Ecology and genetics of *Myotis* spp., Natterer's, Whiskered and Brandt's bats in Ireland

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Ecology and genetics of *Myotis* spp., Natterer's, Whiskered and Brandt's bats in Ireland

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Cover photo: *Myotis nattereri* © Billy Clarke

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EXECUTIVE SUMMARY

The Centre for Irish Bat Research (CIBR) was established with funding from NPWS in 2008 to conduct a diverse, multi-disciplinary research project on *Myotis* bat sp. in Ireland. This project, as was proposed, is now complete and has achieved its objectives greatly adding to the understanding of the ecology of *Myotis* species in Ireland and across their range. All existing historic records of Whiskered bat (*Mytois mystacinus)*/ Brandt's bat (*Myotis brandtii)* and Natterer's bat (*Myotis nattereri)* were collated and potential maternity roosts were identified for future resurvey; 22 maternity roosts of *M. mystacinus*/ *M. brandtii* and 19 maternity roosts of *M. nattereri* were confirmed. Genetic identification of cryptic species, using novel genetic techniques was developed successfully. All *M. mystacinus*/ *M. brandtii* captured were confirmed as *M. mystacinus*. We found no evidence that *M. brandtii* is a resident species in Ireland indicating that the species should be considered a vagrant / Data deficient.

We developed multi-scale species distribution models to examine habitat associations of *M. mystacinus* and *M. nattereri* at a landscape scale. These models revealed complex patterns of habitat associations. For *M. nattereri,* marked differences in the most relevant spatial scales for specific habitat types indicated significant contrasts between roosting/ emergence habitat in woodland, and foraging habitat associated with grassland agriculture. The landscape associations of *M. mystacinus* are dominated by affinities with woodland. These patterns were corroborated through radio-tracking at maternity colonies of both species. Radio-tracked *M. nattereri*, selected grassland in which to forage, whilst *M. mystacinus* foraged in mixed woodland, in close proximity to their roost. Further links with the wider scale agricultural landscape were evident in faecal analysis of diet and stable isotope analysis indicating the trophic position of *M. nattereri* and *M. mystacinus*.

Radio-tracking also revealed strong contrasting roosting behaviour in the two species, with *M. mystacinus* utilising a matrix of roosts (up to 8) whilst female *M. nattereri* appeared to exhibit a high level of roost fidelity, with all but one individual utilising only one roost throughout the tracking study. During tracking, a novel protocol was developed to monitor the effect of disturbance events associated with the behavioural study. This confirmed that impacts on the roosting bats were minimised ensuring a high level of animal welfare.

Past surveys of *Myotis* bats using ultrasonic detectors have previously been limited by the high degree of uncertainty that is attributed to echolocation call parameters. Through the course of this project we investigated the use of a morphometric technique to identify the calls of emerging bats from roosts of known species. This method provided an excellent rate of reliable identification. Future development of this method may allow identification of *Myotis spp.* in other habitats. Trial surveys of woodland sites using an acoustic lure, were attempted to further elucidate the ecology of Irish *Myotis*, but these proved inefficient in capturing bats.

The autumnal swarming sites on *Myotis* bats in Ireland were identified for the first time. This was achieved, through a systematic survey of under-ground sites using a novel protocol. Swarming sites are key conservation areas for *Myotis* bats, and are postulated to have a role in hibernation and mating. These swarming sites are known to have larger catchment areas and may be crucial conservation units for these species.

Examination of the molecular genetic diversity and spatial distribution of this diversity for the mitochondrial *Dloop* and *Cytb,* suggested that the Irish populations of *M. mystacinus* are likely to have originated from continental Europe, sharing many haplotypes with Britain and Europe. In contrast, Irish populations of *M. nattereri* have distinctive *Dloop* haplotypes, most united by a 23bp indel, and sharing only three haplotypes with Britain and Europe. *Cytb* sequences show little divergence within and between Irish and other European populations, again making a European origin likely. Examining nuclear DNA, Irish populations of both *M. mystacinus* and *M. nattereri* are genetically diverse and distinct from both British and European populations. Within Ireland both species show low levels of genetic differentiation among nursery sites, and swarming sites of *M. nattereri*, and no signal of isolation by distance, suggesting levels of gene flow are high among nursery colonies of both *M. mystacinus* and *M. nattereri* with no apparent barriers to dispersal in the Irish landscape.

The combination of genetic, ecological and novel techniques developed specifically to address gaps in our knowledge has provided significant new information regarding the ecology and conservation of *Myotis* bats in Ireland. These insights are not only relevant to Irish *Myotis* but also provide insights into the species ecology across their range. Through this research we are able to provide up-to-date conservation recommendations for both species.

A review of potential population monitoring methods for these species was carried out, leading to the development of a novel technique using DNA extracted from faecal samples (bat droppings). Given that these species are found in low densities in the environment and have cryptic echolocation calls, concentrating efforts to monitor these bats at aggregations, such as nursery sites is deemed most appropriate. The use of DNA collected non-invasively, allows populations to be monitored by capture-mark-recapture techniques, giving accurate population counts and trends as well as information on survival, recruitment and changes in genetic diversity.

This multi-disciplinary research carried out by CIBR has greatly added to our core knowledge of Irish *Myotis* bats. Applying this level of rigour to other species and research questions is essential to fully understand the conservation requirements of Irish bats. We have identified areas requiring further research that include: determining the role and importance of swarming behaviour and swarming sites for *Myotis* bats; a better understanding of landscape and seasonal trends in bat abundance and distribution; a grasp on the economic services provided by bats; and knowledge of the effects of environmental change on bat populations.

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INTRODUCTION

Ten bats species were previously considered to occur frequently in Ireland, from two families, the Vespertilionidae - common pipistrelle (*Pipistrellus pipistrellus*), soprano pipistrelle (*P. pygmaeus*), Nathusius pipistrelle (*P. nathusii*), Leisler's bat (*Nyctalus leisleri*), Daubenton's bat (*Myotis daubentoni*), Natterer's bat (*M. nattereri*), Brandt's bat (*M. brandtii*), whiskered bat (*M. mystacinus*), brown long-eared bat (*Plecotus auritus*) and a single member of the family Rhinolophidae – the lesser horseshoe (*Rhinolophus hipposideros*). All species are generally widespread expect *R. hipposideros* and *P. nathusii*, the former is restricted to karst landscape of the west and south west whilst *P. nathusii* is a rarely recorded species with a more northerly distribution (REFS?).

A 'conservation plan for Irish Vesper bats' was developed for all vespertilionid bats in Ireland in 2006 (McAney 2006). This plan collated the knowledge base for all species and identified priorities for future investigation. Three species of *Myotis* bats in particular were identified as requiring focus; *M. mystacinus*, *M. nattereri* and *M. brandtii*. At that time *M. brandtii* had only just been considered to 'potentially occur frequently'. *M. brandtii* and *M. mystacinus* are considered to be very difficult to distinguish in the field given morphological similarities; this may have resulted in historical misidentification of these two species. O'Sullivan (1994) found only 12 roosts of *M. mystacinus* records during the National Bat Survey with more than five bats present and only 13 roosts of *M. nattereri* with more than 5 bats present. *Myotis* species are additionally difficult to distinguish using echolocation call parameters (Parsons & Jones 2000), a frequently adopted survey method.

The paucity of records and difficulty distinguishing between species has resulted in significant knowledge gaps for these species in Ireland. In May 2008 the NPWS set up a new Centre of excellence for bat research in Ireland, formed by a joint team from University College Dublin and Queens University, Belfast. This Centre for Irish Bat Research (CIBR) was tasked with investigating the ecology and population biology of *M. nattereri*, *M. brandtii* and *M mystacinus* and developing novel ways of monitoring these species with a view to developing and informing best international practise in this field. This report details the research outputs of the centre.

Major achievements:

1. Determined the population and conservation status of Irish populations of *M. nattereri*, *M. mystacinus*
2. Developed molecular techniques to differentiate *M. mystacinus* from *M. brandtii* and confirmed all suspected *M. mystacinus* roosts as *M. mystacinus.*
3. Determined habitat associations, home range size and roosting behaviour of *M. mystacinus* and *M. nattereri* in Ireland.
4. Found no evidence that *M. brandtii* is a resident species in Ireland indicating that the species should be considered a vagrant / Data deficient.
5. Modelled habitat associations and distribution of *M. mystacinus* and *M. nattereri*.
6. Identified the first swarming sites in Ireland.
7. Developed novel methods to discriminate echolocation calls of *Myotis* bats.
8. Determined dietary and trophic ecology of *M. mystacinus* and *M. nattereri* in Ireland.
9. Applied novel analysis to determine roosting activity patterns.
10. Uncovered the European phylogeography and the origin(s) of Irish populations of *M. mystacinus* and *M. nattereri*.
11. Elucidated the population genetic diversity and structuring of Irish *M. mystacinus* and *M. nattereri*.

12. Assessed the quality of population genetic data obtained from faecal DNA for population genetic analysis.
13. Developed a non-invasive, genetic, population monitoring scheme for rare *Myotis* spp. in Ireland using faecal DNA.
14. Established collaborations with leading bat researchers from the UK, Switzerland, Czech Republic, including Prof. Paul Racey, Dr. Manuel Ruedi, Prof. John Altringham and Dr. Pavel Hulva.
15. Disseminated results through peer reviewed publications, conference presentations and the media.

In this report we outline and discuss the individual projects conducted as part of this research tender in three Chapters: 1) Population and conservation status; 2) Roosting and foraging ecology; 3) Conservation genetics and population structure. We then consider the conservation implications in Chapter 4 and review scientific methods for monitoring bat populations in Chapter 5, proposing a novel method for these species.

1. POPULATION AND CONSERVATION STATUS

1.1 Identification and confirmation of historical roost records

Occurrence records from three sources were utilised: roost records, woodland surveys and swarming site records. Of these, potential woodland sites within which surveys could be conducted were identified by expert judgment (Section 2.5) and a systematic survey of potential swarming sites was planned (Section 2.4).

All existing records were collated: NPWS National Bat Survey 1980 – 1990, Bat Conservation Ireland records and miscellaneous records from individual bat worker. All maternity roosts were identified as those records with 1 or more bats present. Records where a single individual was found late in summer season were also treated as potential maternity roosts. For *M. mystacinus*/ *M. brandtii* bat, 27 potential maternity roosts were identified. A further 37 potential maternity roosts were identified for *M. nattereri*.

Surveys consisted of species identification of bats within roosts with internal counts and survey at emergence. In some cases, only survey at emergence was possible where individuals were not visible during internal visits. A complete survey of all sites was limited by permission for access by land owners. Of the 27 identified *M. mystacinus*/ *M. brandtii* sites, 22 of 25 surveyed, were confirmed as *Mytois* maternity colonies. Both internal and emergence counts were carried out where conditions allowed. The average roosts size of *M. mystacinus*/ *M. brandtii* was 15.1 (s.d. = 13.5). For *M. nattereri* 19 of 25 surveyed roosts were confirmed, with an average roost size of 36.4 individuals (s.d. = 35.3), when accurate roost counts could be achieved.

Sites were confirmed by visual or genetic identification of *M. nattereri* and by genetic identification of *M. mystacinus*/ *M. brandtii* (Section 1.2).

1.2 Molecular species identification

Introduction

Brandt's bat (*M. Brandi*) is a cryptic species, difficult to separate morphologically from *M. mystacinus* (whiskered bat), which are found across Europe, including Ireland and *Myotis alcathoe* (Alcathoe's bat) and *Myotis aurascens* (Steppe whiskered bat) which are found in continental Europe (Dietz *et al*. 2009). Separating *M. mystacinus* and *M. brandtii* based on morphology alone requires the use of unreliable characters such as the premolars (Berge 2007) and penis shape (Harris & Yalden 2008; Dietz *et al*. 2009). However, genetically, these two species are quite distinct, with *M. brandtii* grouping with the New World *Myotis*, rather than the Old World *Myotis* which is typical of all other European myotid species (Ruedi & Mayer 2001). The recent identification of the second member of this group, *M. brandtii*, in Ireland (Mullen 2006; Kelleher 2005; Harris 2006), raised the possibility that many roosts previously identified as *M. mystacinus* may have been misidentified *M. brandtii*. Thus, the distribution and population estimates for *M. mystacinus* may have been over-estimated, while *M. brandtii* may have been under-estimated.

Our aim was to genetically distinguish cryptic *M. brandtii* from *M. mystacinus* in Ireland and determine the conservation status of both species.

Materials and Methods

All 22 known maternity colonies of *M. mystacinus* were investigated (Figure 1). Bats were caught using hand nets and harp traps during 2008 and 2009. Morphological identification of the bats was carried

out using diagnostic features following Dietz & von Helversen (2004) and a tissue biopsy was taken from each wing (Licence No X-XX). At one roost it was not possible to capture the bats, instead 10 faecal samples were taken from this site. In 2009, surveys of woodlands using an acoustic lure (Hill and Greenaway 2005) were carried out in two woodlands where suspected *M. brandtii* had been previously caught, Glendalough, County Wicklow, and Killarney National Park, County Kerry, one of the largest areas of native woodland in Ireland (Cabot 1999) (Figure 1). Capture, biopsy and release of bats were performed under licence from the National Parks and Wildlife Service (Licence No.74 C/2008) and the Northern Ireland Environment Agency (Licence No. TSA/12/08).

A 'DNA barcoding' technique was used for species confirmation. We used the 5' end of the Cytochrome C oxidase Subunit 1 mitochondrial region (COI; Ratnasingham and Hebert, 2007). DNA extractions from tissue and faecal samples were carried out using DNeasy and QIAamp DNA Stool Mini kits (Qiagen) respectively. PCR amplifications of the COI region were performed with 2 μmol/L of each primer (forward, ATACTTCGGGGTGGCCGAAGAATCA; reverse, TYTCAACCAAYCACAAAGATATYGG) labelled with an M13 tail, 1.5 mmol/L $MgCl_2$, 1 U of Platinum Taq DNA polymerase (Invitrogen), and 10 ng of DNA. The touchdown PCR reaction consisted of: denaturation at 95°C for 3 minutes, 10 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds minus 1°C per cycle, extension at 72°C for 60 seconds; followed by 35 cycles with 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 60 seconds. The PCR products were separated and visualised in a 1% agarose gel. All PCR products were sequenced in both directions with M13 primers, assembled on Sequencher v4.9 (Gene Codes Corporation).

Samples of known *M. mystacinus* (20 Germany, four France) and *M. brandtii* (two Germany) were included, along with 11 individuals from four other representative European *Myotis* spp. (one *Myotis emarginatus*, Italy; two *Myotis daubentoni*, France; two *Myotis nattereri*, UK; two *Myotis bechsteini*, France; four *M. alcathoe*, France) to demonstrate the phylogenetic relationships and establish the genetic identification of the sampled bats. One, *Nyctalus leisleri* was included in the analysis as an outgroup for phylogenetic reconstructions. The sequences were aligned using ClustalW v2.0.12 (Larkin *et al.* 2007) and then collapsed into haplotypes (Table 1). The generated sequences were deposited in GenBank (Table 1).

Maximum likelihood (ML), minimum evolution (ME) and maximum parsimony (MP) were used to reconstruct phylogenetic relationships and confirm species identity using PAUP v4.0b10 (Swofford 1991). Modeltest v3.7 (Posada & Crandall 1998) was used to estimate the most suitable model of sequence evolution [GTR + Γ; Base pair frequencies = (0.3409, 0.1243, 0.2388); R-matrix = (1.0000, 24.3779, 1.0000, 1.0000, 18.7990); Shape parameter of gamma distribution = 0.1351)]. All searches were performed using heuristic searches with tree-bisection and recombination branch swapping. Starting trees were obtained via Neighbor-joining in ML and ME analyses. One hundred bootstrap replicates were performed for ML and 1,000 for ME and MP (Figure 2).

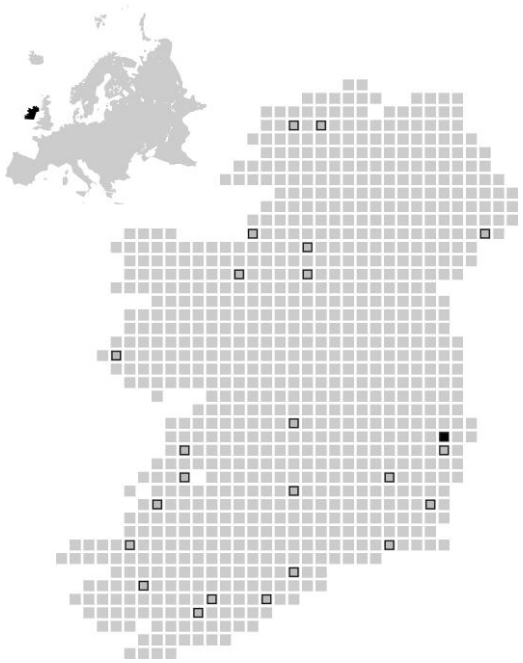


Figure 1: Gridmap of Ireland (10 × 10 km squares) plotted by IrishGrid v0.4 (Bekaert, 2009). Locations of confirmed *M. brandtii* records (■, Glendalough, County Wicklow) and known maternity roosts of *M. mystacinus* sampled (□)

Results

One hundred and forty-five bats from 22 suspected *M. mystacinus* maternity roosts across Ireland were sampled (Figure 1). All specimens were morphologically identified as *M. mystacinus* according to dentition and penis shape. Over 6 nights woodland sampling using the acoustic lure, 10 bats were caught, representing 6 species, including *Plecotus aurtius*, *Nyctalus leisleri*, *Pipistrellus pygmaeus*, *M. daubentonii*, *M. nattereri*, and a single *M. mystacinus* in Glendalough, County Wicklow.

For all suspected Irish *M. mystacinus* samples a 556 bp section of the COI gene was examined. When compared with the sequences of known *M. brandtii* samples, there were 76 parsimony informative sites between the *M. mystacinus* and *M. brandtii* haplotypes. All phylogenetic analyses placed the 146 *M. mystacinus*/ *brandtii* sequences into a well-supported monophyletic *M. mystacinus* clade (100% bootstrap support all analyses), with no *M. brandtii*, confirming that the sampled bats were all *M. mystacinus* (Figure 2). In total, five COI haplotypes of *M. mystacinus* were identified, all separated from each other by a single mutation (Table 1). The most common *M. mystacinus* haplotype occurred in samples from Ireland, Germany and France. While two haplotypes were found only in Irish samples, one of which was unique to a single sample. One was unique to a German sample and another to a French sample (Table 1). All known *M. brandtii* sequences formed a well supported monophyletic clade (97-100% bootstrap support; Figure 2).

Table 1: COI species haplotypes, GenBank accession numbers, country of origin with number of samples sequenced in parentheses and sample collector.

Haplotype	Accession No.	Origin	Collector
M. emarginatus	GU270553	IT (1)	John Altringham & Camille Jan
M. daubentonii	GU270554	FR (2)	Sébastien Puechmaille
M. mystacinus H1	GU270555	IE (107), FR (4), DE (19)	Sébastien Puechmaille; Christian Dietz
M. mystacinus H2	GU270556	IE (38)	Current study
M. mystacinus H3	GU270557	IE (1)	Current study
M. mystacinus H4	GU270558	DE (1)	Christian Dietz
M. mystacinus H5	GU270559	FR (1)	Sébastien Puechmaille
M. alcathoe	GU270560	FR (1)	Sébastien Puechmaille
M. nattereri	GU270561	UK (2)	John Altringham & Camille Jan
M. bechsteini H1	GU270562	FR (1)	Sébastien Puechmaille
M. bechsteini H2	GU270563	FR (1)	Sébastien Puechmaille
M. brandtii H1	GU270564	DE (1)	John Altringham & Camille Jan
M. brandtii H2	GU270565	DE (1)	John Altringham & Camille Jan
N. leisleri	GU270566	FR (1)	Sébastien Puechmaille

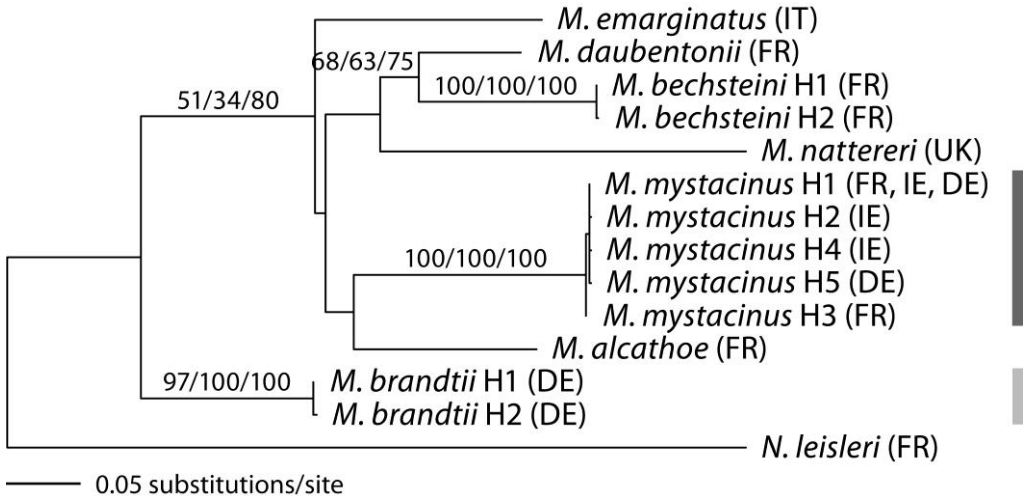


Figure 2: Maximum likelihood tree (lnL = -1753.7) of 556 bp fragment of COI. Bootstrap values from maximum likelihood, distance and parsimony are shown in this order above branches

Discussion

The island-wide genetic survey of all known *M. mystacinus* roosts, confirmed that there has not been a long-term misidentification of *M. brandtii* as *M. mystacinus* in Ireland. This study showed that *M. mystacinus* is widespread throughout Ireland.

The woodland survey in Killarney National Park and Glendalough provided no further *M. brandtii* records. These results suggest that *M. brandtii* are rare and possibly endangered in Ireland. Little is known about their ecology, but they are generally thought to be associated with broadleaf woodland (Taake 1984). Approximately 1% of Ireland is covered in native deciduous woodland due to historical deforestation (Perrin *et al.* 2008). If *M. brandtii* is reliant on this habitat then this could limit its distribution in Ireland, analogous to the situation with *M. bechsteini* in Britain (Harris and Yalden, 2008). It is possible that *M. brandtii* has been resident in Ireland since the early Holocene and the destruction of Irish forests may have caused a major decrease in its distribution and abundance. Alternatively, the recent confirmed records of *M. brandtii* in Ireland may have been vagrants. Since no breeding colony has been found we cannot confirm this species is a resident breeder. However, *M. brandtii* is not thought to be a long distance migrant in Europe (Dietz *et al*. 2009).

The present results may also reflect our survey approach. We targeted known *M. mystacinus* roosts, which are biased towards the larger maternity roosts in dwellings. These sites may not reflect the full range of roost types available. *M. brandtii* have been found to roost in trees, bat boxes and buildings (Sachanowicz & Ruczyński 2001; Dietz *et al*. 2010). Yet, differences in building architecture across Europe may produce intraspecific variation in roost usage across a species range (Marnell & Presetnik 2010). If *M. brandtii* utilise roost sites differently than *M. mystacinus* in Ireland, for example, primarily selecting tree roosts, they could have been missed in the present survey. This would also explain the conspicuous absence of *M. brandtii* roost records to date. Currently there has been no comprehensive study of the usage of tree roosts by Irish bats (McAney 2006). Additionally, our limited woodland surveys using an acoustic lure and mist nets proved unsuccessful in catching *M. brandtii*, however, catch rates were low for all species.

This study, confirmed the known distribution of *M. mystacinus* in Ireland, however, more research needs to be done to investigate the occurrence/ presence of *M. brandtii*, whether this be through more intensive sampling or through new survey techniques. For further details refer to Boston *et al.* 2010.

1.3 A rapid PCR-based assay for identification of cryptic *Myotis spp*.

Introduction

Cryptic species present a problem for conservation. Since distinguishing *M. mystacinus*, *M. alcathoe* and *M. brandtii* is easy using genetically based fixed differences, our aim was to develop primers to identify species specific single nucleotide poylmorphisms (SNP's) between *M. mystacinus*, *M. brandtii* and *M. alcathoe*. This would provide an easy and fast PCR based protocol to distinguish these three most common cryptic species without the need and expense of sequencing, using single nucleotide polymorphisms (SNP's) in ND1 and 12S.

Materials and Methods

Genomic DNA was extracted from wing biopsies from living and dead specimens of 33 *M. mystacinus*, 16 *M. brandtii* and 15 *M. alcathoe* (see details Boston *et al*. 2011), using Qiagen DNeasy tissue extraction kit, following the manufacturers' protocol. Ten faecal samples of *M. mystacinus* were extracted using QIAamp DNA Stool Mini kits (Puechmaille *et al*. 2007). Both 12S and ND1 were amplified for all tissue samples using primer-pairs L1091/H1478 (Kocher *et al.* 1989) and ER65/ER66 (Petit *et al.* 1999). Amplification was performed using a 25µl PCR solution consisting of 0.8 ng genomic DNA, 10x PCR buffer (Invitrogen), 1.5-2.4 mM $MgCl_2$ (Invitrogen), 0.1 mM dNTPs, 0.2 µM primers and 1U Platinum

Taq (Invitrogen). Each PCR cycle consisted of 95°C for 5mins, followed by 10 cycles of 95°C for 30secs, 60°C for 30secs and 72°C for 1min; 35 cycles of 95°C for 30secs, 50°C for 30secs and 72°C for 1min. Products were purified using ExoSAP-IT (Affymetrix Inc.) and sequenced in both directions using the PCR primers at Macrogen Inc, Korea.

Twenty-one *M. mystacinus*, 16 *M. brandtii* and 15 *M. alcathoe*, along with sequences for ND1 and 12S for species of interest from GenBank were used in primer design (see details Boston *et al*. 2011). Informative single nucleotide polymorphisms (SNP's) were identified in each species. Species-specific primers were designed using Primer3 (Rozen & Skaletsky 2000) ensuring the species-specific SNP lay within the five bases at the 3' end of the primer, most important for successful annealing. All seven primers were assessed for primer dimers and hairpins using AutoDimerv1 (Vallone & Butler 2004). Positive control primers were designed in conserved regions of 12S.

All primer-pairs were assessed for species specificity with 5 samples of each species and optimized within a multiplex. The 25µl multiplex consisted of 0.8ng of genomic DNA, 10x PCR Buffer, 1.5mM $MgCl_2$, 0.14mM dNTPs, 0.2 µM ND1Bra02_F, 0.1µM ND1Mys04_F, 0.3µM ND1Alc01_F, 0.28µM ND1Alc06_F, 0.2 µM 12S_Myo3_F, 0.2 µM 12S_Myo4_R, 0.96µM ER66, 1U Platinum Taq (Invitrogen). The PCR protocol was as above, with annealing temperatures of 63°C for 10 cycles; 57°C for 30 cycles.

To verify robustness of the PCR-based identification method, 14 *M. mystacinus* from across the geographic range were amplified, in addition to 10 feacal samples of *M. mystacinus* collected from two maternity roosts in Ireland. Cross amplification with other western European Vespertilionidae bat species was tested (see details in Boston *et al.* 2011).

Results

In the 12S region, 19 species-specific SNPs were identified but no primer-pairs of distinctive size between species could be designed. However a 350bp band could be amplified in each and served as a control-band ensuring successful PCR. In ND1, we identified 7 informative sites for *M. brandtii*, 9 for *M. mystacinus* and 9 for *M. alcathoe*. Only forward primers were designed for each SNP to be compatible with the universal primer ER66 (Petit *et al*. 1999). Four SNP primers were multiplexed with the 12S primer-pair (control-band) producing a bright and distinctive banding for each species (Table 2 & Figure 3).

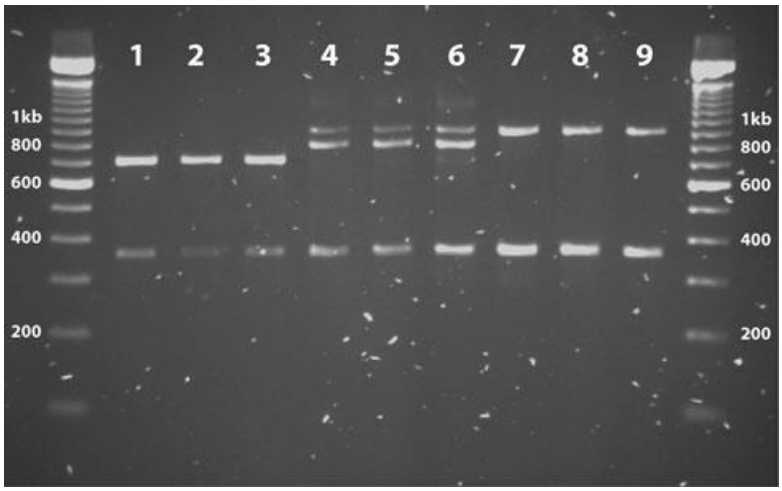


Figure 3: Species-specific amplification of ND1 from tissue samples of *M. brandtii* (1-3), *M. alcathoe* (4-6) and *M. mystacinus* (7-9) with a 12S control-band (near 400bp-size marker).

