

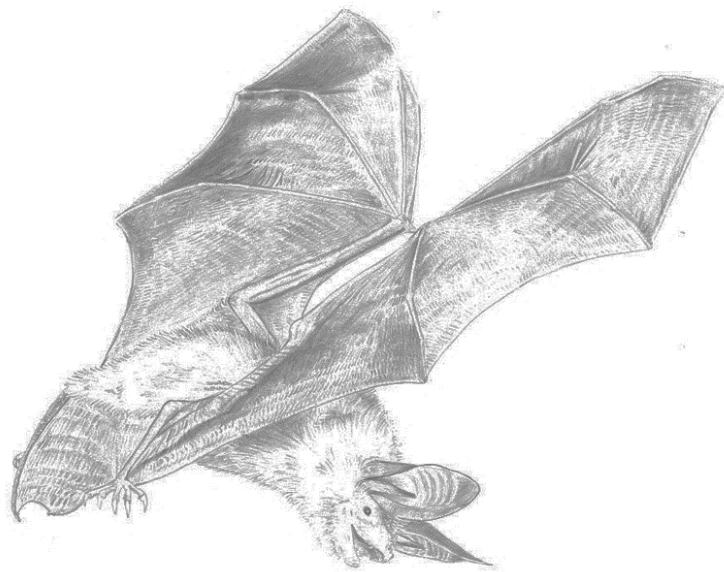
**Monitoring population size, structure and change in
Bechstein's bat (*Myotis bechsteinii*): combined approaches
using molecular and landscape ecology**

Submitted by Patrick Wright to the University of Exeter

as a thesis for the degree of

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Abstract

The Bechstein's bat, *Myotis bechsteinii*, is known as one of Britain's most elusive mammals. Critical information on the species is lacking, hindering evidence-based conservation and management in a human-dominated landscape. In this thesis, I used a combination of molecular and landscape approaches to assess the genetic health and population genetic structure of *M. bechsteinii* and understand how the British landscape affects the species habitat and its connectivity. I also aimed to develop new molecular tools, such as non-invasive genetic sampling and molecular ageing, which could then be used to better monitor the species.

Data from nuclear markers (microsatellites) showed high levels of genetic diversity and little inbreeding across the species range, though genetic diversity was slightly lower in Britain than in mainland Europe. Bayesian and spatial Principal Components (sPCA) analysis showed a clear separation between British and European populations. This analysis also revealed that in Europe the Italian population south of the Alps was found to constitute a different group from other sites. In Britain, there was genetic structuring between the northern and southern part of the species range. Despite there being little genetic divergence in mitochondrial DNA (mtDNA) sequences throughout most of Europe, the mtDNA patterns in Britain confirmed this separation of northern and southern populations. Such genetic structuring within Britain — in the absence of any obvious physical barriers — suggested that other features such as land-use may limit gene-flow. To better understand how the species interacts with

the British landscape, I used a landscape genetic approach, habitat suitability modelling using presence-only data and a landscape connectivity analysis. The negative association of *M. bechsteinii* presence with distance from woodland was identified as the main variable determining habitat suitability, while the landscape genetics results highlighted the importance of woodlands for gene flow. *M. bechsteinii* habitat was highly fragmented and only showed good connectivity if the species was able to disperse over 5,000 m. These results subsequently highlight the importance of woodlands not only for providing suitable habitat, but also in maintaining genetic connectivity between populations.

Then, I investigated the use of non-invasive capture-mark-recapture (CMR) and demographic history models to estimate the population size and changes of *M. bechsteinii*. Bat droppings were collected below roosting sites of a single colony. After species identification, the 123 droppings belonging to *M. bechsteinii* were genotyped at nine DNA microsatellite loci in order to differentiate all individuals. All microsatellites showed very low amplification rates indicating low quality samples. However, at a larger scale, the use of population demographic models to assess effective population size variation using a dataset of 260 bats of the British population gave an estimate of the effective population size of 6,569 (CI: 5,307-8,006) and suggested that the British population of *Myotis bechsteinii* is stable and possibly expanding. Finally, I developed an epigenetic assay to estimate the age of individual bats. For this, I measured DNA methylation on bats of known age at seven CpG sites from three genes. All CpG sites from the tested genes showed a significant relationship between DNA methylation and age and provided reliable age estimates.

The findings presented in this thesis show that despite exhibiting high levels of genetic diversity throughout its range, the genetic structure, habitat and connectivity of *M. bechsteinii* populations is highly influenced by woodlands. It also offers a novel method to monitor the species by developing an assay which can provide information on the age structure of an entire colony from a single sampling session. Such approaches are much needed in the field of conservation and could in the future help preserve a wider range of species.

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All the field work undertaken over the past three years would not have been possible without the help of some amazing volunteers! I would first like to thank the Wiltshire, Surrey, Sussex and Dorset Bat Groups, DBCRG, Herefordshire Mammal Group and the Wiltshire, Dorset and Worcestershire Wildlife Trusts. A few volunteers often went the extra mile to help me with my research. Keith Cohen, Gareth Harris, Johnny Birks, Lis Weidt, Nick Tomlinson, James Shipman, Daniel Whitby, Adrian Bailey, Ian Davidson-Watts and Danielle Linton I am particularly grateful for all your help.

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À Justine

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All illustrations at the start of each chapter and on the thesis title page were made by Anita Long and have been used with her permission.

Chapter 1 Introduction



1.1 Introduction to bats

The order Chiroptera is the second most diverse of all mammals after Rodentia. It comprises 20% of all classified mammals and a total of approximately 1,300 species (Dietz & Kiefer, 2016; Dietz, Nill, & von Helversen, 2009). The first bats evolved after the mass extinction of dinosaurs approximately 65 million years ago (Simmons, Seymour, Habersetzer, & Gunnell, 2008). Their ability to fly, which is unique in mammals, has allowed them to establish themselves in most continents and habitats with the exception of Antarctica. Bats have also evolved a unique ability among land mammals to use echolocation as a tool for orientation (Griffin, 1944). Both adaptations allowed them to take advantage of a volant nocturnal lifestyle and utilise a vast diversity of ecological niches. This diversity is reflected by a wide variety of roosting, feeding, reproductive strategies and social behaviours (Simmons & Conway, 2003). Their diets, for example, are hugely varied and although 70% of bat species are insectivorous, others are frugivorous, nectarivorous, piscivorous or hematophagous (Dietz & Kiefer, 2016). They have adapted to roosting in tree crevices, buildings, leaves or buildings and will perform a wide range of migration and dispersal strategies (Moussy *et al.*, 2013). Many species will also provide important ecosystem services, such as plant pollination, seed dispersal or pest control (Dietz *et al.*, 2009). However, bats are particularly hard to study as they are small, nocturnal and highly vagile, (O'Shea & Bogan, 2003; Puechmaille & Petit, 2007). Despite their abundance and important role in nature, very limited information is available for most species.

1.2 Threats to bats

Dramatic declines of bat populations were reported during the 20th century in Europe. These declines were mainly observed on colonies of building-dwelling bats (Mitchell-Jones, Cooke, Boyd, & Stebbings, 1989) and at winter hibernation sites where thousands of horseshoe, greater mouse-eared bats and other species were rapidly reduced to a few individuals (Dietz *et al.*, 2009) (Figure 1.1). These declines, as for many other species, are all associated with increased human pressures. Indeed, human populations have increased from 2.5 billion in 1950 to 7.3 billion in 2015 in 70 years and are predicted to reach 11.2 billion by 2050 (UN, 2015).

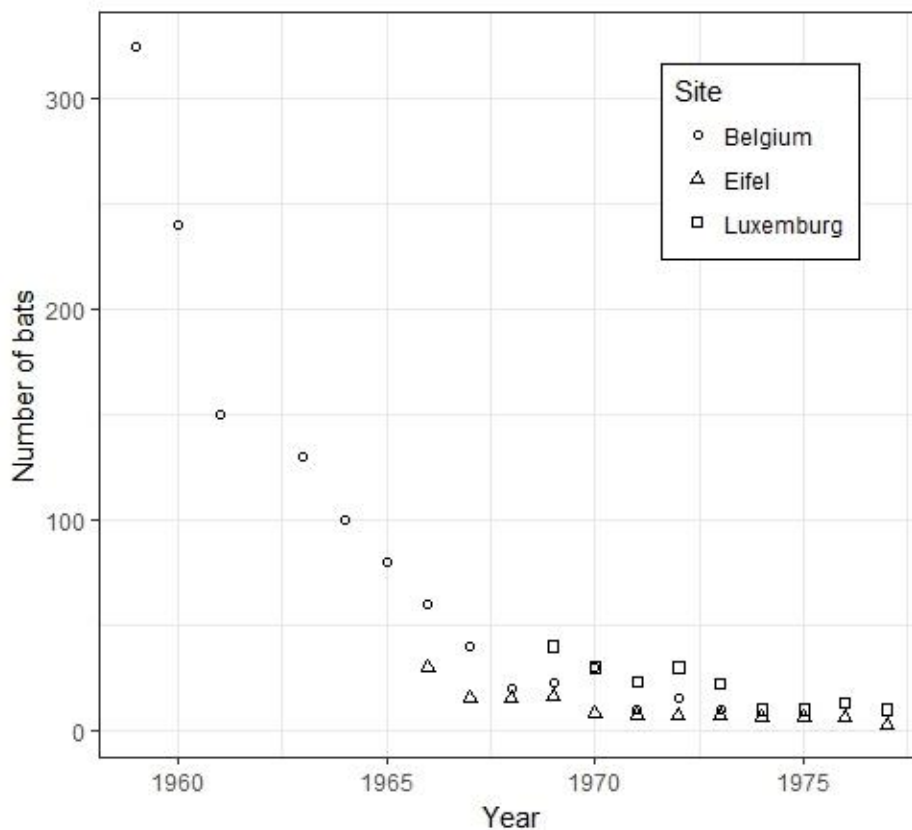


Figure 1.1 Reported decline of *Rhinolophus hipposideros* from sites in Europe from 1959 to 1977 as described by the journal *Myotis* (volume 15) (data reported from Dietz *et al.*, 2009)

The exponential growth of human populations has had a profound effect on land use and subsequently on its biodiversity. For example, habitat fragmentation represents the transformation of large expanses of habitat, which can be rich in biodiversity, into smaller, disconnected patches of habitat (Turner, Gardner, & O'Neill, 2001; Wilcove, McLellan, & Dobson, 1986). This process can impact wildlife as it results in the loss and isolation of suitable habitats (Fahrig, 2003). Its effect will depend on the ability of each species to survive and move through a fragmented landscape (With & Crist, 1995). Fragmentation can sometimes have a positive impact by increasing functional connectivity or habitat diversity (Fahrig, 2017). However, the combination of multiple deterministic (e.g. habitat fragmentation, poaching) and stochastic (e.g. genetic and environmental stochasticity) processes in a human dominated landscape is more likely to impact populations in a negative way (Lande, Engen, & Saether, 2003; O'Grady *et al.*, 2006).

In Britain, habitat fragmentation is mainly associated with increased urbanisation and changes in agricultural practices (Chaverri & Kunz, 2011; Mickleburgh, Hutson, & Racey, 2002) and natural habitats, such as woodlands and heathlands, only cover minimal areas of land cover (Cordingley, Newton, Rose, Clarke, & Bullock, 2015; Watts, 2006). Recent changes in agricultural practices from mixed farming to intensive farming with widespread monocultures have not only affected prey availability by using more pesticides, but also the amount and connectivity of suitable habitats (loss of hedgerows and removal of field boundaries) for many bat and bird species (Hayhow *et al.*, 2016; Stoate *et al.*, 2001). Habitat fragmentation is also strongly associated with roads, as these can result in direct collisions or the disturbance of flight paths

and reduced hunting success by artificial lighting or noise disturbance (Berthinussen & Altringham, 2012; Lesiński, 2007; McClure, Ware, Carlisle, Kaltenecker, & Barber, 2013; Stone, Jones, & Harris, 2009).

The mid-20th century also saw a significant increase in the use of organic pesticides such as dichlorodiphenyltrichloroethane (DDT) and pentachlorophenol (PCP), in agriculture, forestry and timberwork which led to a decline in prey availability and the poisoning of many colonies (Luckens & Davis, 1964). In addition, the destruction and increased disturbance of roosts or hibernation sites in heavily managed woodlands, modernised buildings or caves has also been reported to affect populations (e.g. Tuttle, 1979). Whilst many of the most directly harmful pesticides have now been banned, and legislation is in place across Europe to protect roosting locations in buildings, new pressures, such as persecution (Mickleburgh, Waylen, & Racey, 2009), climate change (Razgour *et al.*, 2017), the increasing use of wind turbines (Kunz *et al.*, 2007) and white-nose syndrome (Blehert *et al.*, 2009), have been identified as important threats likely to affect bat populations at a local and global scale.

1.3 Bat conservation & monitoring

As a direct consequence of these major declines all bat roosts are protected under European Union legislation (Habitats Directive 1992, Annex IV) and for some species, key commuting and foraging areas are also protected (Habitats Directive 1992, Annex II).

The legal protection of bats has allowed certain populations to recover by implementing varied conservation programmes and interventions. For example, the protection of summer roosts and winter hibernation sites has often been

followed by significant increases in population size in horseshoe and greater mouse-eared bats (Schofield, 2008). Along with this, the increase in landscape connectivity between foraging habitats can also have positive effects on species as these result in an increase in habitat and prey availability (Frey-Ehrenbold, Bontadina, Arlettaz, & Obrist, 2013; Verboom & Spoelstra, 1999; Wickramasinghe, Harris, Jones, & Vaughan, 2003). The legal protection of bats in Europe means that the impact of any type of disturbance on bats, such as the destruction of roosts by new developments, has to be minimised (Collins, 2016). Although the implementation of mitigation measures are often criticised as their effectiveness is rarely assessed, such protection has allowed bats to persist in areas where they would have once disappeared.

The current legal status of bats also recognises the importance of monitoring populations in order to have a better understanding of population trends and changes in distribution over time (Battersby, 2010). Bat populations can be monitored by counting individuals emerging from roosts or in hibernacula (O'Shea & Bogan, 2003) and by performing acoustic surveys in order to determine the presence of certain species and quantify their activity (e.g. Fuentes-Montemayor, Goulson, Cavin, Wallace, & Park, 2013). Although valuable long term data is still collected using these methods, monitoring is very much restricted to common species that are easily identified by their echolocation calls.

In woodlands, bats emit quieter echolocation calls which reduces detection rate and they are also very hard to differentiate for the case of *Myotis* species (Russ, 1999). Such limitations often justify the need to capture and examine bats by using more invasive methods, such as mist-netting and harp-trapping, to better

inform the species composition and populations of an area. However, these methods are time consuming and can also be influenced by differences in capture rates (Berry, O'connor, Holderied, & Jones, 2004) and avoidance behaviour (Larsen *et al.*, 2007). As a result most woodland species in Europe, such as *Myotis bechsteinii* or *Myotis alcathoe*, remain under-recorded and poorly understood. The lack of information on these species can subsequently lead to poor wildlife management practices have a negative impact on populations.

1.4 Future challenges in bat conservation

There is a pressing need to implement effective conservation and mitigation measures as urbanisation and human pressures continue to expand and impact wildlife. Today, maintaining habitat connectivity has become a key component of conservation as it can be applied at a local or grand scale and potentially mitigate the impact of climate change and major developments (Bennie, Davies, Inger, & Gaston, 2014; Frey-Ehrenbold *et al.*, 2013; Razgour, 2015). New research must also be targeted on how anthropogenic stresses can be minimised on bats. A better usage of street lighting (Mathews *et al.*, 2015) or detecting conditions where they are most at risk on wind farms (Richardson, Lintott, Hosken, & Mathews, in press) may all potentially have a positive impact on bats. The implementation of such measures is dependent on reliable data collection and information on local population sizes. Errors and limitations in these key stages can in certain cases lead to important casualty rates (Lintott, Richardson, Hosken, Fensome, & Fiona, 2016) or the impossibility to infer the impact of such casualties on local populations. The importance of reliable data for effective conservation means that most research is currently targeted on

common species or Annex II species which are easily monitored. Lesser and greater horseshoe bats, for example, are both listed under Annex II and show strong roost fidelity and can easily be identified through their echolocation calls (e.g. Schofield, 2008). However, woodland species are often neglected as they exhibit more complex roosting behaviours by regularly switching roosts and splitting colonies (Dietz & Pir, 2011). As mentioned in Section 1.3, *Myotis* species can remain undetected because rare and common species cannot be differentiated by their echolocation calls as these are very similar (Russ, 1999; Schnitzler & Kalko, 2001). Subsequently, novel techniques are needed in order to detect population trends and assess the efficacy of conservation management for many species of concern, such as woodland bats.

1.5 Using molecular techniques for conservation

1.5.1 Conservation genetics

The field of conservation genetics deploys an array of molecular genetic tools with the aim to better understand and protect wildlife. These techniques can be used to detect hybridization, resolving taxonomic uncertainties and, most importantly, to assess the genetic health of rare species. The fitness and viability of populations along with their ability to adapt to environmental changes is strongly influenced by their genetic diversity and structure (Frankham, Briscoe, & Ballou, 2002). This is most commonly assessed by using mitochondrial DNA, microsatellites and Single Nucleotide Polymorphisms (SNPs). Genetic diversity represents the amount of variation, while the genetic structure is representative of the distribution of such variation in populations (Balkenhol, Cushman, Storfer, & Waits, 2016). A lack of genetic diversity in a population is often illustrated by individuals with high levels of homozygosity,

which can then affect reproduction, survival (inbreeding depression) and the ability for populations to evolve and adapt to changes within their environment. Both genetic diversity and structure are found to be increasingly impacted by a number of anthropogenic actions, such as habitat fragmentation (Gerlach & Musolf, 2000), poaching (Harley, Baumgarten, Cunningham, & O'RYAN, 2005), climate change (Razgour, 2015) and the introduction of invasive species (McDevitt *et al.*, 2009). While the extinction of wild populations are driven by a combination of deterministic (e.g. habitat destruction, poaching) and stochastic processes (e.g. genetic and environmental stochasticity), the impact of genetic processes, such as inbreeding depression, on extinction have been shown to strongly increase the extinction risk of mammalian and avian taxa (O'Grady *et al.*, 2006). In isolated populations, levels of inbreeding may be so high that most conservation actions are unable to prevent population declines. In these extreme circumstances, genetic rescue, which consists in the introduction of new alleles into a population by introducing individuals from a different population, can provide an effective way to increase population fitness (Whiteley, Fitzpatrick, Funk, & Tallmon, 2015b). For example, the translocation of panthers (*Puma concolor*) from Texas into the small inbred population of Florida panthers occasioned a 4% annual growth rate as opposed to a 5% population decline before translocation (Benson *et al.*, 2011; Johnson *et al.*, 2010; Pimm, Dollar, & Bass, 2006).

1.5.2 Non-invasive genetic sampling

Accurate information on the presence and population size of rare and elusive species is essential for effective wildlife management. Such estimates can be made by counting animals directly or by using more invasive techniques (e.g.

trapping), but these methods can be particularly invasive, time consuming and expensive when focusing on elusive species.

Advances in molecular techniques through the use of PCR allowed the use of new non-invasive sample types: hairs, faeces, urine, feathers, saliva, eggshells, and sloughed skins were found to contain sufficient DNA to allow the amplification of mitochondrial and nuclear loci (Morin & Woodruff, 1996). The approach of non-invasive genetic sampling has been used for monitoring wildlife by using capture-mark-recapture analysis (CMR). These techniques provide insight on species distribution, densities and sex ratios of species, such as mountain goats (Poole, Reynolds, Mowat, & Paetkau, 2011), chimpanzees (Arandjelovic *et al.*, 2011), caribous (Hettinga *et al.*, 2012) or brown bears (Kindberg *et al.*, 2011). Although many of these estimates have proven to be trustworthy, difficulties in collecting samples, combined with the high risk of DNA degradation and contamination in non-invasive methods, can result in low amplification success rates and genotyping errors (Herrmann & Henke, 1999; Lampa, Henle, Klenke, Hoehn, & Gruber, 2013; Taberlet & Luikart, 1999). These in turn can produce over- (Creel *et al.*, 2003) or underestimations (Mills, Citta, Lair, Schwartz, & Tallmon, 2000) of population size.

1.5.3 Landscape genetics

Threats, such as habitat loss and fragmentation, reduces the connectivity and distribution of key resources and environmental conditions (Fischer & Lindenmayer, 2007). This can also affect movement and gene flow of populations and further impact their genetic fitness (Balkenhol *et al.*, 2016). Although major barriers affecting gene flow can sometimes be associated to obvious landscape features, such as mountains and oceans (McLeod, Burns,

Frasier, & Broders, 2015; Moussy *et al.*, 2015), the structure and composition of most landscapes will affect population processes in a more complex way. Therefore, rigorous methods of analysis are needed in order to quantify the effect of such interactions on wildlife.

The field of landscape genetics was first introduced by Manel, Schwartz, Luikart, and Taberlet (2003) and defined as “*research combining population genetics, landscape ecology and spatial analytical techniques to quantify the effects of landscape composition on evolutionary processes*” (Balkenhol *et al.*, 2016). Studies have answered various questions regarding the effect of climate and landscape change on species (Wasserman, Cushman, Shirk, Landguth, & Littell, 2012), the spread of diseases or invasive species (Lecis, Ferrando, Ruiz-Olmo, Manas, & Domingo-Roura, 2008) or the comparison of genetic patterns between contemporary and historic landscapes (Spear & Storfer, 2010; Storfer, Murphy, Spear, Holderegger, & Waits, 2010). The combined use of mapping tools, such as resistance surfaces which represent the cost of movement through the landscape (Figure 1.2) and Circuitscape a software used to calculate all possible pathways connect multiple points (McRae & Nürnberger, 2006), has allowed visualisation of areas of important connectivity between populations, which can then be used to better target areas of conservation importance (e.g. Razgour, 2015; Ruiz-Lopez *et al.*, 2016).



Figure 1.2 Example raster representing a landscape with three different habitat types (white, grey and dark grey) and the resistance values for each of these habitats. High resistance values represent habitats that are difficult to traverse as opposed to low values for which movement is easy.

Although the field of landscape genetics has grown exponentially since the early 2000s (Manel & Holderegger, 2013), limitations are still observed when calculating resistance surfaces. Many studies rely in some way on expert opinion, this dependence can be misleading for elusive species as these can describe biased anecdotal sightings rather than actual biological processes (Balkenhol *et al.*, 2016). Therefore, there is a pressing need to use methods that move away from expert opinion (Peterman, 2018).

1.5.4 Molecular ageing

Information on the age of individuals in wildlife populations is critical for the study of population structure (Dunshea *et al.*, 2011; Oli & Dobson, 2003). Such data can provide information to predict the impact of habitat, climate change or hunting pressure on population viability estimates (Botsford, Holland, Samhouri, White, & Hastings, 2011; Sand *et al.*, 2012; Tella, Rojas, Carrete, & Hiraldo, 2013). However marking, for example through the use of tags or rings, is often

time-consuming and can have negative impacts on animal welfare (Nelson, 2002). Recaptures throughout the lifetime of the animals are required to gather the required information, making the process time-consuming and difficult to execute for rare species (Brunet-Rossini, Wilkinson, Kunz, & Parsons, 2009). Non-invasive approaches such as the unique identification of individuals via markings (e.g. cetaceans, tigers) (Mizroch, Beard, & Lynde, 1990; Speed, Meekan, & Bradshaw, 2007) are suitable only for a limited number of species.

Molecular tools for producing reliable age estimates have received little attention. Most research has focused on understanding the biological process of ageing rather than on the development of routinely applicable techniques to estimate chronological age. Telomere length has been observed to decline over successive mitotic cycles for many vertebrate species (Benetti, García-Cao, & Blasco, 2007; Greider, 1996). This observation highlighted the importance of telomeres in the ageing process of vertebrates and the possibility to use them as age markers (Hausmann & Vleck, 2002). However, despite promising results with birds, where several lifespan stages have been detected on most individuals (Hausmann & Vleck, 2002; Hausmann *et al.*, 2003), studies on other vertebrates have concluded that there is no relationship between telomere length and age suggesting the method cannot be used for age estimation as attrition rate and telomere length varies at birth (Aviv *et al.*, 2009; Bize, Criscuolo, Metcalfe, Nasir, & Monaghan, 2009; Dunshea *et al.*, 2011; Hall *et al.*, 2004; Reichert *et al.*, 2014).

More recently, DNA methylation on CpG sites of certain genes appears to be strongly linked with biological ageing process. The presence or absence of methyl groups at the C5 position of cytosines followed by guanines ('CpG sites')

has an important role in the control of gene expression as they can affect transcription factor binding sites, insulator elements, and chromatin conformation (Jones, 2012). DNA methylation can differ extensively across tissues (e.g. Byun *et al.*, 2009); and while levels of DNA methylation at promoter regions have been found to have a positive effect on the expression of certain genes they largely tend to be negatively associated with gene expression (Gutierrez-Arcelus *et al.*, 2013; Lam *et al.*, 2012). On the other hand, DNA methylation in the gene body is more likely to increase gene expression (Jones, Goodman, & Kobor, 2015; Lister *et al.*, 2009). It has also been found that certain CpG sites within the genome will increase or decrease levels of methylation with age (Horvath, 2013). Therefore, the measure of DNA hypo- and hypermethylation of specific sites has been used as an age predictor on humans (*Homo sapiens*) (Christensen *et al.*, 2009b; Grönniger *et al.*, 2010; Horvath, 2013) and mice (*Mus musculus*) (Maegawa *et al.*, 2010; Stubbs *et al.*, 2017). Polanowski, Robbins, Chandler, and Jarman (2014) developed an age assay for humpback whales (*Megaptera novaengliae*) by measuring DNA methylation from three CpG sites from different genes. Their results were far more accurate than previously developed techniques using telomere length (Dennis, 2006; Olsen, Bérubé, Robbins, & Palsbøll, 2012; Olsen, Robbins, Bérubé, Rew, & Palsbøll, 2014).

1.6 The Bechstein's bat (*Myotis bechsteinii*)

1.6.1 Status and distribution

The Bechstein's bat (*Myotis bechsteinii*) is a non-migratory species widespread throughout central and western Europe; ranging from the Iberian Peninsula, to

southern Scandinavia and to the Caucasus (Dietz & Pir, 2011; Vergari, Dondini, & Ruggieri, 1998).

It is considered rare throughout its range and is now listed as Near Threatened (IUCN, 2015). The species is also protected in the European Union and listed on Annex II of the European Council Habitats and Species Directive (Council Directive 92/43/EEC 21/5/1992).

1.6.2 *Ecology and behaviour*

1.6.2.1 Roosting ecology

The roosting of *Myotis bechsteinii* (and other species) offers a safe place to rear young, rest, digest and socially interact with other members of the colony (Kunz, Lumsden, Kunz, & Fenton, 2003; Napal, Garin, Goiti, Salsamendi, & Aihartza, 2009; Vonhof & Barclay, 1996). Maternity roosts are predominantly found in deciduous semi-natural or ancient woodlands with a diversity of tree ages and vegetative structure (Dietz & Pir, 2011; Napal *et al.*, 2009; Schofield & Morris, 2000). In Britain, studies to date suggest that these woodlands are often greater than 25 hectares and have a high proportion of oak in the canopy mix with a well-developed understorey (Schofield & Morris, 2000). However, it is not uncommon to find them roosting in small woodlands, hedgerows or isolated trees in areas where broadleaved woodland is sparse (Damant & Cohen, 2016). *M. bechsteinii* colonies tend to roost principally in woodpecker-made cavities or natural cavities in old deciduous trees (Dietz & Pir, 2011; Napal *et al.*, 2009). Such crevices are typically dome-shaped and tend to provide stable microclimatic conditions (Dietz & Pir, 2011; Kerth, Weissmann, & König, 2001). Colonies tend to switch roosting sites after a few days and can use up to 60 tree roosts over a breeding period (Dietz & Pir, 2011). Regular switching of roosting sites is thought to reduce parasite load and provide more favourable

microclimatic conditions (Kerth *et al.*, 2001; Reckardt & Kerth, 2007). Over summer, colonies perform fission-fusion behaviour where the colony frequently splits into subgroups which occupy different roosts (Kerth & König, 1999). As opposed to females, male *M. bechsteinii* tend to roost solitarily or in small groups inside crevices for periods of up to 2 weeks and are more likely to be found in suboptimal feeding sites, due to minimal energy demands (Dietz & Pir, 2011; Schofield & Morris, 2000).

M. bechsteinii swarms in late summer near potential hibernacula, such as caves, but also possibly at other sites, such as woodland glades (Kerth, Kiefer, Trappmann, & Weishaar, 2003). Such behaviour in bats is thought to have an important role in the learning of hibernacula locations and may also be part of mating behaviours and subsequently facilitate gene flow (Furmankiewicz & Altringham, 2007; Kerth *et al.*, 2003; Rivers, Butlin, & Altringham, 2005).

1.6.2.2 Foraging ecology

A number of radio-tracking studies indicate that *M. bechsteinii* are short-distance foragers, rarely flying more than 1,500 m to feeding areas (Dietz & Pir, 2011; Schofield & Morris, 2000). These distances can be as short as 300 m depending on woodland. However, smaller forest fragments tend to result in longer distances between roosting sites and core foraging areas (Kerth, Mayer, & Petit, 2002). During post-lactation periods, *M. bechsteinii* can also be found foraging longer distances from their roosting sites in orchards, meadows and barns (Dietz & Pir, 2011). Each breeding female will usually have its own foraging areas of approximately 2-3 hectares, whilst making repeated returns to preferred trees (Bayerl, 2004; Brinkmann, Niermann, & Steck, 2007; Schofield & Morris, 2000). Although certain colonies have been found foraging in sparse woodland cover, coniferous woodlands and open fields (Damant & Cohen,

2016), optimal foraging habitat, in Britain, tends to be associated with mature broadleaf woodland with a well-developed understorey near water courses (Schofield & Morris, 2000).

1.6.2.3 Dietary ecology

Bechstein's bats are agile fliers and have long ears which allow them to focus on prey through passive acoustic detection. This lets them exploit foraging areas efficiently by catching prey on the wing, from foliage and from the ground (Dietz & Pir, 2011). Dietary studies on *M. bechsteinii* indicate that the species feeds mainly on *Lepidoptera* and *Diptera*, but also on a broad spectrum of other invertebrates, such as *Orthoptera*, *Coleoptera*, *Dermaptera* and *Araneae* (Dietz, 2013; Poulton, 2008).

1.6.3 *History of Myotis bechsteinii in Britain*

In Britain, post-glacial fossil deposits suggests that the species was once more widespread and common, but forest clearance during the Neolithic phase, and changes in climatic conditions, may have resulted in population declines across its range (Yalden, 1999). The presence of *Myotis bechsteinii* was highly under-recorded in Britain as only 84 records from 14 sites were available before 1989 and the majority of these were from underground sites (Miller, 2011; Stebbings, 1989). It was not until the late 1990s that the first maternity colonies were found (Schofield & Morris, 2000; Stebbings, 1989) (Figure 1.3).

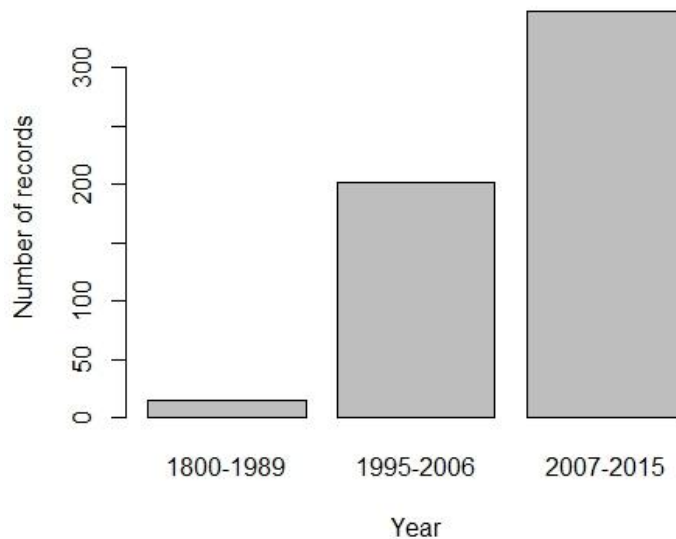


Figure 1.3 Bar plot summarising the total number of *M. bechsteinii* records in Britain from 1800 to 2015 using records from Stebbings (1989) and the records used in Chapter 3.

The development of acoustic lures proved to be an effective technique for identifying the presence of the species in different woodlands (Hill & Greenaway, 2005). These methods were successfully used to determine the species distribution more accurately across Britain for the Bat Conservation Trust (BCT) Bechstein’s Bat Survey from 2007 to 2011 (Miller, 2011). Its core range in Britain appears to be centred in southern England from Gloucestershire to Sussex, but it has recently been found breeding as far north as Herefordshire and Worcestershire. There are also recent records from Kent in the east to Devon in the west (Schofield & Morris, 2009) (Figure 1.4).



Figure 1.4 Distribution of *M. bechsteinii* across Britain (Couzens, Swash, Still, & Dunn, 2017)

The sedentary lifestyle of this species along with its dependence on ancient woodlands makes it particularly vulnerable to changes in its environment and previous studies suggest that the British population may be more inbred than in mainland Europe (Durrant, Beebee, Greenaway, & Hill, 2009). The high legal protection of Bechstein's bats in the UK and in the EU makes it essential to quantify population size and structure, detect changes and protect key habitats. However, conventional approaches cannot achieve these requirements as the species echolocation calls are hard to differentiate from other *Myotis* species and roost count along with trapping are both particularly time consuming.

1.7 Thesis aims

The exponential growth of the human population continues to have a profound effect on land use and on its biodiversity. The high legal status of bats in Europe entails the need to better monitor populations. However, in the case of elusive species, like *M. bechsteinii*, conventional approaches for monitoring cannot be applied. The aim of this thesis was to use genetic tools to assess the genetic health and structure of *M. bechsteinii* and better understand the threats that affect connectivity of different populations in the landscape at a national scale. I also aim to develop new molecular tools, such as non-invasive genetic sampling and molecular ageing, which could then be used to better monitor the species throughout its range.

Chapter 2: Genetic structure and diversity of a rare woodland bat, *Myotis bechsteinii*: comparison of continental Europe and Britain

In this study, I used 14 microsatellites and a 747 bp fragment of the cytochrome b gene (mtDNA) to investigate the genetic diversity and structure of *Myotis bechsteinii* in Britain in comparison with the Continental population. I hypothesised that the British population would show significant differentiation from the Continental population. I also predicted that the British population would show lower levels of genetic diversity and more structure than their European counterparts given that this population is an edge of range population isolated from the rest of Europe where suitable habitat is sparser.

Chapter 3: Assessing habitat quality and connectivity of *Myotis bechsteinii* in Britain through the use of habitat suitability modelling and landscape genetics

In Chapter 3, I hypothesised that woodlands play a key role for the habitat of *M. bechsteinii* but also in maintaining connectivity. For this, I first aimed to identify key variables affecting the distribution of *M. bechsteinii* for both males and females using presence records. Then, I used measures of connectivity to assess the levels of connectivity of suitable *M. bechsteinii* habitat. Finally, I measured the effect of landscape features on genetic connectivity.

Chapter 4: Testing the use of non-invasive genetic sampling and demographic history analysis to monitor woodland bat populations

The aim of Chapter 4 was to evaluate the use of non-invasive genetic sampling techniques to estimate the number of individual bats within a colony of *M. bechsteinii* and to assess the demographic history of the species at a local (colony) and national scale. For this I collected droppings from underneath bat boxes over the course of the months of June 2015 and June 2016 and genotyped them at nine microsatellite loci. Then, I used a demographic history analysis to investigate variations in population size over time.

Chapter 5: Molecular ageing by measuring DNA methylation: a new tool for bat conservation

The aim of Chapter 5 was to develop an epigenetic age-estimate assay for *M. bechsteinii* by measuring levels of DNA methylation. In this work, I used a population of ringed bats ranging from 0 to 14 years old from a single *M.*

bechsteinii colony and measured levels of DNA methylation at seven CpG sites from three genes.

Chapter 6: General Discussion

Finally, in Chapter 6, I summarise the main findings of the thesis and assess their implications for the conservation of *M. bechsteinii* and other woodland species. I further discuss the need for improved monitoring of such species and new techniques that may be implemented in the future.

Chapter 2 Genetic structure and diversity of a rare woodland bat, *Myotis bechsteinii*: comparison of continental Europe and Britain



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2.1 Abstract

The Bechstein's bat (*Myotis bechsteinii*) is a rare sedentary bat considered to be highly reliant on the presence of ancient woodland. Understanding the genetic connectivity and population structure of such elusive mammals is important for assessing their conservation status. In this study, I report the genetic diversity and structure of *Myotis bechsteinii* across Britain and Europe assessed using 14 microsatellites and a 747 bp region of mitochondrial cytochrome b gene. Nuclear DNA (microsatellites) showed high levels of genetic diversity and little inbreeding across the species range and genetic diversity was slightly lower in Britain than in mainland Europe. Bayesian and spatial PCA analysis showed a clear separation between the British and European sites. Within Europe, the Italian population south of the Alps was isolated from the other sites. In Britain, there was genetic structuring between the northern and southern part of the species range. Despite there being little genetic divergence in mitochondrial DNA (mtDNA) sequences throughout most of Europe, the mtDNA patterns in Britain confirmed this separation of northern and southern populations. Such genetic structuring within Britain in the absence of any obvious physical barriers suggests that other factors such as land-use may limit gene-flow.

2.2 Introduction

The fitness and viability of populations along with their ability to adapt to environmental change is strongly influenced by genetic diversity (Reed & Frankham, 2003). In Europe, urbanisation and industrial agriculture have significantly increased during the 20th century leading to further habitat loss and fragmentation (Baker & Harris, 2007). These practices have resulted in a sharp decline of mammal, bird, amphibian and invertebrate populations across a diverse array of habitats (e.g. Marzluff, 2001; Potts *et al.*, 2010) and have contributed towards the loss of genetic diversity and increased differentiation in protected species, such as *Lutra lutra* (Stanton, Hobbs, Chadwick, Slater, & Bruford, 2009), *Rhinolophus ferrumequinum* (Rossiter, Jones, Ransome, & Barratt, 2000) and *Bombus sylvarum* (Ellis, Knight, Darvill, & Goulson, 2006).

Despite their abundance and important role in ecosystems, very limited information is available on the biology and conservation status of most bat species. Bats, being small, nocturnal and highly vagile, are particularly hard to study directly (O'Shea & Bogan, 2003; Puechmaille, Mathy, & Petit, 2007).

However, the use of molecular techniques has provided valuable information on a number of species by contributing towards a better understanding of mating behaviour (Bryja, Kaňuch, Fornůsková, Bartonička, & Řehák, 2009), social behaviour (Kerth, Mayer, & König, 2000) and other aspects of the biology of a number of species (e.g. the spread of diseases in Moussy *et al.*, 2015). The high mobility of bats in comparison to other terrestrial mammals along with the expression of behaviours such as migration (Ahlén, Baagøe, & Bach, 2009; Bryja *et al.*, 2009) and autumnal swarming (Glover & Altringham, 2008; Kerth *et al.*, 2003; McDevitt *et al.*, 2009; Moussy *et al.*, 2013; Parsons & Jones, 2003),

has allowed them to maintain high levels of gene flow and little genetic differentiation throughout their range despite environmental pressures and changes in land management (Ibáñez, García-Mudarra, Ruedi, Stadelmann, & Juste, 2006). The consequence of these pressures is, however, more likely to be apparent in Britain than mainland Europe since populations on highly inhabited islands are more susceptible to genetic drift and inbreeding which can subsequently lead to extinction (Frankham, 1997).

