The population genetics and breeding biology of the European nightjar

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PhD

University of York

Environment and Geography

June 2023

Abstract

Ecological and life history traits influence a species' vulnerability to population decline, with long-distance migratory birds and resource specialists at particular risk. Population genetic variation is imperative for population persistence and is ultimately maintained and regulated through population size, connectivity, and breeding behaviour. In this thesis I investigate the demographic history, spatio-temporal population genomics, genetic mating system and mate choice in a resource specialist, long-distance migratory bird, the European nightjar (*Caprimulgus europaeus*). Using Pairwise Sequentially Markovian Coalescence analysis applied to two nightjar genomes, I show that *C. europaeus* has been subject to significant climate driven changes in population size over the last 5 million years. Genomic analysis of museum and modern samples showed a significant 34.8% decline in genome-wide heterozygosity and shift from panmixia to weak spatial structuring in the British population over the last two centuries, likely owing to habitat loss and fragmentation. Nightjar were found to exhibit social monogamy and moderate extra-pair paternity rates (EPP; 23% of offspring were extra-pair) at two breeding populations in Britain, with EPP significantly higher where male density was elevated. As in most Caprimulgids, male nightjars display prominent white spots on the outer primary and rectrices. White spot size was found to correlate with age in male nightjars. Sires of extra-pair offspring possessed significantly smaller (16%) spots than birds who secured paternity with their social partners. My results suggest that EPP in nightjars is likely secured by lower-quality floater males, which is in alignment with my finding that male but not nest density correlated with EPP rate. My work reveals the genomic signature of population decline likely shared among long-distance migrants and resource specialists, highlighting the need for a wider application of genomic analysis across other species with similar life histories, particularly at range extremes. My results suggest that increasing connectivity between breeding grounds could reduce the genetic structuring and reverse the decrease in genetic diversity that has arisen in the last 200 years. With appropriate management, conserving range extreme populations may be valuable in preserving adaptive variation imperative for range expansion under future climate change scenarios.

" *A strange and somewhat mysterious bird, often heard but rarely seen except as a silent mothlike creature, the nightjar deserves to be better known than just as a disembodied sound in the twilight with a mythical reputation for stealing the milk of cows and goats*"

Tate P, 1989

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Acknowledgements

Firstly, I would like to thank my supervisors, **Professor Kathryn Arnold**, **Dr Dean Waters**, **Dr Kate Durran**t and **Professor Terry Burke** for their support throughout. Kate A has supported me hugely throughout the PhD and has always provided me with constructive advice and support whenever I needed it, despite being hugely busy whether under secondment to DEFRA or as dean of the Graduate Research School. Kate, your insight, and support has been invaluable, thank you. Dean came onboard my supervisory team during my first year and has provided me with invaluable modelling and statistics assistance, insight, and emotional support. One of my favourite memories from working with Dean, is early on in my project Dean suggested I include museum specimens in my population genomic analysis. This was quite quickly shot down by me and later hastily (and humbly) readopted once I had gained experience and realised the value in this sampling approach/research. Sorry, and thank you Dean for persuading me to produce what is now probably my favourite chapter! Kate D gave me a warm welcome when starting my PhD for which I was very grateful as a new and nervous first year! Kate introduced me to DNA collection via buccal swabs, a sampling method which was fundamental in enabling my citizen science based sampling strategy! Kate helped me manage the logistics of running a nation-wide sampling program for which I am very grateful. Throughout the multiple COVID lockdown myself and Kate authored a book chapter, forming Chapter 4 of this thesis. This was a hugely beneficial experience for me, and I appreciate Kate's endless patience with my spelling and grammar! I am very grateful for Terry's unparalleled experience and advice throughout my PhD. Terry's experience was invaluable when constructing the aims and direction of the thesis. I have enjoyed our face-to-face meetings, where the topic usually drifted towards birds and bird ringing quicker than it probably should have done. Split across three universities, managing four supervisors wasn't always easy, but I am very grateful for the diverse experience and perspective each have brought to the project.

My PhD would not have been possible without access to nightjar breeding populations across the country. I am particularly thankful to Natural England, Forestry Commission and Forestry Scotland who provided me with access to sites in South and North Yorkshire and Dumfries and Galloway. Particular thanks must be extended to the team at Humberhead Peatlands NNR, where the majority of my personal fieldwork was undertaken. The team (**Tim Kohler**, Neil Pike, Jannet Canning, David Blackham, Bryan Wainwright) were incredibly accommodating, providing me with accommodation on site, access, and 4x4 vehicle use throughout the two full field seasons I conducted there. Without access to these resources my field work and data

collection would not have been possible. I have been fortunate enough to have had a fantastic team of field workers help me over the last few years. Thank you to **Alex Brighton**, **Will Scott** and **Richard Cooper** for putting up with the baking heat, long nights, swarms of midges, mosquitoes and horseflies and hundreds of ticks over the last three years! Special thanks has to go to **Colin Neale** and **Paul Shawcroft** also for their tireless assistance in the field, having helped me over the last three years after assisting **Dr Lucy Mitchell** (a previous University of York PhD student) for four years beforehand. Thank you both. I am proud of all our hard work and am grateful to call you all friends. My gratitude is extended to 39 volunteer ringers and field workers (listed below) who helped collect data for me across 13 different nightjar breeding sites in the UK. Without the tireless work of these fieldworkers much of the research in this thesis would not have been possible. I cannot thank you all enough!

Ana Cowie	**Graham Scott**	**Lucy Mitchell**
Andy Lowe	**Greg Conway**	**Malcom Richardson**
Ann Ward	**Hugh Hanmer**	**Matilda Dolan**
Ben Dolan	**Ian Henderson**	**Mervyn Greening**
Chris Blakely	**Ian Nicholson**	**Mike Shearing**
Chris Brown	**Jim Baldwin**	**Nik Ward**
Colin Beale	**John Eyre**	**Oliver Padget**
Colin Neale	**Jon Avon**	**Paddy Jenks**
David Aitken	**José M. Zamora-Marin**	**Paul Hopwood**
Eleanor Ness	**Joshua Saunders**	**Paul Shawcroft**
Eric Wood	**Judith Read**	**Tom Bolderstone**
Fraser Rush	**Justin Walker**	**Tony Cross**
Graham Austin	**Kim Kirkbride**	**Tony Davis**

A huge amount of my time during the PhD was spent working in biomolecular laboratories. Not a natural lab worker, I am incredibly grateful to all the technicians for their patience and assistance throughout. Most of my lab work took place at the NERC Environmental Omics Training facility (NEOF), originally the NERC Biomolecular Analysis Facility - giving some idea of

how long I was there for! Indeed, I think I may hold the record for the longest staying visitor at the facility, a crown I wear proudly. **Dr Gavin Horsburgh** at NEOF Sheffield in particular trained me in all of my lab techniques and helped me throughout the countless trials and tribulations associated with sequencing and microsatellite genotyping. **Dr Deborah Dawson** also provided invaluable assistance when processing my microsatellite data. I am grateful also to **Dr Jamie Thompson** and **Rachel Tucker** for their help with sample extraction and preparation for sequencing. I extend my thanks to **Dr Graeme Fox** and **Dr Kathryn Maher** for their help and patience over the last year in training and helping me with my bioinformatics, along with the various meltdowns along the way. Again, I am not a natural bioinformatician but now feel confident in my abilities and find the process immensely rewarding. Thank you both again! My time at NEOF has provided me with new skillets in lab work and bioinformatics I will take with me into my future career. I am very grateful for the training I have received and the friends made. However, I will not miss the commute. During 2020, when I could not gain access to the NEOF facility, **Dr Thorunn Helgason** was kind enough to allow me to use her lab facility in the Biology Department at the University of York. Many months were spent extracting DNA here and I am extremely grateful for the help received from Thorunn and **Hannah Walker** during this time. Finally, I extend my thanks to the eight museums and respective curators who contributed tissue samples from nightjar skins to the project. These museums include Birmingham Museum, Liverpool World Museum, Natural History Museum, National Museum of Ireland, National Museum of Scotland, Royal Albert Memorial Museum, Cambridge University Museum and the York Museum Trust.

Throughout the PhD I have been fortunate enough to receive mentoring and support from a number of highly experienced people outside of my supervisory team. At the very start of my PhD, I was introduced to Lucy Mitchell, who worked on nightjar at Humberhead Peatlands prior to my project starting. Lucy has been a rock for me throughout my PhD and has provided me with help and support since meeting all the way back in 2018. **Dr Greg Conway** provided invaluable mentorship and help with fieldwork throughout the PhD, overseeing data collection at Thetford Forest. **Dr Andy Lowe** and **Ann Ward** were introduced to me by Kate Durrant in my first year, after which I was promptly invited round to their house for tea. There started the hospitality both have shown me throughout the PhD. Their knowledge of nightjar ecology and behaviour as well as their sites in Sherwood and Dorset are unparalleled, and I thank them both dearly for their help and support. **Tony Cross** is a ringer from mid-Wales and spearheaded the data collection at my Welsh sites. Tony is an incredible ornithologist and ringer and has been and continues to be an inspirational character in my life. **Dr Carlos Camacho** is a nightjar expert and an incredible font of knowledge and has provided me with valuable mentorship,

advice, and support in our meetings. **Professor Jon Slate** has provided expert input and advice during my last year of working on my project, his input with the bioinformatics feeding in Chapters 2 and 3 were invaluable. Finally, **Craig Ralston** has been a constant source of encouragement and support throughout the PhD, thank you Craig.

I thank my friends who have supported me and have put up with my tiredness, late night work sessions, and complaining, this particularly the case for my current and former housemates **Robert Kirby**, **James Worth**, **Jenny Close** and **Rebecca Leake**. I am incredibly grateful for the friends I've made during this Phd; **Lauren Rawlins**, **Lucy McMahon, Katey Valentine**, **Nat Anderson**, **Alex Burkitt**, **Ruochan Ma** and **Abby Mycroft**. Thank you all for being such wonderful friends, I honestly couldn't have asked for a better bunch to see this PhD through with. Finally, and most importantly I would like to thank my family and my loving girlfriend, Sarah. My mum and dad have always supported me. They steered me arguably down the right direction way back at the end of A Levels when I was contemplating pursuing life as a 'professional angler' (!), convincing me to perhaps study ecology at university instead. All I can say is I am happy I chose to listen and follow the latter path. My love for birds was sparked by my dad, having spent many a morning bird watching together as a child. Those mornings may have been lost on young George, but I am certainly grateful for them now. Cheers Dad! My mum has provided me with support and motivation at every turn and has convinced me more than once to stick at it when the going got tough! Thank you, mum, I needed that! My parents have always been my biggest supporters, although this crown has now arguably been taken by my girlfriend Sarah. I met Sarah during the first year of my PhD. I now can't imagine my life without her and her daughter Holly. You have both done so well to deal with my bizarre working hours, long absences, and stress. I am eternally grateful for your love and understanding. Thank you and I love you both.

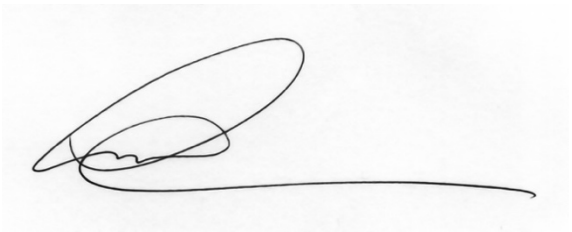
Authors Declaration

I declare that this thesis is original work produced by me as a PhD student at the University of York's Geography and Environment Department. This work has not been presented before for an award at the University of York or any other institution prior to this date. All sources are cited and referenced at the end of the document. The research presented in this thesis was funded by the Natural Environment Research Council (NERC) Adapting to the Challenges of Changing Environment (ACCE) Doctoral Training Partnership.

Chapter 2 has been submitted for publication in an international peer-reviewed journal (*Journal of Avian Biology*) and is currently undergoing review. All author contributions are outlined in in the 'Statement of Contributions' section. The manuscript from this work has been reformatted and adjusted for incorporation in the thesis as **Chapter 2**.

Chapter 4 was written as a book chapter, synthesising current knowledge surround Caprimulgid mating systems, sexual selection, and visual communication, presenting Caprimulgids as a study system for addressing central hypotheses in these sectors. The work has been peer-reviewed and accepted for publication in "*Nightjars: From Mystery to Model in Ecology and Evolution*" (Camacho, in press). Again, author contributions have been outlined in the 'Statement of Contributions' section. The manuscript from the chapter has been reformatted for inclusion in the thesis as **Chapter 4**.

Signed:



Date: 25/06/2023

Statement of Contributions

Chapter 2

This chapter was submitted to '*The Journal of Avian Biology*' and has been reformatted for inclusion in this thesis. Idea conceptualisation was carried out by me, Jon Slate (University of Sheffield) and Terry Burke (University of Sheffield). Sample collection was undertaken by me. Initial lab work was conducted by me, Rachel Tucker (University of Sheffield), Gavin Horsburgh (University of Sheffield) at the NERC Environmental Omics Facility at the University of Sheffield. Sequence library perpetration and sequencing as well as initial read cleaning was undertaken by the Centre for Genomics Research facility at the University of Liverpool. Bioinformatics was undertaken by me with assistance from Graeme Fox (University of Sheffield, now University of Nottingham), Helen Hipperson (University of Sheffield) and Jon Slate. Manuscript preparation was completed by me and reviewed by all co-authors.

Authors: George Day, Prof. Kathryn Arnold, Dr Kate Durrant, Dr Dean Waters, Prof. Terry Burke, Dr Gavin Horsburgh, Dr Kathryn Maher, Dr Graeme Fox, Dr Helen Hipperson, Rachel Tucker, Prof. Jon Slate

Chapter 3

Idea conceptualisation was carried out by me, Dean Waters (University of York), Terry Burke and Kathryn Arnold (University of York). Sample collection was undertaken by me as well as multiple citizen scientist teams (see Acknowledgements) which collected nightjar DNA samples from across the country. Access to museum samples was facilitated by museum curators (see Appendix Table 7.2 for list of museums), with tissue samples collected by curators in all cases with the exception of the Birmingham Museum and York Museum Trust where tissue samples were collected by me. Initial lab work (sample extraction, quantification, and initial sample preparation for sequencing) was undertaken by me, Jamie Thompson (University of Sheffield), Rachel Tucker and Gavin Horsburgh at NERC Biomolecular Analysis Facility at the University of Sheffield. Library preparation, sequencing and initial read trimming was performed by the Centre for Genomics Research facility at the University of Liverpool. Bioinformatics was performed by me with assistance from Kathryn Maher (University of Sheffield), with data analysis conducted by myself. Manuscript preparation was conducted by me, with review and

amendment suggestions made by Kathryn Arnold, Dean Waters, Kate Durant (University of Nottingham), Jon Slate and Terry Burke.

Authors: George Day, Dr Kate Durrant, Dr Dean Waters, Prof. Terry Burke, Prof. Jon Slate, Dr Gavin Horsburgh, Dr Kathryn Maher, Dr Jamie Thompson, Prof. Kathryn Arnold

Chapter 4

This chapter was written initially as a book chapter for '*Nightjars: From Mystery to Model in Ecology and Evolution*' (Camacho, in press). Idea conceptualisation was carried out by myself, Kate Durrant and Carlos Camacho (Lund Univeristy, now Spanish National Research Council). Literature synthesis and manuscript preparation was carried out by me with assistance from Kate Durrant. Manuscript was amended after peer-review, with final amendments made by Carlos Camacho.

Authors: George Day and Dr Kate Durrant

Chapter 5

Myself, Kathryn Arnold, Kate Durrant, Dean Waters and Terry Burke were responsible for Initial conceptualisation. Data collection was carried out by me and field assistants at Humberhead Peatlands, and Greg Conway and team at Thetford Forest. Lab work and initial analysis was carried out by me, with assistance from Gavin Horsburgh and Deborah Dawson (University of Sheffield). Data analysis and manuscript preparation was then conducted by me, with feedback provided by Kathryn Arnold, Dean Waters and Kate Durrant.

Authors: George Day, Dr Kate Durrant, Dr Dean Waters, Prof. Terry Burke, Dr Gavin Horsburgh, Dr Deborah Dawson, Prof. Kathryn Arnold

Chapter 6

Myself, Kathryn Arnold, Kate Durrant, Dean Waters and Terry Burke were responsible for conceptualisation. Data collection was carried out by me and field assistants at Humberhead Peatlands, and Greg Conway and team at Thetford Forest. Lab work and initial analysis was carried out by me, with assistance from assistance from Gavin Horsburgh and Deborah Dawson

(University of Sheffield). Data analysis and manuscript preparation was then conducted by me, with feedback provided by Kathryn Arnold, Dean Waters and Kate Durrant.

Authors: George Day, Dr Kate Durrant, Dr Dean Waters, Prof. Terry Burke, Dr Gavin Horsburgh, Dr Deborah Dawson, Prof. Kathryn Arnold

Chapter 1 General introduction

Declines in biodiversity worldwide are well documented (Cardinale et al., 2012; Lanz, Dietz and Swanson, 2018; Isbell et al., 2023), although this decline is heterogeneously distributed across taxa, with differing ecological and life history characteristics ultimately affecting species vulnerability (Reynolds, 2003; Clavel, Julliard and Devictor, 2011). Genetic and now genomic approaches provide valuable tools in species conservation, enabling insight into historic demography, population connectivity (i.e. gene flow), species mating systems and breeding behaviour and importantly population genetic variation (Frankham, Ballou and Briscoe, 2010; Allendorf et al., 2022). A great deal of effort has been paid to understanding the mechanisms underlying population decline in highly threatened species in order to staithe future reductions in population size (e.g. Cortes-Rodriguez et al., 2019; Feng et al., 2019; Ramstad and Dunning, 2021; Dussex et al., 2021; Cavill et al., 2022; Santos et al. in press). However, considerably less attention has been paid to species that are showing shallower but inexorable population declines. This thesis seeks to apply behavioural, genomic and genetic approaches to European nightjar (*Caprimulgus europaeus*), a species which is data poor and has experienced a recent history of population decline (Conway et al., 2007; Langston et al., 2007). Specifically, I aim to reveal the species' ancient demographic history, more recent (1840-present) spatio-temporal changes in genetic diversity and structure and characterise European nightjars' mating system and mate choice. In this chapter I broadly outline biodiversity and specifically avian population declines, introducing the key topics underlying my work, the methodological approaches commonly used and current knowledge surrounding the species.

