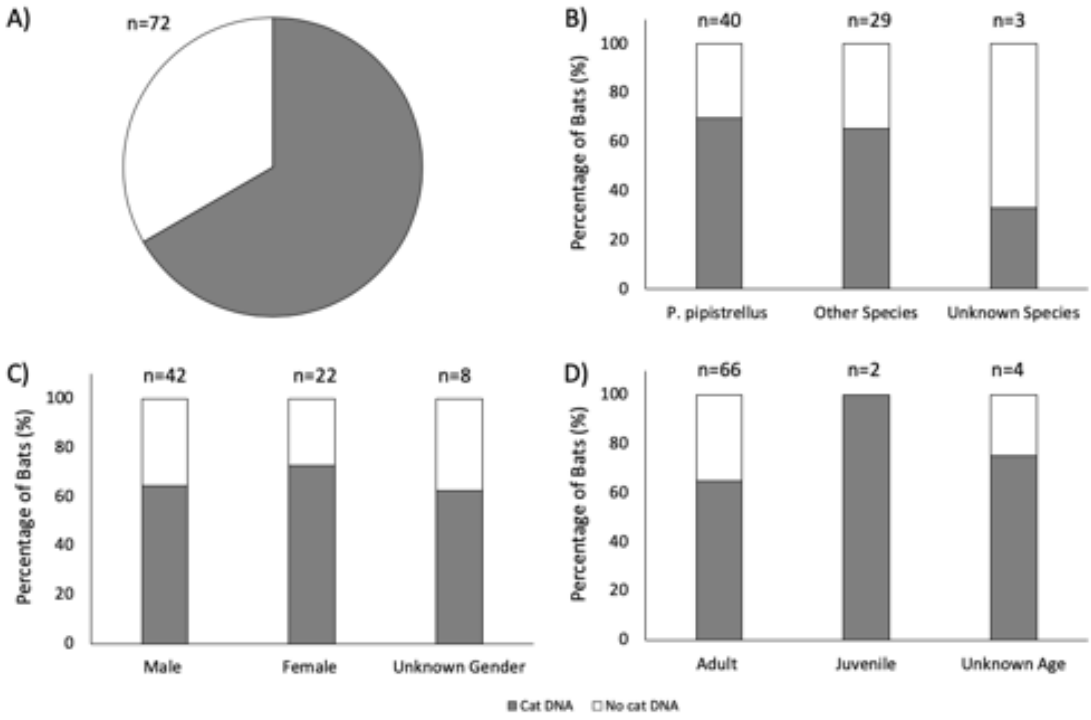


Figure 5.6: A) The percentage of swabs showing presence/ absence of cat DNA. B) Distribution of cat DNA with respect to UK bat species. C) Distribution of cat DNA with respect to bat gender. D) Distribution of cat DNA with respect to the age of the bat.

Table 5.2: Free-text comments on suspected cause of bat wing injuries provided by bat carers, correlated with presence or absence of cat DNA.

Free Text Comments	Cat DNA
'Bat bought in by a cat'	Absent
'Householder has 2 cats, bat with older cat attack scars in wings. On this occasion he was bitten left neck/ear and right shoulder. Signs of cat'	Present
'Householder has 2 cats - cat attack'	Present
'Householder has 2 cats - cat attack'	Present
'Owner of cat witnessed bat in cat's mouth'	Present
'seen brought into home in cat's mouth'	Present
'Seen with cat circling around on ground'	Present
'Found grounded by dog. Injuries appear to be consistent with cat'	Present
'Householder has known roost in attic and owns 3 'well behaved' cats'	Present
'Property owner has 3 cats which catch birds often including woodpeckers'	Present
'Cat at finder address'	Present
'Bad head wound as well as claw holes in wings'	Present
'Seen in cat's mouth'	Present
'Almost certainly cat damage'	Present

Samples were received from 5 counties in the UK: Cheshire, Devon, Dorset, Greater Manchester and Kent (Figure 5.7, Table 5.3). All these locations were categorised as mostly rural areas, except Greater Manchester, which was classified as an urban area (Figure 5.7, Table 5.3). There was a significant difference in the amount of rural and urban areas in each county with sample sizes of 5 or more bats (including Devon, Kent and Dorset) ($\chi 2$= 8.925, df=2, p=0.01) (Figure 5.8), and also the percentage of samples with cat DNA in each county ($\chi 2$=24.128, df=2, p<0.001) (Table 5.3). However, the relationship between urban and rural areas, and the percentage of samples with cat DNA, was not clear. Kent and Greater Manchester both had low amount of rural areas, but Greater Manchester had 100% of samples with cat DNA, and Kent only had 35%.

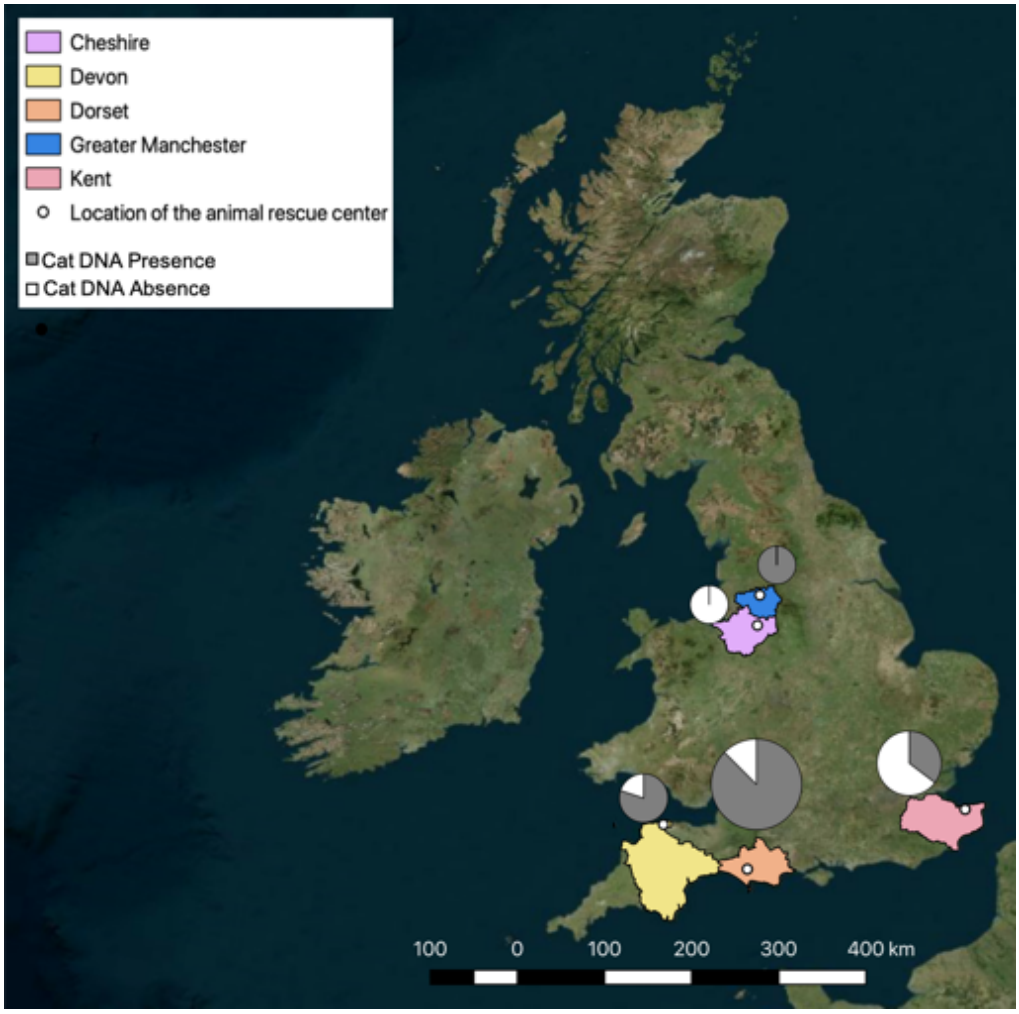


Figure 5.7: Map showing the locations where the swab samples came from, and the percentage of positive and negative samples for cat DNA at each location. The size of the pie chart represents the sample size. In addition, there were 22 samples for which the location was unknown, of these 15 showed the presence of cat DNA.

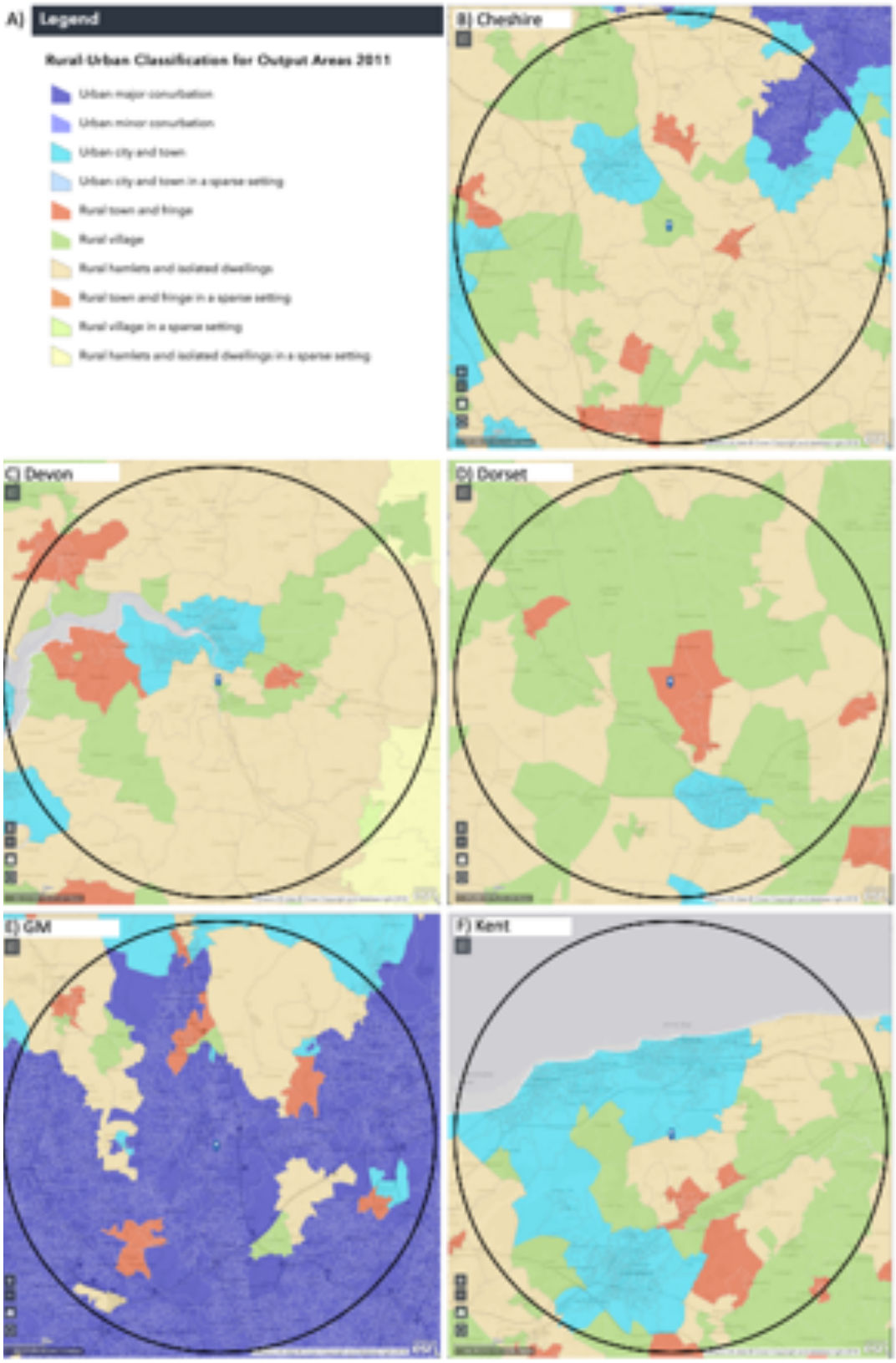


Figure 5.8: A) The rural-urban classification of the five locations: B) Cheshire, C) Devon, D) Dorset, E) Greater Manchester, F) Kent. The scale bar is 2 Km.

Table 5.3: The percentage of samples with presence cat DNA in each location, and the percentage of rural and urban areas in each location

County	Number of samples	Sample with presence cat DNA%	Rural area%	Urban area%
Cheshire	2	0	87.92	12.07
Devon	5	80	93.57	6.43
Dorset	24	88	97.49	2.69
Greater Manchester	2	100	25.80	74.20
Kent	17	35	62.21	35.93

5.3.2 Comparisons with wing tear classifications

The photographic information provided by bat carers on the nature of the wing injuries sustained by the bats was compared to the DNA analysis results. Bats with and without the presence of cat DNA had a similar number of tears when sample size was controlled for (as a percentage) ($\chi 2$= 1.922, df=1, p=0.166). For bat wing swabs where cat DNA was present, there were fewer tears in CI section compared to CII and P sections ($\chi 2$ = 9.324, df=2 p= 0.009) (Figure 5.9A, C, E). In the samples where no cat DNA was found, the P section of the bat had more tears than the CII and CI sections ($\chi 2$ = 13.500, df=2 p= 0.001) (Figure 5.9B, D, F). As in previous chapters, holes were the most common tears overall (63.72%) (Figure 5.9C-F). There were significantly more total tear types (%) in the bat samples with cat DNA present, compared to those without cat DNA ($\chi 2$= 8.758, df=1. P= 0.003). The amount of other tear types (holes, contained tears, trailing edge tears) did not significantly differ between the samples with cat DNA present and those without cat DNA (all ps>0.5). As per previous chapters most tears were oriented rostro-caudally from the wing membrane to the trailing edge (Figure 5.9 A-D).

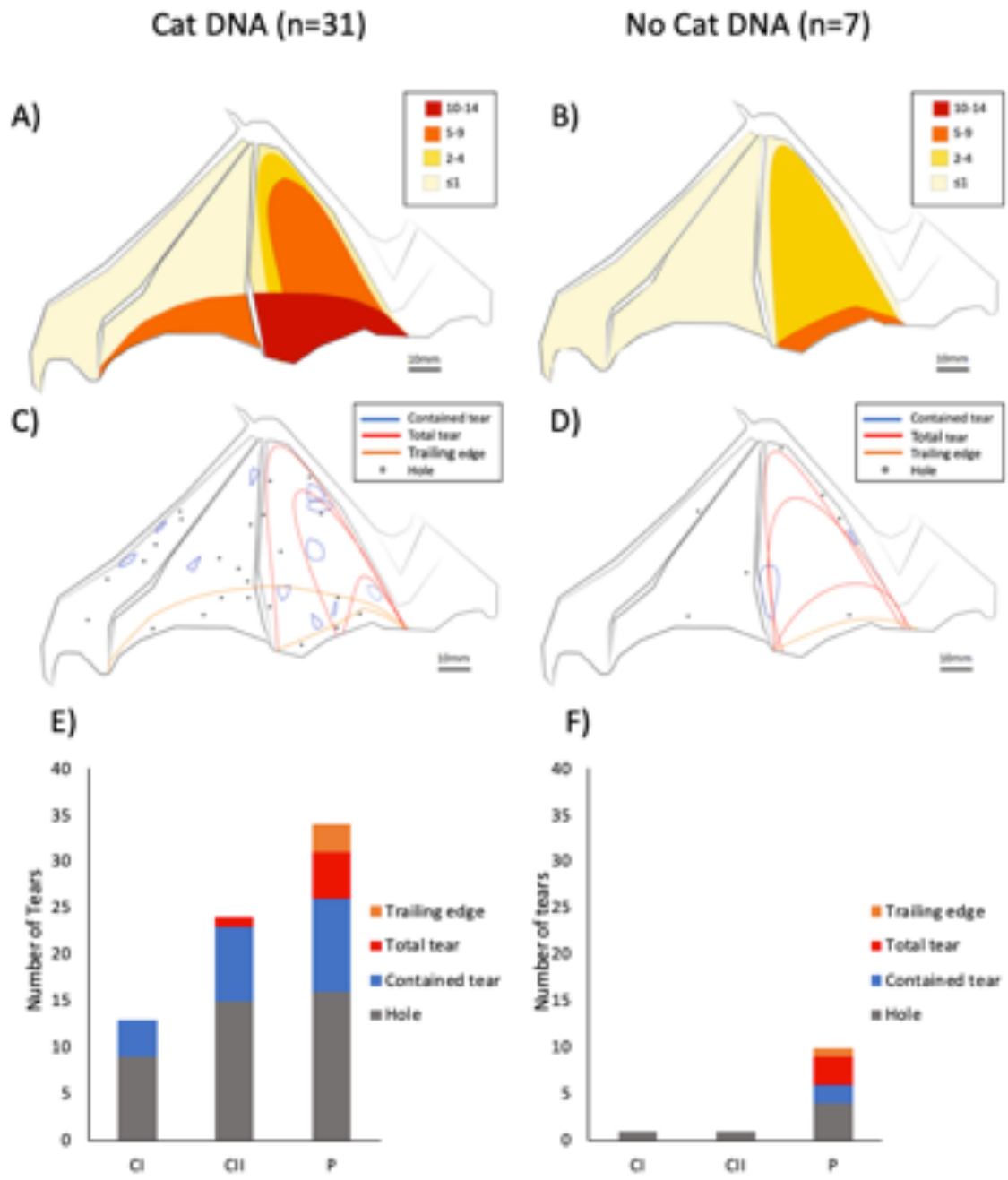


Figure 5.9: The wing tear distribution over the wing section in the samples with cat DNA present and absent. A, B) Diagrams of the wing, presenting the number and the location of the tears over the three sections. C, D) Diagrams of the wing presenting all tears found in bat wing images, over the three sections of the wing. E, F) Graphs representing the number of different types of wing tears in each section of the wing. The wing sections are CI) the first chiropatagium section; CII) the second chiropatagium section; and P) the plagiopatagium section.

5.3.3 Fungal DNA Analysis

The bat swab samples with suspected fungus were analysed and showed the presence of fungus in 24 out of 25 samples (96% of cases). The gel results showed PCR products in 24 samples with a size range from 265bp to 297bp (Figure 5.10, Appendix 3), which is expected for the ITS1 and ITS2 regions (Khodadadi et al., 2017). The positive control showed a PCR product at 450 bp, also within the expected size range of *S. cerevisiae* (Arlorio et al., 1999; Khodadadi et al., 2017), with the sequencing results confirming *S. cerevisiae spp.,* with a 90% similarity match. The negative control was clear indicating no contamination.

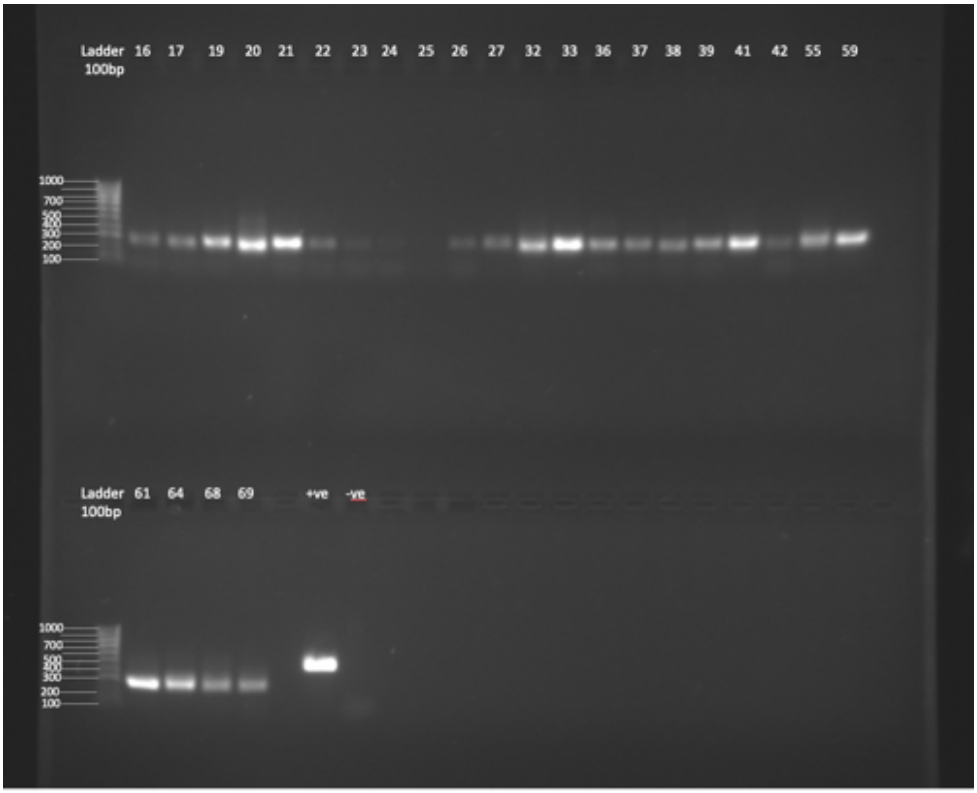


Figure 5.10: Photo of agarose gel electrophoresis of the PRC products from amplified fungal DNA. The numbers on each lane refer to the bat swab number (PCR No. Appendix 3), +ve is the positive control with S. cerevisiae DNA, and -ve is the negative control.

DNA sequencing of these samples showed the presence of 12 different fungal species (Figure 5.11), which are commonly found in the environment, such as in the air, water, soil, food and plants. *Cladosporium* is the most common species, being present in a quarter of samples, particularly those from Southern England (Figure 5.11).

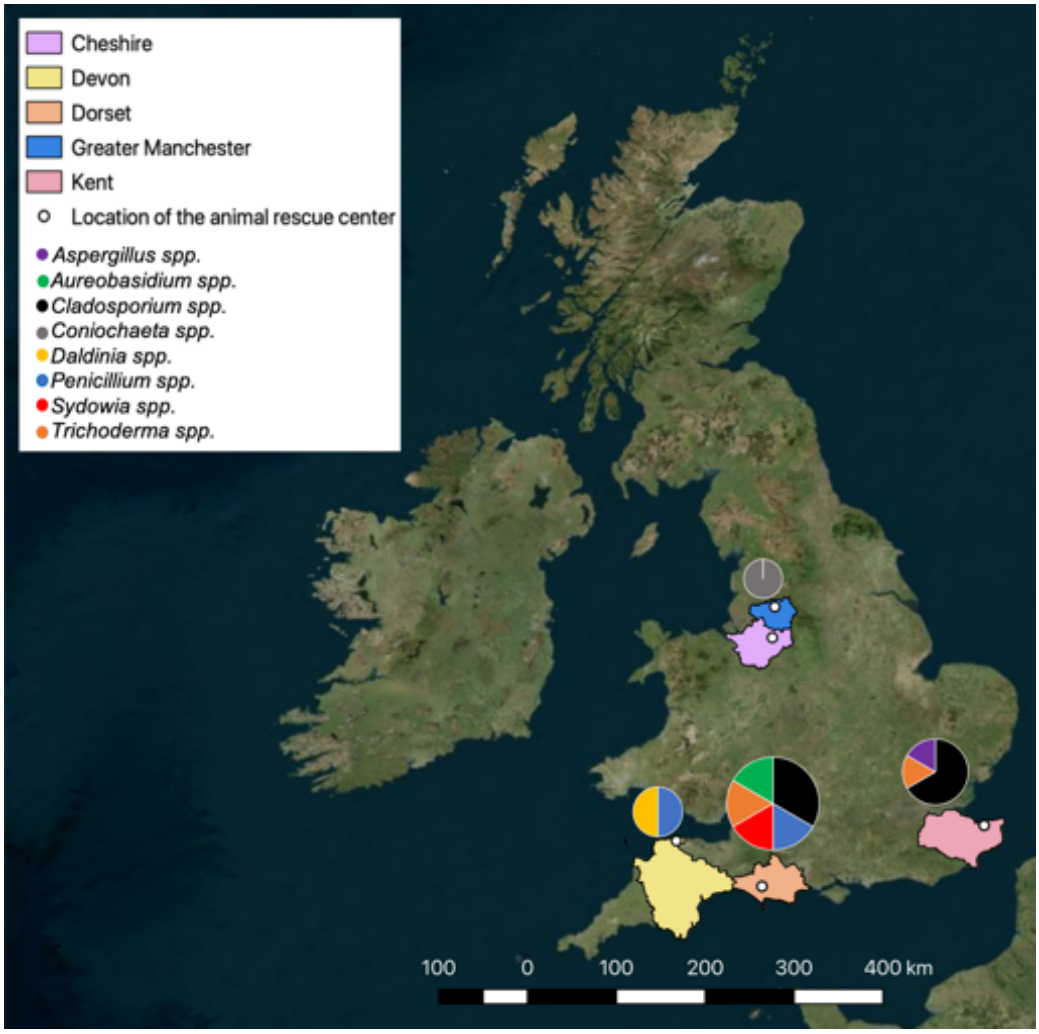


Figure 5.11:Geographical distribution of the fungal species obtained. No fungal growth was observed on the samples obtained from Cheshire. The size of the pie chart is proportional to the sample size. In addition, there were 7 samples for which the location was unknown. These were found to contain 3 instances of Aureobasidium spp. and single instances of Debaryomyces spp., Fusarium spp., Septoria spp., and Talaromyces spp.