The Bechstein's bat (*Myotis bechsteinii*) is a woodland specialist, widespread throughout central and western Europe; ranging from the Iberian Peninsula, to southern Scandinavia and the Caucasus (Dietz & Pir, 2011; Vergari *et al.*, 1998). Its current distribution however is highly insular, and is influenced by the presence of old growth oak and beech woodland. In Britain, post-glacial fossil deposits suggest that it was once more widespread, but forest clearance during the Neolithic phase might have restricted their distribution to southern Britain (Yalden, 1992; Yalden, 1999). The species roosts predominantly in tree holes, particularly those made by woodpeckers, and whilst males are solitary, females breed in maternity groups which are thought to be closed. Foraging usually occurs within 3 km of the day roost, suggesting that the species is highly sedentary (Dietz & Pir, 2011). *M. bechsteinii* is considered Near Threatened by the IUCN and "in need of strict protection" by the European Habitats Directive (92/43/CEE). Autumn swarming plays a key role in maintaining low genetic differentiation in *M. bechsteinii* (Kerth *et al.*, 2003), but the isolation of essential mating sites may have resulted in a loss of genetic diversity (Durrant *et al.*, 2009). Despite the importance of understanding interactions between the British and Continental populations of *M. bechsteinii* as climate change is expected to

impact the distribution of many species (Razgour *et al.*, 2017; Rebelo, Tarroso, & Jones, 2010), current knowledge is mostly restricted to continental studies (e.g. Kerth *et al.*, 2008) whilst information on the British population remains limited to coastal populations (Durrant *et al.*, 2009).

In this study, I used 14 microsatellites and a 747 bp fragment of the cytochrome b gene (mtDNA) to investigate the genetic diversity and structure of *Myotis bechsteinii* in Britain in comparison with the Continental population. I

hypothesised that the British population would show significant differentiation from the Continental population. I also predicted that the British population would show lower levels of genetic diversity and more structure than their European counterparts given that this population is an edge of range population isolated from the rest of Europe where suitable habitat is sparser.

2.3 Materials and methods

2.3.1 Sample collection

A total of 328 individuals from 15 sites were included in this study. All British sites were unique maternity colonies with the exception of the Bernwood population which comprised three maternity colonies within a 3 km radius, and all samples from the Isle of Wight were grouped into one population (Figure 2.1). Samples in Britain were collected during woodland surveys and samples from continental Europe were collected at swarming sites. As *M. bechsteinii* rarely travel over 30 km to reach swarming sites (Dekeukeleire, Janssen, Haarsma, Bosch, & Schaik, 2016), it was judged that at a continental scale, where study sites are separated by over 100 km, swarming sites would be representative of the sampled area since individuals are likely to originate from

a nearby population. A wing tissue sample was taken from each bat using a 3mm biopsy punch (Stiefel Laboratories, Wooburn Green, UK) and stored in absolute ethanol at -4°C until DNA extraction. In addition, 9 bat droppings were also directly collected from individual bats to provide additional information on the Bernwood population. All bats were described (sex, age and other biometric measurements) and released within a few minutes of the procedure at their location of capture. Sampling was conducted under licence from the Home Office (ref no PPL 3003431) and Natural England (2016-24055-SCI-SCI) and was approved by the Ethics Committee of the College of Life Sciences, University of Exeter.

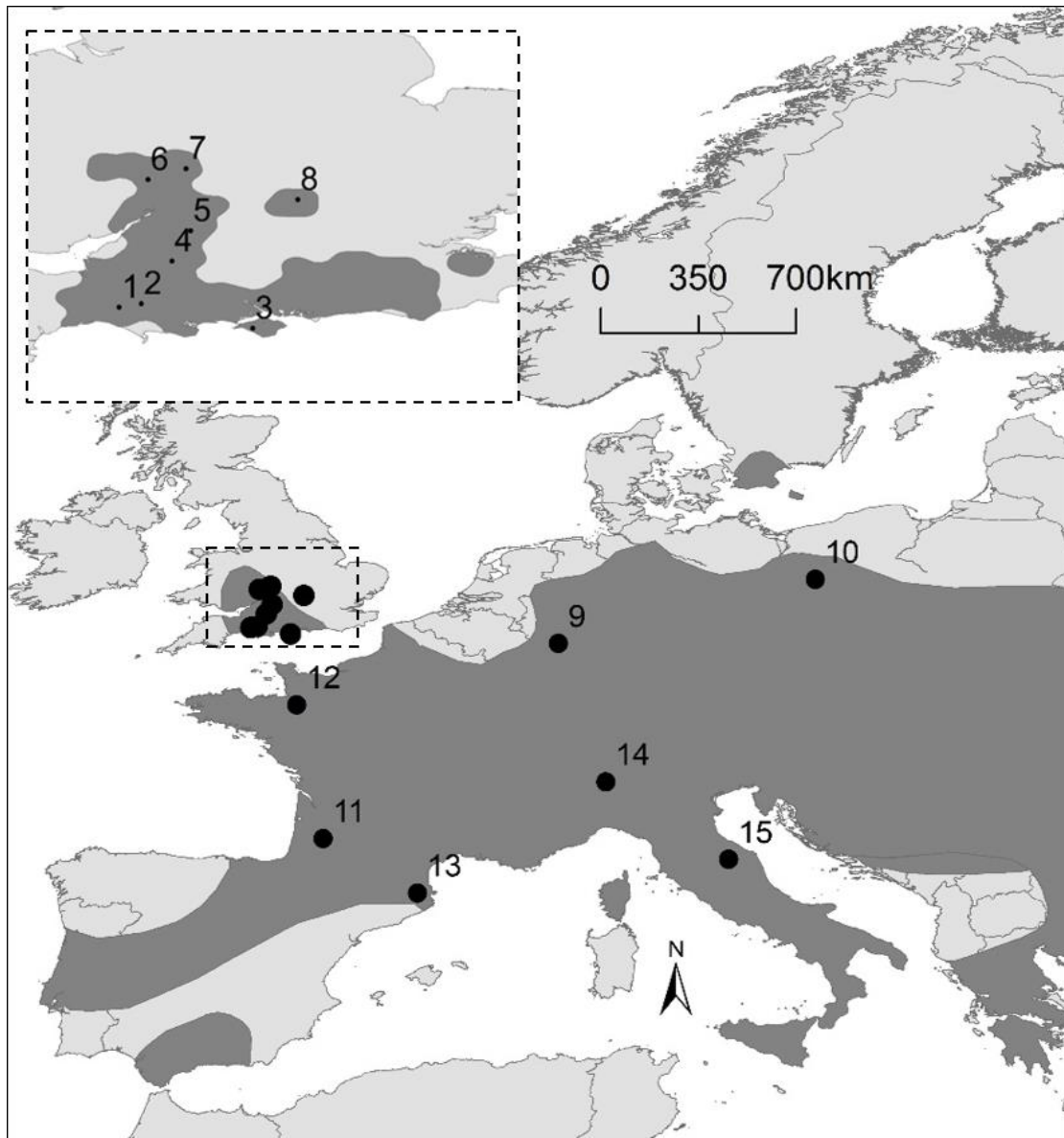


Figure 2.1 Distribution map of *Myotis bechsteinii* in Britain and Europe (Couzens *et al.*, 2017; Paunović, 2016) with location of sampled sites. See Table 2.1 for details on population identity.

2.3.2 DNA extraction, PCR and Microsatellite genotyping

DNA was extracted from each wing biopsy with DNeasy blood & tissue kits (QIAGEN). Samples were then incubated twice for 5 minutes in 80 μ l and 40 μ l of buffer AE to form a final solution of 120 μ l. DNA extraction from bat droppings was performed using a QIAmp DNA Stool Mini Kit (QIAGEN) following the protocol recommended by Puechmaille *et al.* (2007). PCR was carried out in 10

µl volume reactions containing 5 µl of Type-it Microsatellite PCR Kit (QIAGEN), 2 µl of RNase free water, 1 µl of extracted DNA and 2 µl of primer mixture.

A set of 14 microsatellite loci primarily designed for the study of other bat species were selected for genotyping *M. bechsteinii* (Appendix 2.2). A touchdown extra-long PCR program was used, consisting of 5 min at 95 C followed by 35 cycles of 30 s at 95°C, 90 s at the annealing temperature [62°C (3 cycles), 58 (4), 55 (5), 53 (10), 51 (5), 49 (5), 47 (5)] and 3 min at 72°C, then 72°C and 60°C for 10 and 35 min respectively followed by a final 10 min extension step at 72°C. Genotyping was performed on a Beckman Coulter CEQ™ 8000 Genetic Analysis System.

2.3.3 Mitochondrial sequencing

A portion of 860 bp of the mtdna cytochrome b gene was amplified in a subset of samples using primers Molcit-F (5'-AATGACATGAAAAATCACCGTTGT-3', (Ibáñez *et al.*, 2006) and MVZ-16 (5'-AAATAGGAARTATCAYTCTGGTTTRAT-3', (Smith & Patton, 1993). PCR was carried out in 20 µl with 10 µl Type-it Microsatellite PCR Kit, 1.5 µM of each primer, and 1 µl of DNA. The remaining volume was made up with RNase-Free water. PCR conditions employed a “touchdown” PCR program consisting of 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at the annealing temperature [62°C (3 cycles), 58°C (4), 55 (5), 53 (10), 51 (5), 49 (5), 47 (5)] and 1 min at 72°C, then 72°C and 60°C for 10 and 35 min respectively. PCR reactions were then sent for clean-up and sequenced from both directions at SourceBioscience (UK) (<https://www.sourcebioscience.com/>). The resulting sequences were assembled, aligned and trimmed into DNAbaser (DNA Sequence Assembler v4, 2013) to create a 747 bp consensus sequence for each individual. A total of five

M. bechsteinii cytochrome b sequences obtained from Genbank (Accession KF218378.1, AF376843.1, DQ120901.1, DQ120900.1, DQ120899.1) were also included in our analysis. Accession numbers from this study are MF182931 - MF183112.

2.3.4 Data quality

For DNA microsatellite data, the presence of scoring inconsistencies, null alleles, large allele dropout and stuttering was tested in MICROCHECKER (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004). Exact tests for departure from Hardy-Weinberg (HW) equilibrium were performed for each roost and each locus in GENEPOP 4.1.4 (Rousset, 2008) under the hypothesis of heterozygote deficit. A sequential Bonferonni correction was applied to the p-values to account for multiple tests (Rice, 1989). Tests of linkage disequilibrium were performed and the significance levels were adjusted by sequential Bonferonni correction.

2.3.5 Genetic diversity

Indices of genetic diversity, such as observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficients (F_{IS}), were calculated using GENALEX 6.5 (Peakall & Smouse, 2012). Allelic richness (A_r) was calculated using the rarefaction method with the software HP-Rare as this method takes into account differences in sample size (Kalinowski, 2005). A Monte Carlo test of 999 permutations was performed to compare differences in expected heterozygosity (H_e) between Britain and mainland Europe Bechstein's bats with the R package ADEGENET (Jombart, 2008).

2.3.6 Genetic structure and isolation by distance

Isolation by distance (IBD) was tested by Mantel test between a matrix of genetic distances (Edward's distance) and a matrix of Euclidean geographic distances. These tests were performed on the full dataset and separately on the continental and British dataset with the R package ADEGENET and Kernel density estimates were applied to the correlation plots (Jombart, 2008).

Genetic differentiation was first examined by calculating the global and pair-wise F_{st} in genalex 6.5 (Peakall & Smouse, 2012) where the significance of estimates was based on 999 permutations of the dataset. Then, to investigate genetic structure, the Bayesian clustering program STRUCTURE v 2.3.3 (Pritchard, Stephens, & Donnelly, 2000) was used with a burn-in period of 50 000 iterations followed by 150 000 iterations with the number of inferred populations (K) ranging from 1 to 12. I performed ten independent runs of the program using the population admixture model and correlated allele frequencies using the LOCPRIOR option. I determined the most likely number of population clusters using the ΔK statistic (Evanno, Regnaut, & Goudet, 2005). The clusters identified were then run separately in order to identify finer levels of structure.

The R package ADEGENET (Jombart, 2008) was used to further investigate spatial genetic patterns at all sites by performing a spatial principal component analysis (sPCA). This method incorporates spatial autocorrelation (Moran's I) to more commonly used PCAs in multivariate analysis. As opposed to STRUCTURE and other Bayesian approaches, sPCAs measure the contribution of all alleles within genotypes and does not assume that populations are in Hardy-Weinberg equilibrium or that there is any linkage equilibrium between loci. Different components are differentiated into global

scores (positive eigenvalues) which distinguish clusters and clines from allele frequencies, whereas local scores (negative eigenvalues) detect differences between neighbouring individuals. I performed a multivariate test with a Monte Carlo procedure of 1,000 permutations to test for the significance of both patterns.

2.3.7 Mitochondrial DNA analysis

Indices of mtDNA diversity (number of haplotypes, haplotype diversity and nucleotide diversity) and haplotype matrices were analysed with MEGA (Tamura, Dudley, Nei, & Kumar, 2007). The frequency of haplotypes was plotted at all sites using the R package MAPLOTS and a median joining network was constructed using POPART (Leigh & Bryant, 2015).

2.4 Results

2.4.1 Data quality

Of the 14 microsatellites genotyped, evidence of homozygote excess and null alleles were detected at 6 loci (Mluc8, CA38, Mnatt6, MS3D02, H23 & Kpa24), but none of these inconsistencies were consistently detected in different populations. None of the microsatellites analysed showed indication of linkage disequilibrium (LD) between all populations. Tests for Hardy-Weinberg equilibrium (HWE) revealed that eight loci showed significant deviation from HWE, but these did not differ from HWE in more than one population. All loci were retained for further analysis as they did not show persistent evidence of inconsistencies across multiple populations.

2.4.2 Genetic diversity

Levels of genetic diversity for *M. bechsteinii* were high across all sites. Allelic richness varied from 5.09 in Braydon, UK to 6.34 in Germany (Table 2.1). Expected heterozygosity (H_e) ranged from 0.739 (Bernwood, UK) to 0.809 (Lombardy) and observed heterozygosity ranged from 0.755 (Malvern, UK) to 0.886 (Lombardy). Expected heterozygosity (H_e) was significantly higher in Europe (Monte-Carlo test, R-squared = -0.04, $p=0.001$). Levels of inbreeding were low for all sites studied with the exception of the Bernwood population (F_{IS} = 0.121).

Table 2.1 Measures of genetic diversity at all sites using 14 microsatellite loci. Sample size (N), allelic richness (Ar), expected (H_e) and observed (H_o) heterozygosity and inbreeding coefficient (F_{IS}).

Pop ID	Population	N	Ar	H_e	H_o	F_{IS}
1	Brackett's Coppice	68	5.44	0.777	0.785	-0.009
2	Sherborne	30	5.45	0.779	0.797	-0.020
3	Isle of Wight	40	5.40	0.771	0.764	0.010
4	Trowbridge	40	5.37	0.769	0.793	-0.030
5	Braydon	28	5.09	0.756	0.765	-0.013
6	Malvern	30	5.47	0.775	0.783	-0.009
7	Grafton	6	5.57	0.742	0.798	-0.069
8	Bernwood	18	5.13	0.739	0.654	0.121
	All samples from Britain	260	5.65	0.796	0.773	0.030
9	Western Germany	8	6.34	0.790	0.804	-0.020
10	Western Poland	10	5.91	0.776	0.743	0.041
11	Aquitaine	10	6.15	0.791	0.814	-0.031
12	Brittany	10	5.67	0.753	0.821	-0.088
13	Catalonia	10	5.86	0.755	0.779	-0.038
14	Lombardy	10	6.23	0.806	0.886	-0.099
15	Umbria	10	5.57	0.746	0.793	-0.072
	All samples from Continental Europe	68	6.31	0.835	0.806	0.036
	All samples	328	5.88	0.811	0.780	0.039

2.4.3 Isolation by distance and genetic structure

The correlation between genetic and geographic distance showed significant spatial structure on the full dataset ($r = 0.616$, $p = 0.002$) (Figure 2.2a); whereas individually, the continental dataset ($r = -0.054$, $p = 0.575$) and the British dataset ($r = 0.267$, $p = 0.169$) showed no such relationship (Figure 2.2b & 2.2c).

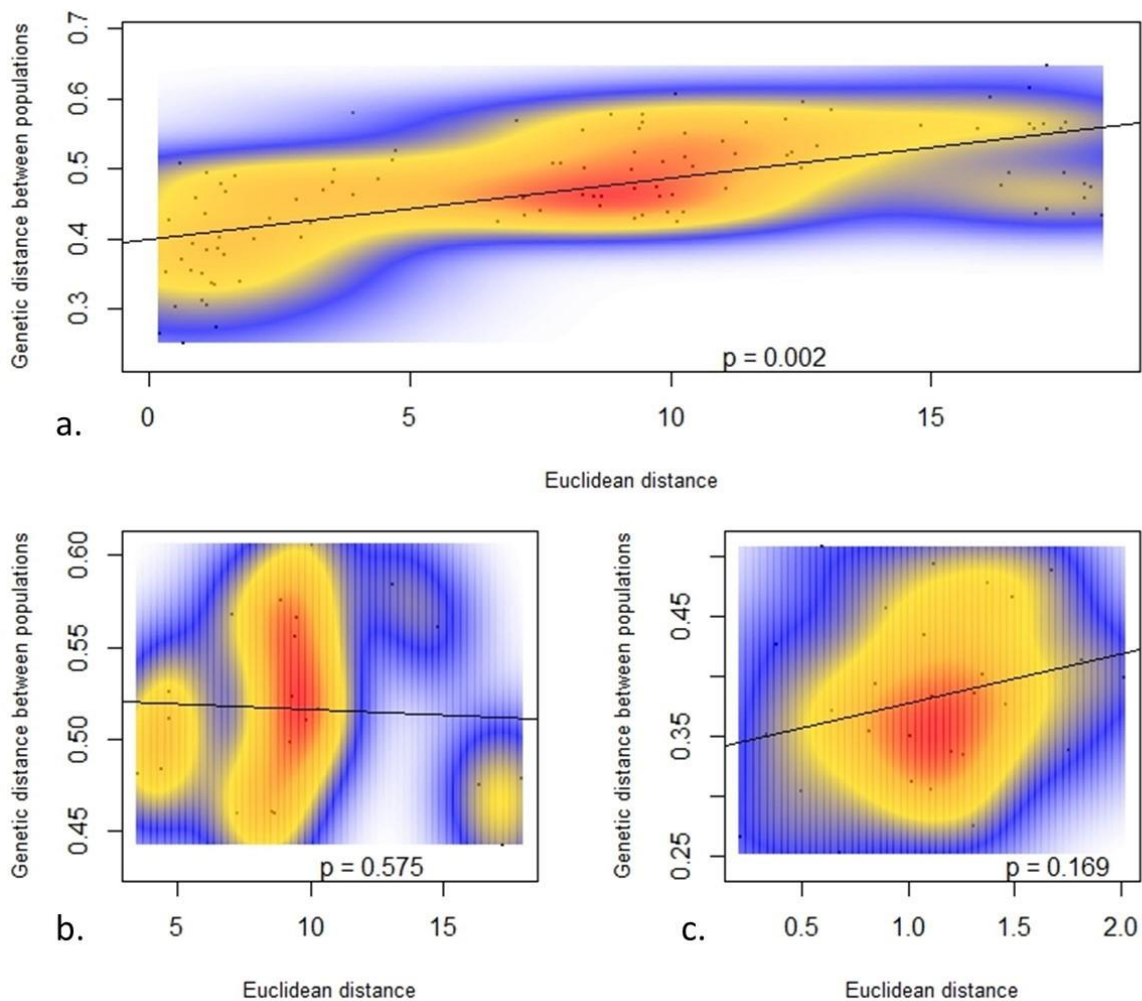


Figure 2.2 Mantel test for isolation by distance on a. the full dataset; b. the continental dataset; c. the British dataset with applied Kernel density estimates to the correlation plots

Global F_{st} for all populations was 0.032 ($p < 0.001$). The highest F_{st} between all populations was between Braydon and Umbria ($F_{st} = 0.106$, $p < 0.001$, Appendix 2.3). The lowest F_{st} was between western Germany and western Poland (F_{st}

= - 0.015, $p = 0.472$). Almost all pairwise F_{st} values were statistically significant ($p < 0.05$), except for those between certain continental sites (Pop 9, 10, 11, 12) and in Grafton (Pop 7).

The number of clusters calculated by STRUCTURE was $K = 2$ based on the ΔK method (Evanno *et al.*, 2005). At $K = 2$, continental sites comprised one group and all sites in Britain were included in the same group (Figure 2.3a). Separate runs on both populations were performed in order to identify higher levels of structure. In Britain, a clear distinction between populations in the south of Britain and the northern part of the species range in Britain was observed (Figure 2.3b). Further runs at higher K showed some differentiation between Braydon (Pop 5) and all other populations (Appendix 2.4). On the continent, differentiation was observed between the Italian populations and the rest of our study sites (Figure 2.3c).

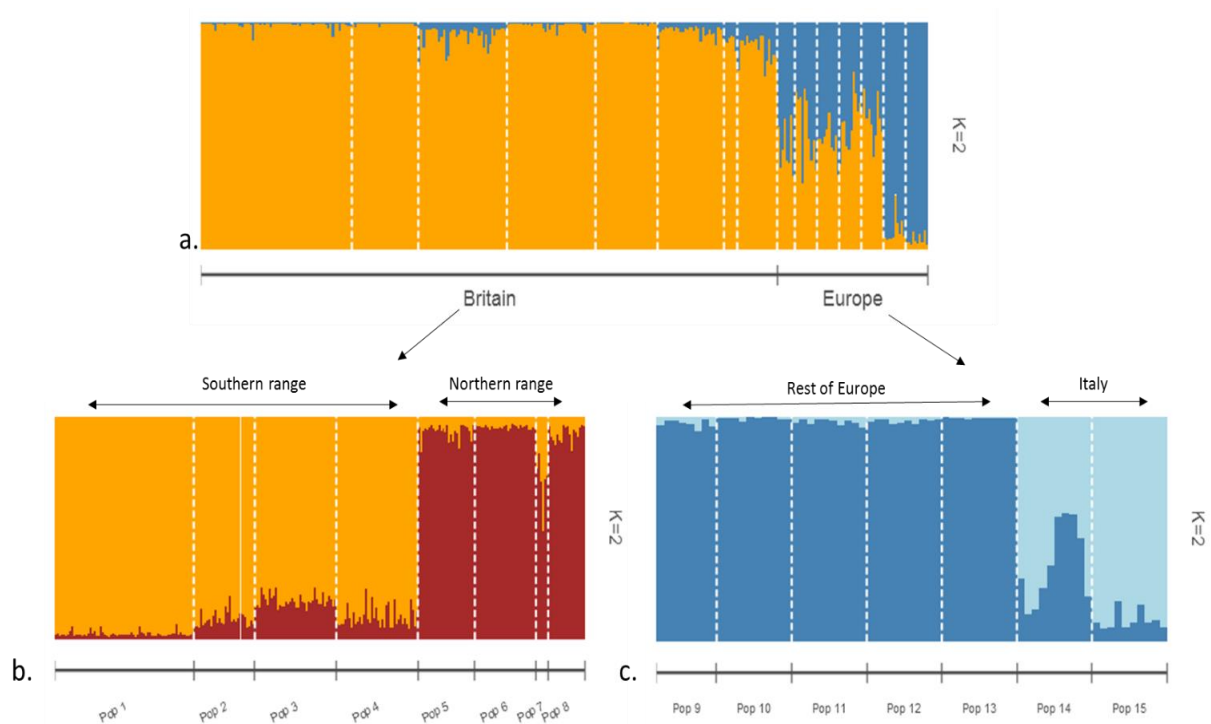


Figure 2.3 Genetic structure diagram produced by STRUCTURE—A. Primary structure of all populations ($K = 2$) B. Independent runs of STRUCTURE on the UK (left) and Continental populations ($K = 2$). Each vertical bar represents an individual, and each colour represents the probability of belonging to one of the genetic clusters. Solid lines separate sites the individuals were sampled from. 1: Brackett’s Coppice, 2: Sherborne, 3: Isle of Wight, 4: Trowbridge, 5: Braydon, 6: Malvern, 7: Grafton Woods, 8: Bernwood, 9: Western Germany, 10: Western Poland, 11: Aquitaine, 12: Brittany, 13: Catalonia, 14: Lombardy, 15: Umbria

For the sPCA, the global test indicated clear global structuring across Britain and Europe (Monte-Carlo test, $R\text{-squared} = 0.55$, $p=0.01$). The representation of the first two eigenvalues confirmed the differentiation between the British and continental bats observed in the STRUCTURE analysis (Figure 2.4a). The first eigenvector (Figure 2.4b), which showed the largest variance and spatial autocorrelation, differentiated the British population from the rest of Europe. Both Italian populations also appeared to show signs of isolation from the rest of Europe. The second eigenvector was weaker and showed no clear structure in

the dataset. No local structuring was found (Monte-Carlo test, R-squared = 0.02, $p=1$). In Britain, the global test indicated significant structuring (Monte-Carlo test, R-squared = 0.49, $p=0.02$) as opposed to the local test which was not significant (Monte-Carlo test, R-squared = 0.06, $p=0.99$). Both the representation of the first two eigenvalues (Figure 2.4c) and the first eigenvector (Figure 2.4d) clearly differentiated the southern populations from the northern populations.

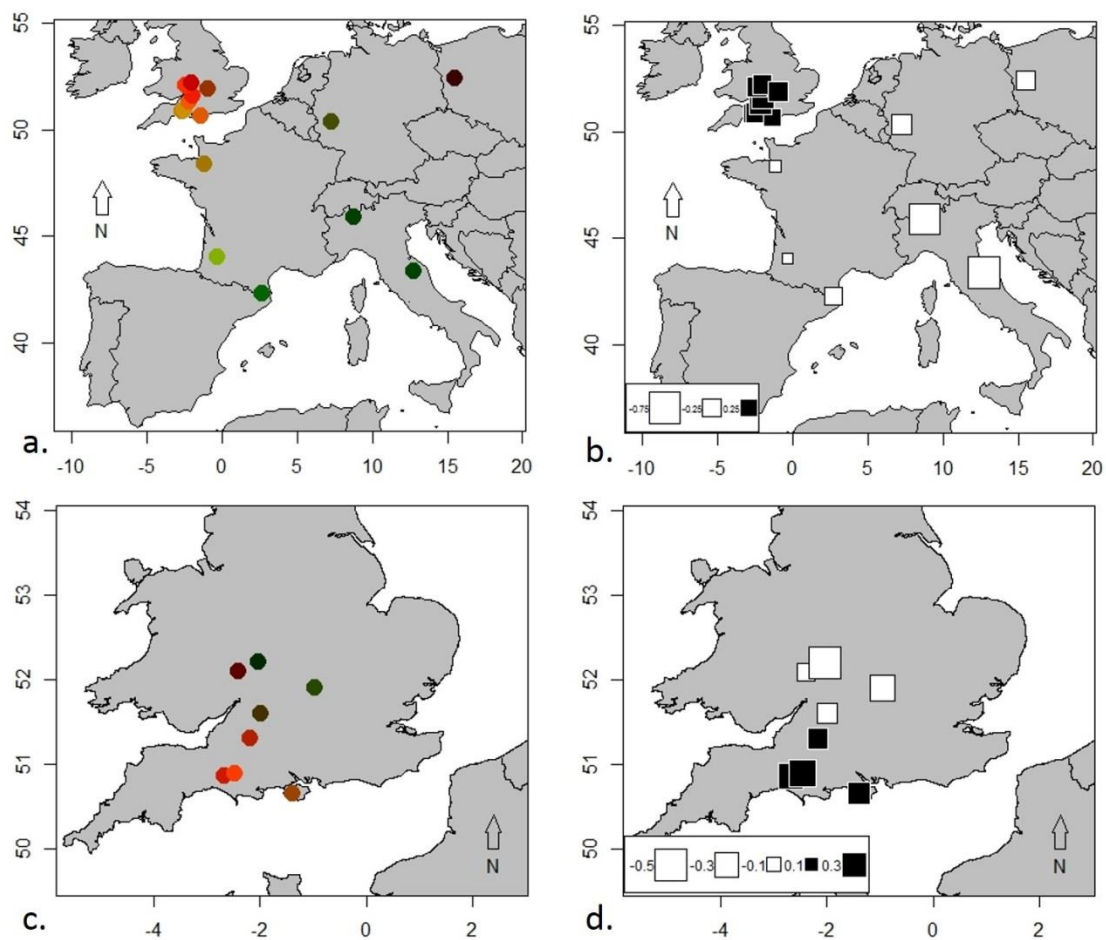


Figure 2.4 Analysis of global structure among *M. bechsteinii* in Europe (a & b) and Britain (c & d) by spatial PCA (sPCA) on 14 microsatellite loci. For a & c: Colours are indicative of the population score for the first and second principal components. For b & d: Square size and colours represent population scores of the first principal component and are positioned according to their location.

2.4.4 Mitochondrial DNA analysis

A total of 12 haplotypes were identified among the subset of 183 samples from 15 sites. The mean genetic diversity across Europe was very low ($d=0.001$, $SE=0.001$) as most *M. bechsteinii* samples across the species range shared one common haplotype (Figure 2.5a). A total of 10 haplotypes observed were comprised of less than 6 individuals and did not differ from the main haplotype by more than 3 base pairs. Bats from southern Britain (Isle of Wight, Brackett's Coppice and Sherborne) all shared a unique haplotype (Figure 2.5b) and the *M. bechsteinii* sequence from Turkey available on Genbank showed the strongest difference with all other haplotypes identified (Figure 2.5c).

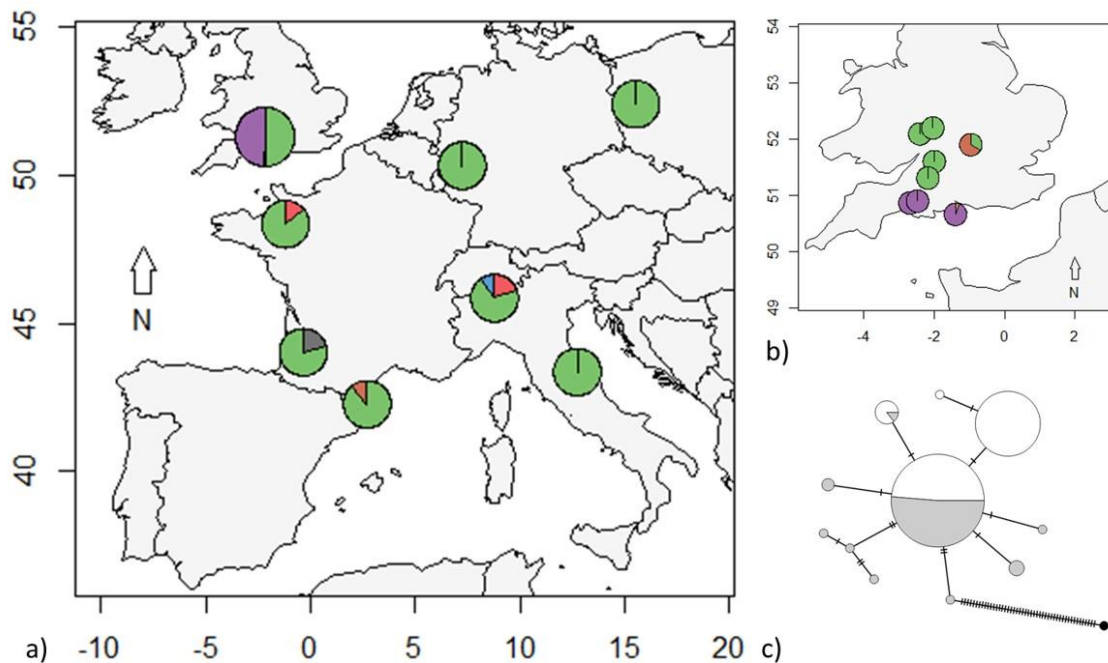


Figure 2.5 Representation of the mtDNA cytochrome b haplotypes for *M. bechsteinii* a) Pie chart representing the haplotype frequencies at each sampling site in Europe; b) Pie chart representing the haplotype frequencies at each sampling site in Britain; c) Median joining network. British haplotypes are represented in white, continental Europe in grey and the Turkish haplotype in black. Mutations are represented as hatch marks and the size of each circle reflects the number of individuals for each haplotype.

2.5 Discussion

This study demonstrates significant population structure between continental Europe and Britain. Although genetic diversity is high across the species range, it is nevertheless lower in Britain than elsewhere in Europe. Most of the genetic structure appears to be influenced by geographical barriers, such as the English Channel and the Alps, but the microsatellite and mtDNA analyses also revealed important genetic structuring between the northern and southern part of the species range in Britain.

2.5.1 Genetic diversity

Levels of diversity are high and similar to more common *Myotis* species found in Europe, such as *M. daubentonii* (Smith *et al.*, 2011), *M. nattereri* (Rivers *et al.*, 2005), *M. myotis* (Ruedi & Castella, 2003), *M. mystacinus* and *M. brandtii* (Bogdanowicz, Piksa, & Tereba, 2012). The use of numerous autumn swarming sites throughout their range is likely to play an important role in maintaining high genetic diversity as it is a frequently observed behaviour in *Myotis* bats (Kerth *et al.*, 2003). However, island populations are more susceptible to factors such as genetic drift, and tend to express lower levels of diversity than continental populations (Frankham, 1997). Although I observed more genetic diversity than previously reported for this species in Britain (Durrant *et al.*, 2009), these levels remained slightly lower than in Europe. The isolation of the Bernwood populations from other populations may also explain why this population is the only one showing higher levels of inbreeding and less genetic diversity.

2.5.2 Genetic structure

The STRUCTURE analysis revealed a clear separation between the British and European populations, which suggests that the English Channel acts as a

barrier to gene flow between Britain and mainland Europe. Such differentiation has been observed in a number of bats, such as *M. daubentonii* (Atterby *et al.*, 2010) , *P. austriacus* (Razgour *et al.*, 2013) and *E. serotinus* (Moussy *et al.*, 2015). Bechstein's bats sampled on the Isle of Wight, located off the British coast, did not show any differentiation compared with the rest of Britain (at $K = 2$); in contrast with the patterns observed for *E. serotinus* (Moussy *et al.*, 2015). Although *M. bechsteinii* forage over much shorter distances than *E. serotinus* (Catto, Hutson, Racey, & Stephenson, 1996; Dietz & Pir, 2011), it appears that they will more readily cross short distances of open water. Such events may well be infrequent but strongly related with mating and autumn swarming events enabling connectivity between populations.

Large geographical barriers, such as the Channel and the Alps, explained genetic differentiation between populations in the STRUCTURE analysis. However, these trends were not apparent when analysing isolation by distance (IBD) with the full dataset. IBD often gives biased results by identifying stepping stone trends of IBD for cases of hierarchical structure (Meirmans, 2012). To control this bias, I performed Mantel tests on the separate clusters identified by STRUCTURE. In this case, the effect of the Alps was clearly identified as a geographic barrier on the continental dataset despite records of Bechstein's bats swarming above 1,500 m (Petrov, 2006). Mountain ranges are also known to be an important geographic barrier and to have a significant effect on the genetic structure of *M. myotis* (Ruedi *et al.*, 2008) and *P. austriacus* (Razgour *et al.*, 2013).

As with Durrant *et al.* (2009), IBD within the British population of *M. bechsteinii* was not detected in this study. However, results from STRUCTURE and sPCA

emphasised a clear separation between the southern and northern part of the species national distribution. The absence of obvious physical barriers (e.g. mountain ranges) between both populations suggests that anthropogenic barriers, such as differences in land-use, may explain the differentiation.

2.5.3 Mitochondrial DNA analysis and genetic history

The cytochrome b gene is known as a good indicator of mitochondrial diversity for mammals (Combe *et al.*, 2016; Stojak *et al.*, 2016) and other vertebrates (Zhu, Cheng, & Rogers, 2014). However, several European bat species show very little mitochondrial divergence throughout their range as observed with *M. bechsteinii* (Ibáñez *et al.*, 2006; Rebelo *et al.*, 2012; Salgueiro, Ruedi, Coelho, & Palmeirim, 2007). Although most British populations shared in common the same haplotype as continental bats, the southern populations shared a unique haplotype that differed by a single base pair from the rest of Europe and Britain. Such differentiation agrees with results from the nuclear microsatellite data. Similar differentiation has also been observed in *E. serotinus* (Moussy *et al.*, 2015). In the absence of clear physical barriers, the break observed may be linked to a lack of essential habitat, such as woodlands and potential swarming sites, which can limit the dispersal abilities of *M. bechsteinii* and further explain the separation between both populations.

Our finding of little diversity across the whole of Europe confirm a recent population range expansion which most likely coincides with post-glacial colonisation as observed in multiple bat species (Moussy *et al.*, 2015; Petit, Excoffier, & Mayer, 1999). Kerth *et al.* (2008) analysed the HV2 and ND1 mitochondrial genes of *M. bechsteinii* from the Balkans and suggested that this population may have been the unique glacial refugium for the species as

genetic diversity was higher than in Europe. Glacial refugia are prone to higher levels of genetic diversity than recently colonised areas. Multiple continental clusters have been identified for *E. serotinus*, *M. myotis*, *R. ferrumequinum* or *B. barbastellus* indicating the recolonization of Europe from separate refugia (Moussy *et al.*, 2015; Rebelo *et al.*, 2012; Rossiter *et al.*, 2000; Ruedi *et al.*, 2008). Although this study lacks samples from the Balkans, it confirms that Italy and Spain were unlikely ancient glacial refugia for the species as they mainly share the same haplotype as the rest of Europe (Kerth *et al.*, 2008). The Balkans has also been identified as an important refugium for *N. noctula* (Petit *et al.*, 1999). This species differs from *M. bechsteinii* as it is known to be migratory and will disperse more than 400km. However, both species highly depend on woodland and tree cavities for roosting as opposed to *E. serotinus*, *M. myotis* or *R. ferrumequinum*. *Fagus sylvatica* (beech) has also undergone an important post-glacial range expansion from a main source population in the Balkans (Magri, 2008; Magri *et al.*, 2006). As discussed by Kerth *et al.* (2008), the increased presence of beech woodlands may have been the main driver behind the post-glacial range expansion of *M. bechsteinii* and *N. noctula* by providing them with suitable habitat and roosting opportunities as both species share strong similarities in range and phylogeographic history.

2.5.4 Conservation implications

Bats often show less genetic differentiation than terrestrial mammals (Ibáñez *et al.*, 2006). However, habitat fragmentation can significantly increase isolation of more sedentary bat species and lead to genetic drift and inbreeding (Meyer, Kalko, & Kerth, 2009). *M. bechsteinii* shows high levels of genetic diversity and little differentiation throughout Europe. However, the British population appears

to be showing less diversity and more genetic structure over a smaller area than on the continent in the absence of obvious physical barriers. Founder effects may explain the lower levels of diversity, but the presence of clear population structure could be the result of anthropogenic factors. The effective dispersal mechanisms of *M. bechsteinii* and genetic exchange at swarming sites may have limited the impact of these threats to date, but continued pressures on landscape connectivity means that long-term monitoring is essential.