1.1 Biodiversity, avifauna, and the Anthropocene

1.1.1 Changes in global biodiversity

Species and populations fluctuate in size over time and space in response to changing climate and habitat availability (Hewitt, 1999; Nadachowska-Brzyska et al., 2015; Caro et al., 2022). Ancient changes in global climate have driven cyclical periods of glacial expansion and retreat, causing shifts in distribution and abundance of animals, ultimately driving speciation and population divergences as populations of the same species are restricted to different refugia (Hewitt, 1999; Hansson et al., 2008; Nadachowska-Brzyska et al., 2016; de Greef et al., 2022). However, dramatic and rapid periods of global environmental change can result in mass extinction events (Bond and Grasby, 2017). It has been widely cited that we are currently within the sixth mass extinction (Wake and Vredenburg, 2008; Barnosky et al., 2011; Ceballos et al., 2015), being the first mass-extinction to be driven solely by anthropogenic causes (Johnson et al., 2017). Human activities have led to an accelerating global loss in biodiversity (Gaston, Blackburn and Goldewijk, 2003; Brooks et al., 2006; Butchart et al., 2010), with the

average abundance of native species in most terrestrial habitats having declined by ≥ 20% since 1900 (Watson et al., 2019). For the first time, a single species (humans) is causing a significant and rapid change in the Earth's climate (Taylor et al., 2013). Increased production of greenhouse gases such as carbon dioxide (CO_2) and methane (CH_4) are leading to increasing global temperatures, placing further pressure on species globally (Habibullah et al., 2022). The increase in temperature and namely CO_2 over short timescales can lead to more extreme weather events (i.e. floods, droughts and cyclones), ocean acidification, aridification, and habitat loss, with all having significant implications for biodiversity (Habibullah et al., 2022). Global warming will likely exceed 1.5°C by 2100 (Masson-Delmotte et al., 2018), exacerbating the aforementioned effects, having significant implications for biodiversity, including birds, driving range shifts (Voskamp et al., 2022), population declines and changes to species assemblages in terrestrial communities (Voskamp et al., 2022).

Further to global warming, habitat loss and fragmentation presents one of the greatest threats to biodiversity (Dirzo and Raven, 2003; Hanski, 2011), including birds (Lees et al., 2022), with the effects exacerbated by climate change (Huntley et al., 2008; Voskamp et al., 2022). Humans have been shaping their environment since the advent of farming approximately ~11 Kya (Ruddiman, 2005). Agriculturalization of society led to widespread clearance of forests, drainage of lowland grasslands, wetlands and peat bogs (Ruddiman, 2005). The rapid urbanisation and agricultural intensification of the 19th and early 20th centuries led to an increase in the intensity of habitat loss, being particularly pronounced throughout much of Europe and North America (Ratcliffe, 1984; Stoate et al., 2001). Globally, deforestation accounts for the most common form of habitat destruction, with the majority of deforested areas (70%) converted to agriculture (Scanes, 2018). In Europe >50% of original forest cover has been lost over the last two centuries (Wallerstein, 2011), with the majority converted to anthropogenic land uses (Bastrup-Birk et al., 2016). Indeed, temperate woodland and heathland underwent significant loss and fragmentation across Europe, and particularly the UK, throughout the 19th and 20th centuries (Stanturf and Madsen, 2002; Laurance, 2010). Whilst some bird species may benefit from urbanisation (e.g. Peregrine falcons *Falco peregrinus*; Kettel et al., 2019), European bird populations have largely suffered significant population declines, with woodland avifauna in particular experiencing losses only surpassed by farmland birds (Gregory, 2006). Focusing on heathland, within the UK lowland heathland underwent ~83% reduction in land coverage between 1800 and 1995 (Department of the Environment, 1995; Newton et al., 2009). Consequently, species specialising in both heath and woodland have been significantly impacted by the decline and fragmentation of the habitats (Langston et al., 2007).

1.1.2 Ecological and life history traits associated with population declines

Patterns of population decline are not homogeneously distributed, with certain ecological and life history traits associated with a heightened vulnerability to population decline (Both et al., 2006; Clavel, Julliard and Devictor, 2011; Stirnemann et al., 2016; Correll et al., 2019). Here I focus on A) dietary and habitat specialisation and B) long-distance migratory behaviour as key ecological and life history traits influencing vulnerability in birds. Species can be broadly split into specialists and 'generalists'. Specialists are typically uniquely adapted to utilise a specific resource, such as a particular habitat or dietary requirement (Correll et al., 2019). Following Ecological Niche Theory (Hutchinson, 1957), niche width can effectively demonstrate the difference between generalist and specialist species, with generalists exhibiting a wider niche width than specialists (see Fig 1.1; Clavel, Julliard and Devictor, 2011). Habitat generalist species are able to use a suite of different habitat types and typically require a less restrictive diet (Wilson et al., 2008). Perhaps unsurprisingly, habitat and dietary specialist species are more vulnerable to anthropogenic pressures (habitat loss, degradation, fragmentation and global warming) than generalists (Correll et al., 2019). Specialist species are less able to cope with changing environments, habitat degradation and loss than generalists (Clavel, Julliard and Devictor, 2011; Correll et al., 2017, 2019). The evolution of specialisation requires relative environmental stability over time (Clavel, Julliard and Devictor, 2011). Rapid environmental change then does not favour specialist species, being less able to adapt than generalists, and historic mass-extinction events have usually led to greater losses of specialist species than generalists (McKinney, 1997). Under current rapid anthropogenic-driven environmental change, habitat and dietary specialists are largely demonstrating more severe changes in population size and distribution than generalists (Bergamini et al., 2009; Clavel, Julliard and Devictor, 2011; Correll et al., 2017, 2019; Bowler et al., 2019). Over large spatial scales the trend in land use change towards habitat homogenisation and simplification (i.e. intensive agriculture; Donald et al., 2001; non-native plantation forests; see Brockerhoff et al., 2008 for review) leads to communities with fewer habitat or dietary specialist species (Beger, 2021).

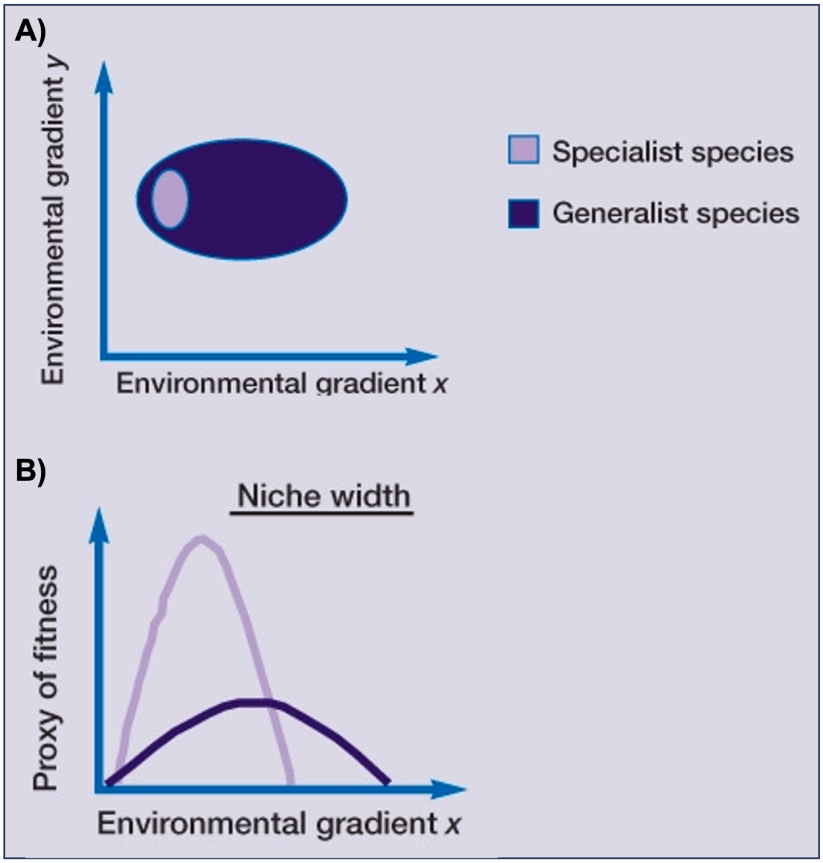


Figure 1.1 Concept of ecological niche occupation by specialist and generalist species across two different measures of ecological niche, A) shows nice width and B) 'fitness' across an environmental gradient. Figure adapted from Clavel, Julliard and Devictor (2011).

In birds, dietary and habitat specialists are typically more threatened than generalist species (Bowler et al., 2019). Within Europe, dietary specialists birds exhibited declining population trends (insectivores; -13%, seedeaters; -28%) throughout the late 20th and early 21st centuries (1995-2015), whilst generalist species exhibited stable trends (Bowler et al., 2019). Dietary specialisation again reduces the ability for a species or population to adapt to rapid changes in prey availability. Many birds are insectivore dietary specialists or demonstrate a high level of insectivory during the breeding season (Bowler et al., 2019). However, invertebrates have shown marked declines globally (Sánchez-Bayo and Wyckhuys, 2019; van der Sluijs, 2020) thought to be broadly driven by changes in land use and increased use of pesticides (van der Sluijs, 2020). Lepidoptera (moths and butterflies) have exhibited particularly significant changes in Europe within the last century (31% decline in Britain 1969-2016; Bell et al., 2020) (see Fig 1.2; Wagner et al., 2021). Declines in prey availability have significant implications on insectivorous specialists avifauna (Bowler et al., 2019). Prey availability ultimately impacts the ability of an individual to meet its energetic requirements, and can ultimately influence population productivity, density, and abundance (Bowler et al., 2019). Climate change is

thought to drive changes in the timing of emergence in many invertebrate species, as well as flowering plants (Both et al., 2009; Brooks et al., 2017; Mayor et al., 2017). This temporal shift in resource availability has been shown to lead to phenological mismatching between the arrival of migratory bird species on breeding grounds with prey availability (e.g. Thackeray et al., 2010; Mayor et al., 2017). This in turn can significantly impact fitness and population persistence (Both et al., 2006; Saino et al., 2011; Mayor et al., 2017).

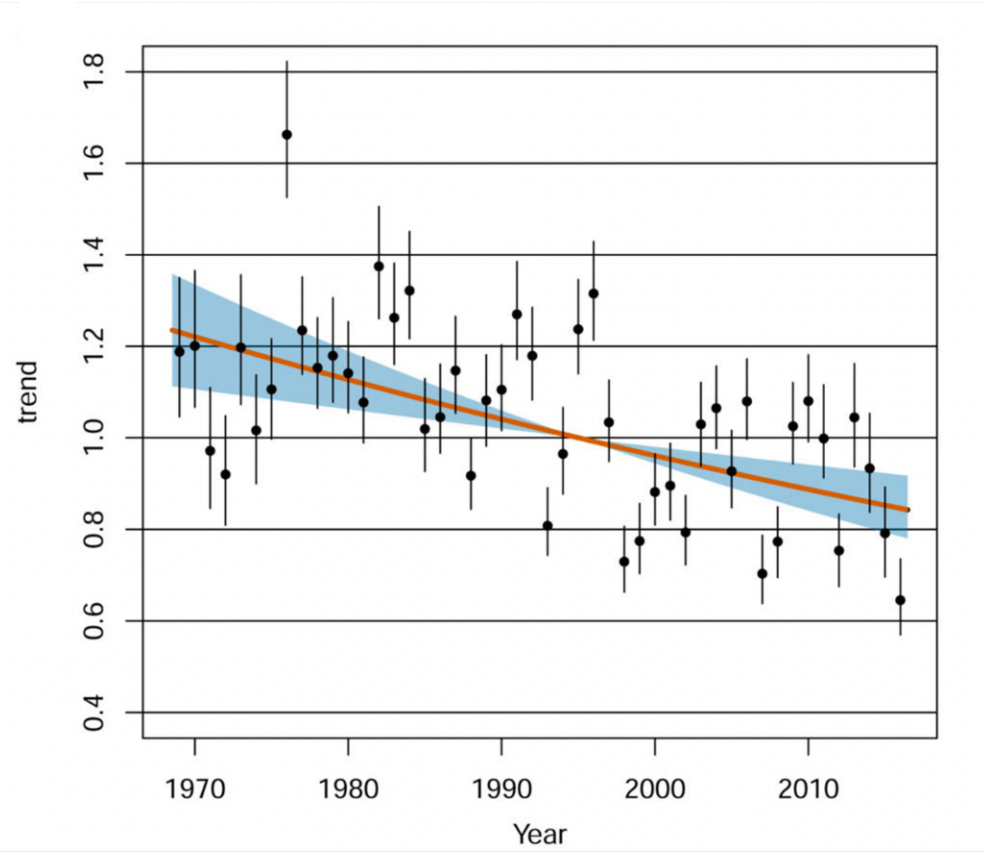


Figure 1.2 Change in moth abundance over time in Britain. Figure shows results of log-linear GLMM for population index of moths with random effects (dots and whiskers) reflecting the yearly mean and variances, with the blue shading showing the 95% confidence intervals. Figure from (Bell, Blumgart and Shortall, 2020). Downloaded from https://shorturl.at/qKNS9 on 20/05/2023.

Species may be typified by the 'speed' of their life history (see the 'slow-fast continuum'; Sæther, 1988; Stearns, 1992), whereby those exhibiting a 'fast' life history are typified by usually a high fecundity (i.e. multiple large broods in birds; Stearns, 1992; Winger and Pegan, 2021) and low inter-annual survival rates (Winger and Pegan, 2021). Conversely, species with slow life histories are typically long-lived (high survival), exhibit a delayed sexual maturity and a lower fecundity (i.e. few, small broods) (Stearns, 1992; Winger and Pegan, 2021). Consequently, the placement of a species along this continuum impacts vulnerability to population decline and subsequent recovery (Sæther, Ringsby and Røskaft, 1996). Species with

fast life histories can show quite rapid recoveries from previous poor breeding seasons (Sæther, Ringsby and Røskaft, 1996; Sæther and Bakke, 2000; Koleček, Albrecht and Reif, 2014), whilst those exhibiting a slow life history may take multiple breeding seasons to recover (Collen et al., 2009; Stirnemann et al., 2016). Indeed, species with slow life histories are usually characterised by low population growth and are more threatened by recent and dramatic environmental perturbations than those exhibiting a fast life history, with extinction risk increased particularly in habitat specialists exhibiting slow life histories (Koleček, Albrecht and Reif, 2014). These life history traits are also important in determining a species breeding behaviour and long-lived 'slow' species of birds are typically characterised by monogamous mating systems, with low levels of infidelity and high levels of parental and specifically paternal, investment (Valcu et al., 2021; Liu et al., 2023; but see Huyvaert and Parker, 2010; Quillfeldt et al., 2012). However, species with fast life histories are usually typified by much higher infidelity rates (Wink and Dyrcz, 1999; Brouwer and Griffith, 2019).

Birds demonstrate unparalleled vagility among vertebrates, including long-distance migration spanning multiple biomes, continents, and hemispheres. Approximately 48% of all bird species globally are exhibiting declining population trends (Lees et al., 2022). Within Europe between 1980 - 2017 breeding bird abundance declined by ~18% across 378 species (Lees et al., 2022). Long-distance migratory species are particularly vulnerable to environmental change (58% of 419 migratory species reviewed by Lees et al. 2022 demonstrated declining population trends), with species affected by environmental stressors across large spatial scales often spanning multiple biomes over continental and global-scales (Vickery et al., 2014; Yong et al., 2021). This elevated vulnerability in migratory birds then also encompasses anthropogenic pressures including global warming, habitat loss, fragmentation, hunting and disturbance (Kirby et al., 2008; Faaborg et al., 2010; Zurell et al., 2018). With disproportionate declines recorded in migrant avifauna (i.e. 23% of long-distance migratory species in Europe showed declining trends between 1980-2009, whilst resident and short distance species declined by 7% over the same time period; (Vickery et al., 2014), long distance migrants with restricted non-breeding ranges, have been shown to suffer more severe declines than non-range restricted or resident species (Sanderson et al., 2006; Gilroy et al., 2016). Subject to environmental constraints at geographically distinct breeding, wintering and migratory stop-over sites, migrants are subject to carryover effects meaning that environmental constraints felt at one location can negatively drive fitness and population dynamics at another (Newton, 2010; Hewson et al., 2016). For example, habitat loss as well as rainfall in the Sahel region of Africa have been shown to significantly impact fitness and survival of migratory passerines breeding in Europe (Vickery et al., 2014; Johnston et al., 2016; Walther, 2016). Illegal persecution along species' migration

routes places further pressure on populations, with illegal killing of migratory avifauna in the Mediterranean resulting in 11-36 million birds killed annually, accounting for 1-1.3% of threatened and near-threatened species populations (Brochet et al., 2016). Long distance migratory avifauna are at greater risk to the effects of global warming, as well as phenological asynchrony (see previous paragraph). Furthermore, migratory species are less able to quickly respond to climate driven conditions (i.e. meteorological, habitat suitability etc.) changes on breeding grounds than resident species (Gilroy et al., 2016). This is owing to spring phenology in migratory species being driven by the conditions experienced on the wintering grounds (Gordo et al., 2005), impairing a species' ability to react to rapid changes in conditions on their breeding grounds often many thousands of kilometres away compared with species resident on the temperate breeding grounds.

Species have been adapting to constantly changing environments for millennia. However, abrupt environmental changes leave many species unable to respond rapidly. Species with highly specialised niches (i.e., habitat and dietary specialists) are further less amenable to change than generalists. Migratory birds, which often exhibit a degree of habitat and dietary specialisation (i.e., insectivores) are also handicapped to respond to rapid environmental change, with less flexible phonologies impacting their ability to respond to environmental change on breeding grounds and along their migratory route. Historic and increasing anthropogenic pressure on habitat availability and climate change have exacerbated these constraints leaving migratory and specialist species under significant threat.

1.2 Conservation genetics

The field of conservation biology has led to fundamental changes in policy, large-scale habitat restoration and protection as well as species captive breeding and reintroduction programs (i.e. California condor *Gymnogyps californianus*; Wallace, 2012). Among birds, conservation efforts have helped at least partially reverse declines and have improved conservation status in 70 bird species (Lees et al., 2022). Nevertheless, this figure is outweighed by 391 species which have exhibited declines in the Red List Index (Lees et al., 2022). The question of how to effectively conserve biodiversity presents a vital but difficult and complex question (Pimm et al., 2001). Conservation biology is constituted of multiple subdivisions, which together provide information pertaining to population trends, threats and status, behaviour, and social science, needed to make effective conservation measures. Conservation genetics is one of these subdivisions and is providing an increasingly vital tool in the conservation biologist's armoury

(Frankham, Ballou and Briscoe, 2010). Conservation genetics can be defined as using genetic methods to solve problems in conservation biology (Allendorf et al., 2022). Since the 1980's the application of genetics to the conservation of wild species gained traction, with the value of conservation genetics now well recognised (Frankham, Ballou and Briscoe, 2010; Allendorf et al., 2022).