Table 2: Primers designed in this study

Gene	Name	Species Amplified	Size fragment	Sequence	Paired Primer
12S	12S_Myo3_F	Control	350bp	AGATACCCCACTATGCTTAGCC	12S_Myo4_R
12S	12S_Myo4_R	Control	350bp	CTGCTAAATCCACCTTTAACTTTTG	12S_Myo3_F
ND1	ND1Bra02_F	*Myotis brandtii*	750bp	ACGAAGTAACCCTGGCCATC	ER66 (Petit *et al*, 1999)
ND1	ND1Mys04_F	*Myotis mystacinus*	950bp	CCCTAACCCTAGCCCTAACAA	ER66 (Petit *et al*, 1999)
ND1	ND1Alc01_F	*Myotis alcathoe*	980bp	GCCCTAACCTTGGCTCTCAT	ER66 (Petit *et al*, 1999)
ND1	ND1Alc06_F	*Myotis alcathoe*	800bp	AGCCGTATACGCTATCTTATGGTC	ER66 (Petit *et al*, 1999)

Bright bands amplified in all 69 samples of living and dead specimens, and clear, but slightly weaker bands from DNA extracted from ten faecal samples. Cross amplification with this primer multiplex, produced bands for the informative SNP primers for four other *Myotis* species. In *M. escalerai* and *M. bechsteinii*, an 800 bp band was amplified but the species can still be distinguished from *M. alcathoe* by the absence of the 980 bp band. The two large species, *M. myotis* and *M. blythii* amplified the 950 bp band of *M. mystacinus*. Of the eight additional species tested, representing six genera (see details in Boston *et al.* 2011), only *Vespertilio murinus* amplified informative SNP primers, with a band at 750bp, and another at 800bp, a species-specific pattern.

Discussion

This SNP array will provide a faster, cost-effective and easy method for the identification of small, cryptic *Myotis* spp., either from samples collected from living or dead specimens *M. mystacinus*, *M. brandtii* and *M. alcathoe*, and from their droppings. This is useful since droppings can be collected from bats in the hand or at roost sites, reducing handling stress. Caution should be used to ensure droppings collected are, according to size, from smaller *Myotis* spp. to avoid confusion with the larger species, *M. myotis* and *M. blythii* shown to amplify with the SNP primers. There is the potential for use also in identifying *Vespertilio murinus*, however further testing with more comprehensive taxon sampling is needed. In conclusion, this method is reliable and easy to perform for routine identification of these small cryptic *Myotis* spp. in various field studies and since sequences from samples spanning the entire range of the three species were used in primer-design, and samples of *M. mystacinus* from a wide geographic spread were tested, this assay should be applicable throughout Europe. Further testing, however, would be needed to determine whether or not these primers amplify in other sympatric Vespertilionid bats.

2. ROOSTING AND FORAGING ECOLOGY

2.1 Habitat selection, home range and roosting behavior of *Myotis nattereri* and *M. mystacinus*.

Introduction

In the temperate zone, human activities have had a major impact on the landscape (Vitousek *et al.* 1997) which in turn has had a negative effect on bat populations (Stebbings 1988). Walsh & Harris (1996) showed that bat abundance in Britain is positively associated with woodland, vegetation corridors and riparian and lacustine habitats, and negatively associated with the area of arable land. Agricultural intensification, has removed important foraging habitats and the use of pesticides has reduced insect prey populations (Aebischer 1991). Woodland habitats are also vulnerable to human activities such as insensitive harvesting practices (Patriquin & Barclay 2003), and conversion to commercial forestry and agriculture (Peterken 1996).

It is difficult to identify bat species of the genus *Myotis* using bat detectors due to overlapping call parameters (Parsons & Jones 2000). Catching bats within habitats may result in bias in habitat assessment due to variation in trapability of different individuals in different habitats. Alternatively, biotelemetry affords a more objective and practical means of assessing foraging and roosting behaviour of bats, although this has been of limited use for small / medium sized bats, due to technological limitations (Aldridge & Brigham 1988). However, technological developments have permitted the foraging behaviour of small / medium bats to be elucidated by radio telemetry. These include relatively small species such as the Lesser horseshoe bat, *Rhinolophus hipposideros* (Bontadina *et al.* 2002), and the pipistrelle bats, *Pipistrellus pipistrellus* and *P. pygmaeus* (Davidson-Watts & Jones 2006). Species of the Vespertilionidae range in size from 3.5 g to 53 g (Dietz *et al.* 2009). At present, radio telemetry is the only feasible method for tracking movements of micro bats without impacting animal welfare (Stebbings 2004).

Foraging behaviour of M. mystacinus *and* M. nattereri

M. mystacinus is a small myotid that is distributed across Europe and northern Morocco (Mitchell-Jones *et al.* 1999). To date, there has been limited research carried out on the habitat use of *M. mystacinus*. Based on morphological features, it is classified as being both an aerial forager and an edge space aerial/ trawling forager (Siemers & Schnitzler 2004). The diet of *M. mystacinus* includes day flying insect groups which indicate gleaning behaviour (Taake 1992). Highly contrasting habitat associations have been described for *M. mystacinus*, such that reliable conservation prescriptions are not available. Taake (1984) found an association with agricultural landscapes and riparian habitats surrounding roosts in Germany, whilst Kanuch *et al.* (2008) suggested *M. mystacinus* to be a woodland generalist with no association with any particular forest type in Slovakia. Berge (2007), in complete contradiction, found that *M. mystacinus* selected pasture with hedgerows in Southern England

M. nattereri bats typically hunt in a variety of habitats across their European range ranging from meadows, orchards , broad leaf wood to open conifer forest and riparian habitats (Arlettaz 1996; Siemers *et al.* 1999; Siemers and Swift 2006; Smith and Racey 2008). *M. nattereri* are likely to select foraging areas which are rich in horizontal and vertical edges (Siemers *et al.* 1999). Foraging areas range from 128 ha to 580 ha (Smith and Racey 2008) and multiple 'partial' foraging areas are used within this area (Smith and Racey 2008). The core of foraging grounds can be up to 4 km from roosts and individuals are faithful to core hunting areas, returning to these on consecutive nights (Siemers *et al.* 1999). Connecting habitats between the roost and core area are also utilised for foraging (Siemers *et al.* 1999).

Study aims

Here we investigate the roost ecology, range behaviour and habitat use of adult bats in typical Irish maternity colonies of *M. mystacinus* and *M. nattereri*.

Methods

M. mystacinus *study site*

The study was conducted between the 15th of May and the 08th July 2009 at a maternity roost in south Co. Cork. Previous emergence counts at this roost estimated the number of individuals present to be 30-40 (Buckley 2004). The roost was located in the attic of an occupied detached house (100+ years old). The colony occupied the space between a timber joist and a brick chimney. The surrounding landscape is predominantly improved pasture with hedgerows and tree lines. The roost is adjacent to forest with conifer, mixed and broadleaf stands. This roost was selected for this study due to the large colony size and because the surrounding landscape is typical of the lowland agricultural landscape that predominates in Ireland.

Eighteen adults consisting of seventeen females and one male *M. mystacinus* were radio-tagged using 0.35g PIP3 radio transmitters (Biotrack Ltd, Devon, UK) and tracked from a maternity roost and associated satellite roost in Co. Cork, Ireland during June - August 2009. Tagging and tracking was not carried out when female bats were in late stages of pregnancy, dependent young were present in the maternity roost or during early lactation (Stebbings 2004). The activity of bats at the maternity roosts was monitored continuously for one week prior to tracking and throughout the study period using ultrasonic recorders which logged activity to ensure behavioural patterns were not disturbed (Section 2.3).

Bats were caught using harp-traps during the emergence period as they exited roosts. *M. mystacinus* have relatively low wing loadings (6.4 Nm^2; Jones & Rydell 1994); species with low wing loadings are capable of carrying tags up to 12% of their body weight without impacting welfare, although avoiding this upper limit is advised (Stebbings 2004). The tags were temporarily attached using skin-bond surgical adhesive between the shoulder blades (Stebbings 2004), allowing tags to drop off after a number of days (mean = 17.6 ± 7.6; Carter *et al.* 2009). Individual bats were held in cotton holding bags and processed immediately after capture. Bats were held in the hand during attachment of tags by licensed individuals.

M. nattereri *study site*

A maternity roost of *M. nattereri* was selected in south east Ireland on the basis of size and accessibility. This roost was not used in the analysis of roost habitat associations but was characteristic of *M. nattereri* roosts across the region, with approximately 60 bats roosting in an open attic of a stone building. The surrounding habitat was dominated by pasture with small woodland blocks dispersed across the landscape characteristic of the predominantly agricultural, rural landscape. The bats roosted openly within the attic allowing selective capture of a small number of individuals. Radio tracking was conducted during May and June 2010, avoiding periods when bats were in late stages of pregnancy or carrying dependent young. Bats were removed from the roost during day time and radio transmitters (0.35g, LB-2N; Holohil Ltd, Canada) attached using surgical adhesive, as above, before being returned to the roost. Bats were tracked in the pre-partum and post-partum periods.

All procedures were carried out under licence from the National Parks and Wildlife Service (NPWS; Licence No.74 C/2008).

Radio-tracking

Up to five *M. mystacinus* were simultaneously tracked during each session by three teams. Bat locations were assessed by triangulation from up to four simultaneously fixed positions. Fixes were taken every 15 minutes when bats were commuting and every 30 minutes during the foraging period. For *M. nattereri*, locations of each individual were assessed using simultaneous triangulation from multiple positions every 30 minutes. Tracking continued for a seven night period after attaching radio-tags, during which up to seven individuals were tracked at one time. Fixes were obtained as above. Kernel analysis, using the Hawth's tools extension for ArcMap 9.2 (version 3.27) was applied to the location of fixes for bats, with the core area calculated as the 50% kernel area (Hooge and Eichenlaub 1997). Kernel analysis is a probabilistic method which accounts not only for the location of radio fix but also density providing a probability of occurrence in an area (Worton 1989). The number of fixes required to accurately calculate core areas was examined by calculating the core area for each individual using increasing number of fixes. The proportional area change of core area was calculated with additional fixes and plotted against number of fixes. The change in core area stabilised when approximately 25 fixes were used. The maximum area over which bats ranged was delineated using Minimum Convex Polygons (MCP). The MCP is produced by drawing the boundary defined by linking the locations of the most disparate fixes.

The locations of these foraging fixes were calculated using Locate (http://www.locateiii.com) using maximum likelihood to allocate fix locations from multiple bearings. This allows an error to be attributed to each fix. To assess the error of locations estimated by triangulation, the locations of transmitters, placed at known points, were calculated. Triangulation of these test locations was carried out from two fixed positions, 1km from a central line, along which transmitters were positioned at intervals of 250m from 0 – 2250m. The accuracy of triangulation was assessed to have a mean error of 30.4m ± 4.3m, at a distance of 1.8km from transmitters.

Roosting behaviour

The daytime roosting locations of all bats were ascertained by tracking on foot. The characteristics of each roost were documented (Table 3)

Results

Sufficient tracking data were collected for 13 *M. mystacinus* and 16 *M. nattereri* to make accurate assessment of home ranges and habitat use.

Table 3: Details of roosts utilised by *M. mystacinus* in the present study.

Roost	Type	Construction material	Roof material	Age (yrs)	Number of tracked bats using roost
Roost 1*	Dwelling house	Stone	Natural slate	100+	9*
Roost 2	Dwelling house	Concrete	Artificial slate	<10	2
Roost 3	Shed	Concrete	Corrugated iron	30+	10*
Roost 4	Dwelling house	Brick	Artificial slate	30+	1
Roost 5	Dwelling house	Stone	Natural slate	100+	1
Roost 6	Beech tree	Not applicable	Not applicable	100+	2
Roost 7	Beech tree	Not applicable	Not applicable	100+	1
Roost 8	Sycamore tree	Not applicable	Not applicable	100+	1

* Roost at which bats were captured for tracking study.

Home ranges

The MCP size for all *M. mystacinus* was 227.8 ha (S.E. ± 55.6). For individual bats this ranged from 71.2 ha to 788 ha (Table 4). Average core area (50% kernel contour) was 58.7 ha (S.E. ± 21.06), ranging from 5.8 ha to 293.8 ha (Table 4). The average commuting distance between roosts and for areas was 0.7km, (ranging from 0.04 km to 3.25 km). There was a large amount of overlap in home ranges and core foraging areas between individuals in the colony. The average core foraging area of *M. nattereri* was 58.1 ± 6.4 ha, with individuals travelling on average 1.46 km to core areas (range .2 km to 3.8 km) (Table 4).

Table 4: Tracking details of all bats. Foraging area is determined as 50% kernel density area for all foraging fixes recorded for an individual. The individual *M. nattereri* which utilised a second roost other than the roost at which it was first captured is marked (*).

M. mystacinus				***M. nattereri***			
Bat	**Nights tracked**	**Mass (g)**	**Foraging area (ha)**	**Bat**	**Nights tracked**	**Mass (g)**	**Foraging area (ha)**
1	5	5.5	28.7	1	5	10.5	13.9
2	6	5	18	2	5	8.8	70.8
3	7	4.9	14.5	3	3	8.7	21.4
4	8	5.5	5.8	4	4	10	95.5
5	9	5.5	43.9	5	5	9	72.5
6	10	5	69.5	6	5	8	44.4
7	11	5	110.1	7	5	9	60.1
8	12	5	55.9	8	3	8.5	74.1
9	13	5	293.8	9*	5	8	14.5
10	14	5.5	29.8	10	5	9.15	34
11	15	5.5	42	11	5	10.3	77.9
12	16	5	33	12	5	9.7	84.6
13	17	5	18.2	13	3	9.9	73.3
-	-	-	-	14	5	8.5	79
-	-	-	-	15	5	8.5	52.6
-	-	-	-	16	5	8	61.2

Roosting behaviour

M. mystacinus individuals were not roost faithful but utilised a network of eight daytime roosts, comprising four dwelling houses, one agricultural shed and two trees (Table 3). One tree roost (7; Table 3) was only used as a night roost by a single bat and bats generally returned to their daytime roosts during the night. On average, females used 1.8 ± 0.2 SE roosts. Bats were observed directly during the day in roost 1 (between rafters) and roost 3 (between the corrugated iron roof and interior bitumen lining) (Table 3). The buildings used as roosts varied in age and structure (Table 3). Two roosts (1 & 3) were the most frequently used as daytime roosts by tracked bats. Roost 1 was 100+ years old, large house with natural stone walls and a natural slate roof and roost 3 was a 30+ year old farm shed with concrete walls and a corrugated roof. However, these roosts were where capture of tracked individuals was carried out, making unbiased assessment of their importance to the local population difficult. All the tree roosts were mature broadleaf species (Beech *Fagus sylvatica* and Sycamore *Acer pseudoplatanus*).

Of the sixteen *M. nattereri* tracked, fifteen returned to the initial study roost. A single bat roosted 1.2 km from the study roost and remained there throughout. This roost was in an active farm building (constructed circa 1970). The core foraging area of this individual was not included in analysis of habitat selection.

Habitat selection:

Habitats in the study area were mapped from aerial photographs (Ordnance Survey Ireland), using ArcMap (www.esri.com) and were divided into discrete categories used by Smith & Racey (2008) using Fossit (2000) as a guide to habitat classification. For habitat use analyses, "scrub" (code WS1, Fossit 2009) and "immature woodland" (WS2) were grouped together. The area within 10m of watercourses was delineated as 'Riparian'. The proportion of available and used habitat was calculated from the surface area of the habitat polygons. Habitat selection was analysed using selection ratios (Manly *et al.* 2002), whereby the observed use of habitat is compared to the expected use. If the selection ratio value ($\hat{w}_i$) of a habitat is = 1, there is no selection; if it is greater than 1, there is positive selection for a habitat; and, if it is less than 1, the habitat is actively avoided. For foraging *M. mystacinus*, mixed woodland and riparian areas were selected (Table 5) whilst pasture, amenity grassland and lake were avoided at both selection levels.

Given the relatively long commuting distance observed for *M. nattereri* and concentrated use of single maternity roost, selection ratios were calculated in distance bands from this central place. The area surrounding the roost was divided into bands with radii 0.25 km 0.5km, 0.75km, 1km, 2km, 3km and 4km. The selection ratios were calculated independently for habitats within these distance bands.

Habitat selection patterns changed with distance from the roost (Table 5). Woodland and developed land cover in the immediate vicinity of the roost were positively selected but selection declined and became negative with increasing distance. There was selection against arable habitat across distance classes from the roost. Pasture was avoided at extremes of the distance range but selected for at intermediate distances. Strongest selection for both woodland and development ratios were observed at 0.5 km. Avoidance of arable habitats was strongest at 0.75 km. The strongest selection for pasture occurred at a distance of 2 km (Table 5).

Table 5: Foraging habitat selection indices ($\hat{w}i$) for *M. mystacinus* and *M. nattereri*. Codes in () refer to Fossit (2000) habitat classifications.

Habitat selection of *M. mystacinus:*		
Habitat	**Description**	**Selection ratio ($\hat{w}_i$) ± s.e.**
Amenity grassland (GA2)	Maintained / managed grassland not associated with agriculture or	0.7±0.16
Lake (FL5, FL7, FL8)	Open freshwater surface	0.62±0.37
Mixed woodland (WD2, WD3)	Mixed deciduous evergreen woodland	2.78±0.38
Pasture (GA1)	Permanent grassland cover	0.74±0.09
Riparian zone	Areas with 5m of rivers	1.76±0.27
Habitat selection of *M. nattereri:*		**Values in () are distance from roost with strongest selection**
Development (BL3)	Urban and manmade land cover classes	1.11±0.01 (0.5 km)
Pasture (GA1)	Permanent grassland cover	$1.25\pm9e^{-3}$ (2.0 km)
Broadleaf woodland (WD1, WN1,WN2, WN6)	Broad leaf and mixed woodland cover	1.72±0.06 (0.5 km)
Arable (BC1)	Arable cropland cover & Complex cultivation patterns	$0.84\pm8e^{-3}$ (0.75 km)

Discussion

The results of the present study suggest that *M. mystacinus* switch roosts frequently during the breeding period. Although roost switching is generally associated with species that utilise ephemeral roosts, such as trees (Lewis 1995), this behaviour is also recorded from species that use more permanent structures, such as buildings. In other parts of their range *M. nattereri* use a network of roosts within the foraging range of a colony and will frequently move between them (Smith & Racey 2005). Tracking female *M. nattereri* in this study revealed high fidelity to a single roost of all tracked bats, only one *M. nattereri* roosted in a location other than the initial study site.

M. mystacinus utilised a range of building types (old and new), as well as mature trees. In a review of roost usage by European bat species, Marnell & Presetnik (2010) recorded that this species mainly roosted in buildings with some records of natural tree roosts and bat boxes. In Southern Britain, a radio telemetry study of 12 individuals of *M. mystacinus* found that the majority roosted in dwelling houses (71.4%) and a minority in unoccupied outbuildings (14.3%) (Berge 2007). In the Lorraine region of France maternity roosts of *M. mystacinus* were recorded mainly from dwelling houses (CPEPESC Lorraine 2009). The use of tree roosts in Ireland by *M. mystacinus* was not well established prior to the present study. Previously the only other roost type known was of single individuals occupying masonry bridges (Smiddy 1991; Shiel 1999)

M. mystacinus foraged in habitats selectively favouring mixed woodland and riparian areas. In continental Europe, *M. mystacinus* has been described as both a forest species (Kanuch *et al.* 2008) and a species of open lowland agricultural landscapes (Taake 1984). Berge (2007) found semi-improved and improved grassland to be the most important habitat for this species. In the present study, pasture grassland was avoided. During tracking, individuals were directly observed foraging over and adjacent to a stream flowing through a stand of trees. Some stands of broadleaf trees, which were available to bats, however, were not utilised. This could indicate that the riparian component of the

woodland is more important to *M. mystacinus* than tree species composition and might explain why broadleaf stands that were not close to water were not selected by tracked individuals. Riparian areas have been shown to be important for bats as a foraging habitat (Russ & Montgomery 2002). Walsh & Harris (1996) found that bat abundance in the landscape of Britain was positively correlated to the availability of woodland, riparian and lacustine habitats. Forest streams also provide suitable foraging environments for bats as they create larger gaps in the forest, which can facilitate travel and hunting by bats adapted to foraging along structural edges (Seidman & Zabel 2001) and provide shelter for insects and for species that have slow manoeuvrable flight like *M. mystacinus,* which may be vulnerable to predation (Warren *et al.* 2000) .

There is a nightly, temporal shift between two behaviours in bats, emergence and foraging. Across their range, *M. nattereri* is regarded as a foraging habitat generalist, gleaning prey from vegetation and aerial hawking insects close to vegetation (Arlettaz 1996; Siemers *et al.* 1999; Siemers & Swift 2006; Smith & Racey 2008) and as a species which emerges relatively late from roosts (Jones & Rydell 1997). Woodland provides this and was selected in the immediate vicinity of the roost, but this selection declined with distance. In contrast, pasture was avoided close to the roosts and selected within the core foraging areas. These conditions provide *M. nattereri* with the desired level of cover in brighter periods (dusk and dawn) and access to foraging habitat, in this case, grassland.

The strong behavioural association with different habitats at different times and scales may lead to misinterpretation of habitat requirements for central placed foragers. For example, if the presence of *M. nattereri* was assessed in the emergence period, it may be concluded that there is a high dependence on woodland habitat. However, observing the species foraging (in grassland) would detract from the importance of woodland close to a roost. During the maternity period it is likely that the effects of spatial scale in habitat association will be most pronounced. At this time, maternity roosts must provide specific thermal conditions for developing young (Lourenço & Palmeirim 2005; Smith & Racey 2005) and females must return to these roosts to provision their young. During the non-maternity period, opportunistic roosting may be in less specific conditions and strong changes in habitat associations related to behaviour may not be observed. The multi-scale habitat selection analysis of the habitat associations of *M. nattereri* indicates that conservation of roosts and their immediate habitat facilitate exploitation of a relatively common habitat by foraging *M. nattereri*.

2.2 The landscape associations of *Myotis mystacinus* and *M. nattereri*

Introduction

Defining the geographical ranges of bat species can be difficult due to their nocturnal and elusive behaviour (Walsh & Harris 1996; Vaughan *et al.* 1997). Species' Distribution Models (SDM) provide a generalisation of species – habitat associations, and are particularly useful for examining the ecology of bat species (Jaberg & Guisan 2001; Rebelo *et al.* 2010). SDMs relate the known occurrence of a species with the environmental character of that area. Using these associations, the suitability of any location and the likelihood that species will occur can be determined. This provides an alternative to direct mapping, of all areas, and allows predictions of a species' current, future and past distributions to be made under the condition that changes in the environment are known (Guisan & Zimmerman 2000; Thomas *et al.* 2004). By establishing the potential range of the species within an area, the models derived can be used to assess a species' conservation status (Cabeza *et al.* 2004; Thomas *et al.* 2004). They can also be used to describe and predict potential changes in species' distribution in response to environmental change such as changing climate variables (e.g. Lundy *et al.* 2010). Additionally, it is possible to define priority conservation areas and sites for reintroductions (e.g. Wilson *et al.* 2010), through identification of highly suitable areas that a species may have previously occupied. The predictions of SDMs are based on estimating the suitability of an area for a species. However, the suitability of an area is not solely related to the availability of habitat but also to the context of the

surrounding landscape structure, size and shape (Virkkala 1991; Jokimaki & Huhta 1996; Bennett *et al.* 2006). Species respond to heterogenous landscapes at multiple scales which combine to determine presence and population processes (e.g. Fryxell *et al.* 2005). Using a multi-scale approach allows the immediate habitat to be placed in the context of the surrounding landscape (Wiens 1989; Jokimaki & Huhta 1996). Multi-scale methods can identify species relationships with aspects of habitat spatial scale (McAlpine *et al.* 2006; Lundy & Montgomery 2010).

Central placed foragers utilise a defined location which provides suitable conditions to rest or protect juveniles and make repeated trips to foraging areas. The character of the central place and foraging area may be highly contrasted for some species. Species distributions can also change significantly during their life cycle in response to changing resource requirements (Law & Dickman 1998). Jaberg and Guisan (2001), for example, demonstrated a seasonal contrast in the habitat associations and predicted distribution of the Greater mouse-eared bat (*Myotis myotis*) in Switzerland shifting from low elevation during summer to higher elevation with structured vegetation cover outside the breeding period. Roosts are crucial in bat ecology and distribution (Kunz & Fenton 1993; Findley 1993), with different species having specific roost requirements (Marnell & Presetnik 2010). Roosts are considered central places particularly during the maternity period (Daniel *et al.* 2008). Roost choice is associated with thermal conditions (Lourenço & Palmeirim 2005; Smith & Racey 2005) and associated behaviour such as avoidance of predators (Jones & Rydell 1997).

We develop a multi-scale SDM (McAlpine *et al.* 2006) to examine land cover associations of *M. mystacinus* and *M. nattereri* at a landscape scale. The use of multi-scale predictive modelling to understand behavioural tradeoffs in habitat selection is of general importance in applying species conservation management strategies. From the predictions of multi-scale SDM it is possible not only to predict where species occur but make predictions about their use of different land covers.

Materials and methods

The study was carried out using all identified maternity roosts (Section 1.1) of *M. mystacinus* (22) and *M. nattereri* (19) spread across Ireland (Figure 4). We select a number of locations at which bat roosts are not known. These are called pseudo-absence records and are generated, at random, in a Geographical Information System (GIS; ArcMap 9.2, ESRI) for both species. This set of locations is used as a comparison for locations at which roosts are known. The foraging areas of *M. nattereri* and *M. mystacinus* can be up to 4 km from roosts (Section 2.1). Hence, we used this distance as the upper limit of spatial scales to model roost occurrence. We calculate the areas of different land covers in areas of increasing radii =0.25km, 0.5km, 0.75km, 1km, 2km, 3km and 4km. Land cover classes are derived from CORINE (EEA 2002; Table 4). The associations of roost occurrence are tested using Independent Generalised Linear Models (GLM) using the area of land covers within each distance band independently (56 models). Using GLM allows consistent associations to be detected, such as increasing likelihood of roost occurrence with increase in a given land cover. Confidence in the associations derived is given by an examination of the regression coefficient (equivalent to the gradient of a straight line) and their associated standard error (variation in estimate of the gradient). A coefficient which encompasses zero, when the standard error is added or taken away, suggests the association is not consistent across all roosts.

We examined associations between different spatial scales and selected the most relevant spatial scale for each land cover variable using a measure called the Akaike Information Criterion, corrected for sample size (AICc; Burnhman & Anderson 2002). AICc gives a measure of how closely the occurrence of roosts matches changes in the area of land cover. Using these selected spatial scales a Maximum Entropy (MaxEnt) model was constructed (Phillips & Dudík 2004) for each species, using linear and quadratic response forms. A MaxEnt model allows the relationships between the occurrence of roosts and land cover to be examined and extrapolated to other regions. Using CORINE land cover allows the model associations to be tested with the occurrence of roosts in other independent areas across Europe.