The identification of a population showing signs of inbreeding and low genetic diversity is of concern. This is particularly relevant to populations on the extreme edges of the British range for instance the Bernwood population, in Buckinghamshire. As such populations are likely to be more sensitive to the continual expansion of developments and other threats leading to habitat fragmentation and loss. It is reasonable to assume that increased pressures are likely to exacerbate inbreeding, reduce genetic diversity and have negative consequences on the viability of such populations.

In conclusion, I observed high levels of diversity across Britain and Europe, although diversity was lower in Britain. Geographic barriers, such as the English Channel and the Alps, explain most of the genetic structure in our dataset. In Britain, I observed important structuring at a nuclear and mitochondrial level between northern and southern populations which may be influenced by anthropogenic factors. The high dispersal abilities and long lifespan of *M. bechsteinii* may result in a delayed response in genetic diversity and structure to recent changes in their environment (e.g. urban development and the intensification of agriculture) and so it is imperative to regularly monitor the effect of potential threats on populations.

2.6 Appendices

Appendix 2.1: Sampling sites of *Myotis bechsteinii* in Britain and Europe

Site	Longitude	Latitude
Brackett's Coppice	-2.68694	50.86285
Sherborne	-2.47335	50.89744
Isle of Wight	-1.39625	50.66029
Trowbridge	-2.17714	51.30878
Braydon	-1.99673	51.60168
Malvern	-2.40752	52.09484
Grafton Woods	-2.04042	52.20087
Bernwood	-0.96141	51.90224
Western Germany	7.24228	50.3359
Western Poland	15.53312	52.40933
Aquitaine	-0.34917	44.03611
Brittany	-1.20126	48.35615
Catalonia	2.697747	42.27699
Lombardy	8.779616	45.86358
Umbria	12.73785	43.3619

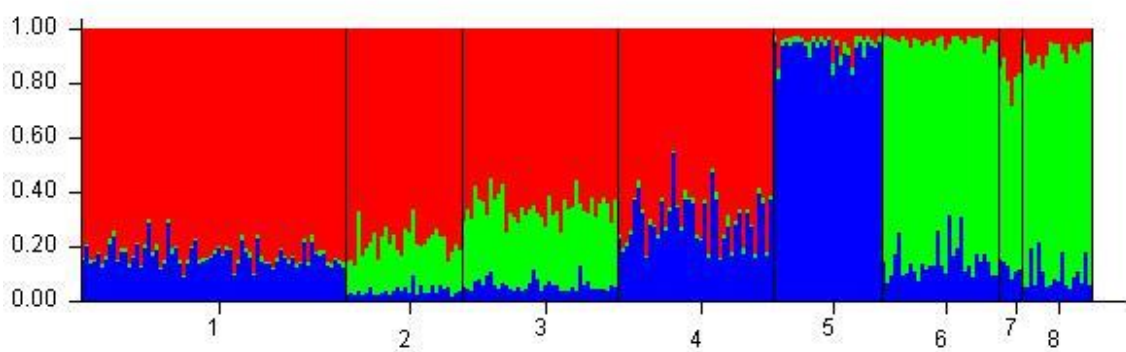
Appendix 2.2 Locus name, primer sequence and repeat motif, number of alleles and size range of all microsatellites tested on *M. bechsteinii*. The reverse primer of Kpa16_2 and the forward primer from C112_125 were modified in order to better fit their own multiplex. *Paur3 is a X-chromosomal microsatellite and was not used for this study.

Multiplex	Locus	Forward primer	Reverse primer	Repeat sequence	Number of alleles	Size range (bp)	Reference
1	A24	D3-GTGGTATGAAATAACCAGTTCACITTTG	GGGTAGTTTACAGAAATAGCAATTGAG	(AC) x	11	268-290	Castella and Ruedi (2000)
	CloneA2	D4-TTTGTTATCAGAAGGAAGG	TCTCAACTGGGTGCTC	(CA) x	15	141-173	O'Brien, McCracken, Say, and Hayden (2007)
	Kpa24	D4-GGCCAGGATGCACAGGAG	CTCCAATCAGCACGTGGATTC	(GA) x T(AG) x	26	238-290	Struebig <i>et al.</i> (2008a)
	B22	D2-CTGATGCAAGACCCCTTACAAC	ACGGCAGCAGTGAAATCAGA	(GT) x	10	151-173	Kerth <i>et al.</i> (2002)
	Mluc8	D3-CCACTCAAGCACCAGATGAATA	AGGAATGAGGGAAGAAAGGAAG	(CTTC) x	11	191-231	Burns, Broders, and Frasier (2012)
2	Kpa16_2	D2-TCAGTGCCATCAGGATTCAG	CAAAGTTTTAGAACTGGAAGTGAAGAA	(GA)xTA(AG) x	18	201-243	Struebig <i>et al.</i> (2008a)
	Paur3*	D3-CTGGAGTGTTGTTTTGCCCTTC	GCTGATGGTGGAGTCTCCTTTTTTC	(GT) x	*	240-256	Burland, Barratt, and Racey (1998)
	CA38	D3-CATCTTTCCAAGCCATTA	GCCTCCTGCAAGAGTTGTTTC	(CA) x	18	173-221	Oyler-McCance and Fike (2011)
	CA11	D4-AGAACCCAGTGCCCTTCTTT	ATGAGAGGGGGATGTGACAG	(CA) x	15	170-206	Oyler-McCance and Fike (2011)
	Mnatt6	D4-TTTCTCAGCCTCCATAATA	ATAGCAAGGCAATAAAAATA		18	250-288	Scott <i>et al.</i> (2013)
3	G6	D4-GGCTTTTTGAAAAGACTGAGG	ACATCAGCCAGTTCCTGTTC	(GT)GA(GT) x	8	95-125	Castella and Ruedi (2000)
	MS3DO2	D2-CTAAGACCCCTTCCAGCTCTCA	GATACCATCACTCTTCCCCTG	(AC) x	25	205-259	Trujillo and Amelon (2009)
	C112_125	CATGCAGGTGTGTGCCTGT	D2-AATGGGTCTCAGAGGGTGAA	(CA) x	16	122-152	Castella and Ruedi (2000)
	B23	D4-CAGTTGGAGGCATGCAGAAA	CCGGAGATACTCTTATTGTTGGTA	(CT) x	19	248-286	Kerth <i>et al.</i> (2002)
	H23_Mluc	D3-TTGTCTACTAGCATTTGTCCAGTG	ATAGCTATGTTGCCTAACCTATTACTC	(GT) x	18	147-187	Castella and Ruedi (2000)

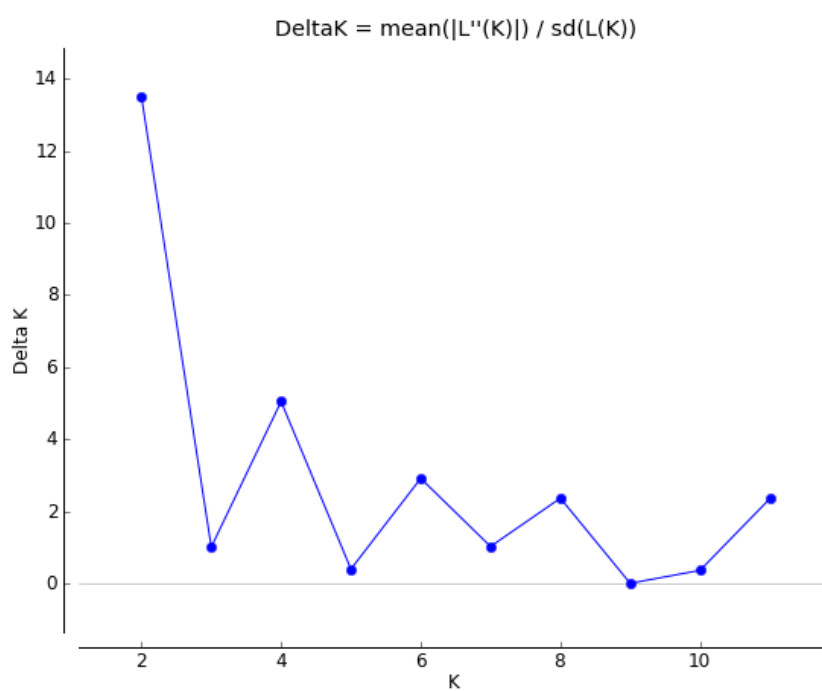
Appendix 2.3. Pairwise F_{ST} results for each of the 15 *Myotis bechsteinii* populations. F_{ST} values are below the black diagonal boxes, significance values are above the black diagonal.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
0.000	***	***	***	***	***	*	***	**	***	**	**	***	***	***		1
0.013	0.000	***	***	***	***	*	***	***	***	**	***	***	***	***		2
0.016	0.015	0.000	***	***	***	**	***	**	**	**	***	***	***	***		3
0.013	0.023	0.024	0.000	***	***	0.076	***	**	***	***	***	***	***	***		4
0.028	0.040	0.037	0.025	0.000	***	**	***	**	***	***	***	***	***	***		5
0.029	0.019	0.021	0.032	0.032	0.000	0.174	***	**	**	**	***	***	***	***		6
0.018	0.017	0.026	0.013	0.036	0.008	0.000	*	*	*	*	**	0.100	***	***		7
0.035	0.032	0.030	0.038	0.039	0.031	0.027	0.000	**	***	**	***	***	***	***		8
0.028	0.032	0.023	0.027	0.024	0.027	0.029	0.034	0.000	0.472	0.240	0.169	*	*	***		9
0.024	0.040	0.022	0.030	0.032	0.029	0.028	0.030	0.000	0.000	0.469	0.233	*	***	***		10
0.018	0.025	0.017	0.028	0.033	0.023	0.024	0.025	0.006	0.015	0.000	0.078	*	**	***		11
0.016	0.035	0.029	0.030	0.034	0.039	0.034	0.040	0.009	0.006	0.014	0.000	*	**	***		12
0.027	0.040	0.033	0.028	0.049	0.047	0.015	0.069	0.022	0.016	0.022	0.017	0.000	***	***		13
0.059	0.057	0.061	0.074	0.074	0.057	0.046	0.060	0.019	0.036	0.029	0.041	0.069	0.000	**		14
0.088	0.073	0.083	0.094	0.106	0.073	0.081	0.084	0.060	0.071	0.066	0.073	0.094	0.032	0.000		15

***= p < 0.001 **= p < 0.01 *= p < 0.05

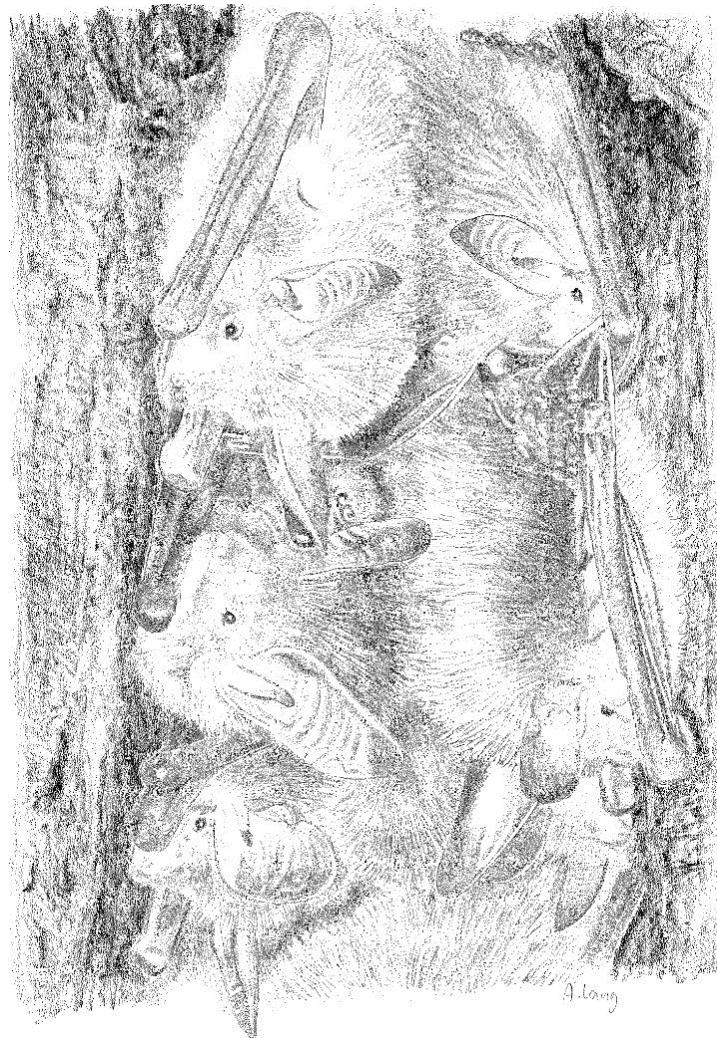


Appendix 2.4 Genetic structure diagram produced by STRUCTURE Independent runs on the British dataset at K=3



Appendix 2.5 DeltaK plot of the structure analyses on the full data set.

Chapter 3 Assessing habitat suitability and connectivity of *Myotis bechsteinii* in Britain through the use of habitat suitability modelling and landscape genetics



3.1 Abstract

The loss and fragmentation of species habitats can negatively impact the viability of populations through inbreeding and a loss in genetic diversity. This may additionally impact on their ability to adapt to environmental changes (e.g. climate change). In this study, I used a combined approach of habitat suitability, landscape connectivity and landscape genetics analysis to identify areas of suitable habitat and assess habitat connectivity of a rare woodland bat, *Myotis bechsteinii*, in Britain. Models were built using occurrence data together with genetic data (14 microsatellite loci) derived from 220 bats sampled at eight geographical locations. The negative association of *M. bechsteinii* presence with distance from woodland was identified as the main variable determining habitat suitability. While the presence of maternity roosts was more influenced by woodland type (broadleaved, mixed and coniferous), males were found to use sub-optimal habitats more readily. Suitable habitat was highly fragmented and only showed good connectivity if the species was able to disperse over 5,000 m. The landscape genetics results indicated that the type of land cover (woodlands, urban areas, pastures, arable, scrub and sea) best explained genetic connectivity between populations and highlighted the importance of woodland cover for maintaining genetic connectivity. These findings strongly highlight the impact of land cover, and more importantly the significance of woodlands in providing suitable habitat and maintaining connectivity for *Myotis bechsteinii*.

3.2 Introduction

The loss and fragmentation of woodlands, combined with the intensification of agriculture and urbanisation has, and continues to contribute towards the decline of biodiversity (Ceballos, Ehrlich, & Dirzo, 2017). These losses may translate into a decline in population size, the loss of genetic diversity, and ultimately species loss (Bailey, 2007). Understanding the effect that landscape structure has on the presence and genetic connectivity of species is essential for wildlife conservation. It can also provide critical information for the conservation of elusive species by identifying isolated populations and contributing to the design of wildlife corridors (Balkenhol *et al.*, 2016).

Habitat suitability modelling (HSM) predicts the distribution of species based on environmental data and occurrence records (Guisan, Thuiller, & Zimmermann, 2017; Guisan & Zimmermann, 2000). It has been increasingly used in conservation as it provides a useful method to predict the distribution of species and to assess the importance of key features for their presence (Bellamy, Scott, & Altringham, 2013; Elith & Leathwick, 2009). It also provides outputs readily accessible to practitioners and hence can lead to improvements in habitat management (Brooks, 1997). The approach is being increasingly used in bat research (e.g. Bellamy & Altringham, 2015; Bellamy *et al.*, 2013; Razgour, Rebelo, Di Febbraro, & Russo, 2016) and is helping to identify how environmental changes, such as climate change, will affect species distribution and diversity (Razgour *et al.*, 2017; Rebelo *et al.*, 2010)

For the case of habitat specialists living in fragmented landscapes, evaluating habitat connectivity may be just as imperative as identifying suitable habitat as

increased fragmentation can affect the ability of species to disperse and subsequently affect genetic diversity and population viability. Therefore, the sole use of HSM may limit its utility, and the combined use of other approaches, such as landscape genetics and landscape connectivity analysis, may improve its usefulness (Razgour *et al.*, 2016). For example, combining methods can help assess the amount of connectivity between habitats in a fragmented landscape (e.g. Clauzel, Bannwarth, & Foltete, 2015; Ramirez-Reyes, Bateman, & Radeloff, 2016), characterise features affecting genetic variation and identify isolated populations or potential wildlife corridors (Razgour *et al.*, 2016).

Although it can be straightforward to relate major barriers to gene flow with certain landscape features, such as mountains or seas (e.g. McLeod *et al.*, 2015; Moussy *et al.*, 2015), the structure and composition of most landscapes will affect population processes in a more complex way. Therefore, rigorous methods of analysis are needed to quantify the effect that landscapes have on wildlife. Landscape genetics, first introduced by Manel *et al.* (2003), combines the use of population genetics, landscape ecology and spatial analysis to quantify the effect of landscape composition on evolutionary processes (Balkenhol *et al.*, 2016). Along with the use of resistance surfaces representing the cost of movement through the landscape and predictive models (See Introduction 1.5.3), such as least cost paths or circuit theory, landscape genetic studies can help design wildlife corridors, detect barriers (Garroway, Bowman, & Wilson, 2011) and predict the impact of landscape changes on wildlife (Razgour *et al.*, 2017). However, there are still limitations when parameterising resistance surfaces as values are often determined by expert opinion or habitat suitability models before undergoing statistical validation. For elusive species,

these methods may be misleading, describing biased anecdotal sightings rather than actual biological processes (Peterman, 2018). To avoid such limitations, Peterman, Connette, Semlitsch, and Eggert (2014) used linear mixed effect models and a nonlinear optimization algorithm to optimize resistance surfaces across a landscape. Such methods were subsequently implemented using genetic algorithms (algorithms that are based on the evolutionary processes of natural selection and genetics) which allows the optimization of resistance surfaces from landscape variables, whilst making no *a priori* assumptions on the effect of these variables (Peterman, 2018). This method has proven to be useful in identifying anthropogenic threats. For example, fire density and villages were found to best explain genetic structure in red colobus monkey (*Procolobus gordonorum*) populations (Ruiz-Lopez *et al.*, 2016).

Male bats tend to use a broader range of habitats than females (Lintott *et al.*, 2014) as they have lower energetic demands than females (pregnancy, lactation) and can save energy by using torpor more regularly when conditions are challenging (Racey & Entwistle, 2000; Ruckstuhl & Neuhaus, 2005). While the mating strategies of most bat species remains poorly understood, the ability for males to use sub-optimal habitats may suggest that they play an important role in maintaining gene flow between populations (e.g. Moussy *et al.*, 2015; Wilmer, Hall, Barratt, & Moritz, 1999). It is subsequently essential to also better understand the characteristic of both male and female habitats in bats to better maintain genetic connectivity between populations.

Myotis bechsteinii is a species largely associated with woodlands (Dietz & Pir, 2011; Napal, Garin, Goiti, Salsamendi, & Aihartza, 2013). While females live in closed maternity groups, males tend to live solitary lives (Kerth & König, 1999).

While post-glacial fossil deposits suggest that the species was once more common in Britain (Yalden, 1999), its presence was only confirmed by a few sporadic records before the 2000s (Schofield & Morris, 2000; Stebbings, 1989), until the use of new trapping techniques helped to provide more detailed information on the species distribution (Hill & Greenaway, 2005; Miller, 2011). It is also known that the British population shows lower levels of genetic diversity and more important genetic structuring in comparison to the rest of the European population (Chapter 2). While such differences in genetic structure are often associated with anthropogenic barriers (e.g. Ruiz-González *et al.*, 2014), it is poorly understood how these may impact genetic differentiation between *M. bechsteinii* populations in Britain.

In this study, I hypothesised that woodlands play a key role for the habitat of *M. bechsteinii* but also in maintaining connectivity. For this, I first aimed to identify key variables affecting the distribution of *M. bechsteinii* for both males and females using presence records. Then, I used measures of connectivity to assess the levels of connectivity of suitable *M. bechsteinii* habitat. Finally, I measured the effect of landscape features on genetic connectivity.

3.3 Materials and Methods

3.3.1 *Habitat suitability model*

I used 11 landscape variables at 100 m resolution for the habitat suitability model, including Corine land cover which was prioritised over other datasets for its transferability to larger scales studies (woodland, urban, arable, pasture, scrub; Aune-Lundberg & Strand, 2010). Ancient woodland (NaturalEngland, 2016; Natural Resources Wales - NRW, 2011) and woodland type

(broadleaved, mixed and coniferous woodlands; Forest Research, 2017) were also included because of the known association of *M. bechsteinii* and woodlands (Schofield & Morris, 2000). I also included elevation (m) (Ordnance Survey System - OSS, 2017) and slope (OSS, 2017) as both variables are known to impact the presence of multiple bat species (Bellamy *et al.*, 2013). Solar index (Suggitt *et al.*, 2014) and topographic wetness (Suggitt *et al.*, 2014) were both used in the analysis despite being very rarely used in habitat suitability models. Solar index was thought to have a possible impact on roosting opportunities for *M. bechsteinii* as they require specific conditions which could be linked to solar radiation (Dietz & Pir, 2011). As for topographic wetness, it has been found to impact forest plant communities (Besnard, La Jeunesse, Pays, & Secondi, 2013) which could subsequently further affect higher trophic levels, such as bats. Finally, distance from specific landscape features (major roads, ancient woodlands, woodlands and urban areas) were included in the model. GIS manipulations (e.g. resampling, reclassifying) were performed in ArcGIS 10.2.2. (ESRI Inc). The correlation of each combination of environmental variables was tested using the R package usdm (Naimi, 2013; Naimi, Hamm, Groen, Skidmore, & Toxopeus, 2014). None of the environmental variables had correlation coefficients > 0.70 and therefore indicated that none of the variables were significantly autocorrelated and that they could all be kept for further analysis.

The model extent was set as the whole of southern Britain up to approximately 50 km north of the most northern *M. bechsteinii* records. A total of 2,877 presence records were collected between 1995 and 2016 at 10 km resolution or higher from the NBN gateway, national and local monitoring schemes, and local

record centres. All duplicate records from the same location and records for which precision was over 100 m in precision were removed from the dataset. Spatial autocorrelation of residuals (1 – predicted Habitat Suitability Index for each presence record) were tested for all three models using Moran's correlograms with the R package *ncf* (Bjørnstad, 2009). I used MaxEnt Version 3.3.3e (<http://www.cs.princeton.edu/~schapire/maxent>), a program which predicts geographical distribution of species by applying the principle of maximum entropy on species presence data by estimating the probability of presence for each cell (pixel). A model was performed using (i) all records, (ii) for all male records collected during the months of April to end of August, and (iii) all confirmed maternity roost records. An initial test model for each set of occurrence data was run using default settings and variables contributing less than 1% to the test run were removed from further analysis. Then, all possible combinations of environmental variables were tested. Default settings for the regularization multiplier and feature class option were maintained as all features are tested in MaxEnt if over 80 presence points are available (Merow, Smith, & Silander, 2013) and the regularization multiplier has been set to give high predictive performance on a large collection of species (Elith *et al.*, 2006). The efficacy of all models were then compared using Akaike Information Criteria (AICc) in ENMTools (Warren & Seifert, 2011). Models were ranked by calculating the difference ($\Delta AICc$) between a model's AICc and the minimum AICc (minAICc). The strength of the final model was evaluated using the AUC score. Five replicated runs were then performed for the best model to obtain response curves of all variables contributing to the final model (Settings Appendix 3.2).

3.3.2 *Habitat connectivity*

All areas showing a high probability of presence (> 0.75) were classified as areas of suitable habitat. These sites were extracted from the habitat suitability model (all records) and resampled to a 500 m resolution for computational purposes. I then used Conefor Sensinode 2.6, a software which calculates multiple connectivity indices based on the graph theory (or network analysis), and the corresponding ArcGIS extensions (Saura & Torne, 2009) to evaluate the connectivity of suitable habitat for *M. bechsteinii*. Since a significant amount of suitable habitat was found outside the species range, connectivity indices were calculated for the total amount of suitable habitat and suitable habitat found within the species known range.

I first calculated the number of links (NL) which represents the number of connections between habitat patches and the number of components (NC). Here, a component is a set of connected patches (or nodes) (i.e. a patch isolated from all others will be counted as a single component) (Saura & Torne, 2009). Since the dispersal abilities of *M. bechsteinii* are poorly understood, these indices were calculated using six dispersal distances (1,000 m; 2,000 m; 5,000 m; 10,000 m; 15,000 m and 20,000 m). Then, I reported the delta of the Integrated Index of Connectivity (dIIC), this index calculates, in percentage terms, the importance of each habitat patch in maintaining connectivity. Higher values are given to the most important patches, while low values may act more as stepping stones. The dIIC calculation requires significant computational power, therefore the number of nodes (habitat patches) was reduced to a manageable amount as recommended by the authors (Saura & Torne, 2009). For this, I grouped components (groups of connected nodes at 5,000 m

dispersal distance) by producing a 1,000 m buffer zone around all connections and merged any overlapping buffer zone into a single polygon. The dispersal distances were calculated at 3,000 m, 8,000 m, 13,000 m and 18,000 m in order to limit the effect of the 1,000 m buffer zone.

3.3.3 *Landscape genetics analysis*

This study is based on the *M. bechsteinii* microsatellite and location records datasets from Chapter 2. Samples were collected from 6-68 individuals at each of 8 geographical locations in Britain. Genotyping was conducted at 14 autosomal loci.

For two of the locations — Bernwood and the Isle of Wight — samples were collected across multiple woodlands, whereas in the other cases the samples were derived from a single woodland. Given that site selection can have a strong effect on the outcomes of landscape genetic analysis (Balkenhol *et al.*, 2016), I therefore selected samples to make them comparable with the other locations. A single woodland was selected and all the individuals sampled within 3 km of the selected woodland. I performed the same data quality check as in Chapter 2 (See section 2.3.4) and the pairwise F_{st} scores were calculated in Genalex (Peakall & Smouse, 2006) along with the distance in km between each site.

I used a landscape genetic approach to test for the importance of environmental variables as barriers using the R package ResistanceGA (Peterman, 2018).

This approach allows the optimisation of resistance surfaces based solely on the pairwise genetic distances and coordinates of sample sites provided. For this, I used a univariate approach as it was thought to be the most effective method to directly detect single variables which may act as barriers to gene flow

whilst also avoiding the influence of expert opinion or the use of habitat suitability analysis. Such biases are important to avoid as habitat selection and gene flow, for example, are governed by very different behavioural processes (Balkenhol *et al.*, 2016), such as juvenile dispersal for gene flow and adult home ranges for habitat selection.

For the analysis, I selected four types of landscape layers, Corine land cover (Aune-Lundberg & Strand, 2010), major roads (classified as 'A road' or motorway EDINA, 2017) and woodlands (Forest Research, 2017) and ancient woodlands (NaturalEngland, 2016), which are all known to affect bats (Table 3.1). The landscape layers were transformed into a grid of 500 x 500 m in order to increase computational speed using the Geographical Information Systems ARCMAP 10.2.2 (ESRI Inc). Rare landscape features were excluded from the land cover layer and only the most dominant features were kept as ResistanceGA may estimate high/nonsensical values because they provide little to no information (Peterman, 2018). Although *M. bechsteinii* is not ordinarily associated with coniferous and mixed woodlands, maternity colonies have been found in these habitats (e.g. Arrizabalaga-Escudero *et al.*, 2014). Therefore, I decided to not differentiate woodland types (broad-leaved, mixed and coniferous) in this study as these are likely to have a similar effect on genetic connectivity.

Table 3.1 List of landscape variables along with their source and the categories used for the landscape genetic analysis.

<u>Landscape variable</u>	<u>Source</u>	<u>Categories</u>
Land cover	Corine Land Cover (Aune-Lundberg & Strand, 2010)	Arable/ Woodland/ Urban areas/ Pastures/ Sea / Scrub
Pasture	Corine Land Cover (Aune-Lundberg & Strand, 2010)	Pasture/ Non-pasture
Scrub	Corine Land Cover (Aune-Lundberg & Strand, 2010)	Scrub/ Non-scrub
Sea	Corine Land Cover (Aune-Lundberg & Strand, 2010)	Sea/ Non-sea
Urban	Corine Land Cover (Aune-Lundberg & Strand, 2010)	Urban/ Non-urban
Arable	Corine Land Cover (Aune-Lundberg & Strand, 2010)	Arable/ Non-arable
Major roads (A road and motorways)	EDINA (2017)	Major roads/ No roads
Ancient woodland	NaturalEngland (2016)	Ancient woodland/ Non-ancient woodland
Woodland	Forest Research (2017)	Woodland / Non-woodland

ResistanceGA was used on each landscape variable using the single surface optimisation function. As recommended by Peterman (2018), I ran ResistanceGA twice on each data set and checked for convergence by using their AIC values. There were only marginal differences in AICc values between

runs and no change in the ranks of the best performing factor (resistance surface, geographical distance or a null model). I used the resistance surface from the highest ranked model to visualise the current flow among the eight sampled populations in Circuitscape (McRae & Nürnberger, 2006). Then, I resampled the habitat suitability model obtained from all records to a 500 m resolution and transformed it so that low habitat suitability was given high resistance scores and low resistance scores were given to good habitat. Finally, to cover a larger area than the sampled populations, I randomly selected 15 points in ArcGIS 10.2.2. within the species British range; and used the resistance surface from the landscape genetics and the habitat suitability model to visualise differences in connectivity in Circuitscape (McRae & Nürnberger, 2006).

3.4 Results

3.4.1 *Habitat suitability model*

From the 2,877 *M. bechsteinii* records in Britain, 620 were left after removal of unprecise and duplicate records from the same locations. These records included 50 confirmed maternity roosts and 84 confirmed male records for analysis.

Four variables (solar index, wetness, distance from major roads and slope) explained less than 1% of the test runs performed on all three model types and were consequently removed from further analyses. AUC values were high for all three final models with values ranging from 0.83 to 0.92 and therefore provided good predictions of the species distribution. Distance from woodland, elevation and land cover were the only variables found to have an important contribution

in all models. This was true whatever record type was used (all records, male records, maternity roost records). Distance from woodland clearly contributed the most to all final MaxEnt models (Table 3.2). In the analysis of all *M. bechsteinii* presence records, the best model include distance from woodlands, distance from ancient woodlands, mean elevation, woodland type, land cover and ancient woodland (AUC=0.876; AICc=17,834.16) (Table 3.2). Analysis of male-only presence records also showed a strong influence of distance to woodland, mean elevation and land cover. However, distance from urban areas was also found to contribute slightly (7.3%) (Appendix 3.3). Conversely, woodland type contributed most to the maternity roost model (40.8%), followed by distance from woodland and land cover and elevation (Table 3.2).

Table 3.2 Best models from MaxEnt runs of *Myotis bechsteinii* in southern Britain using all records, male records and maternity roost records showing the contribution (%) of each variable to the final model.

	Best model		
	All records	Male records	Maternity roost records
AICc	17834.2	2409.5	1501.9
minΔAIC_c	4.4	3.4	4.7
AUC	0.88	0.92	0.83
<u>Contribution (%)</u>:			
Distance from woodlands	50.1	66.3	28.8
Distance from ancient woodland	16.6	NA	NA
Woodland type	15.2	NA	40.8
Elevation	12.7	16.8	14.6
Ancient woodland	3.2	NA	NA
Land cover	2.2	9.6	15.8
Distance from urban area	NA	7.3	NA

Probability of *M. bechsteinii* presence increased with proximity to woodland: the response curves declined to a probability of < 0.05 at distances greater than 1,000 m (Figure 3.1). Similar trends were found for elevation where the highest probability of presence for *M. bechsteinii* was found near 20 m, then a decrease was observed to finally reach zero at approximately 300 m elevation. The land cover variable highlights the strong probability of presence of the species in woodlands (Figure 3.1). Although habitat of average quality (~ 0.5) was commonly found throughout southern Britain, highly suitable habitat remained sparse and predominantly found in the southeast of England while other highly suitable areas remained patchy throughout southern Britain (Figure 3.2).

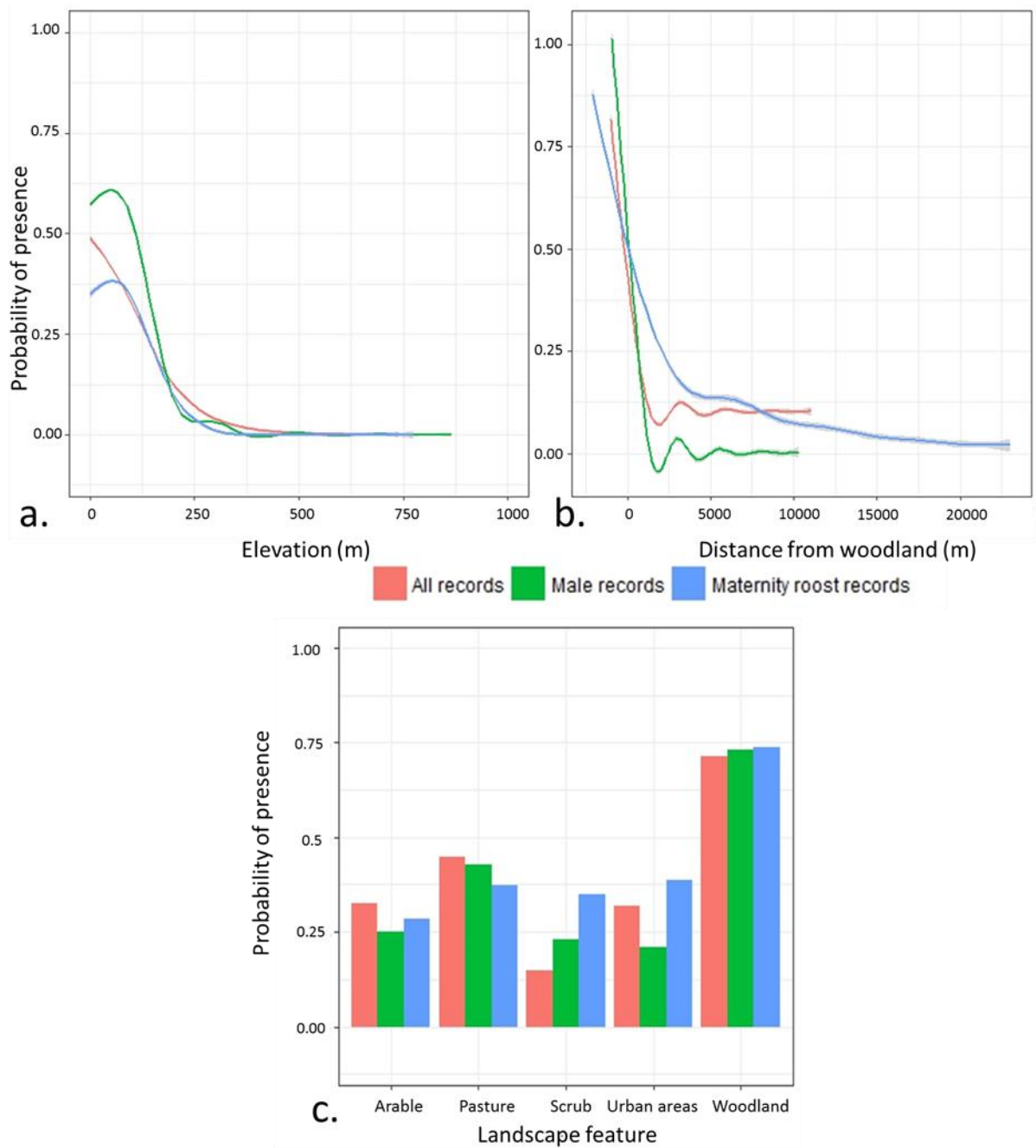


Figure 3.1 Graphs showing the response curves of the probability of presence of *M. bechsteinii* for the three environmental variables (a. Elevation (m); b. Distance from woodland (m); c. Land cover) which contributed towards the final models.

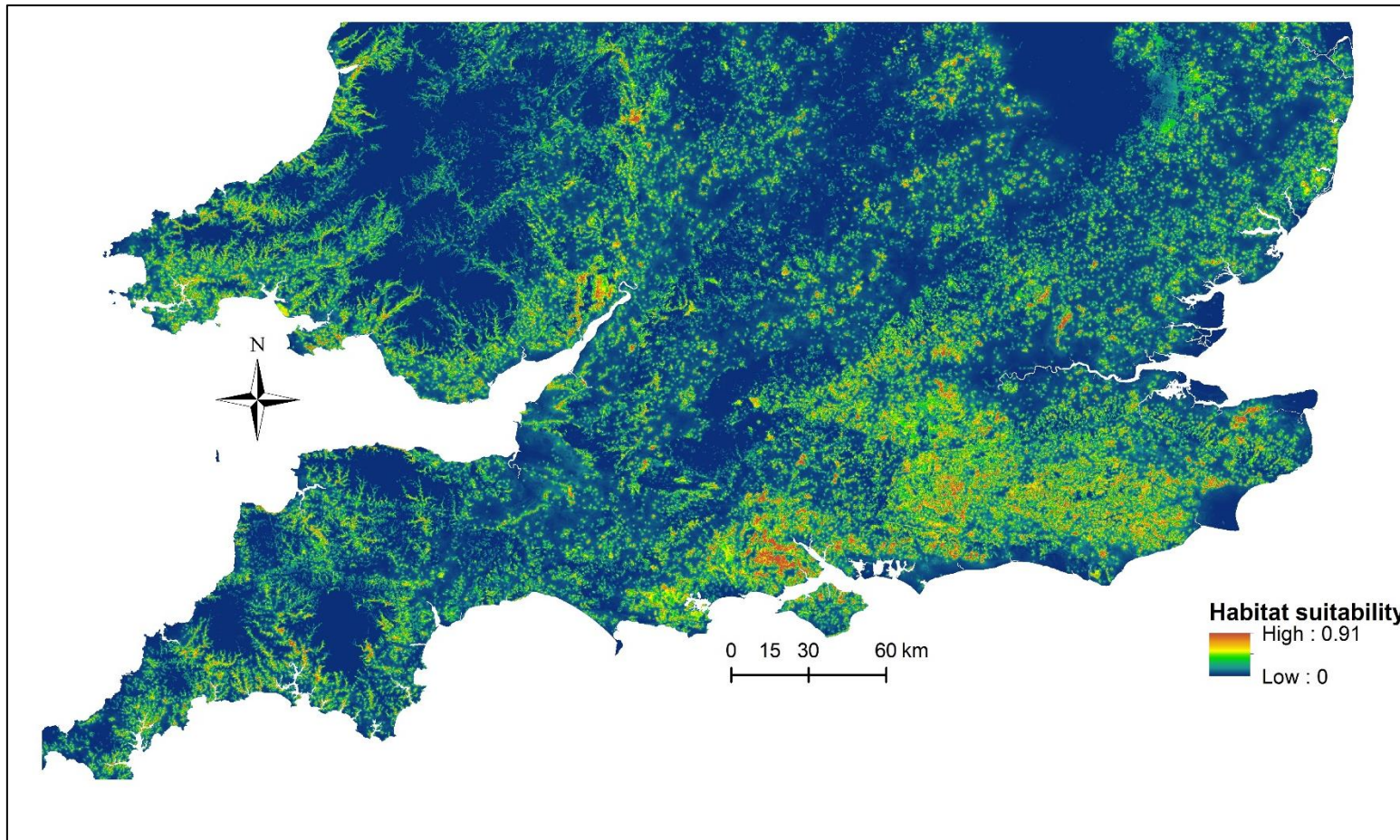


Figure 3.2 Map showing habitat suitability for *Myotis bechsteinii* in southern Britain using the best model from the use of all records. Areas of high suitability are in red, while dark blue represents areas of low suitability.