To determine if fungal growth affected the ability to analyse any cat DNA present, the results from swabs with and without fungal DNA were compared. The percentage of samples showing the presence of cat DNA did not significantly differ between the samples with and without fungal growth (χ^2=2.597, df=1, p=0.107). The average number of loci that amplified in the samples with and without fungal growth were also counted. The results showed no significant difference between the samples with and without fungal growth (Z=243.5, p =0.487). These findings indicated that fungal growth had no effect on the profiling of cat DNA.

5.4 Discussion

5.4.1 Cat DNA Presence/ Absence Analysis

This study revealed that forensic DNA analysis can be used as a potential tool to evaluate whether cats are interacting with bats. By extracting DNA from injured bat wing swabs and amplifying the DNA using qPCR and specific probes, the presence of cat DNA can be identified.

Presence of Cat DNA

Two-thirds of the tested samples (66.7%) showed the presence of cat DNA, which provides additional evidence to observations of bats being preyed upon by cats, which has been documented in previous studies (Woods et al., 2003; Ancillotto et al., 2013). For example, Ancillotto et al. (2013), reported cat predation in an estimated 28.7% of bats being brought into rehabilitation centres, and Mühldorfer et al., (2011a) demonstrated that cat predation accounted for 19.5% of bat deaths based on post-mortems. Results presented in this chapter (Figure 5.6) suggest that cat predation events may be far more common than previously suggested. A recent study by Vlaschenko et al., (2019) reported a similar result that 68% of the Common noctule (*Nyctalus noctula*) bats found dead were killed by cat predation, during the winter when bats hibernated in a large concrete building. The value of two-thirds of bats being predated upon by cats still may be considered an underestimation due to factors such as i) injured bats not always being brought to the attention of carers; ii) insufficient DNA quantity transferred from cat to bat during the predation event; and iii) potential variability in the swabbing technique and sample storage by participating bat carers. It was observed that in one of the swab samples, the bat was bought in to the house by a cat, but this sample failed to show cat DNA (Table 5.2). This could relate to the swabbing area, as just the wings were swabbed whereas the cat may have held the bat by its body. There might also just be low amounts of cat DNA present, although qPCR is considered to be a very sensitive technique for the detection of DNA, with typical limits of detection as low as 1-10 copies of targeted DNA per reaction (Bustin et al., 2009).

A study in mainland Southwest Finland, proposed the idea of 'super predator cats', where just 6 cats (9%) accounted for 40% of all observed captures, with no difference between male and female cats (Kauhala et al., 2015). Therefore, the same cat might be repeatedly attacking bats. Developing the DNA profiling MeowPlex method (Butler et al., 2002) for the bat swabs would be an excellent way to identify repeat-offending cats. Failure of the method trialled here could be due to poor optimisation techniques or low DNA quality (Schneider et al., 2004; Swango et al., 2006; Huang et al., 2017). Low DNA quality most commonly occurs as a result of DNA degradation, which leads to fragmentation of DNA (McCord et al., 2011). This is particularly an issue with attempting to amplify longer alleles, and studies have found DNA fragment ≤200bp in length are more stable (Grubwieser et al., 2006; Regnaut et al., 2006). Several factors could lead to DNA degradation such as the amount of time in between the predation event and the bat wing being swabbed and room temperature storage during transport (Grubwieser et al., 2006; Karni et al., 2013).

The swabs of injured bat wings that were received were 58.33% male, 30.55% female, and 11.10% of an unknown gender. Although there was no significant effect of gender, most of the swabs received here were from injured males. This might be because males spend more time out of the roost (Audet, 1990), so they are probably more likely to receive tears on the wing from several factors including collisions or been attacked by a cat. Females usually spend months (June to July) within a maternity roost to give birth and feed their young for 4–5 weeks, until they become strong enough to fly (BCT, 2019). Therefore, females may be less likely to receive injuries. In my data, the exact time of the year of finding the injured bat was unknown and carers could post samples some time after the injured bat was found.

While data collected in this chapter suggested that more males received wing tears, a higher percentage of females had cat DNA present on their wing tears (although this was not significant). Many previous studies have observed more cat attacks on female bats, although these findings were based only on observational data. More female *N. noctula* bats were killed by cats over the winter in an urban environment (Vlaschenko et al., 2019), and during the summer in the reproductive season

(Ancillotto et al., 2013). Pregnant females might be more vulnerable to predation owing to reduced flight performance and manoeuvrability (Russo et al., 2007). Therefore, females could be more prone to attack by cats during the reproductive season (Ancillotto et al., 2013) and hibernation (Vlaschenko et al., 2019), when they are not in the maternity roost. Investigating the seasonality of wing tear casualties and associating this with the gender of the bats would help us to further understand the extent of the problem, and the long-term effects on bat populations.

There was no significant effect of age (juveniles and adult) on whether more samples had cat DNA present. Ancillotto et al. (2013) and Vlaschenko et al. (2019) also observed juveniles and adults to be just as likely to be targeted by cat attacks. There was also no significant effect of species (*P. pipistrellus* and other species) on whether more samples had cat DNA present. This could be because all the bat species in the study have similar roosts. Indeed, all species studied here may use buildings for roosts (BCT, 2015), at least for breeding periods, in rural and semi-urban areas which could mean encountering free-ranging cats at a similar rate (Ancillotto et al., 2013).

While the exact location of where the wing tear injury occurred was unknown for each sample, an area of 20 miles around the rehabilitation centre was approximated as a likely area of injury (Figure 5.8). Cat predation events have been documented as occurring more frequently in rural and sparse-urban areas, compared to dense urban areas (Ancillotto et al., 2013). This may be because cats in urban areas are often kept indoors at night (Ancillotto et al., 2013). There was no clear pattern in the percentage of samples with cat DNA and their geographical area, but a decrease in the percentage of rural areas, such as in Kent, corresponded to a decrease in the percentage of the samples with cat DNA present (Figure 5.7, 5.8); however, Greater Manchester was the most urban area, and had 100% of the samples containing cat DNA.

5.4.2 Comparison to Wing Tear Classification

Of the bats with cat DNA present, more tears were found in the CII and P sections, which are the closest to the body, and total tears were also more prevalent. As previously discussed in Chapter 2, these tears may be due to cat strikes targeting the body of the bat, and claws raking in the rostral-caudal direction. The presence of cat DNA supports this idea. However, samples which revealed no cat DNA also had more tears in the P section. It may be that some samples with cat DNA present are not being detected by the method (due to swabbing procedures and storage time), or it might be that other causes contribute to tears in the P section. While collisions are probably more likely to affect the distal sections of the wing, perhaps tears during take-off (as the wing unfurls) or grounding might be a possible cause of these. Vegetation, such as brambles or other thorns, being present in take-off and land sites is a likely cause, but hard to identify objectively. The P section is the largest section of the wing so it may just be more likely to receive tears than the most distal section of the wing CI. Findings here match with what was previously found in Chapter 2.

As well as causing wing tears, cat attacks can also lead to bacterial diseases in bats (Mühldorfer et al., 2011a; Mühldorfer et al., 2011b), which can be transmitted to bats from cat saliva (Mühldorfer et al., 2011b). Cats may also receive a viral infection from the bats, such as Nipah virus (NiV) and European bat lyssaviruses (EBLVs), which could lead to cat mortality (Epstein et al., 2006; Dacheux et al., 2009). Future work identifying cat DNA in more bat samples will help further our understanding of the scale of the association between cats and bats.

5.4.3 Fungal DNA Analysis

It was found that the fungal growth on some of the samples had no significant effect on the presence of cat DNA. Despite the presence of fungal growth not affecting the quality of the DNA, in comparison to those samples without fungal growth present. Fungal growth may have happened due to reasons such as: i) some samples took a long time to arrive at the university; ii) the samples not kept in the freezer until being sent.

Implications

This study evaluates the possibility of cat predation upon bats by using forensic DNA analysis techniques as an alternative to observational data. By swabbing the site of bat wing tear injuries, the presence of cat DNA can be determined. The results presented here suggest that cat predation on bats, at least in the UK, is likely to be higher than previously estimated.

Chapter 6 Discussion

Chapter summary

This chapter presents the key findings and implications from each chapter of the thesis. It then identifies the limitations of the work and, finally, it provides recommendations for bat carers and cat owners. The crucial recommendations for bat carers on releasing rehabilitated bats are to: i) understand that the wing tear healing capacity is different depending on the tear location on the wing, ii) monitor wing movement and body orientation during flight to assess if a bat is ready to be released, and iii) to conduct post-release monitoring to truly assess the long-term effect of wing tears. It is recommended that cat owners attach a bell on the cat's collar, and keep cats indoors during the night when bat activity is highest and bat/cat interactions most likely.

6.1 Summary of Findings

Chapter 2 provides the first quantitative description of wing tears in Common pipistrelles (*Pipistrellus pipistrellus)* and other bat species from the United Kingdom. Classification of the wing tears showed that the plagiopatagium (P), the wing section closest to the body, had the most tears. These tears tended to be oriented rostro-caudally, from the wing membrane to the trailing edge. I suggest that investigating the extensive problem of wing tear injuries in bats will help to improve rehabilitation plans and has significant implications for bat conservation and welfare.

Chapter 3 investigated the anatomy of the *P. pipistrellus* wing, specifically whether wing anatomy was associated with tear position and orientation. The results indicated that while the anatomy might be associated with tear healing, it was not sufficient to explain the tear positions and orientations. Indeed, material testing did not identify the plagiopatagium as being significantly weaker than the chiropatagium, despite its having the most tears. Rather, I suggest that the position of the plagiopatagium, rather than its anatomy, is an important factor in determining the

number, location and orientation of wing tears. The position of the tears, close to the body and towards the trailing edge, suggests that they are caused by a predator attack, such as by a cat, rather than by a collision. Consistent with this, 38% of *P. pipistrellus* individuals had confirmed wing tears caused by cats, with an additional 38% identified by carers as suspected cat attacks. Further exploration of the possibility of cats causing wing tears is presented in chapter 5.

Chapter 4 examined the effect of wing tears on flight in *P. pipistrellus*. Comparing the wing movements and body orientation between bats with and without wing tears showed that the bats with bilateral wing tears had impacted wing movements. In particular, bats with bilateral wing tears had the lowest maximum angles, highest minimum angles and highest wing beat frequencies compared to those with no wing tears or with unilateral wing tears. In addition, body orientation was also affected by wing tears, such that bats tilted towards the healthy wing and away from the most injured side. I recommend that further work investigating the survival and fitness of animals post-release is needed in order to make strong predictions about the long-term effects of wing tears on flight, survival and reproductive success.

Finally, chapter 5 investigated whether cats may be responsible wing tears observed. Forensic DNA analysis found the presence of cat DNA in 67% of the wing tears swabbed. Previous studies that reported cat predation on bats have been based on observational data only (Phillips et al., 2001; Woods et al., 2003; Ancillotto et al., 2013; Kauhala et al., 2015; Vlaschenko et al., 2019), and this is the first convincing evidence of cats being a major cause of wing tears in bats.

6.2 Scientific Implications of the Thesis

6.2.1 Cats Cause Many Wing Tears

Chapter 2 revealed that the highest number of tears were found in the plagiopatagium section. The position of the plagiopatagium, being close to the body, is prone to tearing by cats targeting the body with paw strikes. This was confirmed by results in chapter 5 that found cat DNA present on bat wing tears in 67% of all

cases. This is likely to be a conservative estimate, as the tears were swabbed by non-experts and DNA might have degraded over time too. Regarding Locard's principle, which states that 'every contact leaves a trace', if a cat attacks a bat, cat DNA will transfer to the bat wing. But for some reason such as inaccurate swabbing or degraded DNA, the cat DNA may not be detectable on all bat wing swab samples. Samples with cat DNA present had more total tears through the wing, which is defined as a tear that runs from the internal membrane to the trailing edge of the wing and affects more than 50% of the total wing section. As well as having more of these large and significant tears, samples with cat DNA also had more tears in the proximal wing sections.

A complementary final-year undergraduate study was conducted to see where cats target a bat-shaped toy (Figure 6.1A) as they played with it (Tabner, 2019). An example of a body strike can be seen in Figure 6.1B. The results found that the centre of the toy bat was significantly targeted by cats in around 70% of all paw strikes (Figure 6.1C). When the paw strike missed the body, it often connected with the bat's wings instead (Figure 6.1C), which accounted for over 20% of all paw strikes. Strikes of this type could well cause the wing tears that I have described throughout my experimental chapters.

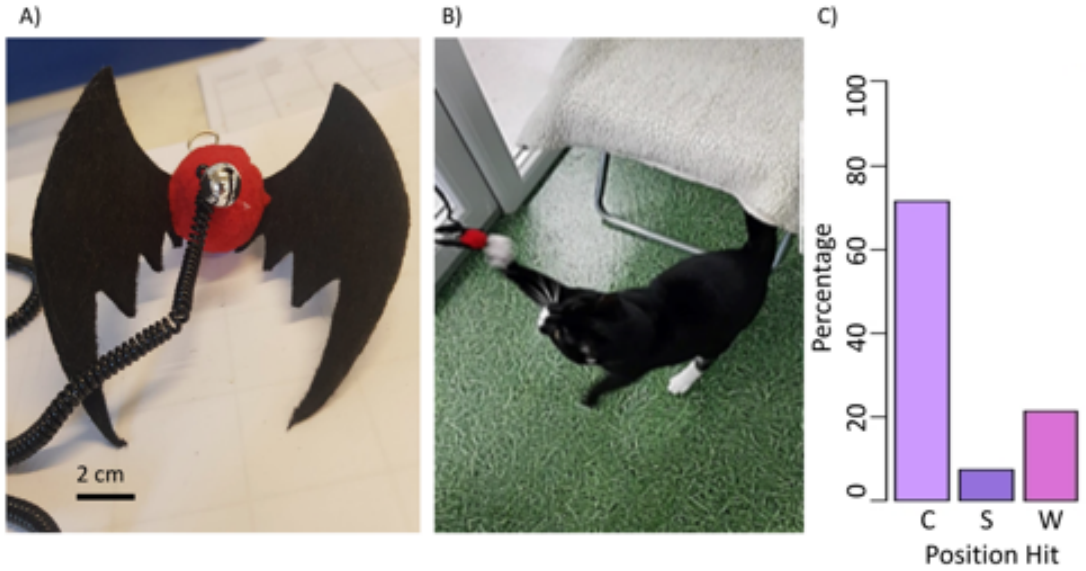


Figure 6.1: A) The toy bat that was used in the study. The toy bat had a similar physical appearance to a real bat with large wings and a small body (with 13 cm in width and 27gm in weight). B) Photo of a cat hitting the centre (body) of the toy bat, C) The graph presenting the number of hits on the toy bat on each position: C: centre (body), S: string, W: wing. (Image modified from Tabner, 2019)

Free-roaming domestic cats cause a significant number of bird and mammal fatalities and, with the number of cats increasing annually (Woods et al., 2003), the effect of cat predation on wildlife is only likely to continue. In the UK, hundreds of bats have to be taken to rescue centres for rehabilitation annually (Kelly et al., 2008). Moreover, several studies based on observational data have reported cat predation on bats (Phillips et al., 2001; Woods et al., 2003; Ancillotto et al., 2013; Kauhala et al., 2015: Vlaschenko et al., 2019). The amount of samples with cat DNA was much higher (at 67%) than those reported previously from the observational data (Mühldorfer et al., 2011a; Ancillotto et al., 2013) at 20% and 29%, respectively. This indicates that cat predation may be much more common than previously thought.

There is no evidence for how cat predation impacts bat populations, however, as cat predation does not affect bird populations overall (Parsons et al., 2006; RSPB, 2019) this might also be true of bats. Investigating the population-level impacts of cats on bat abundance is outside the scope of this thesis. However, I consider that wing tears are likely to primarily be a welfare issue for bats, rather than a conservation one. While this thesis has focussed on the effect of wing tears in UK bat species, and specifically common pipistrelles, it is likely that these findings will also apply to bat species world-wide, especially those that come in to contact with urban predators and are commonly found with wing tears, such as the Big Brown bat in North America (Faure et al., 2009; Ceballos-Vasquez et al*.,* 2015; Pollock et al., 2016).

6.2.2 Healing and Post-release Monitoring

Even though hundreds of bats in the UK are rehabilitated in rescue centres annually as a result of suffering wing tears, there has been little evaluation of bat wing tear classification and placement. Also, the anatomy of the bat wing and how that could affect wing tear position, type, and healing have not been studied before. The findings in chapter 2 illustrate that tears in the plagiopatagium section took longer to heal, with the anatomy of the wing vessels explaining why. Moreover, the finding here supports that found recently in a study by Greville et al. (2018), who noted that

wounds in the plagiopatagium section in the Egyptian fruit bat (*Rousettus aegyptiacus*) took 50% longer to heal than wounds in the chiropatagium sections (Greville et al., 2018). Samples with cat DNA present also had large total tears, that are likely to take longer to heal than smaller holes and contained tears. If cats cause large tears in the plagiopatagium, these are likely to be the longest healing tears. Even though the understanding of tear location and healing has direct implications for bat welfare and rehabilitation strategies, there have been no studies to track how bats with healed wing tears fare post-release.

Chapter 4 provides a quantitative method to evaluate the flight in *P. pipistrelles* by testing wing movement and body orientation. Findings from this chapter may be used to improve release guidelines, as bat carers will be better able to judge if injured bats are ready to be released based on wing movements and body orientation measurements (see section 6.3 on recommendations for more information). Based on the similarities of flight features among bat species (Hedenström et al., 2007), and the agreement of my findings with previous studies of bird flight (Swaddle et al., 1996; Thomas, 1997), this method could easily be applied to other bat species, and even birds, to assess flight.

6.2.3 Effect on Population (Gender, Age, Species)

Female bats play a significant role in the population in regard to their role in reproduction. However, several studies found that the female *P. pipistrelles* have a higher survival rate than males (Gerell and Lundberg, 1985; Gerell and Lundberg, 1990; Sendor and Simon, 2003), probably because females often stay within roosts, especially when the females move to the maternity roost for giving birth and feeding their young (BTC, 2019) and do not put themselves at risk as often as males. However, other studies have observed that, when specifically considering cat predation, female bats were more significantly preyed upon than male bats (Ancillotto et al., 2013; Vlaschenko et al., 2019), and this may be due to cats targeting roost sites (Ancillotto et al., 2013). The results from chapter 5 showed the samples from female bats with the presence of cat DNA to be slightly higher than the male bat samples, although

this was not significant. A decrease in the number of female bats will significantly affect bat populations.

While juvenile bats generally have lower survival rates than adults (Sendor and Simon, 2003; Pryde et al., 2005), the findings in chapter 5 indicted that there was no significant difference between adult and juvenile bats with cat DNA present on the injured wing swabs. Other studies with a larger sample size (n= 1012) have also found that juveniles are less frequently preyed on by cats than adult bats (Ancillotto et al., 2013).

Even though studies have provided the survival rates of some bat species (Gerell and Lundberg, 1985; Gerell and Lundberg, 1990; Sendor and Simon, 2003; Pryde et al., 2005), there are few which report a reliable estimation of long-term survival rates (4-7 years), or the difference in survival rates between species or within populations. In order to better understand the effect of wing tears on bat populations, improved population models need to be constructed taking these factors in to account.

6.3 Limitations

There are some limitations associated with the work completed in this thesis. Firstly, for the anatomy work (chapter 3) the bat wings were a very challenging material to work with. They were very small, thin, folded easily and dried out very quickly. Therefore, the histology images (Figures 3.6) were not as clear as those which can be obtained from other tissue types, and it took many samples to get even these images.

The material testing was also challenging, mainly due to the small size of the wing sections in *P. pipistrellus*. In particular, it was difficult to cut consistent sizes of wing strips for material testing (Figure 3.2) and it was impossible to cut an ideal 'dog-bone' shape, which is usually used for material testing. This caused transverse forces at the grips, which affected some material samples. To overcome these limitations, the wing strips were angled to get the longest possible strips from each wing section. The

wing strip samples that failed at the grips due to transverse forces were discarded from the analyses. In future work, a specialised dog-bone cutter could be created to ensure dog-bone-shaped samples. However, this would be expensive and not allow for variation in wing sizes. A stencil or other sort of cutting pattern would ensure a greater agreement in sample size, although controlling for size in calculations, as I did here, also accounts for variation in sample size.

Other limitations were mostly associated with data and swabs collected from bat carers, especially in terms of collecting information about each bat, and swabbing techniques. The swab samples received from bat carers showed fungal growth on 34.72% of the samples, however, this did not appear to affect the quantity or quality of DNA extracted. It was not possible to profile the DNA from the swab samples, with no swabs producing any allele in the DNA profiles, despite the DNA from positive control samples from cat blood showing full STR profiles. This is likely to be related to the low DNA quality of the swab samples (Schneider et al., 2004; Swango et al., 2006; Huang et al., 2017). Indeed, the low DNA quality produced as a result of DNA degradation, leads to breakdown of large DNA fragments (McCord et al., 2011). Hence, the DNA fragment in length up to 200bp could be relatively stable and less sensitive to the degradation, while the larger DNA could not be amplified which lead to partial STR profile (Grubwieser et al., 2006; Regnaut et al., 2006).

There are several factors that could have led to low DNA quality, such as DNA degradation in samples that have been exposed to temperature, light, humidity and bacterial and fungal contamination (Grubwieser et al., 2006). In this project, incorrect swabbing by bat carers, samples not being sent directly after swabbing and not being kept in a freezer prior to sending could have led to DNA degradation. In future work, the DNA quality could be assessed by using a single-cell gel electrophoresis (COMET) assay to measure DNA strand breaks (Collins, 2004). Moreover, FTA® paper (Whatman, UK) should be used in the bat packs to increase the preservation of the DNA. FTA paper is typically used to collect biological samples in a forensic analysis situation for purification of DNA and for the long term storage of biological samples over several years with no concern of DNA degrdation, even at room temperature

(Smith and Burgoyne, 2004; Picard-Meyer et al., 2007). The paper is impregnated with chemicals, which work to lyse cells, which: i) kills most pathogens, ii) inhibits saprophyte growth and iii) prevents bacteria growth, thus enabling purer DNA (Smith and Burgoyne, 2004).

The exact location the injured bats were found is unknown in all swab samples. In future, it could be better if the bat carers were requested to identify the exact location that they were found, using GPS. This would enable accurate location identification, and the identification of rural and urban areas would be more accurate.