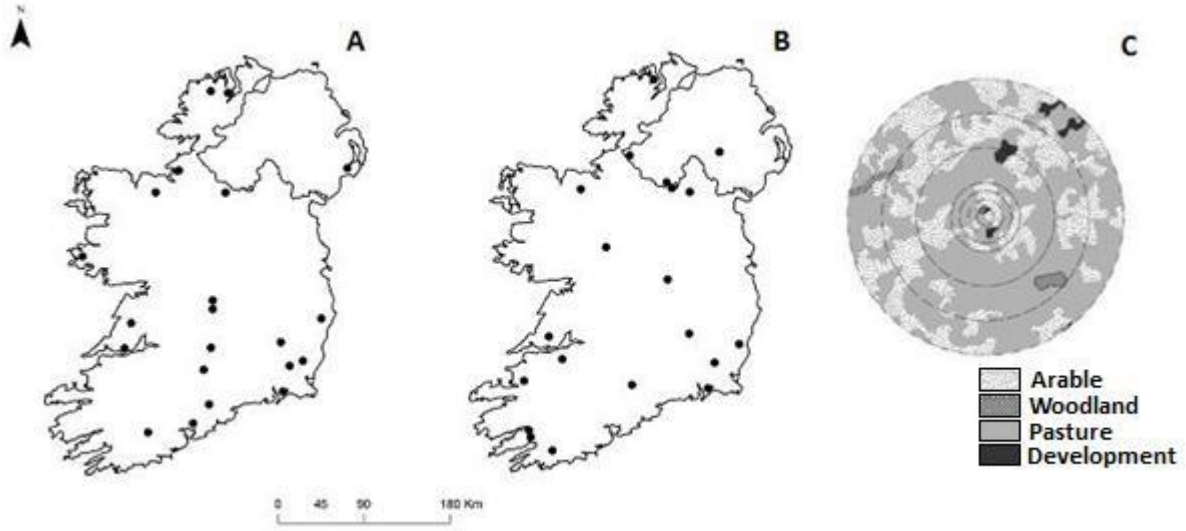


Figure 4: A) *Myotis mystacinus* maternity roost records used in the construction of the Species Distribution Model (SDB) B) *Myotis nattereri* maternity roost records used in the construction of the Species Distribution Model (SDB) C) The habitat surrounding the roost within set distance bands.

Results

Land cover association of the multi-scale SDM

No single distance was selected as the most relevant for all land cover classes. Regression coefficients and their standard errors revealed consistent associations with roost presence for woodland, developed and pasture land covers (Table 6). The spatial scale, percentage contribution to the model and relationship for each land cover class with both species is summarised in Table 6.

The models constructed (Table 6) have an 82% and 78% correct classification rate, within Ireland, for *M. nattereri* and *M. mystacinus* respectively. When the model is tested on an independent geographic region (Wales) the correct rates of classification are 61% and 71% for *M. nattereri* and *M. mystacinus* bat respectively. Records for the occurrence of the species in Wales were obtained from the National Biodiversity Network (NBN) (http://data.nbn.org.uk/). This suggests that there is a small degree of regional specialisation to the data but that the model generalisations describe biologically relevant patterns.

From the model projections across Ireland the suitability of all 20km squares is collated in for those squares where records exist. The average suitability of these areas is calculated and the standard deviation (SD) calculated. A conservative threshold of the minimum suitability for the species to occur is calculated as the average suitability – 1*SD. The area with predicted suitability above this threshold is delineated for across the entire area, as the core suitable range. Some grid squares can have average values below this threshold but still have positive records. This core range is used as a generalised range and only as a guide to broad scale favourability. The predictions of landscape suitability show that suitable areas are available in all regions with no broad patterns of unsuitable areas for *M. nattereri*, but a trend to more favourable areas in southern regions for *M. mystacinus* (Figure 5).

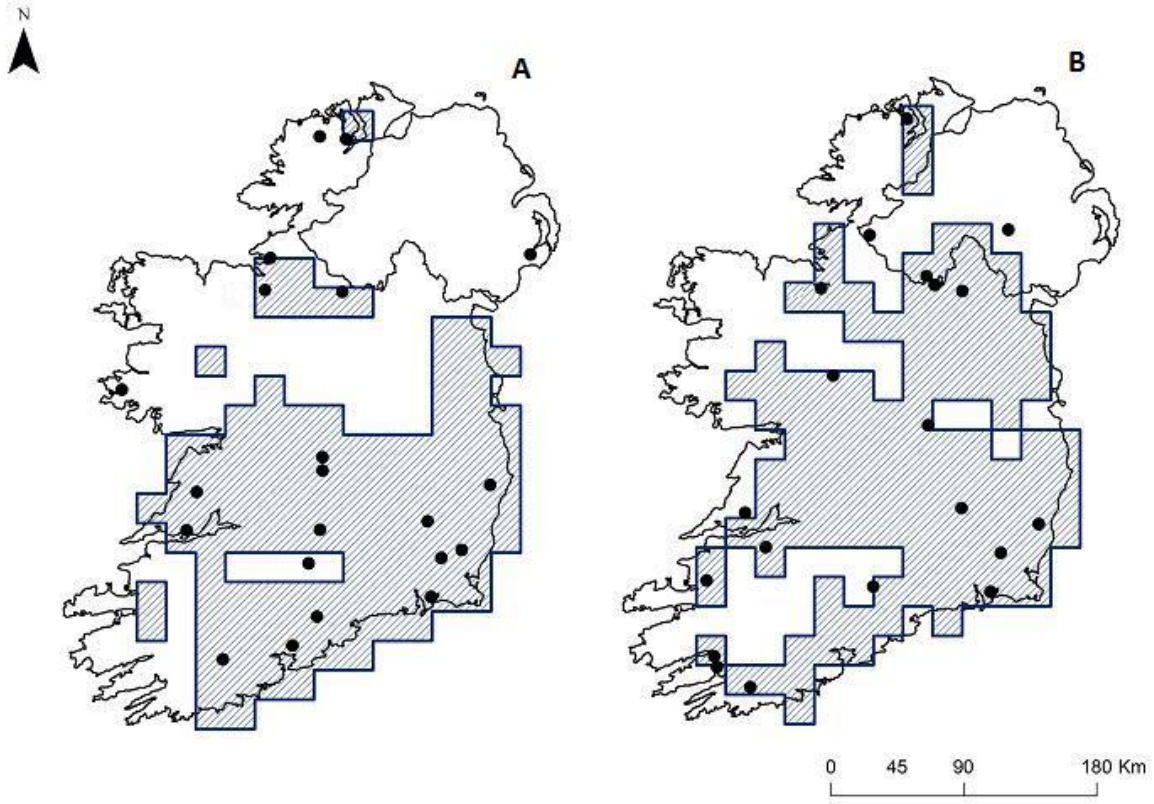


Figure 5: Landscape favourability for A) *M. mystacinus* and B) *M. nattereri*. The area delineated represents the core suitable area determined by MaxEnt distribution modelling; the average suitable area for squares with positive records -1 SD.

Table 6: Relationships with land cover categories for *M. mystacinus* and *M. nattereri*. The contribution to the MaxEnt model and distance selected as most relevant are given. The direction of the relationship can be negative (-ve), positive (+ve) or second or (+ve/-ve).

	M. mystacinus			***M. nattereri***		
Land cover	**Distance**	**Contribution (%)**	**Relationship**	**Distance**	**Contribution (%)**	**Relationship**
Agriculture	2km	23.1	+ve	2km	24.5	+ve/-ve
Bog/Heath	.25km	0.2	-ve	1km	0.9	-ve
Coastal	.5km	1.6	+ve/-ve	.5km	1	-ve
Conifer	2km	8.4	+ve/-ve	.25km	0.2	+ve/-ve
Development	4km	4.7	+ve/-ve	4km	10.3	-ve
Freshwater	.5km	0.3	+ve	2km	5.6	+ve/-ve
Natural grassland	.25km	0.2	-ve	.25km	2	-ve
Pasture	4km	0.5	-ve	4km	39.1	+ve
Woodland	4km	61.1	+ve	.5km	16.4	+ve

Discussion

The multi-scale SDM of roost occurrence identifies the relevant spatial scale of specific land covers related to roost occurrence. Spatial scale processes are related to levels of habitat quality (McAlpine *et al.* 2006) and to biological differences between species (Lundy &Montgomery 2010). Identifying aspects of spatial scale processes increases our understanding of what makes otherwise similar habitat patches heterogeneous with regard to suitability.

The SDM applied to maternity roosting revealed complex patterns of land cover associations. There were marked differences in the most relevant spatial scales for specific land cover classes. We argue that these are related to a trade-off between selection for roosting and selection for foraging areas. There are particularly strong contrasts for the associations of *M. nattereri*. Across their range, *M. nattereri* is regarded as a foraging habitat generalist, gleaning prey from vegetation and aerial hawking insects close to vegetation (Arlettaz 1996; Siemers *et al.* 1999; Siemers & Swift 2006; Smith & Racey 2008) and as a species which emerges relatively late from roosts (Jones & Rydell 1997). Roosting behaviour is associated with habitats providing cover from predators. Woodland provides this habitat and was selected in the immediate vicinity of the roost but the strength of selection declined with distance, with woodland avoided by foraging bats. In contrast, pasture was avoided close to the roosts and selected within the core foraging areas. These conditions provide *M. nattereri* with the desired level of cover in brighter periods (dusk and dawn) and access to foraging habitat, in this case, grassland. These results are concordant with those habitat selection patterns observed previously (Section 2.1).

The strongest positive association for *M. mystacinus* is with woodland. From the radio-tracking of both *M. mystacinus* and *M. nattereri* we observed that these species forage in woodland and pasture respectively. From modelling, these land covers are most relevant at the broadest scale. Of other land covers used to explain the landscape associations of *M. mystacinus,* conifer and areas of development were the only others to contribute markedly to the models. A second order relationship with conifer suggests that a small area of this land cover may be beneficial for the species but areas with large areas of conifer may be unsuitable. Whilst there was a negative association with development for *M.*

nattereri, *M. mystacinus* again had a second order relationship suggesting small levels of development were positive predictors of occurrence, perhaps providing roosts.

With increasing availability and development of GIS and remote sensing data, more comprehensive understanding of patterns and processes of species occurrence may be incorporated into predictive models. At present the CORINE land cover datasets provide a general representation of available land covers. With increasingly accurate land cover mapping, considerable new insights may be forthcoming in the future. SDMs may be perceived as static generalisations of species occurrences. In the face of global environmental change, SDMs have increasingly become essential to predict distributional responses at species and community levels. Mechanistic models of occurrence are used to predict range of species with respect to physiological parameters (Kearney *et al.* 2010). The use of multi-scale techniques to construct SDMs facilitates more than static predictions of species' occurrence. These methods are particularly relevant for centrally placed foragers which occupy patchy environments and can deliver invaluable insights for management.

2.3 Developing a methodology to detect disturbance effects on the activity of maternity roosting bats.

Introduction

Conservation and management of rare and endangered species is a primary concern in many aspects of ecological research. A range of field techniques are used to investigate the ecological requirements and population status of these species, often requiring collection of invasive samples. The impact of such invasive procedures on behaviour of wild animals should be assessed to ensure correct biological interpretation of results and limit possible impacts on animal welfare, ensuring that the information obtained justifies the means of collection (Putman 1995).

Bats and their roosts are legally protected from human disturbance in Europe (EU Directives 92/43 and 97/62). However, researchers are permitted to carry out a range of activities under license to study bats. These research activities often result in at least some degree of disturbance, ranging from entering roosts to count or identify bats visually, to capturing and handling bats to mark, collect tissue samples for molecular analysis or to attach transmitters for tracking studies (Fenton 1997). Whilst these activities are highly regulated in order to minimise or at least to keep disturbance to a minimum, these actions do represent a spectrum of disturbance levels to roosting bats.

Bats select and move between distinct types of roosts throughout the year depending on requirements at specific stages of life history (Dietz *et al*. 2009). Of particular importance is the selection of maternity roosts, where female bats congregate to give birth and raise young. Typically, mating roosts are formed in late spring and can last until July in north temperate species (Dietz *et al*. 2009). These roosts are characteristically dominated by female bats with young. Adults generally occupy the roost until the young are independent (Henry *et al.* 2002). Individual bats frequently return to the same maternity roost year after year (Veilleux & Veilleux 2004). It is common practice to avoid capturing bats at such sites when female bats are likely to be heavily pregnant or likely to be carrying young (Mitchell-Jones & McLeish 2004).

The initial study was carried out during the postpartum period of *M. mystacinus* at a typical maternity roost in Co Cork, Ireland, when dependent young may be present within the roost, but are too large to be carried by adult females. This was applied as part of a tracking study (Section 2.1). The normal activity of bats at the roost was recorded continuously, prior to, and after the disturbance events associated with attempts to capture emerging bats. It was expected that activity at the maternity roost would decline across the study period as juvenile bats became independent and adult bats are no longer required to return to feed and care for juveniles. Wavelet analysis was used to identify activity patterns over the study period. Wavelet analysis is becoming a common tool for analyzing non-

stationary variance at many different frequencies within a geophysical time series (Labat *et al.* 2000). For example, it has been applied successfully to identify inter-annual variability in climate series (Lucero & Rodriguez 1999). In the current research, wavelet analysis was used to assess short term activity patterns from high resolution data collected using continuous ultrasonic data loggers. This is the first time that activity patterns of bats have been examined using these emerging techniques. The method was subsequently applied to ensure the activity *M. nattereri* was not impacted by similar disturbance events during a radio-tracking study (Section 2.1).

Methods

During the prepartum period, two sampling events (26th of June 2009 and 3rd July 2009) carried out a week apart, successfully resulted in the capture of four emerging *M. mystacinus* individuals within the attic space. The maximum number of individuals counted during emergence this period was eight bats. The roost was left undisturbed for five weeks covering the period that this species gave birth (Mitchell-Jones & McLeish 2004). Activity of the roost was monitored over this period by brief diurnal surveys, when roosting bats were visually counted. Accumulation of droppings below the roosting crevice was assessed on a weekly basis. Attempts to capture bats for the radio tracking study, in the postpartum period, recommenced when 'furred', active juvenile bats were observed outside the roost crevice during daylight hours, as per standard bat conservation protocols (Stebbings 2004). An emergence count at this time estimated that the roost contained approximately 16 bats.

Five days prior to the first capture event, on the 21st of June 2009, an ultrasonic activity recorder was placed immediately outside the roosting crevice. This device consisted of a heterodyne bat detector, a Skye electronics transducer (http://www.skyeinstruments.com), and a Tinytag count data logger (http://www.geminidataloggers.com). Ultrasonic activity was monitored continuously, with the number of bat passes (ultrasonic events separated by 0.1 sec) per five minute period recorded. Ultrasonic activity of this type represents a range of behaviours from echolocation in flight, to social interaction. During the prepartum period, bats were observed flying within the attic prior to emergence. It was during these flights that bats were captured using harp-traps (Mitchell-Jones & McLeish 2004) and removed from the building to be fitted with radio tags prior to release in the immediate vicinity of the roost. Two capture events were attempted at the roost in the postpartum period (Figure 6).

Wavelet analysis was used to identify significant activity of different periodicity across the study period (Figure 7). This was done by transforming the one dimensional time series data to a two-dimensional time-frequency image (Torrence & Compo 1998). Wavelet analysis was used to allow analysis of non-stationary activity periods within time series data, carried out using the package 'dplr' in R (http://cran.r-project.org). Trends in activity levels across the study period were examined by fitting a linear regression to the de-trended activity (i.e. to emphasise short-term changes), with time as the independent variable. A linear regression was fitted to the entire period, with three further fitted regression lines to sub-periods A to C (Figure 6). Comparison of resulting regression gradients allowed for the assessment of significant changes in activity level over time.

Results

Continuous activity recording revealed clear peaks in activity of bats at emergence, and also considerable activity during daylight hours. There was a decline in bat activity across the study period (Figure 6). Five bats were captured during the first capture/ disturbance event. While no bats were captured at the second event, they were observed within the roost. The plot of activity suggests that the first disturbance event had little impact on the activity. However, activity on days 12 and 13, immediately after the second disturbance event on day 11, was markedly reduced. Little activity was recorded after day 18 of the study. The activity traces shows that bats were also active within the roost during the hours of darkness in sub periods A and B, but, this behaviour was reduced during period C.

Wavelet analysis clarifies the significant activity cycles apparent from the raw time series data (Figure 7). Three cycles were observed, clearly representing significant behavioural patterns occurring on a 16 - 32 hr, 8 - 16 hr and 2 - 4 hr cycle. These cyclical patterns persisted throughout the time period encompassing the first disturbance event. The behaviour corresponding to the 2 - 4 hr cycle diminished over the course of the study, and was completely absent by day 11. These significant bouts of activity occurred predominantly during daylight hours. The second disturbance event, however, resulted in a noticeable breakdown of all cyclical patterns and roost abandonment for approximately three days. This observation is concordant with the raw activity trace in Figure 6.

Regression of the activity for the three sub periods indicates that there was no increased decline in activity due to disturbance events (Figure 8). Independent regression of the three distinct sub periods (A – C; Figure 6), suggests that the fastest decline in activity occurred prior to the first disturbance event in the postpartum period. Data from days 12 and 13 were not included in the analysis, as there was no activity observed in the roost immediately after the second capture event. The rate of decline during the first period was significantly faster than the following two periods, with a regression coefficient of -5.23 ± 1.37 compared to -2.88 ± 0.60 and -2.32 ± 0.10.

Discussion

Results of this investigation clearly demonstrate both the need and the importance to assess the impact that disturbance events associated with field based techniques may have on subject population, particularly those involving behavioural investigations. Over the course of this study, normal activity at the maternity roost of *M. mystacinus* declined. This may reflect a natural occurring biological process related to the maturation of juvenile bats and resulting reduced dependence on maternal care at maternity roosts. Novel application of Wavelet analysis indicates that first disturbance event had no significant impact on cyclical activity patterns among bats at the roost. In contrast, the second disturbance event had an impact but normal patterns returned shortly after, even though no bats were captured. However, prior to the end of the study, all activity at the roost ceased. The increased impact of the second capture event might be a consequence of the lower biological cost involved with switching roosts because, at this stage, juvenile bats are becoming more independent.

Wavelet analysis has also identified previously unreported significant cyclical activity patterns, involving bouts of activity during the daytime within the roost. These correspond to small peaks in activity at approximately midday (cf. Figure 6 & Figure 7). Roosting bats have specific temperature regime requirements (Smith & Racey 2005). For instance, to conserve energy, roosts that do not vary considerably in temperature from their optimum physiological requirements are selected (Kerth *et al.* 2001). However, juvenile development is facilitated by selecting roosts with higher ambient temperatures (Smith & Racey 2005). Thus, maternity roosts are more likely to reflect external temperatures, which during the summer, may rise above optimal conditions during the warmest portion of the day. At the present site, bats used other crevices within the attic space, in addition to the primary roosting crevice. It is likely that the ability to select and move between roosts affects maternity site choice (Smith & Racey 2005). Continuously recorded bat activity also indicated that, during the initial ten days of the study, activity peaks in the roost were spread across darkness. This suggests that many bats remained within the roost or returned during the night. Among the reasons explaining this observation are a lack of foraging behaviour of juveniles and/ or adults returning to provision young (Henry *et al.* 2002). This observation was not apparent during days 14 – 17, when activity peaked at the time of emergence with little activity within the roost during the night. This suggests that the second disturbance event may have resulted in or coincided with the remaining juvenile bats leaving the roost and becoming fully independent.

Capture of bats in the immediate vicinity does not appear to accelerate the rate at which activity at the roost declined. Thus, the fastest rate of activity decline was observed prior to the first disturbance event. The apparent significant impact on behaviour after the second disturbance event, may have resulted from a proportionally higher impact on individual bats, resulting from the reduced number of bats occupying the roost. Newly volant young could also potentially follow adults to other roost if

disturbed (Wilkinson 1992). Whilst the impacts on the overall roost activity may not be apparent when a large number of bats are using the roost, disturbance may entail a small number of individuals being adversely affected.

Wildlife researchers, particularly those investigating animal behaviour, should consider the impact that their studies may have on their study species. Few studies attempt to quantify their impacts, which may have critical implications for interpretation of results as well as raising significant welfare concerns. Of particular interest to bat ecologists are rates of roost switching and roost choice (Smith & Racey 2005). Here, we demonstrate that rates of roost switching assessed by tracking studies in which bats are captured at roosts may be artificially high due to the impact of disturbance. The impact on roost activity may last for as long as three days which may represent a significant portion of the tracking period of small bat species, as the tracking devices may only last a few days. Emerging technologies, such as data logging ultrasonic records, and analytical techniques, such as wavelet analysis, can greatly aid our understanding of these effects through monitoring and quantifying the disturbance integrated into an ecological study. Torrence & Compo (1998) suggested that wavelet analysis is often regarded as an interesting diversion but suffers from a lack of quantitative results. Here we clearly demonstrate its application in an animal behavioural study providing quantitative insights into both behaviour and welfare.

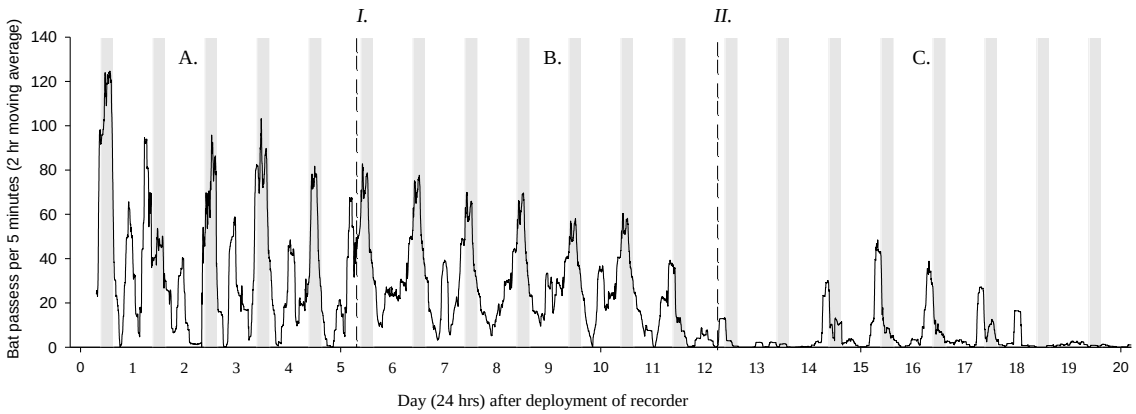


Figure 6: Bat activity across the study period. The grey areas represent hours of darkness. The timing of the two disturbance events are shown (I. & II.).

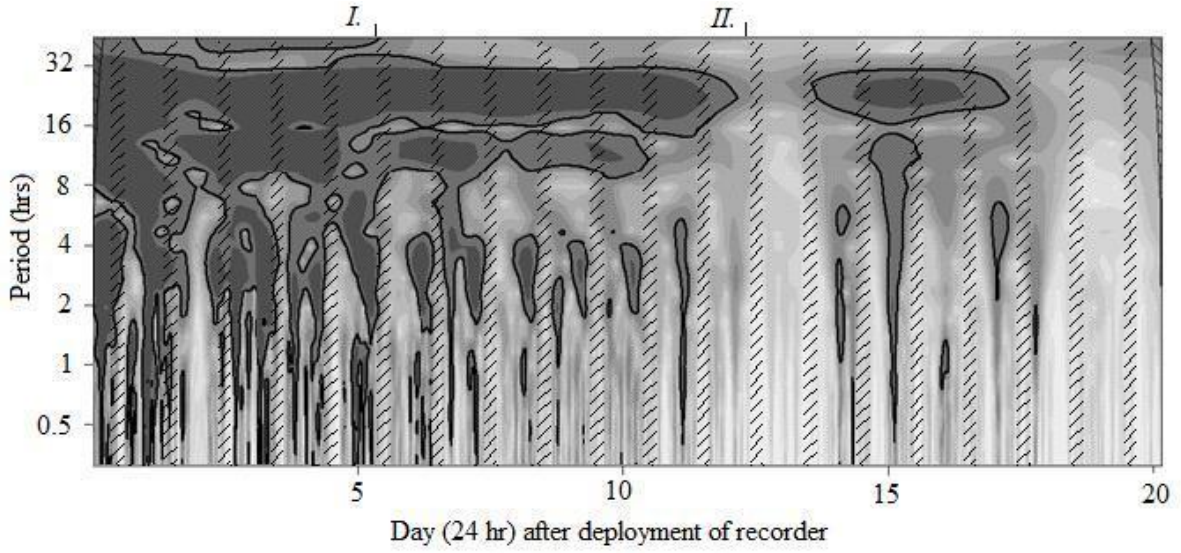


Figure 7: Wavelet analysis of bat activity. Areas bounded by a black line represent areas of significant activity across the study period (P<0.05). Hours of darkness are represented by hashed bars. The timing of the two disturbance events are shown (*I.* & *II.*)

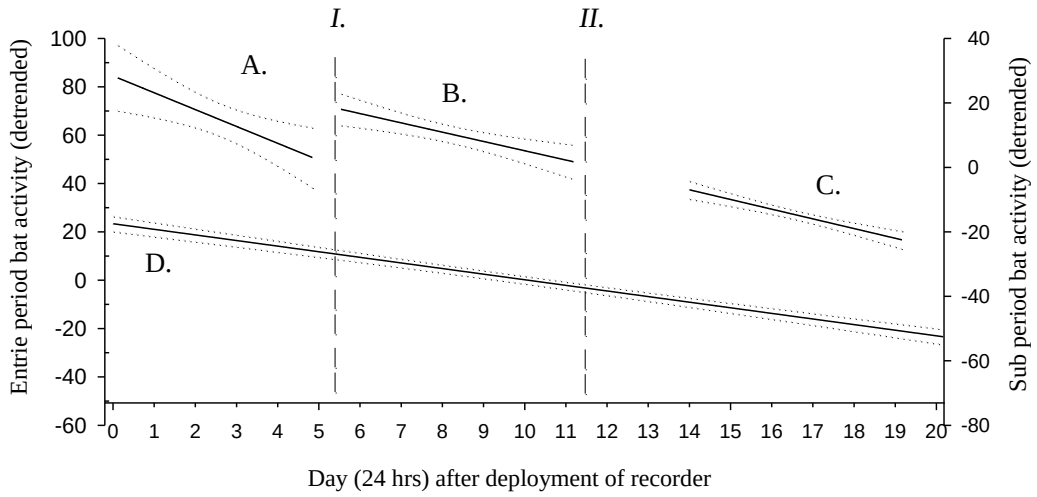


Figure 8: Regression of bat activity against time. The lines A, B and C correspond to the activity trends in the three periods before and after the two disturbance events (*I.* & *II.*) (Figure 3). Line D, shows a regression of bat activity against time for the entire study period. Confidence limits (99%) are shown for each regression.

2.4 Identification of swarming sites

Introduction

The habitat requirements of bat species change significantly between seasons (Jaberg & Guisan 2001). In winter, bats hibernate in roosts which are required to provide a constant environment, remaining cool throughout the hibernation period (Dietz *et al.* 2009). The majority of mating behaviour occurs prior to the onset of hibernation with fertilisation delayed until spring (Dietz *et al.* 2009).

Behaviours which are associated with large aggregations and high levels of aerial activity are termed swarming (Fenton 1969). Swarming activity is typically associated with return to communal roosts at dawn and has also been observed in some bat species during autumn at caves and other underground sites not associated with return to roosts. Late summer and autumn activity or swarming at underground sites is a characteristic behaviour of bats of the genus *Myotis* (Parsons *et al.* 2003). The behaviour is suggested to be related to mating (Parsons & Jones 2003; Rivers *et al.* 2005) or possibly the introduction of the young bats to potential hibernation sites (Humphrey & Cope 1976). During swarming, bats may assess the condition of this underground site as a possible hibernation site before winter (Fenton 1969). Whilst debate remains over the exact role of autumn swarming, protection of swarming sites is essential for the protection of bat biodiversity and to help maintain the favourable conservation status of bat populations.

Although *Myotis* are the most species rich genus of bats occurring in Ireland autumnal swarming behaviour had not be confirmed and limited information of hibernation sites is known. Given the postulated relationship with hibernation the occurrence of swarming behaviour, the lack of knowledge of the locations of swarming sites poses a serious question about the ecology of the Irish

Myotis population. Identification of these sites has potentially significant implications for the conservation of species as mating and undisturbed hibernation is key to maintaining a favourable conservation status.

During late summer and early autumn 2008 and 2009 we conducted a targeted survey of natural underground sites. Using the known preferences for these sites we characterised typical swarming sites and conducted surveys using unmanned ultrasonic recording equipment to identify swarming sites. At identified potential sites focal observation of bat activity was conducted to confirm the occurrence of swarming behaviour. Where conditions allowed, bats were captured to assess species composition and sex ratios. Here we present the findings of this first systematic study of bat swarming in Ireland.

Methods

Four factors, namely degree of chamber formation, hydrological activity, shelter at entrance and cave length are known to be important factors determining if a cave is likely to be a swarming site (Glover & Alteringham 2008). It was possible to obtain measures of all of these variables except shelter from a database of all known caves (http://www.ubss.org.uk/). Three core areas where identified from known roost records with a radius of 20km. Within these areas hydrologically inactive, large well developed caves were prioritised as potential sites. Swarming surveys were conducted between mid August - October in 2008 and 2009. Initial surveys of 17 potential swarming sites were conducted during the '08 field season and a further 6 in 2009. Initial surveys were conducted by observers using ultrasonic detectors at cave entrances to record levels of bat activity and identify the calls of *Myotis* bats. Repeat surveys of all sites at which bat activity was recorded were carried out over a prolonged period of up to 7 nights using ultrasonic logging bat detectors. From these activity traces, activity could be assessed for indicative patterns of swarming.