3.4.2 Habitat connectivity

A total of 6,777 nodes (habitat patches) were identified in southern Britain and 2,677 within the species range at a 500 m resolution (Appendix 3.4). Low numbers of components (NC) and high numbers of links (NL) are representative of a well-connected landscape. While the number of links (NL) constantly increases with dispersal distance, a very strong decline in number of components (NC) is observed between 1,000 m and 5,000 m (Figure 3.3).

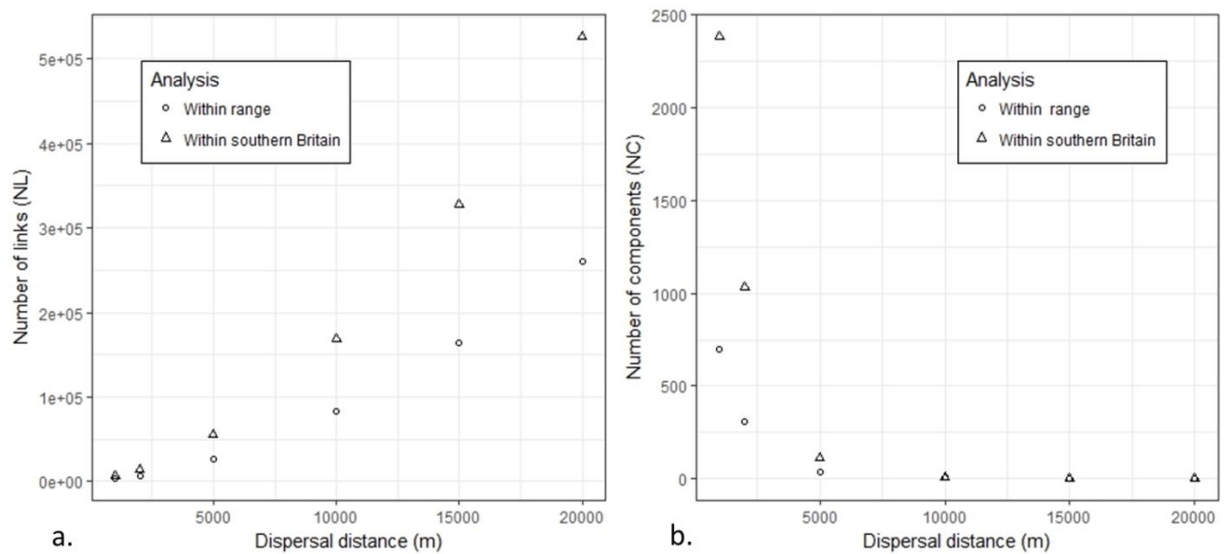


Figure 3.3 Overall Indices of (a) the number of links (NL) and (b) number of components (NC) for *M. bechsteinii* within southern Britain and the species known range for six dispersal distances.

In order to calculate dIIC connectivity values, the number of nodes was reduced to 67 within southern Britain and 20 within the species range by grouping components. The calculation of dIIC connectivity values ranges from 0 to 100 where high scores are given to patches that contribute highly to the overall connectivity. In this study, dIIC values ranged from 0.11 to 43.57 and higher scores were generally given to larger patches (Figure 3.4). Changes in dispersal distance did not affect dIIC values strongly (Table 3.3). While both

types of analysis (Britain and range) calculated similar minimum and maximum values (Table 3.3), the range analysis showed overall higher dIIC values as a consequence of a lower number of nodes.

Table 3.3 Median, minimum and maximum dIIC values for *M. bechsteinii* within southern Britain and the species known range for four dispersal distances (See section 3.3.2)

Measure of connectivity	Dispersal distance within southern Britain (m)				Dispersal distance with species range (m)			
	3,000	8,000	12,000	18,000	3,000	8,000	12,000	18,000
dIIC median	-	3.38	3.13	3.14	-	10.19	9.82	9.68
dIIC min	-	0.13	0.11	0.37	-	1.14	2.97	2.66
dIIC max	-	43.57	42.71	39.81	-	34.59	38.83	42.52

The importance of most nodes within the species range remained similar in both analyses (Figure 3.4b). However, the south eastern patch was identified as being a lot more important in the Britain analysis than in the range analysis as this patch became significantly larger and connected numerous habitats where the species has not yet been recorded (Figure 3.4a).

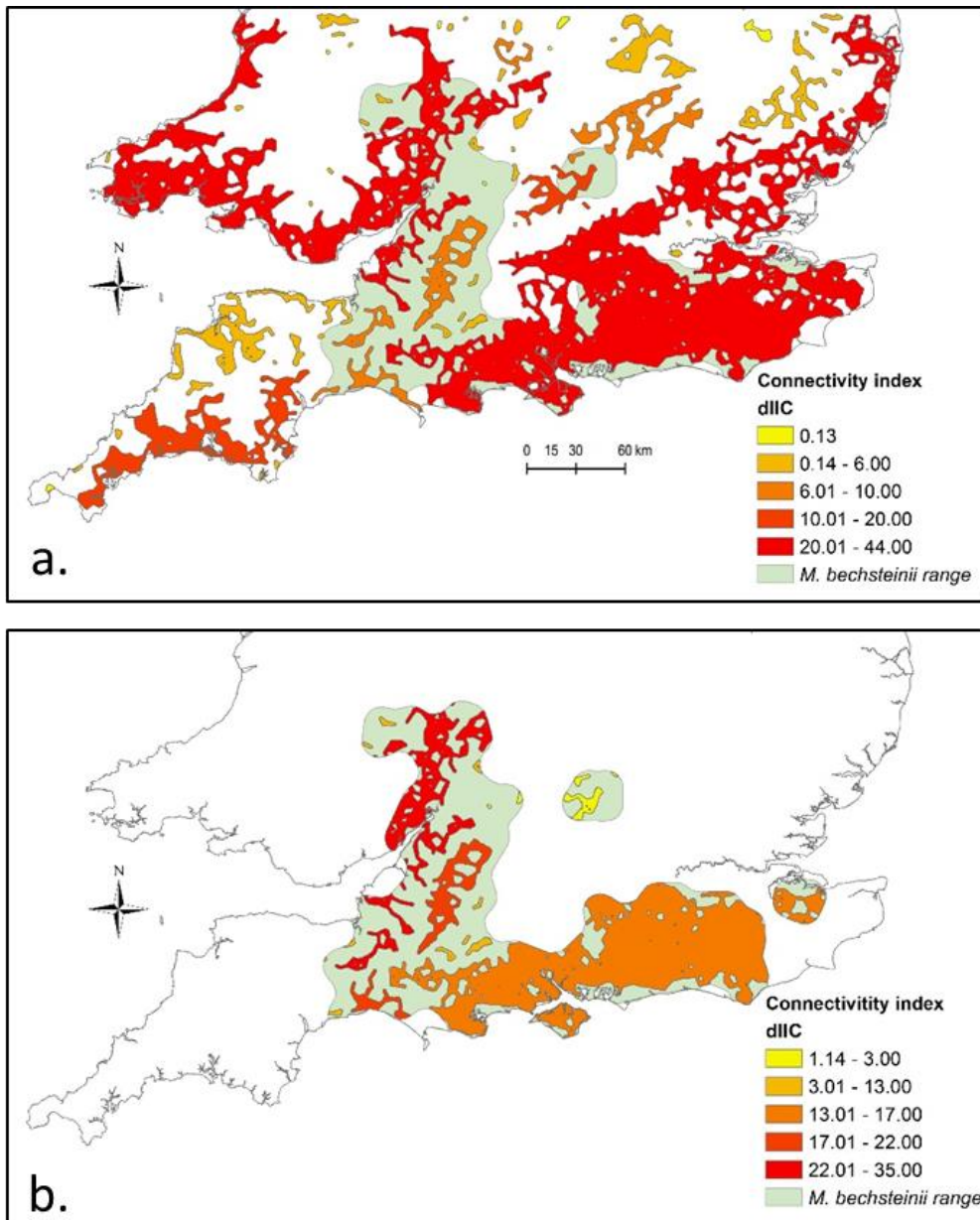


Figure 3.4 Map representing the distribution of connected habitat and their importance in maintaining connectivity using an 8,000 m dispersal distance throughout southern Britain (a) and within the species known range (b). High dIIC scores (red) indicate important habitat patches in maintaining connectivity throughout the landscape while low scores (yellow) indicate habitat patches of low importance.

3.4.3 Landscape genetics

A total of 220 bats were genotyped from 8 sites using 14 microsatellites. There was no evidence of homozygote excess or null alleles detected. One pair of alleles of the microsatellites analysed showed indication of linkage disequilibrium (LD), however this was not detected on the full dataset (Wright *et al.*, 2018) justifying its retention. Tests for Hardy-Weinberg equilibrium (HWE) revealed that three loci showed significant deviation from HWE, but they did not differ from HWE in more than one of the 8 populations. All loci were retained for further analysis as they did not show persistent evidence of inconsistencies across multiple populations.

Table 3.4 Pairwise F_{st} values (lower half of table) and distance (km) (upper half of table) for 8 colonies: 1: Brackett's Coppice, 2: Sherborne, 3: Isle of Wight, 4: Trowbridge, 5: Braydon, 6: Malvern, 7: Grafton Woods, 8: Bernwood,

	1	2	3	4	5	6	7	8
1	-	15.5	104.4	61.1	95.3	138.4	155.5	166.7
2	0.013	-	90.4	50.2	85.1	133.3	148.1	153.5
3	0.022	0.029	-	94.9	113.7	175.4	176.1	134.9
4	0.011	0.019	0.029	-	34.9	88.9	99.7	107.0
5	0.020	0.029	0.035	0.020	-	61.7	66.7	78.9
6	0.021	0.018	0.030	0.024	0.025	-	27.7	101.6
7	0.031	0.033	0.043	0.030	0.043	0.030	-	81.1
8	0.047	0.049	0.062	0.053	0.050	0.046	0.068	-

Landscape genetic analysis using data from eight populations and multiple landscape variables identified land cover as the most important determinant of genetic connectivity between populations ($AIC_c = -334.787$). Resistance values in this model were low for woodlands (Resistance = 1), while all other variables showed high resistance values (Resistance = 52.1 – 3341.35) (Figure 3.5). The null and distance models were ranked second ($\Delta AIC_c = 133.17$) and third ($\Delta AIC_c = 134.06$) respectively (Table 3.5).

Table 3.5 Landscape genetic model rankings for the different resistance surfaces tested on *Myotis bechsteinii*

Landscape feature	AIC _c	ΔAIC _c	R ² _m	R ² _c
Land cover	-334.79	0	0.14843	0.92659
Null model	-201.62	133.17	0	0.90148
Distance model	-200.72	134.06	0.01435	0.90792
Roads	-186.72	148.07	0.39343	0.9353
Urban	-186.07	148.72	0.02393	0.90739
Scrub	-185.76	149.03	0.016081	0.90858
Woodlands	-185.21	149.58	0.02208	0.91148
Ancient woodlands	-185.19	149.6	0.021599	0.91135
Arable	-184.64	150.15	0.15011	0.9296
Sea	-184.6	150.19	0.17281	0.92923
Pastures	-183.24	151.54	0.05642	0.90367

The use of Circuitscape for both the landscape genetics (Land cover) (Figure 3.5 and 3.6a) and the habitat suitability models (3.6b) showed good connectivity in the southeast of Britain and reduced connectivity further north. While similar outputs were retrieved from the use of Circuitscape on both resistance surfaces, the landscape genetic model (3.6a) displayed higher connectivity than the habitat suitability model (3.6b).

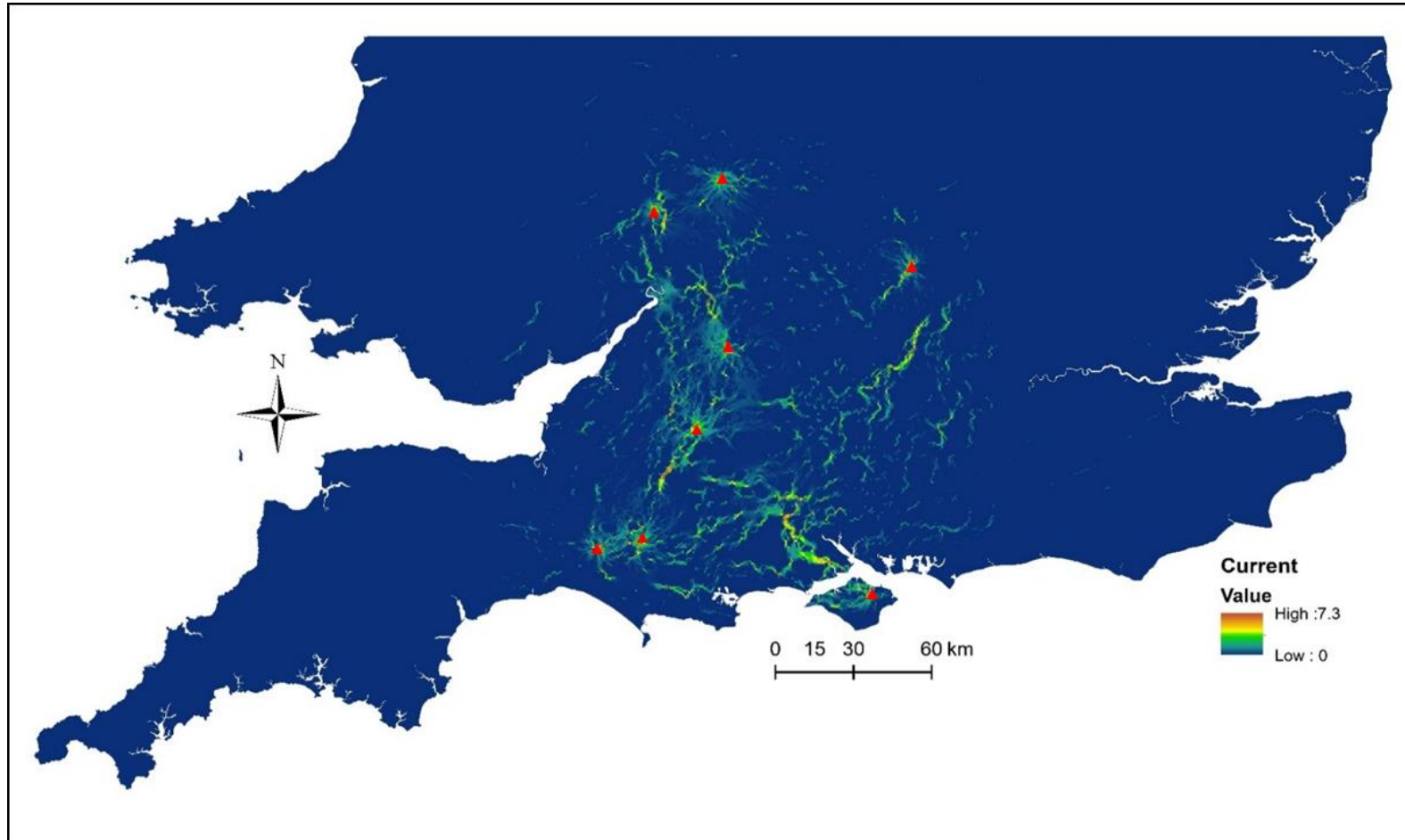


Figure 3.5 Map showing current flow according to the land cover model and demonstrating areas of potential *M. bechsteinii* gene flow across Britain between eight sampled populations (marked with red triangles) using 14 microsatellites.

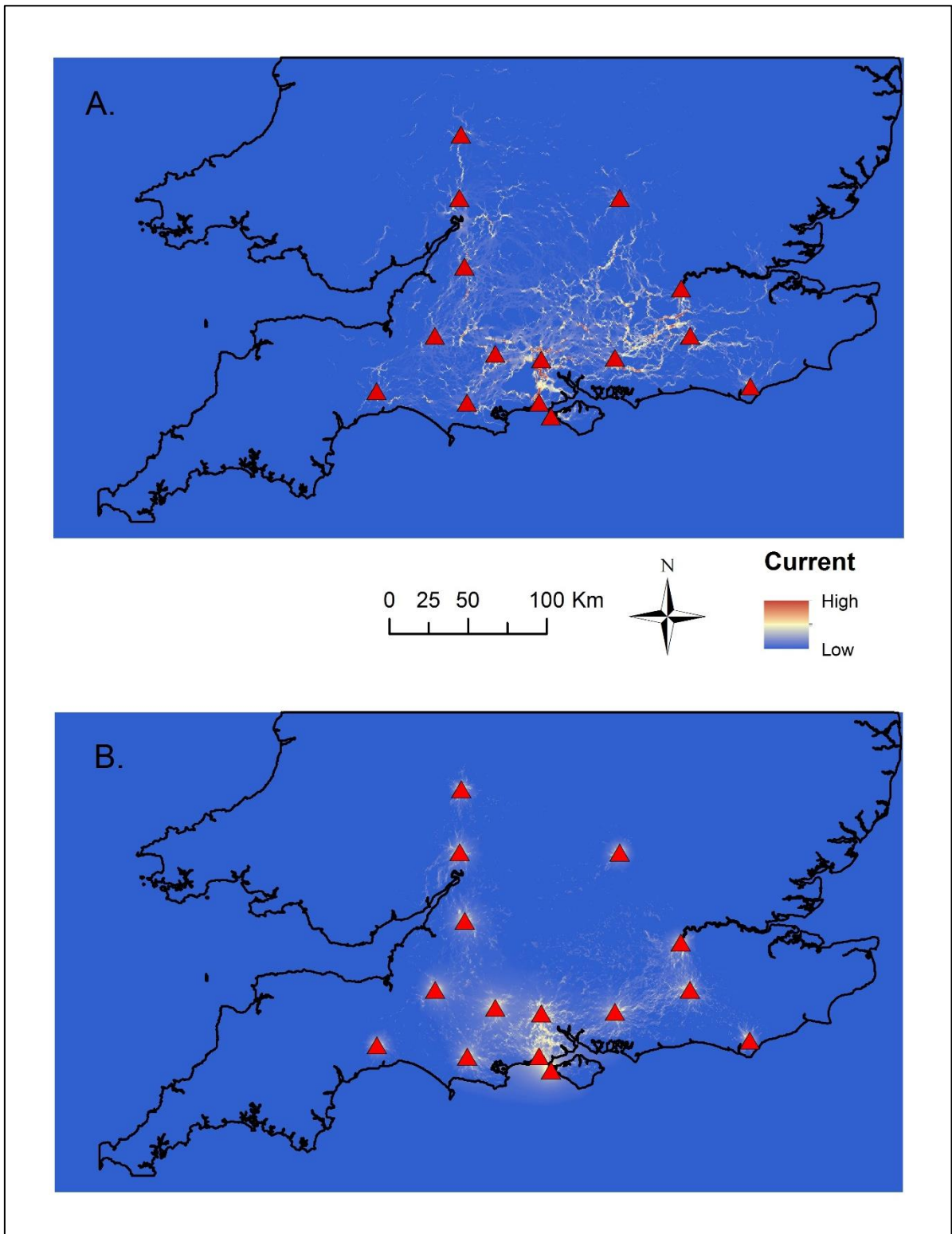


Figure 3.6 Map showing current flow between 15 points (marked with red triangles) chosen randomly within *M. bechsteinii* range according to a) the landscape genetics model and b) the habitat suitability model for all presence data (high habitat suitability with low resistance scores)

3.5 Discussion

Both the habitat suitability modelling and landscape genetics analysis indicated that woodland is a key contributor towards the presence and increased genetic connectivity of *Myotis bechsteinii* in Britain. Woodlands and their connectivity have long been recognised as a key component of bat conservation as they provide substantial food and roosting opportunities (e.g. Russo, Cistrone, Jones, & Mazzoleni, 2004; Smith & Racey, 2008). As a woodland specialist, *M. bechsteinii* depends almost entirely on the presence of old growth broad-leaved woodlands for roosting and foraging (Arrizabalaga-Escudero *et al.*, 2014; Becker & Encarnação, 2012; Dietz, Dawo, Krannich, & Pir, 2015; Dietz & Pir, 2011; Eggers & Köhler, 2012). The species presence was subsequently expected to be positively influenced by such habitats in Britain. However, aside from the importance of woodlands, only elevation appeared to have a clear adverse impact on the species presence as the probability of presence reduced to almost 0 at 250 m. This trend may slightly be influenced by sampling bias as low elevation woodlands are often targeted for *M. bechsteinii* (Miller, 2011). Nonetheless, it has already been shown that mountainous habitats, such as the Alps, have impacted the genetic structure of this species (Chapter 2) and that maternity colonies are rarely found above 250 m in continental Europe (Dietz & Pir, 2011). In Britain, such altitudes are often associated by upland heathland and therefore support large areas of land devoid of trees which are unsuitable for *M. bechsteinii*.

While the preservation of woodlands for maternity roosts is important, this study also highlighted the importance of all woodlands in the conservation of *M. bechsteinii*. Maternity roosts were associated with high quality habitat. This is

most likely explained by the high energetic demands of pregnancy and lactation (Racey & Entwistle, 2000; Ruckstuhl & Neuhaus, 2005). The presence of males, however, was associated with “sub-optimal” habitat characterised by small fragmented patches of woodland or coniferous plantations. Such woodlands may not be able to host maternity colonies, however the landscape genetics analysis suggested that all woodlands have a positive influence on gene flow as opposed to other landscape features (e.g. urban areas and agricultural land). Genetic studies have previously identified woodland fragmentation as a driver for genetic isolation and population decline for certain bat species due to their strong site fidelity and slow reproduction rate (Struebig, Kingston, Zubaid, Mohd-Adnan, & Rossiter, 2008b). In this study, *M. bechsteinii* habitat was found to be highly fragmented (> 7,000 habitat patches at a 500 m resolution); and dispersal distances below 5,000 m showed very high numbers of components (groups of connected patches). Such fragmentation could negatively impact the species and also provided better insight on the species genetic structure between the northern and southern populations (See Chapter 2) as distinct breaks corresponding to the difference in genetic structure was identified. This suggests that *M. bechsteinii* depends highly on suitable habitat to reach breeding sites and that individuals will rarely disperse over 5,000 m of unsuitable habitat.

Future work must try to better understand and integrate landscape connectivity between colonies and these breeding sites. For example, improvements in GPS radio-telemetry and the usage of resistance surfaces may soon provide information on the commuting routes bats take to travel from maternity roosts to swarming sites. Such methods would also provide more informative results than the graph theory connectivity analysis as habitat found between patches could

be included. For example, the use of high resolution data (e.g. LiDAR,) may provide information on the importance of small features, such as hedgerows or the prevalence of mature oaks, in areas with limited habitat.

The negative association of *M. bechsteinii* presence with distance from woodland was identified as the main variable determining habitat suitability, but it may also be a key determinant factor explaining the species current distribution. The post-glacial range expansion of the species may be dependent on the presence of woodland nearby as *M. bechsteinii* has limited dispersal abilities in comparison to similar species (e.g. *M. nattereri*) (Parsons & Jones, 2003). The colonisation of new habitats and subsequent range expansion would have occurred at a slower rate. This may have an important impact on the species ability to adapt to threats, such as climate change (Razgour *et al.*, 2017; Rebelo *et al.*, 2010). Therefore, it is important to identify areas of high suitability outside the known range of *M. bechsteinii* and to ensure the species ability to colonise new areas by increasing their connectivity. The habitat suitability model identified abundant habitat outside the species known range that was well connected to the species current range. This method suggests that the species could possibly expand further west in southern England and Wales and that the south eastern population may also expand north-eastwards.

Identifying potential threats to *M. bechsteinii* and understanding how these affect populations is essential for conserving this elusive species. In this study, I used a combined approach of habitat suitability, landscape connectivity and landscape genetics analyses to identify key environmental features affecting the species presence and connectivity through the landscape. This study gives a better understanding on how human dominated landscapes affect connectivity between populations of this elusive species. The findings highlighted the

importance of woodlands not only for the presence of *M. bechsteinii*, but also for maintaining genetic connectivity between populations as isolation can lead to a loss of genetic diversity and threaten the viability of certain populations.

Therefore, it is essential to also develop methods to efficiently monitor at risk populations.

3.6 Appendices

Appendix 3.1 List of landscape variables along with their source used for the habitat suitability model.

<u>Landscape variable</u>	<u>Source</u>
Ancient woodland	(NaturalEngland, 2016; NRW, 2011)
Distance to ancient woodland	(NaturalEngland, 2016; NRW, 2011)
Distance to major road	EDINA (2017)
Distance to urban areas	Corine land cover (Aune-Lundberg & Strand, 2010)
Distance to woodland	Corine land cover (Aune-Lundberg & Strand, 2010)
Elevation	OS Terrain 50 (OSS, 2017)
Land cover	Corine land cover (Aune-Lundberg & Strand, 2010)
Slope	OS Terrain 50 (OSS, 2017)
Solar index	NERC162 (Suggitt <i>et al.</i> , 2014)
Wetness	NERC162 (Suggitt <i>et al.</i> , 2014)
Woodland type	Forest Research (2017)

Main window:

Output: Logistic

Make pictures of predictions: Unchecked to save memory

Feature type: Autofeatures

Basic tab:

Random test percentage: 0

Regularization multiplier: 1

Replicates: 5

Replicated run type: Cross validation

Advanced tab:

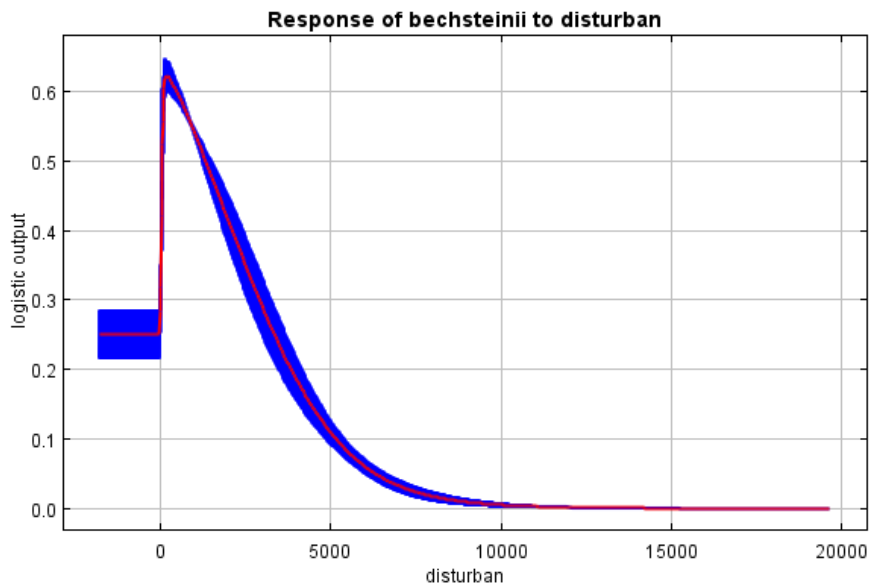
Model iterations: 500

Write output grids: Unchecked to save memory

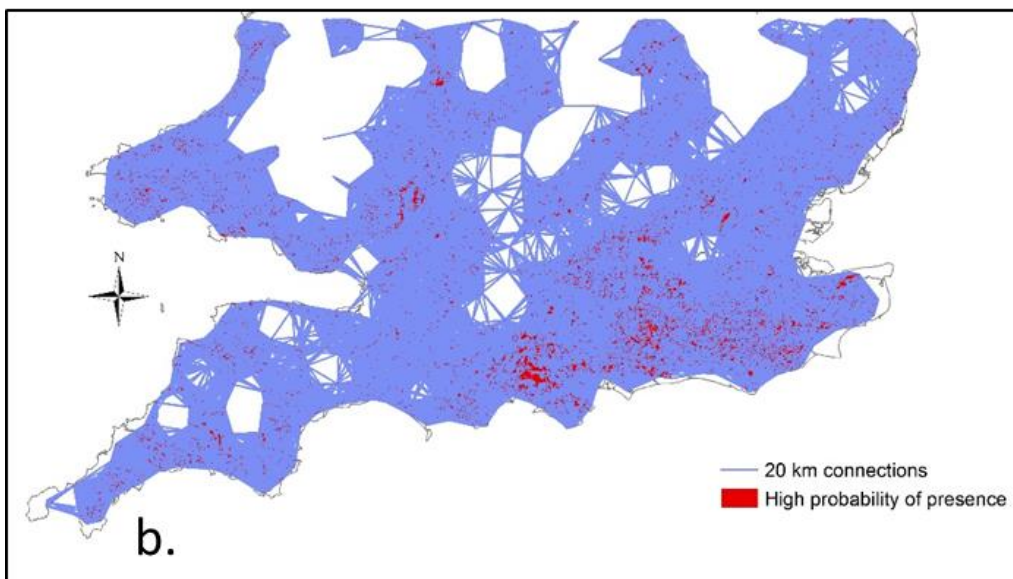
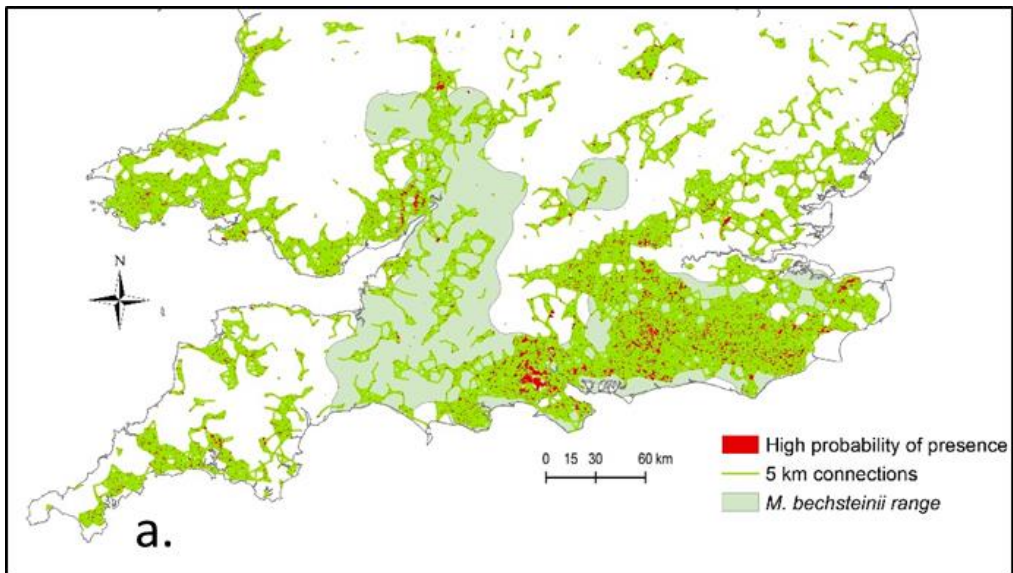
Random seed: Unchecked

All other settings can be kept at their default

Appendix 3.2: Settings used for the top scoring MaxEnt models



Appendix 3.3: MaxEnt output of the response curve showing the probability of presence of male *M. bechsteinii* for distance from urban areas.



Appendix 3.4 Map representing connections between habitat patches (nodes) with a) a 5,000m m dispersal distance and b) a 20,000 m dispersal distance.

Chapter 4 Testing the use of non-invasive genetic sampling and demographic history analysis to monitor woodland bat populations



4.1 Abstract

Information on population size is crucial for the effective management of wild populations. However, such data can be particularly difficult to collect as certain species will only very rarely be captured or detected. Although colony size of certain bat species can be estimated by simple counts (e.g. horseshoe bats), making estimates of colony size is much more challenging for woodland bat species as they can use over 60 roosting sites and perform complex fission-fusion behaviour. Here, I investigated the use of non-invasive capture-mark-recapture (CMR) and demographic history models to estimate the population size and changes of a rare woodland bat (*Myotis bechsteinii*) at a local site based scale (single woodland) and national scale. I collected droppings from underneath five known roosting sites from a single colony. All droppings belonging to *M. bechsteinii* were then genotyped at nine loci in order to differentiate individuals. Among the 294 droppings collected, 123 belonged to *M. bechsteinii* but all microsatellites showed very low amplification rates (<25%) indicating very low quality samples. However, models based on the correlation between sample size and the number of alleles at each locus allowed the production of an estimate of 8 for the minimum number of individuals. Then, I used Migraine (Version 0.4.1) using a dataset of 260 genotypes from across the species range in Britain to assess effective population size and variation of the British populations of *M. bechsteinii* over time. The demographic models estimated the effective population size at 6,569 (CI: 5,307-8,006) and suggested that the British population of *Myotis bechsteinii* is stable and possibly spreading further west.

4.2 Introduction

Accurate information on both the presence of a species and its population size is essential for effective wildlife management. Estimates of population size often depend on how well individuals can be detected. The study of elusive and cryptic species remains limited as methods are often intrusive and time consuming (e.g. Zylstra, Steidl, & Swann, 2010), compared with those suitable for long-term monitoring of more readily visible species such as butterflies (Fox *et al.*, 2015), birds (Gregory *et al.*, 2005), reptiles (Sewell, Guillera-Aroita, Griffiths, & Beebee, 2012) and certain mammals (Croose, Birks, Schofield, & O'Reilly, 2014; Wright, Newson, & Noble, 2014). In addition, the methods often deployed, such as direct counts and capture-mark-recapture studies, often fail to yield the information required because of the difficulty of observing a sufficiently high proportion of the population.

Advances in molecular techniques have been used to identify species and individuals from non-invasive samples, such as faeces, shed hair or feathers (Morin & Woodruff, 1996). Such methods can provide insight into distribution, densities and sex ratios of a number of species, such as mountain goats (Poole *et al.*, 2011), chimpanzees (Arandjelovic *et al.*, 2011), caribous (Hettinga *et al.*, 2012) or brown bears (Kindberg *et al.*, 2011). Many of the estimates made through the use of capture-mark-recapture analysis (CMR) have proven to be efficient and reliable. However, difficulties in collecting sufficient samples combined with the high risk of DNA degradation and contamination in non-invasive methods often results in low amplification success rates and genotyping errors (Herrmann & Henke, 1999; Lampa *et al.*, 2013; Taberlet & Luikart, 1999). Such errors can subsequently result in the over- (Creel *et al.*,

2003) or underestimation (Mills *et al.*, 2000) of population size. Hence, there is a need to apply rigorous sampling and laboratory protocols in order to minimize such errors.

The calculation of effective population size (N_e) by using neutral genetic markers has been increasingly applied in conservation biology, giving a representation of the idealised population size (the number of individuals in a population that contribute to the next generation) and past demographic changes (Frankham, Bradshaw, & Brook, 2014). For example, effective population size estimates have provided insights into pre-whaling populations which appear to be strongly underestimated by historical records (Roman & Palumbi, 2003).

Bats fill a wide range of ecological niches and are valued for the ecosystem services they provide as pollinators, seed dispersers and pest consumers (Kunz, Braun de Torrez, Bauer, Lobo, & Fleming, 2011). However, it has been recognised that increased human pressures, such as habitat loss and modification (Chaverri & Kunz, 2011; Mickleburgh *et al.*, 2002), have led to a global decline of bats (Jones, Mickleburgh, Sechrest, & Walsh, 2009). Despite representing one fifth of mammalian species, their small size and nocturnal lifestyle means that the monitoring of bat populations remains a significant challenge, which non-invasive genetic monitoring may help to meet.

Bat populations are most commonly measured by performing emergence or hibernacula counts (e.g. Warren & Witter, 2002). These can provide valuable information on species presence, population size and activity when coupled with extensive acoustic surveying (e.g. Wickramasinghe *et al.*, 2003). However, these methods also have important drawbacks in cluttered habitats, such as

woodlands, as unlike building dwelling bats, where roosts are frequently known, there is a lack of information on the location of tree roosts. Furthermore, bats are harder to detect in cluttered habitats using acoustic surveying techniques (Russ, 1999; Schnitzler & Kalko, 2001). There is also considerable difficulty in distinguishing species such as *M. bechsteinii* from several other bats in the genus *Myotis* based on acoustic records. While more invasive methods, such as mist-netting and harp trapping, may better inform the species composition and populations of an area, these methods are time consuming and can also be biased by avoidance behaviour (Larsen *et al.*, 2007) or interspecies differences in capture rates (Berry *et al.*, 2004).

The use of non-invasive genetic sampling CMR studies has only been successfully tested with bats for lesser horseshoe bats, *Rhinolophus hipposideros*, by genotyping DNA from droppings on a single sampling session (Puechmaille & Petit, 2007; Zarzoso-Lacoste *et al.*, 2017). This species is an ideal candidate for CMR studies as they roost at a single site and droppings are easily preserved in sheltered areas (caves, barns...) which reduces the rate of DNA degradation and facilitates the collection of samples. On the other hand, woodland bats frequently change roosts. For example, *Myotis bechsteinii*, will regularly switch roosts and use over 60 roosting sites within a single summer whilst undergoing fission-fusion behaviour within the colony (Kerth & König, 1999; Kerth & Reckardt, 2003; Reckardt & Kerth, 2007; Weir *et al.*, 1997). The accumulation of droppings in small enclosed spaces provides an important limitation for CMR studies as all samples are likely to be strongly contaminated by other individuals, and there may also be high rates of degradation.

The aim of this study was (1) to evaluate the use of non-invasive genetic sampling techniques to estimate the number of individual bats within a colony of

M. bechsteinii and (2) to assess the demographic history of the species at a local (colony) and national scale. For this I collected droppings from underneath bat boxes over the course of the months of June 2015 and June 2016 and genotyped them at nine microsatellite loci. Then, I used a demographic history analysis software Migraine Version 0.4.1 (Leblois, Beeravolu Reddy, & Rousset, 2013) to investigate variations in population size over time by using the genetic data from Chapter 2.

4.3 Materials and Methods

4.3.1 *Non-invasive capture-mark-recapture*

4.3.1.1 Study site

All samples were collected from Brackett's Coppice (Dorset, England), a 38 ha ancient woodland nature reserve. A total of 80 Schwegler 2FN boxes and 5 1FW hibernation boxes are present in this woodland. A single *M. bechsteinii* maternity colony has used these boxes since 1998 and has been monitoring using banding techniques since that time.

4.3.1.2 Sample collection and DNA extraction

Bats frequently defecate on emergence from their roost. Therefore, 1mm white insect screening mesh was placed beneath all five 1FW hibernation boxes (box A, B, C, D & E) to catch the droppings before they reached the ground. The mesh makes droppings more visible and keeps them drier. The amount of insect mesh placed under each box varied due to the proximity of trees and vegetation but approximated 1.5 x 2 m. Sampling took place during the month of June in 2015 and 2016. The population was most likely closed during this month because all adult females were likely to be present in the hibernation boxes (large boxes) and no juveniles would yet be born. The mesh traps were checked every 2-3 days. Droppings were collected using sterile tooth picks and

stored individually in absolute ethanol in 1.5 ml microtubes. Samples were then stored at -4°C. DNA extraction was performed using a QIAmp DNA Stool Mini Kit (QIAGEN) following the protocol recommended by Puechmille *et al.* (2007).