1.2.1 Genetic diversity

The study of genetic diversity is central to conservation genetics (Frankham, Ballou and Briscoe, 2010; Allendorf et al., 2022). Genetic diversity represents an indicator of biodiversity (Martínez-Jauregui et al., 2021), being the most basal form of biodiversity recognised by the IUCN deserving of conservation, following species and ecosystem diversity (Allendorf et al., 2022). It is then firstly important to understand what genetic diversity is. Genetic diversity refers to the magnitude of genetic variability within a population (Hughes et al., 2008). Genetic variability ultimately originates from variations in nucleotide sequences within a DNA molecule arising largely through mutations and recombination (Ellegren and Galtier, 2016). Mutations may be advantageous, deleterious, or neutral (i.e. not subject to selection; Neale and Wheeler, 2019), with the majority being neutral (Kimura, 1983; Loewe and Hill, 2010). Alongside mutations, recombination acts as a vital source of genetic variation, producing novel allelic combinations (Bromham, 2016). Recombination occurs during meiosis, specifically during prophase 1 of meiosis where the DNA strands within the paired homologous chromosomes exchange genetic material with one another, effectively producing chromatids of both maternal and paternal heritage. During recombination multiple alleles may swap from one chromosome to another. The result of recombination is that each chromosome after anaphase 1 possesses a unique combination of alleles, and thus provides a source of genetic variation (Bromham, 2016). Ultimately, genetic variation provides the fundamental source of evolution (Frankham, Ballou and Briscoe, 2010; Allendorf et al., 2022). At an individual level, a low genetic diversity is linked with low fitness, including reduced fertility and increased mortality (e.g. increased disease risk and higher probability of developing some cancer; (Spielman et al., 2004; Ujvari et al., 2018). At a population and species level a low genetic diversity can contribute towards extinction risk, limiting population growth and reducing the ability of a population to adapt to stochastic change (Bürger and Lynch, 1995; Frankham, Ballou and Briscoe, 2010; Hohenlohe, Funk and Rajora, 2021).

At a population level, genetic diversity is mediated via immigration and emigration (gene flow) as well as genetic drift (Bromham, 2016). Genetic drift describes the random change in alleles (variants of genes) within a population over time, with variation lost via death and emigration (Frankham, Ballou and Briscoe, 2010). The effects of genetic drift are more pronounced in small populations (Frankham, Ballou and Briscoe, 2010). Therefore the size and distribution of a population, as well as gene flow, influence genetic diversity, with these factors mediated by individual behaviour (Allendorf et al., 2022). Across a species range the distribution of genetic diversity is typically not consistent. Variations in gene flow among breeding populations can lead to genetic structuring, and in extreme cases complete cessation of gene flow between breeding populations and ultimately isolation (Frankham, Ballou and Briscoe, 2010). Populations at the periphery of a species range usually exhibit lower genetic diversity than those at the range centre (Lesica and Allendorf, 1995; Eckert, Samis and Lougheed, 2008). This can be broadly attributed to reduced and mostly uni-directional gene flow toward range extremes from range centre populations (Lesica and Allendorf, 1995; Eckert, Samis and Lougheed, 2008). Conversely, high gene flow among populations usually leads to an overall elevated and homogenised levels of genetic diversity among a population. The directionality (i.e. whether gene flow operates in a uni- or multi-directional manner) and strength of gene flow are then important in influencing a population's genetic diversity (Frankham, Ballou and Briscoe, 2010). When studying population genetic diversity it is therefore important to consider the dispersal potential and vagility of the study species. A higher dispersal potential is often associated with increased gene flow, as species are able to move greater distances and traverse potential barriers to gene flow (i.e., unsuitable habitats, oceans, mountain ranges; Frankham, Ballou and Briscoe, 2010). Migratory birds provide a good example of this, being highly vagile, the majority of long distance migratory species show no or weak genetic structure across their breeding ranges (Pârâu and Wink, 2021; Ralston et al., 2021; Shephard et al., 2022; but see **Chapter 3**). Conversely, where a species dispersal ability is low, populations are more likely to become isolated, reducing gene flow and thus genetic diversity within the affected populations (Allendorf et al., 2022). A low dispersal potential is then inversely related to the differentiation among populations, with lower gene flow increasing differentiation.

Changes in population size over time arguably account for the greatest factor influencing genetic diversity in wild populations. Large, stable populations mostly show consistent levels of genetic variation over time, whilst small populations typically demonstrate reducing trends in genetic diversity over time (Allendorf et al., 2022). Dramatic reductions in population size can lead to genetic bottlenecking (Frankham, Ballou and Briscoe, 2010). For example, the genetic bottleneck experienced by the Mauritius kestrel (*Falco punctatus*) saw the species reduced to a

single pair in 1974, losing approximately 57% of the heterozygosity in the contemporary population, despite subsequent demographic recovery (Nichols, Bruford and Groombridge, 2001). A key detrimental impact of genetic bottlenecks can be the proliferation of rare deleterious alleles within a population owing to increased homozygosity rates (Allendorf et al., 2022). Following population bottlenecks inbreeding rates can increase, having further consequences for population genetic diversity and population persistence (Frankham, Ballou and Briscoe, 2010). Whilst not exclusively associated with small population sizes, bottlenecks and severely reduced population size most commonly drive inbreeding in wild populations (Keller and Waller, 2002). Inbreeding can further reduce the number of alleles able to be passed on to the next generation, reducing heterozygosity and increasing homozygosity (Charlesworth and Willis, 2009). Again, elevated homozygosity rates can lead to the expression of deleterious recessive alleles and in turn inbreeding depression (the reduced survival and fitness of inbred offspring; Charlesworth and Willis, 2009) in the population (Kardos et al., 2016). The effects of inbreeding depression can accumulate to constrain the population growth rate and elevate extinction risk (Kardos et al., 2016). For example, in the New Zealand Hihi (*Notiomystis cincta*) Brekke et al., (2010) and Duntsch et al., (2023) demonstrated that inbreeding depression impacted fitness, reducing offspring survival. The study population of Hihi had undergone a recent significant bottleneck owing to the population being translocated as well as several historic bottlenecks prior to translocation (Brekke et al., 2010).

Many of the world's threatened species are characterised by low genetic diversity (Willoughby et al., 2015; Kleinhans and Willows-Munro, 2019), showing negative trends over time where temporal data are available (e.g. Feng et al., 2019). Despite their inherent vagility and high dispersal potential, birds have not escaped this pattern (Fig 1.3; Li et al., 2014). Severely threatened and isolated avifauna, such as small island endemics (i.e. Seychelles magpie-robin, *Copsychus sechellarum*) and those having suffered significant bottlenecks (i.e. Crested ibis, *Nipponia nippon*) exhibit low genetic diversity in remaining populations, with heterozygosity in these populations being significantly lower than non-threatened avian taxa (Fig 1.3; Feng et al., 2019; Cavill et al., 2022).

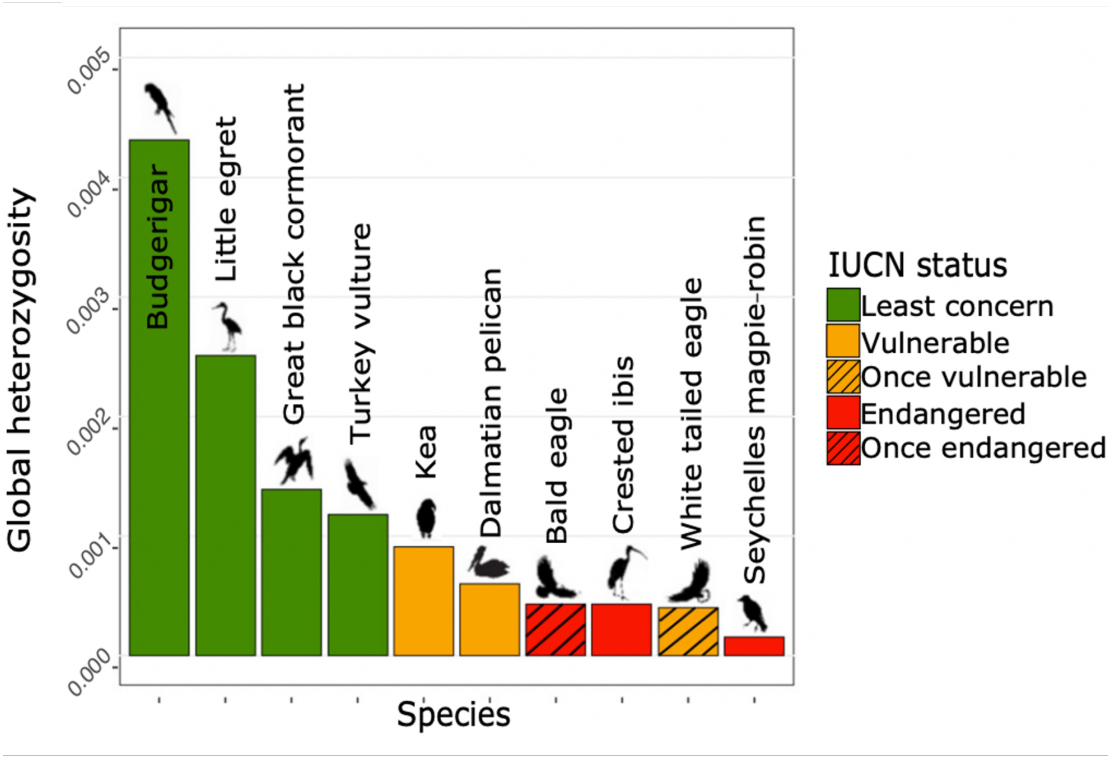


Figure 1.3 Global (genome wide) heterozygosity from 10 birds across different IUCN status categories. Figure taken from Cavill et al. (2022). Downloaded from https://shorturl.at/gruUY on 20/05/2023.

1.2.2 Studying genetic variation in wild population

Understanding the evolutionary, environmental, and behavioural forces shaping the distribution of genetic variation among populations forms the fundamental basis of conservation genetics. Below, I outline the techniques used in studying genetic variation in wild populations, from commonly used sampling materials and genetic markers through to the methodological approaches employed by conservation geneticists to studying genetic structure in wild populations.

1.2.2.1 Sampling material

Perhaps the most fundamental prerequisite of genetic analysis is access to genetic material (DNA, RNA, mtDNA etc.). The invention of the Polymerase Chain Reaction (PCR) (Mullis et al,. 1986) was transformative in the field of molecular biology, amplifying target DNA (loci) by many thousands or millions of times, allowing for samples hosting relatively small DNA yields to be taken (Burke et al., 1991; Bromham, 2016). Genetic sampling can be broadly separated into invasive, less invasive and non-invasive methods and these are addressed below;

Invasive Methods – Typically the highest DNA yields can be attributed to invasive methods, including blood and tissue sampling. Blood sampling remains the most commonly used approach to collect genetic material from birds (Voss, Shutler and Werner, 2010). Birds possess nucleated red blood cells, enabling very high DNA yields (i.e. 257 ng/µl; Handel et al., 2006) and amplification success (Funk, Mullins and Haig, 2007). Soft tissue may also be taken from deceased birds or via a biopsy, again providing high yields (Turcu et al., 2023). However, both blood sampling and biopsies are limited by the stress caused to the sampled individual and elevated risk of injury or mortality (Voss, Shutler and Werner, 2010; Turcu et al., 2023). Furthermore, both methods require a high level of expertise, rigorous training and specialised equipment, also contained by legislature (depending on the sampling country), imposing logistical and ethical limitations on their use for large-scale data collection.

Less invasive methods – Less-invasive sampling methods are increasingly providing viable sources of DNA in population genetics and evolutionary biology studies (Handel et al., 2006; Yannic et al., 2011; Pande et al., 2018; Vilstrup et al., 2018). In birds, the most used less-invasive methods include feather and epithelial cell sampling. Feathers may be collected via plucking or collection of moulted feathers from the environment (e.g. from nest sites; Rudnick et al., 2009), with the former resulting in greater yield and amplification successes (Turcu et al., 2023). Swabs can be used to collect epithelial cell samples from either the mouth (buccal swabs) or cloaca (cloacal swabs). Both can enable relatively high DNA yields and amplification success rates (Handel et al., 2006; Turcu et al., 2023). The method has the advantage of high match certainty between sampling and the targeted individual, making it appropriate for studies where individual identity must be certain such as in studies assigning genetic parentage. An issue with either buccal or cloacal swabbing is contamination, for example from prey DNA in mouth swabs, with the inclusion of PCR inhibitors in faeces common when using cloacal swabs (Asawakarn et al., 2018). Of the two methods, buccal swabs appear to present the most viable, with fewer PCR inhibitors and comparatively higher yields and amplification success rate (Asawakarn et al., 2018; Turcu et al., 2023).

Whilst training is required to conduct either sampling method the cost, expertise and specialised equipment required is less in swab and feather sampling than the invasive methods outlined. This enables for potentially large-scale sample collection using these methods.

Non-Invasive Methods – Non-invasive methods for sampling avian genetic material include the collection of eggshells, faecal samples and environmental DNA (eDNA). Whilst these methods

provide viable sources of DNA, contamination and inclusion of PCR inhibitors remain key limiting factors (Asawakarn et al., 2018; Neice and McRae, 2021). Nevertheless, contamination by prey items also highlights the use of faecal samples for characterising the diet of the target species through molecular barcoding analysis (e.g. Mitchell et al., 2022). However, such non-invasive methods rarely allow for certain associations to be made between the sample and the sampled individuals in the population, reducing the method's value in studies when individual identification is required. This is also an obvious limitation of eDNA, which involves extracting DNA from substrates. However, the method has so far been primarily used to simply identify presence/absence of species, providing a potentially valuable and cost-effective tool for monitoring difficult to study and rare taxa (Neice and McRae, 2021). Finally, DNA may be extracted from historic or ancient museum specimens (i.e. bones, feathers, study skins etc.; Stronen et al., 2019; Tsai et al., 2019). Tissue samples, such as superficial skin scrapes from foot and toe pads are the most common method for sampling historic DNA (hDNA) from preserved avian museum specimens, providing the highest yields, often without compromising the taxonomic value of the specimen (Sigurðsson and Cracraft, 2014; see Section 1.2.2.3.2 for more details).

1.2.2.2 Genetic markers and sequencing

Genetic markers are a valuable tool for estimating genetic variation within and between individuals. A variety of molecular markers have been used in population and conservation genetic studies to measure genetic variation, including allozymes, mtDNA, microsatellites and in more recent years single-nucleotide polymorphisms (SNPs) derived from reduced capture and full-genome sequencing methods (Fig 1.4; Allendorf et al., 2022).

Mitochondrial DNA - Mitochondrial DNA (mtDNA) (Fig 1.4) in animals is arranged in a closed circular loop within the Mitochondria and is highly ubiquitous (Fig 1.4). mtDNA is predominantly non-recombining, maternally inherited and evolves at a faster rate than nuclear DNA (Arif and Khan, 2009). mtDNA has and continues to be used readily in population genetics and phylogenetics studies alike (e.g. Larsen et al., 2007; Han, Robbins and Braun, 2010; Prakas et al., 2021; Nagai and Tokita, 2022). Parts of the entire mtDNA molecule are usually sequenced to determine genetic variation (Frankham et al., 2017). Owing to mtDNA representing a single non-combining unit, the value of mtDNA in characterising within-population genetic variation is limited, and for such studies nuclear markers such as microsatellites and SNPs are usually used instead (Frankham et al., 2017).

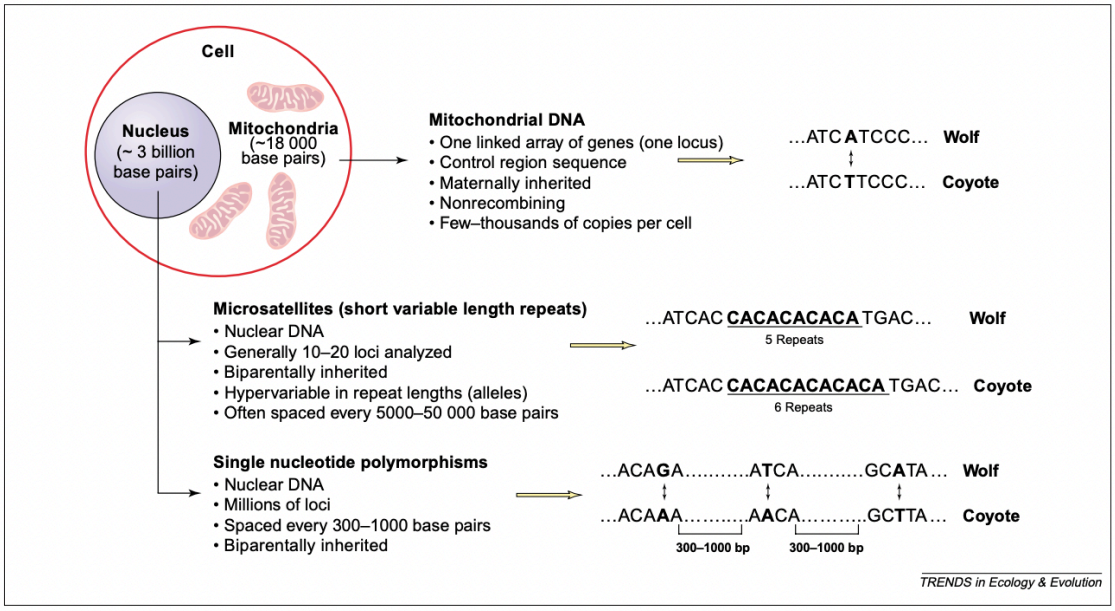


Figure 1.4 Comparison of key characteristics of commonly used markers in conservation genetics, with examples of typical deviations in DNA sequence between two closely related species (Wolf and Coyote). Original figure presented in (Morin et al., 2004). Downloaded from http://tiny.cc/wds9vz on 10/08/2023.

Microsatellites – Microsatellite markers are lengths of simple sequence repeats (SSRs) along the genome, consisting of DNA sequences between 1 – 6 base pairs in lengths repeated between 5 - 100 times (Allendorf and Luikart, 2009), with mutations thought to originate from slippage during replication (Bromham, 2016). Microsatellites are largely thought to be neutral with the majority found outside of coding regions (Vieira et al., 2016). The ubiquity, high polymorphism rate and general neutrality of microsatellite markers make them a valuable marker for characterising genetic diversity and for individual identification (Wan et al., 2004). Genotyping of microsatellite loci primarily relies on variation of within-loci sizes to discern between alleles, with different alleles differentiated by numbers of repeats (Fig 1.4). Primers can be developed for targeted amplification of specific microsatellite loci by PCR and the amplified product used for agarose gel electrophoresis or capillary electrophoresis to discern each individual's genotype (Vieira et al., 2016). Microsatellites have been and are still used extensively in parentage (Flanagan and Jones, 2019) and population genetic studies (Balloux and Lugon-Moulin, 2002; Coates et al., 2009; Abdul-Muneer, 2014), although the markers are limited by low coverage across the genome and low granularity of data compared with many thousands of SNP's (see below; Coates et al., 2009; Allendorf et al., 2022; Formenti et al., 2022) .