Out of all the tear photographs collected from bat carers, only 24% also had rehabilitation outcome data. In future, it will be important to collect more data about rehabilitation outcomes alongside images of healed tears, in order to identify any associations between tear type and position with healing rates and survival. Bat age and gender were also not reported in many of the samples. Gender was unknown in 56%, and the age was unknown in 53%, of all the bat samples (including both photographs and swabs, n=111). If gender and age had been reported, the comparisons between males and females, and adults and juveniles, in terms of tear type and position, rehabilitation outcome and flight movement, could have been further investigated. Predictions could also be made concerning the effect of wing tears on bat populations, if the gender and age of the bats with wing tears were known.

6.4 Recommendations for Welfare

6.4.1 Long-term Monitoring

In order to further progress this work and improve records of bat wing injuries, bat carers should always identify the bat species, gender and age, and take pictures of any injured wings when they have a bat in care. They can identify the tear type and orientation by using the tear coding system that was developed in this thesis (chapter 2), by filling in a modified version of the questionnaire included on the bat packs or

an electronic version of the questionnaire, which is provided on the project website (http://bat-research-mmu.weebly.com/). If rescue centres and rehabilitators continue to record the tear positions and healing rates, future work will be able to formulate a much better understanding of the extent of the problem, as well as the impact of tear type and location on healing. Bat carers need to be aware that tears in the P section take longer to heal, so they can commit to having bats in care for longer periods of time. When the tear is healed, the healing outcome should be identified in terms of the period that the bat spent in care before release, and an image of the healed tear should be taken. This could be used to form rehabilitation recommendations for bat carers, based on tear types and positions.

6.4.2 Flight Assessment

Chapter 4 provides a quantitative method to measure wing movements and body orientation. I suggest that bat carers can start assessing bat flight by looking at the tears, as bats with tears on both wings are likely to have more affected wing movements, than bats with tears on only one wing. Following this, carers could start to assess wing-beat frequency and body orientation, as these variables are significantly altered by wing tears and are fairly easy to measure compared to approximating wing angles. My specific recommendation to bat carers is to test the bat flying indoors or in a flight cage, and to film the bat flying straight-on relative to the camera. They could use a phone camera with a slow-motion feature and count the number of wing beats per second to measure wing frequency. Also, to observe the body orientation during the flight. If wingbeats are more than 13 per second and the body orientation is more than four degrees, this might suggest that flight is being affected in *P. pipistrellus*, and the bat might not be ready for release. Afterwards, when the tear is healed, but before releasing the bat, bat carers should film the bat again and identify the wingbeat frequency and body orientation to compare the wing movement to the previous film clip. If the wingbeat frequency is less than 13Hz and body orientation is around the horizontal (less than 1 degree), the bat may be ready for release. In this way, bat carers will be able to assess the bat's flight based on reliable evidence. However, this flight assessment needs to be coupled with post-

release monitoring in order to reliably say whether wing movements and body orientation measurements are at an appropriate level for release.

6.4.3 Communications to Cat Owners

For cat owners, there is now strong evidence that cats interact with bats, based on the genetic analysis results in chapter 5, and the observational results in chapter 2 and previous studies (Phillips et al., 2001; Woods et al., 2003; Ancillotto et al., 2013; Kauhala et al., 2015: Vlaschenko et al., 2019). The foremost recommendation to cat owners is to keep cats inside at night when bats are foraging. This is especially important during the Spring and Summer seasons when the bats are active (not in hibernation), and from dusk to dawn when the bats usually forage (Audet, 1990; Rydell, 1991; Levin et al., 2013; BTC, 2019). Also, if the cat wears a bell, this could alert the bats to their presence, resulting in an increase in the chance of escaping (Ruxton et al., 2002; Woods et al., 2003; Ancillotto et al., 2013). Indeed, studies have found that the average number of prey items captured by cats and delivered to owners is reduced when the cats wear bells (Ruxton et al., 2002; Woods 2003). These recommendations are especially important in rural and semi-urban areas where cat predation is most frequent (Ancillotto et al., 2013; Russo and Ancillotto, 2015).

References

Amelon, S. K., Hooper, S. E. and Womack, K. M. (2017) 'Bat wing biometrics: using collagen-elastin bundles in bat wings as a unique individual identifier.' *Journal of Mammalogy*, 98(3) pp. 744-751.

Ancillotto, L., Serangeli, M. T. and Russo, D. (2013) 'Curiosity killed the bat: Domestic cats as bat predators.' *Mammalian Biology*, 78(5) pp. 369-373.

Arlettaz, R. (1999) 'Habitat Selection as a Major Resource Partitioning Mechanism between the two Sympatric Sibling Bat Species Myotis myotis and Myotis blythii.' *Journal of Animal Ecology*, 68(3) pp. 460-471.

Arlorio, M., Coïsson, J. D. and Martelli, A. (1999) 'Identification of *Saccharomyces cerevisiae* in bakery products by PCR amplification of the ITS region of ribosomal DNA.' *European Food Research and Technology*, 209(3) pp. 185-191.

Arnett, E. B., Brown, W. K., Erickson, W. P., Fiedler, J. K., Hamilton, B. L., Henry, T. H., Jain, A., Johnson, G. D., Kerns, J., Koford, R. R., Nicholson, C. P., O'Connell, T. J., Piorkowski, M. D. and Roger, D. T. J. R. (2008) 'Patterns of Bat Fatalities at Wind Energy Facilities in North America.' *Journal of Wildlife Management*, 72(1) pp. 61-78.

Asif, M. and Ali, M. (2019) 'Chinese Traditions Folk Art, Festivals and Symbolism.' *International Journal of Research*, 6(1) pp. 1-20.

Association of Forensic Science, P. and Assoc Forensic Sci, P. (2009) 'Standards for the formulation of evaluative forensic science expert opinion.' *Science and Justice*, 49(3) pp. 161-164.

Audet, D. (1990) 'Foraging Behavior and Habitat Use by a Gleaning Bat, *Myotis myotis* (Chiroptera: Vespertilionidae).' *Journal of Mammalogy*, 71(3) pp. 420-427.

Baerwald, E. F., D'Amours, G. H., Klug, B. J. and Barclay, R. M. R. (2008) 'Barotrauma is a significant cause of bat fatalities at wind turbines.' *Current Biology*, 18(16) pp. R695-R696.

Baker, P. J., Molony, S. E., Stone, E., Cuthill, I. C. and Harris, S. (2008) 'Cats about town: is predation by free-ranging pet cats Felis catus likely to affect urban bird populations?' *Ibis*, 150(1) pp. 86-99.

Banfi, G., Salvagno, G. L. and Lippi, G. (2007) 'The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes.' *Clinical Chemistry and Laboratory Medicine*, 45(5) pp. 565-576.

Barratt, D. G. (1997) 'Predation by house cats, *Felis catus* (L), in Canberra, Australia .1. Prey composition and preference.' *Wildlife Research*, 24(3) pp. 263-277.

Bat Consevation Trust. (2010) *Common pipistrelle Pipistrellus pipistrellus*. [Online] [Accessed on 25/03/2017] https://cdn.bats.org.uk/pdf/About%20Bats/commonpipistrelle_11.02.13.pdf?mtime=20181101151257

Bat Consevation Trust. (2015) *Bats and Buildings*. [Online] [Accessed on 29/03/2019] https://cdn.bats.org.uk/pdf/Bats_and_Buildings.pdf?mtime=20181101151310

Bat Conservation Trust. (2016) *Bat Care Guidelines: A Guide to Bat Care for Rehabilitators*. [Online] [Accessed on 20/11/2017] https://www.bats.org.uk/resources/guidance-for-professionals/bat-care-guidelines-a-guide-to-bat-care-for-rehabilitators

Bat Consevation Trust. (2017) *UK Bats*. [Online] [Accessed on 20/04/2017] http://www.bats.org.uk/pages/uk_bats.html#Barbastelle

Bat Consevation Trust. (2018a) *Threats to bats* [Online) [Accessed on 01/09/2018] https://www.bats.org.uk/about-bats/threats-to-bats

Bat Consevation Trust. (2018b) *National Bat Monitoring Programme Annual Report 2017.* [Online[[Accessed on 21/06/2018] https://www.bats.org.uk/our-work/national-bat-monitoring-programme/reports/nbmp-annual-report

Bat Consevation Trust. (2019) *A year in the life of a bat*. [Online] [Accessed on 20/01/2019] https://www.bats.org.uk/about-bats/a-year-in-the-life-of-a-bat

Beckerman, A. P., Boots, M. and Gaston, K. J. (2007) 'Urban bird declines and the fear of cats.' *Animal Conservation*, 10(3) pp. 320-325.

Belwood, J. J. and Fullard, J. H. (1984) 'Echolocation and foraging behaviour in the Hawaiian hoary bat, *Lasiurus cinereus semotus*.' *Canadian Journal of Zoology*, 62(11) pp. 2113-2120.

Berg, C. V. d. and Rayner, J. M. V. (1995) 'The moment of inertia of bird wings and the inertial power requirement for flapping flight ' *Journal of Experimental Biology*, 198(8) pp. 1655-1664.

Berge, M. v. d. and Sijen, T. (2016) 'A male and female RNA marker to infer sex in forensic analysis.' *Forensic Science International: Genetics*, 26 pp. 70-76

Berntson, G. G., Berntson, G. G., Hughes, H. C., Hughes, H. C., Beattie, M. S. and Beattie, M. S. (1976) 'A comparison of hypothalamically induced biting attack with natural predatory behavior in the cat.' *Journal of Comparative and Physiological Psychology*, 90(2) pp. 167-178.

Blehert, D. S., Hicks, A. C., Behr, M., Meteyer, C. U., Berlowski-Zier, B. M., Buckles, E. L., Jeremy, T. H. C., Darling, S. R., Gargas, A., Niver, R., Okoniewski, J. C., Rudd, R. J.

and Stone, W. B. (2009) 'Bat White-Nose Syndrome: An Emerging Fungal Pathogen?' *Science*, 323(5911) pp. 227-227.

Boyce, M. S. (1992) 'Population Viability Analysis.' *Annual Review of Ecology and Systematics*, 23(1) pp. 481-497.

Breviglieri, C. P. B., Piccoli, G. C. O., Uieda, W. and Romero, G. Q. (2013) 'Predation-risk effects of predator identity on the foraging behaviors of frugivorous bats.' *Oecologia*, 173(3) pp. 905-912.
Brooks, A., Creighton, E. K., Gandolfi, B., Khan, R., Grahn, R. A. and Lyons, L. A. (2016) 'SNP Miniplexes for Individual Identification of Random-Bred Domestic Cats.' *Journal of Forensic Sciences*, 61(3) pp. 594-606.

Bullen, R. D. and McKenzie, N. L. (2002) 'Scaling bat wingbeat frequency and amplitude.' *Journal of Experimental Biology*, 205(17) pp. 2615-2626.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. and Wittwer, C. T. (2009) 'The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.' *Clinical Chemistry*, 55(4) pp. 611-622.

Butler, J. M. (2001) *Forensic DNA typing: biology, technology, and genetics of STR markers.*, London; San Diego: Academic Press.

Butler, J. M. (2010) *Fundamentals of forensic DNA typing.* London; Amsterdam: Academic Press.

Butler, J. M. (2012) *Advanced topics in forensic DNA typing: methodology., Waltham; San Diago; London:* Academic Press.

Butler, J. M. (2015) *Advanced topics in forensic DNA typing.* Amsterdam: Academic Press

Butler, J. M., David, V. A., and O'Brien, S. J. (2002). The MeowPlex: A new DNA test using tetranucleotide STR markers for the domestic cat. *Profiles in DNA*, 5(2) pp. 7–10.

Ceballos-Vasquez, A., Caldwell, J. R. and Faure, P. A. (2015) 'Seasonal and reproductive effects on wound healing in the flight membranes of captive big brown bats.' *Biology Open*, 4(1) pp. 95-103.

Cheney, J. A., Allen, J. J. and Swartz, S. M. (2017) 'Diversity in the organization of elastin bundles and intramembranous muscles in bat wings.' *Journal of Anatomy*, 230(4) pp. 510-523.

Cheney, J. A., Konow, N., Bearnot, A. and Swartz, S. M. (2015) 'A wrinkle in flight: The role of elastin fibres in the mechanical behaviour of bat wing membranes.' *Journal of the Royal Society Interface*, 12(106) pp. 20141286-20141286.

Collins, A. R. (2004) 'The comet assay for DNA damage and repair: Principles, applications, and limitations.' *Molecular Biotechnology*, 26(3) pp. 249-261.

Corten, G. P., & Veldkamp, H. F. (2001). Insects Cause Double Stall. In European Wind Energy Conference 2001 (pp. 470–474). Copenhagen.

Coyle, H.M., 2007. *Nonhuman DNA typing: theory and casework applications*. CRC Press.

Crowley, G. V. and Hall, L. S. (1994) 'Histological observations on the wing of the Grey-Headed Flying-Fox (*Pteropus-Poliocephalus*) (Chiroptera, Pteropodidae).' *Australian Journal of Zoology*, 42(2) pp. 215-231.

Cryan, P. M. and Barclay, R. M. R. (2009) 'Causes of Bat Fatalities at Wind Turbines: Hypotheses and Predictions.' *Journal of Mammalogy*, 90(6) pp. 1330-1340.

Cryan, P. M., Meteyer, C. U., Boyles, J. G. and Blehert, D. S. (2010) 'Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology.' *BMC Biology*, 8(1) pp. 135-135.

Daamen, W. F., van Moerkerk, H. T. B., Hafmans, T., Buttafoco, L., Poot, A. A., Veerkamp, J. H. and van Kuppevelt, T. H. (2003) 'Preparation and evaluation of molecularly-defined collagen–elastin–glycosaminoglycan scaffolds for tissue engineering.' *Biomaterials*, 24(22) pp. 4001-4009.

Dacheux, L., Larrous, F., Mailles, A., Boisseleau, D., Delmas, O., Biron, C., Bouchier, C., Capek, I., Muller, M., Ilari, F., Lefranc, T., Raffi, F., Goudal, M. and Bourhy, H. (2009) 'European bat lyssavirus transmission among cats, Europe.' *Emerging Infectious Diseases*, 15(2) pp. 280-284.

Davidson-Watts, I. and Jones, G. (2006) 'Differences in foraging behaviour between *Pipistrellus pipistrellus* (Schreber, 1774) and *Pipistrellus pygmaeus* (Leach, 1825).' *Journal of Zoology*, 268(1) pp. 55-62.

Davis, R. (1968) 'Wing Defects in a Population of Pallid Bats.' *The American Midland Naturalist*, 79(2) pp. 388-395.

Davis, R. and Doster, S. E. (1972) 'Wing Repair in Pallid Bats.' *Journal of Mammalogy*, 53(2) pp. 377-378.

Dawson, A., Hinsley, S. A., Ferns, P. N., Bonser, R. H. C. and Eccleston, L. (2000) 'Rate of moult affects feather quality: a mechanism linking current reproductive effort to

future survival.' *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 267(1457) pp. 2093-2098.

Duberstein, K. J. (2012) 'Evaluating Horse Conformation.' *UGA Cooperative Extension* Bulletin 1400 pp. 1-11.

Eberson, L. S., Sanchez, P. A., Majeed, B. A., Tawinwung, S., Secomb, T. W. and Larson, D. F. (2015) 'Effect of lysyl oxidase inhibition on angiotensin ii-induced arterial hypertension, remodeling, and stiffness.' *PLoS ONE*, 10(4) p. e0124013.

Eiting, T. P. and Gunnell, G. F. (2009) 'Global completeness of the bat fossil record.' *Journal of Mammalian Evolution*, 16(3) pp. 151-173.

Entwistle, A. C., Racey, P. A. and Speakman, J. R. (1998) 'The reproductive cycle and determination of sexual maturity in male brown long-eared bats, *Plecotus auritus* (Chiroptera: Vespertilionidae).' *Journal of Zoology*, 244(1) pp. 63-70.

Epstein, J. H., Rahman, S. A., Zambriski, J. A., Halpin, K., Meehan, G., Jamaluddin, A. A., Hassan, S. S., Field, H. E., Hyatt, A. D., Daszak, P. (2006) 'Feral cats and risk for nipah virus transmission.' *Emerging Infectious Diseases*, 12(7) pp. 1178-1179.

Faure, P. A., Re, D. E. and Clare, E. L. (2009) 'Wound Healing in the Flight Membranes of Big Brown Bats.' *Journal of Mammalogy*, 90(5) pp. 1148-1156.

Foutz, T. L., Stone, E. A. and Abrams Jr, C. F. (1992) 'Effects of freezing on mechanical properties of rat skin.' *American Journal of Veterinary Research*, 53(5) pp. 788-792.

Frey-Ehrenbold, A., Bontadina, F., Arlettaz, R. and Obrist, M. K. (2013) 'Landscape connectivity, habitat structure and activity of bat guilds in farmland-dominated matrices.' *Journal of Applied Ecology*, 50(1) pp. 252-261.

Fuller, N. W., Reichard, J. D., Nabhan, M. L., Fellows, S. R., Pepin, L. C. and Kunz, T. H. (2011) 'Free-Ranging Little Brown Myotis (*Myotis lucifugus*) Heal from Wing Damage Associated with White-Nose Syndrome.' *EcoHealth*, 8(2) pp. 154-162.

Fullmer, H. M. and Lillie, R. D. (1956) 'The Staining of Collagen With Elastic Tissue Stains.' *Journal of Histochemistry and Cytochemistry*, 5(1) pp. 11-14.

Gardiner, J. D., Dimitriadis, G., Codd, J. R. and Nudds, R. L. (2011) 'A potential role for bat tail membranes in flight control.' *PLoS ONE*, 6(3) p. e18214.

Gargas, A., Trest, M. T., Christensen, M., Volk, T. J. and Blehert, D. S. (2009) 'Geomyces destructans sp. nov. associated with Bat white-nose syndrome.' *Mycotaxon*, 108 pp. 147-154.

Gerell, R. and Lundberg, K. (1985) 'Social Organization in the Bat *Pipistrellus pipistrellus*.' *Behavioral Ecology and Sociobiology*, 16(2) pp. 177-184.

Gerell, R. and Lundberg, K. (1990) 'Sexual Differences in Survival Rates of Adult Pipistrelle Bats (*Pipistrellus pipistrellus*) in South Sweden.' *Oecologia*, 83(3) pp. 401-404.

Greville, L. J., Ceballos-Vasquez, A., Valdizon-Rodriguez, R., Caldwell, J. R. and Faure, P. A. (2018) 'Wound healing in wing membranes of the Egyptian fruit bat (*Rousettus aegyptiacus*) and big brown bat (*Eptesicus fuscus*).' *Journal Of Mammalogy*, 99(4) pp. 974-982.

Grover, C. N., Cameron, R. E. and Best, S. M. (2012) 'Investigating the morphological, mechanical and degradation properties of scaffolds comprising collagen, gelatin and elastin for use in soft tissue engineering.' *Journal of the Mechanical Behavior of Biomedical Materials*, 10 pp. 62-74.

Grubwieser, P., Mühlmann, R., Berger, B., Niederstätter, H., Pavlic, M. and Parson, W. (2006) 'A new "miniSTR-multiplex" displaying reduced amplicon lengths for the analysis of degraded DNA.' *International Journal of Legal Medicine*, 120(2) pp. 115-120.

Gupta, B. B. (1967) 'The histology and musculature of plagiopatagium in bats.' *Mammalia*, 31(2) pp. 313-321.

Gurtner, G. C., Werner, S., Barrandon, Y. and Longaker, M. T. (2008) 'Wound repair and regeneration.' *Nature*, 453 pp. 314-321.

Hall, T. (2004) *BioEdit version 7.0.0.* Ibis Therapeutics, a division of Isis Pharmaceuticals, Inc.

Hambly, C., Harper, E. J. and Speakman, J. R. (2004) 'The energetic cost of variations in wing span and wing asymmetry in the zebra finch Taeniopygia guttata.' *Journal of Experimental Biology*, 207(22) pp. 3977-3984.

Harris, S, Morris, P, Wray, S and Yalden, D 1995, A review of British mammals: population estimates and conservation status of British mammals other than cetaceans. Peterborough: The Joint Nature Conservation Committee.

Hedenström, A. and Sunada, S. (1999) 'On the aerodynamics of moult gaps in birds.' *Journal of Experimental Biology,* 202(1) pp. 67-76.

Hedenström, A., Evolutionary, e., Evolutionär, e., Lund, U. and Lunds, u. (2003) 'Flying with Holey Wings.' *Journal of Avian Biology*, 34(4) pp. 324-327.

Hedenström, A., Johansson, L. C., Wolf, M., Busse, R. v., Winter, Y., Spedding, G. R., Evolutionary, e., Evolutionär, e., Lund, U. and Lunds, u. (2007) 'Bat Flight Generates Complex Aerodynamic Tracks.' *Science*, 316(5826) pp. 894-897.

Hewitt, B. M., Yap, M. H. and Grant, R. A. (2016) 'Manual Whisker Annotator (MWA): A Modular Open-Source Tool.' *Journal of Open Research Software*, 4(1) pp. e16-e16.

Holbrook, K. A. and Odland, G. F. (1978) 'A collagen and elastic network in the wing of the bat.' *Journal of Anatomy*, 126(1) pp. 21-36.

Huang, L. H., Lin, P. H., Tsai, K. W., Wang, L. J., Huang, Y. H., Kuo, H. C. and Li, S. C. (2017) 'The effects of storage temperature and duration of blood samples on DNA and RNA qualities.' *PLOS ONE*, 12(9) p. e0184692.

Hubel, T. Y., Hristov, N. I., Swartz, S. M. and Breuer, K. S. (2016) 'Wake structure and kinematics in two insectivorous bats.' *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371(1704) p. 20150385.

Hutson, A.M., Spitzenberger, F., Aulagnier, S., Coroiu, I., Karataş, A., Juste, J., Paunovic, M., Palmeirim, J. and Benda, P. 2008. *Pipistrellus pipistrellus*. The IUCN Red List of Threatened Species 2008: e.T17317A6968203.

INCN. (2017) *The IUCN Red List of Threatened Species.* [Online] [Accessed on 23/04/2017] http://www.iucnredlist.org.

Iriarte-Díaz, J. and Swartz, S. M. (2008) 'Kinematics of slow turn maneuvering in the fruit bat Cynopterus brachyotis.' *Journal of Experimental Biology*, 211(21) pp. 3478-3489.

Johnson, G. D., Erickson, W. P., Strickland, M. D., Shepherd, M. F., Shepherd, D. A. and Sarappo, S. A. (2003) 'Mortality of Bats at a Large-scale Wind Power Development at Buffalo Ridge, Minnesota.' *The American Midland Naturalist*, 150(2) pp. 332-342.

Jung, T. S., Lausen, C. L., Talerico, J. M. and Slough, B. G. (2011) 'Opportunistic Predation of a Little Brown Bat (*Myotis lucifugus*) by a Great Horned Owl (*Bubo virginianus*) in Southern Yukon.' *Northwestern Naturalist*, 92(1) pp. 69-72.

Karni, M., Zidon, D., Polak, P., Zalevsky, Z. and Shefi, O. (2013) 'Thermal degradation of DNA.' *DNA and Cell Biology*, 32(6) pp. 298-301.

Kasso, M. and Balakrishnan, M. (2013) 'Ecological and Economic Importance of Bats (Order Chiroptera).' *ISRN Biodiversity*, 2013 p. 9.

Kauhala, K., Talvitie, K. and Vuorisalo, T. (2015) 'Free-ranging house cats in urban and rural areas in the north: Useful rodent killers or harmful bird predators?' *Folia Zoologica*, 64(1) pp. 45-55.

Kaye, B., Randall, C., Walsh, D. and Hansma, P. (2012) 'The effects of freezing on the mechanical properties of bone.' *Open Bone Journal*, 4(1) pp. 14-19.

Kazlouskaya, V., Malhotra, S., Lambe, J., Idriss, M. H., Elston, D. and Andres, C. (2013) 'The utility of elastic Verhoeff-Van Gieson staining in dermatopathology: Review.' *Journal of Cutaneous Pathology*, 40(2) pp. 211-225.