4.3.1.3 Species identification and microsatellite genotyping

I performed a duplex-qPCR using species specific primers for *Myotis bechsteinii* (MbeccytbF: ACAATCCAATAGGAATCCCCTCTA; MbeccytbR: CTAATAGG-CCGAGGATGTCTTTG) and *Nyctalus noctula* (NnoccytbF: GCCGACCTT-GTTGAGTGAATTTGA; NnoccytbR: AAGTGAAAGGCGAAAAATCGAGT-TAGG) (Harrington, 2015), a species expected to be commonly found in these boxes. Species identification was carried out in 10 µl volume reactions using 5 µl of Faststart Universal SYBR Green Master (ROX) (Roche), 0.2 µM of each primer and 1 µl of extracted DNA. Samples were analysed in duplicates and each plate included two negative controls with H₂O instead of DNA, along with positive controls (two *M. bechsteinii* samples, two *N. noctula*, one *M. nattereri* and one *Plecotus auritus*). The multiplex was also tested on *M. daubentonii* and *M. mystacinus* prior to analysis in order to ensure the specificity of the duplex. All amplifications were carried out using a BioRad iCycler and qPCR conditions consisted of 2 minutes (m) at 50°C, 10 m at 95°C, followed by 30 cycles of 15 seconds (s) at 95°C and 60 s at 60°C. Fluorescence was measured and recorded at the end of each cycle. A final dissociation step was added for melt curve analysis to distinguish *M. bechsteinii* and *N. noctula* using a temperature gradient starting at 60°C and gradually increasing by 0.5°C every 15 s to finally reach 95°C. All samples for which the cycle threshold was over 20 were considered species other than *M. bechsteinii* or *N. noctula* or poor quality samples and were discarded.

All *Myotis bechsteinii* samples were then genotyped in duplicate using a set of nine microsatellite loci previously tested on *M. bechsteinii* droppings (Chapter 2, Appendix 4.3). The microsatellites were amplified in two PCR multiplexes before being pooled together for genotyping (Appendix 4.1). PCR was carried out in 10 µl volume reactions containing 5 µl of Type-it Microsatellite PCR Kit (QIAGEN), 1 µl of Q solution, 1 µl of RNase free water, 1 µl of extracted DNA and 2 µl of primer mixture. A touchdown extra-long PCR program was used, consisting of 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 90 s at the annealing temperature [62°C (3 cycles), 58 (4), 55 (5), 53 (10), 51 (5), 49 (5), 47 (5)] and 3 min at 72°C, then 72°C and 60°C for 10 and 35 min respectively followed by a final 10 min extension step at 72°C. Genotyping was performed on a Beckman Coulter CEQ™ 8000 Genetic Analysis System.

4.3.1.4 Data analysis

I planned two potential ways to analyse the data depending on the results from the sample collection (Figure 4.1). A capture-mark-recapture analysis (Analysis A) would be undertaken if sufficient *M. bechsteinii* droppings provided reliable genotypes (> 20) and an estimate based on the relationship between the number of individuals and number of alleles detected for each locus (Analysis B) would be undertaken if insufficient data were collected.

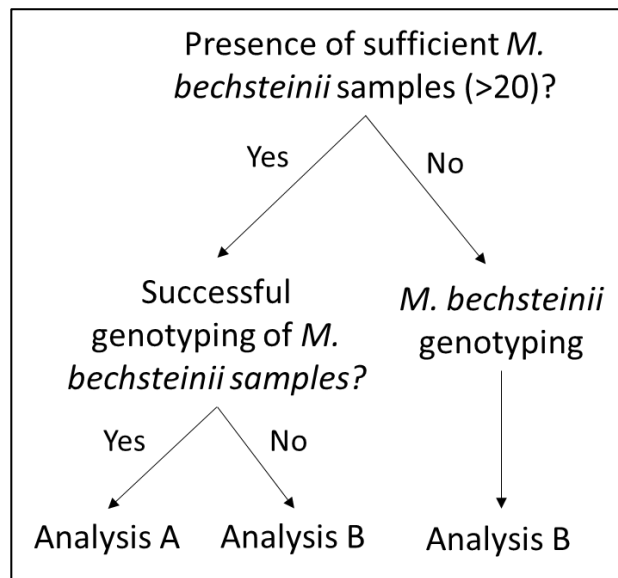


Figure 4.1 Decision flowchart for the type of analysis to undertake depending on sample collection and genotyping results.

Analysis A: After genotyping all *M. bechsteinii* samples twice, I would use the comparative multi-tube approach to reduce genotyping error rates (Frantz *et al.*, 2003; Paetkau, 2003; Puechmaille & Petit, 2007). In this approach, I would discard samples if a consensus genotype was obtained at fewer than four loci as they would be considered to be low-quality samples. Samples with four to six loci accepted would be re-amplified twice more in order to complete the genotype. Samples completed at seven to nine loci would be reamplified once if they differed at one allele or twice if they differed at two alleles. Then, samples offering complete genotypes at six or more loci would be reamplified once to complete the genotype (Figure 4.2).

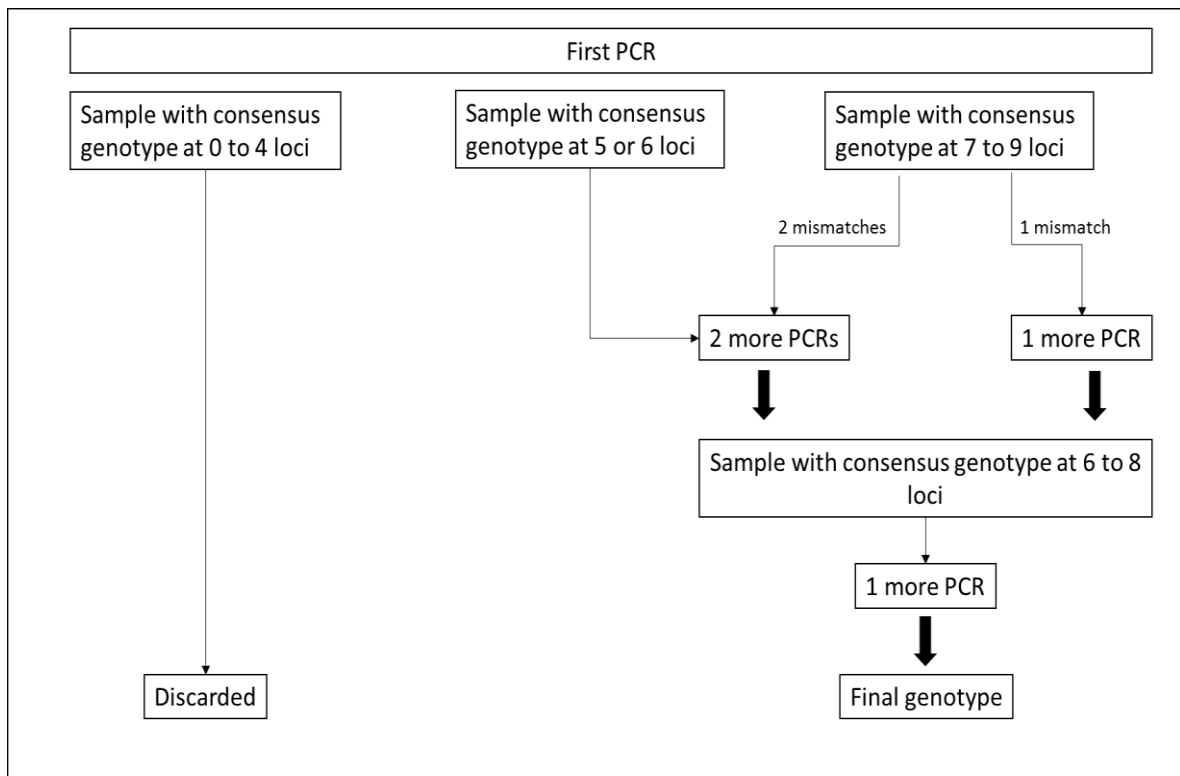


Figure 4.2 Flow chart illustrating the multi-tube procedure to reliably genotype all *M. bechsteinii* droppings.

Allelic dropout and false alleles was calculated using the same methods as Puechmaille and Petit (2007), while P_{ID} was calculated using CERVUS 3.0 (Kalinowski, Taper, & Marshall, 2007). GENALEX 6.5 (Peakall & Smouse, 2012) was used to calculate indices of genetic diversity, such as observed heterozygosity (H_o), expected heterozygosity (H_e), number of alleles (N_a) for each locus. Then, I used the maximum likelihood estimator (Miller, Joyce, & Waits, 2005) from the Capwire software (<http://www.cnr.uidaho.edu/lecg>) as this model assumes a closed population, a similar capture and recapture probability and an equal capture probability for all individuals.

Analysis B: In the case that too few samples amplified to perform a capture-mark-recapture analysis (e.g. failing to give a consensus genotype at a minimum of four loci), I directly reported amplification success and indices of genetic diversity, such as observed heterozygosity (H_o), expected

heterozygosity (H_e), number of alleles (N_a) for each locus using GENALEX 6.5 (Peakall & Smouse, 2012). Then, I would perform a Generalised Additive Model (GAM) based on the non-linear relationship between the number of alleles and the number of individuals in order to estimate the minimum number of individuals identified from the droppings collected. For this, I used the genotypes of 60 individuals from the same colony (Chapter 1). Then, I selected 20,000 times a random number of individuals ranging from 1 to 60 and counted the number of alleles at each locus. The number of individuals was then used as the response variable and the number of alleles at each locus as predictor variables. The model would then be logged to achieve normality and model validation would be conducted by the examination of residuals.

4.3.2 *Demographic history*

We investigated changes in the effective population size of *M. bechsteinii* over time using the software Migraine Version 0.4.1 (Leblois *et al.*, 2013). Migraine uses the class of importance sampling algorithms (IS) (De Iorio & Griffiths, 2004) and was further extended by Leblois *et al.* (2014) by using a generalized stepwise-mutation model (GSM) allowing the insertion or deletion of multiple microsatellite repeats. Although the likelihood-based method is more mathematically and computationally demanding, this method is an improvement on other frequently used alternative programs such as MSVAR (Beaumont, 1999) which assumes a strict stepwise mutation model.

To test for population size variations, Migraine estimates contemporary and ancestral scaled population sizes ($\theta = 2N\mu$ and $\theta_{anc} = 2N_{anc}\mu$, where N and N_{anc} are the contemporary and ancestral diploid population sizes and μ is the mutation rate per generation) and the timing of demographic changes ($Dg\mu$).

The strength of the demographic events is characterised using the N_{ratio}

parameters ($N_{\text{ratio}} = \theta/\theta_{\text{anc}}$). Population declines are represented by $N_{\text{ratio}} < 1$ and expansion by $N_{\text{ratio}} > 1$. Significant changes in population size are indicated where 1 lies outside the 95% confidence intervals (95% CIs) of the N_{ratio} parameter.

To interpret the results from Migraine into effective population size (N_e) and timing of the event in generations, I used a common mutation rate of 5×10^{-4} for microsatellites (Sun *et al.*, 2012; Yue, David, & Orban, 2007). The conversion of timing to years was defined by using a period of 5 years for a generation.

Analyses were conducted with Migraine on seven British populations separately to test for local population changes. The Grafton population was excluded as it had insufficient samples for reliable demographic analysis (<15). The same analysis was then performed on the entire British dataset, but also separately on the northern and southern populations which were found as being genetically distinct (Chapter 2). All runs in Migraine were made using 20 000 trees, 2400–5000 points and 3–10 iterations. I also calculated N_e using the OnePop model for the British population from Migraine as it gives more reliable results when no variation in population size is detected. The OnePop model infers a single parameter (current N_e) as opposed to the prior analysis which infers an ancestral and contemporary population size.

4.4 Results

4.4.1 Capture-mark recapture

DNA was extracted from 294 bat droppings and *Myotis bechsteinii* was identified from 123 (42%) of them while only 14 (0.05%) were identified as *Nyctalus noctula*. All other samples were classified as unidentified, although it is thought that 23 samples belonged to *Myotis nattereri* as controls revealed a

distinguishable non-specific amplification for *M. nattereri* (Ct ~ 25 cycles) (App 4.2). Most of the droppings were collected from box B (256), while box A, C & D had less than 15 droppings and box E had none (Figure 4.3).

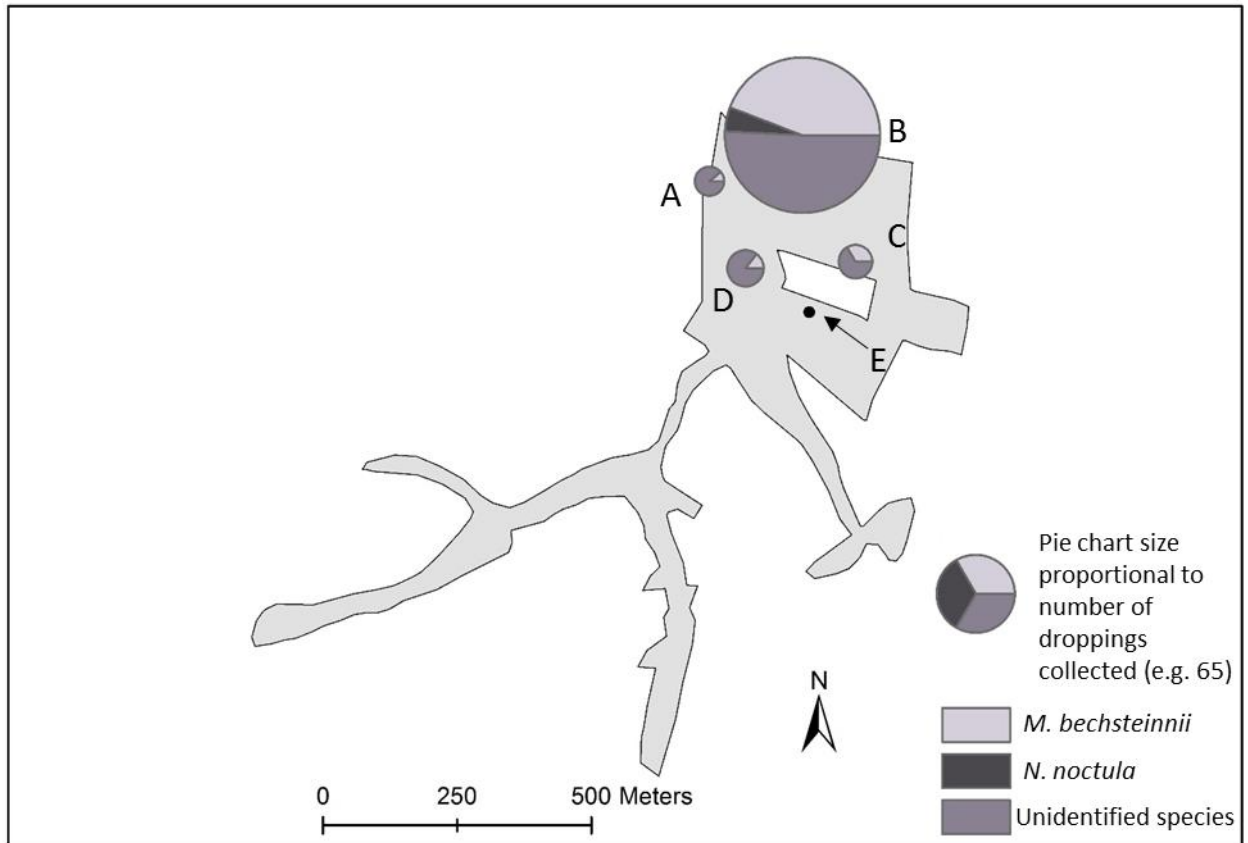


Figure 4.3 Map of the study site, Brackett's coppice, with pie charts at the location of each bat box representing the amount of droppings collected for *M. bechsteinnii*, *N. noctula* and other species (size of pie charts are proportional to the number of droppings collected).

Data analysis B was performed as of the 123 *M. bechsteinnii* droppings only 42 amplified at least one locus. The amplification success rate of all loci was low (<25 %) and was at 0 % for both A24-Mluc and CloneA2 and only five droppings were successfully genotyped at five loci or more and from these five different individuals were successfully identified. Despite low amplification success rates, the number of alleles (N_a & N_e) and levels of heterozygosity (H_e

& Ho) at each locus were only slightly lower than those from the 60 wing samples genotyped from the same colony (Table 4.1).

Table 4.1 Genotyping results from the 123 *Myotis bechsteinii* compared to the full genotypes of 60 individuals from the same colony using wing punches showing the number of droppings successfully genotyped at each loci (N), the percentage amplification, the number of alleles (Na), the number of effective alleles (Ne), observed (Ho) and expected heterozygosity (He).

Locus	CMR samples						Wing samples			
	N	%	Na	Ne	Ho	He	Na	Ne	Ho	He
<i>MS3D02</i>	27	22.0	10	3.79	0.78	0.74	15	3.91	0.75	0.74
<i>G6</i>	26	21.1	4	2.05	0.54	0.51	4	2.81	0.57	0.64
<i>B23</i>	14	11.4	9	6.6	0.64	0.85	13	8.30	0.83	0.88
<i>B22</i>	12	9.75	5	3.03	0.67	0.70	7	3.36	0.67	0.70
<i>C112</i>	7	5.69	5	3.77	1	0.74	8	3.10	0.78	0.68
<i>H23-Mluc</i>	6	4.87	5	3.60	0.67	0.72	11	6.02	0.84	0.83
<i>Mluc8</i>	1	0.813	2	2.00	1	1	8	5.40	0.82	0.81
<i>A24-Mluc</i>	0	0	0	0	0	0	7	4.3	0.85	0.77
<i>CloneA2</i>	0	0	0	0	0	0	9	4.41	0.77	0.77

The Generalised Additive Model designed to estimate the minimum number of individuals from the number of alleles identified at each locus from the 42 droppings showed high predictive power ($R^2=0.95$, $p<0.0001$; Appendix 4.4) and estimated the minimum number of individuals in the box at 8.3 (SE 0.15).

4.4.2 Demographic history

According to the N_{ratio} estimates produced by Migraine, only two of the seven sampled sites showed significant demographic changes in the past. A significant population contraction ($N_{ratio}= 0.019$) dating despite strong confidence intervals most likely from the past century was detected in the population from Malvern using a mutation rate of 0.0005 and a generation period of five years. An expansion was identified in the Brackett's Coppice population ($N_{ratio}= 684$) (Table 4.2). This expansion is more ancient than the

Malvern contraction but cannot be dated accurately due to large confidence intervals. All other populations show overlap between Anc θ and θ which indicates stable populations.

The British population and Cluster B (northern population in Britain) which was identified in Chapter 2 both appeared to be stable. On the other hand, the southern population in Britain (Cluster A) is showing a significant expansion which cannot be dated precisely due to the large confidence intervals. Point estimates and confidence intervals for effective population size estimates have no meaning for stable populations; therefore I inferred the scaled population size (θ) for each of the British populations under the OnePop model as it only infers a single parameter. The effective population size of the British population was estimated at 6,569 (CI: 5,307-8,006) using the OnePop model.

Table 4.2 Migraine outputs of demographic analysis expressed by the current Act θ and ancestral Anc θ population sizes, N_{ratio} (Act θ / Anc θ) and the scaling of time by the mutation rate ($Dg\mu$) since the demographic change occurred for *M. bechsteinii*.

Cluster A: Brackett's Coppice, Sherborne, IoW and Trowbridge; Cluster B: Braydon, Malvern, Bernwood, Grafton.

	Anc θ	Act θ	N_{ratio}	$Dg\mu$	Past population variation
Brackett's Coppice	0.010 (0.004 - 4.5)	6.9 (5.0 – 9.0)	684 (1.2 - 1533)	11 (0.49 - 66)	Significant expansion
Sherborne	8.0 (4.3 - 14)	0.0079 (0 - 10)	0.00099 ($3.5e^{-7}$ - 1.2)	0.00082 ($1.2e^{-9}$ - 0.87)	Stable population
IoW	0.087 (0.00010 - 6.7)	7.3 (5.2 - 11)	84 (0.017 - 65205)	7.0 (0.28 - 278)	Stable population
Trowbridge	7.2 (1.1 - 14)	0.016 (0 - 19)	0.0022 ($5.5e^{-7}$ - 5.2)	0.00092 (0 - 0.79)	Stable population
Braydon	6.4 (2.4 - 17)	0.50 (0 - 39)	0.078 ($6.0e^{-7}$ - 9.9)	0.034 (0 - 55)	Stable population
Malvern	9.3 (5.1 - 35)	0.18 (0 - 4.8)	0.019 ($4.6e^{-5}$ - 0.83)	0.014 ($2.7e^{-7}$ - 0.56)	Significant contraction
Bernwood	6.3 (0.022 - 84)	$6.8e^{-8}$ ($2.1e^{-8}$ - 758)	$1.1e^{-8}$ ($2.8e^{-9}$ - 210)	$1.5e^{-8}$ ($1.6e^{-13}$ - 12265)	Stable population
Cluster A	0.13 (0.058 - 2.4)	7.0 (5.4 – 9.0)	54 (1.6 - 105)	7.5 (0.68 - 33)	Significant expansion
Cluster B	7.5 (5.1 - 14)	2.3 (0 - 12)	0.31 ($1.0e^{-6}$ – 2.0)	0.059 ($8.3e^{-9}$ - 0.64)	Stable population
All Britain	3.2 (1.2 - 6.6)	7.5 (5.9 - 9.7)	2.3 (0.90 - 6.5)	1.8 (0.057 - 5.5)	Stable population

4.5 Discussion

4.5.1 Non-invasive capture-mark-recapture

Despite the successful identification of 123 *M. bechsteinii* droppings by amplifying an 83 bp fragment of mitochondrial DNA, the amplification success of all microsatellites was very low (<25 %). Non-invasive samples have long been recognised as being harder to use as they are often associated with DNA degradation, contamination or with the presence of inhibitors (Herrmann & Henke, 1999; Lampa *et al.*, 2013; Taberlet & Luikart, 1999). The presence of inhibitors (or contaminants) was most likely the main contributor towards the lack of amplification of so many samples. Indeed, inhibitors, such as glycogen, cellulose, non-target DNA, fats and heavy metals, are known to be more common in droppings than other tissues and subsequently affect PCR efficiency (Löfström, Knutsson, Axelsson, & Rådström, 2004; Monteiro *et al.*, 1997). The use of Inhibitex tablets (Qiagen) during the DNA extraction process proved to be insufficient to limit the effect of such contaminants and ensure successful amplification. In addition, the complexity of PCR multiplexes for microsatellite genotyping may also limit the amplification of microsatellites as inhibitors do not affect all primers equally (Opel, Chung, & McCord, 2010; Schrader, Schielke, Ellerbroek, & Johne, 2012). For example samples with few inhibitors may have amplified at multiple loci, samples with a “medium” amount of inhibitors only amplified at certain loci, while samples with high amounts of inhibitors would not amplify. Previous capture-mark-recapture studies on bats using non-invasive genetic samples have shown much higher amplification success (Puechmaille & Petit, 2007). However, this study differs from others as the focal species is a woodland specialist as opposed to horseshoe species which roost

predominantly in caves or barns and subsequently might not be subject to as many inhibitors or UV and water which are known to rapidly increase the process of DNA degradation (Murphy, Kendall, Robinson, & Waits, 2007). DNA degradation is also often associated as a limiting factor when using non-invasive samples (Brinkman, Schwartz, Person, Pilgrim, & Hundertmark, 2010; Murphy *et al.*, 2007) and might have also had an effect on the amplification success of the droppings collected in this study. Testing the effect of time and rainfall in controlled conditions has helped to understand variations in PCR amplification on deer faecal pellets (Brinkman *et al.*, 2010). A similar approach could also be tested on bat droppings to better understand the degradation process of bat droppings in damp outdoor conditions and further improve sampling methods. In addition, the use of new primers with higher melting temperatures along with improved purification methods could also provide better results (Opel *et al.*, 2010).

While the use of capture-mark-recapture studies remain the most precise and methodical technique for estimating the number of individuals, the estimate of the minimum number of individuals based on the number of alleles detected can also be a useful last resort tool. Allelic richness is known as one of the most significant and commonly used estimators of genetic diversity in populations. These measures are highly influenced by sample size as a low number of samples can lead to strong underestimates in allelic diversity of the study species (Bashalkhanov, Pandey, & Rajora, 2009). In population genetic studies, different methods have been developed to avoid potential sample size bias when estimating allelic diversity (e.g. Kalinowski, 2005), but never to estimate sample size from the number of alleles. The poor amplification success rate of

all microsatellites only allowed the identification of five individuals, but a higher minimum number of bats was calculated by counting the number of alleles identified at each locus. From the 42 droppings which provided at least one confirmed genotype I could estimate a minimum number of 8 individuals. If extrapolated, I can assume a minimum number of 23 bats from 123 droppings.

Although the methods used in this study require prior knowledge of the genetic diversity of the population at each locus before making any estimate, they have the advantage of providing similar information with pooled samples as the identification of individuals is not essential. Subsequently, improvements in the field of genetics may provide new opportunities for the estimate of individuals directly from pooled samples as the use of next-generation sequencing can be used to amplify microsatellites as short as 40 bp and can subsequently be used on highly degraded samples (De Barba *et al.*, 2017) and estimates may be done using a pile of droppings from a bat roost.

The use of non-invasive genetic sampling provided unexpected insight into the social life of this colony and highlighted the difficulty of collecting adequate data for robust social network analyses. Indeed, this colony has been monitored on a monthly basis from March to October since 1999 and during the month of June most of the colony is found in a large 1FW hibernation boxes. The results from this study suggest that the colony was most likely using box B in mid-June 2015. However, results from previous monitoring suggests that this box has never been used in the past and suggests that this colony uses areas of the woodland for roosting that were previously unknown despite regular monitoring. Such findings raise a question on the frequency of box checks for social network analyses as regular monthly checks may lack the necessary

information to provide a good understanding of the social interactions between bats. Therefore, in order to perform a rigorous analysis I suggest to check boxes every two weeks (e.g. August, Nunn, Fensome, Linton, & Mathews, 2014) as this would double the number of checks whilst limiting disturbance on the bats.

4.5.2 Demographic history

The results of the demographic analysis shows that despite important changes in the landscape over time and their dependence on woodlands for gene flow (See Chapter 3), the British population of *M. bechsteinii* appears to have remained stable as no population expansions or contractions were detected. The effective population size of the British population ($N_e = 6,569$; CI: 5,307-8,006) seems to correspond relatively well with the most recent population size estimate (21,875: CI 10,294-55,588) (Mathews & Kubasiewicz, 2017) bearing in mind N_e tends to be into a factor of 10 times less than population sizes (Frankham *et al.*, 2014). Moreover, the effective population size is also sufficiently high to avoid inbreeding depression for the whole population (Frankham *et al.*, 2014).

As observed in Chapter 2, the British population shows less genetic diversity and more structure than their European counterparts and despite being dependent on woodlands for foraging, roosting and genetic connectivity population size appears to be stable (See Chapter 3). These results may illustrate the species colonisation history in Britain or the resilience of colony survival to changes in their environment. Indeed, certain demographic changes in this study may have remained unnoticed as the detection rate of variations

with Migraine is highest when these are not too recent, too old or too weak (Leblois *et al.*, 2014).

Durrant *et al.* (2009) identified a population bottleneck in Britain, but this study used a lower number of loci, samples and sites as few populations were known at the time of the study. This study also used polyacrylamide gels for genotyping which can sometimes be imprecise and subsequently lead to genotyping errors (Durrant, 2016). Although no population size changes were identified, *M. bechsteinii* populations have probably gone through local demographic changes in Britain at some point in time as fossil records have been recorded in areas where no populations are currently known (Norfolk and East-Anglia) (Yalden, 1992). Therefore, the interpretation of our results may also relate to the colonisation history and range shifting patterns of *M. bechsteinii*. The species most likely colonised Britain from the southeast of Britain while it was still attached to continental Europe and the species range would have expanded across southern Britain. Changes in their environment (e.g. metropolisation of London) would have resulted in population decreases of the eastern population, whilst their range continued to expand further west.

In the face of current climate change many bat species are expected to show important range shifting (Rebelo *et al.*, 2010). The increase of the southern population may illustrate such changes as the most western records of *M. bechsteinii* in Britain are currently from mid-Devon; and the presence of suitable habitat (See Chapter 3) with good connectivity along with a more favourable climate suggests that the species range may still be expanding further west.

The stability of *M. bechsteinii* populations in Britain suggested by the results of this study could also be associated with their ability to adapt to sub-optimal habitats. It is thought that this species shows very strong site fidelity over generations and maternity colonies will continue to use the same sites after strong habitat disturbances (e.g. felling of oak trees). Although *M. bechsteinii* maternity colonies are usually reported to use broad-leaved woodlands for foraging and roosting, recent records have shown the species using “sub-optimal” habitats. They have been found roosting and breeding in coniferous woodlands (Arrizabalaga-Escudero *et al.*, 2014), roof voids (Schofield & Morris, 2009) or hedgerow trees (Damant & Cohen, 2016). While these colonies appear to travel longer distances to forage, their diet remained characteristic of the species (Damant, 2017; Poulton, 2008). However, whilst colonies manage to persist and successfully breed, the dispersal abilities of future generation’s remain limited and could result in more genetic structure.

Sample size is known as a significant limiting variable when it comes to the calculation of effective population sizes. Indeed, it has been shown multiple times in simulations or directly in natural and managed populations that insufficient samples will provide low precision estimates and strongly underestimate effective population size (e.g. Ardren & Kapuscinski, 2003; England, Cornuet, Berthier, Tallmon, & Luikart, 2006; Wang, Santiago, & Caballero, 2016). For the use of Migrate Version 0.4.1, Leblois *et al.* (2014) indicated that bottlenecks and expansions are best detected with large samples sizes (>100). Subsequently, it is possible that certain population changes for our colonies may have gone undetected. Nonetheless, despite such limitations, Brackett’s Coppice and Malvern display respectively a population expansion

and contraction. The Malvern woodland was felled after World War II and the use of bat boxes by *M. bechsteinii* was reported less than 10 years ago.

Population variations less than 10-20 generations are hard to detect using the Migrate analytical approach (Leblois *et al.*, 2014). However, the contraction of the Malvern population may reflect the period before the use of bat boxes, whereas the more ancient population expansion of Brackett's Coppice may directly result from the population size increase of the southwestern population (Cluster A) as they both show similar results.

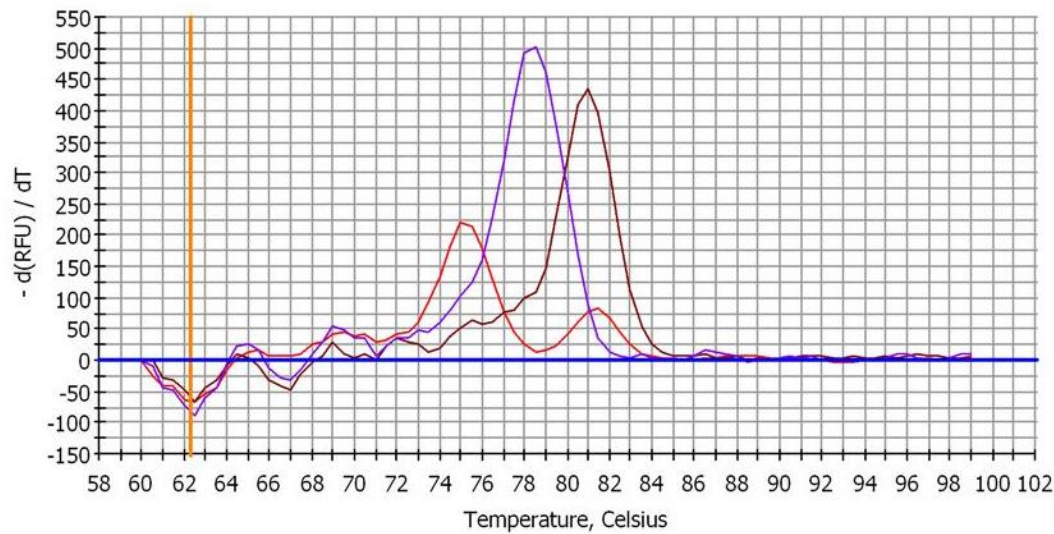
It is important to detect population changes rapidly as alterations in the environment may lead to strong population declines and potentially lead to the extinction of local populations. While the use of non-invasive capture-mark-recapture techniques may in the future provide a way to monitor these changes effectively, it has been shown that the estimation of effective population size by collecting samples from individuals of different generations (temporal samples) can detect much more recent changes without the need for large sample sizes (Antao, Pérez-Figueroa, & Luikart, 2011; Waples, Antao, & Luikart, 2014).

Therefore, regular sampling (e.g. every five years) from populations or the collection of samples from juveniles and old bats would be required in order to better detect population changes and allow rapid and effective conservation measures.

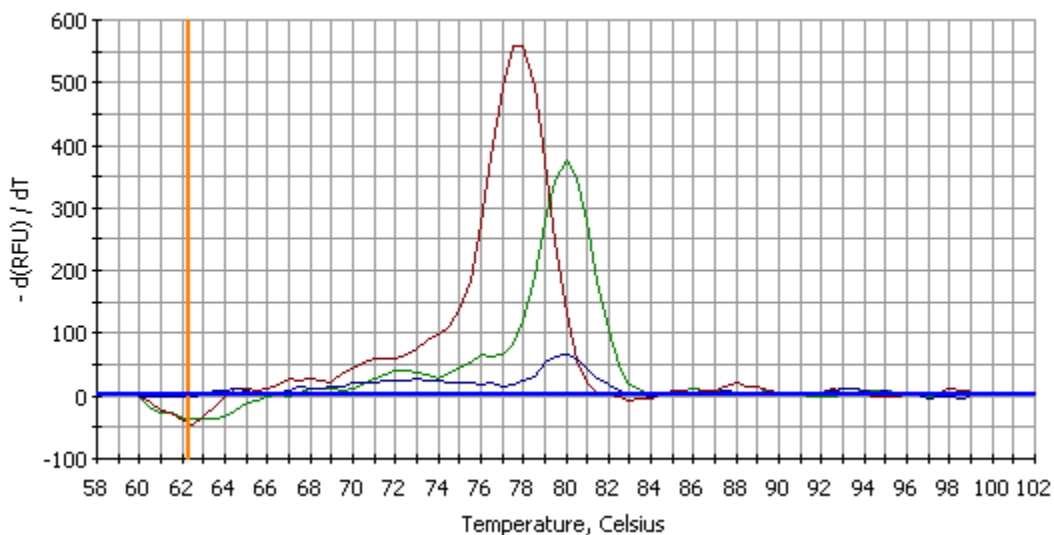
To summarise, the use of non-invasive genetic sampling to perform capture-mark-recapture population size estimates on *M. bechsteinii* was unsuccessful, however with the collected information it was still possible to calculate the minimum number of individuals present. Furthermore, the use of non-invasive genetic sampling provided useful insight on the use of bat boxes throughout the

woodland. Demographic models indicated that the British population of *M. bechsteinii* has remained stable despite the important loss of woodlands and degradation of their habitat in the past. This may indicate that the species range may still be shifting and while certain population might have gone extinct new areas have been colonised. In order to detect population trends more efforts should be focused on calculating temporal effective population size as these could detect changes within a single generation.

4.6 Appendices



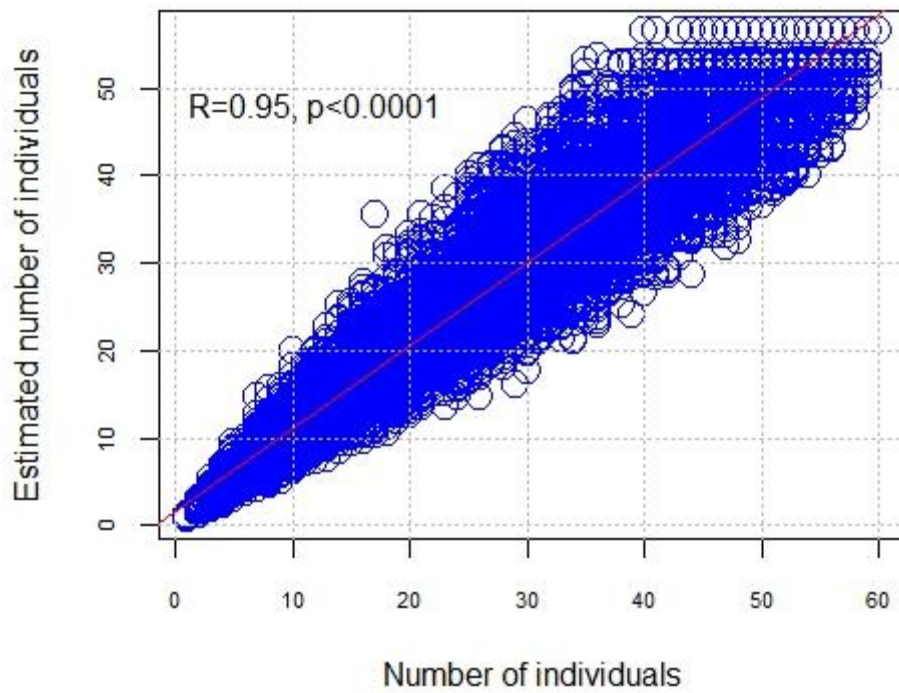
Appendix 4.1 Real time PCR melt curve of *M. bechsteinii* (purple: Ct ~ 16.3), *N. noctula* (brown: Ct ~ 15.8) and *M. nattereri* (red: Ct ~ 25.1) from the species identification duplex qPCR. Positive controls of *P. auritus* did not amplify, while *M. nattereri* showed evidence of primer dimer or non-specific amplification. However, this did not affect the ability to detect both targeted species as this always occurred after 20 cycles.



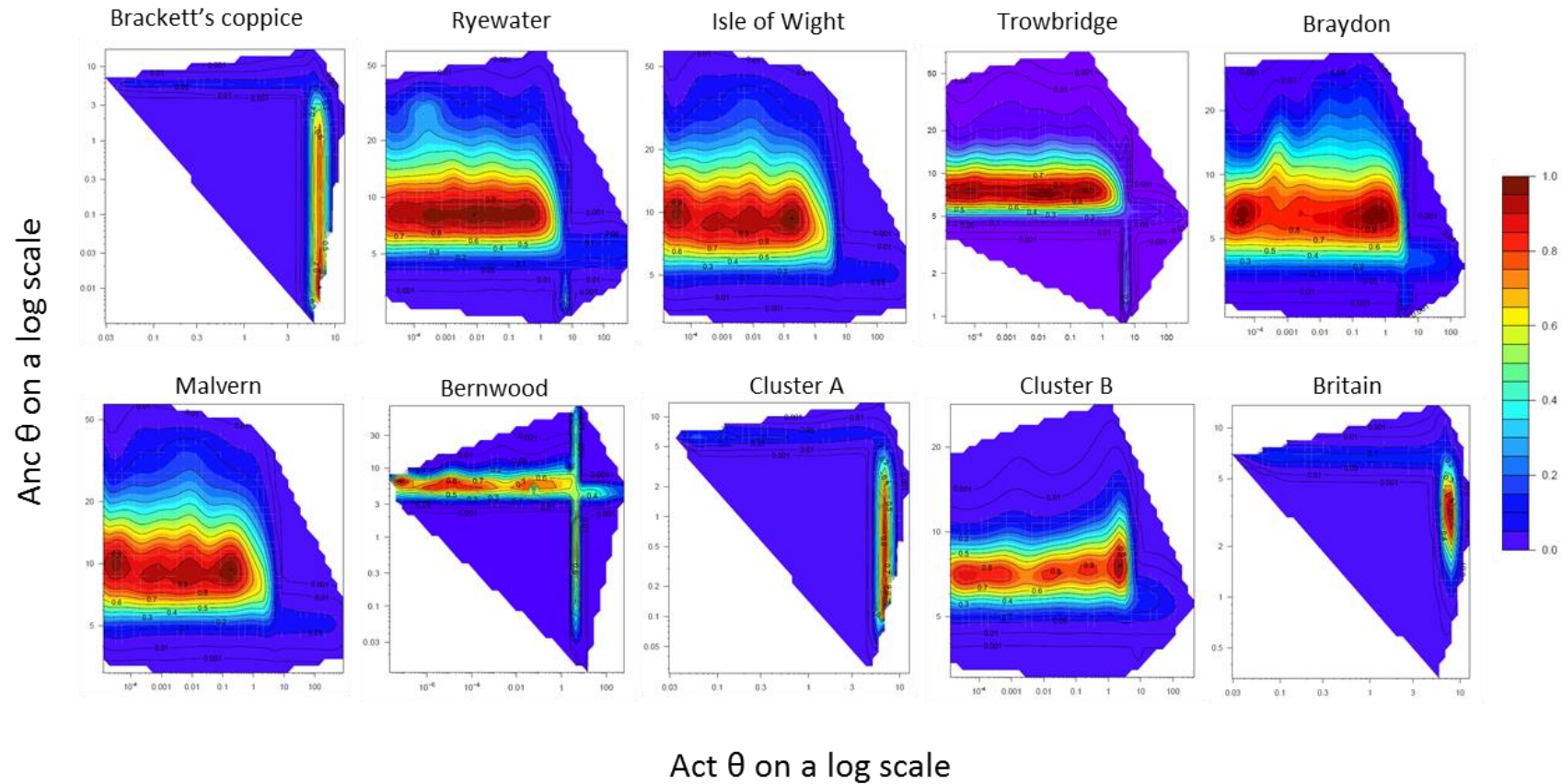
Appendix 4.2 Real time PCR melt curve of *M. bechsteinii* (brown: Ct ~ 16.3), *N. noctula* (green: Ct ~ 15.8) and *M. daubentonii* (dark blue: Ct ~ 25.1) from the species identification duplex qPCR. Positive controls of *M. mystacinus* did not amplify.