Single Nucleotide Polymorphisms – Single nucleotide polymorphisms (SNPs) span only a single base-pair, occurring where there are two or more alternative bases occurring at a frequency of >1% (Frankham et al., 2017), with SNPs usually biallelic (containing two alleles at a locus; Fig 1.4). Some of the more common ways in which SNP's can be identified and called are by using allele specific PCR methods, DNA microarray chips, restriction site associated DNA (RAD) sequencing and full genome sequencing/resequencing methods (Frankham et al., 2017). SNP's can cover both coding and non-coding regions across the genome (Allendorf et al., 2022) Whilst compared to microsatellites SNPs show low levels of polymorphism (i.e. typically 2 alleles per SNP compared with 10's of alleles per microsatellite loci; Morin et al., 2004; Coates et al., 2009), the ubiquity and even coverage across the genome demonstrated by SNPs enable for powerful high-resolution inferences of genetic variation (Fola et al., 2020; Pârâu and Wink, 2021). Indeed, SNP sets of >100,000 and > 1 million are common in current population genomic studies (Allendorf et al., 2022). SNP's are being increasingly utilised in population genomics studies, providing increased granularity compared with microsatellite markers (Feng et al., 2019; Flanagan and Jones, 2019; Cavill et al., 2022; Formenti et al., 2022; Duntsch et al., 2023), also presenting an increasingly viable option for parentage studies (Flanagan and Jones, 2019).

Next Generation Sequencing – Next generation sequencing (NGS) refers to DNA sequencing technologies able to produce millions to billions of short reads (i.e. 25 - 500 base pairs in length) over short time scales (Allendorf et al., 2022). Advances and cost reductions in such high-throughput sequencing (HTS) have led to increased uptake in NGS, including RAD (Narum et al., 2017), and full-genome sequencing methods in conservation and population genetics studies alike (Fuentes-Pardo and Ruzzante, 2017; Formenti et al., 2022). Where traditional population genetics studies have been held back by a small number of neutral markers, full genome sequencing/resequencing allows for genetic variation to be characterised from across the genome through the application of thousands or millions of SNPs, enabling a high resolution when studying spatiotemporal patterns of genetic diversity (e.g. Feng et al., 2019) and structuring (Funk et al., 2012). Furthermore, adopting a genomic approach allows for questions surrounding the genomic basis of adaptive and functional traits to be addressed in natural populations (Primmer, 2009; Ouborg et al., 2010). The field of conservation genomics (using genome-wide data to address conservation problems; Allendorf et al., 2022), is now gaining traction with full genome resequencing being increasingly applied to natural populations and in non-model species (e.g. Feng et al., 2019; Pârâu et al., 2022; see **Chapters 2 and 3**).

1.2.3 Approaches in studying genetic variation in wild populations

Characterising patterns in genetic variation across space and time is important in order to understand population level genetic diversity (See Box 1.1 for the key metrics used to characterise genetic diversity), the extent and directionality of gene flow and levels of population differentiation. As such, population and conservation geneticists have developed a multitude of approaches to characterise and quantify spatio-temporal patterns in genetic variation (genetic structure) and relate these to environmental and anthropogenic factors. Below I outline the key approaches used to; 1) characterise genetic structure in avifauna, 2) understand the role of environmental and anthropogenic factors in influencing spatio-temporal distribution of genetic variation and 3) study historic changes in population demography. In this section I will describe some key methodological approaches applied to genetic and genomic data to investigate spatio-temporal patterns in genetic variation and structure. I will also briefly review the approaches used to link these patterns to anthropogenic pressures (i.e., habitat loss, fragmentation, changes to landscape features etc.).

Box 1.1: Commonly Used Genetic Diversity Metrics

Below I outline the definitions of commonly used metrics for measuring genetic diversity used in this thesis and in the wider evolutionary and conservation genetics/ genomics literature. Definitions are as per Hughes et al., (2008) and Allendorf et al., (2022).

Diversity Metric	Definition
Nucleotide diversity (π)	The average number of nucleotide differences per site between two DNA sequences in all possible pairs in the sample population.
Observed Heterozygosity (H_o)	Observed proportions of heterozygotes at a given locus.
Expected Heterozygosity (H_E)	Expected proportions of heterozygotes at a given locus, under Hardy-Weinberg Equilibrium if the population is mating at random.
Global Heterozygosity	Genome-wide proportion of heterozygous sites (typically SNPs)

Allelic Diversity	The average number of alleles for a given locus present within a sampled population.
Allelic Richness	Number of alleles per locus.
Genotypic Richness	Number of genotypes within a population.

1.2.3.1 Methods for characterising genetic structure:

In this section I will describe a selection of the key approaches used to character spatial and temporal population genetic structure.

Fixation Index (F_{ST}) – Wright's F_{ST} represents one of the most commonly used structure metrics in population genetics and is an estimate of the proportion of genetic diversity among populations, with high F_{ST} values (measured on a 0 – 1 scale) indicating high differentiation. However, F_{ST} assumes that the population is at a state of migration drift equilibrium (the balance between genetic variation in a population loss by genetic drift and gained through migration), which may reduce its suitability when studying structuring over short time scales (Whitlock and McCauley, 1999; Paetkau et al., 2004). Furthermore, as with other population level assessments of structure, the requirement of *a priori* associations of individuals to subpopulations (i.e. sampling locations) has been criticised for imposing subjective pre-existing structure (Pearse and Crandall, 2004). Other genetic distance metrics are available, although are similarly constrained (e.g. Chord distance and Nei's chord distance; Cavalli-Sforza and Edwards, 1967; Nei, 1972).

Individual Based Population Assignment – Individual based population assignment typically uses Bayesian clustering methods (e.g. STRUCTURE, NGSADMIX; Pritchard, Stephens and Donnelly, 2000; Skotte, Korneliussen and Albrechtsen, 2013) to probabilistically assign individuals to *K* populations (where *K* is unknown) based on individual multi-locus genotypes to maximise conformance to Hardy-Weinberg equilibrium (the state in which a populations' genotype frequency are equal to those expected under a binomial distribution; Allendorf et al., 2022) (Pritchard, Stephens and Donnelly, 2000; Skotte, Korneliussen and Albrechtsen, 2013; Paetkau et al., 2004). Where assignment patterns appear random, populations may be considered panmictic, whilst clustering of individuals denotes structuring. Importantly, Bayesian clustering methods are able to define clusters, or genetic structure, without the prerequisite of pre-defined subpopulations or sampling locations (Pearse and Crandall, 2004).

Principal Component Analysis – Principal component analysis (PCA) is a multivariate approach which clusters populations based on allele frequencies, (Menozzi, Piazza and Cavalli-Sforza, 1978; Elhaik, 2022). PCA reduces multidimensional data to a few principal components which, in this case, best explain genetic variation. Based on allele frequencies, individuals can then be clustered along axes. PCA has similar power to capture population structure as Bayesian clustering techniques (Patterson, Price and Reich, 2006) and provides a method whereby statistical significance can be assigned to levels of population structure (Patterson, Price and Reich, 2006). Again, this method does not require individual assignment to pre-defined subpopulations or sampling locations.

Isolation By Distance – Spatial contexts are readily applied to genetic structure analysis, most commonly in the form of isolation by distance (IBD), whereby spatial distances are regressed against pairwise genetic distances (i.e., F_{ST} or chord distance). In its simplest form IBD refers to the regular increase in genetic differentiation among individuals with geographical distance. IBD is a commonly used metric for expressing genetic differentiation in a spatial context (van Strien, Holderegger and Van Heck, 2015), although the method does not account for environmental and landscape effects on dispersal and is typically reliant on large population sample sizes to effectively explain variation (population N of 9 = 50%, N of >23 = 90% probability of significance; Jenkins et al., 2010).

Commonly, all or a selection of the above approaches are used when characterising genetic structure in wild populations, with synthesis of all approaches providing a reliable reflection of genetic structure in the study population (Manel et al., 2003; i.e. see Feng et al., 2019; Beckmann et al., 2021; Blanco et al., 2021; Cavill et al., 2022; Pârâu et al., 2022; see also **Chapter 3**).

1.2.3.1.1 Understanding the role of environment and anthropogenic factors in shaping genetic variation

Increasingly, the additional effects of environmental and geographical factors are being applied to population genetics models, enabling the processes underlying patterns of gene flow and genetic variation in wild populations to be better understood (Manel and Holderegger, 2013). Indeed, the study of the effects of landscape characteristics and environmental factors in influencing population genetic patterns has led to the development of the interdisciplinary field of 'landscape genetics' (Manel and Holderegger, 2013).

Understanding the role of environmental factors and particularly anthropogenic changes in land use on genetic variation is especially poignant in cases of threatened taxa which have been subject to significant anthropogenic land use change, persecution, pollution or disturbance (Storfer et al., 2007). For example, the Golden-cheeked warbler (*Dendroica chrysoparia*) is a small North American passerine which has been subject to severe demographic decline owing to loss and fragmentation of suitable breeding habitat (Juniper woodland) (Rappole et al., 2005; Duarte et al., 2013). Lindsay et al., (2008) tested for associations in proportions of habitat types between sampled Golden-cheeked warbler populations and genetic differentiation (F_{ST} and Chord distance). The authors found that genetic differentiation among Golden-cheeked warbler populations was negatively associated with habitat connectivity. Moreover, associations between genetic diversity and environmental variables can be determined with simple regression models. Vandergast et al., (2019) used linear and non-linear regression models to determine the effects of the proportion of suitable habitat at different spatial scales relative to mean dispersal distances, on within patch genetic diversity among populations of the California gnatcatcher (*Polioptila californica californica*). The authors highlighted varying association between suitable habitat availability and genetic diversity at different spatial scales, with no association at ≤ 5km, but a strong negative association at 30 km from populations (aggregations of sampled individuals). Further examples of landscape genetics approaches exist, specifically within less-vagile taxa, namely mammals (reviewed in Montgelard et al., 2014; Kozakiewicz, Carver and Burridge, 2018) and herpetofauna (e.g. Spear et al., 2005; Wang, 2009; Emaresi et al., 2011; Gutiérrez-Rodríguez et al., 2017; Kozakiewicz, Carver and Burridge, 2018), with birds typically underrepresented in studies of landscape genetics (Kozakiewicz, Carver and Burridge, 2018; Lindsay et al., 2008; Vandergast et al., 2019). Whilst the vagility of many bird species may enable individuals to cross landscape features that would otherwise lead to population fragmentation and differentiation in less-vagile taxa, spatial genetic variation in birds may be behaviourally driven and dictated by decision making based on habitat availability. For example, where birds are habitat specialists, even in the absence of traditional landscape obstructions (e.g. roads, water bodies, mountain ranges), spatial fragmentation of suitable habitat could lead to fine-scale genetic differentiation (Spear et al., 2005; Wang, 2009; Emaresi et al., 2011; Gutiérrez-Rodríguez et al., 2017; Kozakiewicz, Carver and Burridge, 2018). In which case, where species are habitat specialists it should be expected that land use, environmental and climatic factors may still ultimately influence gene flow patterns over measurable spatial scales, even in highly vagile species (e.g. Caizergues et al., 2003; Pavlacky et al., 2009; Adams and Burg, 2015; Brewer et al., 2020).

Studies investigating the effects of habitat loss and fragmentation on genetic diversity and structuring, typically utilise the landscape genetics approaches described above or a comparative approach, comparing population genetic patterns between continuous and fragmented landscapes on either intra- (Mossman and Waser, 2001; Caizergues et al., 2003; Keller and Largiadèr, 2003) or inter-specific levels (Bates, 2000; Mech and Hallett, 2001; Brown et al., 2004). Whilst landscape genetics studies concerning contemporary population genetics and landscape features can provide insight into the genetic implications of habitat fragmentation, the temporal-lag between demographic responses to fragmentation and genetic structuring can make results challenging to interpret and reduce their usefulness in informing conservation measures (Epps and Keyghobadi, 2015). Moreover, the lack of landscape level replication and inability to control for different environmental and ecological constraints in either comparison group limits the ability of the method (comparing fragmented with continuous habitats) to accurately reflect the impact of fragmentation on contemporary genetic patterns (Keyghobadi, 2007).

An alternative, less commonly utilised, approach is to perform a temporal comparison of population genetic diversity and structuring before and after periods of habitat loss and fragmentation (Martínez-Cruz, Godoy and Negro, 2007). This approach requires historic DNA (hDNA) from museum specimens (e.g., skins, subfossils etc.) from known locations, collected prior to the period of habitat loss being studied. As such, the spatiotemporal distribution of specimens available for DNA sampling presents a key constraint when considering hDNA in population genetics and genomics studies, leading to incomplete time series or spatial sampling distributions (Gutiérrez-Rodríguez et al., 2017; Irestedt et al., 2022). This is typically owing to specimens in museum collections being collected and donated on an ad-hoc basis, with samples often spatially or temporally clustered. Therefore, flexibility is usually required in the sampling and experimental design to account for sample availability in studies employing a spatio-temporal sampling approach (Billerman and Walsh, 2019). Second, whilst museum specimens have been demonstrated as a viable source of hDNA (examples in; Leonard, 2008; Billerman and Walsh, 2019; see also **Chapter 3**), poor quality DNA and contamination (i.e. degradation by shearing, depurination, and deamination of cytosines to uracils; Briggs et al., 2007; Dabney, Meyer and Pääbo, 2013) represent perhaps the main issue facing hDNA studies (e.g. Wandeler, Hoeck and Keller, 2007; Billerman and Walsh, 2019; Irestedt et al., 2022). Low yields from hDNA have previously limited the application of full genome sequencing approaches (Card et al., 2021). However, the development of optimised wet lab methods (dedicated clean rooms, extraction, and library preparation protocols, PCR amplification etc.; Irestedt et al., 2022) and bioinformatics applications dedicated to working with low-quality

DNA (e.g. damage mapping, SNP calling by genotype likelihoods etc.) have helped mitigate these issues (Irestedt et al., 2022). As costs continue to decrease, full-genome resequencing is becoming increasingly utilised, having been used to reveal temporal demographic and genetic patterns in threatened taxa (Feng et al., 2019; Cavill et al., 2022) as well as extinct species (e.g. Murray et al., 2017).

The application of hDNA from museum samples holds great potential for determining whether contemporary patterns of genetic structure and variation represent natural processes or are a result of more recent anthropogenic pressures (i.e. land use, habitat loss and fragmentation etc.; Martínez-Cruz, Godoy and Negro, 2007; Shepherd and Lambert, 2008; Tracy and Jamieson, 2011; Stronen et al., 2019). Nevertheless, there is a distinct lack of temporal analysis applied to highly vagile species, such as long-distance migratory birds (Klinga et al., 2020). This is perhaps owing to the perception that the majority of migratory birds exhibit high levels of panmixia among populations, limiting the worth of significant population genomics projects (Pârâu and Wink, 2021; but see Calderón et al., 2016; Pârâu et al., 2022). However, apparent panmixia shown in previous studies of migratory birds might be an artefact of using low-resolution markers (Pârâu and Wink, 2021). Historic conclusions of population admixture are likely to be reconsidered as the increased adoption of NGS enables fine-scale structuring to be detected (Pârâu and Wink, 2021), even in highly mobile migratory taxa (e.g. Larison et al., 2021; but see Calderón et al., 2016; Pârâu et al., 2022). Full genome sequencing presents a valuable method in ascertaining spatio-temporal population genetic trends in migratory avifauna in order to understand the mechanisms driving contemporary genetic patterns.

1.2.3.1.2 Characterising historic changes in population demography

Current levels and distribution of genetic diversity across the genome are ultimately a result of a species' historic demography, across both recent history and ancient timescales. Applying genetic and genomic methods to study historic demography has inherent value in explaining current genetic diversity in populations as well as resolving taxonomic uncertainties, alongside identifying whether species have been subject to past bottlenecks. Multiple approaches can be taken to determine a species historic demography (i.e. demographic reconstruction by Bayesian Skyline methods such as BEAST; Ho and Shapiro, 2011), with genomic data enabling patterns in ancient demography to be elucidated (i.e. PSMC, MSMC; Li and Durbin, 2011; Mather, Traves and Ho, 2019). It is possible to detect if a contemporary population has suffered a recent genetic bottleneck. This can be signalled by a heterozygosity excess, where

population excess heterozygosity is greater than expected if the population were at equilibrium between genetic drift and mutation (Frankham, Ballou and Briscoe, 2010) and is frequently examined using software such as BOTTLENECK (Luikar, Piry and Cornuet, 2020). However, if mutation-drift equilibrium (used to determine likelihood of bottleneck) is quickly re-established (e.g., via migration, enhanced connectivity etc.) no evidence of a historic bottleneck will be detectable in contemporary samples. In this case, the inclusion of hDNA samples collected before the suspected population decline can be compared with contemporary samples, changes in order to to truth bottleneck analysis over short (~200 years) timescales (e.g. Brown et al., 2007; Kempe, 2008; Larsson et al., 2008; Bergner et al., 2016; Klinga et al., 2020). Furthermore, use of hDNA for temporal comparison may reveal changes in heterozygosity not detectable solely through bottleneck analysis on contemporary samples (Larsson et al., 2008). Given the problems associated with bottleneck analysis, where possible a combination of temporal population genetic analysis (i.e. studying changes in population genetic diversity and structure over time using museum and modern samples) provides the most reliable method to capture recent genetic bottlenecks in species where high dispersal potential may lead to the rapid establishment of the mutation-drift equilibrium (Larsson et al., 2008).

Genomics has provided researchers with the opportunity to utilise genome-wide heterozygosity patterns to characterise historic demographic change over ancient time scales. Pairwise Sequentially Markovian Coalescent (PSMC) analysis is a powerful tool which infers ancestral changes in effective population size (N_e) from a single genome. The analysis estimates changes in N_e over ancient time scales (~10Kya - 5Mya) through applying hidden-Markov modelling to the coalescence framework, treating a genome as multiple historic genealogies partitioned by recombination events (see Li and Durbin, 2011; Mather, Traves and Ho, 2019; see **Chapter 2** for detailed description of the method). PSMC can be applied to combined pseudo genomes of individuals from different populations, subspecies or species to estimate the timing of divergence between contemporary populations (see **Chapter 2** for detailed description; Li and Durbin, 2011; Prado-Martinez et al., 2013; Sato et al., 2020), enabling powerful inferences on ancient climatic drivers of contemporary population structure (e.g. Sato et al., 2020). Multiple Sequentially Markovian Coalescent (MSMC) analysis utilises a similar approach to PSMC when reconstructing demographic histories, but requires multiple genomes enabling for more recent changes in N_e to be estimated (Mather, Traves and Ho, 2019). However, both PSMC and MSMC remain poor at reconstructing recent (~2,000 ybp) demographic histories (Li and Durbin, 2011; Prado-Martinez et al., 2013; Sato et al., 2020). Bayesian techniques (PopSizeABC; Boitard et al., 2016) provide an alternative for more recent

changes in N_e (e.g. < 2,000 ybp), but are typically limited in more ancient N_e reconstruction (i.e. > 10 Kya; Boitard et al., 2016; Feng et al., 2019). Characterising ancient changes in population demography, particularly across multiple individuals and populations has a number of applications in conservation genetics. These include, understanding the response of the study species to past climate change, as well as the ability to understand the basis of current phylogenetic patterns and to resolve taxonomic uncertainties. Furthermore, the approaches allow for current genetic diversity and N_e to be understood relative to past levels, which is valuable in providing a 'baseline' from which current decisions on population trends and threats as well as conservation decisions can be made (Hohenlohe, Funk and Rajora, 2021).