Kelly, A., Goodwin, S., Grogan, A. and Mathews, F. (2008) 'Post-release survival of hand-reared pipistrelle bats (*Pipistrellus spp*).' *Animal Welfare*, 17(4) pp. 375-382.

Kendall, H. W. (1990) 'Population Regulation.' *In* Bear, R., Rintoul, D., Snyder, B., Smith-Caldas, M., Herren, C. and Horne, E. (eds.) *Principles of Biology*. Manhattan: The Authors, pp. 89-91.

Kiat, Y., Izhaki, I. and Sapir, N. (2016) 'Determinants of wing-feather moult speed in songbirds.' *Evolutionary Ecology*, 30(4) pp. 783-795.

Konow, N., Cheney, J. A., Roberts, T. J., Iriarte-Diaz, J., Breuer, K. S., Waldman, J. R. S. and Swartz, S. M. (2017) 'Speed-dependent modulation of wing muscle recruitment intensity and kinematics in two bat species.' *Journal of Experimental Biology*, 220(10) pp. 1820-1829.

Korner-Nievergelt, F., Brinkmann, R., Niermann, I. and Behr, O. (2013) 'Estimating bat and bird mortality occurring at wind energy turbines from covariates and carcass searches using mixture models.' *PloS One*, 8(7) pp. e67997-e67997.

Khodadadi, H., Karimi, L., Jalalizand, N., Adin, H. and Mirhendi, H. (2017) 'Utilization of size polymorphism in ITS1 and ITS2 regions for identification of pathogenic yeast species.' *Journal of Medical Microbiology*, 66(2) pp. 126-133.

Kovalyova, I. M. (2014) 'Key morphofunctional transformations in the evolution of bats (Mammalia, Chiroptera).' *Russian Journal of Developmental Biology*, 45(6) pp. 324-336.

Kunz, T. H., de Torrez, E. B., Bauer, D., Lobova, T. and Fleming, T. H. (2011) 'Ecosystem services provided by bats.' *Annals of the New York Academy of Sciences*, 1223(1) pp. 1-38.

Kunz, T. H., Arnett, E. B., Erickson, W. P., Hoar, A. R., Johnson, G. D., Larkin, R. P., Strickland, M. D., Thresher, R. W. and Tuttle, M. D. (2007) 'Ecological Impacts of Wind Energy Development on Bats: Questions, Research Needs, and Hypotheses.' *Frontiers in Ecology and the Environment*, 5(6) pp. 315-324.

Lee, L., Cui, J. Z., Cua, M., Esfandiarei, M., Sheng, X., Chui, W. A., Xu, M. H., Sarunic, M. V., Beg, M. F., Van Breemen, C., Sandor, G. G. S. and Tibbits, G. F. (2016) 'Aortic and cardiac structure and function using high-resolution echocardiography and optical coherence tomography in a mouse model of Marfan syndrome.' *PLoS ONE*, 11(11) p. e0164778.

Levin, E., Roll, U., Dolev, A., Yom-Tov, Y. and Kronfeld-Shcor, N. (2013) 'Bats of a Gender Flock Together: Sexual Segregation in a Subtropical Bat.' *PLoS ONE*, 8(2) p. e54987.

Lewis, S. E. (1995) 'Roost Fidelity of Bats: A Review.' *Journal of Mammalogy*, 76(2) pp. 481-496.

Lind, J., Naturvetenskapliga, f., Avdelningen för, e., Zoologiska, i. and Stockholms, u. (2001) 'Escape Flight in Moulting Tree Sparrows (Passer montanus).' *Functional Ecology*, 15(1) pp. 29-35.

Lintott, P. R., Bunnefeld, N., Minderman, J., Fuentes-Montemayor, E., Mayhew, R. J., Olley, L. and Park, K. J. (2015) 'Differential responses to woodland character and landscape context by cryptic bats in urban environments.' *PLoS ONE*, 10(5) p. e0126850.

Lorch, J. M., Palmer, J. M., Lindner, D. L., Ballmann, A. E., George, K. G., Griffin, K., Knowles, S., Huckabee, J. R., Haman, K. H., Anderson, C. D., Becker, P. A., Buchanan, J. B., Foster, J. T. and Blehert, D. S. (2016) 'First Detection of Bat White-Nose Syndrome in Western North America.' *MSPHERE*, 1(4) pp. e00148-00116.

Loss, S. R., Will, T. and Marra, P. P. (2013) 'The impact of free-ranging domestic cats on wildlife of the United States.' *Nature Communications*, 4 p. 1396.

Loss, S. R. and Marra, P. P. (2017) 'Population impacts of free-ranging domestic cats on mainland vertebrates.' *Frontiers in Ecology and the Environment*, 15(9) pp. 502-509.

Lowe, S., Browne, M., Boudjelas, S. and De Poorter, M. (2000) '100 of the World's Worst Invasive Alien Species. A Selection From the Global Invasive Species Database (IUCN).'

Luo, J., Jiang, T., Lu, G., Wang, L., Wang, J. and Feng, J. (2013) 'Bat conservation in China: should protection of subterranean habitats be a priority?' *Oryx*, 47(4) pp. 526-531.

Madej, J. P., Mikulová, L., Gorošová, A., Mikula, Š., Řehák, Z., Tichý, F. and Buchtová, M. (2012) 'Skin structure and hair morphology of different body parts in the Common Pipistrelle (*Pipistrellus pipistrellus*).' *Acta Zoologica*, 94(4) pp. 478-489.

Makanya, A. N. and Mortola, J. P. (2007) 'The structural design of the bat wing web and its possible role in gas exchange.' *Journal of Anatomy*, 211(6) pp. 687-697.

Mathews, F., Roche, N., Aughney, T., Jones, N., Day, J., Baker, J. and Langton, S. (2015) 'Barriers and benefits: Implications of artificial night-lighting for the distribution of common bats in britain and ireland.' *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370(1667) pp. 20140124-20140124.

McCord, B., Opel, K., Funes, M., Zoppis, S. and Meadows Jantz, L., 2011. An investigation of the effect of DNA degradation and inhibition on PCR amplification of single source and mixed forensic samples. *National Criminal Justice References Report*, (236692).

Medina, F. M., Bonnaud, E., Vidal, E., Tershy, B. R., Zavaleta, E. S., Josh Donlan, C., Keitt, B. S., Corre, M., Horwath, S. V. and Nogales, M. (2011) 'A global review of the impacts of invasive cats on island endangered vertebrates.' *Global Change Biology*, 17(11) pp. 3503-3510.

Menotti-Raymond, M., David, V. A., Weir, B. S. and O'Brien, S. J. (2012) 'A Population Genetic Database of Cat Breeds Developed in Coordination with a Domestic Cat STR Multiplex: Domestic Cat STR Population Genetic Database.' *Journal of Forensic Sciences*, 57(3) pp. 596-601.

Menotti-Raymond, M. A., David, V. A., Wachter, L. L., Butler, J. M. and O'Brien, S. J. (2005) 'An STR forensic typing system for genetic individualization of domestic cat (*Felis catus*) samples.' *Journal of Forensic Sciences*, 50(5) pp. 1061-1070.

Mickleburgh, S. P., Hutson, A. M. and Racey, P. A. (2002) 'A review of the global conservation status of bats.' *Oryx*, 36(1) pp. 18-34.

Mitchell-Jones, A. J., and Mcleish, A. P. (2004) 'Bat workers manual'. *Joint Nature Conservation Committee.*

Muchhala, N. and Jarrin-V, P. (2002) 'Flower visitation by bats in cloud forests of Western Ecuador.' *Biotropica*, 34(3) pp. 387-395.

Muijres, F. T., Johansson, L. C., Barfield, R., Wolf, M., Spedding, G. R., Hedenström, A., Evolutionary, e., Evolutionär, e., Lund, U. and Lunds, u. (2008) 'Leading-Edge Vortex Improves Lift in Slow-Flying Bats.' *Science*, 319(5867) pp. 1250-1253.

Mühldorfer, K., Speck, S. and Wibbelt, G. (2011a) 'Diseases in free-ranging bats from Germany.' *BMC Veterinary Research*, 7(1) pp. 61-61.

Mühldorfer, K., Speck, S., Kurth, A., Lesnik, R., Freuling, C., Müller, T., Kramer-Schadt, S. and Wibbelt, G. (2011b) 'Diseases and causes of death in European bats: Dynamics in disease susceptibility and infection rates.' *PLoS ONE*, 6(12) p. e29773.

Neuweiler, G. (2000) *The biology of bats.* New york: oxford university press.

Norberg, U. M. L. and Norberg, R. Å. (2012) 'Scaling of wingbeat frequency with body mass in bats and limits to maximum bat size.' *Journal of Experimental Biology*, 215(5) pp. 711-722.

Nudds, R. L., Taylor, G. K. and Adrian, L. R. T. (2004) 'Tuning of Strouhal number for high propulsive efficiency accurately predicts how wingbeat frequency and stroke

amplitude relate and scale with size and flight speed in birds.' *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 271(1552) pp. 2071-2076.

O'Shea, T. J., Cryan, P. M., Hayman, D. T. S., Plowright, R. K. and Streicker, D. G. (2016) 'Multiple mortality events in bats: a global review.' *Mammal Review*, 46(3) pp. 175-190.

Patterson, B.D., Willig, M.R. and Stevens, R.D. (2003). 'Trophic strategies, niche partitioning, and patterns of ecological organization'. *In* Kunz, T.H and Fenton, M.B. (ed.) *Bat Ecology*, Chicago: University of Chicago Press, pp.536–579.

Parsons, H., Major, R. E. and French, K. (2006) 'Species interactions and habitat associations of birds inhabiting urban areas of Sydney, Australia.' *Austral Ecology*, 31(2) pp. 217-227

Pavisse, R., Vangeluwe, D. and Clergeau, P. (2019) 'Domestic Cat predation on garden birds: an analysis from European ringing programmes.' *ARDEA*, 107(1) pp. 103-109.

Pavlinić, I., Tvrtković, N. and Holcer, D. (2008) 'Morphological identification of the soprano pipistrelle (*Pipistrellus pygmaeus* Leach, 1825) in Croatia.' *Hystrix*, 19(1) pp. 47-54.

Phillips, S., Coburn, D. and James, R. (2001) 'An observation of cat predation upon an eastern blossom bat syconycteris Australis.' *Australian Mammalogy*, 23(1) pp. 57-58.

Picard-Meyer, E., Barrat, J. and Cliquet, F. (2007) 'Use of filter paper (FTA ®) technology for sampling, recovery and molecular characterisation of rabies viruses.' *Journal of Virological Methods*, 140(1) pp. 174-182.

Polak, T., Korine, C., Yair, S. and Holderied, M. W. (2011) 'Differential effects of artificial lighting on flight and foraging behaviour of two sympatric bat species in a desert.' *Journal of Zoology*, 285(1) pp. 21-27.

Pollock, T., Moreno, C. R., Sánchez, L., Ceballos-Vasquez, A., Faure, P. A. and Mora, E. C. (2016) 'Wound healing in the flight membranes of wild big brown bats.' *The Journal of Wildlife Management*, 80(1) pp. 19-26.

Powers, L. E., Hofmann, J. E., Mengelkoch, J. and Francis, B. M. (2013) 'Temporal variation in bat wing damage in the absence of white-nose syndrome.' *Journal of Wildlife Diseases*, 49(4) pp. 946-954.

Pryde, M. A., O'Donnell, C. F. J. and Barker, R. J. (2005) 'Factors influencing survival and long-term population viability of New Zealand long-tailed bats (*Chalinolobus tuberculatus*): Implications for conservation.' *Biological Conservation*, 126(2) pp. 175-185.

Raub, C. B., Mahon, S., Narula, N., Tromberg, B. J., Brenner, M. and George, S. C. (2010) 'Linking optics and mechanics in an in vivo model of airway fibrosis and epithelial injury.' *Journal of Biomedical Optics*, 15(1) p. 015004.

Regnaut, S., Lucas, F. S. and Fumagalli, L. (2006) 'DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations.' *Conservation Genetics*, 7(3) pp. 449-453.

Reichard, J. D. and Kunz, T. H. (2009) 'White-nose syndrome inflicts lasting injuries to the wings of little brown myotis (*Myotis lucifugus*).' *Acta Chiropterologica*, 11(2) pp. 457-464.

Rich, C. and Longcore, T. (2006). *Ecological consequences of artificial night lighting*. Washington, DC: Island Press.

Riskin, D. K., Willis, D. J., Iriarte-Díaz, J., Hedrick, T. L., Kostandov, M., Chen, J., Laidlaw, D. H., Breuer, K. S. and Swartz, S. M. (2008) 'Quantifying the complexity of bat wing kinematics.' *Journal of Theoretical Biology*, 254(3) pp. 604-615.

Rodríguez-Durán, A., Pérez, J., Montalbán, M. A. and Sandoval, J. M. (2010) 'Predation by free-roaming cats on an insular population of bats.' *Acta Chiropterologica*, 12(2) pp. 359-362.

Romanelli, A. M., Fu, J., Herrera, M. L. and Wickes, B. L. (2014) 'A universal DNA extraction and PCR amplification method for fungal rDNA sequence-based identification.' *Mycoses*, 57(10) pp. 612-622.

Russ, J. M., Briffa, M. and Montgomery, W. I. (2003) 'Seasonal patterns in activity and habitat use by bats (*Pipistrellus spp*. and *Nyctalus leisleri*) in Northern Ireland, determined using a driven transect.' *Journal of Zoology*, 259(3) pp. 289-299.

Russo, D. and Ancillotto, L. (2015) 'Sensitivity of bats to urbanization: a review.' *Mammalian Biology*, 80(3) pp. 205-212.

Russo, D., Cistrone, L. and Jones, G. (2007) 'Emergence time in forest bats: the influence of canopy closure.' *Acta Oecologica*, 31(1) pp. 119-126.

Russo, D., Bosso, L. and Ancillotto, L. (2018) 'Novel perspectives on bat insectivory highlight the value of this ecosystem service in farmland: Research frontiers and management implications.' *Agriculture, Ecosystems and Environment*, 266 pp. 31-38.

Ruxton, G. D., Thomas, S. and Wright, J. W. (2002) 'Bells reduce predation of wildlife by domestic cats (*Felis catus*).' *Journal of Zoology*, 256(1) pp. 81-83.

Rydell, J. (1991) 'Seasonal Use of Illuminated Areas by Foraging Northern Bats Eptesicus nilssoni.' *Holarctic Ecology*, 14(3) pp. 203-207.

Sanderson, E. W. (2006) 'How Many Animals Do We Want to Save? The Many Ways of Setting Population Target Levels for Conservation.' *BioScience*, 56(11) pp. 911-922.

Sankaran, R. (2001) 'The status and conservation of the Edible-nest Swiftlet (*Collocalia fuciphaga*) in the Andaman and Nicobar Islands.' *Biological Conservation*, 97(3) pp. 283-294.

Santago, A. C., Kemper, A. R., McNally, C., Sparks, J. L. and Duma, S. M. (2009) 'Freezing affects the mechanical properties of bovine liver.' *Biomedical Sciences Instrumentation*, 45 pp. 24-29.

Schaub, A., Ostwald, J. and Siemers, B. M. (2008) 'Foraging bats avoid noise.' *The Journal of experimental biology*, 211(Pt 19) pp. 3174-3180.

Schneider, P. M., Bender, K., Mayr, W. R., Parson, W., Hoste, B., Decorte, R., Cordonnier, J., Vanek, D., Morling, N., Karjalainen, M., Marie-Paule Carlotti, C., Sabatier, M., Hohoff, C., Schmitter, H., Pflug, W., Wenzel, R., Patzelt, D., Lessig, R., Dobrowolski, P., O'Donnell, G., Garafano, L., Dobosz, M., de Knijff, P., Mevag, B., Pawlowski, R., Gusmão, L., Conceicao Vide, M., Alonso Alonso, A., García Fernández, O., Sanz Nicolás, P., Kihlgreen, A., Bär, W., Meier, V., Teyssier, A., Coquoz, R., Brandt, C., Germann, U., Gill, P., Hallett, J. and Greenhalgh, M. (2004) 'STR analysis of artificially degraded DNA—results of a collaborative European exercise.' *Forensic Science International*, 139(2) pp. 123-134.

Schofield, H. (2002) *A Guide to the identification of pipistrelle bats.* [Online] [Accessed on 09/09/2016] http://www.warksbats.co.uk/pdf/pipistrelleID.pdf

Schulze, M. D., Seavy, N. E. and Whitacre, D. F. (2000) 'A Comparison of the Phyllostomid Bat Assemblages in Undisturbed Neotropical Forest and in Forest Fragments of a Slash-and-Burn Farming Mosaic in Peten, Guatemala.' *Biotropica*, 32(1) pp. 174-184.

Schunk, C., Swartz, S. M. and Breuer, K. S. (2017) 'The influence of aspect ratio and stroke pattern on force generation of a bat-inspired membrane wing.' *Interface Focus*, 7(1)

Sears, K. E., Behringer, R. R., Rasweiler, J. J. and Niswander, L. A. (2006) 'Development of Bat Flight: Morphologic and Molecular Evolution of Bat Wing Digits.' *Proceedings of the National Academy of Sciences of the United States of America*, 103(17) pp. 6581-6586.

Sendor, T. and Simon, M. (2003) 'Population Dynamics of the Pipistrelle Bat: Effects of Sex, Age and Winter Weather on Seasonal Survival.' *Journal of Animal Ecology*, 72(2) pp. 308-320.

Siemers, B. M. and Schnitzler, H.-U. (2000) 'Natterer's Bat (*Myotis nattereri Kuhl*, 1818) Hawks for Prey Close to Vegetation Using Echolocation Signals of Very Broad Bandwidth.' *Behavioral Ecology and Sociobiology*, 47(6) pp. 400-412.

Siemers, B. M. and Swift, S. M. (2006) 'Differences in Sensory Ecology Contribute to Resource Partitioning in the Bats *Myotis bechsteinii* and *Myotis nattereri* (Chiroptera: Vespertilionidae).' *Behavioral Ecology and Sociobiology*, 59(3) pp. 373-380.

Simmons, N.B. and A.L. Cirranello. 2019. *Bat Species of the World: A taxonomic and geographic database.* Accessed on 08/06/2019. www.batnames.org.

Skulborstad, A. J., Swartz, S. M. and Goulbourne, N. C. (2015) 'Biaxial mechanical characterization of bat wing skin.' *Bioinspiration & Biomimetics*, 10(3) p. 036004.

Smith, L. M. and Burgoyne, L. A. (2004) 'Collecting, archiving and processing DNA from wildlife samples using FTA® databasing paper.' *BMC Ecology*, 4(1) pp. 4-4.

Sokolov, V. (1982) Mammal skin. Berkeley: University of California Press.

Speakman, J. R. (1991) 'The impact of predation by birds on bat populations in the British Isles.' *Mammal Review*, 21(3) pp. 123-142.

Sterbing-D'Angelo, S., Chadha, M., Chiu, C., Falk, B., Xian, W., Barcelo, J., Zook, J. M. and Moss, C. F. (2011) 'Bat wing sensors support flight control.' *Proceedings of the National Academy of Sciences of the United States of America*, 108(27) pp. 11291-11296.

Stevens, A. (2010) Dynamics of Predation. *Nature Education Knowledge*, 3(10):46.

Stone, E. L., Jones, G. and Harris, S. (2009) 'Street Lighting Disturbs Commuting Bats.' *Current Biology*, 19(13) pp. 1123-1127.

Studier, E. H. (1972) 'Some Physical Properties of the Wing Membranes of Bats.' *Journal of Mammalogy*, 53(3) pp. 623-625.

Swaddle, J. P., Witter, M. S., Cuthill, I. C., Budden, A. and McCowen, P. (1996) 'Plumage Condition Affects Flight Performance in Common Starlings: Implications for Developmental Homeostasis, Abrasion and Moult.' *Journal of Avian Biology*, 27(2) pp. 103-111.

Swango, K. L., Timken, M. D., Chong, M. D. and Buoncristiani, M. R. (2006) 'A quantitative PCR assay for the assessment of DNA degradation in forensic samples.' *Forensic Science International*, 158(1) pp. 14-26.

Swartz, S. M. and Konow, N. (2015) 'Advances in the study of bat flight: The wing and the wind.' *Canadian Journal of Zoology*, 93(12) pp. 977-990.

Swartz, S. M., Groves, M. S., Kim, H. D. and Walsh, W. R. (1996) 'Mechanical properties of bat wing membrane skin.' *Journal of Zoology*, 239(2) pp. 357-378.

Tabner, H. (2019) *Lateralization in shelter cats (Felis catus). Do Cats show Handedness like Humans?* Manchester Metropolitan University.

Tapanes, E., Detwiler, K. M. and Cords, M. (2016) 'Bat Predation by *Cercopithecus* Monkeys: Implications for Zoonotic Disease Transmission.' *EcoHealth*, 13(2) pp. 405-409.

Taylor, G. K., Nudds, R. L. and Thomas, A. L. R. (2003) 'Flying and swimming animals cruise at a Strouhal number tuned for high power efficiency.' *Nature*, 425(6959) pp. 707-711.

Teeling, E. C., Madsen, O., Ronald, A. V. D. B., Wilfried, W. d. J., Stanhope, M. J. and Springer, M. S. (2002) 'Microbat Paraphyly and the Convergent Evolution of a Key Innovation in Old World Rhinolophoid Microbats.' *Proceedings of the National Academy of Sciences of the United States of America*, 99(3) pp. 1431-1436.

The Royal Society for the Protection of Bird RSPB. (2019) The RSPB, Cats and Birds | Are Cats Causing Bird Declines. [Online] [Accessed on 30 July 2019] https://www.rspb.org.uk/birds-and-wildlife/advice/gardening-for-wildlife/animal-deterrents/cats-and-garden-birds/are-cats-causing-bird-declines/.

Thomas, A. L. R., Jones, G., Rayner, J. M. V. and Hughes, P. M. (1990) 'Intermittent gliding flight in the pipistrelle bat (*Pipistrellus pipistrellus*) (Chiroptera: Vespertilionidae).' *Journal of Experimental Biology*, 149 pp. 407-416.

Thomas, A. L. R. (1993) 'The Aerodynamic Costs of Asymmetry in the Wings and Tail of Birds: Asymmetric Birds can't Fly round Tight Corners.' *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 254(1341) pp. 181-189.

Tian, X., Iriarte-Diaz, J., Middleton, K., Galvao, R., Israeli, E., Roemer, A., Sullivan, A., Song, A., Swartz, S. and Breuer, K. (2006) 'Direct measurements of the kinematics and dynamics of bat flight.' *Bioinspiration and Biomimetics*, 1(4) pp. S10-S18.

Toju, H., Tanabe, A. S., Yamamoto, S. and Sato, H. (2012) 'High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples.' *PLoS ONE*, 7(7) p. e40863.

Townsend, C. R., Begon, M. and Harper, J. L. (2008) *Essentials of ecology.* 3rd ed., Oxford: Blackwell.

Tuladhar-Douglas, W. (2008) 'The use of bats as medicine among the Newars.' *Journal of Ethnobiology*, 28(1) pp. 69-91.

Vable, M. (2012) *Mechanics of Materials*. 2nd ed. [ebook] Michigan: Michigan Technological University, pp.1-87. [Accessed on 01/09/2017] https://archive.org/details/ost-engineering-vable_mom_2nd_edition_aug_2012.

Van Ee, C. A., Chasse, A. L. and Myers, B. S. (2000) 'Quantifying skeletal muscle properties in cadaveric test specimens: Effects of mechanical loading, postmortem time, and freezer storage.' *Journal of Biomechanical Engineering*, 122(1) pp. 9-14.

van Heezik, Y., Smyth, A., Adams, A. and Gordon, J. (2010) 'Do domestic cats impose an unsustainable harvest on urban bird populations?' *Biological Conservation*, 143(1) pp. 121-130.