Appendix 4.3 Locus name, primer sequence and repeat motif, number of alleles and size range of all microsatellites tested on *M. bechsteinii*. The forward primer from C112_125 was modified in order to better fit their own multiplex.

Multiplex	Locus	Forward primer	Reverse primer	Repeat sequence	Number of alleles	Size range (bp)	Reference
1	A24	D3-GTGGTATGAAATAACCAAGTTCACCTTG	GGGTAGTTTACAGAAATAGCAATTGAG	(AC) _x	11	268-290	Castella and Ruedi (2000)
	CloneA2	D4-TTTGTTATCAGAAGGAAGG	TCTCAACTGGGTGCTC	(CA) _x	15	141-173	O'Brien <i>et al.</i> (2007)
	B22	D2-CTGATGCAAGACCCCTTACAAC	ACGGCAGCAGTGAAATCAGA	(GT) _x	10	151-173	(Kerth <i>et al.</i> , 2002)
	Mluc8	D3-CCACTCAAGCACCAGATGAATA	AGGAATGAGGGAAGAAAGGAAG	(CTTC) _x	11	191-231	Burns <i>et al.</i> (2012)
2	G6	D4-GGCTTTTTGAAAAGACTGAGG	ACATCAGCCAGTTCCTGTTC	(GT)GA(GT) _x	8	95-125	Castella and Ruedi (2000)
	MS3DO2	D2-CTAAGACCCTTCCAGCTCTCA	GATACCATCACTCTTCCCCTG	(AC) _x	25	205-259	Trujillo and Amelon (2009)
	C112_125	CATGCAGGTGTGTGCCTGT	D2-AATGGGTCTCAGAGGGTGAA	(CA) _x	16	122-152	Castella and Ruedi (2000)
	B23	D4-CAGTTGGAGGCATGCAGAAA	CCGGAGATACTCTTATTGTTGGTA	(CT) _x	19	248-286	(Kerth <i>et al.</i> , 2002)
	H23_Mluc	D3-TTGTCTACTAGCATTGTCCAGTG	ATAGCTATGTTGCCTAACCTATTTACTC	(GT) _x	18	147-187	Castella and Ruedi (2000)



Appendix 4.4 Results from the GAM (General Additive Model) showing the predicted number of individuals against the actual number of individuals using the genotype data from Brackett's coppice



Appendix 4.5: Likelihood surfaces for indicators of scaled effective population size (ancestral (anc) and actual (act) θ). The point estimates and 95% coverage confidence intervals values are shown in Table 4.2. The expansion of Brackett's coppice and Cluster A are visible in these graphs. Cluster A represents pooled samples from Brackett's Coppice, Sherborne, Isle of Wight and Trowbridge; Cluster B groups Braydon, Malvern, Bernwood and Grafton. The Britain dataset includes all combined sites + Grafton.

Chapter 5 Molecular ageing by measuring DNA methylation: a new tool for bat conservation



An adapted version of this chapter has been published as:

Wright, P.G.R, Mathews, F., Schofield, H., Morris, C., Burrage, J., Smith, A., Dempster, E.L., Hamilton, P.B. (2018) Application of a novel molecular method to age free-living wild Bechstein's. *Molecular Ecology Resources*.

5.1 Abstract

Age-structure is one of the fundamental parameters affecting the conservation status of populations. Such information is highly valued in wildlife conservation yet it is exceptionally difficult to measure, and the only recognised methods are very time consuming. Here, I assessed the use of DNA methylation of homologous genes as a novel way of establishing the age structure of a rare and elusive wild mammal: the Bechstein's bat, *Myotis bechsteinii*. I collected 62 wing punches from individuals whose ages were known as a result of a long-term banding study. DNA methylation was measured at seven CpG sites from three genes (Aspartoacylase - ASPA; Tet methylcytosine dioxygenase 2 - TET2 & Glutamate ionotropic receptor AMPA type subunit 2 - GRIA2) which have previously shown age-associated changes in humans and laboratory mice. All CpG sites from the tested genes showed a significant relationship between DNA methylation and age, both individually and in combination (multiple linear regression $R^2=0.58$, $p<0.001$). The assay reliably distinguished three biologically-meaningful age groups (sub-adult, adult and old bats) ($\kappa=0.65$). This method is of considerable practical benefit as it can reliably age individual bats. It is also much faster than traditional capture-mark-recapture techniques, with information on the age structure of an entire colony being available from a single sampling session, facilitating prompt conservation action when required. By identifying three genes which can provide useful epigenetic data across distantly related species, this study also suggests that the techniques can potentially be applied across a wide range of mammals.

5.2 Introduction

Information on the age of individuals in wildlife populations is essential for the study of population structure (Dunshea *et al.*, 2011; Oli & Dobson, 2003). Such data can provide information to predict the impact of habitat, climate change or hunting pressure on population viability estimates (Botsford *et al.*, 2011; Sand *et al.*, 2012; Tella *et al.*, 2013). However, estimating age through marking (e.g. using tags and rings) and recapture can be challenging and can have negative impacts on animal welfare (Nelson, 2002). Recaptures throughout the lifetime of the animals are required to gather the required information, making the process time-consuming and difficult to execute for rare species (Brunet-Rossini *et al.*, 2009). Non-invasive approaches to age wildlife, such as the unique identification of individuals via markings (e.g. cetaceans, tigers) (Mizroch *et al.*, 1990; Speed *et al.*, 2007), are suitable only for a limited number of species. Molecular tools for producing reliable age estimates have, by contrast, received little attention. Most research has focused on understanding the biological process of ageing, for example, in studies of telomere shortening (e.g. Turbill, Ruf, Smith, & Bieber, 2013), rather than on the development of routinely applicable techniques to estimate chronological age.

The biological process of ageing combines both environmental and programmed processes (Horvath, 2013; Koch *et al.*, 2011). The presence or absence of methyl groups at the C5 position of cytosines followed by guanines ('CpG sites') has an important role in the control of gene expression; as increased levels of methylation at CpG sites are associated with reduced gene transcription rates (Hannum *et al.*, 2013; Horvath, 2013). The multitude of DNA methylation loci linearly correlated to age and the possibility of using multiple

tissues (e.g. blood, skin...) means that DNA methylation is now used in human forensic science with great effect (e.g. Bekaert, Kamalandua, Zapico, Van de Voorde, & Decorte, 2015; Horvath, 2013; Zbieć-Piekarska *et al.*, 2015). The measure of DNA hypo- and hypermethylation of specific sites has been used as an age predictor on humans (*Homo sapiens*) (Christensen *et al.*, 2009b; Grönninger *et al.*, 2010; Horvath, 2013) and mice (*Mus musculus*) (Maegawa *et al.*, 2010; Stubbs *et al.*, 2017). However, this approach had not been attempted with wild animals until Polanowski *et al.* (2014) developed an age assay for humpback whales (*Megaptera novaengliae*) by measuring DNA methylation from 3 CpG sites from different genes. Their results were far more accurate than previously developed techniques using telomere length (Dennis, 2006; Olsen *et al.*, 2012; Olsen *et al.*, 2014).

Bats live substantially longer than other mammals of similar size and metabolic rate (Wilkinson & South, 2002). Certain species, such as Brandt's bat (*Myotis brandtii*), have been recorded surviving in the wild for over four decades (Gaisler, Hanák, Hanzal, & Jarský, 2003). The long lifespan of bats is thought to have evolved from a lower risk of extrinsic mortality due to the evolution of flight along with the use of roosting and hibernation sites (Munshi-South & Wilkinson, 2010). Age estimates of bat populations are particularly important for their conservation, because their long lifespan often results in a delayed response to recent changes in their habitat. Up until now, such estimates depended solely on long-term ringing studies (Gaisler *et al.*, 2003; Wilkinson & South, 2002). Whilst a variety of additional methods give some indication of whether an animal is a juvenile, such as analysing linear growth of bones (Kunz & Hood, 2000), epiphyseal-diaphyseal fusion (De Paz, 1986), chin spots (Richardson, 1994), body mass and pelage coloration (Cheng & Lee, 2002), these are helpful

for only the first few months of life. For adult bats, tooth wear and incremental dentin may give an indication of age (Batulevicius, Pauziene, & Pauza, 2001; Storz, Bhat, & Kunz, 2000) but examination is invasive and the results are imprecise, so the methods are not widely adopted.

In this study, I examined the potential of measuring DNA methylation to age a rare woodland bat, *M. bechsteinii*. I used a population where the age of all bats was known to test our method. Our finding of genes in which the level of methylation relates to age opens the possibility of developing rapid age estimates of bats. The use of such assays can rapidly provide information on the age structure of previously unstudied populations, while ringing bats would take over a decade to provide any information.

5.3 Materials and methods

5.3.1 Sample collection

Wing tissue samples were collected from *M. bechsteinii* using 3 mm wing biopsy punches (Stiefel Laboratories, Wooburn Green, UK) and stored in absolute ethanol at -4°C. Genomic DNA was extracted from the biopsy punches with the DNeasy blood & tissue kit (Qiagen) and samples were eluted in 120 µl of buffer AE. I used 60 female samples collected from Brackett's Coppice (Dorset; 50.860456, -2.6918909), a maternal colony monitored since 1999. I also included two juveniles which were found dead in bat boxes. These were stored in ethanol after being found and were included in the study as DNA methylation is chemically stable and can be measured on ancient DNA (Briggs *et al.*, 2009; Llamas *et al.*, 2012). No male samples were collected as they tend to lead solitary lives and are very rarely observed after they are weaned, while females maintain tight social bonds throughout their lives (Kerth & König, 1999).

The bats included in this study represent an even distribution of ages ranging from 0 to 14 years old. Eight droppings were also collected from individuals of known age from the same colony and DNA was extracted using a QIAmp DNA Stool Mini Kit (QIAGEN) following the protocol recommended by Puechmaille *et al.* (2007).

5.3.2 PCR and pyrosequencing

Genes with age-related epigenetic changes in humans, mice and Humpback whales were identified through literature searches (Appendix 5.1). Candidate 5' regulatory region sequences were taken from GenBank and used as queries for BLASTN (Altschul *et al.*, 1990) searches of Chiroptera sequences in GenBank and BLAT searches of the Little brown bat (*Myotis lucifugus*) genome (Cunningham *et al.*, 2014). Sequences were then matched to a *M. bechsteinii* genome which was sequenced using 100bp paired-end sequencing on an Illumina HiSeq 2500 (EBI access N°: PRJEB23351). The paired-end sequencing was prepared by extracting DNA from a male *M. bechsteinii* using a DNeasy blood & tissue kit (QIAGEN). Then, the concentration and purity of the DNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Library construction was prepared using the NEXTflex® Rapid DNA Sequencing Library Prep Kit. Contaminating adaptors and low quality sequences were removed using fastq mcf tools (Aronesty, 2013); and BlastN was used to select genomic reads that matched the target sequences.

PCR and pyrosequencing assays were designed using the PyroMark Assay design software (Qiagen, Hilden, Germany). The target sequences analysed comprised CpG sites in genes known to undergo age-related epigenetic

changes in other species (Koch *et al.*, 2011; Polanowski *et al.*, 2014; Weidner *et al.*, 2014). Template preparation and pyrosequencing was carried out as described by Tost and Gut (2007). Genomic DNA was treated with sodium bisulfite using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) according to the manufacturer's standard protocol. Samples were then incubated twice for 5 minutes in 15 μ l and 10 μ l of M-Elution buffer to form a final solution of 25 μ l. Water negative controls were run to verify the absence of DNA contamination. Bisulfite-PCR amplification was performed using the primers in Table 5.1. Water controls were included to confirm the absence of DNA contamination, and unmodified DNA samples from wing punches were included during primer optimisation to confirm primer specificity for bisulfite-modified DNA.

Amplification reactions consisted of 3 μ l of 5x HOT FIREPol® Blend Master Mix, 0.75 μ l (0.075 μ M) of forward and reverse primers, 1 μ l of bisulfite converted template DNA and 9.5 μ l of RNase-free water. PCR conditions were 15 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C and a final extension step of 10 min at 72 °C. Before pyrosequencing, all samples were diluted in 15 μ l of water. Pyrosequencing was performed on a PYROMARK 24 Pyrosequencing System (Qiagen). The PYROMARK Q24 software gave percentage methylation values for each CpG site and I used eight duplicates to test for Pyrosequencing precision.

5.3.3 Data analysis

All statistical analysis was undertaken using R studio (RCoreTeam, 2016; RStudio, 2012). All CpG sites reported in this study have previously been shown to undergo linear age-associated methylation changes. Therefore, I maintained a linear analysis throughout the study and assumed non-linear

trends as a likely consequence of sample size. The differences in percentage methylation between bats of varying ages was initially explored using univariate linear regression in methylation percentages for each CpG site separately. All individuals with missing data were omitted from the analysis. All CpG sites showing a significant relationship with age were considered for developing the multiple linear regression whilst including the interactions between sites from the same gene. This model was then used as an epigenetic age assay for *M. bechsteinii*. The predicted age of each bat was plotted against their known age to test the consistency of the model. In addition, I assessed the practical utility of the assay in classifying individuals into three meaningful groups; young bats (0-3 years); mature bats (4-8 years); and old bats (≥ 9 years). The predictive power in achieving the correct classification was assessed by calculating kappa values using the CIA 2.0.0 software where values close to 1 indicate a good predictive power (Trevor Bryant, University of Southampton, Southampton, UK).

I assessed the precision of the age estimate assay by performing a Leave One Out Cross Validation (LOOCV) analysis as performed by Polanowski *et al.* (2014) Here, the multiple linear regression was tested by using all wing samples but one (N-1) to estimate the individuals age; the predicted age was then plotted against the known age of the individual.

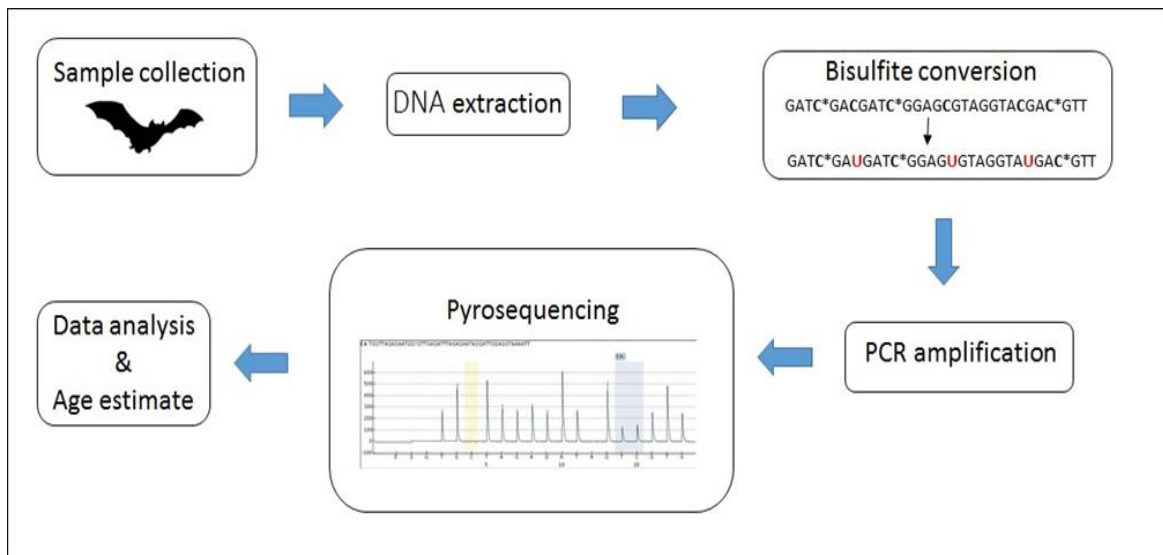


Figure 5.1 Summary of the key steps for estimating the age of bats by measuring DNA methylation. Methylated cytosines are represented by *

5.4 Results

From the 13 sequences identified from different genes containing CpG sites known for undergoing age-associated CpG methylation changes in other species (Appendix 5.1), I successfully designed assays and amplified the regulatory region of three genes for *M. bechsteinii* (Aspartoacylase – ASPA; Tet methylcytosine dioxygenase 2 - TET2 & Glutamate ionotropic receptor AMPA type subunit 2 - GRIA2; Table 5.1). Seven CpG sites from the regulatory regions of these genes were assayed and all CpG sites showing a significant regression relationship with age were used for the age assay. Of the 62 wing samples four samples had at least one failed amplicon out of the three genes tested, and 58 successfully amplified for all three assays.

Table 5.1 PCR and sequencing primers of the three analysed assays along with the GenBank reference sequences and previous studies analysing age associated changes in DNA methylation for each assay.

Gene (Accession number)	References	Primer name	Sequence (5' to 3')	T _m (°C)
TET2 (MF322927)	Polanowski <i>et al.</i> (2014), Grönniger <i>et al.</i> (2010)	Tet2_Koch_F1b	Biotin-GAAATTTTGGTTTTTT TTATAATAGAGGTT	57
		Tet2_Koch_R1	CCAAAAAATTTCTCAATA ACTCTACTT	59
		Tet2_Koch_Seq1	TTCTCAATAACTCTACTTCT	44
GRIA2 (MF322926)	Polanowski <i>et al.</i> (2014), Koch <i>et al.</i> (2011), Chakrabarti, Bandyopadhyay, and Poddar (2001)	GRIA2_F1	GTGTATGGGAGGGTGTGA ATATTTTAA	61
		GRIA2_R1b	Biotin-AACAAAAAATTCCTA TTCCCAAATCC	61
		GRIA2_Seq1	GTTGAATATTTTAAGTTTTGG GATTAT	47
ASPA (MF322925)	Bekaert <i>et al.</i> (2015), Weidner <i>et al.</i> (2014)	ASPA_F1	GAGTTAATAGGAGTATTTTG GTTAAGTA	58
		ASPA_R1B	Biotin-AAATAATTTTACCTCCA ATCCTATTCT	58
		ASPA_Seq1	GGAGTATTTTGGTTAAGTAT	44

All seven CpG sites tested in this study showed a significant relationship with the age of *M. bechsteinii* (Figure 5.2). For sites from the TET2 and GRIA2 genes increasing age was associated with increased methylation, whereas the reverse was true for ASPA (Figure 5.2g). The average difference in percentage methylation between duplicates was 3.9 and did not affect the results of the multiple linear regression model (Appendix 5.2). The multiple linear regression performed included all CpG sites, whilst taking into account interactions between sites from the same gene. This full model explained 58% of age variation ($R^2 = 0.576$, $p < 0.001$) and the overall precision of the predictions was 2.08 years by calculating the root mean-square error (Figure 5.3). Our model slightly overestimates the age of young bats whilst underestimating the older

individuals. However, the split of our data in three categorical age groups (Figure 5.3b) shows that the developed age prediction model could effectively differentiate all age classes with very little overlap (one-way Anova: $F = 39.6$, $p < 0.001$) and a kappa value of 0.646 (0.467-0.824) which indicates a good level of prediction.

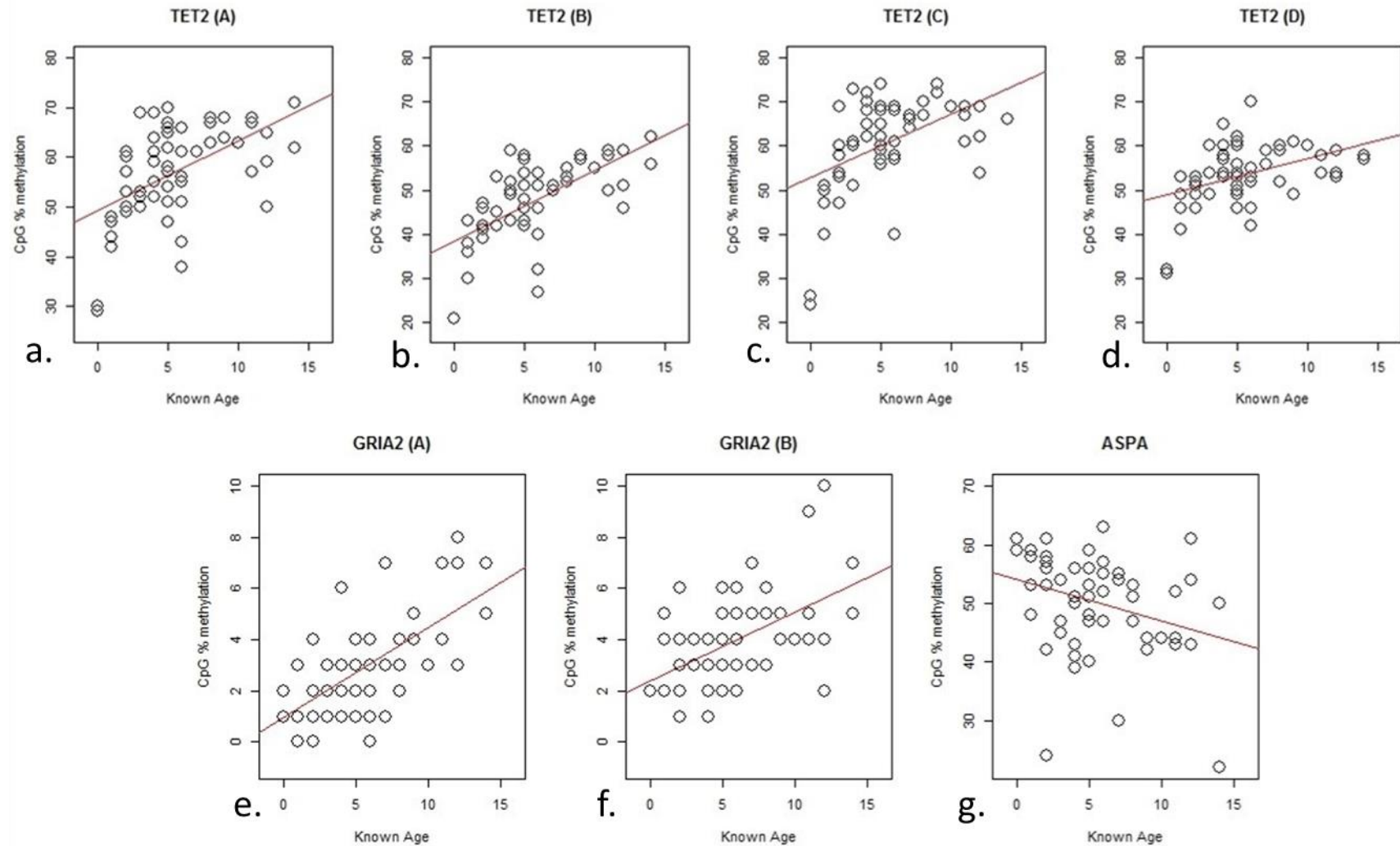


Figure 5.2 Percentage methylation versus age for seven markers validated in three different genes. TET2 (A): $R^2=0.263$, $p < 0.001$; TET2 (B): $R^2=0.384$, $p < 0.001$; TET2 (C): $R^2=0.221$, $p < 0.001$; TET2 (D): $R^2=0.168$, $p < 0.001$; GRIA2 (A): $R^2=0.419$, $p < 0.001$; GRIA2 (B): $R^2=0.256$, $p < 0.001$; ASPA: $R^2=0.0711$, $p = 0.0241$

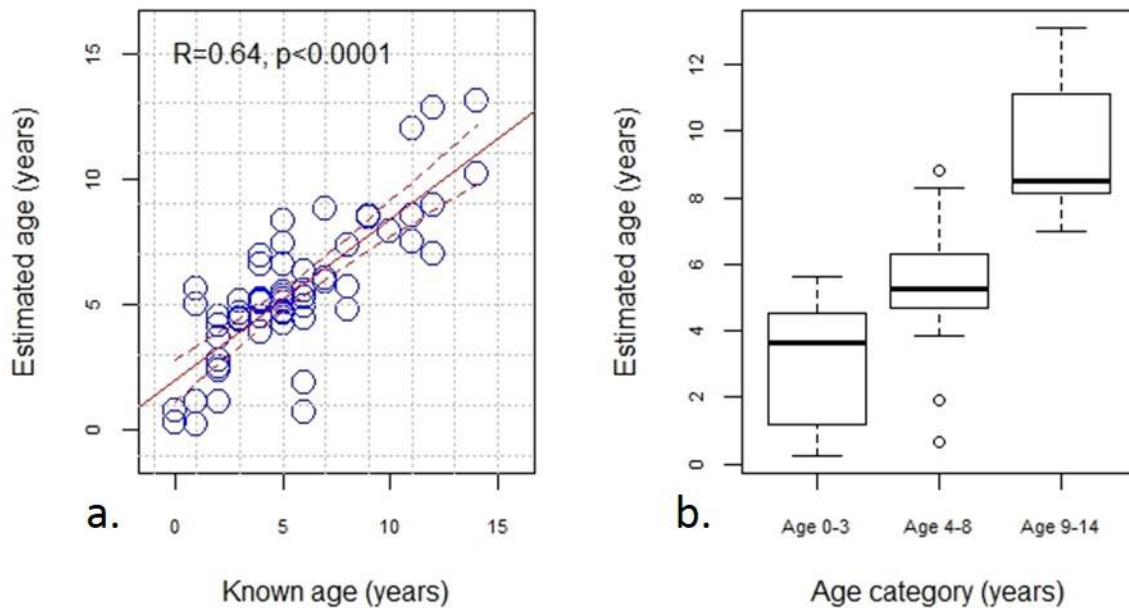


Figure 5.3 a) Multiple linear regression for the predicted age *M. bechsteinii* from measurement of CpG methylation at seven CpG sites with 95% confidence limits b) boxplots representing the known age of bats in three distinct categories of known age (0-3 years old, 4-8 years old, 9-14 years old)

The leave-one-out cross validation analysis (LOOCV) was performed to provide an unbiased estimate of the accuracy of the Bechstein's bat age estimates. The overall precision of the LOOCV was estimated at 1.52 years by calculating the standard deviation of the mean difference between known and estimated ages (Figure 5.4). Methylation levels of the eight droppings tested fell outside the range of the epigenetics age assay designed for wing punches. Methylation values in droppings appeared to vary more between individuals than wing punches (TET2 (A) for droppings ranged from 1 to 96% compared to 29 to 71% for wing punches).

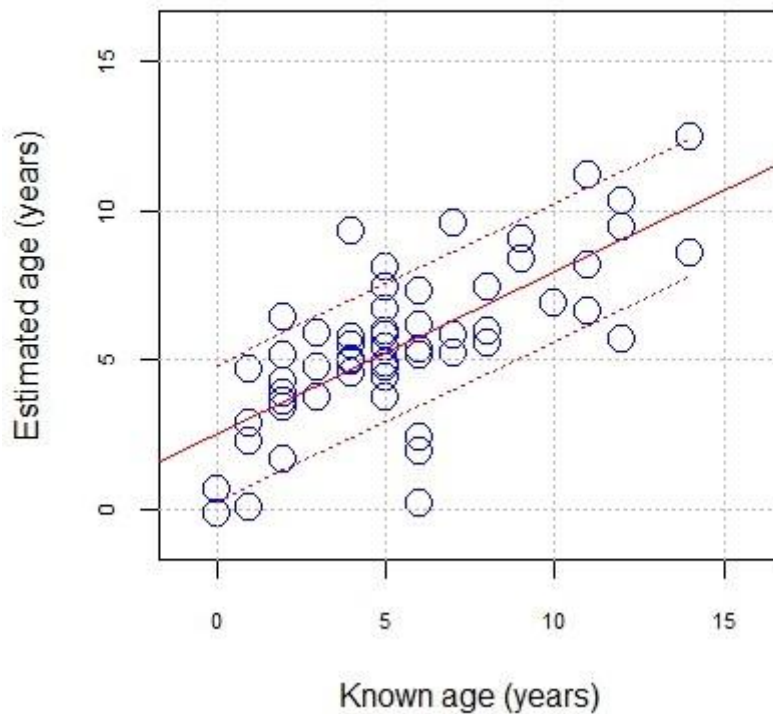


Figure 5.4 Results of ‘Leave One Out Cross Validation’ (LOOCV) analysis. The estimated ages of every bat when the predictive model is based on data for the other N = 57 bats are plotted with together with 95% confidence limits.

5.5 Discussion

This is the first epigenetic age assay developed for bats, a taxon which forms a third of all mammalian species, and only the second designed for use on a wild species. The age prediction model developed explained 64% of variance and predicted age from wing punches with a standard deviation of 1.52 years.

Although the accuracy of this assay is lower than human epigenetic age assays which have gradually tested a greater number of CpGs to improve model accuracy, this method is of considerable practical value in being able to

precisely age *M. bechsteinii* and give indications on population trends. For example, colonies lacking bats aged 0 to 3 years old might indicate recent poor breeding success which could subsequently lead to a delayed population decline. This information could then be used to assess the impact of recent environmental changes in their environment (e.g. felling of large roosting trees, weather) on their breeding success.

The Bechstein's bat age assay provides a novel tool offering necessary insight on the age structure of bat colonies, but will require further development and validation prior to widespread use. The elusive nature of bats makes the collection of known age samples time-consuming and labour intensive. The oldest bat in this study was 14 years. Yet evidence suggests that individual *M. bechsteinii* can live for more than 20 years (Dietz *et al.*, 2009). It may therefore be possible to further improve this epigenetics age assay by including older bats. Additionally, I cannot confirm that this assay is suitable for males as only female bats were used. However, it is reasonable to assume that **sex** would not affect these results as most studies appear to show very similar trends in DNA methylation between males and females (Polanowski *et al.*, 2014; Zbieć-Piekarska *et al.*, 2015). The sampling of a single colony could also potentially bias results as bats within a colony may have been subjected to similar environmental stresses which can sometimes impact levels of methylation (Teschendorff, West, & Beck, 2013).

Although I only tested a few bat droppings, I detected important variations in methylation levels which would not allow us to accurately estimate the age of bats. A separate dropping age estimate assay is required as changes in DNA

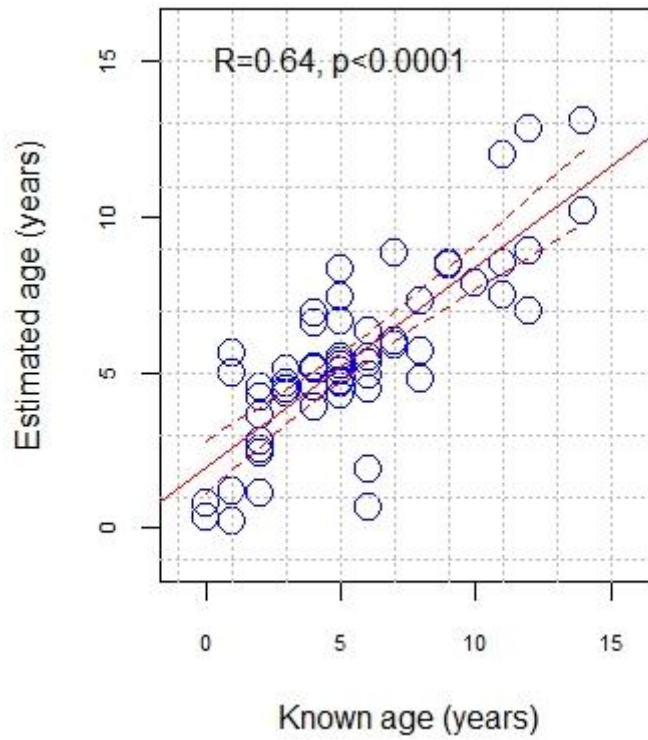
methylation are often tissue specific (Christensen *et al.*, 2009b). The development of such an assay would also need to consider all points mentioned above whilst using a larger sample size. Indeed, unlike tissue samples from wings, droppings may be more indicative of biological age and show high levels of instability, as these would comprise cells from the intestinal tract (Jones & Laird, 1999; Maegawa *et al.*, 2010). Additionally, the quality and quantity of DNA along with a higher risk of contamination from droppings could also affect results, as these vary significantly more than wing punches (Puechmaille *et al.*, 2007).

This study demonstrates that useful and rapid age estimates can be derived from an epigenetic assay. Our methods provide sufficient sensitivity to confidently estimate the age of bats ranging from 0 to 14 years old at an affordable cost (<20 GBP per sample). Such techniques can be used to inform about the age structure of bat colonies and further improve their conservation. The use of three genes applicable for age assays across distantly related species, such as humans, whales, mice and bats, suggests the potential for a widespread use of these techniques for mammal conservation in the future.

5.6 Appendices

Appendix 5.1: List of genes considered for the development of the Bechstein's age estimate assay, but not used in this study as they were not identified in the *M. bechsteinii* genome or unsuitable for the design of high quality PCR assays.

Gene	Species	GenBank SeqID	Reference
CDKN2A	<i>M. novaengliae</i>	KF791964.1	Polanowski <i>et al.</i> 2014; Koch <i>et al.</i> 2011; Horvath 2013; Maegawa <i>et al.</i> 2010
ELN	<i>M. davidii</i>	XM_015570262.1 (9-182)	Bocklandt <i>et al.</i> 2011
HOXA9	<i>M. novaengliae</i>	KF791967.1	Polanowski <i>et al.</i> 2014; Gronniger <i>et al.</i> 2010; Koch <i>et al.</i> 2011
PDE4C	<i>M. brandtii</i>	XM_005873865.2 (1136-1308)	Weidner <i>et al.</i> 2014
TRIM58	<i>M. novaengliae</i>	KF791966.1	Polanowski <i>et al.</i> 2014; Koch <i>et al.</i> 2011
EDARADD	<i>M. novaengliae</i>	KF791968.1	Polanowski <i>et al.</i> 2014; Bocklandt <i>et al.</i> 2011
ITGA2B	<i>H. sapiens</i>	AH002749.2	Weidner <i>et al.</i> 2014
ELOVL2	<i>H. sapiens</i>	AL121955.20	Zbieć-Piekarska <i>et al.</i> 2015; Spiers <i>et al.</i> 2016
TOM1L1	<i>H. sapiens</i>	AC090824.6 (51020-51239)	Bocklandt <i>et al.</i> 2011
NPTX2	<i>M. lucifugus</i>	XM_006097686.1 (29-155)	Bocklandt <i>et al.</i> 2011



Appendix 5.2 Multiple linear regression for the predicted age of *M. bechsteinii* from CpG methylation measurements at seven CpG sites with 95% confidence limits using data from duplicates.

Chapter 6 General Discussion



6.1 Overview

The effective monitoring of elusive species continues to challenge wildlife conservationists. The Bechstein's bat, *Myotis bechsteinii*, is one of Britain's most elusive mammals and critical information on the species is lacking, hindering evidence-based conservation and management in a human-dominated landscape. Through the course of this thesis, I have combined molecular approaches and landscape ecology to provide information on the conservation status and threats of *M. bechsteinii* and develop novel methods to better monitor this species.

6.2 Discussion of key findings

6.2.1 Molecular and landscape approach to Myotis bechsteinii conservation

I have undertaken analysis to assess the genetic diversity and structure of the British and European population of *M. bechsteinii*; and further assess the effect of the British landscape on genetic connectivity and habitat suitability.

In Chapter 2, I reported the genetic diversity and structure of *M. bechsteinii* across Britain and Europe using 14 microsatellite markers and a 747 bp region of mitochondrial cytochrome b gene. Nuclear DNA (microsatellites) showed high levels of genetic diversity and little inbreeding across the species range, though genetic diversity remained slightly lower in Britain than in mainland Europe.

Bayesian and spatial PCA analysis showed a clear separation between the British and European sites. Within Europe, the Italian population south of the Alps was isolated from the other sites, in Britain, there was genetic structuring

between the northern and southern part of the species range. Despite there being little genetic divergence in mitochondrial DNA (mtDNA) sequences throughout most of Europe, the mtDNA patterns in Britain confirmed this separation of northern and southern populations. Such genetic structuring within Britain in the absence of any obvious physical barriers suggests that other factors, such as land-use, may limit gene-flow.

These results highlighted the need to better understand how the British landscape impacts *M. bechsteinii* as this can help determine potential threats and better inform conservation management. A combined approach of habitat suitability, connectivity and landscape genetics analyses provided a valuable framework to assess these impacts (Chapter 3). I used MaxEnt modelling, landscape connectivity analysis and a landscape genetic approach to assess how the species is affected by different landscape features. The habitat suitability model identified distance from woodlands as being the main variable for determining habitat suitability. While maternity roosts were more influenced by woodland type, males were found to use sub-optimal habitats more readily. Suitable habitat was highly fragmented and only showed good connectivity if the species was able to disperse over 5,000 m. The landscape genetics analysis indicated that land cover (woodlands, urban areas, pastures, arable, scrub and sea) best explained genetic connectivity between populations and highlighted the importance of woodland cover for maintaining genetic connectivity. This was also observed in the connectivity analysis as it helped better understand the difference in genetic structure between the southern and northern populations.

Overall these results showed that the British population of *M. bechsteinii* only showed slightly lower levels of genetic diversity than in Europe, but showed more important genetic structuring in the absence of obvious geographical barriers. These findings also strongly highlighted the impact of land cover and more importantly the significance of woodlands and their connectivity in providing and maintaining not only habitat but also genetic connectivity for *M. bechsteinii* at a national scale. Such information should encourage conservation managers to consider the overall landscape connectivity rather than woodland management alone to inform on the health and viability of *M. bechsteinii* populations. This is particularly important as I found that the isolation of populations, such as Bernwood, can lead to lower genetic diversity and increased inbreeding.

6.2.2 Novel methods for monitoring Myotis bechsteinii

The study of elusive animals can be particularly time consuming and ineffective. It is, therefore, necessary to develop novel techniques often inspired from other fields of study (e.g. forensic science) to protect wildlife from threats that may hinder populations. In Chapter 4 and 5, I developed novel methods to better monitor *M. bechsteinii* populations more effectively by using non-invasive genetics, demographic history analysis and epigenetics.