At sites where swarming was identified bats were captured to assess species composition and sex ratios of swarming bats. To minimise potential disturbance sampling was attempted from onset of swarming until behaviour began to decline. All bats captured were identified to species. Tissue samples from all bats were used to confirm species identification. Males and females of all species where enumerated and time of capture recorded and examined for signs of sexual activity.

Results / Discussion

During initial surveys *Mytois* spp. activity was recorded at nine of the twenty cave sites. Inspection of the logged activity from follow up surveys showed that five caves had patterns indicative of swarming (Figure 9). One of these sites had five unconnected caves within 1km of limestone escarpment, at which swarming activity was noted at all cave entrances.

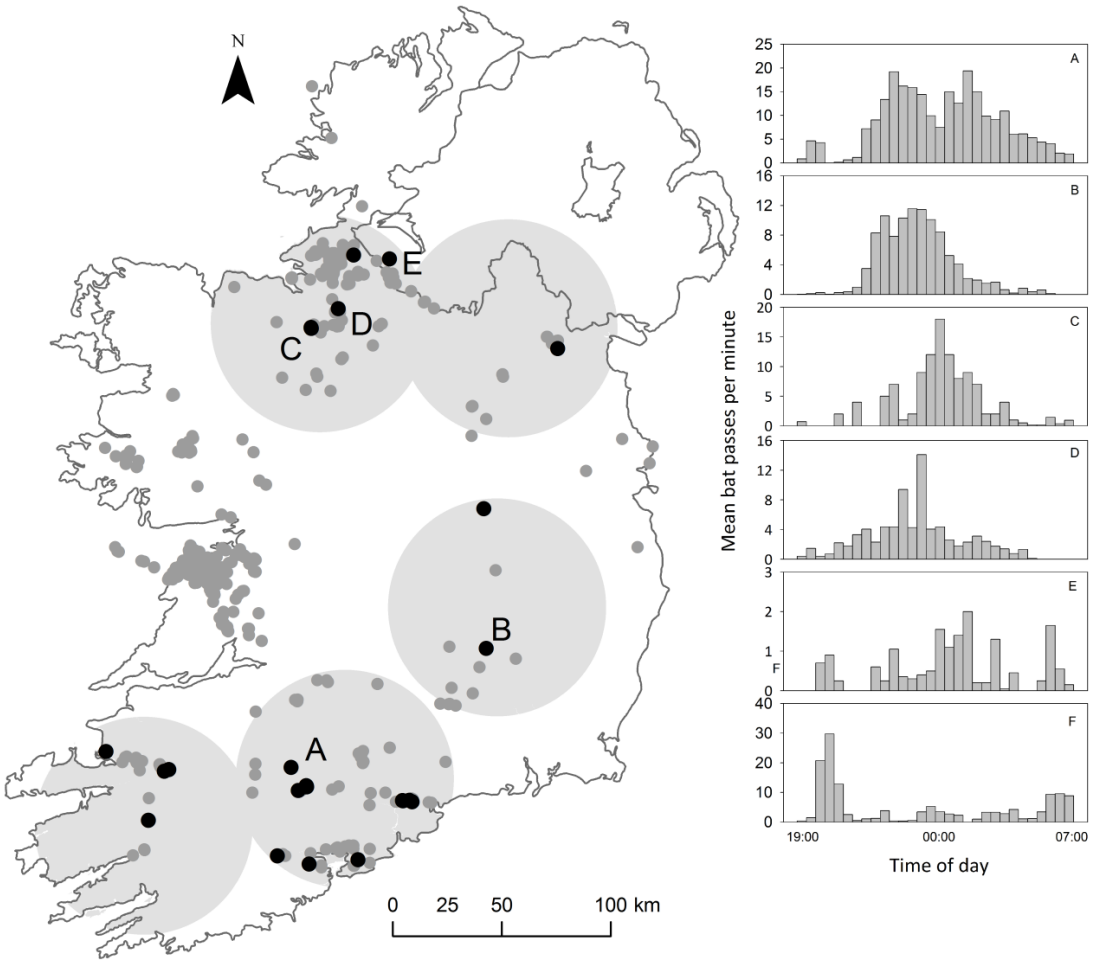


Figure 9: Distribution of known (grey circles) and surveyed (black circles) underground sites in Ireland. All sites with observed swarming activity are represented with a letter code. A single site in Northern Ireland (E) where swarming was suspected is also shown. Panels A – F show traces of activity across a single night for each identified site, panel F shows an activity trace from a roost for comparison.

Sampling bats during swarming behaviour was possible at 3 of the five identified sites, namely; Kesh caves Sligo, Castlepooke cave, Cork and Dunmore cave, Kilkenny. At all sites a higher ratio of males to females was recorded (Kesh = 28:13; Castlepooke = 32:8; Dunmore = 97:32). To assess trends in the capture of bats against time an index of capture rate was calculated; as the ratio of the number of individuals captured in a five minute period to the average number captured in each five minute period (Figure 10). This was calculated for both sexes and shows that male bat captures peaked earlier than captures of female bats (Figure 10). This may reflect a behavioural difference between the sexes with males arriving earlier to 'compete' to occupy optimal areas, perhaps in competition for access to females for mating. Examination of the testes of males suggested all to be sexually active.

Kesh caves, Co. Sligo, was the most species diverse swarming site with Brown long eared, Natterer's, Daubenton's, whiskered and even pipisterelle bats, species not traditionally thought to swarm.

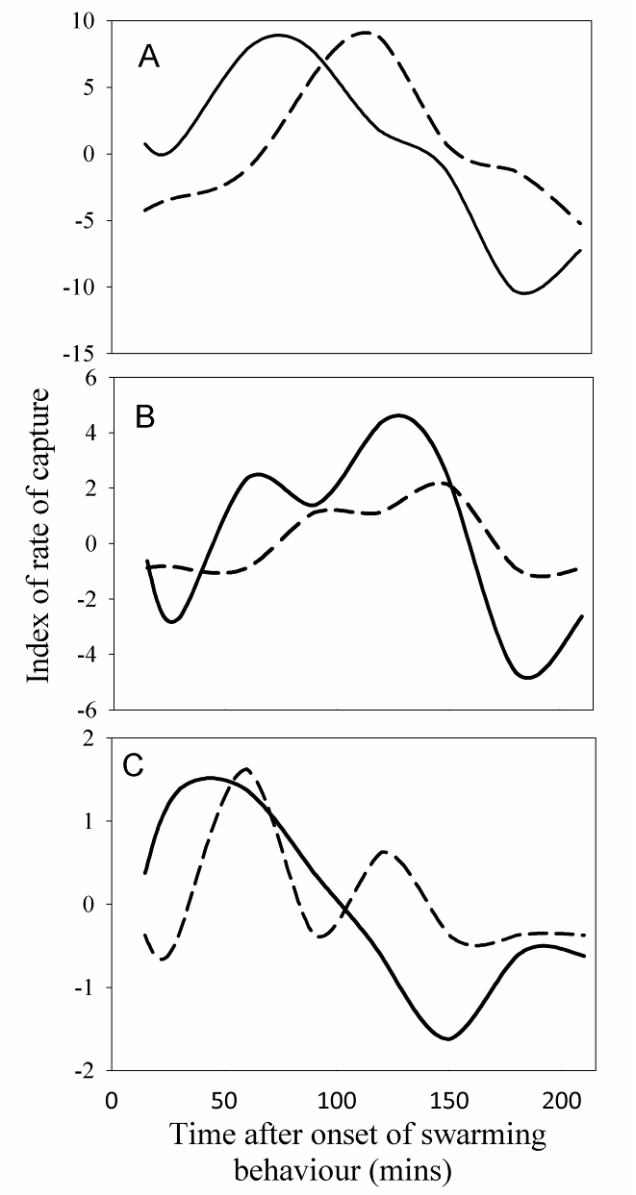


Figure 10: Index of capture rate during initial 4 hour period after the onset of swarming for male and (solid line) female (dashed line), a) *Myotis nattereri*, b) *Myotis daubentonii* and all other species

Summary

Previously limited data existed for swarming activity of bats in Ireland. The behaviour has now been confirmed in *M. nattereri* and *M. daubentonii*. The situation for *M. mystacinus* remains poorly understood. Future work should aim to utilise the protocol described above to find new sites and better understand the function and regularity of use of those sites identified.

2.5 Effectiveness of an acoustic lure to catch bats in woodlands

Introduction

M. mystacinus and *M. nattereri* are considered to be associated, across their range, with woodland habitats (Kanuch *et al.* 2008; Smith & Racey 2008). Systemic survey of woodlands for *Myotis* bats is made difficult due to the difficultly in species identification using ultrasonic detectors (Section 2.7). The development of an acoustic lure for target surveys designed to catch specific species is proposed and has been successfully applied to catch *Mytois* in Irish woodlands. To evaluate the effectiveness of the acoustic lure to catch bats in Irish woodlands a standardised survey of woodlands was conducted.

Methods

Catching bats with an acoustic lure was trialled at 6 broadleaf woodlands over 11 nights. Killarney National Park (3 nights), Glendalough (3 nights) where *M. brandtii* bat has been previously found and Cahercon (1 night) adjacent to a suspected *M. brandtii* roost were surveyed. Two other woodlands Glengarriff (2 nights) and Portumna (1 night) were also surveyed.

At each woodland site, three locations within characteristic foraging habitat, at least 150m apart, were selected. The acoustic lure was used in conjuction with mist nets and harp traps set around the lure. Following the advice of Jon Flanders (*pers. coms.*) the acoustic lure was used at each location for 13 minutes, during which the lure was alternated between 'on' for 3 minutes and 'off' for 2 minutes. This was followed by a period of approximately 45 minutes during which the lure was used at the 2 other sites. Sampling began 45 minutes after sunset and continued for 3 rotations of each location. The calls used were social calls or mixed echolocation and social calls from 4 species : *M. mystacinus*, *M. nattereri*, *Nyctalus leisleri* and *Pipistrellus nathusii*.

Results

In total, 21 bats were caught with significant improvement due to the lure in comparison to capture at location when the lure was not used (χ^2=5.76, p<0.02). During the survey 8 bat species were captured (Figure 11).

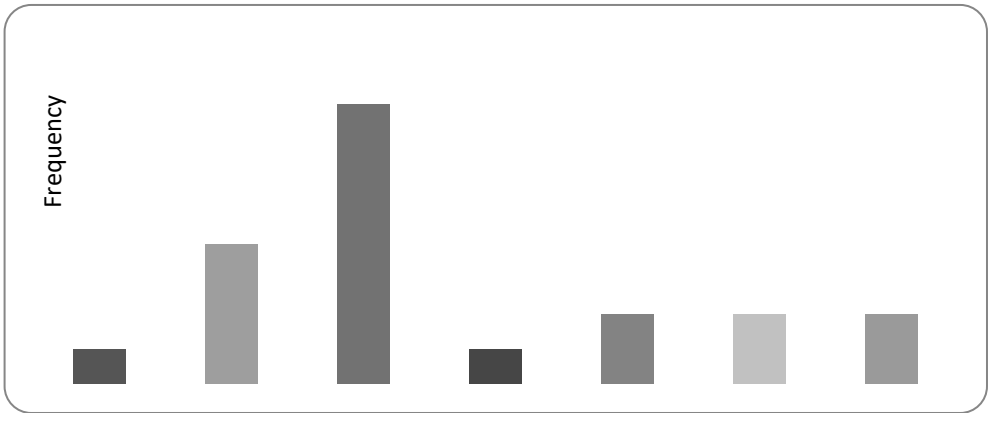


Figure 11: Species of bat captured during trail of acoustic lure.

Summary

Although bat capture was significantly improved by use of the lure, target species (*M. mystacinus* and *M. nattereri*) only represented 3 of the 21 captures. The method appears to be effective for catching

Plecotus auritus. Further trials of the lure technique should be attemped to improve species specific capture. Trials in a greater range of habitats specific to those identified as important to *M. mystacinus* and *M. nattereri* in Ireland (Section 2.2) may, further elucidate the habitat associations identified for these species.

2.6 Trophic ecology of *M. mystacinus* and *M. nattereri* in Ireland

Introduction

With a global distribution, diverse feeding and reproductive strategies and important ecological roles, bats are ideal indicators of environmental stress. All the species of bats in Europe are considered to be insectivorous (Dietz *et al.* 2009); although, carnivory has been recorded in noctule bat (*Nyctalus lasiopterus*). Boyles *et al.* (2011) suggest that bats are amongst the most economically important, non-domesticated animal groups in North America due to their impact on pest arthropod populations.

Both *M. mystacinus and M. nattereri* are relatively rarely recorded in Ireland. We have shown that there are differences in the habitat use (Section 2.1 & 2.2) and landscape associations between the species. To explore further the ecology of the species, we undertook an analysis of the diet and trophic ecology of the species. Previous analysis of *M. nattereri* in Ireland from a maternity roost revealed that large Diptera, such as dung fly belonging to the family Scathophagidae, were the main food source for *M. nattereri* along with Trichoptera, Hymenoptera and Arachnida (Shiel *et al.* 1991). No previous analysis of the diet of *M. mystacinus* has been conducted in Ireland. European populations are known to forage on Diptera and Arachnida (Safi & Kerth 2004).

Analysis of faecal material provides a snap shot of diet. It is unavoidable that a number of fragments cannot be identified. In order to account for this high degree of uncertainty and establish longer terms patterns of trophic patterns we apply analysis of stable isotopes. Stable isotope analysis (SIA) is a means of assessing trophic relationships and longer term assessment of foraging as the isotopic signal within the tissue is developed over a number of weeks/months. The basic principle of this technique is that the isotopic composition of organisms reflects aspects of their diet, such as the main sources of energy and the mean trophic level occupied by each species (Fry 2006; Crawford *et al.* 2008). For example, isotope ratios of carbon ($^{13}C/^{12}C$ expressed as $\delta^{13}C$) vary substantially among primary producers with different photosynthetic pathways (C3 vs C4) and also between aquatic and terrestrial systems (Lajtha & Marshall, 1994). However, $\delta^{13}C$ varies little with each trophic transfer and so can be used to determine the principal sources of carbon and reflect differences in habitat use (Inger *et al.* 2006). Stable nitrogen isotope ratios ($^{15}N/^{14}N$ expressed as $\delta^{15}N$) are robust indicators of trophic position, as proportionally more of the light isotope is lost with each trophic exchange (DeNiro & Epstein, 1978, 1981). Measures of $\delta^{15}N$ can also identify areas affected by anthropogenic inputs of nitrogen, such as grassland fertilisers (Oelbermann & Voroney, 2007).

Methods

Dietary analysis

Droppings were collected from roosts of known species composition; 12 *M. mystacinus* and 8 *M. nattereri*. Insect fragments were dissected from droppings and mounted on slides. These were then classified to family level using the methods of McAney *et al.* (1991). A total of 330 fragments were identified from droppings of *M. nattereri* and 512 from droppings of *M. mystacinus*.

Stable Isotope analysis

A sample of bat wing tissue (n= 48, for both species) from selected roosts with broad geographical spread across the island, collected within the same year and season, were analysed for stable carbon and nitrogen isotope ratios. Tissue samples were grouped within roosts to provide samples of >0.12mg. Each sample was loaded into tin-capsules for the simultaneous determination of stable carbon and nitrogen isotope ratios. Analysis was performed using continuous flow isotope ratio mass spectrometry at the Chronos Centre at Queen's University Belfast. Stable isotope ratios were expressed as $\delta^{15}N$ and $\delta^{13}C$ (McKinney *et al.* 1950).

Results

For both species, Diptera contributed the greatest proportion of the diet (Figure 12). However, there was significant difference in the family composition of this order. Of the fragments identified in the faecal material of *M. nattereri* Muscoidea (superfamily) largely dominated the diet, this superfamily includes the family Scathophagidae (dung-flies). In contrast, the dominant family of Diptera eaten by *M. mystacinus* was Ceratopogonidae, commonly called biting midges. The Levin's niche breadth was calculated for each species (Krebs 1999). The niche breath of *M. mystacinus* was 3.81 c.f. with 4.45 ± 4.25 from published studies in Europe (Safi & Kerth 2004) and 4.91 for *M. nattereri* c.f. with 2.66 ± 0.49 from other studies (Safi & Kerth 2004).

The results of SIA reflected this greater niche breath in *M. nattereri* detected in fragment analysis (Figure 12). The $\delta^{15}N$ range for *M. nattereri* was 10.7 to 13.3 and $\delta^{13}C$ ranged -25.9 to – 24.9 compared to a $\delta^{15}N$ range of 10.0 to 12.4 and a $\delta^{13}C$ range of -26.9 to 25.5 (Figure 13). A commonly utilised characteristic of stable nitrogen isotopes is the predictable increase in $\delta^{15}N$ (usually between 2‰ to 4‰) between trophic level (Kelly 2000; Fry 2006). This suggests that there may be significant trophic variation across the range of bats sampled. However, nitrogen isotope ratios can vary along several other potentially useful gradients (Rubenstein & Hobson 2004) such as soil enrichment due to application of fertilisers or anthropogenic inputs such as sewage outfalls into aquatic systems (Anderson & Cabana 2006). SIA suggested that there was some evidence for niche partitioning between *M. nattereri* and *M. mystacinus* with a trend towards depleted $\delta^{13}C$ ($F=4.271$; $df=1,14$; $P=0.058$) and depleted $\delta^{15}N$ ($F=3.324$; $df= 1,14$; $P=0.09$) in *M. mystacinus.* The $\delta^{13}C$ signature of *M. mystacinus* may reflect a concentration on aquatic sources of carbon, as systems often have depleted $\delta^{13}C$. A trend toward depleted N possible reflects use of habitats with reduced anthropogenic activity such as the mixed woodland areas which tracked *M. mystacinus* foraged in (Section 2.1).

We explored the trophic patterns of both species in response to the habitat available at roosts. The habitat surrounding the roosts used for SIA was delineated (within 5km) and the area covered by pasture, extensive agriculture and woodland (EEA 2000) calculated. These were independently regressed against $\delta^{13}C$ and $\delta^{15}N$ for each species. There was no significant relationship of any land cover variable with the isotopic signal of *M. mystacinus*. For *M. nattereri* carbon enrichment increased significantly with the area of pasture (ß=1.44±0.5; $F=7.0$; $df= 1,7$; $P<0.05$) and nitrogen depletion related to the area of other agricultural land (ß=3.25±1.3; $F=5.9$; $df= 1,7$; $P<0.05$). These patterns reflect the close link between anthropogenically modified habitats and *M. nattereri* at a broad scale supporting previous results (Section 2.1 & 2.2).

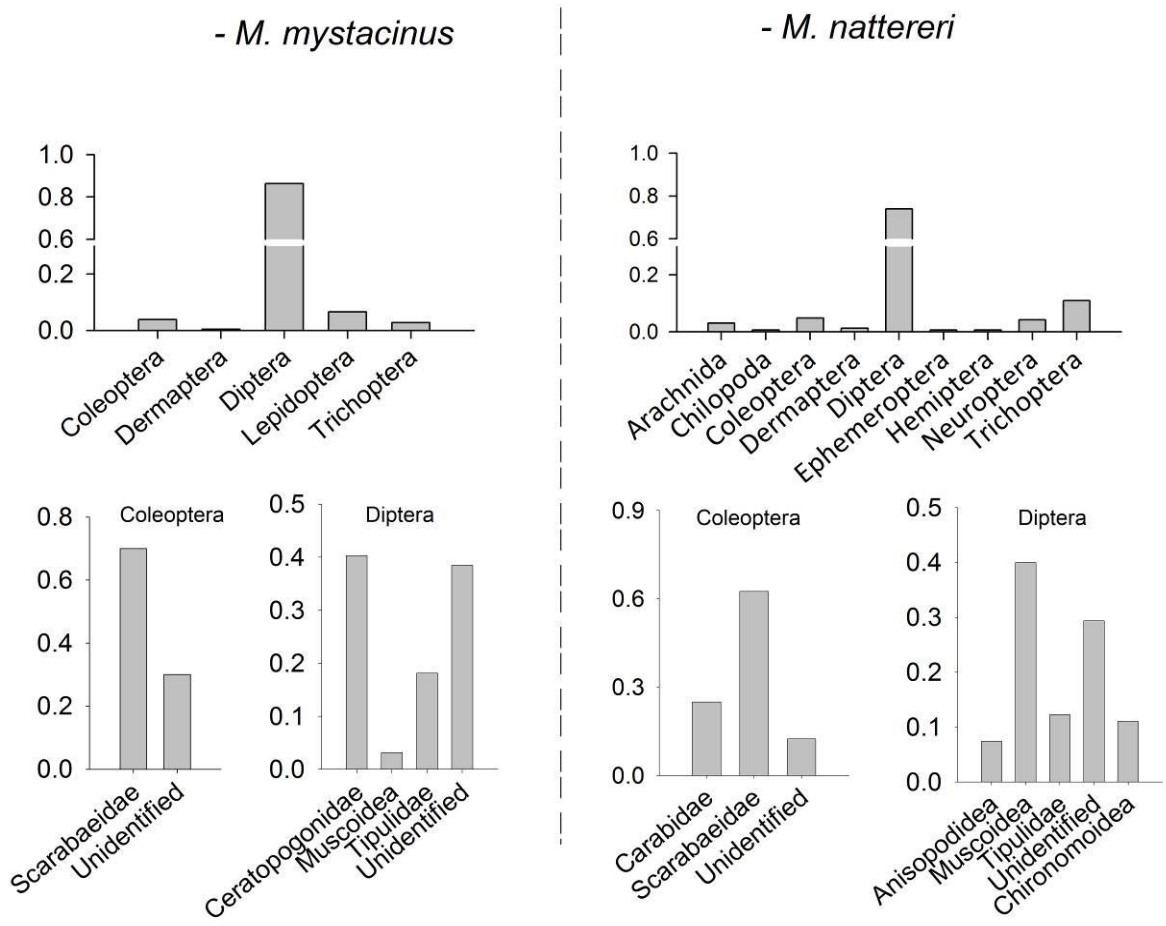


Figure 12: Dietary composition of *M. mystacinus* and *M. nattereri*. The proportion composition of insect fragments identified in faecal material is shown for identified orders. For Coleoptera and Diptera these are further divided into family / superfamily.

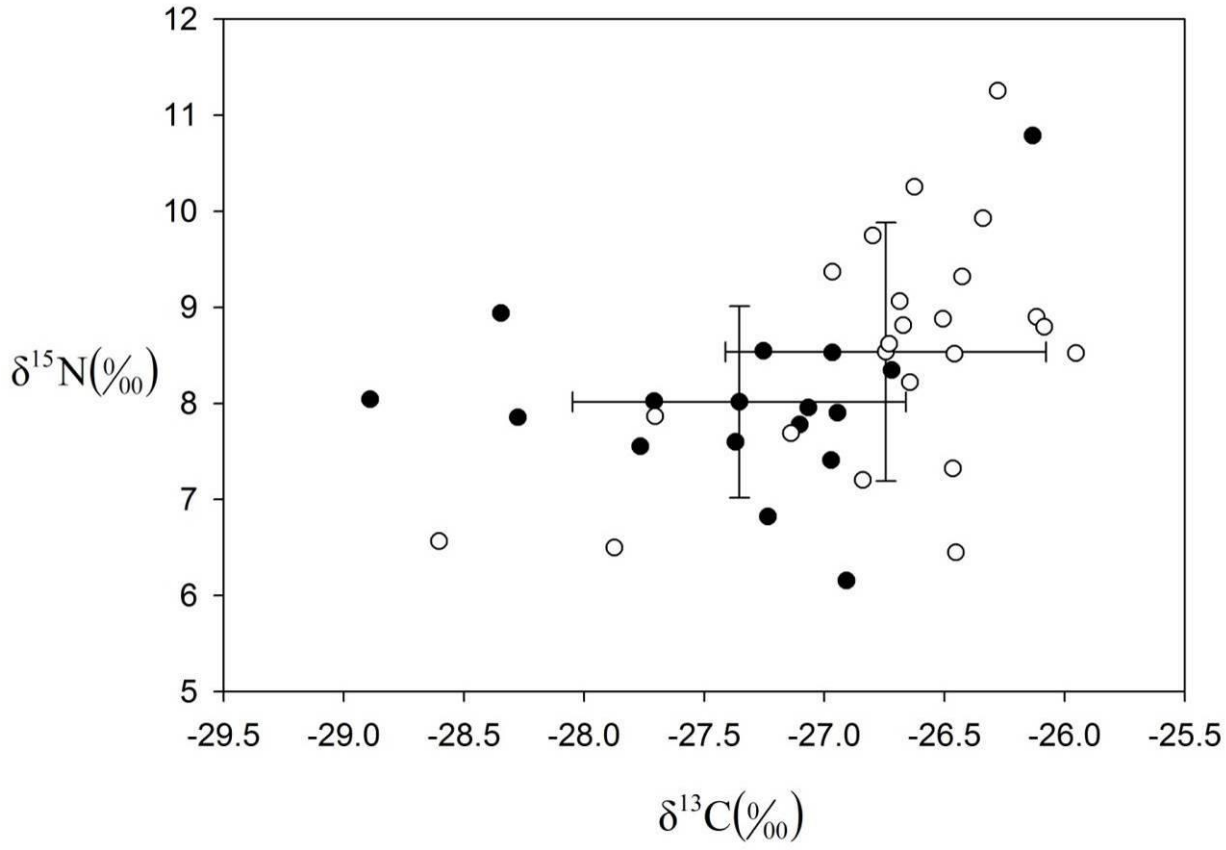


Figure 13: Mean $\delta^{13}C$ and $\delta^{15}N$ ratios (±SD) for *M. nattereri* (open circles) and *M. mystacinus* (filled circles). The values for each individual bat for both species are also shown. These values have been corrected appropriately for fractionation.

Summary

The results of dietary analysis support the findings of the previous ecological studies carried out in Sections 2.1. It appears that *M. nattereri* diet is closely linked to agricultural processes, both in terms of insect families consumed and the trophic relationship detected by SIA. In contrast to the dietary niche reviewed by Safi & Kerth (2004), we find an increased niche breath for *M. nattereri*. This may reflect the unique Irish landscape which in comparison to Europe has a reduced grain size with small field sizes and a high density of linear boundary features (Mitchell & Ryan 2001).

M. mystacinus appeared more reliant on species associated with aquatic habitats having a depleted $\delta^{13}C$ in comparison to *M. nattereri* and foraging on insects of the family Ceratopogonidae which commonly have aquatic larval stages (Chinery 1995). Radio-tracking of *M. mystacinus* (Section 2.1) revealed that foraging ranges were concentrated in a small area relatively close to the roosts, in comparison to *M. nattereri* which ranged much more widely. The reliance on a relatively small foraging area may explain why trophic patterns were not found to be related to the general habitat around roosts.

Although both *M. mystacinus and M. nattereri* remain relatively rarely recorded in Ireland the reasons for their rarity are not shared; habitat use, roosting behaviour and now the results of dietary and trophic analysis support the view that these species have contrasting conservation requirements in Ireland.

2.7 A method to identify echolocation calls of *Myotis* bats.

Introduction

Bat species use ultrasonic echolocation to orientate in their environment. In many cases, these are species specific calls that can be used to easily identify a species. However, identification of a number of species using recorded echolocation calls can be difficult, due mainly to the overlap between species and plasticity of echolocation calls within species (Kalko & Schintzler 1993; Parsons & Jones 2000). Adaption of echolocation calls due to immediate habitat structure or foraging can introduce problems when identifying species (Broaders *et al.* 2004). Depending on environmental conditions a bat species can modify calls in the immediate vicinity, such as reduction of call intensity (Brinkløv *et al.* 2010) or increase in minimum frequency and call rate when foraging in more cluttered environments (Kalko & Schnitzler 1993).