Vaughan (1970) 'The muscular system.' *In* Wimsatt, W. A. (ed.) *Biology of Bats*. New York: Academic Press, pp. 140-194.

Verboom, B. and Spoelstra, K. (1999) 'Effects of food abundance and wind on the use of tree lines by an insectivorous bat, *Pipistrellus pipistrellus*.' *Canadian Journal of Zoology*, 77(9) pp. 1393-1401.

Videler, J.J., 2006. *Avian flight*. Oxford University Press.

Vlaschenko, A., Kovalov, V., Hukov, V., Kravchenko, K. and Rodenko, O. (2019) 'An example of ecological traps for bats in the urban environment.' *European Journal of Wildlife Research*, 65(2) pp. 1-5.

Voigt, C. C. (2013) 'Bat flight with bad wings: Is flight metabolism affected by damaged wings?' *Journal of Experimental Biology*, 216(8) pp. 1516-1521.

Voigt, C. C., Borrisov, I. M. and Voigt-Heucke, S. L. (2012) 'Terrestrial locomotion imposes higlh metabolic requirements on bats.' *The Journal of experimental biology*, 215(Pt 24) pp. 4340-4344.

Voigt, C. C., Schneeberger, K., Voigt-Heucke, S. L. and Lewanzik, D. (2011) 'Rain increases the energy cost of bat flight.' *Biology letters*, 7(5) pp. 793-795.

Walker, M. (2002) *Identification of British Bats*. South Nottinghamshire Bat Group. (Online) (Accessed on 05/06/2016) http://nottsbatgroup.org.uk/resources/training/

Wang, D.-y., Ma, W., Niu, Y.-h., Chang, X.-x. and Wen, Z. (2007) 'Effects of cyclic freezing and thawing on mechanical properties of Qinghai–Tibet clay.' *Cold Regions Science and Technology*, 48(1) pp. 34-43.

Weaver, K. N., Alfano, S. E., Kronquist, A. R. and Reeder, D. M. (2009) 'Healing Rates of Wing Punch Wounds in Free-Ranging Little Brown Myotis (*Myotis lucifugus*).' *Acta Chiropterologica*, 11(1) pp. 220-223.

Weier, S. M., Grass, I., Linden, V. M. G., Tscharntke, T. and Taylor, P. J. (2018) 'Natural vegetation and bug abundance promote insectivorous bat activity in macadamia orchards, South Africa.' *Biological Conservation*, 226 pp. 16-23.

Wells, G., Prest, H. and Russ, C. W. (2011) *Signal, Noise, and Detection Limits in Mass Spectrometry.* USA: Agilent Technologies.

Wheeler, J. B., Mukherjee, R., Stroud, R. E., Jones, J. A. and Ikonomidis, J. S. (2015) 'Relation of Murine Thoracic Aortic Structural and Cellular Changes With Aging to Passive and Active Mechanical Properties.' *Journal of the American Heart Association*, 4(3) pp.1-9.

Wibbelt, G., Kurth, A., Hellmann, D., Weishaar, M., Barlow, A., Veith, M., Prüger, J., Görföl, T., Grosche, L., Bontadina, F., Zöphel, U., Seidl, H. P. and Blehert, D. S. (2010) 'White-nose syndrome fungus (*Geomyces destructans*) in bats, Europe.' *Emerging infectious diseases*, 16(8) p. 1237.

Wiedeman, M. P. (1963) 'Dimensions of Blood Vessels from Distributing Artery to Collecting Vein.' *Circulation Research*, 12(4) pp. 375-378.

Williams, E. V. and Swaddle, J. P. (2003) 'Moult, Flight Performance and Wingbeat Kinematics during Take-Off in European Starlings Sturnus vulgaris.' *Journal of Avian Biology*, 34(4) pp. 371-378.

Woods, M., McDonald, R. A. and Harris, S. (2003) 'Predation of wildlife by domestic cats Felis catus in Great Britain.' *Mammal Review*, 33(2) pp. 174-188.

Ängeby Möller, K., Kinert, S., Størkson, R. and Berge, O.-G. (2012) 'Gait Analysis in Rats with Single Joint Inflammation: Influence of Experimental Factors.' *PLoS ONE*, 7(10) p. e46129.

Appendix

Appendix 1: The abstract of the published paper and the full paper:

Khayat, R. O., Shaw, K. J., Dougill, G., Melling, L. M., Ferris, G. R., Cooper, G., and Grant, R. A. 'Characterizing wing tears in common pipistrelles (*Pipistrellus pipistrellus*): investigating tear distribution, wing strength, and possible causes.' *Journal of Mammalogy*, pp. 1-13.

Abstract:

Bats have large, thin wings that are particularly susceptible to tearing. Anatomical specializations, such as fiber reinforcement, strengthen the wing and increase its resistance to puncture, and an extensive vasculature system across the wing also promotes healing. We investigated whether tear positioning is associated with anatomy in common pipistrelles (*Pipistrellus pipistrellus*). Wing anatomy was described using histological techniques, imaging, and material testing. Tear information, including type, position, time in rehabilitation, and possible causes, was collected from rehabilitators of injured bats across the United Kingdom. Results suggest that the position of the plagiopatagium (the most proximal wing section to the body), rather than its anatomy, influenced the number, location, and orientation of wing tears. While material testing did not identify the plagiopatagium as being significantly weaker than the chiropatagium (the more distal sections of the wing), the plagiopatagium tended to have the most tears. The position of the tears, close to the body and toward the trailing edge, suggests that they are caused by predator attacks, such as from a cat (*Felis catus*), rather than collisions. Consistent with this, 38% of *P. pipistrellus* individuals had confirmed wing tears caused by cats, with an additional 38% identified by rehabilitators as due to suspected cat attacks. The plagiopatagium had the lowest number of blood vessels and highest amounts of elastin fibers, suggesting that healing may take longer in this section. Further investigations into the causes of tears, and their effect on flight capabilities, will help to improve bat rehabilitation.

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Characterizing wing tears in common pipistrelles (*Pipistrellus pipistrellus*): investigating tear distribution, wing strength, and possible causes

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Bats have large, thin wings that are particularly susceptible to tearing. Anatomical specializations, such as fiber reinforcement, strengthen the wing and increase its resistance to puncture, and an extensive vasculature system across the wing also promotes healing. We investigated whether tear positioning is associated with anatomy in common pipistrelles (*Pipistrellus pipistrellus*). Wing anatomy was described using histological techniques, imaging, and material testing. Tear information, including type, position, time in rehabilitation, and possible causes, was collected from rehabilitators of injured bats across the United Kingdom. Results suggest that the position of the plagiopatagium (the most proximal wing section to the body), rather than its anatomy, influenced the number, location, and orientation of wing tears. While material testing did not identify the plagiopatagium as being significantly weaker than the chiropatagium (the more distal sections of the wing), the plagiopatagium tended to have the most tears. The position of the tears, close to the body and toward the trailing edge, suggests that they are caused by predator attacks, such as from a cat (*Felis catus*), rather than collisions. Consistent with this, 38% of *P. pipistrellus* individuals had confirmed wing tears caused by cats, with an additional 38% identified by rehabilitators as due to suspected cat attacks. The plagiopatagium had the lowest number of blood vessels and highest amounts of elastin fibers, suggesting that healing may take longer in this section. Further investigations into the causes of tears, and their effect on flight capabilities, will help to improve bat rehabilitation.

Key words: bat wing, collagen, elastin, healing, material testing, plagiopatagium, wing tear

Bats have thin wing membranes well adapted to generate appropriate lift and thrust to be maneuverable during flight (Vaughan 1970; Swartz et al. 1996; Neuweiler 2000). However, the large area and the thin membranous material of the wings make them particularly susceptible to injuries, such as holes and tears (Ceballos-Vasquez et al. 2015). Davis (1968) found over 40% of pallid bats (*Antrozous pallidus*) in one rural roost had wing injuries or abnormalities. While bats can fly with large wing tears (Davis 1968; Voigt 2013), hundreds of bats are taken to rescue centers for rehabilitation annually in the United Kingdom, especially the common pipistrelle, *Pipistrellus pipistrellus* (Kelly et al. 2008). Indeed, 748 *Pipistrellus* spp. were admitted to just one rescue center in the United Kingdom between 1997 and 2006 (Kelly et al. 2008). Tears are considered significant and severe injuries (Molony et al. 2007; Kelly et al. 2008). Rehabilitation in captivity can also result in increased stress (Moorhouse et al. 2007); therefore, the tear and resulting rehabilitation can significantly affect animal health and welfare (Molony et al. 2007; Kelly et al. 2008). Even though several studies have investigated wing tears in bats (Davis 1968; Powers et al. 2013; Voigt 2013; Greville et al. 2018), there is little characterization of their form (position, orientation, size) and what causes them, although collisions (Davis 1968), fungal infections (Reichard and Kunz 2009; Cryan et al. 2010; Fuller

et al. 2011), and predator attacks (Speakman 1991; Woods et al. 2003; Ancillotto et al. 2013; Loss et al. 2013) are all likely.

Urbanization is likely to increase the occurrence of wing tear injuries in bats, due to a greater likelihood of collisions with man-made structures and the increase in abundance of urban predators, such as cats (*Felis catus*). Urbanization is one of the most dramatic forms of land-use change (Lintott et al. 2015) and it is difficult to predict how it will affect individual species (Mehr et al. 2011; Hale et al. 2012; Lintott et al. 2015; Jung and Threlfall 2018; Santini et al. 2019). Many bats, such as *P. pipistrellus*, exploit urban environments (Mendes et al. 2014; Hale et al. 2015), especially for roosting, water, and foraging under lights (Russo and Ancillotto 2015). However, this also exposes them to urban risks, including predation (Woods et al. 2003). For example, in the United Kingdom, domestic cats are the most abundant carnivores (Woods et al. 2003), and their numbers are concentrated around urban areas (Aegerter et al. 2017). Evidence has suggested that cats target house-roosting bats in both rural and semi-urban areas, with repeated predation events having the capacity to wipe out entire roosts (Ancillotto et al. 2013). However, many of these observations are only occasional and not based on strong evidence (Woods et al. 2003; Ancillotto et al. 2013). A better description of wing tears and their causes is needed in order to understand the scale of the problem in the short term, and to develop management practices in the long term, in terms of treatment and rehabilitation practices.

Bat wings can heal from tears (Davis and Doster 1972; Faure et al. 2009; Weaver et al. 2009), and it has been proposed to use fruit bat wings as a model to study wound healing and contraction (Church and Warren 1968). Bat wings also have an extensive blood supply to enable wound cleaning, prevention of infection, and tissue reformation (Faure et al. 2009). Faure et al. (2009) found that the uropatagium (interfemoral membrane) healed faster than the chiropatagium in big brown bats (*Eptesicus fuscus*), and attributed it to increased vasculature in that area. Moreover, while bat wings are thin and susceptible to tearing, anatomical specializations, such as a net-like fiber system containing collagen and elastin, reinforce the wings and increase their resistance to puncture (Studier 1972; Holbrook and Odland 1978; Madej et al. 2012; Cheney et al. 2017). This is especially true in ground-foraging bats, whose wings are more resistant to puncture and less elastic than bats who forage in more open habitats (Studier 1972). The complex anatomy of the wing, including wing fibers and strength, might affect the position and type of wing tears.

We characterized wing tear injuries in the common pipistrelle, and provide a quantitative summary of tear types, distributions, and rehabilitation outcomes. We examined the anatomy of *P. pipistrellus* wings to identify whether wing vasculature, strength, and fiber distribution are associated with the position and type of wing tears. In particular, we investigated vasculature, material properties, fiber type, and fiber orientation. If anatomy influences tearing, we expect 1) more tears to occur in the weakest wing section (based on material property data); 2) tears should not have a specific orientation because net-like fibers should reinforce equally in all orientations; and 3) tears should heal fastest in the section with the most blood vessels, which should transport factors for wound cleaning and new tissue formation. We collected data from bat rehabilitators across the United Kingdom to characterize wing tear injuries, and discuss some likely causes of tears based on first-hand observations. Our results suggest that the position of the plagiopatagium, rather than its anatomy, influenced the number, location, and orientation of wing tears. Predator attacks were the likely cause of many of the tears, and we suggest that predators directing their attacks toward the bat's body caused many of the rostro-caudal tears in the plagiopatagium.

Materials and Methods

We refer to the anatomy of *P. pipistrellus* wings over three sections (Fig. 1a). The most distal section of the chiropatagium (CI) is the membrane between digits iii and iv. The second section of the chiropatagium (CII) is the membrane between digits iv and v. The most proximal section of the wing is the plagiopatagium (P), which is the membrane between digit v and the body. Ethical approval for the study was obtained through the Research Ethics and Governance Committee at Manchester Metropolitan University, and all tissue was held under a Natural England license (2014-4322-SCI-SCI). Methods conformed to guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes et al. 2016).

Ten adult whole-animal *P. pipistrellus* specimens from euthanized animals were donated by bat rehabilitators for vessel tracing (two bats, right side wings), and histology (two bats used in Masson's Trichrome staining and two bats used in Verhoeff-Van Gieson [VVG] staining, right side wings) and material testing (10 bats in total, left side wings, including the six bats from the vessel and histology work). These animals were admitted to care following injury and grounding; although exact details were not known by the rehabilitators, they likely had many internal injuries and complications. All individuals had intact wings so we could examine their anatomy. All photography was undertaken from live adult animals during usual husbandry and rehabilitation procedures carried out by bat rehabilitators (eight *P. pipistrellus* bats for vessel tracing, 55 *P. pipistrellus* bats, and 22 other United Kingdom bats for characterization of tears). Bat rehabilitators were trained individuals registered with the Bat Conservation Trust (BCT).

Vessel tracing.—Ten bat specimens were used to identify the major blood vessels in *P. pipistrellus* wings. Two whole-bat euthanized specimens were donated by bat rehabilitators. Their right wings were removed whole and stored in 4% paraformaldehyde (PFA), at 4°C. The wings were stretched out over a bright lightbox (LEDW-BL-100/100-SLLUB-Q-1R24, Phlox, Aix-en-Provence, France), and photographed using a digital camera (D3200, Nikon, Tokyo, Japan; Fig. 1a). Another eight photographs were collected from bat rehabilitators, who had stretched the wings of live bats, admitted for rehabilitation, over a white piece of gridded card. All wings were intact and did not contain any holes or scarring.

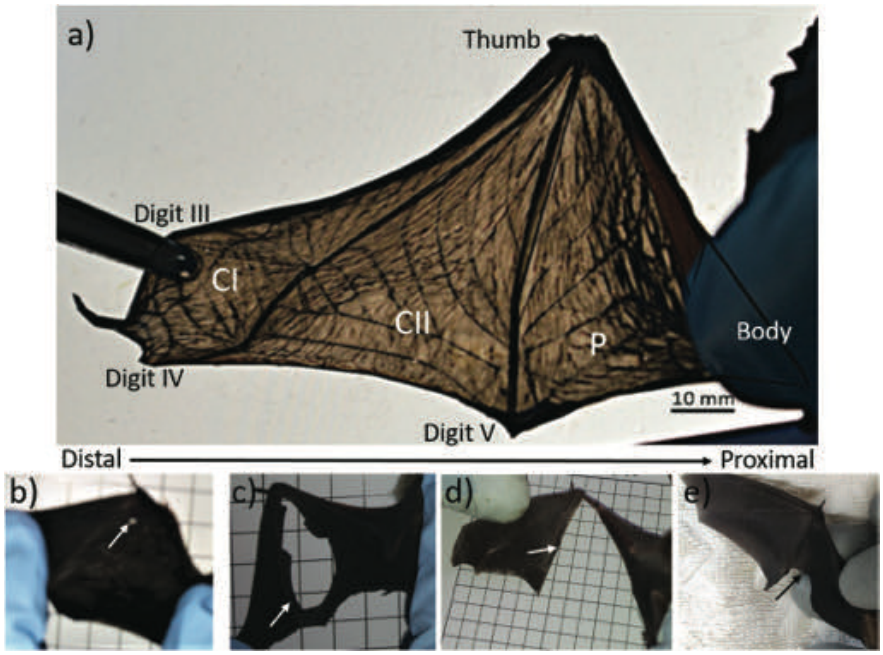

Fig. 1.—Example images of bat wing and tears of common pipistrelles (*Pipistrellus pipistrellus*). a) The wing was stretched over a lightbox to image the blood vessels. The sections of the wing are indicated (CI: the first chiropatagium section; CII: the second chiropatagium section; and P: the plagiopatagium). Examples of the different types of tears are shown, including hole (b), contained tear (c), total tear (d), and trailing edge tear (e).

Inkscape software (https://inkscape.org/en/) was used to trace the blood vessels in all photographs. Some vessels were approximately twice as thick as other vessels, and these were traced with thicker lines. Once the vessels had been drawn in all the photographs, they were combined into one figure to give a summary of all possible blood vessels. This figure was validated against descriptions in the literature (Pavlinić et al. 2008). The number of vessels were counted in each section of the wing (CI, CII, P), and any bifurcations were counted as another vessel. The area of each section of the wing was also measured using Inkscape, to give an approximation of blood vessel density (count/wing area).

Wing histology.—We used Masson's Trichrome staining to identify wing fiber orientation. Two wings from euthanized *P. pipistrellus* were stored in 4% PFA, at 4°C. A fragment from each section of the wing (approximately 10 mm^2) was dissected and sliced tangential to the wing at 30 µm thickness using a freezing cryostat (CM3050, Leica, Wetzlar, Germany) at −20°C. This thickness was selected to reduce the curling and wrinkling of slices that occurred in thinner sections. The slices were transferred to a solution of 10% phosphate-buffered saline (PBS) overnight, mounted on microscope slides (Menzel-Glaser, Thermo Scientific, Braunschweig, Germany), and left to dry for an additional 24 h. The slices were then stained using Masson's Trichrome (Trichrome Stain Kit, Sigma-Aldrich, St. Louis, Missouri). Slides were put in a fixative solution (4% paraformaldehyde in 0.1 M PBS) for 1 h, and introduced to Bouin's Solution for 3 h. They were then cleared with xylene, rehydrated with ethyl alcohol (100, 90, 80, and 70%), and moved through a sequence of solutions for the Masson's Trichrome staining (Biebrich Scarlet Acid, Phosphotungstic and Phosphomolybdic Acids, Aniline Blue, and Acidified Water), with multiple washes of distilled water between each stage. The slices were then dehydrated with ethyl alcohol (70, 90, and 100%) and xylene, towel dried, and cover-slipped using Distyrene Plasticizer Xylene (DPX; Sigma-Aldrich).

To measure relative amounts of collagen and elastin within the sections of the wing, VVG staining was used. Two wings from two euthanized *P. pipistrellus* were stored in 4% PFA, at 4°C. A sample (approximately 10 mm^2) was removed from each section of the wing and placed in 4% PFA overnight at 4°C. Each sample was embedded in 2% agar in PBS and transferred in a histology cassette for tissue processing (Shandon Citadel 2000, Thermo Scientific). Subsequently, the samples were placed in 70% Industrial Methylated Spirits (IMS) for 3 h, 80% IMS for 60 min, 90%

IMS for 60 min, 100% IMS for 2 h twice, 100% IMS for 60 min, then xylene for 90 min twice, xylene for 2 h, and finally in paraffin wax twice for 3 h. Afterwards, the samples were embedded in paraffin wax for slicing. Each sample was sliced across the wing axis (perpendicular to tangential) at 5 µm thickness on an automatic, rotary microtome with water bath (microtome HM355S, Thermo Scientific), collected on glass slides (Superfrost Plus, Thermo Scientific), and incubated in an oven at 37°C overnight before staining. The slides were then cleared with xylene and rehydrated with ethyl alcohol (100%, 90%, 80%, 70%, and distilled water) prior to staining. To stain the elastin, the slides were placed for 10 min in working elastic stain solution, that consisted of 20 ml of hematoxylin solution (HT 251, Sigma-Aldrich), 3 ml ferric chloride solution (HT252, Sigma-Aldrich), 8 ml of Weigert's iodine solution (HT253, Sigma-Aldrich), and 5 ml deionized water. The slides were then rinsed in deionized water and differentiated in ferric chloride solution, comprising of 3 ml ferric chloride solution (HT252, Sigma-Aldrich) and 37 ml of deionized water. Next, the slides were rinsed in tap water, and placed in 95% ethyl alcohol to remove the iodine and then in deionized water. Subsequently, slides were stained for collagen in Van Gieson's solution (Sigma-Aldrich) for 1–3 min, then rinsed in 95% alcohol. Finally, they were dehydrated (100% ethyl alcohol), placed in xylene, and cover-slipped with DPX.

All slices were visualized using a Zeiss Stereo Lumar V12 light microscope (Zeiss, Oberkochen, Germany). Figures were captured using Zeiss Axiovision, version 4.8. Occasional adjustments to exposure and white balance were made. The fiber orientation was described qualitatively for each section stained with Masson's Trichrome. The relative amounts of collagen and elastin were approximated quantitatively using image processing in Matlab from each section stained with VVG. VVG is a standard histological stain used to identify collagen and elastin fibers (Fullmer and Lillie 1956; Kazlouskaya et al. 2013; Cheney et al. 2017), and has been used to quantify amounts of collagen and elastin in stained tissues (Daamen et al. 2003; Raub et al. 2010; Eberson et al. 2015; Wheeler et al. 2015; Lee et al. 2016). Images were selected for image processing when the section was clear and not folded so that all fibers could be seen in the image. Ten to 12 slices were taken

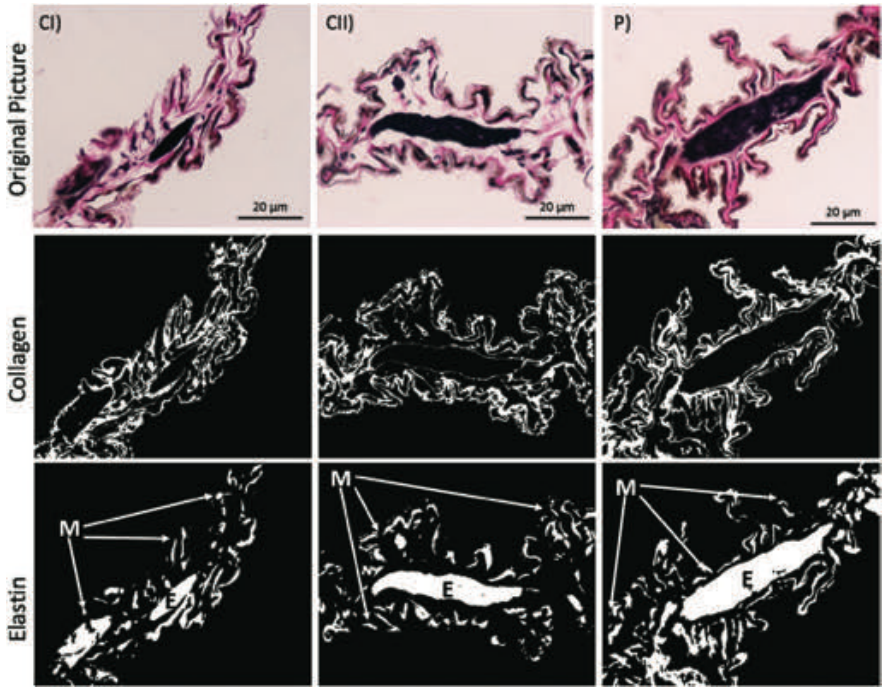

Fig. 2.—Example images demonstrating the processing of elastin and collagen fibers. The top panel shows the original images collected from the microscope following Van Gieson staining. These were processed to find the red-pink collagen colors (middle panels) and the dark elastin colors (bottom panels) for the first chiropatagium section (CI), the second chiropatagium section (CII), and the plagiopatagium section (P). All red-pink pixels were counted for the collagen fibers. For the elastin (E), only the internal elastin fibers were included in the pixel counts; the edges also appeared black in the slices but mainly contained melanin (M), therefore these were cropped from the elastin pixel counts.

for each wing section and then two to three images captured from each slice, giving a total of 35 images for each section.