1.2.3.2 Conservation genomics in migratory avifauna

The field of conservation genetics has made huge strides in understanding genetic variation in the world's threatened taxa, the evolutionary and anthropogenic drivers driving this, and in developing targeted conservation measures to help mitigate and increase genetic diversity in wild populations (Beaumont and Wang, 2019; Holderegger et al., 2019; Wright et al., 2019). Temporal sampling strategies (i.e., use of historic staples from museum collections alongside contemporary samples) have enabled for changes in genetic diversity and thus the anthropogenic impact on contemporary populations to be accurately calculated (see Section 1.2.3.1.2 and **Chapter 3**). Furthermore, the application of genomic (i.e. full genome resequencing) over genetic (i.e. microsatellite genotyping) approaches has provided increased resolution when characterising patterns of genetic diversity and structure, also providing increased opportunity for researchers to elucidate a species ancient demography (i.e. PSMC analysis; Li and Durbin, 2011) (Allendorf et al., 2022). Nevertheless, population genomic studies tracking recent and ancient N_e changes have been largely restricted to model taxa, or geographically isolated highly threatened species for which the genomic footprints of demographic change are severe (e.g. Feng et al., 2019; Robinson et al., 2021; Cavill et al., 2022; Westbury et al., 2022). In such cases, information on historic bottlenecks and contemporary structure are often imperative for effective conservation (e.g., translocation of individuals, delineating conservation units etc.; Frankham, Ballou and Briscoe, 2010). However, comparatively little attention has been paid to non-model taxa or species which have likely avoided severe bottlenecks or are distributed across a large geographic range. Indeed, the majority of migratory avifauna have avoided severe population bottlenecks, and instead exhibit moderate population declines typified by local extinctions and fragmentation across large ranges (Payevsky, 2006; Cox, 2010). The genomic impacts of this pattern of decline may

have been missed by previous studies which have overwhelmingly found little genetic structure, owing to the use of low resolution markers (i.e. microsatellite loci) compared with high resolution genome wide approaches (Pârâu and Wink, 2021). The application of genomics in such species will be valuable in characterising the genomic signature of a pattern of population decline common across migratory avifauna, providing an accurate picture of the extent and impact of population decline on genetic diversity.

1.3 Mating systems, mate choice, and infidelity

Below I briefly outline the background surrounding mating systems and sexual selection in birds, a detailed review of these topics and as they relate to Caprimulgiformes can be found in **Chapter 5**.

The mechanisms underpinning mating systems in animals have long fascinated biologists. Social monogamy, characterised by a stable breeding pair (two individuals of opposite sex), is the most common mating system in birds, recorded in~ 90% of all avian taxa (Griffith, Owens and Thuman, 2002). Comparatively few birds exhibit true polyandry (~1%) or polygyny (~2%), with the former describing systems whereby females mate with multiple males and the latter where males mate with multiple females (Griffith, Owens and Thuman, 2002). However, with the advent of molecular techniques in the late 20th century our understanding of avian mating systems has been rapidly transformed (e.g. Burke and Bruford, 1987). It is now clear that whilst birds commonly exhibit 'social monogamy', genetic monogamy is the exception rather than the rule (Brouwer et al., 2017; Brouwer and Griffith, 2019). Instead, >75% of studied avian taxa are socially, rather than genetically monogamous (Arct, Drobniak and Cichoń, 2015; Reitsma et al., 2018), engaging in extra-pair paternity (EPP; Ju et al., 2014; Wells et al., 2015; Grunst et al., 2017; Grinkov et al., 2018), intraspecific brood parasitism, and quasi-parasitism (Blomqvist et al., 2002). Understanding the evolutionary and ecological drivers behind the ubiquity of infidelity, namely EPP, has received a lot of attention (e.g. see synthesis of studies in Brouwer and Griffith, 2019). Whilst the benefits to males appear clear (i.e., higher fecundity) the apparent benefits to females remain unclear; why would females choose to mate with multiple males, when their fitness is ultimately limited by the number of eggs? The topic of female mate choice has and continues to be a topic of great interest (Darwin, 1871; Mays et al., 2008; Jones and Ratterman, 2009; Hasegawa, 2018). Female benefits can be dissected into direct (e.g., fecundity, parental care, protection, resource acquisitions; Jones and Ratterman, 2009) and indirect benefits (e.g. offspring sexual quality, offspring viability; Andersson and Simmons,

2006). The selected upon sex (usually males) can exhibit elaborate ornamentation (i.e. striking structural and plumage features; i.e. Fig 1.5) thought to reflect individual quality and ultimately selection on by the 'choosy' sex (usually females) (e.g. *Ficedula* flycatchers; Fig 1.5; Pärt and Qvarnström, 1997; Siitari and Huhta, 2002; Török, Hegyi and Garamszegi, 2003).

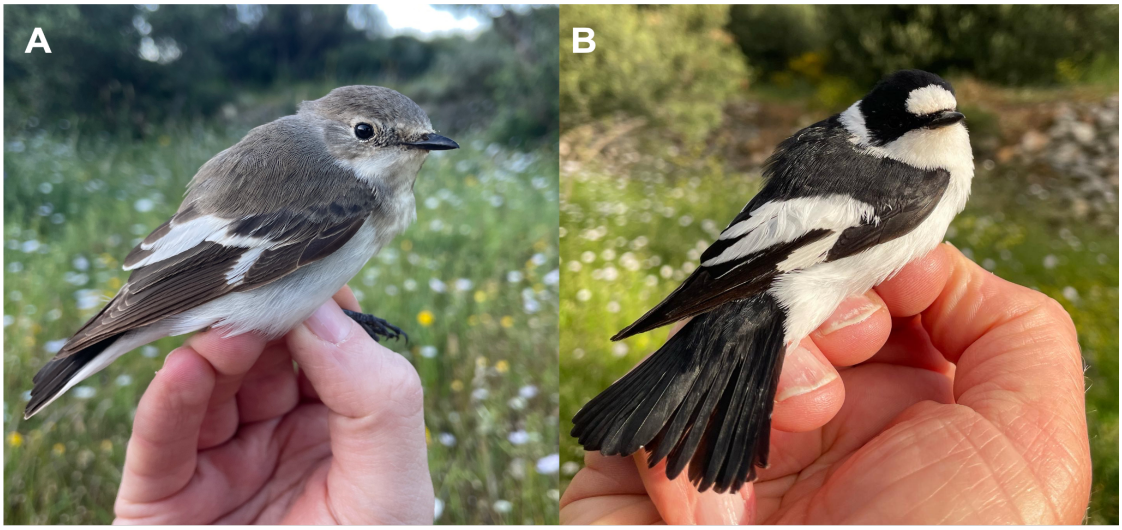


Figure 1.5 plumage of a female (left) and male (right) Collared flycatcher (*Ficedula albicollis*) showing sexual dichromatism of achromatic ornamentation, exhibited by male birds, a feature thought to be under sexual selection (Pärt and Qvarnström, 1997; Siitari and Huhta, 2002; Török, Hegyi and Garamszegi, 2003).

Understanding the ecological and evolutionary drivers of mate choice and infidelity have received a lot of attention (Birkhead and Møller, 1995; Westneat, 1990; Westneat and Stewart, 2003; Wan, Chang and Yin, 2013; Mingju et al., 2017). However, despite a wealth of research there is a lack of consensus regarding the drivers of infidelity and female choice in avian mating systems (Brouwer and Griffith, 2019). One limiting factor in effectively testing central hypotheses (see **Chapters 4 - 6**) has been the taxonomic bias in previous studies (Brouwer and Griffith, 2019), comparatively lacking in data from non-passerines and particularly nocturnal avifauna, a group which has received little attention (Brouwer and Griffith, 2019; but see Roulin et al., 2004; Saladin et al., 2007; Horníček et al., 2017). Research on mating systems and visual signalling in as yet unstudied families and orders will be valuable if we are to fully understand the evolution and ecological drivers of infidelity and mate choice in Aves.

Understanding a species' mating system carries substantial applied value. Effective population sizes are significantly influenced by differential and skewed amounts of breeding success between individuals (Sutherland, 1998) so that the productivity and viability of populations are ultimately influenced by a species' mating system (Nunney, 1993; Hogg, 2000; Schindler et al.,

2013). Under different mating systems the number of individuals contributing gametes to the next generation (N_e) can vary significantly. For example, under polygyny successful reproduction is typically restricted to relatively few males in the breeding population. In such cases, N_e and census population sizes would differ greatly, particularly if population census data were determined from male display activity such as territorial singing, as it is for many birds (Gilbert, Gibbons and Evans, 1998). A lack of understanding of a species mating system may thus lead to flawed decision-making regarding population sizes, viability, and conservation practices.

1.4 Study species

The European nightjar (*Caprimulgus europaeus*), henceforth nightjar, is a nocturnally foraging and crepuscular displaying bird (Cleere, 1998; Cleere, Christie and Rasmussen, 2021). Here I provide an overview of the species taxonomy, biology and ecology and present current knowledge gaps.

1.4.1 Nightjar taxonomy and range

The species belongs to the family Caprimulgidae, of the order Caprimulgiformes. Caprimulgids are typified by their cryptic and nocturnal behaviour and cryptic contour plumage, and anatomical adaptations which enable them to effectively hunt insects at night (i.e. large gape and very large eyes; Cleere, 1998; Jackson, 2003; Cleere, Christie and Rasmussen, 2021). Caprimulgids demonstrate a near global distribution, found on all continents, excluding Antarctica (Cleere, 1998; Cleere, Christie and Rasmussen, 2021). Nightjar are the only species of Caprimulgid breeding across Northern temperate Europe, with Red-necked nightjar (*Caprimulgus ruficollis*) restricted to Iberia and North Africa and Grey nightjar (*Caprimulgus jotaka*) to Eastern and Central Asia (Cleere, 1998; Cleere, Christie and Rasmussen, 2021). Nightjar breeding distribution ranges from Britain in the NW to Mongolia and Western China in the East (Fig 1.6). The highest diversity of Caprimulgids can be found in the tropics and sub-tropics. Indeed, multiple species of Caprimulgids can be found in tropical and subtropical Africa, from which nightjar were thought to have originated, with the most closely related extant species being an Afrotropic resident (Rufous-cheeked nightjar; *Caprimulgus rufigena*) (Han, Robbins and Braun, 2010). It has been suggested that the species evolved migratory

behaviour during the last glacial period (Larsen et al., 2007), although this has not been investigated.

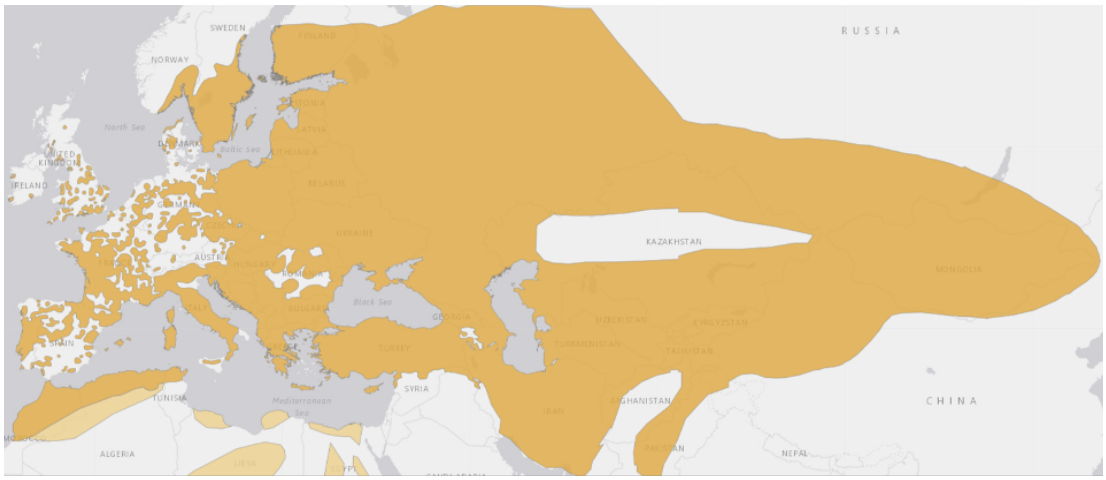


Figure 1.6 breeding range (dark orange) of European nightjar. Light orange represents sites occupied only on passage migration. Map from rom (IUCN, 2023), downloaded 22/02/2023.

European nightjar is currently composed of six subspecies (*C.e. europaeus*, *meridionalis*, *sarudnyi*, *unwini*, *plumipes*, *dementievi*), broadly following an East-West clinal distribution (Cleere, 1998; Cleere, Christie and Rasmussen, 2021). However, subspecies have been largely defined by plumage characteristics, with mtDNA analysis finding little association between genetic variation and current sub-species classification (Schweizer et al., 2020; Cleere, Christie and Rasmussen, 2021). Moreover, nightjar appear to demonstrate a clinal change in plumage colour, becoming paler in Easterly populations (Schweizer et al., 2020). Whilst there have been a number of phylogenetic studies investigating the phylogeny of Caprimulgidae (Larsen et al., 2007; Han, Robbins and Braun, 2010) and Caprimulgiformes (Mariaux and Braun, 1996; Braun and Huddleston, 2009), there is a need for a detailed phylogeny of European nightjar and it's subspecies in order to resolve current taxonomic classifications (see **Chapter 2**).

1.4.2 Nightjar biology

Nightjar are a moderately long-lived species (up to 12 years; Robinson et al., 2022), able to breed after their first wintering period (Cramp and Simmons, 1985). The specialist anatomical adaptations of nightjar allow them to exploit low light levels associated with crepuscular and nocturnal behaviour. For example, the species possess very large eyes and a proportionally large gape and specialised rictal bristles, which are thought to aid tactile recognition of prey among other functions (Cleere, 1998; Delaunay et al., 2020). These adaptations enable the

species to effectively visually hunt flying insects in low light levels (Cramp and Simmons, 1985; Cleere, 1998; Jackson, 2003). Nightjar primarily hunt either from a perch (hawking) or on the wing, whereby they typically approach moths from underneath, using the silhouette against the night sky to locate their prey (Evens et al., 2017a). Indeed, elevated lunar luminance has been shown to lead to increased feeding behaviour in the species (Norevik, Åkesson and Hedenström, 2017). Nightjar feed predominantly on moths (Lepidoptera), with recent dietary analysis highlighting a preference towards large, abundant moth species but little preference with regards to specific species (Evens et al., 2020a; Mitchell et al., 2022).

Nightjar are characterised by their highly cryptic plumage. Contour plumage of either sex is largely monotypic (Fig 1.7 A-C), with birds typified by highly-patterned brown and grey feathers, resembling brash or dead wood, enabling them to camouflage against their surroundings when roosting or nesting on the ground (Cramp and Simmons, 1985; Cleere, 1998; Fig 1.7C). Nightjar however do exhibit sexual dimorphism, with males most notably exhibiting white spots on the outer three primaries and the tips of the outer two tail feathers (Fig 1.7A, B). The functioning of these plumage features have been hypothesised to be important during display and territorial disputes. Indeed during territorial flights and display behaviour nightjar extend their wings and fan their tails to apparently display white spots to conspecifics (Cramp and Simmons, 1985; Aragones, Reyna and Recuerda, 1999; Roth, Argyros and Browning, 2003). This behaviour usually takes place in twilight and increases during nights of high lunar luminance (Holyoak, 2001; Reino et al., 2015), with the environmental light perhaps enabling increased conspicuity of the spots. Moreover, sexually dichromatic achromatic plumage appears to be a feature commonly shared among Caprimulgidae (see **Chapter 4**). Variations in the size of the white plumage features among other species has been shown to vary with individual age, highlighting their role as a potential signalling mechanism. However, as yet there has been no published work investigating the function of the white spots in European nightjar (but see **Chapter 6**).

Figure 1.7 A) male nightjar exhibiting dichromatic white spots and B) female nightjar without white spots. C) female nightjar brooding 2 chicks. Photos A by Helge Sorensen (downloaded on 10/08/2023 from http://tiny.cc/ses9vz) and B from Ove Ferling (downloaded on 10/08/2021 from https://www.birdforum.net/opus/File:Nightjar15.jpg).

Nightjar are ground nesting. Nest sites lack any structure, instead consisting of a scrape usually amongst brash, leaf litter or bare ground, with location seemingly dictated by background complexity in order to ensure camouflage of the brooding bird and eggs (Fig 1.7C; Camacho, 2014; Troscianko et al., 2016). Nests are typically located at the base of a small bush or tree (≤ 3m; unpublished data). Nightjar produce on average two eggs per clutch, with a clutch of 3 exceptional (Cramp and Simmons, 1985; Cleere, 1998). The majority of incubation is done by the mother although the father will periodically incubate the eggs during the night. Both sexes brood and provision the chicks, with females accounting for the majority of diurnal brooding (Fig 1.7C; Cramp and Simmons, 1985; Cleere, 1998). Chicks are semi-precocial and will typically move away from the nest site prior to fledging 15-17 days after hatching (Cramp and Simmons, 1985; Cleere, 1998). Nightjar typically produce two broods within a breeding season, with the male accounting for the soul share of chick care from the first brood shortly before and after

fledging, enabling the female to begin egg laying and brooding the second brood. Whilst females usually demonstrate within-season pair fidelity, between brood mate switching has been recorded (Lowe pers comm.).

Nightjar have long been characterised as monogamous due to their biparental care and records of inter- and intra-seasonal pair fidelity, although mate switching has been recorded (Cramp and Simmons, 1985; Cresswell and Alexander, 1990). Observations of suspected polyandry (Padget et al. 2019) and polygyny (Jensen, 2013) arising from single cases and anecdotal records have been noted, although the extent of and drivers for this intraspecific variation are unknown. Despite a wealth of literature on other aspects of European nightjar biology and ecology (e.g. migration; Cresswell and Edwards, 2013; Evens et al., 2017b; Norevik, Åkesson and Hedenström, 2017; Lathouwers et al., 2022; habitat preferences and foraging ecology; Sharps et al., 2015; Evens et al., 2017a, 2020a; Mitchell et al., 2020, 2022), a full observational and molecular study of the species mating system is lacking (see **Chapter 5**).