Multivariate analyses of call parameters, such as linear Discriminant Function Analysis (DFA), and Artificial Neural Networks (ANN), are used to identify unknown echolocation calls using spectral and temporal parameters from recorded calls of known species. These techniques have been shown to achieve high rates of correct species identification (Zingg 1990; Obrist 1995; Vaughan *et al.* 1997; Parsons & Jones 2000; Papadatou *et al.* 2008). High rates of misclassification, however, particularly for the *Myotis* spp. are common. (Vaughan *et al.* 1997)

In Ireland and Britain *Myotis* spp. represent 30 – 40% of the resident bat species (Harris & Yalden 2008; Marnell *et al.* 2009). Monitoring schemes often rely on bat echolocation detector surveys (Ahlén & Baagøe 1999), whereby the species are identified based on their echolocation calls. Therefore, the high degree of uncertainty associated with classification of *Myotis* spp. remains a significant issue for bat conservation and ecological studies. Ultrasonic calls of *Myotis* bats are often unresolved to species when calls cannot be confidently assigned to a single species allowing only generalisations of habitat use for the genus to be made (Hayes 1997; Russo & Jones 2003; Lundy & Montgomery 2010b).

Morphometric analyses can be applied to identify species (Neto *et al.* 2006) and explore important shape variations within species (Silvia 2003; Tracey *et al.* 2006). Elliptic Fourier Descriptors (EFDs), proposed by Kuhl & Giardina (1982), can delineate any type of shape with a closed two-dimensional contour and have been applied effectively to the evaluation of various biological shapes (Bierbaum & Ferson 1986; Innes & Bates 1999; Neto *et al.* 2006). Such analyses may allow more information concerning shape to be captured than curve fitting, and lead to parameterisation of asymmetries and a description of shape dimensional ratios which are lost through curve fitting methods. Given the potential difficulties in classification of echolocation calls of *Myotis* spp. from call parameters alone, the present study concerns the potential for analysis of shape to classify echolocation calls of bats. This is the first attempt to classify echolocation calls of bat species using morphometrics.

Method

Recordings of emerging bats were made from roosts (Section 1.1) occupied by a single identified *Myotis* species. All roosts of *M. mystacinus* in the current study were occupied houses, a location considered typical of this species in Ireland (O'Sullivan 1994). Roosts from which *M. nattereri* were recorded ranged from crevices in stone walls and occupied houses to disused churches. *M. daubentonii* roosts consisted of disused stone buildings and a single active church. A balanced sample of individual bat calls was taken from roosts of each species. A single echolocation call was extracted from a call sequence for an individual bat during emergence from a roost (Figure 14). In total, 113 individual calls were identified; *M. mystacinus* – 33 calls from 11 roosts, 3 individuals from each roost; *M. nattereri* – 50 calls from 10 roosts, 5 individuals from each roost and *M. daubentonii* – 30 calls from 3 roosts, 10 individuals from each. It was not possible to collect equal samples of calls for each species due to differences in the numbers of bats which occupy roosts. Recordings were made during July and

August 2009, starting as bats began to emerge from roosts and continuing until all emergence was complete. Calls were recorded using a Peterson D240X time expansion (x10) bat detector using an Edirol R-09 digital audio recorder from a distance within 15 m of a roost exit.

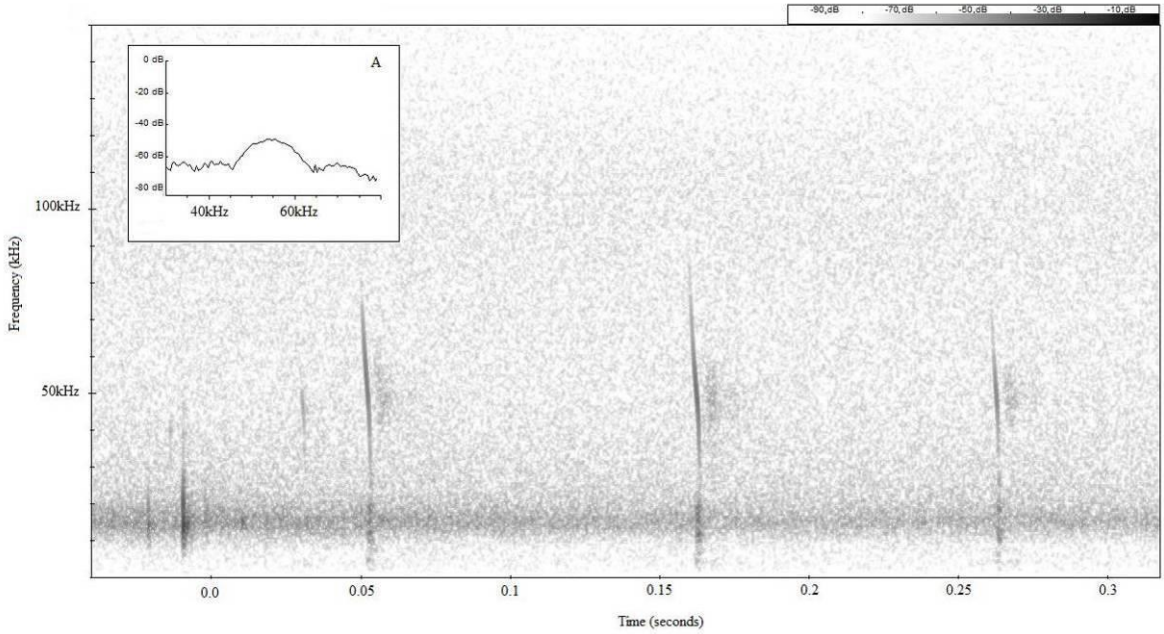


Figure 14: A spectrogram of the frequency modulating echolocation calls of a whiskered bat, *Myotis mystacinus*. The spectrogram shows 3 discrete echolocation calls of a call sequence. Insert A. Shows the power spectrum of the call.

Recorded calls were examined in BatSound Pro version 3, at a sampling rate of 44 Khz, 16 bit precision. A single call from each pass with the highest signal-to-noise ratio without being overloaded from each sequence was obtained by applying a Hanning window spectrogram, with a fast Fourier transform (FFT) of 1024. A lower threshold of 10 dB below the maximum intensity (dB) was applied to a monochrome spectrogram. Intensity levels were obtained from power spectrum analysis with an FFT of size 1024 and the maximum frequency measured (Vaughan *et al.* 1997). The maximum frequency recorded in a call of *Myotis* spp. was shown by Parsons and Jones (2000) to have the single greatest classification ability, when using DFA to classify *Myotis* to species level. Only ultrasound between 30 kHz and 80 kHz was retained, to limit disturbance from low frequency background noise and to ensure that morphology of the most commonly recorded call portions were those analysed. Parallel vertical bars were superimposed at the beginning and end of the call, and a reference scale box of 10 KHz added (Figure 15). These are required to control for effects of image scaling and rotation in later image processing.

Images were transferred to SHAPE v1.03 (Iwata & Ukai 2002). Four coefficients of the first 20 EFD harmonics were extracted and normalised by the radius of the first harmonic. Average harmonic coefficients for calls recorded at each roost were calculated. A Mahalanobis typicality model (Lanitis *et al.* 1995) was constructed independently for each harmonic coefficient for each species. All Mahalanobis typicality models were applied to each call, providing twelve scores of morphology. These scores were used within stepwise DFA, with selection based on Wilks' λ, to identify the optimum combination and weighting of morphological descriptors to classify species correctly. The typicality values of each harmonic for each species were examined with a quantile-quantile plot to ensure that the underlying distribution did not deviate significantly from normal (Fernandez 2002). A second stepwise DFA was applied including maximum frequency of each call in addition to morphology parameters. Fisher's linear discriminant coefficients were obtained for each species extracted for both DFAs. The classification success of DFAs was tested using correct classification rates

based on the data used to construct the DFA and set of calls excluded from morphological typicality modelling. The test set comprised one call from each roost which was not used in construction of the model (N=24). The DFA equation outputs were averaged for each roost to test the correct classification rate of roosts.

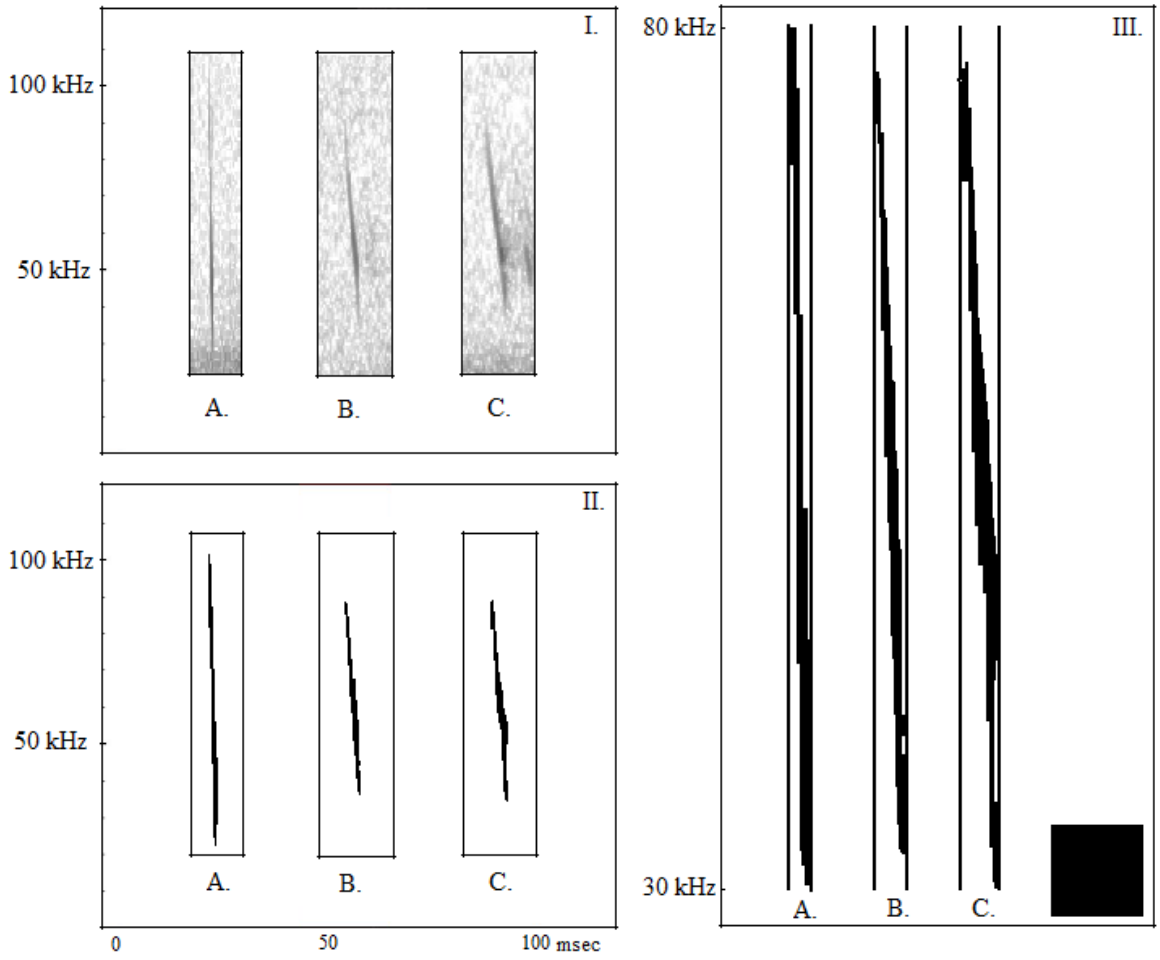


Figure 15: Steps in call processing to provided images for morphological analysis. I. Spectrograms of a call extracted from emergence call sequences of: A – *Myotis nattereri*, B – *Myotis mystacinus* and C – *Myotis daubentonii*. II. A filtered spectrogram of 10dB below maximum intensity of the call. III. Extracted calls with reference bars and scale box (10 kHz x 10 kHz).

Results

The average maximum frequency (± standard error) for each species was: *M. daubentonii* – 83.9 ± 7.8 kHz (N=30); *M. nattereri* – 114.1 ± 5.5 kHz (N=50); *M. mystacinus* 97.8 ± 5.35 kHz (N=33). The typical morphology model of *M. mystacinus* provided the highest baseline typicality values for calls of all species tested (Table 7). *M. daubentonii* and *M. mystacinus* calls had an average typicality across the four coefficients of 88% and 86% with their own typical outline, respectively. *M. nattereri* calls had morphologies with highest typicality for using the *M. mystacinus* typical outline model, (77%) compared to 21% typical for the *M. nattereri* model. However, the highest value of another species using the *M. nattereri* typical morphology model was only 8% for calls of *M. myscatinus*.

Seven of the twelve morphological descriptors were selected by stepwise DFA as having the greatest discriminating capability between species (Table 8). The DFA selected typicalities of calls specific to

all species. The classification success was 100% for both *M. daubentonii* and *M. mystacinus* and 78% for *M. nattereri* using all calls and the seven selected parameters (Table 9). Correct classification based on the test set of calls was 100% for *M. daubentonii* and 80% and 91% for *M. nattereri* and *M. mystacinus* respectively. Correct classification rates of test calls with the inclusion of maximum frequency in the stepwise DFA, was 100% in *M. mystacinus* and *M. daubentonii* and 90% in *M. nattereri* (Table 10). The DFA, including maximum frequency, did not include morphological descriptors specific to *M. nattereri*. Clustering of species calls on the DFA plot is clearly evident (Figure 16). This plot identifies calls of *M. nattereri* which were potentially misclassified as *M. mystacinus*. Using either the DFA classification equation, including or excluding maximum frequency, there was 100% correct classification of roosts when an averaged output of the DFA equation was calculated for all calls from a single roost.

Table 7: Mahalanobis typicality matrix. Average typicalities across for the four coefficients of each harmonic are shown for each species when applied to its own species and the Mahalanobis typicality model of other species.

	M. daubentoni	*M. nattereri*	*M. mystacinus*
M. daubentoni	88	19	22
M. nattereri	5	21	8
M. mystacinus	44	77	86

Table 8: Parameters selected using Wilks' λ from two stepwise discriminant function analysis (DFA) to correctly classify calls of each species using twelve morphology typicalities calculated for each call. Lower values of Wilks' λ, indicate the more important variables for discriminating between calls of each species.

A. Morphology				
		Classification function coefficients		
Variable	**Wilks' λ**	***M. daubentoni***	***M. mystacinus***	***M. nattereri***
M. nattereri Harmonic 3	0.091	-0.103	-0.027	0.005
M. mystacinus Harmonic 1	0.077	-0.022	0.016	-0.017
M. mystacinus Harmonic 2	0.086	0.078	0.172	0.181
M. mystacinus Harmonic 3	0.071	0.074	0.228	0.176
M. mystacinus Harmonic 4	0.075	0.099	0.235	0.238
M. daubentonii Harmonic 2	0.104	0.198	0.002	-0.015
M. daubentonii Harmonic 3	0.169	0.266	-0.089	-0.111
Constant		-29.4	-29.6	-24.3
B. Morphology & maximum frequency				
Maximum frequency	0.089	3.128	3.967	4.587
M. mystacinus Harmonic 1	0.033	0.047	0.116	0.077
M. mystacinus Harmonic 2	0.032	0.154	0.272	0.344
M. mystacinus Harmonic 3	0.036	0.089	0.332	0.291
M. daubentonii Harmonic 2	0.034	0.209	0.024	0.015
M. daubentonii Harmonic 3	0.046	0.114	-0.179	-0.181
M. daubentonii Harmonic 4	0.029	0.078	-0.156	-0.210
Constant		-148.8	-216.3	-284.6

Table 9: Results of discriminant analyses using morphological typicality values. Values in parenthesise are the ratio of test calls classified and values in italics are the percentage of roosts classified to each group.

	True group (%)		
Classified as	***M. daubentoni***	***M. nattereri***	***M. mystacinus***
M. daubentoni	100; (3/3); *100*	0	0
M. nattereri	0	79.6; (8/10); *100*	21.4 (2/10)
M. mystacinus	0	0 (1/11)	100; (10/11); *100*

Table 10: Results of discriminant analyses using both morphological typicality values and maximum frequency of the each call. Values in parenthesis are the ratio of test calls classified and values in italics are the percentage of roosts classified to each group.

	True group (%)		
Classified as	***M. daubentoni***	***M. nattereri***	***M. mystacinus***
M. daubentoni	100; (3/3); *100*	0	0
M. nattereri	0	96.7; (9/10); *100*	4.3 (1/10)
M. mystacinus	0	0	100; (11/11); *100*

Discussion

The present study demonstrates how morphometric analysis can be used to classify echolocation calls of *Myotis* bats to species level. Given that *Myotis* spp. are considered to have morphologically similar calls the potential for application to other bat species groups is promising. Morphometric analysis is widely used to describe shape independent of size and orientation (Zelditch *et al.* 2004). In the present study, calls are scaled by intensity and are placed in the context of a standard frequency and time window. It is important that calls are not rescaled and orientation changed during morphological analysis as these relate to frequency range, call duration and the frequency time course. These aspects are maintained by inclusion of temporal and frequency baselines and fixed frequency time scales. In doing so, the EFDs and morphological typicalities created, capture aspects of call duration, frequency ranges, energy distribution and aspects of the frequencies time course which independent measurements and curve fitting do not fully describe.

The morphometric analyses of echolocation calls also demonstrate differing levels of call plasticity within species. A greater level of call variation in *M. nattereri* than *M. mystacinus* and *M. daubentonii*, results in a greater difficulty in classifying calls. This may arise from the having a broader range of roost types and, generally, a wider foraging niche (Shiel *et al.* 1991; Smith & Racey 2008; Dietz *et al.* 2009). However, it may also result as a direct response to relatively larger sample size and the increased environmental variability encountered (Rydell 1990; Kalko & Schnitzler 1993; Jones, 1995). *M. nattereri* produced calls which were most difficult to identify by call morphology alone. The inclusion of the maximum frequency of the call significantly improved rates of the correct classification for *M. nattereri*. The increased classification powers of this parameter are reduced when EDFs are examined alone as only frequencies up from 30 kHz to 80 kHz are examined, to reduce the impact of frequency attenuation and disturbance form background noise.

Both *M. mystacinus* and *M. nattereri* forage by gleaning prey off surfaces and catching prey in flight (Dietz *et al.* 2009) whereas *M. daubentonii* typically forages over water collecting prey from the water surface (Jones & Rayner 1988; Kalko & Schnitzler 1989). Of the three species examined here, the typical morphology model created for *M. mystacinus* gave the highest baseline for calls of other species, suggesting that the typical morphology of *M. mystacinus* described is contained within the morphological parameters of other species. Therefore, it may be that it is the extreme elements of other calls which lead to high rates of successful classification, particularly the morphology of *M. daubentonii* calls.

Summary

The present analyses demonstrate that there is a sufficient level of conservation of call morphology within species to allow reliable species identification. In the present study all calls were collected from bats emerging from roosts. This limits the environmentally induced variability within the recorded calls but the effect of environmental conditions, such as clutter, should be considered in future applications of this technique and other species classification studies (Broders *et al.* 2004). Collection of

calls from a greater range of environments is often hindered by difficulties in accurately identifying calls to species level recorded away from known roosts or from release of captive bats. Further novel techniques, therefore, may be required to fully characterise the call shape of bats under different environmental conditions (Hiryu *et al.* 2007). It remains a priority that classification and identification of bat species by echolocation calls is explored to develop effective monitoring tools to establish conservation status.

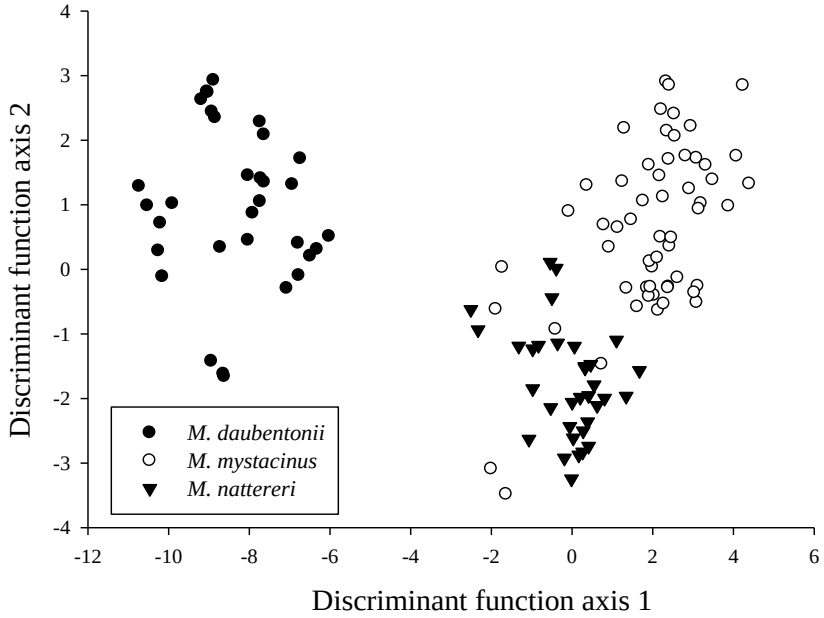


Figure 16: Clustering of echolocation calls of three *Myotis* bat species following discriminant function analysis (DFA) conducted on typical call morphologies and maximum frequency of call (Table 8; B).

3. CONSERVATION GENETICS AND POPULATION STRUCTURE

3.1 Phylogeographic origins of Irish populations of *M. mystacinus* and *M. nattereri*

Background

The current distribution of Europe's flora and fauna is the result of its geological history, in particular the succession of ice ages during the Pleistocene (1.8 million - 11.5 thousand years before present). During this time ice caps at the poles expanded over much of Eurasia and North America. These ice sheets expanded and contracted cyclically recurring every 100,000 years, to the current interglacial period, beginning around 10,000 yrs BP. Throughout the Last Glacial Maximum (LGM) approximately 20,000yrs BP, sea levels were lower than their current position, and northern Europe was largely covered in ice and permafrost, forcing species to retreat into refugia (Hewitt, 2000).

The origin of the Irish terrestrial fauna and flora has been a topic of considerable debate (Yalden 1999). Like the rest of northern Europe, most of Ireland was covered by ice at the height of the last LGM. Ireland is species poor compared to Britain and continental Europe, which has been attributed to the 'steeple chase' effect, as the retreating ice cut off Ireland from Britain and continental Europe around 7,500yrs BP (Mitchell & Ryan 1997). However, geophysical models simulating the land mass response to glaciohydro-isostatic adjustment (Brook *et al*. 2007, 2008), indicate that there is no evidence for land-bridges between Ireland and Britain during the Holocene. Biogeographic studies of species such as the Kerry slug (*Geomalacus maculosus*) and the strawberry tree (*Arbutus unedo*) (Corbet 1961; Vincent 1990), suggest a possible link between Ireland and south-western Europe. Additionally, several phylogeographic studies of Irish flora and fauna, have demonstrated that Irish populations are genetically distinct and diverse, in relation to their European counterparts, including published work on the stoat (Martinkova *et al.* 2007) and common frog (Teacher *et al.* 2009), but also several unpublished works, including that of the Leisler's bat (*Nyctalus leisleri*). Boston *et al.* (2007) identify two distinct and diverse mtDNA haplotypic lineages among Irish *N. leisleri* populations, likely to have diverged in separate refugia during the LGM. These results suggested two colonisation events, and raised the possibility of the existence of novel refugia, now submersed, along the western fringes of Europe during the LGM. This was the first study to examine the mtDNA phylogeographic signal within an Irish bat population. Since then, Dool *et al.* (2011) examining the mtDNA phylogeography of the lesser horseshoe bat (*Rhinolophus hipposideros*) across Europe, suggested a single origin of European recolonisation most likely from the Balkan Peninsula, and observed low diversity in Britain and Ireland. These two species tell two different stories about recolonisation of Ireland, and may reflect their contrasting life histories, yet to fully understand the fate of these volant mammals during the LGM and recolonisation routes to Ireland, further species need to be studied.

Aims

To determine the phylogeographic relationships and origin(s) of populations of *M. mystacinus and M. nattereri* in Ireland

Methods

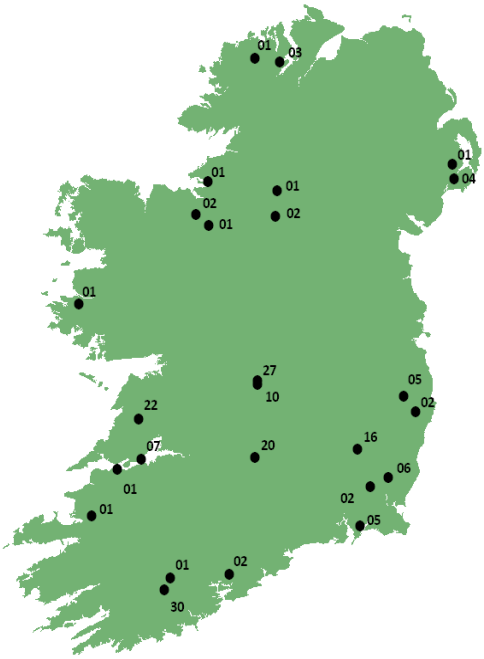
To examine the genetic status of these *Myotis* species it was necessary to collect genetic material from across Ireland. Wing-biopsy tissue samples were taken from bats caught in 2008 and 2009, under licence from the National Parks and Wildlife Service (Licence No.74 C/2008) and the Northern Ireland Environment Agency (Licence No. TSA/12/08) from nursery colonies and also swarming sites and from bats caught during woodland catches where possible. Information on nursery colonies was provided by Bat Conservation Ireland. All sites were visited, and the presence of a roost determined.

Wing tissue was stored in 90% ethanol prior to use in genetic analysis. In addition to the samples collected in Ireland, samples were donated from a number of collaborators in Europe. Details on the number of sites and samples taken are given below:

***M. mystacinus*:** A total of 171 samples from 22 sites across Ireland were obtained (Figure 17a). 18 samples were also obtained from Greece, along with 2 samples from Albania, 22 samples from north-west France, 10 samples from southern France, 10 samples from Germany and 10 samples from Britain (Figure 14).

***M. nattereri*:** A total of 576 samples were obtained (1) 323 specimens representing 19 nursery sites, and three swarming sights throughout Ireland, plus a single dead specimen (Figure 17b); (2) 40 specimens from 5 locations in Britain; 5 from three sites in Isle of Man, 20 specimens from two locations in Germany; 20 specimens from two sites in France, 20 specimens from two sites in Italy, plus single samples from sites in Switzerland, Turkey, Greece, and China (Figure 18).

a.



b.

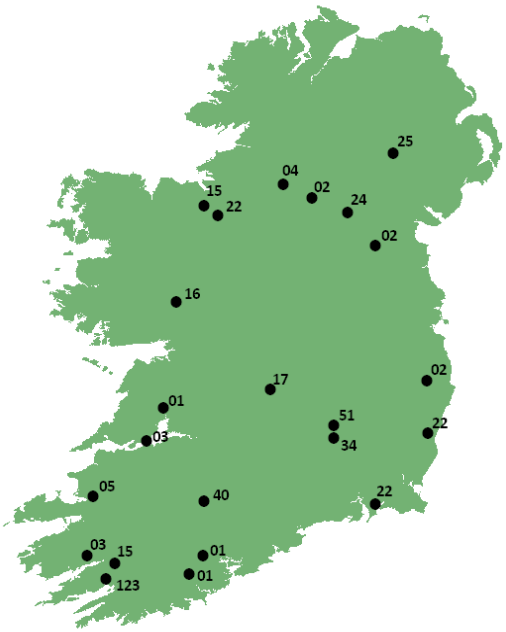


Figure 17: Locations and numbers of genetic tissue samples collected for a) *M. mystacinus* and b) *M. nattereri* in Ireland over the two field seasons 2008 and 2009.