Original 8-bit color images of the slides reveal collagen as a red-pink color and elastin as a dark, near black color (Fig. 2, top panel; see online version for color). Filters were applied to create two black and white images from each original image, one that showed only collagen and a second showing only elastin (Fig. 2, middle and bottom panels, respectively). A range of filter strengths were tested on the sample images and inspected for accuracy by three independent observers. The position and presence of collagen fibers was also validated by comparing the VVG slices to a subset of slices that were stained with Sirius Red. The settings that provided the most accurate separation of collagen and elastin fibers were then applied to the full image set. Elastin images were created by filtering out pixels with a moderate or high 8-bit color intensity in any RGB channel. Some black color could be seen at the edges of the slices, consisting of melanin in the bat wing skin. Although some elastin was also likely to occur in this area, the edges of the sample were cropped to focus on measuring the internal elastin fibers only (Fig. 2, bottom panels). Collagen images were created by filtering out any pixel with a high green or blue intensity or a low red channel intensity. The red channel threshold was determined automatically (graythresh in Matlab using Otsu's method) on an image-by-image basis taking into account the overall color spectrum of the image. Relative percentages of collagen and elastin were calculated by counting the number of white pixels in each of the generated images.

Material testing.—Ten left wings were used to test the material properties of each wing section; these were all from euthanized bats. Each wing was kept in a freezer at −18°C, and then defrosted in 10% PBS for 10 min. Freezing may have affected the mechanical properties of the samples, and previous studies have shown mixed results (Wang et al. 2007; Kaye 2012) with freezing not having an effect in some cases (Foutz et al. 1992; Van Ee et al. 2000; Santago et al. 2009). As all samples were frozen, we were able to compare between and within samples and observe relative differences, but the absolute values may vary from other studies. After defrosting, long strips were cut out from each wing section, from the digit joint to the trailing edge. The length and width of each strip sample were measured with a ruler (Table 1). The wing thickness was calculated as the mean of three measurements by placing the sample on glass beads and using a microscope (Lumar.V12, Zeiss) with a calibrated camera (AxioCam MRc, Zeiss). Samples were kept hydrated in 10% PBS and tested before drying. Each sample was gripped in a tensometer (Hounsfield H10KS, Tinius Olsen, Horsham, Pennsylvania) using pneumatic grips ensuring consistent grip pressure across all tests. A gauge length of 5 mm was used for all samples, with approximately 11 mm in each grip (refer to average length in Table 1). Each sample was stretched at 10 mm/min until failure, along its longest axis (from the digit joint to the trailing edge). Due to the small size and the delicate nature of the wing of *P. pipistrellus*, the aspect ratios of the tested samples were fairly small and it was not possible to cut a dog-bone shape. Therefore, there were some transverse stresses at the clamps, resulting in wing breakage at the clamp in 30% of the samples, rather than in the middle of the sample. Samples that failed at the grips were removed from further analyses to reduce the effect of incidental bias of transverse stresses on failure stress results. The maximum force at failure (N) and maximum extension (mm) was

Table 1.—Comparing anatomical and material properties of the three wing sections (CI, CII, and P) of common pipistrelles (*Pipistrellus pipistrellus*). Values are mean ± *SD*, *n* refers to the number of bats, and n.a refers to when it was not appropriate to run statistical tests. *P* was considered significant at the < 0.01 level, with a Bonferroni correction (indicated in bold).

	Wing section			
	CI	CII	P	Statistics
Blood vessels				
Number	14.00 ± 1.70	12.60 ± 1.51	9.00 ± 1.56	$\chi^2 = 18.686$, $P <$ **0.001** (CI, CII > P), $n = 10$
Density (no./cm^2)	2.16 ± 0.99	0.98 ± 0.54	0.63 ± 0.48	$\chi^2 = 13.628$, $P =$ **0.001** (CI > CII, P), $n = 10$
Fibers				
% Collagen	79.92 ± 13.73	43.09 ± 21.78	52.47 ± 26.95	$\chi^2 = 35.922$, $P <$ **0.001** (CI > CII, P), $n = 2$
% Elastin	20.08 ± 13.73	56.91 ± 21.78	47.53 ± 26.95	$\chi^2 = 35.922$, $P <$ **0.001** (CI < CII, P), $n = 2$
Material properties				
Section length (mm)	33.60 ± 7.97	26.66 ± 7.45	26.02 ± 5.79	n.a
Section width (mm)	2.83 ± 1.18	4.59 ± 0.96	5.60 ± 1.38	n.a
Section depth (mm)	0.22 ± 0.04	0.24 ± 0.02	0.33 ± 0.04	$\chi^2 = 13.569$, $P =$ **0.001** (CI, CII < P), $n = 7$
Failure stress (N/mm^2)	2.32 ± 0.81	2.48 ± 0.94	1.58 ± 0.61	$\chi^2 = 4.364$, $P = 0.113$ $n = 7$
Failure strain (mm/mm)	0.37 ± 0.110	0.61 ± 0.182	0.63 ± 0.20	$\chi^2 = 9.062$, $P =$ **0.011** (CI < CII, P), $n = 7$
Young's modulus (N/mm^2)	8.07 ± 2.19	6.22 ± 3.12	4.45 ± 1.97	$\chi^2 = 6.033$, $P = 0.049$ (CI > P), $n = 7$
Component stiffness (N/mm)	1.37 ± 0.29	1.10 ± 0.61	0.93 ± 0.55	$\chi^2 = 2.879$, $P = 0.237$ $n = 7$

recorded. From these values, failure stress (force at failure divided by the cross-sectional area), failure strain (maximum extension divided by the original sample length of 5 mm), and Young's modulus (change in stress divided by the change in strain) were all calculated for each sample from each wing section. As strips from the P section tended to be wider than those from the other sections (Table 1), component stiffness (force at failure divided by sample width, divided by failure strain) was also calculated to control for sample width but not thickness. Results were quasi-linear and did not exhibit the 2-part loading curve, with "toe" and "upturn" regions, demonstrated by Skulborstad et al. (2015); therefore, a single gradient was calculated from the major linear region of each stress–strain curve.

Wing tear photographs.—Data on bat wing tears were collected over 20 months between March 2016 and October 2017 from live, rehabilitating animals. Photographs of torn wings were collected from bat rehabilitators soon after the bat was admitted to care. Bat rehabilitators were recruited by advertising the project at the Mammal Society Easter Meetings, the National Bat Conference, the National Bat Care Conference, and in Bat Care News, as well as from Facebook groups across the United Kingdom (UK Bat Workers, Cambridgeshire Bat Group, Kent Bat Group, and South Lancashire Bat Group). Soon after admittance, bat rehabilitators were also asked to describe how the bat was found and the possible cause of the tear. Bat rehabilitators emailed comments on the possible cause, describing any evidence for their decision. Fifty-five pictures of *P. pipistrellus* and 21 pictures of other United Kingdom bat species were collected, including two brown long-eared bats (*Plecotus auritus*), three Natterer's bats (*Myotis nattereri*), one serotine bat (*Eptesicus serotinus*), 12 soprano pipistrelles (*Pipistrellus pygmaeus*), and three whiskered bats (*Myotis mystacinus*). The wing tears were photographed while the bat was awake (not during torpor nor under anesthetic), and its wing was extended and held against a gridded card for scale. From each image, every tear was traced as it appeared in the photograph using Inkscape onto a wing diagram, and coded by color for the frequency of its occurrence in that location. The total number of all tears in each section of the wing was also determined. In addition, the tears were categorized into four major types, based on criteria of classification that were developed during this study: holes, contained tears, total tears, and trailing edge tears. A hole is a small puncture, it can be round or oval, and is usually not more than 2% of a wing segment (Fig. 1b). A contained tear is larger than a hole. It is a tear, rather than a puncture, that is still entirely contained within the wing (Fig. 1c). It can also be round or oval, with 5–50% of the membrane missing from a wing segment. A total tear is a tear that runs from the internal membrane to the trailing edge of the wing (Fig. 1d), thus not being contained within the wing. It often has a vertical appearance (like a triangle), and the bones are often affected or missing; more than 50% of the membrane tends to be missing from the wing segment. A trailing edge tear is horizontal in appearance and occurs only at the trailing edge of the wing (Fig. 1e). Some variation existed in how much the wing was stretched in each photograph (Figs. 1b–e). For example, sometimes other injuries prevented the rehabilitator from fully extending the wing. This may have influenced some classifications of holes and contained tears. However, holes did not have any further ripping, and were puncture wounds (Fig. 1b), whereas contained tears tended to be much larger and ragged around the edges, from ripping (Fig. 1c).

Bat rehabilitators were approached 9–12 months after submitting their photographs and asked what the outcome of the rehabilitation was. No further photographs were collected at this follow-up. Recommendations for bat rehabilitators for release, rehabilitation, and euthanasia practices are provided by the BCT and the Department for Environment, Food and Rural Affairs (DEFRA—Mitchell-Jones and McLeish 2004; Miller 2016), but are not quantitative, and rely on the experience and opinions of the individual rehabilitators. Bat rehabilitators emailed comments detailing how long the bat was in care before release, whether they were still in care, or were euthanized. These data were collected and, upon review, fell naturally in to four categories: released after 2 weeks, released within 2–3 months, still in care after 6 months, and euthanized. These follow-up data were collected from 13 common pipistrelles (*P. pipistrellus*), and 15 other species of United Kingdom bats, including 12 soprano pipistrelles (*P. pygmaeus*), one Natterer's bat (*M. nattereri*), one brown long-eared bat (*P. auritus*), and one serotine bat (*E. serotinus*).

Statistical considerations.—The three sections of the wing were compared for the following variables: number of blood vessels, density of blood vessels (number/cm^2), section thickness (mm), stress (N/mm^2), strain (mm/mm), Young's modulus (N/mm^2), component stiffness (N/mm), % collagen, and % elastin. They were compared using a Kruskal–Wallis test, with wing section as the dependent variable. Pairwise comparisons were undertaken using Mann–Whitney tests in SPSS (IBM SPSS Statistics, version 24, Armonk, New York), and are all summarized in Table 1, with a Bonferroni correction applied at the $P < 0.01$ level of significance. Total tear numbers and tear types were compared for *P. pipistrellus* between each of the three wing sections using a chi-square test. In other United Kingdom bat species, only the total tear numbers were tested with a chi-square test for each of the wing sections, and there were many zero scores in the tear type data. Sample sizes for the follow-up healing data were too small for statistical analysis, but are presented graphically for comparison.

Results

Blood vessels.—Section P had significantly fewer blood vessels than sections CI and CII of the bat wing (Table 1, $P < 0.01$); from our observations, it also appeared to have the thickest blood vessels, as indicated by the thicker lines in Fig. 3 (see also Fig. 1a). However, section P was also the largest section. When the number of blood vessels was normalized to the area of each wing section, there was no significant difference in blood vessel density between sections P and CII (Table 1), but section CI had the densest arrangement of blood vessels (Table 1, $P < 0.01$).

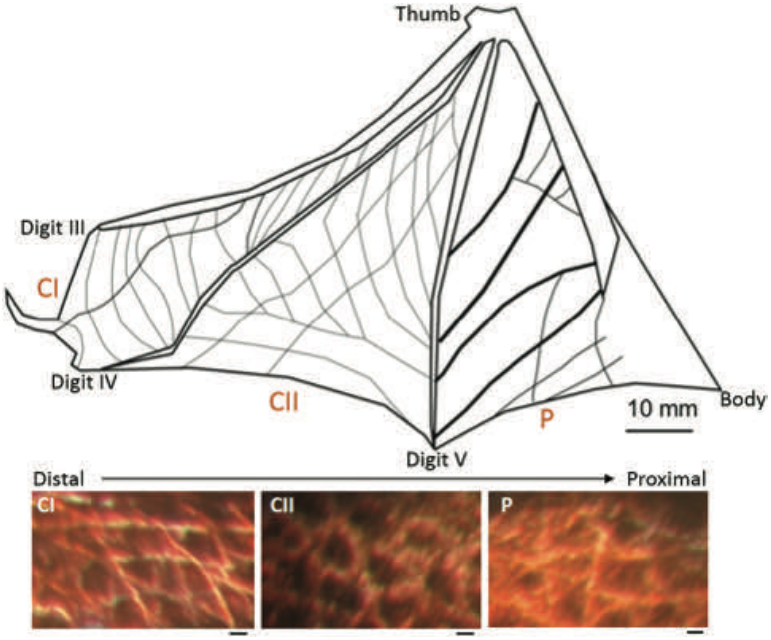

Fig. 3.—Bat wing anatomy. Top: Blood vessel tracing for common pipistrelle (*P. pipistrellus*) wings; thicker lines correspond to vessels that are twice as thick as others. Digits III, IV, and V are also indicated on the figure; digit II is not visible as it is folded against digit III, and digit I is the thumb. Bottom: Fiber orientations (elastin and collagen) for the first chiropatagium section (CI), the second chiropatagium section (CII), and the plagiopatagium section (P). Scale bars are 0.1 mm.

Wing fibers.—The orientation of fibers within the wing tended to be multidirectional and distributed in a net-like fashion throughout the membrane, with a similar appearance in each wing section (Fig. 3). The appearance of the fibers at many orientations indicates the material is, at least visually, isotropic. The amount of collagen (%) was significantly higher in section CI and the amount of elastin (%) was significantly lower in section CI, compared to sections CII and P (Table 1, $P < 0.01$).

Material testing.—Section P was the thickest section (Table 1, $P < 0.05$). No significant differences were observed between the wing sections for any of the material-testing measurements (Table 1), although CI tended to have the smallest deformation (failure strain) and highest Young's modulus (compared to section P), and section P tended to have the lowest failure stress and component stiffness (Table 1).

Characterizing wing tears.—There were more wing tears in the P section than in CI and CII in *P. pipistrellus* ($\chi^2 = 18.951$, $P < 0.01$, Figs. 4a, c, and e). The types of tears did not differ significantly between wing sections in *P. pipistrellus* ($\chi^2 = 3.647$, $P = 0.161$, Figs. 4c and e). Holes were the most common tear type in all wing sections and appeared distributed fairly evenly in each wing section. The contained and total tears tended to be oriented rostro-caudally, from an internal part of the wing membrane toward the trailing edge (Fig. 4c), and occurred more prevalently in the proximal wing sections (Figs. 4c and e). Bat rehabilitators gave possible causes for 11 of 55 individuals. One individual was bought in to the house by a cat, four were seen being attacked by a cat (Fig. 5a), and five were suspected by the rehabilitators to be cat attacks. One individual was found on the ground and was likely to have sustained tears from brambles on the ground surface (Fig. 5b), and had tears throughout each section of the wing.

Other bat species (all species pooled) also had significantly more wing tears in the P section than in CI and CII ($\chi^2 = 8.773$, $P = 0.012$; Figs. 4b, d, and f). Holes and contained tears were common tear types, and section P was the only section to reveal all the possible tear types. Tear types were commonly oriented in the rostro-caudal direction, from the membrane to the trailing edge, with only section P revealing one trailing edge tear that was oriented distal-proximally. Bat rehabilitators gave possible causes for seven of 21 individuals. One was seen being attacked by a cat, and two were suspected by the rehabilitators to be cat attacks (Fig. 5c). One bat was caught in flypaper and three were seen in a cat's mouth (Fig. 5d).

Sample numbers reporting rehabilitation outcomes were low and not analyzed statistically. Larger tears in CI did not affect the length of time that *P. pipistrellus* spent in care (Fig. 6).

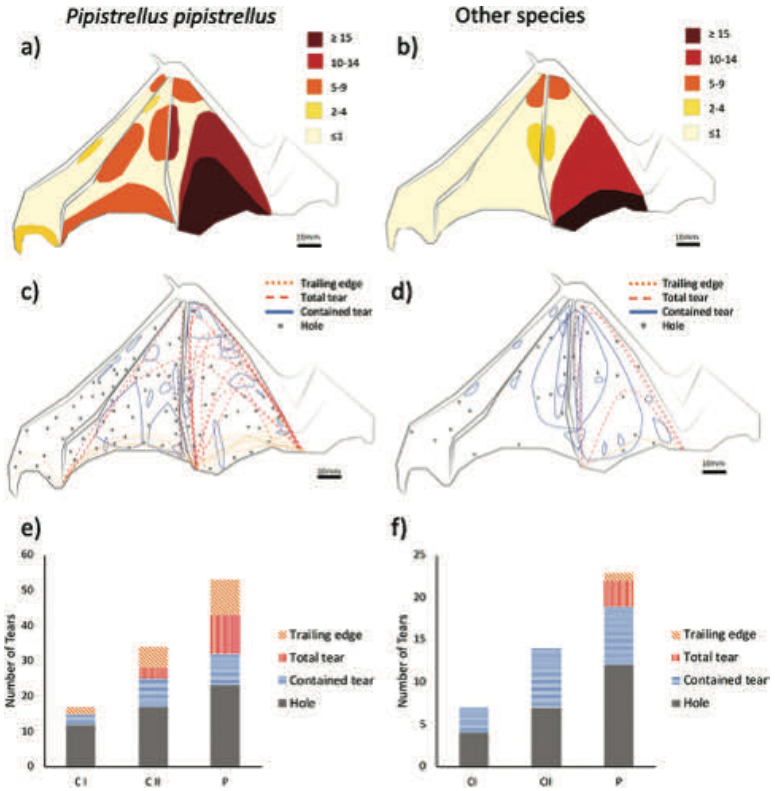

Fig. 4.—Characterization of bat wing tears. Left hand panels are data from *P. pipistrellus*, and the right hand panels are from other United Kingdom bat species. Panels (a) and (b) show the numbers of tears in each section of the wing. Panels (c) and (d) show all the wing tear positions of all tear types on each section of the wing, with dashed lines indicating total tears, dotted lines indicating trailing edge tears, solid lines indicating contained tears, and gray dots as holes. Panels (e) and (f) show the total numbers of different tear types in each wing section for the first chiropatagium section (CI), the second chiropatagium section (CII), and the plagiopatagium section (P)).

However, larger tears in P could be seen in the bat that was still in care after 6 months (Fig. 6a). In addition, large tears in both CII and P were found in the euthanized *P. pipistrellus* (Fig. 6a). In other species, large tears in section P were found in the two bats that were still in care after 6 months (Fig. 6b), but tear size did not vary much between the other wing sections in euthanized bats, or those released after 2 weeks and 2–3 months (Fig. 6b).

Discussion

The plagiopatagium section (P) sustained the most injuries. We suggest that section P, being close to the body, is likely to be torn by predators targeting the body. Furthermore, cat attacks might be causing many of the rostro-caudal tears in the P section. We consider tearing capacity and suggest that, according to its anatomy, section P should not be more prone to tearing than any other section. Therefore, the position of section P, rather than its anatomy, is an important factor in determining the number, location, and orientation of wing tears.

Position of tearing.—Across all species, section P contained the highest number and most varied types of tears (Fig. 4). Most figures in Davis (1968) also revealed that torn wings or large holes in Pallid bats (*A. pallidus*) were common in section P, with CI and CII having more trailing edge tears. There are several reasons for the greater number of tears in section P. It is the largest section of the wing and perhaps more likely to tear. It also contains the fewest bones (Fig. 1a), which may act to stop tearing. Section P is extended first before flight and might get caught or snagged during flight preparation (see figure 1 in Gardiner et al. 2011). Our consideration of anatomical properties (fiber type and material testing data) within section

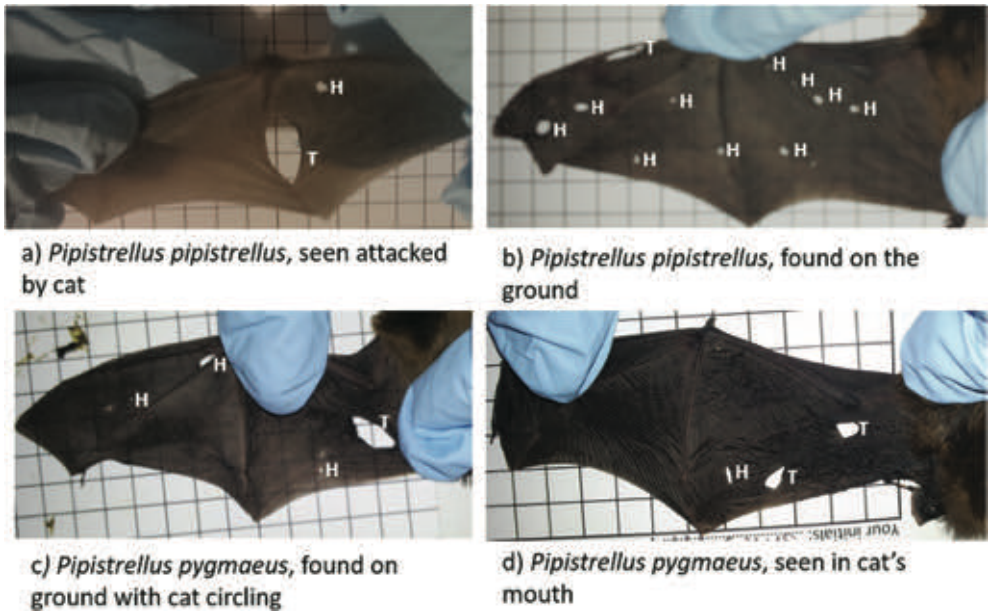

Fig. 5.—Example wing tears, with associated causes. Confirmed cat attacks cause damage to the proximal wing sections (section P) in *P. pipistrellus* (a) and *P. pygmaeus* (c and d). Grounded bats have damage to other areas of the wing in *P. pipistrellus* (b) and *P. pygmaeus* (c). Tears in these photographs were categorized as holes (H) and contained tears (T). Some pale marks can also be seen in image (b) and (c), which are healed tears.

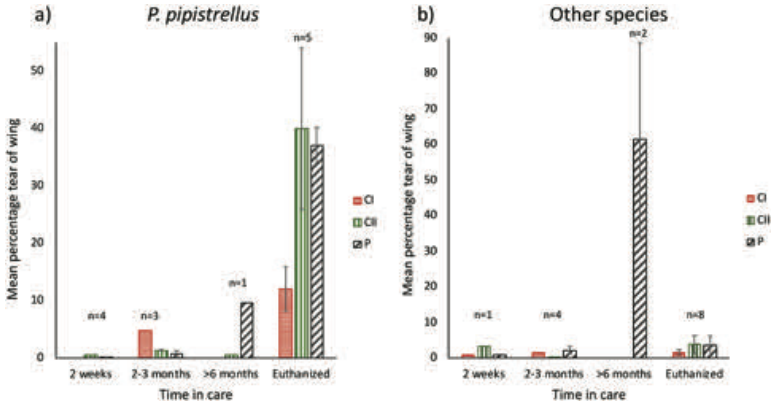

Fig. 6.—Time in rehabilitation from follow-up data. Panels (a) and (b) show the length of time that bats were in care, when they received a tear in a wing section. Percentage of the wing section torn or missing is on the y-axis, and mean values are presented with *SE* bars. *n* represents the number of bats in that classification.

P suggests it should not be more prone to tearing than sections CI and CII.

Within a bat wing, elastin fibers run perpendicular to the wing bones, and collagen fibers create a network parallel and perpendicular to the elastin, which has been described in a number of studies (Holbrook and Odland 1978; Madej et al. 2012; Cheney et al. 2015, 2017). This net-like fiber array provides tensile strength and limits extension of the wing membranes, which is important for flight (Holbrook and Odland 1978). We observed a fibrous net that has a similar appearance in each section of the

wing (Fig. 3). This net might act to limit tears from extending, and maintain holes as small holes rather than tearing further. Its equal distribution reinforces the entire wing surface (Holbrook and Odland 1978). That this net does not appear to differ between the three wing sections indicates that they should each be similarly resistant to tearing.