1.4.3 Nightjar migration and habitat requirements

Nightjar are long distance migrants. Recent migration tracking studies have revealed that nightjar predominantly winter in the southern subtropical central and south Africa and exhibit a loop migration strategy, whereby birds migrate through Western Africa on spring (return) migration and exhibit a more direct route in Autumn (Fig 1.8; Cresswell and Edwards, 2013; Evens et al., 2017b; Norevik, Åkesson and Hedenström, 2017; Lathouwers et al., 2022). On their breeding grounds nightjar are habitat specialists typically requiring Heather (*Calluna*) and Birch (*Betula pendula*) dominated habitats, such as heathland and woodland clearfell for nesting and display (Conway et al., 2007). Birds may exhibit different preferences for nesting versus foraging habitat (Conway et al., 2007) and will regularly use grazed grassland pastures, waterbody margins as well as woodland edge for foraging (Sharps et al., 2015; Evens et al., 2018; Mitchell et al., 2020). As such, nightjar can exhibit large home ranges during the breeding season, although home range size can vary among individuals with nesting and foraging habitat availability and distribution (Evens et al., 2018; Mitchell et al., 2020). Nightjar show varying levels of site and nest site fidelity (Lowe, Rogers and Durrant, 2014; Raymond et al., 2020). Limited cases of natal philopatry have also been recorded (unpublished data), although this appears to vary among breeding sites (Conway pers comm.). Nevertheless, despite significant inter-annual ringing (mark and recapture) effort at a number of breeding sites, few cases of between site movements in nightjar have been recorded, suggesting high

site fidelity. Little is known regarding inter-site movements and thus the extent of gene flow between breeding sites, and further work here is needed.

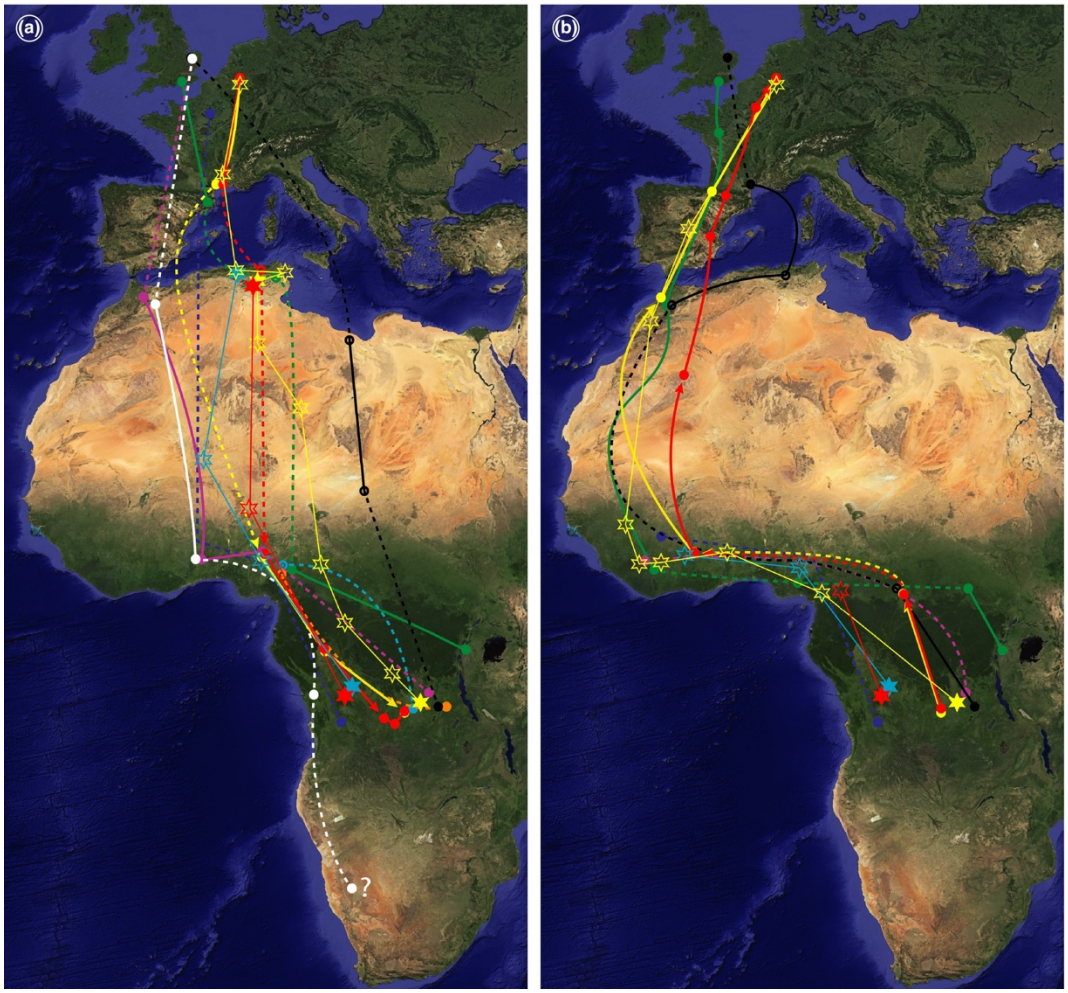


Figure 1.8 Nightjar migration tracks during A) Autumn and B) Spring migration (n = 12 tracks from 11 birds). Originally presented in (Evens et al., 2017b). Downloaded from https://shorturl.at/hAPUV on 19/05/2023.

1.4.4 Nightjar conservation status and population trends

Nightjar populations have broadly exhibited a declining trend across Europe over the last two centuries. This trend however has not be uniform across the species range, with some countries showing severe declines (i.e. Switzerland; Sierro and Erhardt, 2019, Austria; Wichmann, 2004), whilst others have seen stable or increasing population sizes in recent years (e.g. Britain; Conway et al., 2007). Nightjar were once a widespread breeding species across the entirety of the British Isles throughout the 1800s (Holloway, 2010). However, the species underwent a population decline throughout the 20th century, undergoing a >50% population reduction between 1966 and 1981 (**Chapter 3**; Fig 3.1), and the species was declared extinct in

Northern Ireland and near-extinct in the Republic of Ireland in the late 20th century (Gribble, 1983; Conway et al., 2007), although undergoing a partial recovery in the late 20th and early 21st century (34% increase between 1992-2004; Langston et al., 2007). The changes in population size have been broadly associated with reduction in breeding habitat, with the recent partial population recovery associated with increased availability of clearfell in coniferous plantations, many of which were planted over areas historically supporting nightjar (Langston et al., 2007). However, recovery has not been consistent across the species range, whilst some populations in the East of England appeared to increase with the availability of plantation clear-fell habitat, populations in Northwest of England continued to withtract, with populations in Northern Ireland and The Republic of Ireland becoming extinct (Conway et al., 2007; Langston et al., 2007).

At a breeding site level, light pollution, habitat degradation, human disturbance and nest site predation limit nightjar populations (Langston et al., 2007; Lowe, Rogers and Durrant, 2014; Sierro and Erhardt, 2019). Whilst ambient light may aid foraging activity and movement in nightjar (Evens et al. 2020), light pollution may limit the recolonization of breeding sites, owing to increased light levels altering prey (moth) behaviour as well as affecting the species sensitive vision (Sierro and Erhardt, 2019). Nightjar also appear to be sensitive to human disturbance, particularly dog walkers (Langston et al., 2007), with increased human activity associated with lower nest densities at some sites (Lowe, Rogers and Durrant, 2014). Nests typically fail at the incubation stage, due to predation by Corvids, Adders (*Vipera berus*), and Red foxes (*Vulpes vulpes*) (Berry, 1979; Berry and Bibby, 1981), with livestock and deer presenting significant trampling risk, owing to the species nesting on the ground (A. Lowe pers comm.) Despite a wealth of ringing effort, there is a paucity of studies investigating nightjar survival in the UK, with the only published survival study in Italy (Silvano and Boano, 2012), with the authors recording annual adult survival rates between 0.65 - 0.75.

1.4.5 Nightjar knowledge gaps

The cryptic and nocturnal nature of nightjar, and indeed all Caprimulgids, has provided a barrier for researchers wishing to study these species. Nevertheless, nightjar are perhaps the best studied Caprimulgid, with Red-necked nightjar and Common nighthawk (*Chordeiles minor*) also accounting for a large proportion of literature surrounding Caprimulgidae (Forero, Tella and García, 1995, Roth, Argyros and Browning, 2003; Camacho, 2013, 2014; Camacho et al., 2019; Vala et al., 2020; McGuire, Boyles and Brigham, 2021). The majority of recent research

on nightjar centres primarily around habitat use and migration, using light weight remote VHF and GPS tracking technology (e.g. Sharps et al., 2015; Evens et al., 2017a, 2017b; Mitchell et al., 2020; Lathouwers et al., 2022), enabling an insight into the species behaviour otherwise obscured by low light levels. This work has dramatically advanced our understanding of the species ecology and enabled targeted and informed conservation measures. However, we are lacking rather basic information surrounding nightjar ecology and behaviour, namely the species breeding biology. This dearth of knowledge can be largely extended across all Caprimulgiformes. At the time of writing, no published data exists describing mating systems or sexual selection in any Caprimulgidae, including nightjar. Indeed, whilst there has been some initial work investigating the role of sexually dichromatic achromatic plumage features in Caprimulgidae (Red-necked nightjar; Forero, Tella and García, 1995; Aragones, Reyna and Recuerda, 1999; Common nighthawk; Roth, Argyros and Browning, 2003), as yet no effort has been made to link this to breeding behaviour to ascertain whether features might be subject to sexual selection (see **Chapter 4** for more information).

Taxonomic assertions surrounding nightjar subspecies are largely based on morphological and variations in plumage characteristics, so there is a need for a molecular phylogeny of nightjar to resolve sub-species categories (Schweizer et al., 2020; Cleere, Christie and Rasmussen, 2021). Indeed recent genetic analysis of Vaurie's nightjar (*Caprimulgus centralasicus*) from central Asia, a nightjar species known from a single type specimen and designated solely from plumage and morphological features, found that the Vaurie's nightjar likely belongs to the *Plumipes* sub species of nightjar (*Caprimulgus europaeus plumipes*) and not a separate species as previously thought (Schweizer et al., 2020). The work highlights the unreliability of taxonomic designations solely by plumage and morphological characters and emphasises the need for molecular analysis of nightjar subspecies and a range-wide phylogenetic study. Furthermore, studying the fine-scale population genetics of nightjar will help understand gene flow and thus connectivity between breeding sites, which ringing data has so far been unable to elucidate. Indeed, the application of a temporal sampling approach would further enable for the true extent and genetic implications of anthropogenic driven demographic change in the British population to be assessed, with census data likely limited by the cryptic nature of the species.

Despite the inherent limitations imposed by the species ecology and behaviour, modern genetic and genomic methods can enable novel and valuable insights into nightjar breeding and population ecology. Full-genome sequencing provides the opportunity to uncover novel information on the historic demography of nightjar, which could elucidate the species'

response to historic climate change, historic distribution, and the timing of divergence among subspecies. The pattern of population change exhibited by nightjar across their European breeding range is similar to that of many other Afro Palearctic long-distance migrants, habitat and dietary specialists (Bowler et al., 2019; PanEuropean Common Bird Monitoring Scheme, 2022). The high-resolution data capabilities of genomic analysis will help highlight spatio-temporal patterns in genetic variation in migratory avifauna which might have been missed by the historic application of low-resolution markers (Pârâu and Wink, 2021). Characterisation of nightjar genetic mating system and role of achromatic ornamentation in mating success, would be the first within the order Caprimulgiformes, providing phylogenetically valuable data towards central hypothesis surrounding mate choice and mating system evolution. Furthermore, the conspicuity of sexually-dichromatic achromatic ornaments (i.e. white spots exhibited by male nightjars) leave nightjar well placed among nocturnal and crepuscular taxa to study the evolution of nocturnal visual signalling, a growing field (see **Chapter 4**), and address the historical bias towards studying diurnal taxa (Penteriani and Delgado, 2017).

1.5 Study sites

Contemporary samples and data for this thesis were collected from a total of thirteen nightjar breeding sites for the data presented in this thesis. The thirteen sites spanned the known contemporary range of European nightjar in Britain (Fig 1.9A). Key site details can be found in Table 1.1. All thirteen sites contributed data towards **Chapter** 3, whilst data were only collected from Humberhead Peatlands and Thetford Forest for **Chapters 5 and 6**, more in depth site descriptions for both sites can be found in **Chapters 5 and 6** (Table. 1.1). Sixty historic (1841 - 1960) nightjar specimens were also sampled (foot and toepad tissue scrapes) for full-genome resequencing (**Chapter 3**; **Appendix** Table 8.2) from across eight museum collections in Britain and the Republic of Ireland (see Fig. 1.9B and for sampling distribution).

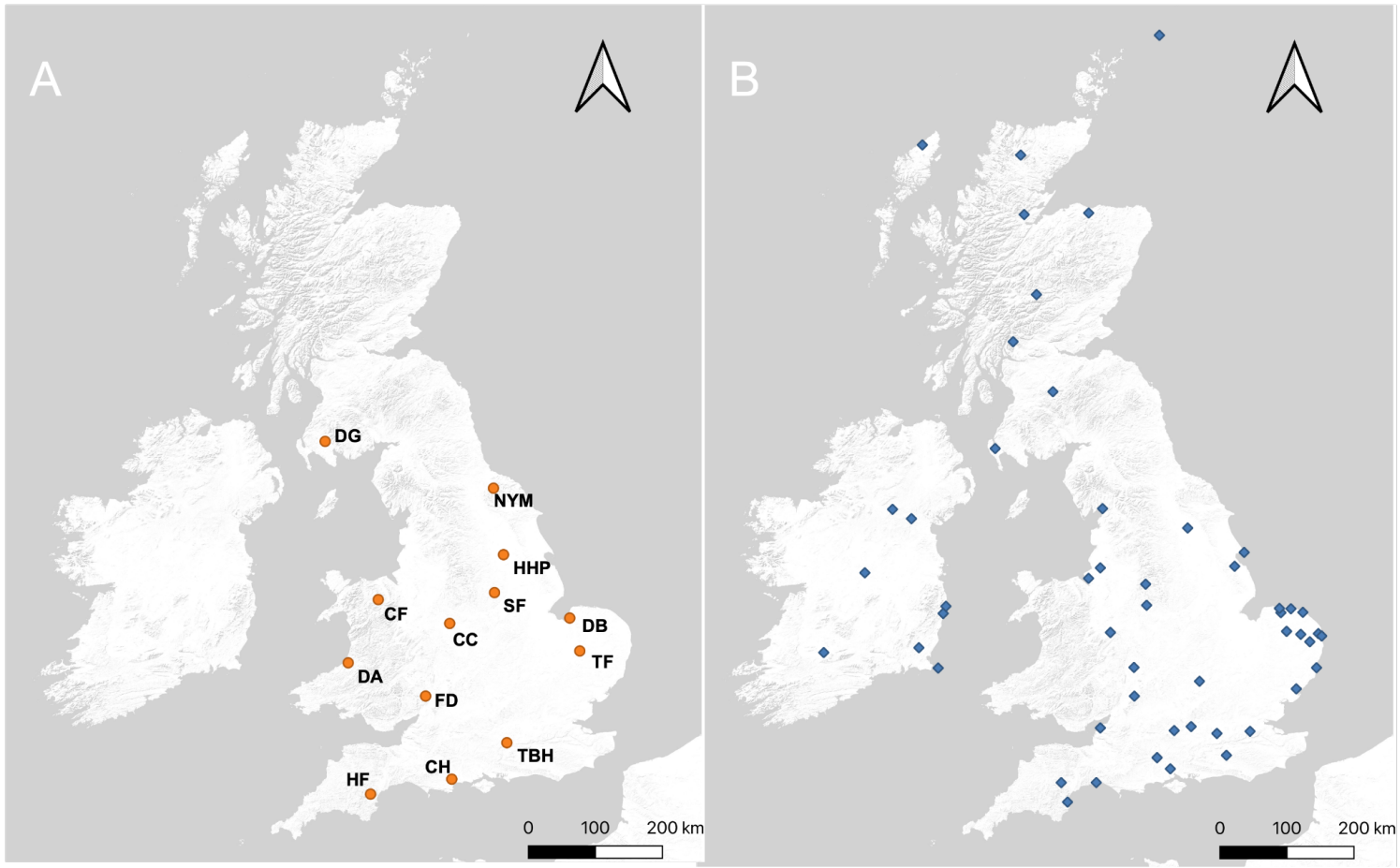


Figure 1.9 Map of A) contemporary sampling site locations (see Table 1.1 for breakdown of chapter contributions per site) denoted by orange dots and B) collection locations of historic nightjar specimens used in **Chapter 3** as shown by blue diamonds. Letter codes in A refer to site ID's which can be found in Table 1.1.

Table 1.1 Contemporary nightjar breeding sites sampled for this thesis, with ID code used in Fig 1.8, description of predominant habitat types, and data chapters data are used in.

Site name	*Code*	*Habitat Description*	*Chapter*
Dumfries and Galloway	DG	Upland coniferous plantation forest.	3
North Yorkshire Moors	NYM	Upland plantation forest	3
Humberhead Peatlands	HHP	Lowland peat bog, birch forest and heathland.	2 ,3, 5, 6
Sherwood Forest	SF	Lowland coniferous plantation and degraded heathland.	3
Cannock Chase	CC	Lowland heathland.	3
Dersingham Bog	DB	Lowland heathland.	3
Thetford Forest	TF	Lowland coniferous plantation forest.	3, 5, 6
Thames Basin Heaths	TBH	Lowland heathland.	3
Canford Heath	CH	Lowland heathland.	3
Haldon Forest	HF	Lowland coniferous plantation forest and heathland.	3
Forest of Dean	FD	Mixed broadleaf woodland, coniferous plantation forest, lowland heathland.	3
Dyffryn Arth	DA	Upland coniferous plantation forest.	3
Clocaenog Forest	CF	Upland coniferous plantation forest.	3

1.6 Ethics Statement

All work conducted in this thesis conforms to the animal welfare ethics standards as outlined by the University of York's Animal Welfare and Ethical Review Body (AWERB) in 2019, where ethics approval was granted for the project. All handling, ringing, and sampling of living birds was undertaken by trained, licensed individuals (licensed ringers by the British Trust for Ornithology; BTO), with DNA sampling and tagging of birds conducted under specific project licences issued by the BTO. Tissue samples were obtained from deceased specimens collected from the environment, with use of museum specimens having received ethics approval under each collection establishment (see **Appendix** 8.2 for list of contributing collections).

1.7 Thesis Outline and Aims

Below I outline a theoretical framework (see Fig 1.10), developed by empirical evidence in the literature (see below), for which my work in this thesis will contribute towards.