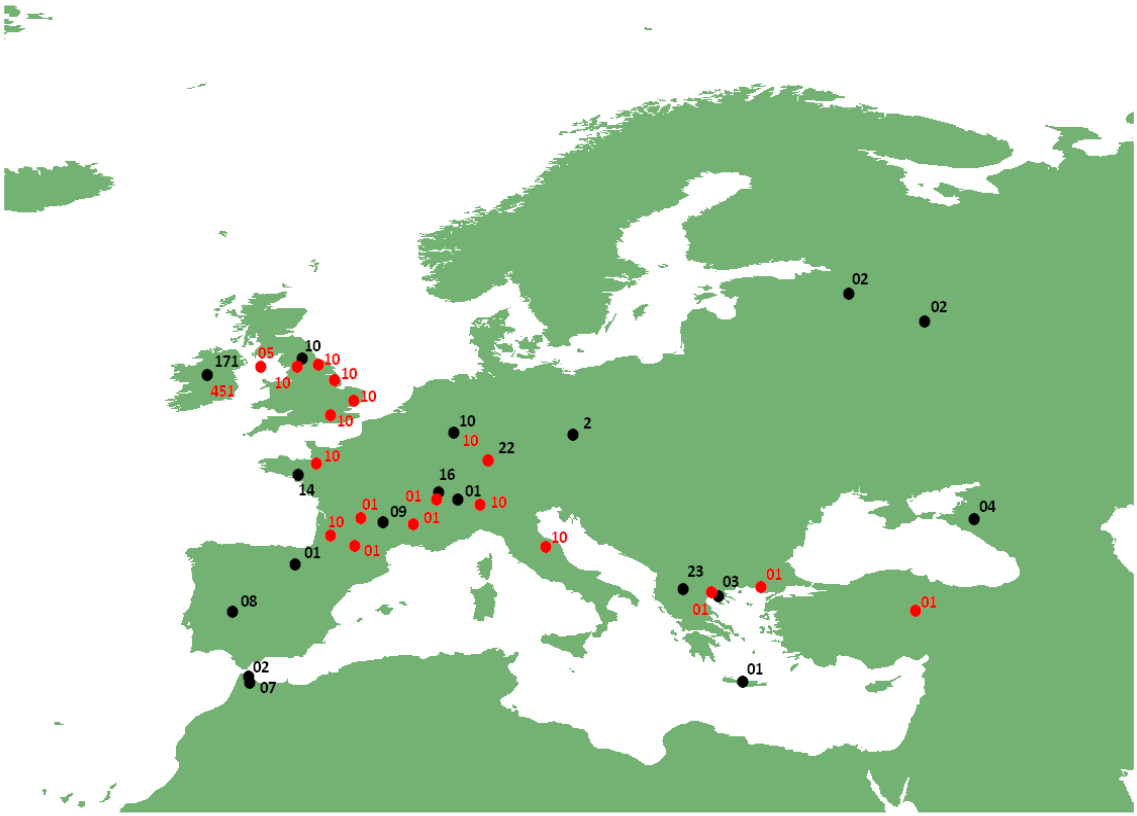


Figure 18: Map of *M. mystacinus* (black) and *M. nattereri* (red) samples/sequences from across Europe.

Sequencing and Data analysis

Genomic DNA was extracted from biopsy tissue samples were extracted using 'Qiagen DNeasy Blood and Tissue' kits. The mitochondrial *Dloop* (HV II) region was amplified using PCR primers L16517 (Fumagalli *et al.* 1996), and sH651 (Castella *et al.* 2001). The mitochondrial *Cytb* region was amplified primers R3 and F3b (Puechmaille, pers com.) for *M. mystacinus*; and primers Molcit-F and MVZ-16 (Smith & Patton 1993) for *M. nattereri*. *M. mystacinus* samples were sequenced at the Macrogen facility and *M. nattereri* sequenced using the ABI 3730xl DNA analyser at QUB. Sequences were aligned, quality checked and used to produce a network displaying the genetic relationships among haplotypes using the program Network 4.510. Summary descriptive statistics for *Dloop* mtDNA for each species and sample locality were calculated using the program ARLEQUIN 3.5 (Excoffier & Lischer, 2010). Analysis of Molecular Variance Analysis (AMOVA) was conducted in ARLEQUIN 3.5 (Excoffier & Lischer, 2010) to examine the proportion of variation within sites, between sites and between countries. Demographic history was examined using mismatch distributions (Slatkin & Hudson 1991) in ARLEQUIN 3.5 (Excoffier & Lischer 2010). Mismatch distribution relies on the assumption that in a scenario of neutral evolution, fluctuations in population size leave distinct signatures in the data when compared with populations of a constant size. The presence of such signatures can be tested by comparing observed and expected distributions of the number of nucleotide differences between each pair of haplotypes within samples. The goodness of fit of both the demographic and spatial population expansion models were investigated by comparing the sum of squared deviations between the observed (our data) and the estimated mismatch distributions. The peak of mismatch distribution provides an estimate of tau (τ), the starting time of the expansion in units of $1/(2\mu)$ generations. From τ, it is possible to estimate the time (t) since the most recent expansion using the equation $\tau = 2\mu t$ (Rogers & Harpending 1992), where μ is the mutation rate per locus (i.e. the product of the mutation rate per site (λ) and the sequence length). A mutation rate of 20% per million years was applied following Petit *et al.* (1999).

Results/Discussion

Myotis mystacinus

In both Ireland and Britain mitochondrial diversity in *M. mystacinus* nursery roosts is low compared to those in France, Germany, Switzerland and Greece (Table 11), despite the smaller sample sizes. This suggests either a smaller overall population size in Ireland, or that they may have experienced a population bottleneck in the past. The AMOVA demonstrated that the majority of variation in *M. mystacinus* was observed within sites (40.9%), then among countries (31.9%), and then between sites within countries (27.2%).

Table 11: Diversity statistics, including, the number of samples (*n*), number of haplotypes (*nh*), number of polymorphic sites (Poly. Sites), haplotypic diversity (*h* +SD), mean number of pairwise difference between sequences and nucleotide diversity (π + SD).

Location	*N*	*nh*	Poly. sites	*h* +SD	Mean no. of pairwise	π + SD
Ireland	171	8	7	0.4349 +/- 0.0456	0.548469 +/- 0.454137	0.001966 +/- 0.001801
Britain	10	3	2	0.3778 +/- 0.1813	0.555556 +/- 0.494259	0.001991 +/- 0.002003
North western France	14	4	4	0.7473 +/- 0.0659	1.142857 +/- 0.786394	0.004096 +/- 0.003164
Southern France	9	4	3	0.7500 +/- 0.1121	0.944444 +/- 0.710412	0.003385 +/- 0.002889
Switzerland	17	6	5	0.5882 +/- 0.1348	0.691176 +/- 0.550672	0.002477 +/- 0.002209
Western Germany	8	6	8	0.8929 +/- 0.1113	3.464286 +/- 1.974020	0.012417 +/- 0.008060
Southern Germany	21	7	9	0.7810 +/- 0.0726	2.247619 +/- 1.287979	0.008056 +/- 0.005153
Czech Republic	2	2	7	1.0000 +/- 0.5000	7.000000 +/- 5.291503	0.025090 +/- 0.026822
Agio Germanos, Greece	23	7	10	0.8024 +/- 0.0619	4.146245 +/- 2.140401	0.014861 +/- 0.008556
Rendina, Greece	3	2	2	0.6667 +/- 0.3143	1.333333 +/- 1.098339	0.004779 +/- 0.004910
Krasnodar, Russia	4	2	1	0.5000 +/- 0.2652	0.500000 +/- 0.519115	0.001792 +/- 0.002222
Novgorod, Russia	2	2	1	1.0000 +/- 0.5000	1.000000 +/- 1.000000	0.003584 +/- 0.005069
Tver, Russia	2	2	1	1.0000 +/- 0.5000	1.000000 +/- 1.000000	0.003584 +/- 0.005069

A total of 36 *Dloop* haplotypes were identified in Europe. Eight of these haplotypes occur in Ireland, three being unique to Ireland. The distribution of the *Dloop* haplotypes is shown in Figure 19. In total 21 *Cytb* haplotypes were identified, two in Ireland, only one of which was unique. A median joining network was constructed from 252 individuals for *Dloop* and *Cytb* are shown in Figure 20 and Figure 21. Both mtDNA networks suggest a west European and east European split in *M. mystacinus*. Haplotypes on either side of this split are not shared between regions, except Germany which acts as a meeting zone between the two, explaining the high diversity found there.

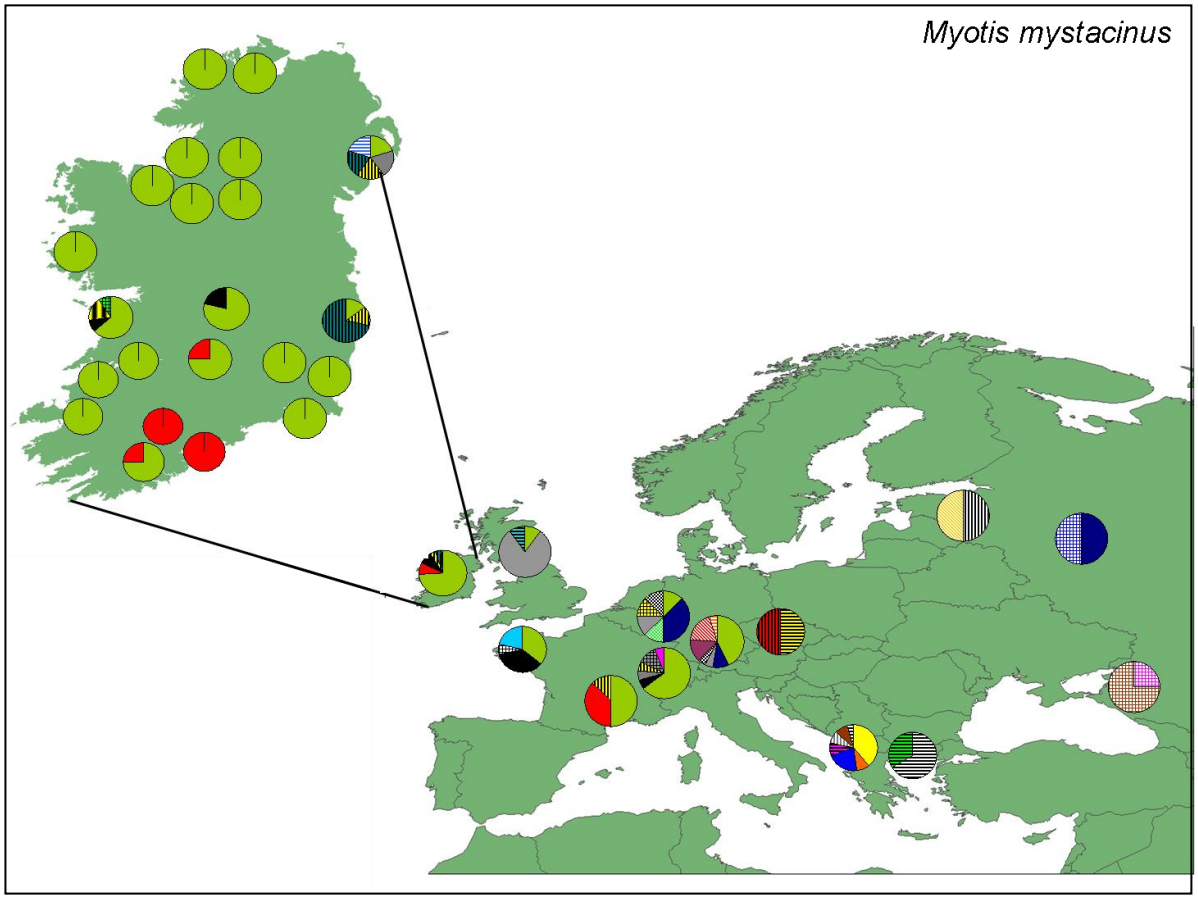


Figure 19: The distribution of Dloop the 36 haplotypes identified in *M. mystacinus* in Europe

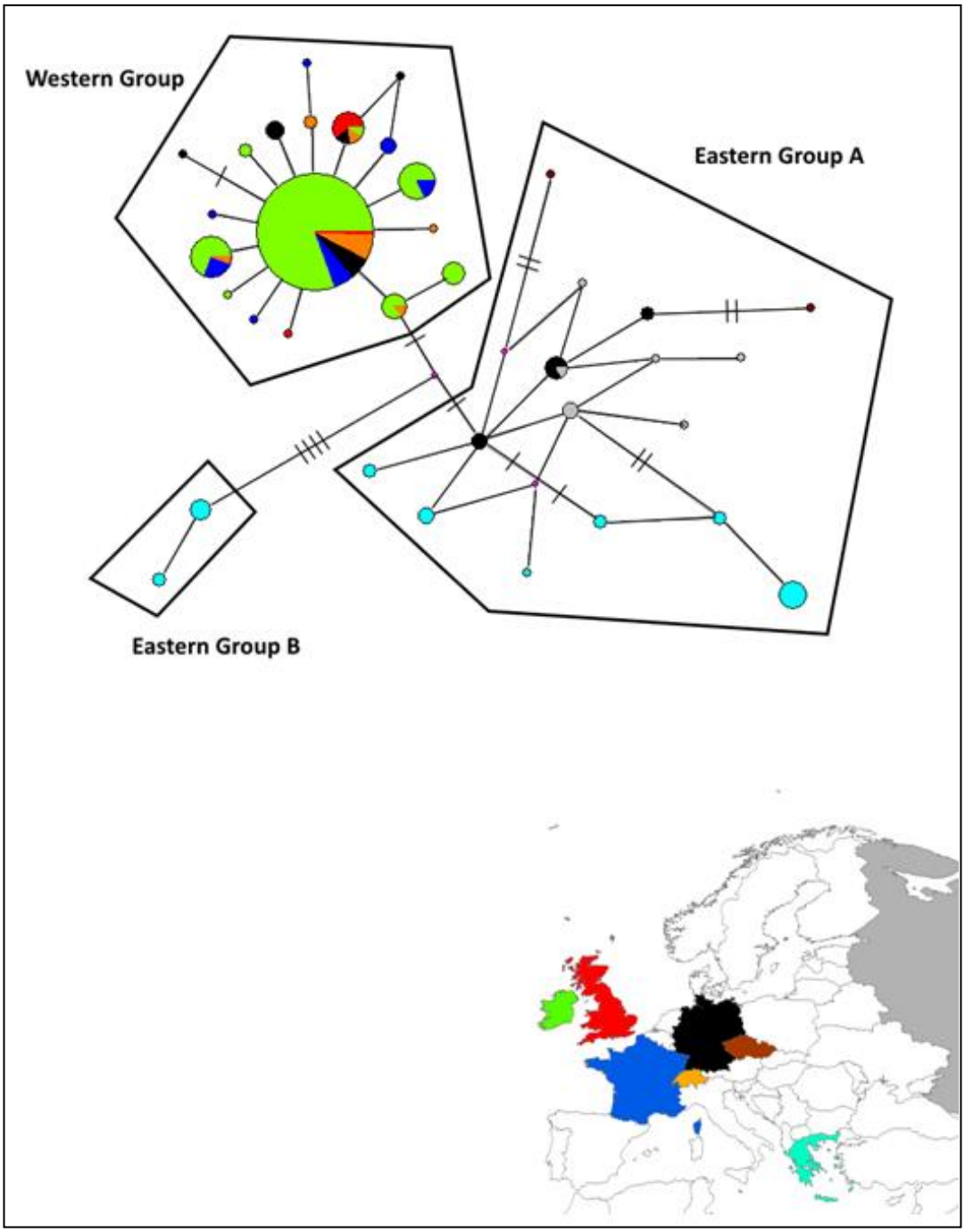


Figure 20: Median joining network showing 36 haplotypes for 279 bp of the hyper variable domain of the control region (D-loop) for *M. mystacinus*. Haplotypes are coloured based on the countries where they occur (see key on bottom right). Single step-wise mutations between haplotypes that are greater than one are marked by the cross-lines. Missing haplotypes are coloured pink.

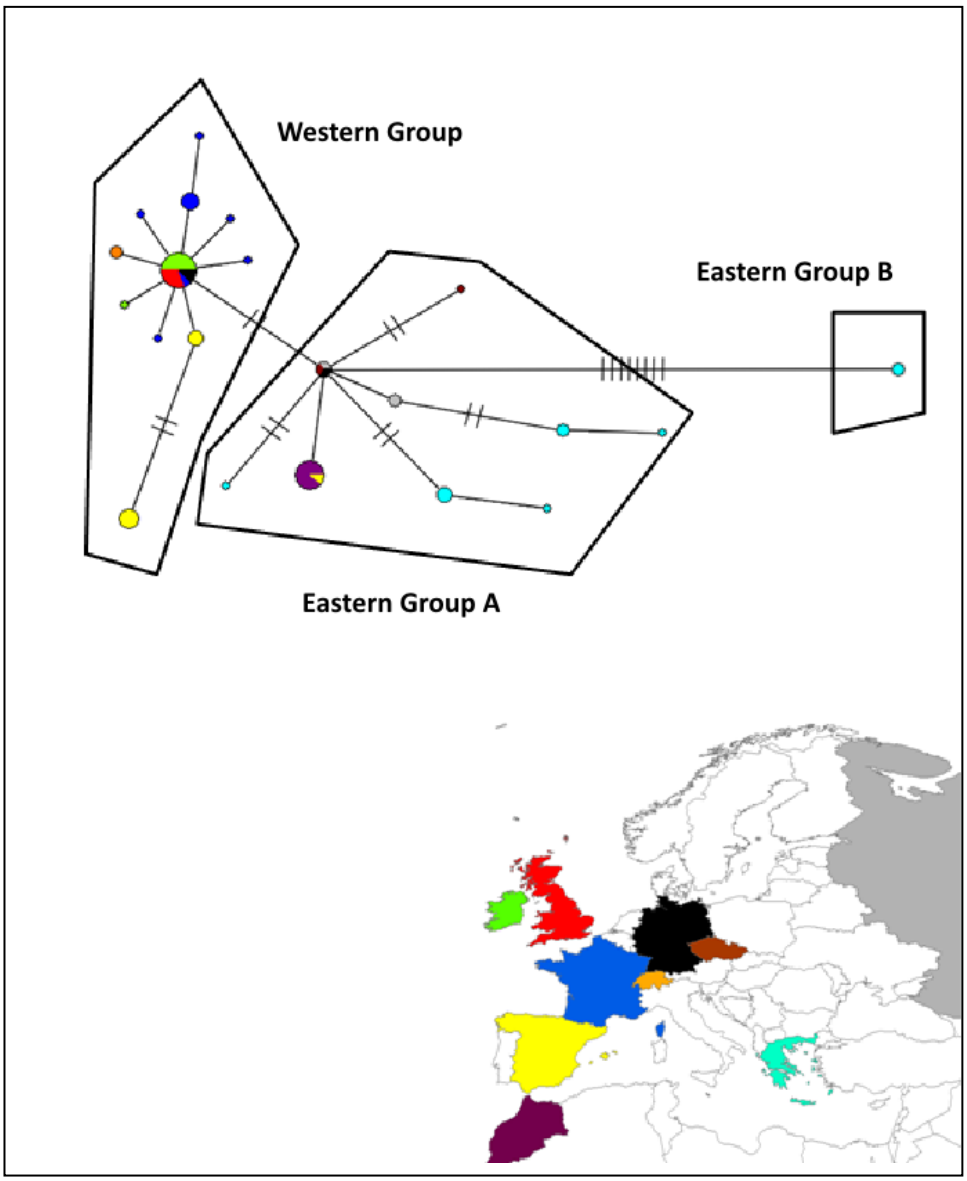


Figure 21: Median joining network showing 19 haplotypes for the 729 bp of *Cytb* for *M. mystacinus*. Haplotypes are coloured based on the countries where they occur (see key on bottom right). Single step-wise mutations between haplotypes that are greater than one are marked by the cross-lines.

Diversity is highest in the eastern European group. Samples from Greece are the most divergent, and include samples from more than one clade. Mismatch distribution analysis with these groups suggest that the western group has undergone demographic expansion, supported by the star shaped pattern in the network. The eastern group (not including the divergent Greek samples) also shows an expansion pattern. Using the approximate mutation rate of 20%/million years, expansion times were calculated for the western group of 10,215yrs BP (ranging from 7,455 – 15,645yrs BP), while the eastern clade expansion dates to 32,974yrs BP (ranging from 16,129 – 51,075yrs BP).

We conclude, based on the network and timing of expansion, that all Irish populations of *M. mystacinus* most likely originated from a European glacial refugia. Given the higher diversity, and

earlier expansion dates in the east, and the lower diversity in western Europe, colonisation to the west from an eastern refugia is the most probable. There is evidence of further divergent groups within Greece and potentially Spain, suggesting there may have been more than one refugium, however, further sampling would be needed to elucidate this. The expansion of the western group ties in with the end of the LGM, 10,000 yrs BP. Irish populations, which have a combination of unique and shared haplotypes with mainland Europe and Britain are likely to have colonised while a land connection still existed and when sufficient habitat was available for roosting and foraging, or at least had to cross a limited water body.

Myotis nattereri

Mitochondrial diversity in Ireland was relatively low in comparison to Britain, but, sample sizes from elsewhere are too small for comparison (Table 12). *Dloop* sequence was obtained for 299 specimens of *M. nattereri*. A total of 23 individual mtDNA haplotypes were identified from the analysis of 299 individuals sequenced. The frequency distribution of mtDNA haplotypes among samples examined is displayed in Figure 22. The AMOVA demonstrated that the majority of variation in *M. nattereri* was observed among sites within countries (47.6%), with 28.3% within sites and 24.2% between countries.

A median joining network constructed from 299 individuals for *Dloop* and *Cytb* are shown in Figure 23 and Figure 24. A total of 23 *Dloop* haplotypes were identified in Europe. Thirteen haplotypes were identified in Ireland, 10 of which were unique to Ireland. Three Irish haplotypes, found in 46 individuals in Cork, were shared with Britain, Switzerland, Germany, France and Greece. All the rest contained a 23bp indel suggesting they derived from a single ancestral haplotype. In total, 4 *Cytb* haplotypes were identified in Ireland, one which was particularly common and shared with Britain, France and Germany, whilst three others were unique to Ireland.

Table 12: Diversity statistics including, the number of samples (*n*), number of haplotypes (*nh*), number of polymorphic sites (Poly. Sites), haplotypic diversity (*h* +SD), mean number of pairwise difference between sequences and nucleotide diversity (π + SD) for *Dloop* mtDNA sequences.

Location	***N***	***nh***	**Poly. sites**	***h* +SD**	**Mean no. of pairwise**	**π + SD**
Ireland	250	14	8	0.7957 +/- 0.0180	1.368000 +/- 0.847373	0.003977 +/- 0.002725
Britain	23	8	9	0.9004 +/- 0.0279	2.528139 +/- 1.413684	0.007349 +/- 0.004585
France	10	3	2	0.5111 +/- 0.1643	0.666667 +/- 0.556156	0.001938 +/- 0.001828
Germany	13	5	6	0.8333 +/- 0.0597	2.410256 +/- 1.397666	0.007007 +/- 0.004567
Switzerland	1	1	7	N/A	N/A	N/A
Greece	1	1	10	N/A	N/A	N/A
Turkey	1	1	2	N/A	N/A	N/A

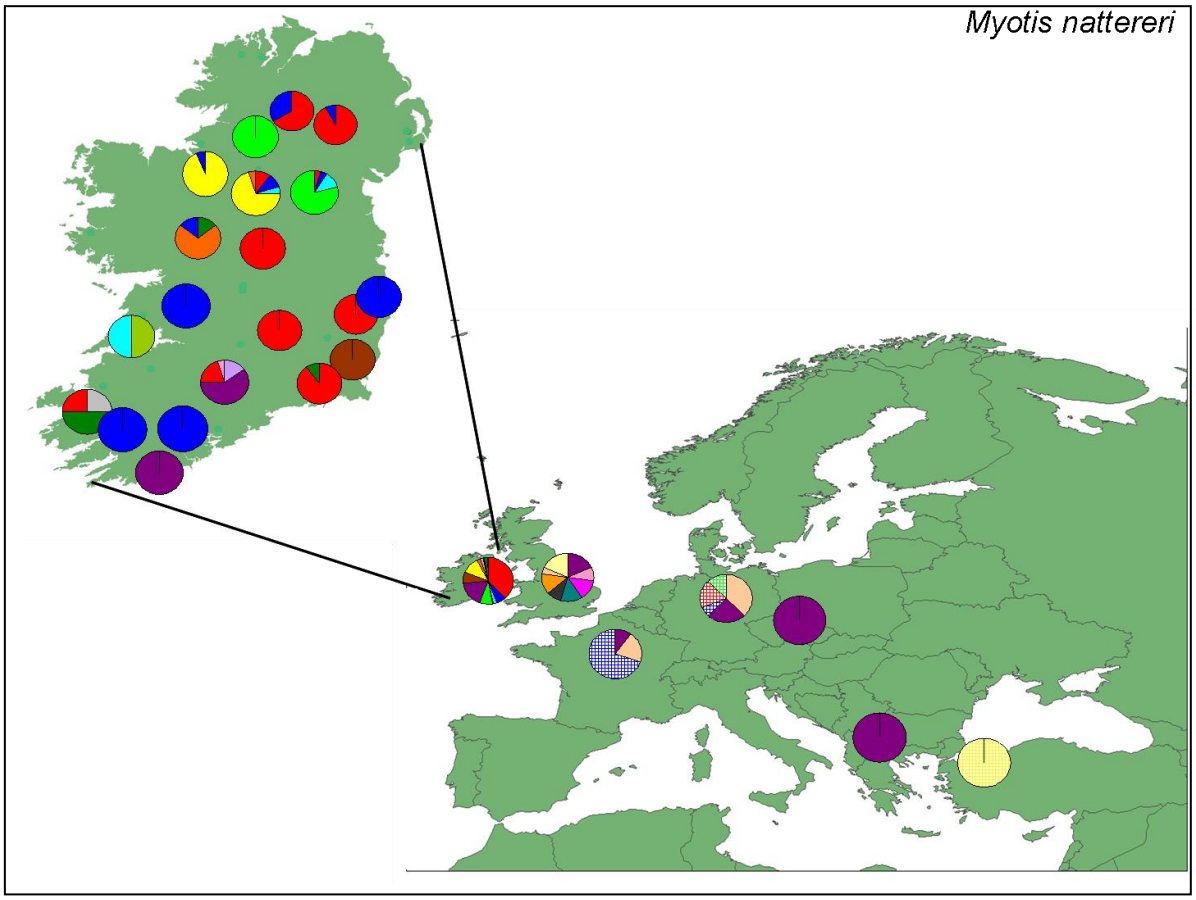


Figure 22: Frequency distribution of mtDNA *Dloop* haplotypes among surveyed sites of *M. nattereri*

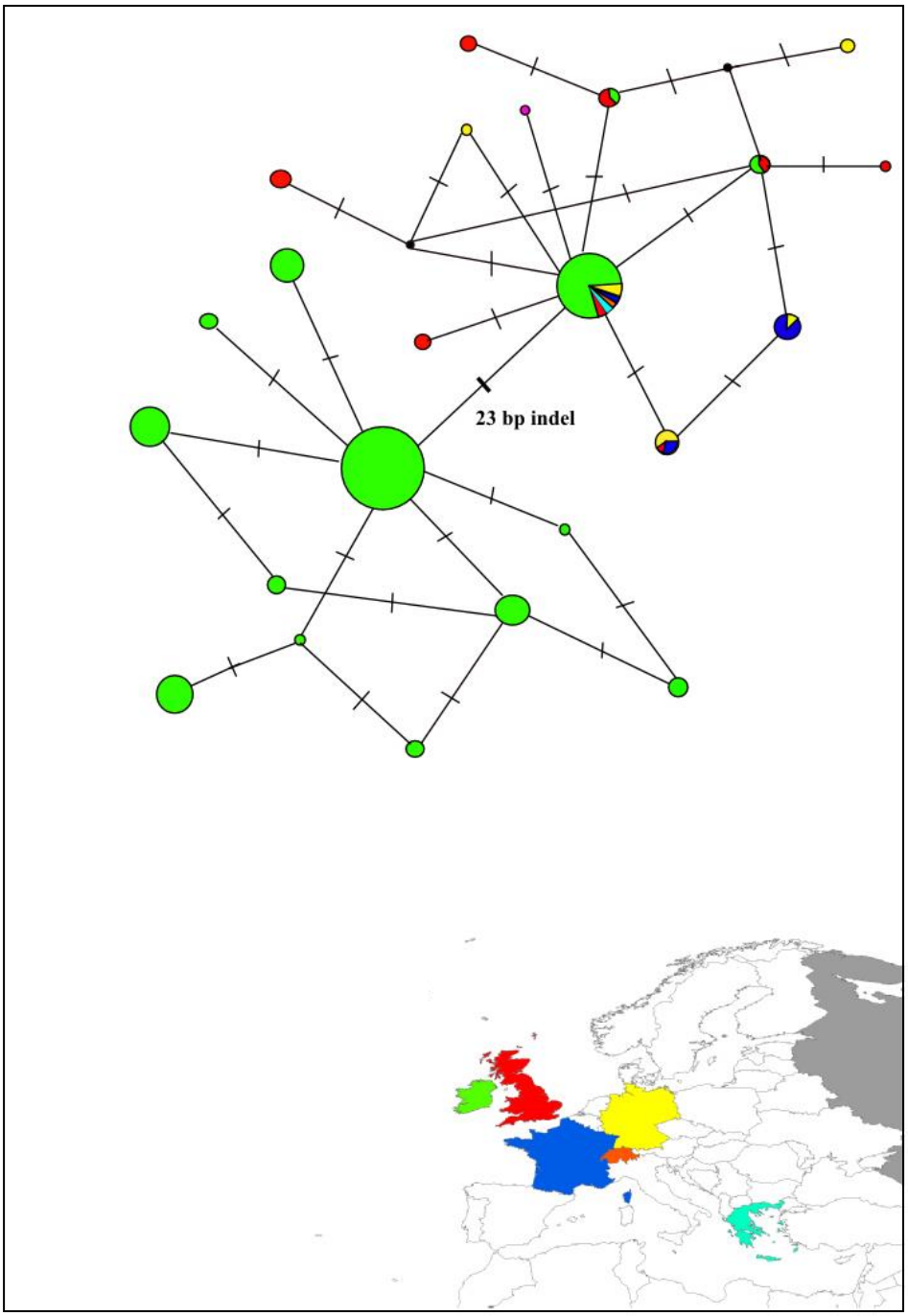


Figure 23: Median joining network showing 23 haplotypes for 366 bp of the hyper variable domain of the control region (D-loop) for *M. nattereri*. Haplotypes are coloured based on the countries where they occur (see bottom right). Single step-wise mutations are marked by the cross lines missing haplotypes are coloured red.