Quasi-static material testing and analysis of collagen-elastin percentages were carried out to compare the three wing sections further. Section CI had significantly higher relative collagen percentages than the other wing sections (% collagen, Table 1) and tended to exhibit the least extension (failure strain, Table 1, although not significant $P > 0.01$). Sections CII and P exhibited more equal collagen-elastin ratios and consequently failed at lower stress values and underwent greater extension. However, when accounting for the increased thickness of the CII and P section, no significant difference was found in the component stiffness of any of the three sections. Component stiffness normalizes for the dissected width of tested samples but allows for the natural variation in thickness of the wing sections. The similarity of values across CI, CII, and P suggests that no one section is inherently "easier" to induce failure in than any other. The P section, while weak as a pure material, requires a similar force to break when viewed as a component. The higher relative percentages of elastin in the CII and P sections may be an adaptation to improve wing folding. As the largest and most proximal sections, they are required to unfurl (stretch out) the most during flight, and higher levels of elastin should benefit this function (Cheney et al. 2015).

A study on seven species of non-*Pipistrellus* bats by Swartz et al. (1996) found that the plagiopatagium was the weakest wing section overall, was thicker, had the lowest Young's modulus, and stretched the most before breaking. This fits the general trend in our data. However, we did not observe significant differences in these parameters. Patterns in material properties varied between the different wing sections across bat genera (see figure 9 in Swartz et al. 1996), which might explain why our results differed, as they did not measure *Pipistrellus* sp. We also observed variation within individuals (note high *SD* values in material property data in Table 1).

Orientation of tears.—Despite presence of the fibrous net in all wing sections, many tears occurred in a rostro-caudal orientation, especially in section P. This coincides with the direction of travel, and might be indicative of the bat wing being snagged while moving forward. Proximal-distal tears tended to occur only around the trailing edges of the wings (Figs. 4c and d), despite being reinforced here by bundles of skeletal muscle fibers (Holbrook and Odland 1978).

If the wing is isotropic when it is stretched out, it should be equally susceptible to tearing in every orientation. Bat wings were thought to be highly anisotropic (Swartz et al. 1996). However, the elastin accounts for much of this difference and once the elastin has "unwrinkled," the wing is isotropic (Cheney et al. 2015). Therefore, our force results are likely representative of the wing as a whole, regardless of orientation of the sample, although displacement will be significantly higher in samples perpendicular with ours, as the elastin stretches out (unwrinkles).

Implications for healing.—While anatomy of the wing is not associated with the position and orientation of tears, it may affect healing. Indeed, Faure et al. (2009) suggested that tears healed quicker in the uropatagium than the chiropatagium due to its extensive vasculature. If so, our results suggest that tears to section P may take the longest time to heal. Section CI has the highest density of blood vessels, while section P has the lowest number of blood vessels (Fig. 3; Table 1), which occurs as the vessels naturally bifurcate from proximal to distal.

Extensive vasculature is associated with increased healing capabilities in bat wings and tails (Faure et al. 2009), with both the wound and scarring healing quicker. Blood carries factors to the wound site to clean the wound, prevent infection, and begin the process of reforming the tissue matrix. Therefore, being close to a vessel is likely to be important for quick healing, which has also been suggested by Faure et al. (2009) and Pollock et al. (2016). As CI had the most extensive vasculature, we expect it to heal quicker than section P. While section P had the lowest numbers of blood vessels, it also had the thickest vessels. These supply blood to the thinner, branched, more densely distributed vessels in the other sections of the wing. Following a tear, these thicker vessels might bleed more, and lead to additional complications. The majority of tears occurred on section P of the wing, which is likely to be the slowest to heal.

We did not measure healing rates in this study. However, our follow-up data on rehabilitation outcomes suggest that larger tears in section P were found in *P. pipistrellus* individuals that spent a long time in care (> 6 months) or were euthanized, although the sizes of tears were large in all wing sections in animals that were euthanized (Fig. 6). In other United Kingdom bat species, the individuals that spent a long time in care (> 6 months) also had large tears in the P section. Decisions about release, rehabilitation, and euthanasia are highly subjective and are dependent not only on the extent of injury, but also on the judgment of the bat rehabilitator, season, and weather conditions. Linking our tear characterization method with detailed information about healing rates and post-release survival will help to develop stricter rehabilitation recommendations.

Greville et al. (2018) found that in the Egyptian fruit bat (*Rousettus aegyptiacus*) wounds took about 1.5 days longer to heal to 50% wound closure in section P, compared to the chiropatagium (sections CI and CII), although this was not found in the big brown bat (*E. fuscus*). They suggest that not just blood vessels, but also collagen and elastin fibers are likely to play a role in healing. Indeed, Greville et al. (2018) suggest that over-stretching of the collagen or elastin fibers during healing can cause the tear to enlarge before healing. This phenomenon also was observed in tail and wing membranes by Pollock et al. (2016). We suggest that proximal-distal orientation of elastin fibers may hold the common rostro-caudal tears apart, thus increasing healing times. This will be especially true in the P section, which has a lot of elastin and is the largest wing section with the most movement. It also undergoes the most wrinkling when

folded against the body. Tears in this section may be stretched and extended the most during the healing process.

Implications for survival.—Section P supports the bat's body during flight and provides lift (Vaughan 1970; Swartz et al. 1996; Neuweiler 2000), while the CI and CII sections provide thrust (Swartz et al. 1996; Neuweiler 2000). Therefore, damage to different sections of the wing can affect flight differently. Tears in the CI or CII sections are likely to affect maneuverability and speed, while tears in the P section may inhibit bats' ability to generate lift. However, bats do fly with large tears in their wings (Davis 1968). Voigt (2013) studied two species of *Myotis* with unilateral trailing edge tears of approximately 20% in section P and found that injured bats made fewer flight maneuvers and had lower metabolic rates than healthy individuals. Thus, foraging success and survival are likely affected by limited flight maneuverability. Large wing tears can prevent flight altogether. Grounded bats are likely to sustain wing tears from thorns and other ground matter (Davis 1968). This may explain the tears in all wing sections in severely injured bats that were euthanized (Fig. 6). Indeed, wound healing is just the first step in a long rehabilitation process. While some studies have monitored *Pipistrellus* species post-release (Kelly et al. 2008, 2012; Serangeli et al. 2012), these have been relatively short term and not focused on wing tears. Further investigations of the effect of wing tears on flight capabilities, foraging, and survival, especially post-release, will help us to understand both the long-term effects of wing injuries and benefits of rehabilitation.

Possible causes of wing tears.—Each section of the wing is equally disposed to tearing in any orientation, despite the prevalence of many rostro-caudal tears in the P section. We suggest that it is the position of section P, rather than its anatomy, that makes it more likely to tear. Wing tears and holes can occur as a result of collisions with objects or plants with thorns (Davis 1968), fungal infections (Reichard and Kunz 2009; Cryan et al. 2010; Fuller et al. 2011), or predator attacks, including those by cats (Ancillotto et al. 2013; Loss et al. 2013; Russo and Ancillotto 2015) and birds of prey (Speakman 1991). Puncture wounds may also be caused by interspecific (Brokaw et al. 2016) or intra-specific aggression (such as in roost sites), which is likely to occur across the whole wing surface, including P, CI, and CII. Collisions are more likely to produce holes or tears on the distal wing sections (i.e., section CI), and may be oriented rostro-caudally, in the direction of flight. Tears in section P can be holes and horizontal trailing edge tears, but many are rostro-caudal, starting from the middle of the wing and extending to the trailing edge. We suggest that holes or tears in wing sections proximal to the body may be caused by predators, including cat attacks and perhaps failed talon strikes by birds like barn owls (*Tyto alba*—Speakman 1991). Indeed, the position and orientation of many tears in the plagiopatagium were consistent with the notion that predators direct their attacks toward the body of the bat, as this is the wing section that is closest to the body. Ancillotto et al. (2013) found that predation by cats accounted for 28.7% of adult bats admitted to rehabilitation centers. Identifying the causes of wing tears will help us to understand both the scale of the problem and enable us to design prevention strategies and management procedures.

Acknowledgments

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Literature Cited

Aegerter, J., D. Fouracre, and G. C. Smith. 2017. A first estimate of the structure and density of the populations of pet cats and dogs across Great Britain. PLoS ONE 12:1–21.

Ancillotto, L., M. T. Serangeli, and D. Russo. 2013. Curiosity killed the bat: domestic cats as bat predators. Mammalian Biology 78:369–373.

Brokaw, A. F., J. Clerc, and T. J. Weller. 2016. Another account of interspecific aggression involving a hoary bat (*Lasiurus cinereus*). Northwestern Naturalist 97:130–134.

Ceballos-Vasquez, A., J. R. Caldwell, and P. A. Faure. 2015. Seasonal and reproductive effects on wound healing in the flight membranes of captive big brown bats. Biology Open 4:95–103.

Cheney, J. A., J. J. Allen, and S. M. Swartz. 2017. Diversity in the organization of elastin bundles and intramembranous muscles in bat wings. Journal of Anatomy 230:510–523.

Cheney, J. A., N. Konow, A. Bearnot, and S. M. Swartz. 2015. A wrinkle in flight: the role of elastin fibres in the mechanical behaviour of bat wing membranes. Journal of the Royal Society Interface 12:20141286.

Church, J. C., and D. J. Warren. 1968. Wound healing in the web membrane of the fruit bat. The British Journal of Surgery 55:26–31.

Cryan, P. M., C. U. Meteyer, J. G. Boyles, and D. S. Blehert. 2010. Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. BMC Biology 8:135.

Daamen, W. F., et al. 2003. Preparation and evaluation of molecularly-defined collagen-elastin-glycosaminoglycan scaffolds for tissue engineering. Biomaterials 24:4001–4009.

Davis, R. 1968. Wing defects in a population of pallid bats. The American Midland Naturalist 79:388–395.

Davis, R., and S. E. Doster. 1972. Wing repair in pallid bats. Journal of Mammalogy 53:377–378.

Eberson, L. S., P. A. Sanchez, B. A. Majeed, S. Tawinwung, T. W. Secomb, and D. F. Larson. 2015. Effect of lysyl oxidase inhibition on angiotensin II-induced arterial hypertension, remodeling, and stiffness. PLoS ONE 10:e0124013.

Faure, P. A., D. E. Re, and E. L. Clare. 2009. Wound healing in the flight membranes of big brown bats. Journal of Mammalogy 90:1148–1156.

Foutz, T. L., E. A. Stone, and C. F. Abrams, Jr. 1992. Effects of freezing on mechanical properties of rat skin. American Journal of Veterinary Research 53:788–792.

Fuller, N. W., J. D. Reichard, M. L. Nabhan, S. R. Fellows, L. C. Pepin, and T. H. Kunz. 2011. Free-ranging little brown myotis

(*Myotis lucifugus*) heal from wing damage associated with white-nose syndrome. EcoHealth 8:154–162.

Fullmer, H. M., and R. D. Lillie. 1956. The staining of collagen with elastin tissue stains. Journal of Histochemistry and Cytochemistry 4:11–14.

Gardiner, J. D., G. Dimitriadis, J. R. Codd, and R. L. Nudds. 2011. A potential role for bat tail membranes in flight control. PLoS ONE 6:e18214.

Greville, L. J., A. Ceballos-Vasquez, R. Valdizón-Rodríguez, J. R. Caldwell, and P. A. Faure. 2018. Wound healing in wing membranes of the Egyptian fruit bat (*Rousettus aegyptiacus*) and big brown bat (*Eptesicus fuscus*). Journal of Mammalogy 99:974–982.

Hale, J. D., A. J. Fairbrass, T. J. Matthews, G. Davies, and J. P. Sadler. 2015. The ecological impact of city lighting scenarios: exploring gap crossing thresholds for urban bats. Global Change Biology 21:2467–2478.

Hale, J. D., A. J. Fairbrass, T. J. Matthews, and J. P. Sadler. 2012. Habitat composition and connectivity predicts bat presence and activity at foraging sites in a large UK conurbation. PLoS ONE 7:e33300.

Holbrook, K. A., and G. F. Odland. 1978. A collagen and elastic network in the wing of the bat. Journal of Anatomy 126(Pt 1):21–36.

Jung, K., and C. G. Threlfall. 2018. Trait-dependent tolerance of bats to urbanization: a global meta-analysis. Proceedings of the Royal Society B: Biological Sciences 285:20181222.

Kaye, B. 2012. The effects of freezing on the mechanical properties of bone. The Open Bone Journal 4:14–19.

Kazlouskaya, V., S. Malhotra, J. Lambe, M. H. Idriss, D. Elston, and C. Andres. 2013. The utility of elastic Verhoeff-Van Gieson staining in dermatopathology. Journal of Cutaneous Pathology 40:211–225.

Kelly, A., S. Goodwin, A. Grogan, and F. Mathews. 2008. Post-release survival of hand-reared pipistrelle bats (*Pipistrellus* spp.). Animal Welfare 17:375–382.

Kelly, A., S. Goodwin, A. Grogan, and F. Mathews. 2012. Further evidence for the post-release survival of hand-reared, orphaned bats based on radio-tracking and ring-return data. Animal Welfare 21:27–31.

Lee, L., et al. 2016. Aortic and cardiac structure and function using high-resolution echocardiography and optical coherence tomography in a mouse model of Marfan syndrome. PLoS ONE 11:1–19.

Lintott, P. R., et al. 2015. Differential responses to woodland character and landscape context by cryptic bats in urban environments. PLoS ONE 10:e0126850.

Loss, S. R., T. Will, and P. P. Marra. 2013. The impact of free-ranging domestic cats on wildlife of the United States. Nature Communications 4:1396.

Madej, J. P., et al. 2012. Skin structure and hair morphology of different body parts in the common pipistrelle (*Pipistrellus pipistrellus*). Acta Zoologica 94:478–489.

Mehr, M., R. Brandl, T. Hothorn, F. Dziock, B. Förster, and J. Müller. 2011. Land use is more important than climate for species richness and composition of bat assemblages on a regional scale. Mammalian Biology 76:451–460.

Mendes, E. S., M. J. R. Pereira, S. F. Marques, and C. Fonseca. 2014. A mosaic of opportunities? Spatio-temporal patterns of bat diversity and activity in a strongly humanized Mediterranean wetland. European Journal of Wildlife Research 60:651–664.

Miller, H. (ed.). 2016. Bat care guidelines. 2nd ed. The Bat Conservation Trust, London, United Kingdom.

Mitchell-Jones, A. J., and A. P. Mcleish. 2004. Bat workers manual. 3rd ed. Joint Nature Conservation Committee, Peterborough.

Molony, S. E., P. J. Baker, L. Garland, I. C. Cuthill, and S. Harris. 2007. Factors that can be used to predict release rates for wildlife casualties. Animal Welfare 16:361–367.

Moorhouse, T. P., M. Gelling, G. W. McLaren, R. Mian, and D. W. Macdonald. 2007. Physiological consequences of captive conditions in water voles (*Arvicola terrestris*). Journal of Zoology 271:19–26.

Neuweiler, G. 2000. The biology of bats. Oxford University Press, New York.

Pavlinić, I. G. O. R., N. I. Tvrtković, and D. R. Holcer. 2008. Morphological identification of the soprano in Croatia. Hystrix 19:47–53.

Pollock, T., C. R. Moreno, L. Sánchez, A. Ceballos-Vasquez, P. A. Faure, and E. C. Mora. 2016. Wound healing in the flight membranes of wild big brown bats. Journal of Wildlife Management 80:19–26.

Powers, L. E., J. E. Hofmann, J. Mengelkoch, and B. M. Francis. 2013. Temporal variation in bat wing damage in the absence of white-nose syndrome. Journal of Wildlife Diseases 49:946–954.

Raub, C. B., S. Mahon, N. Narula, B. J. Tromberg, M. Brenner, and S. C. George. 2010. Linking optics and mechanics in an in vivo model of airway fibrosis and epithelial injury. Journal of Biomedical Optics 15:015004.

Reichard, J. D., and T. H. Kunz. 2009. White-nose syndrome inflicts lasting injuries to the wings of little brown myotis (*Myotis lucifugus*). Acta Chiropterologica 11:457–464.

Russo, D., and L. Ancillotto. 2015. Sensitivity of bats to urbanization: a review. Mammalian Biology 80:205–212.

Santago, A. C., A. R. Kemper, C. McNally, J. L. Sparks, and S. M. Duma. 2009. Freezing affects the mechanical properties of bovine liver. Biomedical Sciences Instrumentation 45:24–29.

Santini, L., M. González-Suárez, D. Russo, A. Gonzalez-Voyer, A. von Hardenberg, and L. Ancillotto. 2019. One strategy does not fit all: determinants of urban adaptation in mammals. Ecology Letters 22:365–376.

Serangeli, M., L. Cistrone, L. Ancillotto, A. Tomassini, and D. Russo. 2012. The post-release fate of hand-reared orphaned bats: survival and habitat selection. Animal Welfare 21:9–18.

Sikes, R. S., and the Animal Care and Use Committee of the American Society of Mammalogists. 2016. 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. Journal of Mammalogy 97:663–688.

Skulborstad, A. J., S. M. Swartz, and N. C. Goulbourne. 2015. Biaxial mechanical characterization of bat wing skin. Bioinspiration & Biomimetics 10:036004.

Speakman, J. R. 1991. The impact of predation by birds on bat populations in the British Isles. Mammal Review 21:123–142.

Studier, E. H. 1972. Some physical properties of the wing membranes of bats. Journal of Mammalogy 53:623–625.

Swartz, S. M., M. D. Groves, H. D. Kim, and W. R. Walsh. 1996. Mechanical properties of bat wing membrane skin: aerodynamic and mechanical functions. Journal of Zoology, London 239:357–378.

Van Ee, C. A., A. L. Chasse, and B. S. Myers. 2000. Quantifying skeletal muscle properties in cadaveric test specimens: effects of mechanical loading, postmortem time, and freezer storage. Journal of Biomechanical Engineering 122:9–14.

Vaughan, T. A. 1970. The muscular system. Pp. 139–194 in Biology of bats. 1st ed. (W. A. Wimsatt, eds). Academic Press, New York.

Voigt, C. C. 2013. Bat flight with bad wings: is flight metabolism affected by damaged wings? Journal of Experimental Biology 216:1516–1521.

Wang, D., W. Ma, Y. Niu, X. Chang, and Z. Wen. 2007. Effects of cyclic freezing and thawing on mechanical properties of Qinghai–Tibet clay. Cold Regions Science and Technology 48:34–43.

Weaver, K. N., S. E. Alfano, A. R. Kronquist, and D. M. Reeder. 2009. Healing rates of wing punch wounds in free-ranging little brown myotis (*Myotis lucifugus*). Acta Chiropterologica 11:220–223.

Wheeler, J. B., R. Mukherjee, R. E. Stroud, J. A. Jones, and J. S. Ikonomidis. 2015. Relation of murine thoracic aortic structural and cellular changes with aging to passive and active mechanical properties. Journal of the American Heart Association 4:e001744.

Woods, M., R. A. McDonald, and S. Harris. 2003. Predation of wildlife by domestic cats *Felis catus* in Great Britain. Mammal Review 33:174–188.

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Associate Editor was John Scheibe.

Appendix 2: The cards in the bat pack, A) Bat wing swabbing instructions, B) Questionnaire, C) Bat wing photographing instructions, D) Gridded card (1cm).

A) Wing swabbing instructions

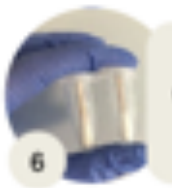
1 With gloved hands, open the packet of one of the swabs & remove using the wooden handle.

2 Place the tip of the swab into the tube containing water (labelled W). **Ensure all water is absorbed.**

3 Gently roll & rotate the swab over the surface of the bat wing at the site of the tear, applying even & moderate pressure, for approximately 10 seconds.

4 Place swab into tube (W) and use fingers to break the stick of the swab, short enough so the lid can be closed securely.

5 Remove second swab from the packet. **This swab should remain dry**. Repeat swabbing procedure in the same location.



6 Once finished, insert swab into the second tube (D), snap stick & shut. Both tubes are now ready to put in the bubble-bag and posted in the pre-paid envelope.

B) Questionnaire

Thank you for your participation!

Please answer these questions and send this card back to us with the swabbing samples, using the pre-paid envelope provided.

1- Your initials .....

Please put your initials on the sample tubes, and on the graph sheet when you take a photo of the bat wing.

2- The bat species that you swabbed the tear on the wing is :

❑ Common pipistrelle ❑ Soprano pipistrelle ❑(other).....

3- The sex of the bat that you swabbed the tear on the wing is :

❑ Male ❑ female

4- The age of the bat that you swabbed the tear on the wing is :

❑ Adult ❑ Juvenile

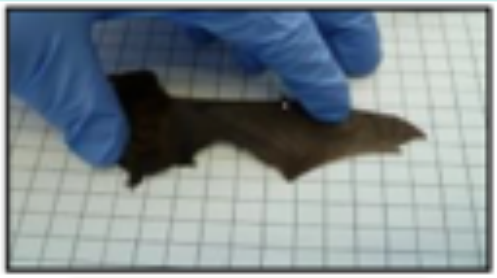
5- If you are in the northwest, would it be possible to visit to film the bat in flight?

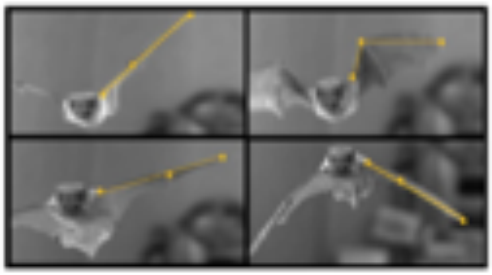
❑ Yes ❑No

If yes, please can you provide your e-mail address?

.....

C) *Photographing instructions*





Spread the bat wing directly on to the squared paper provided and take a picture. Please send a picture of both the torn and healthy wing (if available) **Please send the picture to the email provided below.**

As part of our study, we are also looking to video bat flight to check their healing. **If you do not mind us visiting to film your bat, please let us know.**

- To send us your picture of the bat wings, please email to: batresearch@mmu.ac.uk
- If you are happy for us to come and film the bats then please let us know.
- Visit us at www.bat-research-mmu.weebly.com to hear our latest findings
- Many thanks again for your help and support.