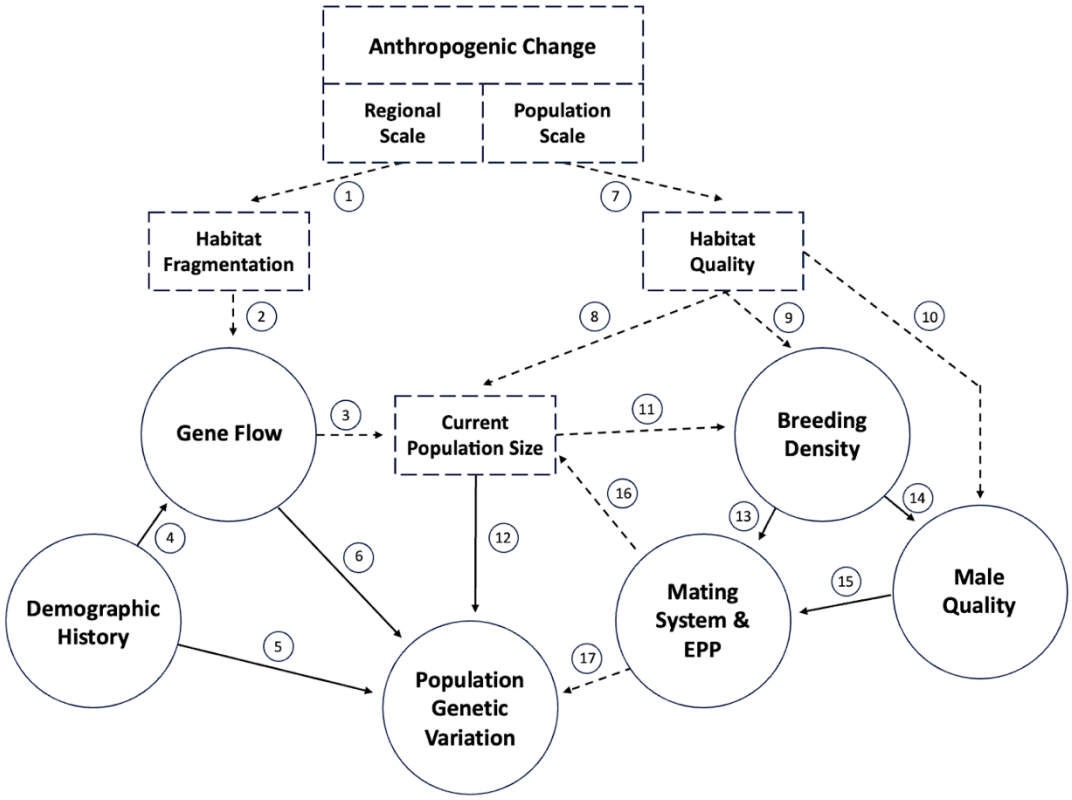


Figure 1.10 Conceptual framework summarising the relationships of ecological and evolutionary processes that drive population genetic variation studied in this thesis (solid circles) and not covered in this thesis (dashed rectangles). Arrows denote directionality of relationship between processes, with

solid lines denoting relationships supported by work in this thesis and dashed support by evidence in the literature. Numbers on arrows are used to identify relationships as they are discussed in the main text.

Population genetic variation can be influenced by gene flow, population size, mating system and geographical distribution (Fig 1.10 - arrows 1 and 7; Frankham, Ballou and Briscoe, 2010), which can ultimately be mediated by ecological factors such as habitat availability, quality and fragmentation (Fig 1.10; Quader, 2005; Segelbacher et al., 2010; Manel and Holderegger, 2013). Specifically, habitat fragmentation can reduce gene flow between breeding sites (Fig 1.10 – arrow 2), ultimately limiting population growth and genetic variation (Fig 1.10 – arrows 3 and 6; Song et al., 2013; Sexton, Hangartner and Hoffmann, 2014). Similarly habitat quality can dictate population abundance, density and breeding behaviour, including mating systems, EPP, mate choice (Fig 1.10 – arrows 8, 9 and 10; Quader, 2005), which again can either facilitate or limit population growth and genetic variation (Fig 1.10 – arrows 16 and 17; Nunney and Elam, 1994; Frankham, 1995; Quader, 2005). The response to changes in habitat quality, composition and fragmentation are particularly exacerbated in resource specialists (e.g. Graham et al., 2022; Pasinelli, 2022), such as nightjar. However, nightjar, like many other species, is typified by a shallow prolonged pattern of demographic decline (Conway et al., 2007; Langston et al., 2007), for which information on population genetics and breeding biology are lacking, potentially limiting our understanding and conservation of the species (Payevsky, 2006; Sanderson et al., 2006; Cox, 2010).

In this thesis I combine genetic and genomic analysis with behavioural observations in the field to reveal fundamental aspects of nightjar breeding and population ecology, with an aim to provide informed conservation recommendations. Until now, no population genetic or genomic studies have been applied to nightjar or indeed any species of Caprimulgiformes. Similarly, my research is the first to classify the genetic mating system and infidelity rates in nightjar or any species within the order Caprimulgiformes. Finally, the research presented in this thesis is the first to study the function of white spots in the European nightjar, and the first across all Caprimulgiformes to study spot characteristics in relation to mating success. The thesis is arranged as a series of independent data chapters (**Chapter 2**, **3**, **5 and 6**) with one literature synthesis (**Chapter 4**) and a final discussion and conclusion chapter (**Chapter 7**). Here I outline the aims addressed by each chapter.

Chapter 2 – Revealing the demographic history of the European nightjar Caprimulgus europaeus

This chapter aims to elucidate the ancient demographic history of nightjar in Europe, determining the response of the species to ancient periods of climate change and provide a preliminary investigation of genetic differentiation and timings of divergence between different European populations. Specifically, this chapter will use the recently assembled reference genome and raw reads of a nightjar from Southern Europe (Secomandi et al., 2021), and a novel nightjar genome from Britain (North West Europe) sequenced by me for analysis of ancient demography using Pairwise Sequentially Coalescent Analysis (PSMC). PSMC analysis will be used to address the following key aims;

2.1) Investigate historic N_e trends of two contemporary nightjar populations relative to past climate fluctuations, including the last glacial maxima (110–10 Kya) as well as the rapid global climate oscillations of the Mid Pleistocene Revolution (~1.2 Mya - 450 Kya), and the mid-Brunhes Event (430–110 Kya);

2.2) Compare N_e trends from the two contemporary populations and a pseudo-diploid combined genome to map genetic divergence between the two populations over the last 5 million years;

2.3) Examine temporal N_e patterns to infer the evolution of migratory behaviour in nightjar over the last 5 million years.

Chapter 3 – Chapter 3: The genomic signature of demographic decline in a long-distance migrant in a range extreme population

Chapter 3 aims to investigate changes in genetic structuring and variation in the British nightjar population between 1841 and 2021 to elucidate the impacts of anthropogenic habitat loss and fragmentation on the species population genetics. Specifically, I use full genome resequencing data across 96 individuals from both historic (n = 60 samples) and modern populations (n = 36 samples), sampled across the historic and extant range of European nightjar in the British Isles. I use this high-resolution data to investigate the genomic signature of recent demographic decline and recovery in a population of nightjar at the limit of the breeding range, to address the following key aims;

3.1) Characterise spatio-temporal genetic structure in the British population and determine whether the historic population (1840-1960) exhibited higher connectivity than the contemporary population (2019-21);

3.2) If genetic structure is found I aim to apply isolation by distance analysis to test the effects of spatial isolation on genetic differentiation among populations;

3.3) Establish whether the British nightjar population exhibited a change in genome-wide heterozygosity over time;

3.4) Determine whether heterozygosity varies across regions across Britain and the Republic of Ireland in both modern and historic temporal groups.

Chapter 4 – Nightjar mating systems and visual communication

This chapter is a synthesis of current avian mating system, sexual selection, and visual communication literature as well as current knowledge in these areas within Caprimulgidae. The work has been accepted as a book chapter in '*Nightjars: From Mystery to Model in Ecology and Evolution*'. The key aims of the chapter are;

4.1) Synthesise current knowledge, and factors limiting the study of mating systems, sexual selection and visual communication in Caprimulgidae;

4.2) Explore how Caprimulgids might be used to test hypotheses relating to mating systems, visual communication and mate choice';

4.3) Identify knowledge gaps and outline valuable future research directions .

This chapter was written when access to facilities was limited and I was unable to collect museum or field samples owing to restrictions related to the COVID pandemic (see Covid Statement for full impacts and further information; **Appendix** Section 8.5).

Chapter 5 – Intraspecific variation in the mating system of European nightjar Caprimulgus europaeus among British breeding sites

Chapter 5 aims to characterise the genetic mating system and extra-pair paternity rates in nightjar for the first time, also testing the effect of nest and male density on extra-pair paternity rates at two breeding sites. Specifically, the chapter combines behavioural (nest site parental associations and breeding density data) and genetic (molecular parentage analysis using microsatellite markers) information from two British nightjar breeding populations (Humberhead Peatlands and Thetford Forest) over three years (2019-21) to address the following aims;

5.1 Characterise the social and genetic mating system and infidelity rates in nightjar for the first time;

5.2 Determine whether infidelity rates vary between and within sites over sampled years;

5.3 Test the hypothesis that breeding density will influence EPP rates at a nest level across populations and years.

Chapter 6 – Are dichromatic white spots sexually selected in European nightjar Caprimulgus europaeus ?

Chapter 6 aims to investigate the role of the sexually dichromatic achromatic white spots exhibited by male nightjar. I investigate whether the white spots act as a quality measure as well as their role in mate choice. Specifically, the chapter uses behavioural (nest site parental associations), morphometric (male wing length, mass), plumage characteristics (size and asymmetry of white spots) and genetic paternity data from two British nightjar breeding populations (Humberhead Peatlands and Thetford Forest) over three years (2019-21) to address the following aims;

6.1 Investigate the role of sexually dichromatic white spots as visual signals and indicator of male a quality in nightjar;

6.2 Establish whether dichromatic white spots are sexually selected features by determining if spot characteristics explain reproductive success variation in paired and cuckolding male nightjars.

Chapter 7 – General discussion and conclusions

In C**hapter 7** I summarise the main results of the thesis and provide a synthesis of the key findings. I address the contributions made by the work to the broader literature, discuss the limitations of my study, highlight directions for future work and finally provide conservation recommendations.

Chapter 2 Revealing the demographic history of the European nightjar *Caprimulgus europaeus*

2.1 Abstract

A species' demographic history provides important context to contemporary population genetics and a possible insight into past responses to climate change. An individual's genome provides a window into the evolutionary history of contemporary populations. Pairwise sequentially Markovian coalescent (PSMC) analysis uses information from a single genome to derive fluctuations in effective population size change over the last ~5 million years. Here I apply PSMC analysis to two European nightjar (*Caprimulgus europaeus*) genomes, sampled in Northwest and Southern Europe, with the aim of revealing the demographic history of nightjar in Europe. I successfully reconstructed effective population size over the last 5 million years for two contemporary nightjar populations. My analysis shows that nightjar are responsive to global climate change, with effective population size broadly increasing under stable warm periods and decreasing during cooler spans and prolonged glacial periods. PSMC analysis on the pseudo-diploid combination of the two genomes revealed fluctuations in gene flow between the populations over time, with gene flow ceasing by the last-glacial maximum. This pattern of differentiation is in line with the species utilising different refugia during glacial maxima. I suggest that nightjar in Europe may show latitudinal (East-West) genetic structuring as a result of reduced gene flow between different glacial refugia. Finally, my results suggest that migratory behaviour in nightjar likely evolved prior to the last-glacial maximum, with long-distance migration seemingly persisting throughout the Pleistocene. However, further genetic structure analysis of nightjar from known breeding sites across the species' contemporary range is needed to fully understand the extent and origins of range-wide differentiation in the species.

2.2 Introduction

Technological and methodological advancements have led to genomics playing an increasingly important role in conservation biology (Allendorf, 2017). There is an inherent value in analysing genetic data, with an individual's genome representing the partial genomes of the individuals who have contributed gametes to the contemporary (sampled) population. Genomes, therefore, provide a repository from which information on historic changes in genetic diversity, effective population size (N_e), speciation, and population structuring can be inferred and used to track adaptations to historic environmental change (Mather, Traves and Ho, 2019; Patil and Vijay, 2021). Specifically, sequence data from a single aligned genome can be used to track historic demographic patterns exhibited by a species or population (Li and

Durbin, 2011). Whilst there are a number of studies showing patterns of interspecific variability in vulnerability to climate and environmental change in extant populations (Gregory et al., 2009; Rodenhouse et al., 2008), genomic analysis is only starting to provide insight into the evolutionary history of species (e.g. Chattopadhyay et al., 2019; Sato et al., 2020). Understanding a species' evolutionary history provides a critical context for evaluating the resilience of species by determining N_e response in context to historic climatic events, to the ongoing global changes of the Anthropocene.

2.2.1 The coalescent and Pairwise Sequentially Markovian Coalescent analysis

Biological phenomena, as reflected in genomic data, can be analysed within a framework known as Coalescent Theory, which is an extension of classical population genetics. Coalescent thinking focuses on viewing populations backward in time, using the divergence amongst alleles observable in a current population or individual to estimate the time or number of generations back to a most recent common ancestor (MRCA) (Rosenberg and Nordborg, 2002; Allendorf et al., 2022). This MRCA is the point where gene genealogies come together, or `coalesce', in a single biological organism (Fig 2.1) (Allendorf et al., 2022).

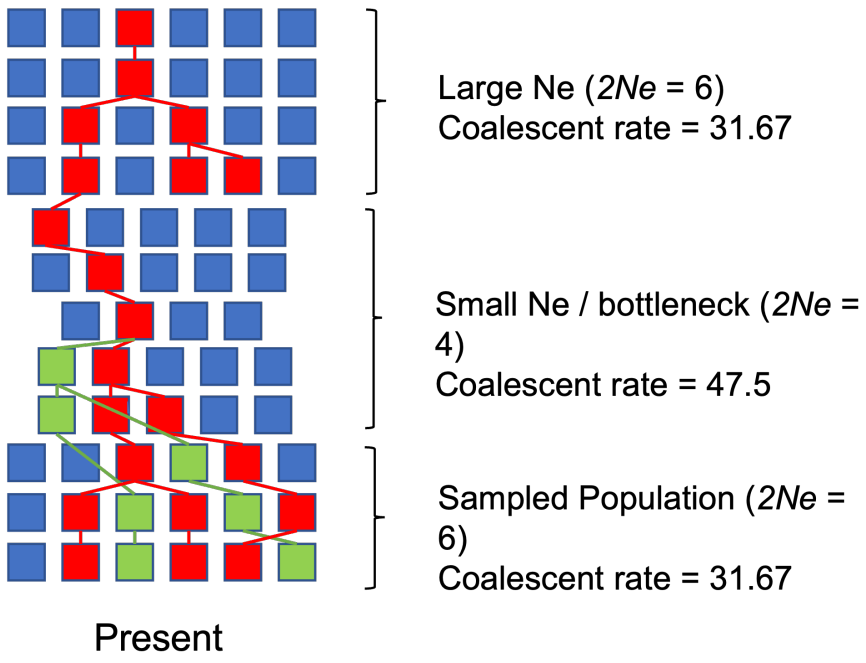


Figure 2.1 Adapted diagram from Buckley, (2018), schematic showing coalescence process of two alleles represented as red and green blocks. The blue blocks here represent unknown alleles. The process works backward from the present (sampled population) through time, with the bottom of the diagram representing the contemporary point of sampling. The diagram shows how small N_e are reflected by the coalescent rate.

Pairwise sequentially Markovian coalescent (PSMC) analysis is a powerful tool that applies a hidden-Markov modelling approach to an aligned genome to infer ancestral changes in N_e (Li and Durbin, 2011). PSMC analysis uses the coalescent theory framework to estimate ancestral N_e change. The rates of coalescence events and N_e are fundamentally linked. Where coalescence rates are elevated, N_e is typically small (i.e. the smaller the past population, the more likely future descendants are to have shared a common ancestor from the focal population), with the converse also true (Mather, Traves and Ho, 2019). Coalescence theory provides a mathematical framework for this process (Box 2.1), enabling historical estimates of N_e to be predicted. Multiple genealogies can be extracted from a single genome owing to the process of recombination, which effectively partitions the genome into distinct segments all with unique evolutionary histories, thus providing unique genealogies across the genome from which coalescent events can be inferred (Fig 2.2). Using this concept, PSMC partitions the genome into blocks determined by the density of mutually inherited heterozygous sites (Li and Durbin, 2011; Mather, Traves and Ho, 2019). The time to the most recent common ancestor (TMRCA) for each block is estimated, with higher densities of heterozygous sites indicative of less recent coalescence intervals (Fig 2.2, Kozma et al., 2016; Mather, Traves and Ho, 2019). Importantly, TMRCA varies between blocks across the genome where recombination has occurred between them, enabling detailed historic genealogies, and thus N_e at different timepoints, to be inferred from a single genome (fig 2.2; Kozma et al., 2016; Mather, Traves and Ho, 2019). PSMC analysis then only requires a single aligned diploid genome (Li and Durbin, 2011; Mather, Traves and Ho, 2019), although genomes of >18x depth coverage are typically needed to accurately infer genotypes at heterozygous sites (Nadachowska-Brzyska et al., 2016; Mather, Traves and Ho, 2019).