Information on population size is critical for the effective management of wild populations. Colony size of certain bat species can be estimated by simple counts (e.g. horseshoe bats). Such counts, however, are more challenging for woodland bats, such as *M. bechsteinii*, as they can use over 60 roosting sites and perform complex fission-fusion behaviour making reliable estimates

extremely difficult. To provide better colony size estimates, I investigated the use of non-invasive capture-mark-recapture (CMR) on *M. bechsteinii* by collecting droppings from below known roosting sites. Species specific primers confirmed the presence of 123 *M. bechsteinii* droppings of the 294 collected but all microsatellites showed very low amplification rates (< 25 %) indicating low quality samples and could subsequently not be used to provide any CMR estimates. However, generalised additive models (GAM) based on the correlation between sample size and the number of alleles at each locus allowed an estimation of the minimum number of individuals (here, n=8). The use of non-invasive sampling also provided unexpected insight into the social life of the colony and highlighted the limitations of human observations which can bias social network analyses, as the species was found roosting predominantly in boxes where it has never been recorded despite regular box checks.

To assess population size and variation at a national and local scale, I also performed demographic analysis. For this, I used Migraine (Version 0.4.1) to assess effective population size and variation of the British populations of *M. bechsteinii* overtime. This gave an effective population size of 6,569 (CI: 5,307-8,006) and suggested that the British population is currently stable and possibly spreading further west. Such information should encourage the search for new populations outside the species known range as sufficient well connected habitat was found in southern Wales and southwestern England. While this method provides information on population size and overall trends, it does not provide sufficient information to detect recent population trends. Therefore, I

suggest more efforts should be focused on calculating temporal effective population change as these can detect changes within a single generation.

Age-structure is also a fundamental parameter related to population size and affecting the conservation status of populations. Such information is highly valued in wildlife conservation yet it is exceptionally difficult to measure, and the only recognised methods are very time consuming. In Chapter 5, I assessed the use of measuring the percentage of DNA methylation at specific CpG sites as a novel way of establishing the age structure of *M. bechsteinii*. I collected 62 wing punches from individuals whose ages were known as a result of a long-term banding study. DNA methylation was measured at seven CpG sites from three genes which have previously shown age-associated changes in humans and laboratory mice. All CpG sites from the tested genes showed a significant relationship between DNA methylation and age, both individually and in combination (multiple linear regression $R^2 = 0.58$, $p < 0.001$). The assay reliably distinguished three biologically-meaningful age groups (sub-adult, adult and old bats) ($\kappa = 0.65$). This method is of considerable practical benefit as it can reliably age an individual bat. It is also much faster than traditional capture-mark-recapture techniques which require multiple years of ringing, with information on the age structure of an entire colony being available from a single sampling session, facilitating prompt conservation action when required. By identifying three genes which can provide useful epigenetic data across distantly related species, this study also suggests that the techniques can potentially be applied to a wider range of mammals. However, to develop these

assays, efforts must first focus on the long-term monitoring of elusive species, such as woodland bats.

While I found that the British population of *M. bechsteinii* was stable, the use of non-invasive capture-mark-recapture techniques was inefficient for population size estimates of woodland bats. More importantly, the development of an epigenetic age assay can now allow the rapid assessment of the age structure of a whole *M. bechsteinii* colony and further improve their conservation by improving population viability estimates or rapidly detecting population trends (as discussed in 6.3.3.).

6.3 Further research and applications

Although the scope of this thesis has provided significant information on *M. bechsteinii* populations and developed novel techniques for improved monitoring of the species, much information on the species ecology is still lacking. Here, I review areas of research that still remain understudied for *M. bechsteinii* and bat conservation and discuss how new technologies can help towards better protecting and monitoring bats.

6.3.1 Information on *Myotis bechsteinii*

In Britain, increased evidence is showing *M. bechsteinii* foraging longer distances from woodlands and roosting outside woodlands more readily (Damant & Cohen, 2016) than reports in continental Europe (Dietz & Pir, 2011). The reasons for these differences in behaviour are not well understood and could be influenced by numerous factors. Colder climate or the prevalence of mature hedgerow trees, for example, could encourage bats to roost outside

woodlands. Woodland size, fragmentation and management (e.g. coppicing and clearance) may also be responsible for this as small British woodlands may not provide sufficiently large areas for whole colonies to forage. Therefore, better knowledge on how woodland size, shape, management and surrounding habitat affects the distance individual bats will forage and how it affects their diet would help towards better protecting colonies.

The use of winter hibernation and autumn mating sites also remains an important gap in the knowledge of *M. bechsteinii* and many other bat species. The protection of these sites alone may not be sufficient as bats often need to travel important distances to reach these sites. Improvements in GPS radio-telemetry and high resolution data, such as LiDAR along with the usage of software's, such as Circuitscape (McRae & Nürnberg, 2006), may soon be able to provide information on the commuting routes bats take to travel from maternity woodlands to swarming sites. More information from swarming sites may also be collected with the use of mtDNA microsatellites, as used by Kerth *et al.* (2003) along with new ones (Jebb, Foley, Kerth, & Teeling, 2017), and provide a useful tool for the identification of new maternity colonies. *M. bechsteinii* live in closed maternal colonies and while the sequencing of fragments mtDNA reveals little genetic diversity, microsatellites are more diverse and have the potential to be used as a "colony identifier tool" by providing a unique barcode for each colony. From this, estimates on the number of colonies surrounding swarming sites could be made by collecting samples from male or female bats.

The identification of an isolated population in Chapter 2 showing higher levels of inbreeding also raises questions on the genetic health of other isolated populations, as these are common at the edge of the species range (e.g. southern Spain, Turkey, Georgia and Scandinavia). For the case of populations in the Middle-East, research must also be focused on identifying whether these may be a cryptic species as they also show very strong mitochondrial divergence in comparison to the European population.

6.3.2 Predicting and mitigating global threats to bats

Most efforts in bat conservation have been targeted on identifying and limiting the impact of current and past threats. Legislation and innovative research in European countries have given a legal status to many species which in some cases has allowed them to thrive. Future efforts must now also be focused on anticipating and limiting the impact of future global threats. Landscape-scale approaches for conservation have become a well-recognised forward-thinking way to better protect species by creating bigger and better connected habitats for a diversity of species (Pressey & Bottrill, 2009). However, the practical application of such approaches is lengthy and threats, such as climate and land cover (e.g. human developments) changes, are expected to have a continual and profound impact in the future on species distribution and abundance (Struebig *et al.*, 2015). These changes may have a highly negative impact on species as ranges can shift or contract, but also impact the dispersal abilities of species to newly suitable habitats and resulting in more isolated populations. The isolation of populations would subsequently reduce genetic diversity and have an important impact on their viability. Although such scenarios remain

largely hypothetical, they highlight the pressing need to better understand the combined impact of climate and land cover changes on bat populations as most research has primarily been focused on the impact of climate alone (Razgour, 2015; Razgour *et al.*, 2013; Razgour *et al.*, 2017; Rebelo *et al.*, 2010).

Emphasis must also be focused on understanding how increasing functional connectivity between known and predicted populations may be used as an effective way to mitigate the impact of such threats on populations.

6.3.3 Establishing woodland bat monitoring schemes

As future pressures may have profound impacts on bat populations, effective monitoring is essential to help towards identifying threats and inform management practices. It has been shown that the estimation of effective population size by collecting samples from individuals of different generations (temporal samples) can detect much more recent changes without the need for large sample sizes (Antao *et al.*, 2011; Waples & Antao, 2014; Waples *et al.*, 2014). These methods are now employed extensively in fisheries monitoring (Charlier, Laikre, & Ryman, 2012; Whiteley *et al.*, 2015a). However, unlike fish, where generations can be identified by size and other visible characteristics, it is difficult to identify distinct generations when studying many wild animals. For example, Mueller, Chakarov, Krüger, and Hoffman (2016) needed to study common buzzard chicks for 12 years to successfully detect population trends over time by calculating effective population sizes. Although such methods are precise, they are also time consuming and difficult to apply to bats. The use of epigenetics techniques, as developed in this thesis, delivers a step-change in the potential to monitor the status of wild bat populations by delivering robust

assessments of the age of individuals as seen in Chapter 5. For the first time, it is now possible to separate *Myotis bechsteinii* bats reliably into distinct age classes. This means that the current status of populations can be understood in much greater detail: colonies predominately composed of older individuals are likely to be in decline whereas those with a high proportion of young animals are expanding. Furthermore, because it is possible to classify individuals into different generations, there is the potential to apply the techniques outlined above: if population declines have occurred then they could be detectable within a single field season using only a limited sample size (~25 animals) (Antao *et al.*, 2011).

6.3.4 Considering genetic rescue

The failing of large scale measures, such as increasing landscape connectivity at a continental scale for isolated populations, may raise the need to undertake more radical actions. Translocations have only rarely been performed on bats as there is a general consensus that the complexity of their social lives may limit the success of these actions (Ruffell, Guilbert, & Parsons, 2009). In addition, translocations need to take into account a multitude of variables (e.g. post-release monitoring or transmission of disease) which are particularly hard to anticipate (IUCN/SSC, 2013). Nonetheless, with improving monitoring technologies bat translocations may be used to increase the genetic diversity and subsequently the viability of isolated populations.

M. bechsteinii may be a species for which genetic rescue may be performed as males are excluded from maternity colonies from a very young age and tend to live solitary lives (Dietz & Pir, 2011). My findings also suggest that males are

less selective in terms of woodland type and that gene flow is more likely to be male-mediated. Therefore, the translocation of juvenile males in September to isolated populations may prove successful as these would almost replicate natural conditions and hopefully increase the viability of isolated populations.

6.3.5 Making the most of new sequencing technologies

The constant and rapid improvements in sequencing technologies (e.g next-generation sequencing) continue to offer more detailed information on wild populations. Although these methods are becoming more affordable, their use in conservation has yet to be more widely used on non-invasive samples.

The recent use of environmental DNA (eDNA), genetic material derived from the habitat of an organism, has provided insight on aquatic but also mammal fauna in woodlands (Ishige *et al.*, 2017; Miya *et al.*, 2015; Ushio *et al.*, 2017). Identifying areas where *M. bechsteinii* and other woodland bats are present is a time consuming task which involves several nights of trapping and often fails to give a clear picture of the bat fauna present within a woodland. Further development of eDNA techniques may allow the rapid detection of bat species within a woodland in a standardised, cost-effective and non-invasive manner (Thomsen & Willerslev, 2015). Improvements may also provide new opportunities for the estimate of individuals directly from pooled samples as the use of next-generation sequencing can be used to amplify microsatellites as short as 40 bp and can subsequently be used on highly degraded samples (De Barba *et al.*, 2017). Estimates may therefore be possible using a pile of droppings from a bat roost.

Epigenetic mechanisms have been found to cause alterations to genetic activity without making any changes to DNA sequences and are associated with many diseases, such as neurological disorders (e.g. Mill *et al.*, 2008). While the study of epigenetics is providing extensive evidence on the negative effect of chemical exposure and other forms of ecological and posttraumatic stresses on human health and model species (e.g. Christensen *et al.*, 2009a; Laing *et al.*, 2016; Marsit & Christensen, 2011; Mehta *et al.*, 2013; Verhoeven, Jansen, Van Dijk, & Biere, 2010; Yehuda *et al.*, 2014), their impact on wildlife remains almost entirely unknown (Richard Pilsner *et al.*, 2010). As these methods become more affordable, more research can be conducted in order to assess the effect of metabolomic impacts, such as air pollution, pesticides (e.g. glyphosate) but also the effect of posttraumatic stress related to sudden habitat changes (e.g. forest clearance) on offspring health and survival. Such techniques could provide rapid information on threats that may not be detected using traditional population genetic techniques.

6.4 Concluding thoughts

Effective conservation of elusive species, such as *M. bechsteinii*, continues to challenge wildlife biologists. Yet it is also such species and challenges that lead to the most significant advances in the field. In this thesis, I used a combination of molecular and landscape approaches to better inform practical conservation strategies for *M. bechsteinii* by assessing the genetic health of the British population, identifying areas of habitat suitability and landscape barriers affecting genetic connectivity and by improving monitoring techniques.

Despite showing high levels of genetic diversity throughout almost its entire range, the data also highlights the importance of woodlands for maintaining habitat and genetic connectivity. The increased interest in landscape scale and evidence-based conservation can help further protect this unique species by effectively protecting woodlands and maintaining their connectivity whilst also acting as an umbrella species for other woodland wildlife. Along with this, the use of novel monitoring techniques will help towards rapidly assessing future threats to the species and also offers the possibility to apply them to a wider range of species. This is especially important as the world's biodiversity still today remains under unprecedented threats from human activities and climate change.

Chapter 7 References

- Ahlén, I., Baagøe, H. J., & Bach, L. (2009). Behavior of Scandinavian bats during migration and foraging at sea. *Journal of Mammalogy*, **90**(6): 1318-1323.
- Antao, T., Pérez-Figueroa, A., & Luikart, G. (2011). Early detection of population declines: high power of genetic monitoring using effective population size estimators. *Evolutionary Applications*, **4**(1): 144-154.
- Arandjelovic, M., Head, J., Rabanal, L. I., Schubert, G., Mettke, E., Boesch, C., . . . Vigilant, L. (2011). Non-invasive genetic monitoring of wild central chimpanzees. *PLoS One*, **6**(3): e14761.
- Ardren, W. R., & Kapuscinski, A. R. (2003). Demographic and genetic estimates of effective population size (N_e) reveals genetic compensation in steelhead trout. *Molecular Ecology*, **12**(1): 35-49.
- Arrizabalaga-Escudero, A., Napal, M., Aihartza, J., Garin, I., Alberdi, A., & Salsamendi, E. (2014). Can pinewoods provide habitat for a deciduous forest specialist? A two-scale approach to the habitat selection of Bechstein's bat. *Mammalian Biology-Zeitschrift für Säugetierkunde*, **79**(2): 117-122.

Atterby, H., Aegerter, J. N., Smith, G. C., Conyers, C. M., Allnutt, T. R., Ruedi, M., & MacNicoll, A. D. (2010). Population genetic structure of the Daubenton's bat (*Myotis daubentonii*) in western Europe and the associated occurrence of rabies. *European journal of wildlife research*, **56**(1): 67-81.

August, T. A., Nunn, M. A., Fensome, A. G., Linton, D. M., & Mathews, F. (2014). Sympatric woodland *Myotis* bats form tight-knit social groups with exclusive roost home ranges. *PLoS One*, **9**(10): e112225.

Aune-Lundberg, L., & Strand, G.-H. (2010). CORINE Land Cover 2006. *The Norwegian CLC2006 project, Report from the Norwegian Forest and Landscape Institute*, **11**(10).

Aviv, A., Chen, W., Gardner, J. P., Kimura, M., Brimacombe, M., Cao, X., . . . Berenson, G. S. (2009). Leukocyte telomere dynamics: longitudinal findings among young adults in the Bogalusa Heart Study. *American journal of epidemiology*, **169**(3): 323-329.

Bailey, S. (2007). Increasing connectivity in fragmented landscapes: an investigation of evidence for biodiversity gain in woodlands. *Forest Ecology and Management*, **238**(1): 7-23.

- Baker, P. J., & Harris, S. (2007). Urban mammals: what does the future hold? An analysis of the factors affecting patterns of use of residential gardens in Great Britain. *Mammal Review*, **37**(4): 297-315.
- Balkenhol, N., Cushman, S. A., Storfer, A., & Waits, L. P. (2016). *Introduction to Landscape Genetics—Concepts, Methods, Applications*. Chichester: Wiley-Blackwell.
- Bashalkhanov, S., Pandey, M., & Rajora, O. P. (2009). A simple method for estimating genetic diversity in large populations from finite sample sizes. *BMC genetics*, **10**(1): 84.
- Battersby, J. (2010). *Guidelines for surveillance and monitoring of European bats*: UNEP/EUROBATS Secretariat.
- Batulevicius, D., Pauziene, N., & Pauza, D. H. (2001). Dental incremental lines in some small species of the European vespertilionid bats. *Acta Theriologica*, **46**(1): 33-42.
- Bayerl, H. (2004). *Raum-Zeit-Nutzungsverhalten und Jagdgebietwahl der Bechsteinfledermaus (Myotis bechsteinii, Kuhl 1817) in zwei Laubmischwäldern im hessischen Wetteraukreis*.
- Beaumont, M. A. (1999). Detecting population expansion and decline using microsatellites. *Genetics*, **153**(4): 2013-2029.

- Becker, N. I., & Encarnação, J. A. (2012). Cost-effectiveness of habitat-suitability maps using low-detailed data for elusive bat species. *European Journal of Wildlife Research*, **58**(6): 945-953.
- Bekaert, B., Kamalandua, A., Zapico, S. C., Van de Voorde, W., & Decorte, R. (2015). Improved age determination of blood and teeth samples using a selected set of DNA methylation markers. *Epigenetics*, **10**(10): 922-930.
- Bellamy, C., & Altringham, J. (2015). Predicting species distributions using record centre data: multi-scale modelling of habitat suitability for bat roosts. *PLoS One*, **10**(6): e0128440.
- Bellamy, C., Scott, C., & Altringham, J. (2013). Multiscale, presence-only habitat suitability models: fine-resolution maps for eight bat species. *Journal of Applied Ecology*, **50**(4): 892-901.
- Benetti, R., García-Cao, M., & Blasco, M. A. (2007). Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. *Nature genetics*, **39**(2): 243-250.
- Bennie, J., Davies, T. W., Inger, R., & Gaston, K. J. (2014). Mapping artificial lightscapes for ecological studies. *Methods in Ecology and Evolution*, **5**(6): 534-540.

- Benson, J. F., Hostetler, J. A., Onorato, D. P., Johnson, W. E., Roelke, M. E., O'Brien, S. J., . . . Oli, M. K. (2011). Intentional genetic introgression influences survival of adults and subadults in a small, inbred felid population. *Journal of Animal Ecology*, **80**(5): 958-967.
- Berry, N., O'connor, W., Holderied, M. W., & Jones, G. (2004). Detection and avoidance of harp traps by echolocating bats. *Acta Chiropterologica*, **6**(2): 335-346.
- Berthinussen, A., & Altringham, J. (2012). The effect of a major road on bat activity and diversity. *Journal of Applied Ecology*, **49**(1): 82-89.
- Besnard, A., La Jeunesse, I., Pays, O., & Secondi, J. (2013). Topographic wetness index predicts the occurrence of bird species in floodplains. *Diversity and Distributions*, **19**(8): 955-963.
- Bize, P., Criscuolo, F., Metcalfe, N. B., Nasir, L., & Monaghan, P. (2009). Telomere dynamics rather than age predict life expectancy in the wild. *Proceedings of the Royal Society B: Biological Sciences*, **276**(1662): 1679-1683.
- Bjørnstad, O. N. (2009). *ncf: spatial nonparametric covariance functions*. R package: <http://cran.r-project.org/web/packages/ncf/>.

- Blehert, D. S., Hicks, A. C., Behr, M., Meteyer, C. U., Berlowski-Zier, B. M., Buckles, E. L., . . . Niver, R. (2009). Bat white-nose syndrome: an emerging fungal pathogen? *Science*, **323**(5911): 227-227.
- Bogdanowicz, W., Piksa, K., & Tereba, A. (2012). Genetic structure in three species of whiskered bats (genus *Myotis*) during swarming. *Journal of Mammalogy*, **93**(3): 799-807.
- Botsford, L. W., Holland, M. D., Samhuri, J. F., White, J. W., & Hastings, A. (2011). Importance of age structure in models of the response of upper trophic levels to fishing and climate change. *ICES Journal of Marine Science: Journal du Conseil*: 1270-1283.
- Briggs, A. W., Stenzel, U., Meyer, M., Krause, J., Kircher, M., & Pääbo, S. (2009). Removal of deaminated cytosines and detection of in vivo methylation in ancient DNA. *Nucleic Acids Research*, **38**(6): e87.
- Brinkman, T. J., Schwartz, M. K., Person, D. K., Pilgrim, K. L., & Hundertmark, K. J. (2010). Effects of time and rainfall on PCR success using DNA extracted from deer fecal pellets. *Conservation Genetics*, **11**(4): 1547-1552.
- Brinkmann, R., Niermann, I., & Steck, C. (2007). Quartiernutzung und Habitatpräferenz von Bechsteinfledermäusen (*Myotis bechsteinii*) in einem Eichen-Hainbuchenwald in der oberrheinischen Tiefebene.

Mitteilungen des Badischen Landesvereins für Naturkunde und Naturschutz, **20**: 181-195.

Brooks, R. P. (1997). Improving habitat suitability index models. *Wildlife Society Bulletin (1973-2006)*, **25**(1): 163-167.

Brunet-Rossini, A., Wilkinson, G., Kunz, T., & Parsons, S. (2009). Methods for estimating age in bats. *Ecological and behavioral methods for the study of bats, 2nd edition (TH Kunz and S. Parsons, eds.)*. The Johns Hopkins University Press, Baltimore: 315-326.

Bryja, J., Kaňuch, P., Fornůsková, A., Bartonička, T., & Řehák, Z. (2009). Low population genetic structuring of two cryptic bat species suggests their migratory behaviour in continental Europe. *Biological Journal of the Linnean Society*, **96**(1): 103-114.

Burland, T., Barratt, E., & Racey, P. (1998). Isolation and characterization of microsatellite loci in the brown long-eared bat, *Plecotus auritus*, and cross-species amplification within the family Vespertilionidae. *Molecular Ecology*, **7**(1): 136-138.

Burns, L. E., Broders, H. G., & Frasier, T. R. (2012). Characterization of 11 tetranucleotide microsatellite loci for the little brown bat (*Myotis lucifugus*) based on in silico genome sequences. *Conservation Genetics Resources*, **4**(3): 653-655.

- Byun, H.-M., Siegmund, K. D., Pan, F., Weisenberger, D. J., Kanel, G., Laird, P. W., & Yang, A. S. (2009). Epigenetic profiling of somatic tissues from human autopsy specimens identifies tissue- and individual-specific DNA methylation patterns. *Human Molecular Genetics*, **18**(24): 4808-4817.
- Castella, V., & Ruedi, M. (2000). Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae). *Molecular Ecology*, **9**(7): 1000-1002.
- Catto, C. M. C., Hutson, A. M., Racey, P. A., & Stephenson, P. J. (1996). Foraging behaviour and habitat use of the serotine bat (*Eptesicus serotinus*) in southern England. *Journal of Zoology*, **238**: 623-633.
- Ceballos, G., Ehrlich, P. R., & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proceedings of the National Academy of Sciences*.
- Chakrabarti, L., Bandyopadhyay, B., & Poddar, M. (2001). Is age-induced decline in immune response associated with hypothalamic glutamate receptor density and dietary protein? *Nutritional Neuroscience*, **4**(5): 375-387.

- Charlier, J., Laikre, L., & Ryman, N. (2012). Genetic monitoring reveals temporal stability over 30 years in a small, lake-resident brown trout population. *Heredity*, **109**(4): 246-253.
- Chaverri, G., & Kunz, T. H. (2011). Response of a specialist bat to the loss of a critical resource. *PLoS One*, **6**(12): e28821.
- Cheng, H., & Lee, L. (2002). Postnatal growth, age estimation, and sexual maturity in the Formosan leaf-nosed bat (*Hipposideros terasensis*). *Journal of Mammalogy*, **83**(3): 785-793.
- Christensen, B. C., Houseman, E., Godleski, J. J., Marsit, C. J., Longacker, J. L., Roelofs, C. R., . . . Nelson, H. H. (2009a). Epigenetic profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome. *Cancer Research*, **69**(1): 227-234.
- Christensen, B. C., Houseman, E. A., Marsit, C. J., Zheng, S., Wrensch, M. R., Wiemels, J. L., . . . Bueno, R. (2009b). Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. *PLoS Genetics*, **5**(8): 1-13.
- Clauzel, C., Bannwarth, C., & Foltete, J.-C. (2015). Integrating regional-scale connectivity in habitat restoration: An application for amphibian

conservation in eastern France. *Journal for Nature Conservation*, **23**: 98-107.

Collins, J. (2016). *Bat Surveys for Professional Ecologists: Good Practice Guidelines (3rd edn)*: Bat Conservation Trust, London.

Combe, F. J., Ellis, J. S., Lloyd, K. L., Cain, B., Wheeler, C. P., & Harris, W. E. (2016). After the Ice Age: The Impact of Post-Glacial Dispersal on the Phylogeography of a Small Mammal, *Muscardinus avellanarius*. *Frontiers in Ecology and Evolution*, **4**(72): 1 - 12.

Cordingley, J. E., Newton, A. C., Rose, R. J., Clarke, R. T., & Bullock, J. M. (2015). Habitat fragmentation intensifies trade-offs between biodiversity and ecosystem services in a heathland ecosystem in southern England. *PLoS One*, **10**(6): e0130004.

Couzens, D., Swash, A., Still, R., & Dunn, J. (2017). *Britain's Mammals: A Field Guide to the Mammals of Britain and Ireland*. Princeton University Press.

Creel, S., Spong, G., Sands, J. L., Rotella, J., Zeigle, J., Joe, L., . . . Smith, D. (2003). Population size estimation in Yellowstone wolves with error-prone noninvasive microsatellite genotypes. *Molecular Ecology*, **12**(7): 2003-2009.

Croose, E., Birks, J. D. S., Schofield, H. W., & O'Reilly, C. (2014). Distribution of the pine marten (*Martes martes*) in southern Scotland in 2013. *Scottish Natural Heritage Commissioned Report, No. 740*.

Damant, C. (2017). Genetic analysis of Bechstein's bat diet in Bernwood.
[Personal communication]

Damant, C., & Cohen, K. (2016). Radio tracking studies of Bechstein's bats.
[Personal communication].

De Barba, M., Miquel, C., Lobréaux, S., Quenette, P., Swenson, J., & Taberlet, P. (2017). High-throughput microsatellite genotyping in ecology: improved accuracy, efficiency, standardization and success with low-quantity and degraded DNA. *Molecular Ecology Resources*, **17**(3): 492-507.

De Iorio, M., & Griffiths, R. C. (2004). Importance sampling on coalescent histories. II: Subdivided population models. *Advances in Applied Probability*, **36**(02): 434-454.

De Paz, O. (1986). Age estimation and postnatal growth of the greater mouse bat *Myotis myotis* (Borkhausen, 1797) in Guadalajara, Spain. *Mammalia*, **50**(2): 243-252.

- Dekeukeleire, D., Janssen, R., Haarsma, A.-J., Bosch, T., & Schaik, J. V. (2016). Swarming behaviour, catchment area and seasonal movement patterns of the Bechstein's bats: implications for conservation. *Acta Chiropterologica*, **18**(2): 349-358.
- Dennis, C. (2006). Conservation at a distance: a gentle way to age. *Nature*, **442**(7102): 507-508.
- Dietz, C., & Kiefer, A. (2016). *Bats of Britain and Europe*. London: Bloomsbury Publishing.
- Dietz, C., Nill, D., & von Helversen, O. (2009). *Bats of Britain, Europe and Northwest Africa*. London: A & C Black.
- Dietz, M. (2013). *Populationsökologie und Habitatansprüche der Bechsteinfledermaus Myotis bechsteinii*. Zarbock.
- Dietz, M., Dawo, B., Krannich, A., & Pir, J. B. (2015). Identifying key habitats in different scales provides a reliable basis for the conservation of forest dwelling Bechstein's bat. (Paper presented to the 4th International Berlin Bat Meeting).
- Dietz, M., & Pir, J. B. (2011). *Distribution, Ecology and Habitat Selection by Bechstein's Bat (Myotis Bechsteinii) in Luxembourg*: Laurenti.

Dunsha, G., Duffield, D., Gales, N., Hindell, M., Wells, R. S., & Jarman, S. N. (2011). Telomeres as age markers in vertebrate molecular ecology. *Molecular Ecology Resources*, **11**(2): 225-235.

Durrant, C. J. (2016). Correspondence with Durrant, C.: [personal communication].

Durrant, C. J., Beebee, T. J., Greenaway, F., & Hill, D. A. (2009). Evidence of recent population bottlenecks and inbreeding in British populations of Bechstein's bat, *Myotis bechsteinii*. *Conservation Genetics*, **10**(2): 489-496.

EDINA. (2017). OS MasterMap Topography Layer, <http://edina.ac.uk/digimap> [accessed March 2017]

Eggers, B., & Köhler, R. (2012) *Perspectives on Nature Conservation-Patterns, Pressures and Prospects* (pp. 141-160). Croatia: InTech.

Elith, J., Graham, C. H., Anderson, R. P., Dudík, M., Ferrier, S., Guisan, A., . . . Lehmann, A. (2006). Novel methods improve prediction of species' distributions from occurrence data. *Ecography*, **29**(2): 129-151.

- Elith, J., & Leathwick, J. R. (2009). Species distribution models: ecological explanation and prediction across space and time. *Annual Review of Ecology, Evolution, and Systematics*, **40**: 677-697.
- Ellis, J., Knight, M. E., Darvill, B., & Goulson, D. (2006). Extremely low effective population sizes, genetic structuring and reduced genetic diversity in a threatened bumblebee species, *Bombus sylvarum* (Hymenoptera: Apidae). *Molecular Ecology*, **15**(14): 4375-4386.
- England, P. R., Cornuet, J.-M., Berthier, P., Tallmon, D. A., & Luikart, G. (2006). Estimating effective population size from linkage disequilibrium: severe bias in small samples. *Conservation Genetics*, **7**(2): 303-308.
- Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**(8): 2611-2620.
- Fahrig, L. (2003). Effects of habitat fragmentation on biodiversity. *Annual Review of Ecology, Evolution, and Systematics*, **34**(1): 487-515.
- Fahrig, L. (2017). Ecological responses to habitat fragmentation per se. *Annual Review of Ecology, Evolution, and Systematics*, **48**(1):1-23.

Fischer, J., & Lindenmayer, D. B. (2007). Landscape modification and habitat fragmentation: a synthesis. *Global ecology and biogeography*, **16**(3): 265-280.

Forest Research. (2017). *National Forest Inventory*. National Forest Inventory, <https://www.forestry.gov.uk/inventory> [accessed March 2017].

Fox, R., Brereton, T., Asher, J., August, T., Botham, M., Bourn, N., . . . Harrower, C. (2015). *The State of the UK's Butterflies 2015: Butterfly Conservation and the Centre for Ecology & Hydrology*, Wareham, Dorset.

Frankham, R. (1997). Do island populations have less genetic variation than mainland populations? *Heredity*, **78**(3): 311-327.

Frankham, R., Bradshaw, C. J., & Brook, B. W. (2014). Genetics in conservation management: revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biological Conservation*, **170**: 56-63.

Frankham, R., Briscoe, D. A., & Ballou, J. D. (2002). *Introduction to Conservation Genetics*. Cambridge: Cambridge University Press.

- Frantz, A., Pope, L., Carpenter, P., Roper, T., Wilson, G., Delahay, R., & Burke, T. (2003). Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA. *Molecular Ecology*, **12**(6): 1649-1661.
- Frey-Ehrenbold, A., Bontadina, F., Arlettaz, R., & Obrist, M. K. (2013). Landscape connectivity, habitat structure and activity of bat guilds in farmland-dominated matrices. *Journal of Applied Ecology*, **50**(1): 252-261.
- Fuentes-Montemayor, E., Goulson, D., Cavin, L., Wallace, J. M., & Park, K. J. (2013). Fragmented woodlands in agricultural landscapes: The influence of woodland character and landscape context on bats and their insect prey. *Agriculture, Ecosystems & Environment*, **172**: 6-15.
- Furmankiewicz, J., & Altringham, J. (2007). Genetic structure in a swarming brown long-eared bat (*Plecotus auritus*) population: evidence for mating at swarming sites. *Conservation Genetics*, **8**(4): 913-923.
- Gaisler, J., Hanák, V., Hanzal, V., & Jarský, V. (2003). Results of bat banding in the Czech and Slovak Republics, 1948–2000. *Vespertilio*, **7**: 3-61.
- Garroway, C. J., Bowman, J., & Wilson, P. J. (2011). Using a genetic network to parameterize a landscape resistance surface for fishers, *Martes pennanti*. *Molecular Ecology*, **20**(19): 3978-3988.

- Gerlach, G., & Musolf, K. (2000). Fragmentation of landscape as a cause for genetic subdivision in bank voles. *Conservation biology*, **14**(4): 1066-1074.
- Glover, A. M., & Altringham, J. D. (2008). Cave selection and use by swarming bat species. *Biological Conservation*, **141**(6): 1493-1504.
- Gregory, R. D., Van Strien, A., Vorisek, P., Meyling, A. W. G., Noble, D. G., Foppen, R. P., & Gibbons, D. W. (2005). Developing indicators for European birds. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **360**(1454): 269-288.
- Greider, C. W. (1996). Telomere length regulation. *Annual review of biochemistry*, **65**(1): 337-365.
- Griffin, D. R. (1944). How bats guide their flight by supersonic echoes. *American Journal of Physics*, **12**(6): 342-345.
- Grönniger, E., Weber, B., Heil, O., Peters, N., Stäb, F., Wenck, H., . . . Lyko, F. (2010). Aging and chronic sun exposure cause distinct epigenetic changes in human skin. *PLoS Genetics*, **6**(5): 1-10.
- Guisan, A., Thuiller, W., & Zimmermann, N. E. (2017). *Habitat Suitability and Distribution Models: with Applications in R*. Cambridge: Cambridge University Press.

Guisan, A., & Zimmermann, N. E. (2000). Predictive habitat distribution models in ecology. *Ecological Modelling*, **135**(2): 147-186.

Gutierrez-Arcelus, M., Lappalainen, T., Montgomery, S. B., Buil, A., Ongen, H., Yurovsky, A., . . . Planchon, A. (2013). Passive and active DNA methylation and the interplay with genetic variation in gene regulation. *eLife*, **2**: e00523.

Hall, M. E., Nasir, L., Daunt, F., Gault, E. A., Croxall, J. P., Wanless, S., & Monaghan, P. (2004). Telomere loss in relation to age and early environment in long-lived birds. *Proceedings of the Royal Society of London B: Biological Sciences*, **271**(1548): 1571-1576.

Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sada, S., . . . Gao, Y. (2013). Genome-wide methylation profiles reveal quantitative views of human aging rates. *Molecular Cell*, **49**(2): 359-367.

Harley, E. H., Baumgarten, I., Cunningham, J., & O'RYAN, C. (2005). Genetic variation and population structure in remnant populations of black rhinoceros, *Diceros bicornis*, in Africa. *Molecular Ecology*, **14**(10): 2981-2990.

- Harrington, A. (2015). *Novel rapid genetic tests for non-invasive identification of bat species of Ireland and Great Britain (unpublished thesis)*: Waterford Insitute of Technology, Ireland.
- Hausmann, M., & Vleck, C. (2002). Telomere length provides a new technique for aging animals. *Oecologia*, **130**(3): 325-328.
- Hausmann, M. F., Winkler, D. W., O'Reilly, K. M., Huntington, C. E., Nisbet, I. C., & Vleck, C. M. (2003). Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proceedings of the Royal Society of London B: Biological Sciences*, **270**(1522): 1387-1392.
- Hayhow, D., Burns, F., Eaton, M., Al Fulaij, N., August, T., Babey, L., . . . Gregory, R. (2016). State of Nature 2016. *The State of Nature Partnership*.
- Herrmann, B., & Henke, W. (1999). DNA preservation: a microsatellite-DNA study on ancient skeletal remains. *Electrophoresis*, **20**: 1722-1728.
- Hettinga, P. N., Arnason, A. N., Manseau, M., Cross, D., Whaley, K., & Wilson, P. J. (2012). Estimating size and trend of the North Interlake woodland caribou population using fecal-DNA and capture-recapture models. *The Journal of Wildlife Management*, **76**(6): 1153-1164.

- Hill, D. A., & Greenaway, F. (2005). Effectiveness of an acoustic lure for surveying bats in British woodlands. *Mammal Review*, **35**(1): 116-122.
- Horvath, S. (2013). DNA methylation age of human tissues and cell types. *Genome Biology*, **14**(10): R115.
- Ibáñez, C., García-Mudarra, J. L., Ruedi, M., Stadelmann, B., & Juste, J. (2006). The Iberian contribution to cryptic diversity in European bats. *Acta Chiropterologica*, **8**(2): 277-297.
- Ishige, T., Miya, M., Ushio, M., Sado, T., Ushioda, M., Maebashi, K., . . . Matsubayashi, H. (2017). Tropical-forest mammals as detected by environmental DNA at natural saltlicks in Borneo. *Biological Conservation*, **210**: 281-285.
- IUCN/SSC. (2013). *Guidelines for Reintroductions and Other Conservation Translocations. Version 1.0.*: Gland, Switzerland: IUCN Species Survival Commission.
- Jebb, D., Foley, N. M., Kerth, G., & Teeling, E. C. (2017). The complete mitochondrial genome of the Bechstein's bat, *Myotis bechsteinii* (Chiroptera, Vespertilionidae). *Mitochondrial DNA Part B*, **2**(1): 92-94.

- Johnson, W. E., Onorato, D. P., Roelke, M. E., Land, E. D., Cunningham, M., Belden, R. C., . . . Shindle, D. (2010). Genetic restoration of the Florida panther. *Science*, **329**(5999): 1641-1645.
- Jombart, T. (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, **24**(11): 1403-1405.
- Jones, K. E., Mickleburgh, S. P., Sechrest, W., & Walsh, A. L. (2009). Global overview of the conservation of island bats: importance, challenges and opportunities. *Island Bats: Evolution, Ecology, and Conservation.*: 496-530.
- Jones, M. J., Goodman, S. J., & Kobor, M. S. (2015). DNA methylation and healthy human aging. *Aging Cell*, **14**(6): 924-932.
- Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, **13**(7): 484-492.
- Jones, P. A., & Laird, P. W. (1999). Cancer-epigenetics comes of age. *Nature genetics*, **21**(2): 163-167.
- Kalinowski, S. T. (2005). hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular ecology notes*, **5**(1): 187-189.

- Kalinowski, S. T., Taper, M. L., & Marshall, T. C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, **16**(5): 1099-1106.
- Kerth, G., Kiefer, A., Trappmann, C., & Weishaar, M. (2003). High gene diversity at swarming sites suggest hot spots for gene flow in the endangered Bechstein's bat. *Conservation Genetics*, **4**(4): 491-499.
- Kerth, G., & König, B. (1999). Fission, fusion and nonrandom associations in female Bechstein's bats (*Myotis bechsteinii*). *Behaviour*, **136**(9): 1187-1202.
- Kerth, G., & König, B. (1999). Fission, fusion and nonrandom associations in female Bechstein's bats (*Myotis bechsteinii*). *Behaviour*, **136**(9): 1187-1202.
- Kerth, G., Mayer, F., & König, B. (2000). Mitochondrial DNA (mtDNA) reveals that female Bechstein's bats live in closed societies. *Molecular Ecology*, **9**(6): 793-800.
- Kerth, G., Mayer, F., & Petit, E. (2002). Extreme sex-biased dispersal in the communally breeding, nonmigratory Bechstein's bat (*Myotis bechsteinii*). *Molecular Ecology*, **11**(8): 1491-1498.