D)

Your initials: _____

Appendix 3: All data from all bat samples received from bat carers. Bat species: C.P.= Common pipistrelle, S.P.=Soprano pipistrelle, Nt= Natterer's bat, Wh= Whiskered, BLE= Brown Long-eared, Se= Serotin. Gender: F= Female, M= Male. Age: A= Adult, J=

Juvenile. Location: Ch=Cheshire, De=Devon, Do= Dorset, K= Kent, GM= Greater Manchester. Wing tear type: H= hole, C= Contained tear, T= Total tear, E=Trailing edge tear. *Wing sections*, P=plagiopatagium, CII= chiropatagium I, and CI= chiropatagium II. Fungal DNA, Cat DNA: P=Presence, A=Absence. Fungus species: AS.= *Aspergillus spp.*, AU. = *Aureobasidium spp*., CL.= *Cladosporium spp*., CO.= *Coniochaeta spp*., DA.=*Daldinia spp*., DE. = *Debaryomyces spp.,* FU. = *Fusarium spp*., BE. = *Penicillium spp.*, SE.= *Septoria spp.,* SY.= *Sydowia spp*., TA. = *Talaromyces spp*., TR.= *Trichoderma spp*.. Blank= No information available

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
1	10-05	C.P.						1E	1T						
2	2015 0614	C.P.						1C	1H						
3	2015 0615	C.P.							1H						
4	2015 0616	C.P.							3H						
5	IMG-2565	C.P.							1T						
6	P1oo o648	C.P.					2H 1C	1H 1C	1H						
7	Tiesto LHa	C.P.							1H						

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
8	TSElR H100 415	C.P.						1H							
9	Gail Armst rong	C.P.							1C						
10	B	C.P.							1T						
11	F	C.P.					4H	7H 1E	1H						
12	F2	C.P.						1H 1E	1H						
13	G	C.P.						1C							
14	H	C.P.							1H						
15	I	C.P.							1H						
16	FB95 17	C.P.			Bat was caught by a cat		1H	1E	1E						
17	FB27 17	C.P.						1co	1H						

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
18	FB11 717	C.P.						1E	1T						
19	FB31 717	C.P.			Badly torn right wing, a couple of broken fingers. No body puncture but really do suspect a cat has had him.				1T						
20	0308 17-A	C.P.							1H						
21	0308 17-C	C.P.							1T						
22	FB11 817	C.P.			cat was just looking at it.		1H	1C	1E						
23	FB17 817	C.P.					1E	1E							
24	FB17 817	C.P.			bats you trapped in the wild				1E						
25	W7	C.P.						2T	2C						
26	W8	C.P.						1E	1H 1E						

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
27	W 10	C.P.					1H	1H	1H						
28	FB14 917	C.P.		J					1E						
29	1509 17-J	C.P.							1H 1E	14 days in care					
30	1509 17-M	C.P.							1H	2 months in care					
31	1509 17-L	C.P.					1H	1H		79 days ion care					
32	1009 17-O	C.P.					2E			Euthanised					
33	1909 17-P	C.P.					1E		1H	Euthanised					
34	1010 17-T	C.P.						1H	1H						
35	1010 17-U	C.P.					1H								
36	1010 17-W	C.P.							1H						

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
37	Jessica Dangerfield	Nt					1H	1C	2H						
38	AF	Wh						1C	1E						
39	FB11717	BLE			Trapped in Flypaper				1C						
40	GQB	C.P.	M	A	Bat bought in by a cat				1H 1C		1	1.9	1.59		A
41	SN	C.P.									2	2.4	1.87		P
42	HR 0	C.P.		A		K					3	1.2	1.09		A
43	AC	S.P.	M	A							4	1.8	1.13		P
44	S.P 1	Wh	M	A		De	1C	1H 1C	1C		5	0.9	7.11		P
45	S.P 2	Nt	M	A		De			1T		6	1.6	1.32		P
46	S.P 3	Wh	M	A		De			1T		7	-0.2	0.14		A

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
47	JD+HS	S.P.	M	A							8	2	1.6		A
48	AG	S.P.	F	A							9	1	1.5		A
49	HR 4	C.P.	M	A		K					10	0.7	6		A
50	HR 5	C.P.	M	A		K					11	0.5	0.7		P
51	HR 1	C.P.	M	A		K					12	-0.6	0.5		A
52	HR 2	C.P.	F	J		K					13	-0.9	0.51		P
53	HR 3	C.P.	M	A		K					14	0	-0.1		A
54	VB		F	A					1E		15	-1.6	0.9		A
55	HR 6	C.P.	F	A		K					16	0	-0.3	P CL.	A
56	HR 7	BLE	M	A		K					17	1	2.6	P	A

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
57	HR 8	C.P.	M	A		K					18	1.4	1.6	A	A
58	RJ	C.P.	M	A	The bat died				1T		19	0.9	1.5	P TA.	A
59	HR 9	C.P.	M	A		K					20	1.1	2.1	P CL.	A
60	HR 10	C.P.	M	A		K					21	0.7	1.3	P TR.	P
61	S.P 4	C.P.	M	A		De					22	-0.7	1.24	P PE.	P
62	SAH10/17	Se	M	A	Bat die after found	Do	1H	1H	1H	Bat was euthanised	23	0.2	0.65	P CL.	P
63	SAH12/17	C.P.	M	A		Do		2H			24	-1.4	0.89	P PE.	P
64	SAH13/17	S.P.	F	A		Do		1H	1H	Bat was euthanised	25	0.4	1.66	A	P
65	KH	C.P.	F	A			5H	1H			26	-1.6	1.12	P AU.	P
66	LB	C.P.							1T		27	0.6	1.95	P AU.	P

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
67	SAH15/17	S.P.	M	A	Householder has 2 cats, bat with older cat attack scars in wings. On this occasion he was bitten left neck/ear and right shoulder. Signs of cat'	Do			1H	Bat died after 14 days despite antibiotics and pain relief.	28	0.4	0.4		P
68	SAH18/17	C.P.	F	A	Householder has 2 cats - cat attack	Do		1H	1H	13 days in care	29	0.1	0.71		P
69	SAH19/17	C.P.	M	A	Householder has 2 cats - cat attack	Do			1H	13 days in care. It flew for a minimum of 20 minutes as par the Protocol at East Do Bat Rescue and Rehabilitation	30	-0.6	2.11		P
70	SAH20/17	S.P.	M	A	Owner of cat witnessed bat in cat's mouth	Do	1C	1H 1C		Died on day of admittance due to flystrike	31	0.1	0.15		P
71	KH+SH	C.P.	M	A					1C 1E		32	-1.1	1.39	P SE.	P
72	SAH21/17	S.P.	F	A		Do		1H	2H	79 days in care. Released back at location found successfully	33	-1.2	1	P SY.	P
73	SAH24/17	S.P.	M	A	seen brought into home in cat's mouth'	Do			3C	32 days in care. Released successfully.	34	-1	0.8	A	P

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
74	SAH2 5/17	S.P.	M	A	Seen with cat circling around on ground	Do	1H 1C		2H 1C	35 days in care. Released successfully	35	0.3	20. 9	A	P
75	SAH2 7/17	C.P.	M	A		Do		1E	1T	Bat was euthanised	36	-0.7	2	P TR.	P
76	SAH2 8/17	C.P.	F	A		Do		1C 1T	2C 1T	Bat was euthanised	37	0.5	1.5	P -	P
77	SAH2 9/17	C.P.	M	A	Found grounded by dog. Injuries appear to be consistent with cat'	Do	4H 2C	4H	5H	48 days in care. Returned successfully to the wild	38	-0.1	6.5	P CL.	P
78	SAH3 0/17	Nt	F	A		Do	1H	1H		died within 24 hrs of admittance	39	0.5	-1.5	P AU.	P
79	SAH3 1/17	S.P.	F	A	Householder has known roost in attic and owns 3 'well behaved' cats'	Do			1H 1C	44 days in care. Released successfully	40	1.1	4.6		P
80	LV	C.P.	M	A							41	1.3	3.5	P AU.	P
81	HR 13	C.P.	M	A		K					42	1.1	3.1	P AS.	P

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
82	JE 1	C.P.	F	A							43	1.2	3.3		P
83	JE 2	C.P.	M	A							44	1.8	2.3		P
84	SAH3 3/17	S.P.	F	A	Property owner has 3 cats which catch birds often including woodpeckers'	Do		1C	1C	euthanised on receipt as bone too badly damaged.	45	0.5	31. 4		P
85	SAH3 4/17	S.P.	M	J	cat at the finder address	Do			1H	died later on day of admittance.	46	0.4	3.1		P
86	SAH4 3/17	C.P	M	A		Do		2H 1C	1H		47	2.1	3.6		P
87	SAH5 0/17	S.P.	F	A		Do			1C	Still in care (more than 6 months).	48	1	4.0		P
88	SAH5 1/17	C.P.	M	A	Bad head wound as well as claw holes in wing	Do			1H	10 days in care. having reached pre-release protocols at EDBRR	49	2.4	2.9		P
89	HR 11	S.P.	F	A		K					50	1.7	1.9		P
90	SAH5 2/17	S.P.	M	A		Do			1H	died on admittance	51	1.1	3.1		A

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
91	0709 17-E	C.P.	F	A		Ch	1H	2H	1H 1C		52	0.8	-2.4		A
92	0709 17-F	C.P.	/	A		Ch			1H		53	-1.1	1.4		A
93	SAH5 6/17	S.P.	F	A	seen in cat's mouth	Do	1H	1H 1C	1H	29 days in care. flew off strongly having maintained flight for 20 minutes, able to fly up from ground and catch insects as par our release protocols for EDBRR.	54	-1.5	0.9		P
94	HR 12	C.P.	M	A		K					55	0.5	-1.4	P CL.	A
95	AF	Wh	F	A							56	-1.5	0.8		P
96	HR 14	S.P.	M	A		K					57	0.1	-0.2		A
97	AJM	C.P.	F	A	Almost certainly cat damage'			3H	1H 1C 1E	Still in care (more than 6 months).	58	1.2	3.8		P
98	SP 5	C.P.	M	A		De					59	2.1	1.7	P DA.	P

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
99	SAH6 5/17	BLE	F	A		Do			1T	Still in care (more than 6 months).	60	1.3	3.7		A
100	KL16/ 01	C.P.	/	A		GM	2H	2H 1C	1C		61	1.4	2	P CO.	P
101	SP16/ 13	C.P.	/	A		GM			1T		62	3.2	2.4		P
102	LE	C.P.	M	A							63	1.3	1.7		P
103	LE-2	C.P.	M	At			1H	2H 1C	2H 1E		64	1.6	3.0	P DE.	P
104	S.T	BLE	M	A			2H				65	-0.8	0.6		P
105	SAH 14/18	Wh	F	A		Do					66	-1	0.7		A
106	LE 3	C.P.	F	A							67	2.3	4.5		P
107	HR 15	C.P.	M	A		K					68	1.7	6.5	P CL.	P

NO	Bat carers initials	Bat species	Gender	Age	Additional Comment	Location	Wing tear type at wing sections			Rehabilitation outcome	PCR NO.	DNA concentration	DNA purity	Fungus DNA- species	Cat DNA
							CI	CII	P						
108					bat found in garden in East Tilbury. Wound on body, broken shoulder, tears in wings.						69	2.2	2.3	P FU.	P
109	1/33 1RF										70	1.9	2.2 8		A
110	JM	BLE	M	A							71	-0.8	0.4		A
111	HR 16	S.P.	M	A		K					72	-0.6	0.4		A

Appendix 4: The peer reviewed abstract:

Khayat, R. O., Shaw, K. J., L. M., and Grant, R. A. (2018) The Effect of Wing Tears on the Flight Behaviour of Common Pipistrelles Bats *(Pipistrellus pipistrellus)*. Abstract, Measuring Behaviour conference, Manchester, UK.

The Effect Of Wing Tears On The Flight Behaviour Of Common Pipistrelle Bats *(Pipistrellus Pipistrellus)*

Bats are in the order Chiroptera and are the only mammals capable of flight, due to them having large flapping wings [1]. However, these wings are also thin, which causes them to be very prone to tearing [2]. This study developed a method to investigate the effect of these tears on the flight behaviour on common pipistrelle Bats (*Pipistrellus pipistrellus*). Bats, both injured and healthy, were filmed using a high-speed camera (phantom camera, MIRO_M110) and the video recordings were used to track bat wing movements [Figure 1] using the Manual Whisker Annotator software (MWA) [3]. The data gathered from the MWA software included the following variables for both the left and right wings: maximum angle, minimum angle, mean angle, amplitude-RMS (Root Mean Square Amplitude) and frequency, as well as the body orientation. Tears significantly affected wing movement during flight, however the body orientation was affected even more. Future methods to measure the effect of wing tears will focus on measuring aspects of the body, rather than tracking the wing movements.

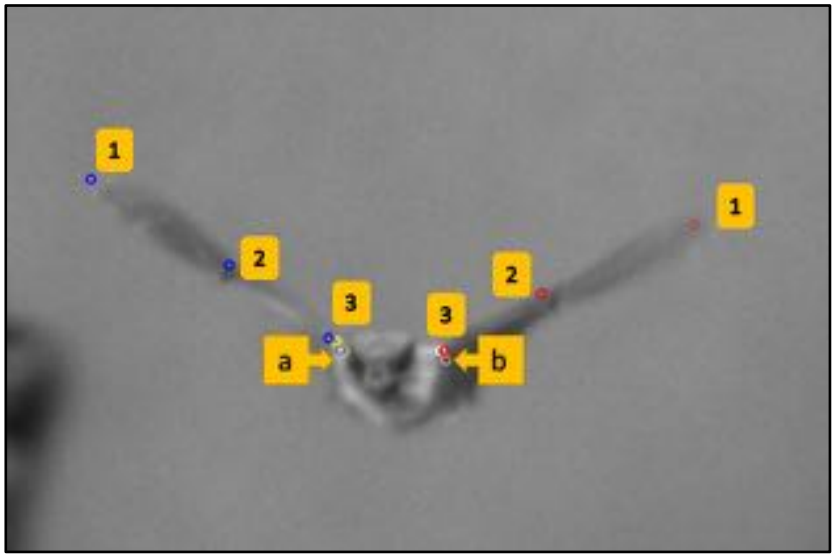
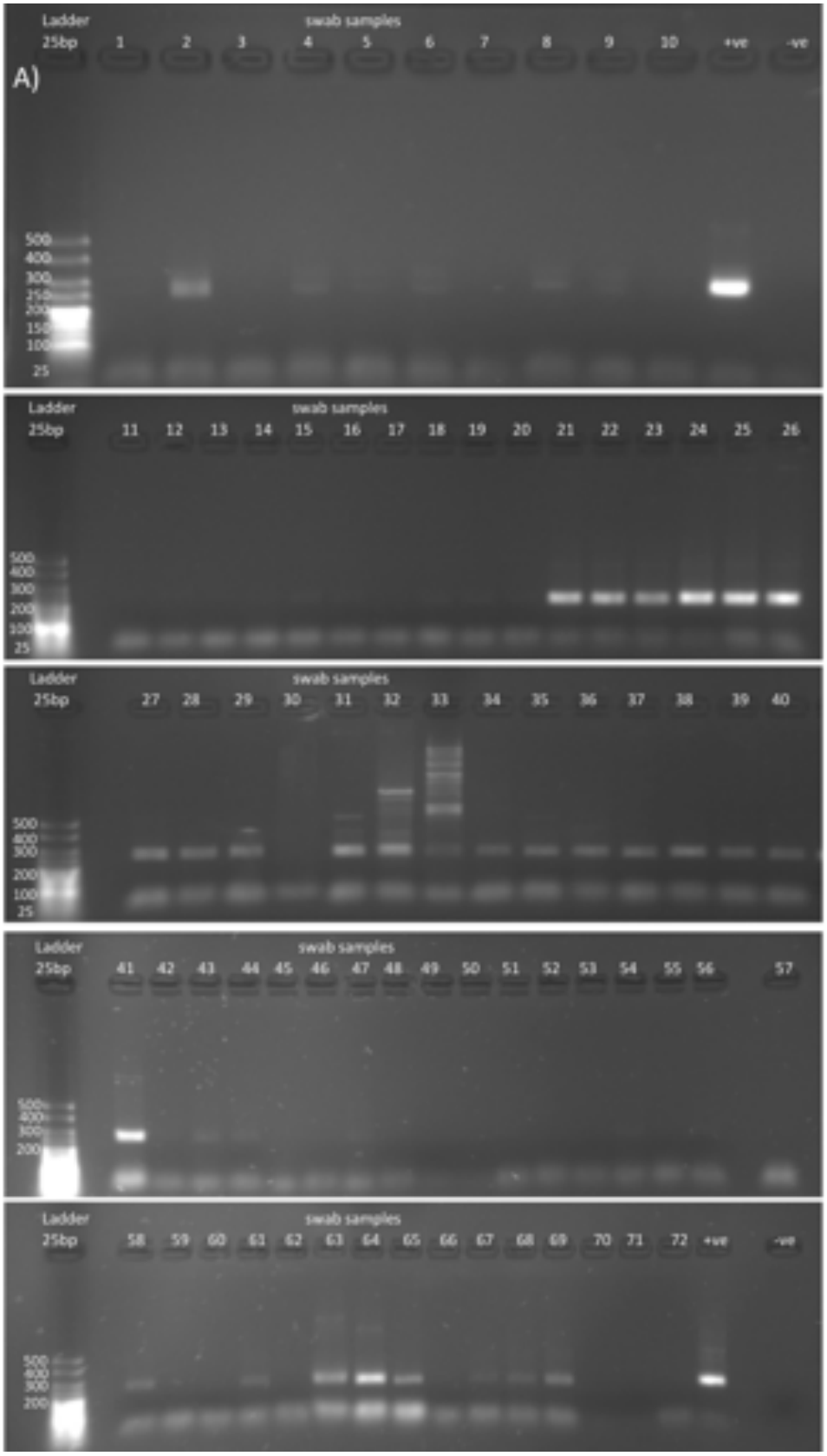


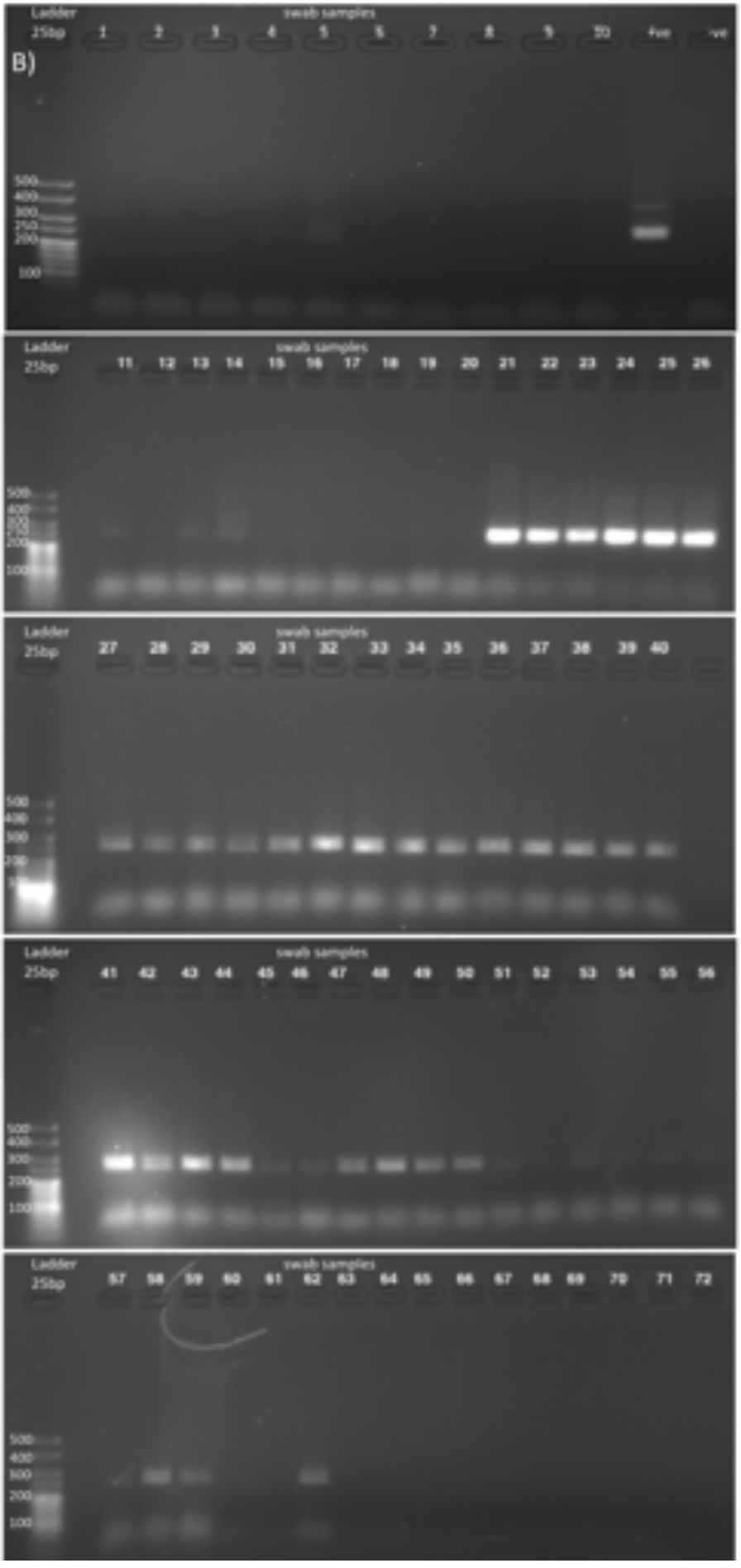
Figure1. The tracking points for the bat movements. The two points at the middle of the bat (a and b) are for the body orientation. The three points on each wing are for tracking the wing movement.

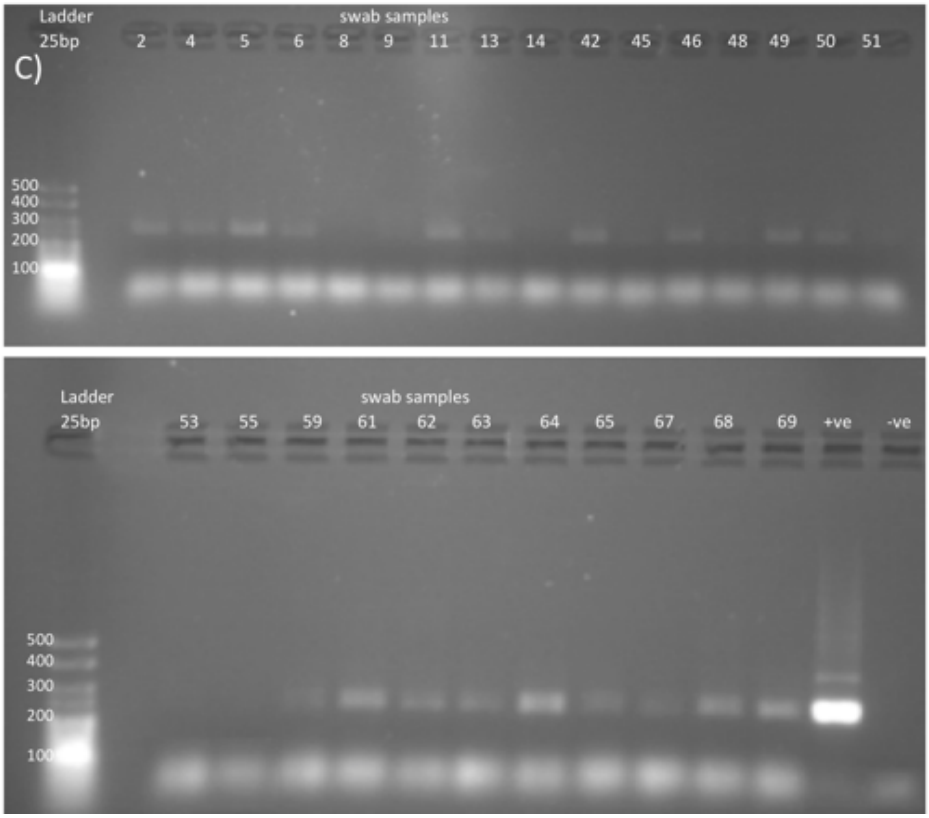
REFERENCES

1. Voigt, C., Borrisov, I. and Voigt-Heucke, S. (2012) 'Terrestrial locomotion imposes high metabolic requirements on bats'. *Journal of Experimental Biology*, 215(24) pp.4340-4344.
2. Ceballos-Vasquez, A., Caldwell, J. and Faure, P. (2014). 'Seasonal and reproductive effects on wound healing in the flight membranes of captive big brown bats'. *Biology Open*, 4(1), pp.95-103.
3. Hewitt, B., Yap, M. and Grant, R. (2016). 'Manual Whisker Annotator (MWA): A Modular Open-Source Tool'. *Journal of Open Research Software,* 4 (16), p.p. 1-7.

Appendix 5: Gel photos of running the qPCR products on the gel for all swab samples, A) first run, B) second run, and C) third run (for the samples that showed different results on the first and second run




B)
Ladder
25bp
swab samples
1 2 3 4 5 6 7 8 9 10 +ve -ve
500
400
300
250
200
100
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 40
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72


C)
Ladder
25bp
swab samples
2 4 5 6 8 9 11 13 14 42 45 46 48 49 50 51
500
400
300
200
100
Ladder
25bp
swab samples
53 55 59 61 62 63 64 65 67 68 69 +ve -ve
500
400
300
200
100