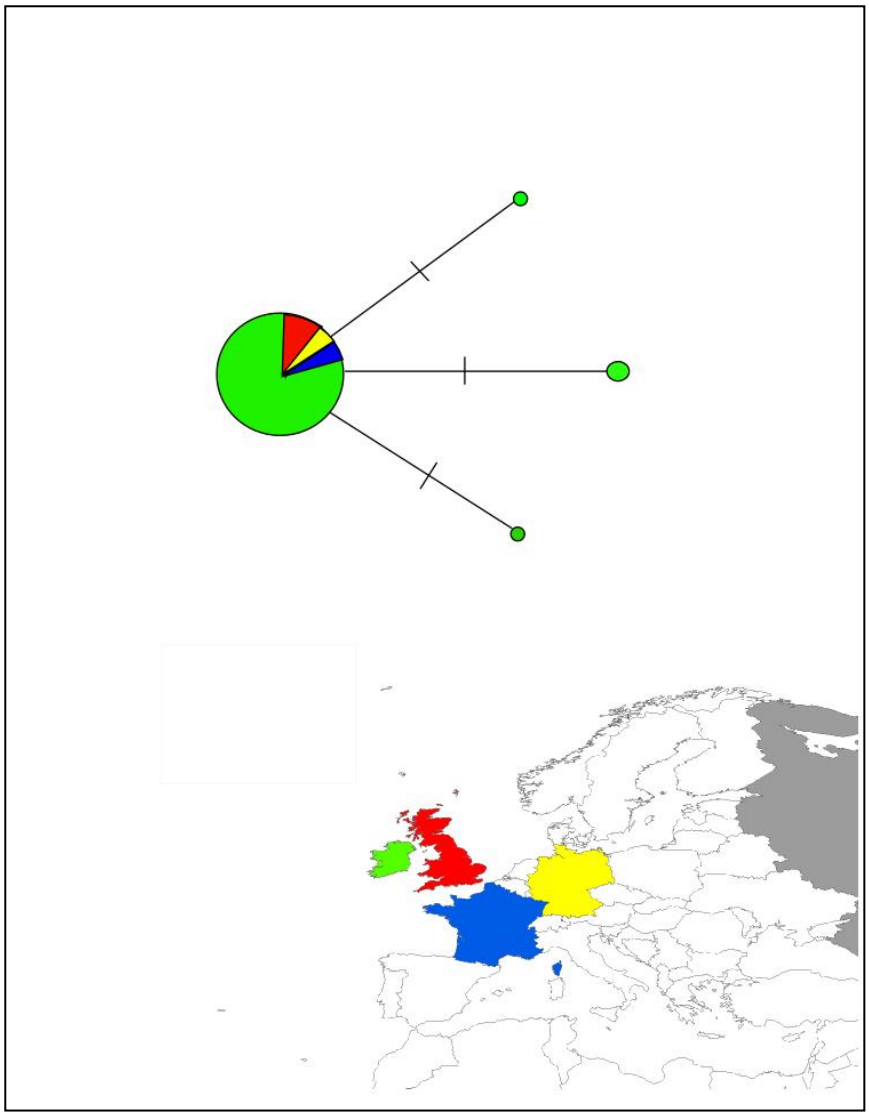


Figure 24: Median joining network showing 4 haplotypes for the 552 bp of *CytB* for *M. nattereri*. Haplotypes are coloured based on the countries where they occur (see bottom right). Single step-wise mutations are marked by the cross lines.

If we consider the *M. nattereri Dloop* haplotypes as a single haplotypic group, mismatch distribution analysis suggests that this group has undergone demographic expansion approximately 12,863yrs BP, ranging from 8,059 – 17,485yrs BP, placing the expansion after the LGM. In order to date the expansion of the group of Irish haplotypes containing the indel we carried out mismatch distribution analysis separately, which also demonstrated a signal of demographic expansion, dating to 10,547yrs BP, ranging from 8,301 – 14,344yrs BP.

The high haplotypic diversity among Irish *Dloop* haplotypes, most of which are unique to Ireland, may be suggestive of a separate glacial refugia, however, the low divergence, the expansion times and the low diversity in *Cytb* as presented here, and as demonstrated in Puechmaille *et al.* (2011), suggests these haplotypes may have arisen following recolonisation of Ireland after the LGM. The three haplotypes in Cork shared with Britain, Switzerland, France, Germany and Greece, may suggest some concurrent gene flow. However, it is possible that these also originated in Ireland, one is the ancestral

haplotype, and the others are derived from this ancestral haplotype from which Irish haplotypes containing the deletion are derived.

Summary

In this study, haplotypic diversity in Ireland is much higher in *M. nattereri* (0.7957+/-0.0180) than in *M. mystacinus* (0.4349+/-0.0456), with *M. nattereri* having many more unique Irish haplotypes, all containing a distinctive indel. This could potentially be explained by differing population sizes in these species. For *M. mystacinus*, based on the *Dloop* network and timing of expansion, all Irish populations are likely to have originated from Europe. While Irish *M. nattereri* have distinctive *Dloop* haplotypes, the *Cytb* does not suggest that Irish populations are deeply diverged from those in Britain and Europe, supporting a European origin for these bats also. Sample sizes from Europe in this study are not large enough to suggest a location of the glacial refugia, however, the current distribution and diversity of *M. nattereri*, and the associated sub-species in Europe, the most likely location was the Balkans (Puechmaille *et al.* 2011).

3.2 Population genetics of *M. mystacinus* and *M. nattereri* in Ireland

Background

The understanding of the population genetic structure of a species can provide important relevant insights into the historical and prevailing factors affecting its distribution in time and space. From a historical perspective, as we have seen in Section 3.1, and on a microgeographic scale, molecular studies can provide unambiguous evidence for the existence of social interactions within populations, and contribute to the identification of ecological and/or environmental factors affecting patterns of dispersal and gene flow (Avise, 1987, 2000). Knowledge of these factors is essential for any conservation or management program to ensure long term viability of populations and species.

Aims

Examine population genetic structure and gene flow among nursery colonies of *M. mystacinus* and among nursery colonies *M. nattereri* across Ireland.

Determine the genetic connectivity between individual *M. nattereri* occupying nursery colonies in spring and those caught at swarming sites in autumn.

Methods

Tissue collection, extraction is described in Section 3.1. A total of 20 microsatellite markers were optimised and screened for *M. mystacinus* on the ABI PRISM 3130xl Genetic Analyser 16 Capillary system (Applied Biosystems). A further ten microsatellite markers were developed at QUB. In total, 30 markers were optimised and screened for *M. nattereri* on the ABI PRISM 3130xl Genetic Analyser 16 Capillary system (Applied Biosystems). Twelve markers which amplified well were selected for analysis for each species (Table 13). Standard diversity indices for microsatellite data (no. of alleles, observed heterozygosity (Ho), expected heterozygosity (He)) were calculated using ARLEQUIN 3.5 (Excoffier & Lischer 2010). The number of clusters among samples was estimated using the program STRUCTURE 2.3.2 (Pritchard *et al.* 2000), and also determined by the lowest Bayesian Information Criterion (BIC) using the population genetics package "adegenet" (Jombart 2008) in R (R Development Core Team 2009). Discriminant Analysis of Principal Components (DAPC), which emphasises variables which maximise group separation, was used to visualise the distribution of diversity using "adegenet" (Jombart 2008) in R (R Development Core Team 2009). Population differentiation between

sites within Ireland was examined by calculating a pairwise F_{ST} (Weir & Cockerham, 1984) matrix and a Fisher's exact test for each pair of sites in Genepop 1.2 (Raymond & Rousset, 1995).

Table 13: List of microsatellite 20 loci amplified, the number of alleles in loci screened in each species, and the source.

	M. mystacinus	*M. nattereri*	
Loci	**No. alleles**	**No. alleles**	**Source**
A2	11	12	John Altringham's Group
C112	8	-	John Altringham's Group
EF15	12	8	John Altringham's Group
G2	-	-	John Altringham's Group
G31	9	8	John Altringham's Group
B8	24	-	Castella & Ruedi (2000)
D15	8	10	Castella & Ruedi (2000)
D9	-	22	Castella & Ruedi (2000)
E24	-	15	Castella & Ruedi (2000)
F19	15	4	Castella & Ruedi (2000)
G30	19	-	Castella & Ruedi (2000)
H23	-	10	Castella & Ruedi (2000)
G25	9	-	Castella & Ruedi (2000)
H19	4	10	Castella & Ruedi (2000)
MN079	-	11	QUB
MN117	14	9	QUB
DSG6	9	4	QUB

Results/Discussion

Myotis mystacinus

Nursery colonies of *M. mystacinus* were genetically diverse with a mean observed heterozygosity (*He*) = 0.73. STRUCTURE and cluster analysis identified two clusters, which geographically corresponded to Greece and the rest of Europe were identified, whilst Irish samples suggested a single grouping. Using DAPC's analysis greater separation can be seen throughout Europe. Once Greece is removed, Irish grouped separate from those of continental Europe and England, which is associated more closely to France. Within Ireland, overall there was very little evidence of population differentiation between sites according to pairwise comparisons, however, there was significant differentiation ($P<0.01$) between the furthest south site (Cork B) and the furthest north site (Down), Wexford A differentiated significantly from all sites, except for Clare A, while Wicklow was significantly different from Clare A. Wexford A also stands apart in the DAPC analysis (Figure 25), however since this site has a relatively small sample size this may be an artefact of sample size.

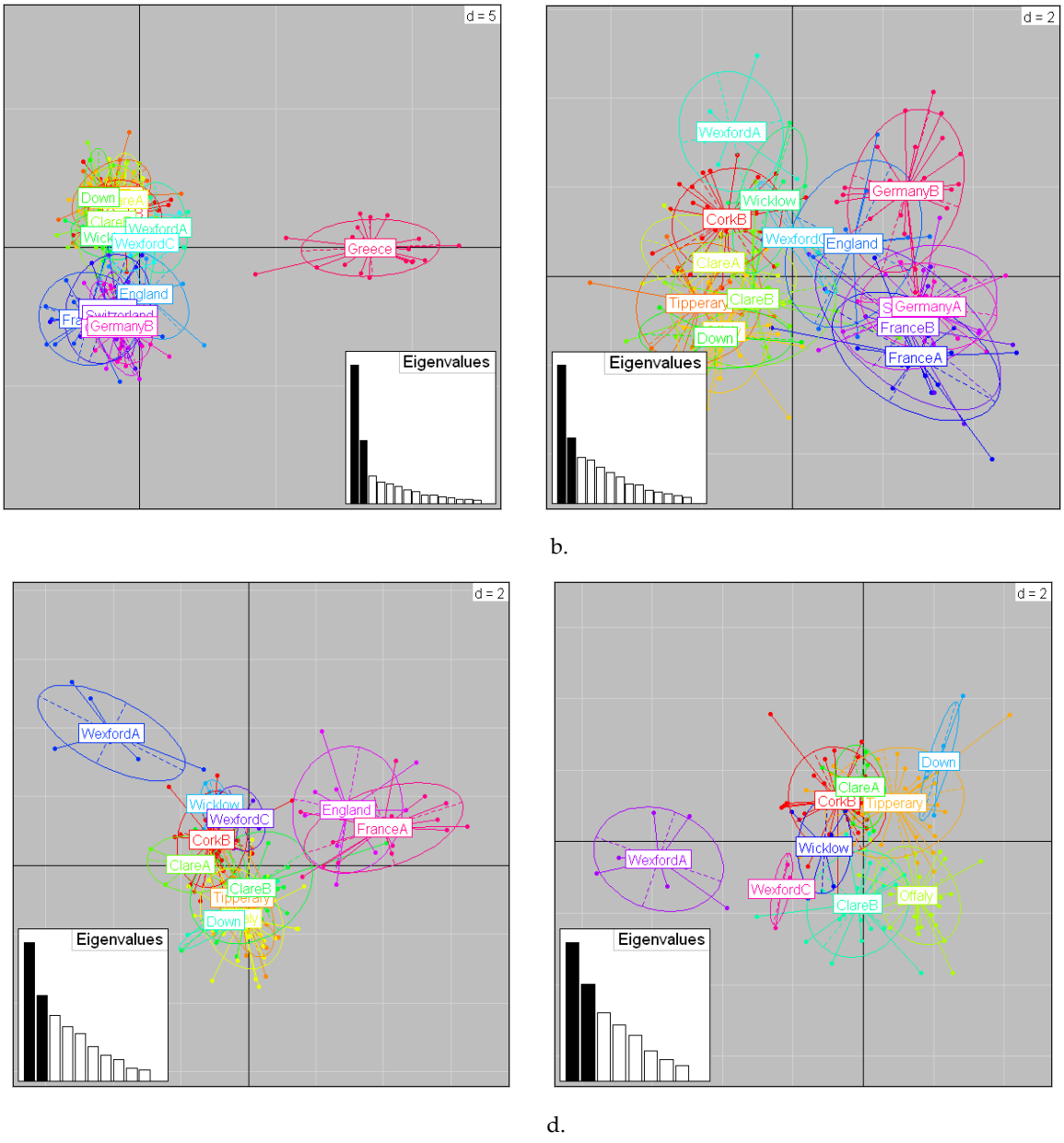
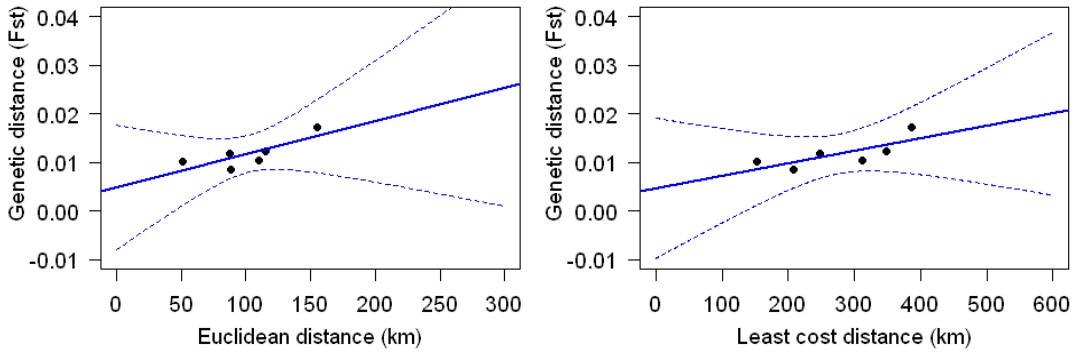


Figure 25: Discriminant Analysis of Principal Components (DAPC) of a) all samples, b) western Europe, c) Ireland, England, France and d) Irish samples only. The names of the country or county (Ireland) in which the roost was located are given.

Genetic distance among sample sites was compared to geographic distance, both Euclidean and least cost path, which was determined using habitat preference information from the radio tracking study in Section 2.1. Only sample sites with more than 10 samples were used in this analysis to avoid sample size bias. Euclidean distance did not differ significantly from least cost path distance, demonstrating that potentially no features in the Irish landscape created a barrier between our study sites. A mantel test between genetic distance and Euclidean; and genetic distance and least cost distance showed a positive relationship, suggesting that genetic distance increased with geographic distance (Figure 26 a,b).



a. b.

Figure 26: a) Mantel test comparing genetic distance among sites within Ireland with Euclidean distance, b) Mantel test comparing genetic distance among site within Ireland with least cost path distance.

Levels of genetic diversity among Irish nursery roosts of *M. mystacinus,* were similar to sites in Britain and continental Europe. STRUCTURE, cluster analysis and DAPC's confirmed the differences between Greece and Western Europe. Within Western Europe, Ireland separated out, with England more akin to French populations than Irish. This suggests that Irish populations are genetically isolated from both continental Europe and Britain. The nursery colonies within Ireland grouped as one population, despite a low signal of isolation by distance. This along with the low levels of genetic differentiation among sites demonstrates that among nursery colonies of *M. mystacinus*, gene flow is high enough to prevent genetic divergence. The comparison of Euclidean distance to least cost path distance, based on the habitat use of this species, demonstrated a lack of barriers to dispersal for this species, which supports our finding of high gene flow within Ireland.

Myotis nattereri

Genetic diversity was similar to that seen among roosts of *M. mystacinus*, with an average expected heterozygosity (*He)* = 0.653 per roost. STRUCTURE identified two clusters among all samples, Ireland, France and Britain, and Germany. PCA analysis determined no structure, while DAPC identified four clusters, which corresponded to Ireland and France, two clusters in Britain, and Germany and then another German site (Figure 27a). France remains clustered with Ireland (Figure 27b) whilst within Ireland two clusters were identified, as the nursery roost at Kerry groups separately (Fig27c). Pairwise comparisons demonstrate that the Kerry roost was significantly different from both the roosts at Armagh and Wexford B. Thus, nursery colonies of *M. nattereri* were genetically distinct from colonies in the Britain and Germany in particular, while more akin to those in France. Within Ireland, gene flow appears to be high, with no strong differentiation between nursery sites.

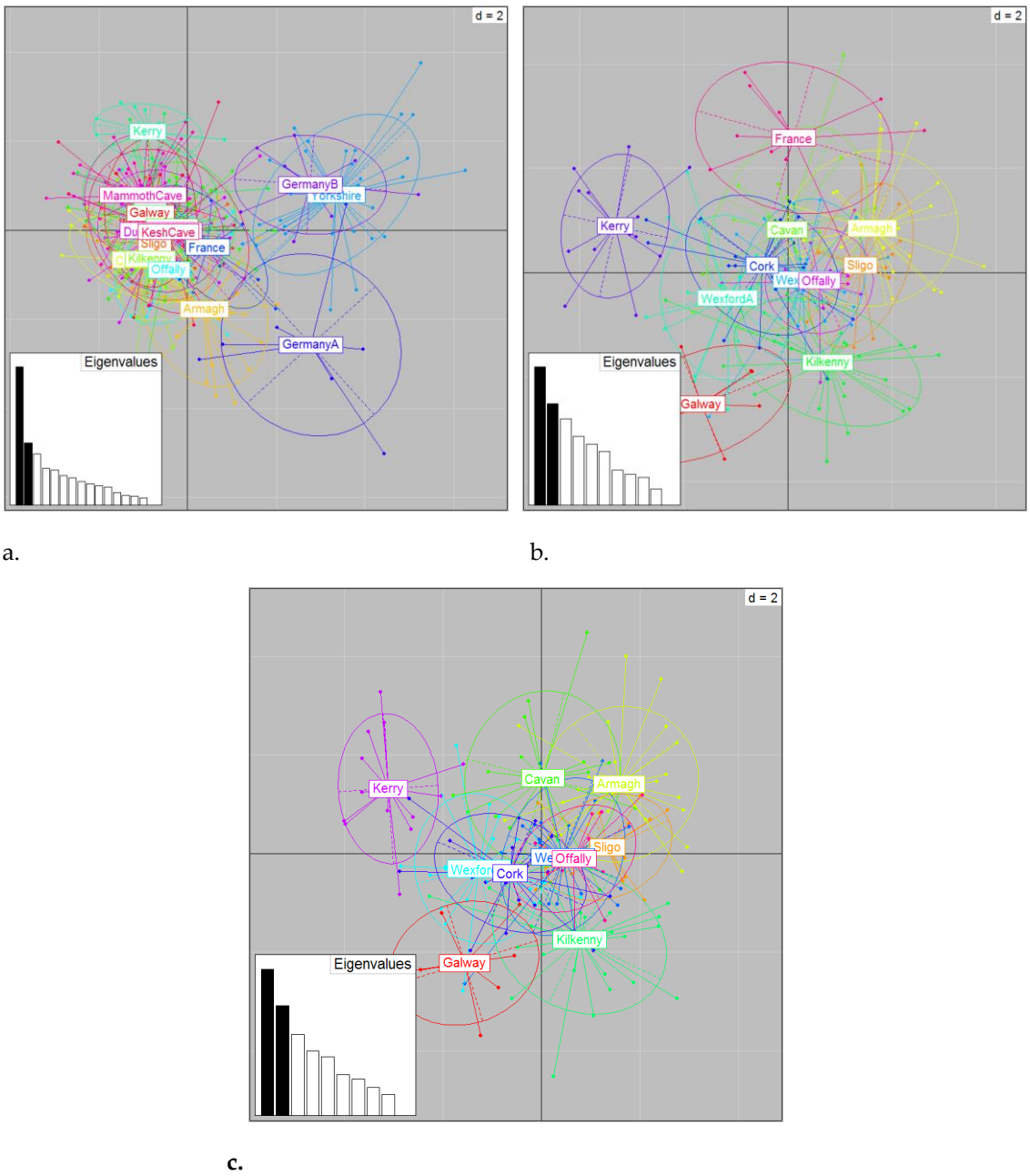
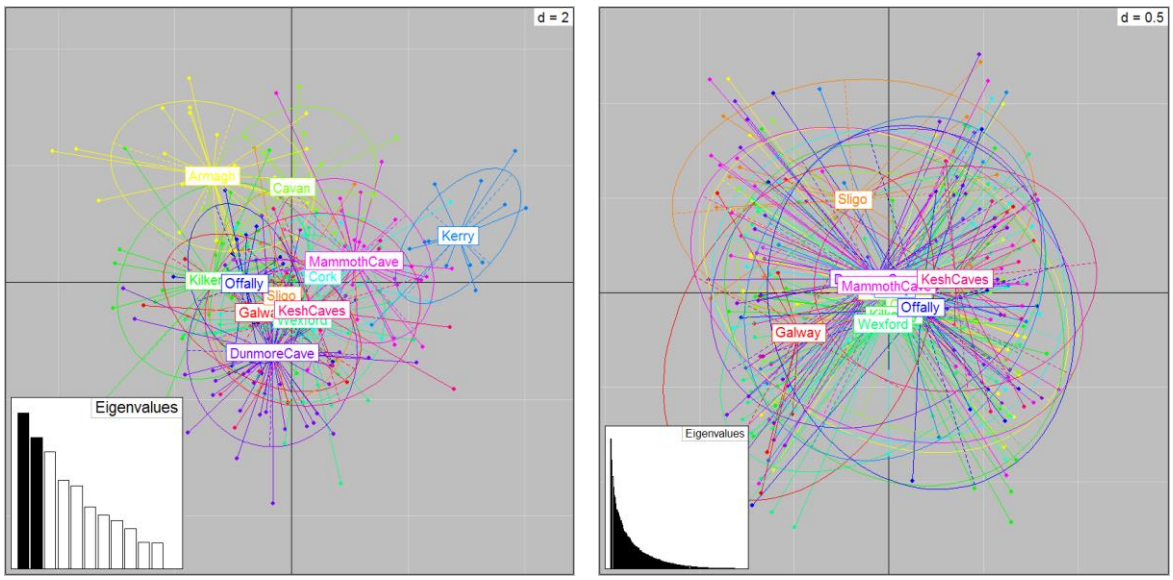


Figure 27: Discriminant Analysis of Principal Components (DAPC) of a) all populations, b) Irish and a French colony and c) all Irish nursery colonies. The names of the country or county (Britain & Ireland) in which the roost was located are given.

Four clusters were identified in Ireland when the three swarming sites were included alongside the nursery roosts of *M. nattereri.* The swarming sites with high diversity overlap with nearly all sites, except Armagh, Cavan and Kerry (Figure 28a) However, again the PCA revealed no distinct clustering of samples from nursery roosts and swarming sites in Ireland (Figure 28b). As in *M. mystacinus*, there was no indication of isolation by distance among the nursery colonies (>n=10) of *M. nattereri* (Figure 29), supporting the conclusion of high gene flow among nursery colonies.



a. **b.**

Figure 28: a**)** DAPC and b) a PCA of all Irish nursery roosts and the three swarming sites, the names of the country or county (Britain & Ireland) in which the roost was located are given.

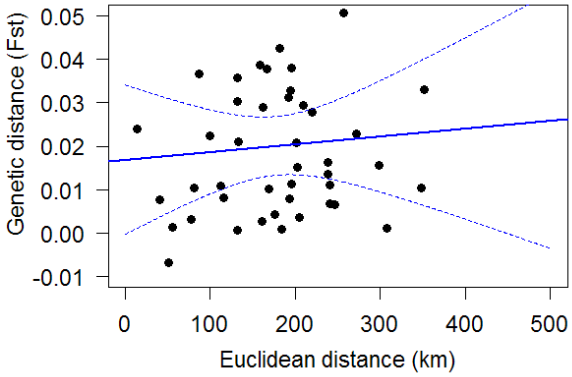


Figure 29: Mantel test comparing genetic distance among sites within Ireland with Euclidean distance

Summary

Irish populations of both *M. mystacinus* and *M. nattereri* are genetically diverse and distinct from both British and European populations. Within Ireland both species show low levels of genetic differentiation among nursery sites, or swarming sites of *M. nattereri*, and no signal of isolation by distance.

4. CONSERVATION IMPLICATIONS

The preceding body of research conducted by the Centre for Irish Bat Research provides updated range, population and overall conservation status data on *M. brandtii*, *M. mystacinus* and *M. nattereri*. This builds on information in previous conservation plans for Irish vesper bats (McAney 2006) and the Threat Response Plan: Vesper bats 2009-2011 (NPWS 2009). In addition, a follow up review of potential population monitoring methodologies for these species, as described in McAney (2006,) has been prepared, and a novel, non-invasive, genetic method for monitoring these bats is proposed below.

Brandt's bat

In the last national conservation assessment for bats under the Habitats Directive (NPWS 2008), *M. brandtii* could not be separated from *M. mystacinus*, given the cryptic nature of the species and lack of data on the newly described *M. brandtii*. Since then, a thorough review of the status of these two species has been conducted using genetic species identification methodologies (Section 1.2; Boston *et al.* 2010). This will allow separate conservation assessments to be prepared for each species for the next Article 17 report (due to the European Commission in 2013).

Range

Molecular genetic surveys across all known nursery roost sites of *M. mystacinus* and at several woodland sites across Ireland demonstrated that no species mis-identification had occurred (Section 1.2; Boston *et al.* 2010). Only a single record of *M. brandtii* in Ireland can be confirmed (using molecular techniques by CIBR, July 2011), from Glendalough, Co. Wicklow in 2003 (Mullen 2006).

Population

Since no breeding colony of *M. brandtii* has been found in Ireland, and the potential that this single specimen identified in Wicklow may be a vagrant, this species cannot be considered one of Ireland's resident bat species. However, it may be that this species is very rare in Ireland, or not easily identified by the methods used in this study, therefore, further effort should be made to determine whether this species, or potentially others, may be present, or colonising Ireland. In the meantime, the most recent Red Data List assessment of data deficient seems appropriate (Marnell *et al*. 2009).

Whiskered bat

M. mystacinus records in Ireland have now been separated from potential *M. brandtii* records. In addition, a review of all known nursery roosts has been conducted and new research has greatly improved what is known about this species in Ireland.

Range

The 'current range' and 'favourable range' of *M. mystacinus* was previously delineated on 50km grid (NPWS 2007). We have now transferred this onto a 20km grid (Figure 30b; green area). This area is based on the grid that bounded all known records. From the analysis applied in Section 2.2 we overlie the modelled core area (Figure 30a; blue hatching). The maternity roost records confirmed (Section 1.1) from all existing sources are shown (Figure 30a; red hatching). These, although sparsely distributed, have a wide geographic distribution spanning the extent of the previously identified 'current range'/ 'favourable range' and the modelled core range. The modelled range is significantly smaller than that of the 'current range'/ 'favourable range' (Table 14). The disparity between the areas and the occurrence of positive records outside the 'current range'/ 'favourable range', and the amount

of records which fall outside of the core area, suggests that more records of the species may exist within these areas and indeed in small areas of suitable habitat outside of these areas.