- Kerth, G., Petrov, B., Conti, A., Anastasov, D., Weishaar, M., Gazaryan, S., . . . Bruyndonckx, N. (2008). Communally breeding Bechstein's bats have a stable social system that is independent from the postglacial history and location of the populations. *Molecular Ecology*, **17**(10): 2368-2381.
- Kerth, G., & Reckardt, K. (2003). Information transfer about roosts in female Bechstein's bats: an experimental field study. *Proc Biol Sci*, **270**(1514): 511-515.
- Kerth, G., Weissmann, K., & König, B. (2001). Day roost selection in female Bechstein's bats (*Myotis bechsteinii*): a field experiment to determine the influence of roost temperature. *Oecologia*, **126**(1): 1-9.
- Kindberg, J., Swenson, J. E., Ericsson, G., Bellemain, E., Miquel, C., & Taberlet, P. (2011). Estimating population size and trends of the Swedish brown bear *Ursus arctos* population. *Wildlife Biology*, **17**(2): 114-123.
- Koch, C. M., Suschek, C. V., Lin, Q., Bork, S., Goergens, M., Jousen, S., . . . Wagner, W. (2011). Specific age-associated DNA methylation changes in human dermal fibroblasts. *PLoS One*, **6**(2): e16679.
- Kunz, T. H., Arnett, E. B., Erickson, W. P., Hoar, A. R., Johnson, G. D., Larkin, R. P., . . . 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): 315-324.

- Kunz, T. H., Braun de Torrez, E., Bauer, D., Lobova, T., & Fleming, T. H. (2011). Ecosystem services provided by bats. *Annals of the New York Academy of Sciences*, **1223**(1): 1-38.
- Kunz, T. H., & Hood, W. R. (2000). Parental care and postnatal growth in the Chiroptera. In E. G. Crichton & P. H. Kruttsch (Eds.), *Reproductive biology of bats* (pp. 415-468). London: Academic Press.
- Kunz, T. H., Lumsden, L. F., Kunz, T., & Fenton, M. (2003). Ecology of cavity and foliage roosting bats. *Bat ecology*, **1**: 3-89.
- Laing, L., Viana, J., Dempster, E., Trznadel, M., Trunkfield, L., Uren Webster, T., . . . Mill, J. (2016). Bisphenol A causes reproductive toxicity, decreases dnmt1 transcription, and reduces global DNA methylation in breeding zebrafish (*Danio rerio*). *Epigenetics*, **11**(7): 526-538.
- Lam, L. L., Emberly, E., Fraser, H. B., Neumann, S. M., Chen, E., Miller, G. E., & Kobor, M. S. (2012). Factors underlying variable DNA methylation in a human community cohort. *Proceedings of the National Academy of Sciences*, **109**(2): 17253-17260.
- Lampa, S., Henle, K., Klenke, R., Hoehn, M., & Gruber, B. (2013). How to overcome genotyping errors in non-invasive genetic mark-recapture

population size estimation-A review of available methods illustrated by a case study. *The Journal of Wildlife Management*, **77**(8): 1490-1511.

Lande, R., Engen, S., & Saether, B.-E. (2003). *Stochastic population dynamics in ecology and conservation*. Oxford: Oxford University Press.

Larsen, R. J., Boegler, K. A., Genoways, H. H., Masefield, W. P., Kirsch, R. A., & Pedersen, S. C. (2007). Mist netting bias, species accumulation curves, and the rediscovery of two bats on Montserrat (Lesser Antilles). *Acta Chiropterologica*, **9**(2): 423-435.

Leblois, R., Beeravolu Reddy, C., & Rousset, F. (2013). *The Migraine project: A user-friendly software for likelihood-based inferences of spatial structure and demographic history from genetic data*. Paper presented at the Summer Research School Software and Statistical methods for population genetics. 2013-06-17/2013-06-21, Aussois, FRA.

Leblois, R., Pudlo, P., Néron, J., Bertaux, F., Beeravolu, C. R., Vitalis, R., & Rousset, F. (2014). Maximum likelihood inference of population size contractions from microsatellite data. *Molecular biology and evolution*, **31**(10): 2805-2823.

Lecis, R., Ferrando, A., Ruiz-Olmo, J., Manas, S., & Domingo-Roura, X. (2008). Population genetic structure and distribution of introduced American mink

- (*Mustela vison*) in Spain, based on microsatellite variation. *Conservation Genetics*, **9**(5): 1149-1161.
- Leigh, J. W., & Bryant, D. (2015). popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, **6**(9): 1110-1116.
- Lesiński, G. (2007). Bat road casualties and factors determining their number. *Mammalia*, **71**(3): 138-142.
- Lintott, P., Richardson, S., Hosken, D., Fensome, S., & Fiona, M. (2016). Ecological impact assessments fail to reduce risk of bat casualties at wind farms. *Current Biology*, **26**(21): 1135-1136.
- Lintott, P. R., Bunnefeld, N., Fuentes-Montemayor, E., Minderman, J., Mayhew, R. J., Olley, L., & Park, K. J. (2014). City life makes females fussy: sex differences in habitat use of temperate bats in urban areas. *Royal Society Open Science*, **1**(3): 140200.
- Lister, R., Pelizzola, M., Downen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., . . . Ngo, Q.-M. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, **462**: 315-322.

- Llamas, B., Holland, M. L., Chen, K., Cropley, J. E., Cooper, A., & Suter, C. M. (2012). High-resolution analysis of cytosine methylation in ancient DNA. *PLoS One*, **7**(1): e30226.
- Löfström, C., Knutsson, R., Axelsson, C. E., & Rådström, P. (2004). Rapid and specific detection of *Salmonella spp.* in animal feed samples by PCR after culture enrichment. *Applied and Environmental Microbiology*, **70**(1): 69-75.
- Luckens, M. M., & Davis, W. H. (1964). Bats: sensitivity to DDT. *Science*, **146**(3646): 948-948.
- Maegawa, S., Hinkal, G., Kim, H. S., Shen, L., Zhang, L., Zhang, J., . . . Issa, J.-P. J. (2010). Widespread and tissue specific age-related DNA methylation changes in mice. *Genome Research*, **20**(3): 332-340.
- Magri, D. (2008). Patterns of post-glacial spread and the extent of glacial refugia of European beech (*Fagus sylvatica*). *Journal of Biogeography*, **35**(3): 450-463.
- Magri, D., Vendramin, G. G., Comps, B., Dupanloup, I., Geburek, T., Gömöry, D., . . . Roure, J. M. (2006). A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. *New Phytologist*, **171**(1): 199-221.

- Manel, S., & Holderegger, R. (2013). Ten years of landscape genetics. *Trends in Ecology & Evolution*, **28**(10): 614-621.
- Manel, S., Schwartz, M. K., Luikart, G., & Taberlet, P. (2003). Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology & Evolution*, **18**(4): 189-197.
- Marsit, C. J., & Christensen, B. C. (2011). Epigenomics in environmental health. *Frontiers in Genetics*, **2**(84): 1-10.
- Marzluff, J. M. (2001). Avian Ecology and Conservation in an Urbanizing World
In J. M. Marzluff, R. Bowman, & D. Roarke (Eds.), *Worldwide urbanization and its effects on birds* (pp. 19-47). Boston: Springer.
- Mathews, F., & Kubasiewicz, L. (2017). Preliminary results from: *Britain's Mammals 2018: The Mammal Society's Guide to their Conservation Status*. [Personal communication]
- Mathews, F., Roche, N., Aughney, T., Jones, N., Day, J., Baker, J., & Langton, S. (2015). Barriers and benefits: implications of artificial night-lighting for the distribution of common bats in Britain and Ireland. *Phil. Trans. R. Soc. B*, **370**(1667): 20140124.

- McClure, C. J., Ware, H. E., Carlisle, J., Kaltenecker, G., & Barber, J. R. (2013). An experimental investigation into the effects of traffic noise on distributions of birds: avoiding the phantom road. *Proceedings of the Royal Society of London B: Biological Sciences*, **280**(1773): 20132290.
- McDevitt, A. D., Edwards, C. J., O'Toole, P., O'Sullivan, P., O'Reilly, C., & Carden, R. F. (2009). Genetic structure of, and hybridisation between, red (*Cervus elaphus*) and sika (*Cervus nippon*) deer in Ireland. *Mammalian Biology-Zeitschrift für Säugetierkunde*, **74**(4): 263-273.
- McLeod, B., Burns, L., Frasier, T., & Broders, H. (2015). Effect of oceanic straits on gene flow in the recently endangered little brown bat (*Myotis lucifugus*) in maritime Canada: implications for the spread of white-nose syndrome. *Canadian Journal of Zoology*, **93**(6): 427-437.
- McRae, B. H., & Nürnberger, B. (2006). Isolation by resistance. *Evolution*, **60**(8): 1551-1561.
- Mehta, D., Klengel, T., Conneely, K. N., Smith, A. K., Altmann, A., Pace, T. W., . . . Mercer, K. B. (2013). Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. *Proceedings of the National Academy of Sciences*, **110**(20): 8302-8307.
- Meirmans, P. G. (2012). The trouble with isolation by distance. *Molecular Ecology*, **21**(12): 2839-2846.

- Merow, C., Smith, M. J., & Silander, J. A. (2013). A practical guide to MaxEnt for modeling species' distributions: what it does, and why inputs and settings matter. *Ecography*, **36**(10): 1058-1069.
- Meyer, C. F., Kalko, E. K., & Kerth, G. (2009). Small-scale fragmentation effects on local genetic diversity in two Phyllostomid bats with different dispersal abilities in Panama. *Biotropica*, **41**(1): 95-102.
- Mickleburgh, S., Waylen, K., & Racey, P. (2009). Bats as bushmeat: a global review. *Oryx*, **43**(2): 217-234.
- Mickleburgh, S. P., Hutson, A. M., & Racey, P. A. (2002). A review of the global conservation status of bats. *Oryx*, **36**(01): 18-34.
- Mill, J., Tang, T., Kaminsky, Z., Khare, T., Yazdanpanah, S., Bouchard, L., . . . Schumacher, A. (2008). Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. *The American Journal of Human Genetics*, **82**(3): 696-711.
- Miller, C. R., Joyce, P., & Waits, L. P. (2005). A new method for estimating the size of small populations from genetic mark–recapture data. *Molecular Ecology*, **14**(7): 1991-2005.
- Miller, H. (2011). *Bechstein's Bat Survey: September 2007 – September 2011*: Report held by the Bat Conservation Trust.

- Mills, L. S., Citta, J. J., Lair, K. P., Schwartz, M. K., & Tallmon, D. A. (2000). Estimating animal abundance using noninvasive DNA sampling: promise and pitfalls. *Ecological Applications*, **10**(1): 283-294.
- Mitchell-Jones, A., Cooke, A., Boyd, I., & Stebbings, R. (1989). Bats and remedial timber treatment chemicals a review. *Mammal Review*, **19**(3): 93-110.
- Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., . . . Araki, H. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *Royal Society Open Science*, **2**(7): 150088.
- Mizroch, S. A., Beard, J. A., & Lynde, M. (1990). Computer assisted photo-identification of humpback whales. *Report of the International Whaling Commission*, **12**: 63-70.
- Monteiro, L., Bonnemaïson, D., Vekris, A., Petry, K. G., Bonnet, J., Vidal, R., . . . Mégraud, F. (1997). Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *Journal of Clinical Microbiology*, **35**(4): 995-998.

- Morin, P. A., & Woodruff, D. S. (1996). Noninvasive Genotyping for Vertebrate. In T. Smith & R. Wayne (Eds.), *Molecular Genetic Approaches in Conservation* (pp. 298). Oxford: Oxford University Press.
- Moussy, C., Atterby, H., Griffiths, A., Allnutt, T., Mathews, F., Smith, G., . . . Hosken, D. (2015). Population genetic structure of serotine bats (*Eptesicus serotinus*) across Europe and implications for the potential spread of bat rabies (European bat lyssavirus EBLV-1). *Heredity*, **115**(1): 83-92.
- Moussy, C., Hosken, D., Mathews, F., Smith, G., Aegerter, J., & Bearhop, S. (2013). Migration and dispersal patterns of bats and their influence on genetic structure. *Mammal Review*, **43**(3): 183-195.
- Mueller, A., Chakarov, N., Krüger, O., & Hoffman, J. (2016). Long-term effective population size dynamics of an intensively monitored vertebrate population. *Heredity*, **117**(4): 290.
- Munshi-South, J., & Wilkinson, G. S. (2010). Bats and birds: exceptional longevity despite high metabolic rates. *Ageing Research Reviews*, **9**(1): 12-19.
- Murphy, M. A., Kendall, K. C., Robinson, A., & Waits, L. P. (2007). The impact of time and field conditions on brown bear (*Ursus arctos*) faecal DNA amplification. *Conservation Genetics*, **8**(5): 1219-1224.

- Naimi, B. (2013). usdm: Uncertainty analysis for species distribution models. *R package version, 1*: 1-12.
- Naimi, B., Hamm, N. A., Groen, T. A., Skidmore, A. K., & Toxopeus, A. G. (2014). Where is positional uncertainty a problem for species distribution modelling? *Ecography, 37*(2): 191-203.
- Napal, M., Garin, I., Goiti, U., Salsamendi, E., & Aihartza, J. (2009). Selection of maternity roosts by *Myotis bechsteinii* (Kuhl, 1817) in the Southwestern Iberian Peninsula. *Acta Chiropterologica, 11*(2): 425-433.
- Napal, M., Garin, I., Goiti, U., Salsamendi, E., & Aihartza, J. (2013). Past deforestation of Mediterranean Europe explains the present distribution of the strict forest dweller *Myotis bechsteinii*. *Forest Ecology and Management, 293*: 161-170.
- NaturalEngland. (2016). Ancient Woodlands (England), <https://data.gov.uk/dataset/ancient-woodlands-england2> [accessed March 2017].
- Nelson, M. E. (2002). The science, ethics, and philosophy of tooth extractions from live-captured white-tailed deer: a response to Festa-Bianchet et al.(2002). *Wildlife Society Bulletin: 284-288*.

- NRW. (2011). Ancient Woodland Inventory 2011, <https://data.gov.uk/dataset/ancient-woodlandinventory-2011> [accessed October 2017].
- O'Shea, T. J., & Bogan, M. A. (2003). Monitoring trends in bat populations of the United States and territories: problems and prospects. *Publications of the US Geological Survey*, 16.
- O'Brien, J., McCracken, G. F., Say, L., & Hayden, T. J. (2007). Rodrigues fruit bats (*Pteropus rodricensis*, Megachiroptera: *Pteropodidae*) retain genetic diversity despite population declines and founder events. *Conservation Genetics*, 8(5): 1073.
- O'Grady, J. J., Brook, B. W., Reed, D. H., Ballou, J. D., Tonkyn, D. W., & Frankham, R. (2006). Realistic levels of inbreeding depression strongly affect extinction risk in wild populations. *Biological Conservation*, 1(133): 42-51.
- Oli, M. K., & Dobson, F. S. (2003). The relative importance of life-history variables to population growth rate in mammals: Cole's prediction revisited. *The American Naturalist*, 161(3): 422-440.
- Olsen, M. T., Bérubé, M., Robbins, J., & Palsbøll, P. J. (2012). Empirical evaluation of humpback whale telomere length estimates; quality control

and factors causing variability in the singleplex and multiplex qPCR methods. *BMC Genetics*, **13**(1): 77.

Olsen, M. T., Robbins, J., Bérubé, M., Rew, M. B., & Palsbøll, P. J. (2014). Utility of telomere length measurements for age determination of humpback whales. *NAMMCO Scientific Publications*, **10**.

Opel, K. L., Chung, D., & McCord, B. R. (2010). A study of PCR inhibition mechanisms using real time PCR. *Journal of Forensic Sciences*, **55**(1): 25-33.

OSS. (2017). Ordnance Survey System, OS Terrain 50, <https://www.ordnancesurvey.co.uk/business-and-government/products/terrain-50.html> [accessed October 2017].

Oyler-McCance, S. J., & Fike, J. A. (2011). Characterization of small microsatellite loci isolated in endangered Indiana bat (*Myotis sodalis*) for use in non-invasive sampling. *Conservation Genetics Resources*, **3**(2): 243-245.

Paetkau, D. (2003). An empirical exploration of data quality in DNA-based population inventories. *Molecular Ecology*, **12**(6): 1375-1387.

- Parsons, K., & Jones, G. (2003). Dispersion and habitat use by *Myotis daubentonii* and *Myotis nattereri* during the swarming season: implications for conservation. *Animal Conservation*, **6**(04): 283-290.
- Paunović, M. (2016). *Myotis bechsteinii*. The IUCN Red List of Threatened Species 2016: e.T14123A22053752.
- Peakall, R., & Smouse, P. (2012). GenAIEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics*, **28**: 2537-2539.
- Peakall, R., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**(1): 288-295.
- Peterman, W. E. (2018). ResistanceGA: An R package for the optimization of resistance surfaces using genetic algorithms. *Methods in Ecology and Evolution*, **9**(6).
- Peterman, W. E., Connette, G. M., Semlitsch, R. D., & Eggert, L. S. (2014). Ecological resistance surfaces predict fine-scale genetic differentiation in a terrestrial woodland salamander. *Molecular Ecology*, **23**(10): 2402-2413.

- Petit, E., Excoffier, L., & Mayer, F. (1999). No evidence of bottleneck in the postglacial recolonization of Europe by the noctule bat (*Nyctalus noctula*). *Evolution*, **53**(4): 1247-1258.
- Petrov, B. P. (2006). Distribution and status of *Myotis bechsteinii* in Bulgaria (Chiroptera: Vespertilionidae). *Lynx*, **37**: 179-195.
- Pimm, S. L., Dollar, L., & Bass, O. L. (2006). The genetic rescue of the Florida panther. *Animal Conservation*, **9**(2): 115-122.
- Polanowski, A. M., Robbins, J., Chandler, D., & Jarman, S. N. (2014). Epigenetic estimation of age in humpback whales. *Molecular Ecology Resources*, **14**(5): 976-987.
- Poole, K. G., Reynolds, D. M., Mowat, G., & Paetkau, D. (2011). Estimating mountain goat abundance using DNA from fecal pellets. *The Journal of Wildlife Management*, **75**(6): 1527-1534.
- Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution*, **25**(6): 345-353.
- Poulton, S. (2008). *Analysis of feeding preferences of Bechstein's bats*: Bioecoss Ltd: The Vincent Wildlife Trust.

- Pressey, R. L., & Bottrill, M. C. (2009). Approaches to landscape-and seascape-scale conservation planning: convergence, contrasts and challenges. *Oryx*, **43**(4): 464-475.
- Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, **155**(2): 945-959.
- Puechmaille, S. J., Mathy, G., & Petit, E. J. (2007). Good DNA from bat droppings. *Acta Chiropterologica*, **9**(1): 269-276.
- Puechmaille, S. J., & Petit, E. J. (2007). Empirical evaluation of non-invasive capture-mark-recapture estimation of population size based on a single sampling session. *Journal of Applied Ecology*, **44**(4): 843-852.
- Racey, P. A., & Entwistle, A. C. (2000). Life-history and reproductive strategies of bats. *Reproductive biology of bats*: 363-414.
- Ramirez-Reyes, C., Bateman, B. L., & Radeloff, V. C. (2016). Effects of habitat suitability and minimum patch size thresholds on the assessment of landscape connectivity for jaguars in the Sierra Gorda, Mexico. *Biological Conservation*, **204**: 296-305.
- Razgour, O. (2015). Beyond species distribution modeling: A landscape genetics approach to investigating range shifts under future climate change, **30**. *Ecological Informatics*.

Razgour, O., Juste, J., Ibáñez, C., Kiefer, A., Rebelo, H., Puechmaille, S. J., . . .

Beaumont, M. (2013). The shaping of genetic variation in edge-of-range populations under past and future climate change. *Ecology Letters*, **16**(10): 1258-1266.

Razgour, O., Rebelo, H., Di Febbraro, M., & Russo, D. (2016). Painting maps

with bats: species distribution modelling in bat research and conservation. *Hystrix*, **27**: 1-8.

Razgour, O., Taggart, J. B., Manel, S., Juste, J., Ibáñez, C., Rebelo, H., . . .

Park, K. (2017). An integrated framework to identify wildlife populations under threat from climate change. *Molecular Ecology Resources*, **18**(1): 18-31.

RCoreTeam. (2016). *R: A Language and Environment for Statistical Computing*.

Vienna, Austria.

Rebelo, H., Froufe, E., Brito, J. C., Russo, D., Cistrone, L., Ferrand, N., &

Jones, G. (2012). Postglacial colonization of Europe by the barbastelle bat: agreement between molecular data and past predictive modelling. *Molecular Ecology*, **21**(11): 2761-2774.

- Rebelo, H., Tarroso, P., & Jones, G. (2010). Predicted impact of climate change on European bats in relation to their biogeographic patterns. *Global Change Biology*, **16**(2): 561-576.
- Reckardt, K., & Kerth, G. (2007). Roost selection and roost switching of female Bechstein's bats (*Myotis bechsteinii*) as a strategy of parasite avoidance. *Oecologia*, **154**(3): 581-588.
- Reed, D. H., & Frankham, R. (2003). Correlation between fitness and genetic diversity. *Conservation Biology*, **17**(1): 230-237.
- Reichert, S., Rojas, E., Zahn, S., Robin, J., Criscuolo, F., & Massemin, S. (2014). Maternal telomere length inheritance in the king penguin. *Heredity*, **114**(1): 10-16.
- Rice, W. (1989). The sequential Bonferroni test. *Evolution*, **43**: 223-225.
- Richard Pilsner, J., Lazarus, A. L., NAM, D. H., Letcher, R. J., Sonne, C., Dietz, R., & Basu, N. (2010). Mercury-associated DNA hypomethylation in polar bear brains via the LUMinometric Methylation Assay: a sensitive method to study epigenetics in wildlife. *Molecular Ecology*, **19**(2): 307-314.
- Richardson, P. (1994). A new method of distinguishing Daubenton's bats (*Myotis daubentonii*) up to one year old from adults. *Journal of Zoology*, **233**(2): 307-309.

Richardson, S., Lintott, P., Hosken, D., & Mathews, F. (*in prep*). An evidence-based approach to specifying survey effort in ecological assessments of bat activity

Rivers, N. M., Butlin, R. K., & Altringham, J. D. (2005). Genetic population structure of Natterer's bats explained by mating at swarming sites and philopatry. *Molecular Ecology*, **14**(14): 4299-4312.

Roman, J., & Palumbi, S. R. (2003). Whales before whaling in the North Atlantic. *Science*, **301**(5632): 508-510.

Rossiter, S. J., Jones, G., Ransome, R. D., & Barratt, E. M. (2000). Genetic variation and population structure in the endangered greater horseshoe bat *Rhinolophus ferrumequinum*. *Molecular Ecology*, **9**(8): 1131-1135.

Rousset, F. (2008). genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular ecology resources*, **8**(1): 103-106.

RStudio. (2012). RStudio: integrated development environment for R. *RStudio Inc, Boston, Massachusetts*.

Ruckstuhl, K., & Neuhaus, P. (2005). *Sexual segregation in vertebrates*. Cambridge: Cambridge University Press.

- Ruedi, M., & Castella, V. (2003). Genetic consequences of the ice ages on nurseries of the bat *Myotis myotis*: a mitochondrial and nuclear survey. *Molecular Ecology*, **12**(6): 1527-1540.
- Ruedi, M., Walter, S., Fischer, M. C., Scaravelli, D., Excoffier, L., & Heckel, G. (2008). Italy as a major Ice Age refuge area for the bat *Myotis myotis* (Chiroptera: Vespertilionidae) in Europe. *Molecular Ecology*, **17**(7): 1801-1814.
- Ruffell, J., Guilbert, J., & Parsons, S. (2009). Translocation of bats as a conservation strategy: previous attempts and potential problems. *Endangered Species Research*, **8**: 25-31.
- Ruiz-González, A., Gurrutxaga, M., Cushman, S. A., Madeira, M. J., Randi, E., & Gómez-Moliner, B. J. (2014). Landscape genetics for the empirical assessment of resistance surfaces: the European pine marten (*Martes martes*) as a target-species of a regional ecological network. *PLoS One*, **9**(10): e110552.
- Ruiz-Lopez, M., Barelli, C., Rovero, F., Hodges, K., Roos, C., Peterman, W., & Ting, N. (2016). A novel landscape genetic approach demonstrates the effects of human disturbance on the Udzungwa red colobus monkey (*Procolobus gordonorum*). *Heredity*, **116**(2): 167-176.

Russ, J. (1999). *Bats of Britain and Ireland: Echolocation calls, sound analysis and species identification*. Exeter: Alana Books.

Russo, D., Cistrone, L., Jones, G., & Mazzoleni, S. (2004). Roost selection by barbastelle bats (*Barbastella barbastellus*, Chiroptera: Vespertilionidae) in beech woodlands of central Italy: consequences for conservation. *Biological Conservation*, **117**(1): 73-81.

Salgueiro, P., Ruedi, M., Coelho, M., & Palmeirim, J. (2007). Genetic divergence and phylogeography in the genus *Nyctalus* (Mammalia, Chiroptera): implications for population history of the insular bat *Nyctalus azoreum*. *Genetica*, **130**(2): 169-181.

Sand, H., Vucetich, J. A., Zimmermann, B., Wabakken, P., Wikenros, C., Pedersen, H. C., . . . Liberg, O. (2012). Assessing the influence of prey–predator ratio, prey age structure and packs size on wolf kill rates. *Oikos*, **121**(9): 1454-1463.

Saura, S., & Torne, J. (2009). Conefor Sensinode 2.2: a software package for quantifying the importance of habitat patches for landscape connectivity. *Environmental Modelling & Software*, **24**(1): 135-139.

Schnitzler, H.-U., & Kalko, E. K. (2001). Echolocation by Insect-Eating Bats: We define four distinct functional groups of bats and find differences in signal

structure that correlate with the typical echolocation tasks faced by each group. *Bioscience*, **51**(7): 557-569.

Schofield, H., & Morris, C. (2000). *Ranging behaviour and habitat preferences of female Bechstein's bat, Myotis bechsteinii (Kuhl, 1818), in summer*: Report held by The Vincent Wildlife Trust.

Schofield, H., & Morris, C. (2009). *Report on the Bechstein's bat colony at Poplar Cottage, Balcombe, West Sussex*: Report held by The Vincent Wildlife Trust.

Schofield, H. W. (2008). *The lesser horseshoe bat: conservation handbook*: Vincent Wildlife Trust.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors—occurrence, properties and removal. *Journal of Applied Microbiology*, **113**(5): 1014-1026.

Scott, D. D., Fitzpatrick, S., Bailie, D. A., Boston, E. S., Lundy, M. G., Buckley, D. J., . . . Prodöhl, P. A. (2013). Isolation and characterization of eight polymorphic microsatellite loci for Natterer's bat, *Myotis nattereri* (Vespertilionidae, Chiroptera). *Conservation Genetics Resources*, **5**(3): 643-645.

- Sewell, D., Guillera-Arroita, G., Griffiths, R. A., & Beebee, T. J. (2012). When is a species declining? Optimizing survey effort to detect population changes in reptiles. *PLoS One*, **7**(8): e43387.
- Simmons, N., & Conway, T. (2003). Evolution and ecological diversity of bats *Bat ecology*. T.H.Kunz & M.B.Fenton (pp. 493-535). Chicago: University of Chicago Press.
- Simmons, N. B., Seymour, K. L., Habersetzer, J., & Gunnell, G. F. (2008). Primitive Early Eocene bat from Wyoming and the evolution of flight and echolocation. *Nature*, **451**(7180): 818-821.
- Smith, G., Aegerter, J., Allnutt, T., MacNicoll, A., Learmount, J., Hutson, A., & Atterby, H. (2011). Bat population genetics and Lyssavirus presence in Great Britain. *Epidemiology and Infection*, **139**(10): 1463-1469.
- Smith, M. F., & Patton, J. L. (1993). The diversification of South American murid rodents: evidence from mitochondrial DNA sequence data for the akodontine tribe. *Biological Journal of the Linnean Society*, **50**(3): 149-177.
- Smith, P., & Racey, P. (2008). Natterer's bats prefer foraging in broad-leaved woodlands and river corridors. *Journal of Zoology*, **275**(3): 314-322.

- Spear, S. F., & Storfer, A. (2010). Anthropogenic and natural disturbance lead to differing patterns of gene flow in the Rocky Mountain tailed frog, *Ascaphus montanus*. *Biological Conservation*, **143**(3): 778-786.
- Speed, C. W., Meekan, M. G., & Bradshaw, C. J. (2007). Spot the match—wildlife photo-identification using information theory. *Frontiers in Zoology*, **4**(2): 1-11.
- Stanton, D., Hobbs, G., Chadwick, E. A., Slater, F. M., & Bruford, M. W. (2009). Mitochondrial genetic diversity and structure of the European otter (*Lutra lutra*) in Britain. *Conservation Genetics*, **10**(3): 733-737.
- Stebbins, R. (1989). The Bechstein's bat (*Myotis bechsteinii*) in Dorset and Britain 1800-1989. *Proceedings of the Dorset Natural History and Archaeological Society*, **110**: 178-180.
- Stoate, C., Boatman, N., Borralho, R., Carvalho, C. R., De Snoo, G., & Eden, P. (2001). Ecological impacts of arable intensification in Europe. *Journal of environmental management*, **63**(4): 337-365.
- Stojak, J., McDevitt, A. D., Herman, J. S., Kryštufek, B., Uhlíková, J., Purger, J. J., . . . Wójcik, J. M. (2016). Between the Balkans and the Baltic: phylogeography of a common vole mitochondrial DNA lineage limited to Central Europe. *PLoS One*, **11**(12): e0168621.

- Stone, E. L., Jones, G., & Harris, S. (2009). Street lighting disturbs commuting bats. *Current Biology*, **19**(13): 1123-1127.
- Storfer, A., Murphy, M. A., Spear, S. F., Holderegger, R., & Waits, L. P. (2010). Landscape genetics: where are we now? *Molecular Ecology*, **19**(17): 3496-3514.
- Storz, J. F., Bhat, H. R., & Kunz, T. H. (2000). Social structure of a polygynous tent-making bat, *Cynopterus sphinx* (Megachiroptera). *Journal of Zoology*, **251**(2): 151-165.
- Struebig, M. J., Horsburgh, G. J., Pandhal, J., Triggs, A., Zubaid, A., Kingston, T., . . . Rossiter, S. J. (2008a). Isolation and characterisation of microsatellite loci in the papillose woolly bat, *Kerivoula papillosa* (Chiroptera: Vespertilionidae). *Conservation Genetics*, **9**(3): 751-756.
- Struebig, M. J., Kingston, T., Zubaid, A., Mohd-Adnan, A., & Rossiter, S. J. (2008b). Conservation value of forest fragments to Palaeotropical bats. *Biological Conservation*, **141**(8): 2112-2126.
- Struebig, M. J., Wilting, A., Gaveau, D. L., Meijaard, E., Smith, R. J., Fischer, M., . . . Consortium, B. M. D. (2015). Targeted conservation to safeguard a biodiversity hotspot from climate and land-cover change. *Current biology*, **25**(3): 372-378.

- Stubbs, T. M., Bonder, M. J., Stark, A.-K., Krueger, F., von Meyenn, F., Stegle, O., & Reik, W. (2017). Multi-tissue DNA methylation age predictor in mouse. *Genome Biology*, **18**(1): 68.
- Suggitt, A., Wilson, R., August, T., Beale, C., Bennie, J., Dordolo, A., . . . Jorieux, P. (2014). *Climate change refugia for the flora and fauna of England*. Peterborough: Natural England.
- Sun, J. X., Helgason, A., Masson, G., Ebenesersdóttir, S. S., Li, H., Mallick, S., . . . Reich, D. (2012). A direct characterization of human mutation based on microsatellites. *Nature genetics*, **44**(10): 1161-1165.
- Taberlet, P., & Luikart, G. (1999). Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society*, **68**(1-2): 41-55.
- Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular biology and evolution*, **24**(8): 1596-1599.
- Tella, J. L., Rojas, A., Carrete, M., & Hiraldo, F. (2013). Simple assessments of age and spatial population structure can aid conservation of poorly known species. *Biological Conservation*, **167**: 425-434.

- Teschendorff, A. E., West, J., & Beck, S. (2013). Age-associated epigenetic drift: implications, and a case of epigenetic thrift? *Human Molecular Genetics*, **7**: 7-15.
- Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, **183**: 4-18.
- Tost, J., & Gut, I. G. (2007). DNA methylation analysis by pyrosequencing. *Nature Protocols*, **2**(9): 2265-2275.
- Trujillo, R. G., & Amelon, S. K. (2009). Development of microsatellite markers in *Myotis sodalis* and cross-species amplification in *M. grisescens*, *M. leibii*, *M. lucifugus*, and *M. septentrionalis*. *Conservation Genetics*, **10**(6): 1965.
- Turbill, C., Ruf, T., Smith, S., & Bieber, C. (2013). Seasonal variation in telomere length of a hibernating rodent. *Biology Letters*, **9**(2): 20121095.
- Turner, M. G., Gardner, R. H., & O'Neill, R. V. (2001). *Landscape ecology in theory and practice: patterns and process*. New York: Springer-Verlag.
- Tuttle, M. D. (1979). Status, causes of decline, and management of endangered gray bats. *The Journal of Wildlife Management*, **43**(1): 1-17.
- UN. (2015). *World urbanization prospects, the 2014 revision*. New York.

- Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., . . . Sato, Y. (2017). Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources*, **17**(6): 63-75.
- Van Oosterhout, C., Hutchinson, W. F., Wills, D. P., & Shipley, P. (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**(3): 535-538.
- Verboom, B., & 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): 1393-1401.
- Vergari, S., Dondini, G., & Ruggieri, A. (1998). On the distribution of *Myotis bechsteinii* (Kuhl, 1817) in Italy (Chiroptera: Vespertilionidae). *Hystrix, the Italian Journal of Mammalogy*, **10**(2): 49-56.
- Verhoeven, K. J., Jansen, J. J., Van Dijk, P. J., & Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytologist*, **185**(4): 1108-1118.
- Vonhof, M. J., & Barclay, R. M. (1996). Roost-site selection and roosting ecology of forest-dwelling bats in southern British Columbia. *Canadian Journal of Zoology*, **74**(10): 1797-1805.

- Wang, J., Santiago, E., & Caballero, A. (2016). Prediction and estimation of effective population size. *Heredity*, **117**(4): 193-206.
- Waples, R. S., & Antao, T. (2014). Intermittent breeding and constraints on litter size: consequences for effective population size per generation (N_e) and per reproductive cycle (N_b). *Evolution*, **68**(6): 1722-1734.
- Waples, R. S., Antao, T., & Luikart, G. (2014). Effects of overlapping generations on linkage disequilibrium estimates of effective population size. *Genetics*, **197**(2): 769-780.
- Warren, D. L., & Seifert, S. N. (2011). Ecological niche modeling in Maxent: the importance of model complexity and the performance of model selection criteria. *Ecological Applications*, **21**(2): 335-342.
- Warren, R. D., & Witter, M. S. (2002). Monitoring trends in bat populations through roost surveys: methods and data from *Rhinolophus hipposideros*. *Biological Conservation*, **105**(2): 255-261.
- Wasserman, T., Cushman, S., Shirk, A., Landguth, E., & Littell, J. (2012). Simulating the effects of climate change on population connectivity of American marten (*Martes americana*) in the northern Rocky Mountains, USA. *Landscape Ecology*, **27**(2): 211-225.

- Watts, K. (2006). British forest landscapes: the legacy of woodland fragmentation. *Quarterly Journal of Forestry*, **100**(4): 273-279.
- Weidner, C. I., Lin, Q., Koch, C. M., Eisele, L., Beier, F., Ziegler, P., . . . Mühleisen, T. W. (2014). Aging of blood can be tracked by DNA methylation changes at just three CpG sites. *Genome Biology*, **15**(2): 1-12.
- Weir, B. S., Triggs, C., Starling, L., Stowell, L., Walsh, K., & Buckleton, J. (1997). Interpreting DNA mixtures. *Journal of Forensic Science*, **42**(2): 213-222.
- Whiteley, A. R., Coombs, J. A., Cembrola, M., O'Donnell, M. J., Hudy, M., Nislow, K. H., & Letcher, B. H. (2015a). Effective number of breeders provides a link between interannual variation in stream flow and individual reproductive contribution in a stream salmonid. *Molecular Ecology*, **24**(14): 3585-3602.
- Whiteley, A. R., Fitzpatrick, S. W., Funk, W. C., & Tallmon, D. A. (2015b). Genetic rescue to the rescue. *Trends in Ecology & Evolution*, **30**(1): 42-49.
- Wickramasinghe, L. P., Harris, S., Jones, G., & Vaughan, N. (2003). Bat activity and species richness on organic and conventional farms: impact of agricultural intensification. *Journal of Applied Ecology*, **40**(6): 984-993.

Wilcove, D. S., McLellan, C. H., & Dobson, A. P. (1986). Habitat fragmentation in the temperate zone. *Conservation Biology*, **6**: 237-256.

Wilkinson, G. S., & South, J. M. (2002). Life history, ecology and longevity in bats. *Aging Cell*, **1**(2): 124-131.

Wilmer, J. W., Hall, L., Barratt, E., & Moritz, C. (1999). Genetic structure and male-mediated gene-flow in the ghost bat (*Macroderma gigas*). *Evolution*, **53**(5): 1582-1591.

With, K. A., & Crist, T. O. (1995). Critical thresholds in species' responses to landscape structure. *Ecology*, **76**(8): 2446-2459.

Wright, L. J., Newson, S. E., & Noble, D. G. (2014). The value of a random sampling design for annual monitoring of national populations of larger British terrestrial mammals. *European journal of wildlife research*, **60**(2): 213-221.

Wright, P., Hamilton, P., Schofield, H., Damant, C., Davidson-Watts, I., Glover, A. M., & Mathews, F. (2018). Genetic structure and diversity of a rare woodland bat, *Myotis bechsteinii*: comparison of continental Europe and Britain. *Conservation Genetics*: 1-11.

- Yalden, D. (1992). Changing distribution and status of small mammals in Britain. *Mammal Review*, **22**(2): 97-106.
- Yalden, D. (1999). *The history of British mammals*. London: A&C Black.
- Yehuda, R., Daskalakis, N. P., Lehrner, A., Desarnaud, F., Bader, H. N., Makotkine, I., . . . Meaney, M. J. (2014). Influences of maternal and paternal PTSD on epigenetic regulation of the glucocorticoid receptor gene in Holocaust survivor offspring. *American Journal of Psychiatry*, **171**(8): 872-880.
- Yue, G., David, L., & Orban, L. (2007). Mutation rate and pattern of microsatellites in common carp (*Cyprinus carpio* L.). *Genetica*, **129**(3): 329-331.
- Zaroso-Lacoste, D., Jan, P. L., Lehnen, L., Girard, T., Besnard, A. L., Puechmaille, S., & Petit, E. (2017). Combining noninvasive genetics and a new mammalian sex-linked marker provides new tools to investigate population size, structure and individual behaviour: an application to bats. *Molecular Ecology Resources*, **18**(2): 217-228.
- Zbieć-Piekarska, R., Spólnicka, M., Kupiec, T., Makowska, Ż., Spas, A., Parys-Proszek, A., . . . Branicki, W. (2015). Examination of DNA methylation status of the ELOVL2 marker may be useful for human age prediction in forensic science. *Forensic Science International: Genetics*, **14**: 161-167.

Zhu, Y., Cheng, Q., & Rogers, S. M. (2014). Genetic structure of *Scomber japonicus* (*Perciformes: Scombridae*) along the coast of China revealed by complete mitochondrial cytochrome b sequences. *Mitochondrial DNA*: 1-9.

Zylstra, E. R., Steidl, R. J., & Swann, D. E. (2010). Evaluating survey methods for monitoring a rare vertebrate, the Sonoran desert tortoise. *Journal of Wildlife Management*, **74**(6): 1311-1318.