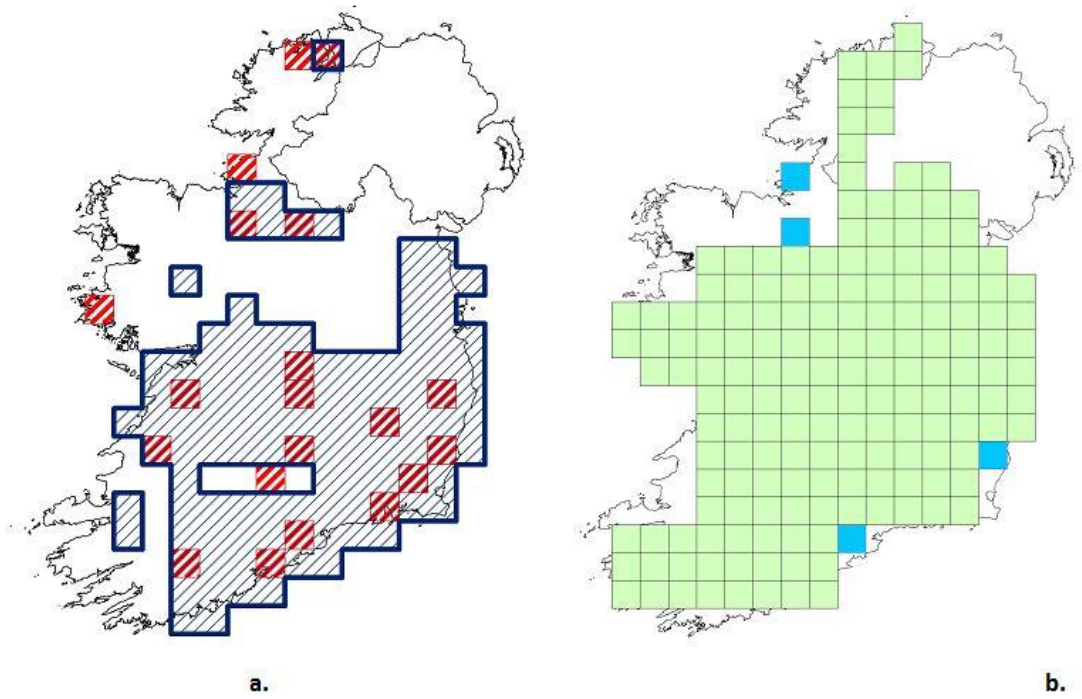


Figure 30: *M. mystacinus* range; a. Core suitable area (Blue hatching) and comfirmed roost records (Red hatching). b. The defined favourable reference range transferred from 20 km grid from previously defined 50 km grid (Green filled cells; NPWS 2007) and added area (Blue filled cells)

Table 14: The area of defined ranges of *M. mystacinus* in Ireland

Range	**Area (Km²) [resolution]**
Favorable reference range (NPWS 2007)	67, 500 [50Km]
Favorable distribution (NPWS 2007)	50, 000 (74.1%) [50Km]
Core suitable area (Section 2.1)(Land only)	37, 555 [20Km]
Occupied core area (using distribution from NPWS (2007))	23, 867 (63.5%) [20Km]
Occupied core area (using confirmed records)	5798 (15.4%) [20Km]

Population

O'Sullivan (1994) reported only 34 roosts during the National Bat Survey in Ireland, 22 of which had less than five bats. In this study, 27 potential maternity sites were identified, 22 of the 25 surveyed were confirmed maternity colonies. The average roost size of *M. mystacinus* was 15.1 (s.d. = 13.5).

Habitat

Roosting habitat

Whiskered bats are typically found in houses during the summer in small numbers, often between the rafters and felt or in narrow slits where the timbers meet (O'Sullivan 1994). This observation was

confirmed by surveys conducted by CIBR in 2008 and 2009. Whiskered bats have also been described roosting in crevices in bridges (Smiddy 1991; Shiel 1999). In addition, radio telemetry (Section 2.1), found that *M. mystacinus* switched roosts frequently throughout the maternity period and utilised a number of roost sites in an area, including other buildings and tree roosts. Tree roosts were in mature broadleaf species (*Fagus sylvatica* and *Acer pseudoplatanus*), whilst four dwelling houses were also used, varying in age from relatively new builds to the typical old (100yrs+) large dwelling house described by O'Sullivan (1994), and an agricultural shed with a corrugated iron roof. These observations suggest that the protection of a single nursery roost in an area may not be sufficient.

Foraging habitat

Irish populations of *M. mystacinus* appear to be reliant on prey species associated with aquatic habitats having a depleted $\delta^{13}C$ in and foraging on insects of the family Ceratopogonidae which commonly have aquatic larval stages (Chinery 1997). Radio-tracking of *M. mystacinus* (Section 2.1) revealed that foraging ranges were concentrated in a small area relatively close to the roosts. The reliance on a relatively small foraging area may explain why trophic patterns were not found to be related to the general habitat around roosts. No previous analysis of the diet of *M. mystacinus* has been conducted in Ireland. However, present data supports the finding of a large proportion of Diptera in the diet in European populations (Safi & Kerth 2004).

Mixed woodland habitats and the riparian zone were selected within the core foraging areas of *M. mystacinus*, supporting observations from the dietary analysis. Arable land and rough grassland were also selected in the home range but this is likely due to these habitats being adjacent to the roosts. During the maternity period home ranges and particularly core foraging areas were small, highlighting the importance of the protection of riparian environments for these bats and good habitat close to maternity roosts.

Nursery colonies of *M. mystacinus* were genetically diverse, and there appeared to be no barriers to gene flow likely due to the homogeneous distribution of suitable habitat for the species across its range.

Hibernation and Swarming

There are only two records of *M. mystacinus* hibernating in underground sites in Ireland, one in a cave in Co. Galway (McAney 1994) and another in a cave in Co. Kilkenny (McAney 1997). Sampling at three confirmed swarming sites, identified only a single *M. mystacinus* at Kesh Caves, Co. Sligo. Whether or not this species swarms in Ireland is inconclusive. They are commonly encountered swarming at underground sites in late summer and autumn in the UK (Parsons *et al.* 2003).

Future prospects

Although not often recorded, *M. mystacinus* is believed to be widely distributed in Ireland, and has been listed as 'least concern' in the Irish Red List (Marnell *et al*. 2009).

Natterer's bat

A review of all existing nursery roost records of *M. nattereri* and research into the population ecology has greatly improved knowledge of this species in Ireland.

Range

The 'current range' and 'favourable range' of *M. nattereri* was previously delineated (NPWS 2007; Figure 31; green area). This area was based on the grid that bounds all known records. From the

analysis applied in Section 2.1we overlie? the modelled core area (Figure 31; blue hatching). The maternity roost records confirmed from all existing sources (Section 1.1) are shown (Figure 31; red hatching). These have a broad geographic distribution spanning both the extent of the previously identified 'current range'/ 'favourable range' and the modelled core. There is high degree of agreement between the previously identified 'current range'/ 'favourable range' and the modelled core range, with some refinement of the boundary of the 'current range'/ 'favourable range' (Table 15).

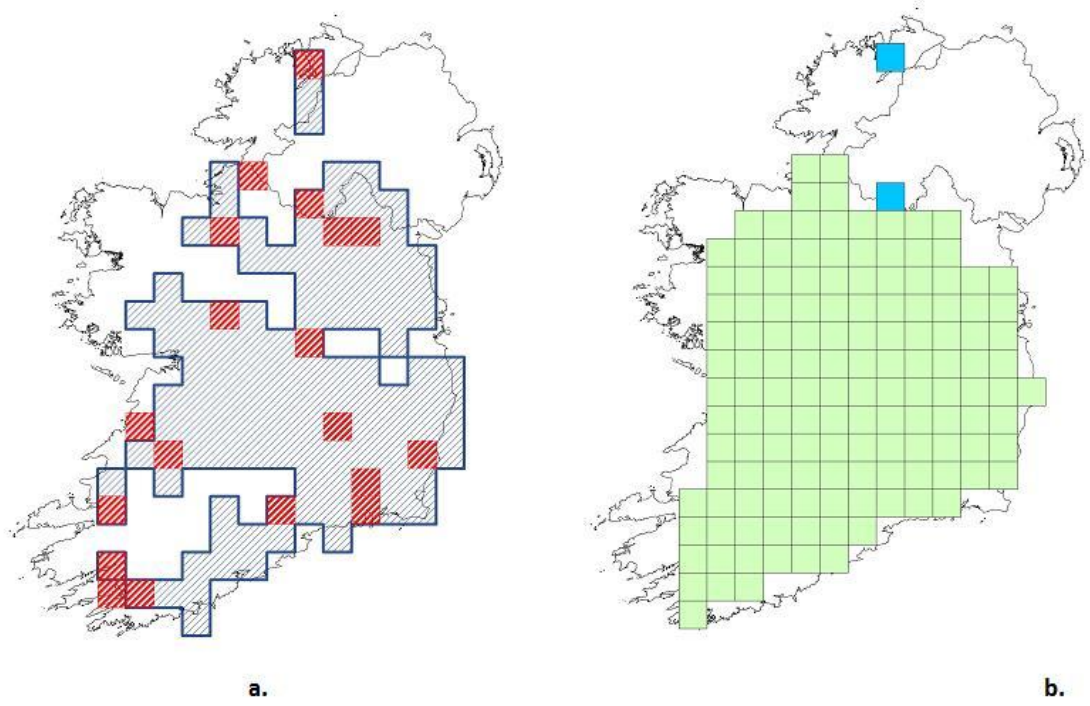


Figure 31. *M. nattererii* range; a. Core suitable area (Blue hatching) and comfirmed roost records (Red hatching). b. The defined favourable reference range (Green filled cells; NPWS 2007) and added area (Blue filled cells)

Table 15**:** The area of defined ranges of *M. nattereri* in Ireland

Range	Area (Km^2) [resolution]
Favorable reference range (NPWS 2007)	54, 000 [20Km]
Favorable distribution (NPWS 2007)	23, 600 (43.7%) [20Km]
Core suitable area (Section 2.1) (Land only)	42, 196 [20Km]
Occupied core area (using distribution from NPWS (2007))	18, 591 (44.0%) [20Km]
Occupied core area (using confirmed records)	5864 (13.7%) [20Km]

Population

O'Sullivan (1994) identified 44 roosts for *M. nattereri* during the National Bat Survey, with 20 containing single bats, and only seven with more than 50 bats. Of the 37 potential nursery roosts of *M.*

nattereri identified from the records, 19 of the 25 surveyed were confirmed, with an average roost size of 36.4 individuals (s.d. = 35.3), when accurate roost counts could be achieved.

Habitat

Roosting habitat

Natterer's are typically found to roost in buildings during the summer, in large roof spaces of dwellings or often in the attics of Church of Ireland churches, with colonies of >50 bats (McAney 2006). Records of these species are also known from bridges (Smiddy 1991; Shiel 1999) with a large maternity roost (n=>80) also known from bat boxes in Glengarriff Nature Reserve, Cork.

Radio-telemetry suggested these bats were roost faithful throughout the maternity period highlighting the importance of these single sites.

Foraging habitat

Previous analysis of *M. nattereri* in Ireland from a maternity roost revealed that large Diptera, such as dung fly belonging to the family Scathophagidae, are the main food source for *M. nattereri* along with Trichoptera, Hymenoptera and Arachnida (Shiel *et al.* 1991). Here it appears that the *M. nattereri* diet is closely linked to agricultural processes, both in terms of insect families consumed and the trophic relationship detected by SIA. In contrast to the dietary niche reviewed by Safi & Kerth (2004) we find an increased niche breath for *M. nattereri*. This may reflect the unique Irish landscape which in comparison to Europe has a reduced grain size with small field sizes and a high density of linear boundary features (Mitchell & Ryan 2001).

Radio-tracking revealed that woodland appears to be selected in the immediate vicinity of *M. nattereri* nursery roosts, but this selection declines with distance. In contrast, pasture was avoided close to the roosts and selected within the core foraging areas, supported by the dietary analysis. This highlights the importance of conserving woodland habitats close to nursery roosts for this species and, given its broad feeding niche and roost fidelity, suggests that for this species roost limitation may be the main limiting factor on abundance.

Nursery colonies of *M. nattereri* were relatively genetically diverse, and there appeared to be no barriers to gene flow between nursery roosts and swarming sites.

Hibernation and Swarming

Only 14 Natterer's bats have been recorded during hibernation surveys in west and south west Ireland; 10 in caves, two in ruined buildings and one each in a mine and bridge (McAney 1994; 1997). *M. nattereri* were found swarming at, at least four of the swarming sites identified by CIBR, potentially providing an opportunity to protect these bats outside of the summer nursery season.

Future prospects

Although seldom recorded, *M. nattereri* is thought to be widely distributed in Ireland, and is currently listed as 'least concern' in the Irish Red List (Marnell *et al*. 2009).

5. SCIENTIFIC MONITORING

Background

All Irish bat species are protected under both Irish (Wildlife Act (1976); Wildlife (Amendment) Act 2000) and EU legislation (EU Directive (92/43/EEC) Habitats Directive). Ireland is a signatory of the European Bats Agreement (EUROBATS), part of the Bonn Convention on the Conservation of Migratory Species of Wild Animals (1979), the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention, 1982) and the Convention on Biological Diversity (1992). Under these agreements Ireland is obliged to develop local biodiversity plans and develop strategies for monitoring all of Irelands bat species. The Irish Bat Monitoring Programme (IBMP) contains four separate schemes which are run on an annual basis to collect data on the lesser horseshoe bat and five of Ireland's nine vesper bat species (McAney 2006; Aughney & Roche 2008; Roche *et al.* 2009; Aughney *et al.* 2009). The Centre for Irish Bat research was tasked to identify a suitable monitoring scheme for the rarer *Myotis*, *M. mystacinus* and *M. nattereri*. This scheme needs to be able to identify any changes in the range of each species as well as providing robust data on population trends (Evans & Arvela, 2011)

In Europe, most *Myotis* sp. are monitored through hibernacula counts, particularly *M. mystacinus* (Battersby 2010). However, in Ireland this is not possible due to the absence of known or large hibernacula. Both species are difficult to distinguish acoustically, and thus cannot be monitored using bat detectors. Furthermore, woodland surveys using mist-nets and harp traps have shown that this species is encountered in low abundance in the environment, even with the assistance of the acoustic lure (Section 2.7). Therefore, a site based monitoring approach appears to be the most appropriate. Annual nursery roost counts have been used to monitor populations of *Plecotus auritus* in Ireland by Bat Conservation Ireland since 2007 (Aughney & Roche, 2008) and by the Bat Conservation Trust in the Britain since 2001. *P. auritus* show a high degree of long-term roost fidelity (Entwistle *et al*., 1997) and this, coupled with the fact that the species roosts within the attic spaces of buildings, provides a means of monitoring emergence counts. Roche & Aughney (2008) assessed the viability of this method for both *M. mystacinus* and *M. nattereri* in Ireland, where both exhibit fidelity to artificial roosts. However, based on simulations used to estimate the number of roosts required to be counted per year for *P. auritus* (> 30) to have enough power in the data to detect change, they suggest, given the comparatively lower roost size of *M. mystacinus* and *M. nattereri*, an even larger number of sites may be required, making this unrealistic as a monitoring tool for these species. In addition, given the late emergence of this species, it is difficult to get accurate estimates from emergence counts (McAney 2006).

The discovery of swarming sites in Ireland (Section 2.5), potentially the main breeding sites for *Myotis* spp., may provide an opportunity to monitor population trends of both male and female *M. nattereri* in the future. However, the distribution, timing and importance of swarming sites are still poorly understood, making inferences difficult at present. Thus, monitoring of *Myotis* bats is an area that requires further investigation.

The conventional approach used on continental Europe is capture-mark-recapture (CMR) using wing banding, whilst newer methods using PIT tags have been adopted for some species (Ellison *et al.* 2006). These methods can provide valuable long term data, but, they are invasive and labour intensive, as well as requiring multiple tagging and recapture events. Collecting the 'mark' data non-invasively, however, may overcome these issues, leading to the proposal of developing a non-invasive genetic monitoring protocol for these species. Genetic monitoring is effectively a CMR (capture-mark-recapture) study. Bats are genotyped to give a 'DNA fingerprint', which is a permanent 'mark', and can be used to identify when individuals are recaptured. This allows counts of individuals within the roost directly each year, but also more accurate cumulative index of abundance based on the CMR data across years within the chosen study sites. In addition, a monitoring scheme such as this can provide much more than estimates of population size. We can get estimates of survival rates, recruitment into the population and simultaneously monitor levels of genetic diversity within the population which could provide estimates of the effective population size (*Ne*), an estimate much

more closely tied to the genetic health of a population. These data will be of greater value in the conservation of these species than estimates of population size alone.

Hence, we propose this as a potential monitoring protocol for both *M. mystacinus* and *M. nattereri* across a subset of the known nursery roost sites, as a portion of the total population of these species in Ireland. As we have seen from the genetic results presented in Section 3.2, *M. mystacinus* and *M. nattereri* nursery colonies in Ireland are genetically separated from those in Britain and mainland Europe, but show high gene flow within Ireland, suggesting that monitoring at a number of roosts can be considered as subsamples of a single population.

Aim

Determine the feasibility of this novel non-invasive genetic method in the field and laboratory.

Carry out population simulations to examine the level of sampling required to determine accurate population estimates using CMR in nursery roosts of *M. mystacinus* and *M. nattereri*.

Estimate the number of sites required across Ireland to allow for accurate monitoring of population trends.

Methods

Field and laboratory methods

In order to explore the feasibility of this method in the field and in the laboratory, we choose five nursery roosts of *M. mystacinus* and *M. nattereri* from across Ireland. In 2008, we visited these sites in order to take tissue samples for Section 1.2, 3.1 & 3.2, and at the same time collected faecal samples, 20/per site (i.e. n=100/species). We returned to these sites in 2010 and this time collected 30/site (i.e. n=150/species) to improve the number of recaptures. Droppings were collected in the attic space, between May and June of each year and placed into individual ependorf tubes containing silca gel. Droppings were no older than the beginning of the current nursery season (early May), and effort was made to collect the freshest, black shiny droppings from across the faecal pile(s).

All samples were extracted, amplified with a mtDNA marker for species ID, then amplified and scored with 12 microsatellite loci. Tissue samples were extracted and amplified as described above in Section 3.1 & 3.2. DNA extraction from faecal samples was carried out using QIAmp DNA Stool Mini Kit (QIAGEN) following the modifications recommended by Puechmaille *et al.* (2007). For faecal samples, a multi-tube approach was used, repeating each PCR three times and a consensus genotype noted for each locus. To identify matching samples from the same individual within the faecal samples we used the program GeneCap 1.3 (Wilberg & Dreher 2004). Those differing at one and two loci pairs were not used in analysis. Genetic profiles from each PCR were compared to the consensus to detect genotyping errors, particularly ADO and FA (Taberlet *et al.* 1996) following the methods detailed in Puechmaille & Petit (2007).

In 2008 we had the opportunity to directly compare the quality of data obtained by both faecal and tissue genetic sampling methods. Allelic frequency distributions, allelic richness and deviations from Hardy-Weinberg equilibrium were calculated for each locus for both tissue and faecal samples from each nursery roost using GeneCap 1.3 (Wilberg & Dreher 2004) and Genepop version 4.0.10 (Raymond & Rousset, 1995, Rousset, 2008). Genetic differentiation between sample tissue and faecal sets per population were calculated using an exact G test and Fst's between each pair were calculated in Genepop on the web (Raymond & Rousset, 1995).

GenCap version 1.3 was used to determine the number of individuals recaptured within and between years at the five nursery roosts for each species. Between year recaptures were used to determine realistic expectations for the number of recaptures given our sampling efforts.

Simulations

Population simulations were run to determine the level of sampling required to get accurate population estimates using CMR in nursery roosts of *M. mystacinus* and *M. nattereri*, using the program R. A population across a 12 year period was simulated, incorporating an average lifespan of 4.6 years with a standard deviation of 0.8 years, parameters estimated from known mortality and survivorship data for *M. mystacinus* in Dietz *et al*. (2009). One hundred simulation replicates were preformed across a range of population sizes (20-100) in stable populations and both increasing and decreasing populations. These simulated populations were then re-sampled across a 10 year period of the study, population size estimates for each year were estimated and the level of sampling effort required to obtain estimates with confidence intervals +/- 1 individual determined, using CMR, implemented in the open source package Rcapture, in R.

Power analysis

Under the Habitats Directive, Member States are required to identify whether species are declining at >1% per year. Such a decline would trigger an "unfavourable" assessment. However, assessing population trends to this level of accuracy, in particular given the potential for natural fluctuation, is not considered to be statistically sound. Power analysis was used to determine the number of roost sites needed to detect a 30% decline over 10 years (equivalent to IUCN's Vulnerable category) or a 50% decline over 10 years (IUCN Endangered) in *M. mystacinus* and *M. nattereri* populations using the CMR method described above. We simulated roosts for both species with starting populations and population sizes which declined by 30%, 50% and 90% over a 10 year period. Initial roosts sizes were generated randomly from negative binomial distribution distributions independently fitted to the empirical roosts sizes (>10 bats) determined in roost surveys for both species (Section 1.1).

Results

Field and laboratory methods

DNA was extracted from 109 tissue samples of both *M. mystacinus* and *M. nattereri*, along with 110 and 100 faecal samples of *M. mystacinus* and *M. nattereri* from 2008. For *M. mystacinus,* 99% (108/109) of tissue samples and 93% (102/110) of faecal samples amplified the ~850bp fragment of the mtDNA *CO1* and for *M. nattereri* 93% (105/109) of tissue samples and 89% (89/100) of faecal samples amplified a ~1200bp fragment of the mtDNA *Dloop*.

A total of 108 individual tissue samples and 102 faecal samples of *M. mystacinus*, and 105 individual tissues samples and 89 faecal samples of *M. nattereri* were then amplified with the twelve microsatellite markers. In both species, there was a higher amplification success for tissue (100%) than for faecal (72-88%), and a higher scoring success at 8+ loci for tissue (91-95%), compared to faecal (70-85%) samples. With a sampling effort of 20 droppings per site in 2008, recapture rates among faecal samples within sites were fairly low (*M. mystacinus* 0-55%; *M. nattereri* 0-16%), as were the number of individuals sampled both from tissue and faeces. Recapture rates were highest when roost sizes were small. The allelic dropout rate, proportion of false alleles and number of samples with no consensus genotype are comparable to those from other non invasive studies. Only three loci showed high rates of dropout and false alleles, and, thus, should be treated with caution in further analysis.

Allelic richness per loci was not significantly different (t-test P=>0.05; df. 22) for tissue and faecal samples. Genetic differentiation between each population faecal/tissue pair for both species demonstrated no significant difference between data collected from faecal or tissue samples for all sites, with the exception of one Natterer's site were five loci differed significantly between these sample sets.

Our results demonstrate that DNA extracted from faecal samples provide results comparable to that obtained through direct tissue sampling of *M. mystacinus* and *M. nattereri*, both for the amplification of mtDNA and nuclear microsatellites. This supports the utility of this non-invasive genetic sampling method for the use in a population monitoring study.

The number of recaptures of individuals recorded from faecal samples was estimated between the sampling years 2008 and 2010. Across the five roosts of *M. mystacinus*, 15% of individuals were recaptured, while for *M. nattereri* 10.9% of individuals were recaptured. From this we can extrapolate that if sampling were to occur each year at these sites we may expect around a 30% recapture rate for *M. mystacinus* and 20% recapture rate for *M. nattereri*.

Simulations

In a simulated stable population sizes the number of samples required to get estimates with CI +/- 2 individuals is approximately 1/3 more samples than the number bats present. The number of samples required to get very accurate estimates with CI +/- 1 rises very steeply, making this only realistic in small populations where it may be more necessary, while in larger populations slightly less accurate estimates of +/- 3 may be more appropriate, requiring less sample effort (Table 16). For a large roost size of *M. mystacinus* (~30 bats) 40 samples would need to be collected to get accurate estimates, while for *M. nattereri* and average roost size (~50 bats) 70 samples would need to be collected, or 50 for slightly less accuracy.

Table 16: Showing the sampling effort required to achieve accurate population estimates with CI +/- 1, +/- 2 and +/- 3 in a range of population sizes within stable populations.

	Sampling effort		
Pop. size	**CI +/- 1**	**CI +/- 2**	**CI+/- 3**
20	40	30	20
30	50	40	30
40	70	50	40
50	90	70	50
60	100	80	60
70	150	100	80
80	170	100	90
90	200	130	110
100	230	160	120

Power analysis

Fifteen roosts were generated for each condition. The decline in population was simulated to occur as follows: A. equally across all simulated roosts with a normal distribution and a standard deviation that determined that no roost would decline by more than the defined 'alert' B. decline in 50% of roosts and; C. decline concentrated in 25% of roosts.

Power analysis was applied using G*Power (http://www.psycho.uni-duesseldorf.de). The number of roosts required to achieve 80% power was determined for a Wilcoxon paired test with alpha = 0.05 (Table 17).

Table 17: The number of roosts of *M. mystacinus* and *M. nattereri* required to confirm population declines of 30%, 50% and 90% when the decline is equally spread across all roosts (condition A) , confined to 50% of roosts (condition B) and confined to 25% of roosts (condition C).

M. mystacinus

	30 % Decline			50% Decline			90% Decline		
Condition	A	B	C	A	B	C	A	B	C
Number or roosts	11	23	29	9	12	17	7	9	10

M. nattereri

	30 % Decline			50% Decline			90% Decline		
Condition	A	B	C	A	B	C	A	B	C
Number or roosts	33	33	35	11	13	21	9	11	13

Financial considerations

Implementing this protocol would involve costs such as petrol expenses of volunteers/NPWS rangers to visit these sites once per year. In this study, using these loci in three multiplexes, the laboratory the cost of consumables, carrying out DNA extraction, amplification and microsatellite screening of non-invasive faceal samples was approximately €34.24 per sample compared to €13.39 incl. VAT per tissue sample. However with considerable optimisation and the advancement of molecular techniques, these 12 loci could be optimised into a single multiplex, lowering the cost to €14.42 per faecal sample. Additionally these costs could be significantly reduced if this monitoring is completed on a larger scale. In addition there will be the cost of paying a skilled person(s) to undertake the laboratory and data analyses as required.

Summary

Both laboratory testing and simulations suggest that monitoring populations of bats from nursery roosts using DNA extracted from faecal samples is feasible, and could provide accurate estimates of roost sizes needed to detect population trends. The accuracy of the method depends on the size of the roosts under study and the amount of samples (sample effort) used. For a typical large *M. mystacinus* roost (n=30), simulations show that around 40 samples would need to be collected yearly to get good estimates with CMR. While for larger roost sizes, like many *M. nattereri* sites (n=50), approximately 70 samples are required to be collected. Power analysis to determine the number of sites per species that would need to be monitored to gain accurate trend analysis again depended on the size of roosts to be surveyed and the sampling effort applied. For *M. mystacinus* to detect a decline of 30% over 10 years, considering a low level of skew among sites, 23 sites would need to be monitored, and this decreases with the rate of decline being detected, so for a 50% decline over 10 years, 12 sites would need to be monitored yearly. Again to monitor larger roosts, a greater number would need to be monitored, so for *M. nattereri*, at 30%/10years 33 roosts would need to be monitored, or 13 to detect a decline of 50%/10years.

The considerations to put a monitoring scheme such as this in place are: a) how many sites are known at which monitoring could be conducted for the said species; and b) given this number and the roost sizes, the cost. Species with smaller roost sizes and a larger number of known roosts are more likely to meet these criteria, in this instance it may be more feasible and cost effective to implement this scheme to monitor *M. mystacinus* than *M. nattereri*. Based on our current knowledge of roost distribution and current laboratory costs, we can estimate the costs of running such a monitoring programme for each

species over a five year period: please give a very simple break down for each species, using the 30% decline target:

23 *M. mystacinus* roosts x 40 samples per year for five years with lab data generation and analysis of all samples after 5 years plus 20% overheads would cost an estimated €79,558.

33 *M. nattereri* roosts x 70 samples per year for five years with lab data generation and analysis of all samples after 5 years plus 20% overheads would cost an estimated €199,861.

To generate and analyse the data we would need to hire a trained postdoctoral researcher at level 1 of the Irish Universities Association for 12 months, estimated at €49,358.

The protocol for sample collection is fairly straight forward, and could be performed with minimal training, by either volunteers or NPWS rangers. However, it would require access to be gained to these roost sites once per year. Both laboratory and data analyses would require a person with a high level of skill to be employed to conduct this analyses yearly, or even at intervals of 5 years, as costed above.

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