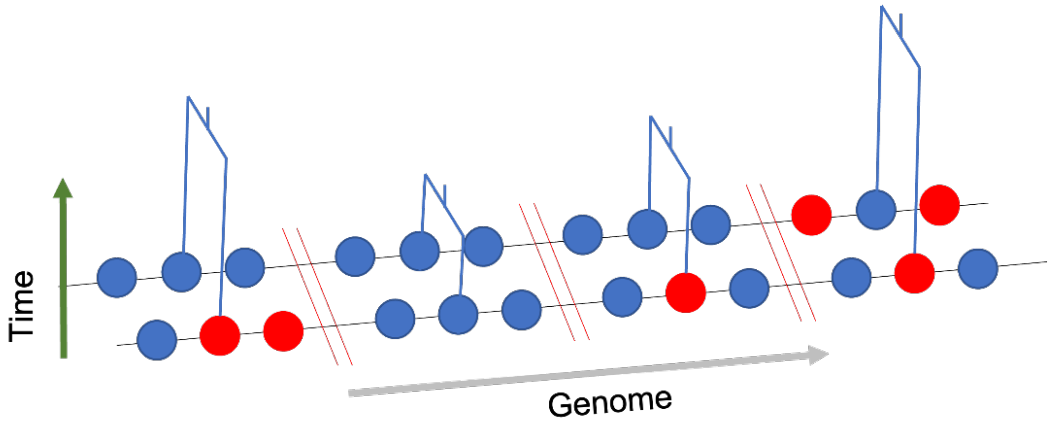


Figure 2.2 The diagram shows alleles along a diploid sequence, with circles representing nucleotide states along the genome. Segments are separated by recombination events (red lines). Different coloured circles at each locus represent heterozygous states, with more heterozygous sites indicating less recent coalescence events. PSMC analysis uses proportions of heterozygous sites along the genome to infer coalescence times, with local trees (blue lines) denoting time to the most recent common ancestor. Note the topology of each genealogy is fixed, but TMRCA differs between segments. (Adapted 3D diagram originally presented in Mather, Traves and Ho, 2019)

PSMC analysis has been used to determine ancestral (up to ~ 5 Mya) population trends and track time scales of species and population divergences (e.g., *Ficedula* flycatchers; Nadachowska-Brzyska et al., 2016; Mather, Traves and Ho, 2019, *Catharus* thrushes; Termignoni-Garcia et al., 2022), as well as shedding light on periods of gene flow among otherwise genetically structured populations. PSMC analysis can be applied to pseudo-diploid combined genomes from individuals from two different populations to investigate changes in gene flow and estimate the timing of divergence. For example, PSMC applied to pseudo-diploid genomes from three Golden eagle (*Aquila chrysaetos*) subspecies revealed the timing of divergence and gene flow change among the subspecies over a time scale of ~11 million years (Sato et al., 2020). When combined with geological and historical climatic data, PSMC analysis can reflect a species' past ability to adapt to environmental change, and particularly on how different populations, subspecies, or species have been affected by broad climatic trends (Nadachowska-Brzyska et al., 2015; Mather, Traves and Ho, 2019). For example, in a comprehensive study of genomes from 38 different bird species, Nadachowska-Brzyska et al., (2015) showed that avian populations typically decreased in N_e under periods of glacial expansion, with recovery during glacial retreat. However, importantly, the authors found that this trend differed between species, highlighting variation in the past ability of different species to cope with environmental change. Understanding a species' response to past environmental change then allows for predictions to be made regarding vulnerability to

contemporary and future climate change and how this may vary interspecifically owing to different life histories (Kozma et al., 2016; 2018; Chattopadhyay et al., 2019), or between populations at different locations across a species range (Sato et al., 2020).

Box 2.1: Neutral Theory and Coalescence:

Information adapted from examples given in Hedrick, (2005) and Frankham, Ballou and Briscoe, (2010). Following neutral theory, the coalescence of two alleles from a contemporary population is predictable. Provided that the loci in question are neutral, the time in generations (T_k) to the previous coalescence event between *K* alleles (e.g., to a generation where there are *K*-1 alleles) can be described as:

$$Tk = \frac{4Ne}{K(K-1)}\ generations$$

Where N_e is the effective population size. The '4' is generated for diploid species, with each potential parent carrying two alleles for a given locus.

N_e then affects the time to coalescence. Under different N_e sizes the time to the most recent coalescence point differs (see examples below from Frankham, Ballou and Briscoe, 2010), with larger effective population sizes leading to longer coalescence times.

Example (A) N_e = 50, *K* = 3:

$$T3 = \frac{4\ Ne}{[K(K-1)]} = \frac{(4*50)}{(3*2)} = 33\ generations$$

Example (B) N_e = 100, *K* = 3

$$T3 = \frac{4\ Ne}{[K(K-1)]} = \frac{(4*100)}{(3*2)} = 67\ generations$$

2.2.2 Historic European climate change

Over the past ~5 million years the global climate has fluctuated dramatically, oscillating between extensive glaciation and interglacial periods of warming. The mid-Pleistocene was characterised by long glacial periods interrupted by short interglacial periods. During this time climate oscillations from glacial to interglacial periods were shortened dramatically from ~100 to ~40 Ky after the Mid-Pleistocene Revolution (MPR) ~ 1 Mya. Climate oscillations were constricted, with temperatures during interglacial periods throughout this time typically lower than those presently recorded (Pisias and Moore, 1981). However, throughout the Mid-Brunhes Event (MBE; ~450-110 Kya), interglaciations were characterised by warmer

temperatures, increasing the amplitude of climate cycles compared with those of the mid and mid-late Pleistocene (Candy et al., 2010; Barth et al., 2018). With these climate cycles, temperate latitudes in Eurasia will have been subject to huge temperature and environmental changes. However, the effects of these oscillations have not been consistent across the continent, with more northerly latitudes subject to greater fluctuations than southerly latitudes (Stroeven et al., 2016). Associated fluctuations in habitat change and availability are also expected to have varied latitudinally (Candy et al., 2010; Lisiecki and Raymo, 2005; Head and Gibbard, 2015). During the last glacial period (~110 Kya) the Fennoscandian ice sheet covered much of Eastern and North Eastern Europe (Denton and Hughes, 1981).

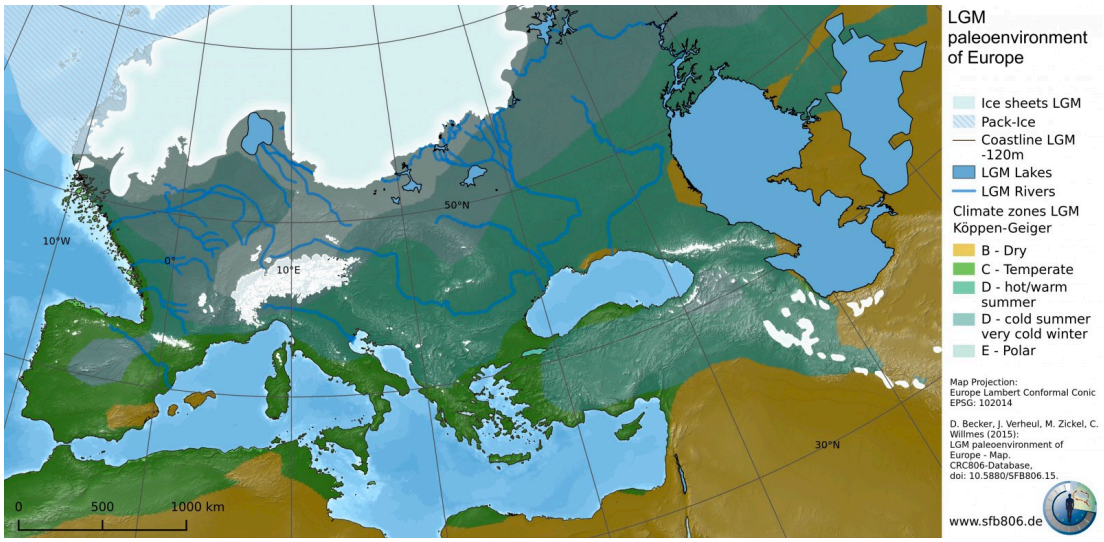


Figure 2.3 map of the extent of glaciation across Eurasia during the last glacial maximum (~110 Kya) showing that the British Isles experienced near complete coverage from the Fennoscandian ice sheet, compared to Italy which avoided any ice sheet coverage and retained a temperate climate. Original map downloaded on 10/12/22 from; https://shorturl.at/OU245)

Temperate climate zones were then restricted to contemporary Southern Eurasia (Fig 2.3). These significant shifts in global climate have been shown to correspond with fluctuations in population size in a number of species (Nadachowska-Brzyska et al., 2015; Kozma et al., 2016, 2018). Over periods of cooling, temperate species will have likely been restricted to southern refugia in Europe (Iberia, Apennines, and Balkans; Hewitt, 1999; but see Thorup et al., 2021). Restriction to different glacial refugia and subsequent northward expansion during interglacial periods have been linked to contemporary population structure and sub-species divergence in multiple species (e.g. Hansson et al., 2008; Nadachowska-Brzyska et al., 2016; de Greef et al., 2022). Indeed many Palearctic birds show an East - West pattern in genetic structure and speciation, hypothesised to be associated with different glacial refugia occupation (e.g. Hansson et al., 2008; Lombardo et al., 2022; Väli et al., 2022).

2.2.3 Study species background

The European nightjar (*Caprimulgus europaeus*), henceforth nightjar, is a long-distance migratory bird with a temperate breeding distribution ranging from Northwest Europe through to Eastern Russia and Mongolia (BirdLife International, 2022). Nightjars are habitat specialists relying primarily on heathlands and clear-fell habitats to breed (Cleere, 1998). The species exhibits a slow life history, being long-lived (up to 12 years; BTO, 2022) and producing only 2 young per brood (maximum of 4 chicks per breeding season over 2 broods; Cramp and Simmons, 1985). Nightjars have been subject to population declines across the NW of their range (Conway et al., 2007; Langston et al., 2007), and to a lesser extent throughout continental Europe (listed under Annex I of the Birds Directive 2009/147/EC) (Evens et al., 2017b; Knaus et al., 2018), owing to the loss and degradation of breeding habitats (Langston et al., 2007; Ashpole et al., 2015). Being a habitat specialist, an aerial insectivore, and strongly migratory, leaves nightjar especially vulnerable to environmental change, with effects of current anthropogenic habitat loss likely to be compounded under future climate change scenarios. While current population trends for nightjar are not a cause for concern (IUCN: Least Concern; BirdLife International, 2022), ancestral demographic history may leave populations vulnerable to contemporary and future anthropogenic-driven environmental change, if populations have been subject to bottlenecks resulting in a loss of genetic variation (Bürger and Lynch, 1995; Frankham, Ballou and Briscoe, 2010; Nadachowska-Brzyska et al., 2015; Hohenlohe, Funk and Rajora, 2021). There is then a value in understanding how the species has responded to historic environmental change to better inform predictions of future population trajectories.

European nightjar likely originated from the Afrotropics, with the most closely related extant species being an Afrotropic resident (Rufous-cheeked nightjar *Caprimulgus rufigena*) (Han, Robbins and Braun, 2010). Nightjar evolved a migratory behaviour, likely utilising breeding habitat across temperate Eurasia as it became available during warm interglacial periods (Larsen et al., 2007), with the authors proposing the evolution of migratory behaviour in nightjar during the last glacial maxima (LGM). The species' breeding range will have likely been restricted to temperate habitat in Southern European refugia during glacial maxima (Fig 2.3) (Ponti et al., 2020), with breeding habitat lost across the Northern and Western extents of nightjar's contemporary range (Ponti et al., 2020). Whilst it is unknown whether nightjar bred across temperate Eurasia prior to the LGM, it seems probable; with evidence of a migratory

phylogenetic divergence in nightjar between Eastern and Western populations, likely originating in the early Pleistocene or late Pliocene (~2.5 Mya) (Schweizer et al., 2020). Indeed, suitable breeding habitats would have been available across temperate Eurasia throughout warm interglacial periods. If nightjars exhibited an Afro-European migration strategy pre-LGM, paleoclimatic-driven periods of population expansion and contraction should be evident, with birds likely constrained to temperate refugia within Southern Eurasia and North Africa during cooling periods and expanding out to breed across temperate Eurasia as habitat availability increased during warm interglacial periods.

European nightjar is currently comprised of six subspecies (*C.e. europaeus*, *meridionalis*, *sarudnyi*, *unwini*, *plumipes*, *dementievi*) broadly following an East-West clinal distribution (Cleere, 1998; Cleere, Christie and Rasmussen, 2021). However, sub-species have largely been defined by plumage characteristics, with mtDNA analysis finding little association between genetic variation and current sub-species classification (Schweizer et al., 2020). Nevertheless, divergence among subspecies is thought to have occurred during expansion post-LGM (Holyoak, 2001). As in other Palearctic migratory species (see Pârâu and Wink, 2021 for review) it is likely that diversification of European nightjar occurred through isolation during breeding periods in spatially separate refugia (i.e. Iberian peninsula, Italy, South East Europe etc.) during historic glacial maxima. Within the European population of nightjars an East-West genetic structure might be expected, with contemporary population differentiation likely occurring during the LGM where breeding populations would have been spatially segregated.

2.2.4 Aims and hypotheses

The reference genome for the European nightjar was sequenced and assembled in 2021 from a bird captured in Southern Europe during the spring migration period (Secomandi et al., 2021). In this chapter I used this published genome alongside a novel Pacbio HiFi reference, sequenced in this study, sampled from a population from the extreme North Western range limit in the UK. I applied PSMC analysis to determine the ancestral demography of nightjar from two contemporary populations in Europe to estimate the historic N_e change over time from 10 Kya to 5 Mya. Specifically, I aimed to;

1) Investigate historic N_e trends of two contemporary nightjar populations relative to past climate fluctuations. I hypothesised that, in both populations, decreases in N_e will have followed periods of global cooling (i.e., LGM (110–10 Kya)), as well as the rapid

global climate oscillations of the Mid-Pleistocene Revolution MPR (~1.2 Mya - 450 Kya). I predict that N_e increased following periods of warming (i.e., the warmer interglacial periods during the Mid-Brunhes Event MBE; 430–110 Kya);

2) Compare N_e trends from the two contemporary populations and a pseudo-diploid combined genome to examine divergence between the two populations, addressing the hypothesis that the two populations will have diverged during the LGM.

3) Examine temporal N_e patterns to investigate the evolution of migratory behaviour in nightjar. I hypothesise that nightjar will have exhibited fluctuating N_e and population divergence before the LGM, suggestive of long-distance migratory behaviour arising prior to the LGM.

2.3 Methods

2.3.1 Sampling genetic material, extraction and sequencing

A female nightjar from a breeding population in the East of England (latitude: 53.531, longitude: -0.953) was used to extract DNA for sequencing (population henceforth referred to as NW Europe or NWE). The bird died on 7th August 2019, so was assumed to have been part of the breeding population and not moving through on migration, with spring and autumn migration for nightjar occurring between April - June and late August and September respectively. High molecular weight DNA was extracted from a blood clot in the individual's heart using a modified version of the phenol-chloroform protocol outlined by (Sambrook, Fritsch and Maniatis, 1989). Full extraction protocol details can be found in **Appendix**, Section 8.2.1. The high molecular weight DNA was then sent to the Centre for Genomics Research facility at the University of Liverpool for PacBio HiFi sequencing library preparation. The reference genome (for assembly details see Secomandi et al., 2021) and 10x Genomics Illumina sequence reads were sequenced from a single female nightjar captured in South West Italy in spring 2021 (latitude: 40.794, longitude: 13.427) provided by Secomandi et al., (2021) (population henceforth referred to as South Europe or SE).

2.3.2 Genome alignment

Minimap (minimap2 v. 2.18-r101; Li, 2018) and BWA mem (arXiv:1303.3997v1 [q-bio.GN]; Li, 2013) software were used to align the reads from the NWE (HiFi reads) and SE (10 x Illumina reads) nightjars to the reference genome, respectively. Unmapped reads were then filtered from both files leaving only mapped reads.

2.3.3 PSMC analysis

To understand ancestral changes in N_e a Pairwise Sequential Markovian Coalescent (PSMC) method was applied to the mapped bam files from the HiFi and 10x Illumina reads, for which the average coverage was 30.5x and 88.1x, respectively. First, consensus sequences were generated from the aligned indexed bam files from the HiFi and 10x reads using SAMtools mpileup command and vcfutils.pl as per Li and Durbin, (2011). Consensus files were generated for each chromosome independently before being combined. For the HiFi data, from the NWE genome, four chromosomes (chromosome numbers; 3, 5, 25, and 32) failed to produce consensus files and reduced representations for two of the four chromosomes (3 and 5) were used, with two chromosomes (25 and 32) excluded from the analysis. This resulted in a loss of only ~1% of genomic material for analysis. Sex chromosomes were also excluded from the analysis for both HiFi and 10x genomes. This resulted in 89.8% of the NWE genome and 90.8% of the SE genome being retained for downstream analysis. Consensus files were then filtered for read depth and quality. In order to reduce the effects of low coverage and collapsed regions, consensus files were filtered by excluding reads ~ < ⅓ and >2x mean depth. This resulted in removing reads <10x and >60x for the HiFi data and <30 and >120x for the 10x reads. Finally, filtering for base quality scores of <20 for the HiFi reads and 10x reads were applied.

The PSMC analysis was then run on the combined consensus fastq files using the PSMC software package Li and Durbin, (2011); https://github.com/lh3/psmc). PSMC parameters used by Nadachowska-Brzyska et al., (2015) for demographic analysis of 38 different bird species were chosen for my analysis, where "-N" (30) is the number of iterations, "-t" (5) is the maximum time to the most recent common ancestor, "-r" (5) is the initial mutation/ recombination rate ($r = \theta/\rho$) and "-p" (4+30*2+4+6+10) denotes the distribution of atomic time intervals. In order to determine variation in PSMC predictions, the data were bootstrapped 100 times.

PSMC analysis can be applied to pseudo-diploid genomes formed from the fusing of haploid genomes from two separate populations or species. When PSMC is applied, deviations in N_e trends of the pseudo-diploid genome from the two parent populations can denote reductions in gene flow and points of divergence between the two populations signified by the N_e of the pseudo-diploid genome tending towards infinity (reducing coalescence events leading to an apparent increase in N_e) (Li and Durbin, 2011; Prado-Martinez et al., 2013; Sato et al., 2020). To determine the timing of divergence between the two sampled populations a pseudo-diploid genome was created by first generating pseudo-haploid genomes through randomly sampling heterozygous alleles using Seqtk V1.3 'randbase' (-r) (https://github.com/lh3/seqtk; accessed Oct 25th, 2022) from both consensus sequence files as generated above. Pseudo-haploid files were then merged using Seqtk 'mergefa' to produce a single pseudo-diploid genome consensus file. PSMC analysis was then applied to the pseudo-diploid genome as described above.

Finally, all PSMC results were plotted using Gnuplot (http://www.gnuplot.info/) with the -R flag applied to export .txt files. In order to plot the PSMC results, the data must be scaled to real-time by using mutation rate and generation time (Li and Durbin, 2011). A generation time of 2 (-g 2) was selected for nightjars following that used for Chuck-will's-widow (*Antrostomus carolinensis*) (Nadachowska-Brzyska et al., 2015), with birds able to breed in their second year (Cramp and Simmons, 1985b). As no species-specific mutation rates were available for European nightjar, a mutation rate of $\mu = 4.6 \times 10^{-9}$ was used as per Sato et al., (2020). The mutation rate was initially estimated for collared flycatchers (*Ficedula albicollis*) (Smeds, Qvarnström and Ellegren, 2016), but has since been successfully applied to other passerines (Ericson, Irestedt and Qu, 2022), raptors (Hanna et al., 2017; Sato et al., 2020) and waterfowl species (Ericson et al., 2017).

2.4 Results and discussion

In this study I explored the demographic history of two modern nightjar populations (NW and S Europe). Using PSMC I found significant fluctuation in N_e in European nightjar over the last 5 million years, coinciding with major paleoclimatic events (Fig 2.4A). The timing of initial divergence between the two nightjar populations was ~1.2Mya (Fig 2.4A), with final divergence found to coincide with the LGM (~110 Kya) (Fig 2.4B).

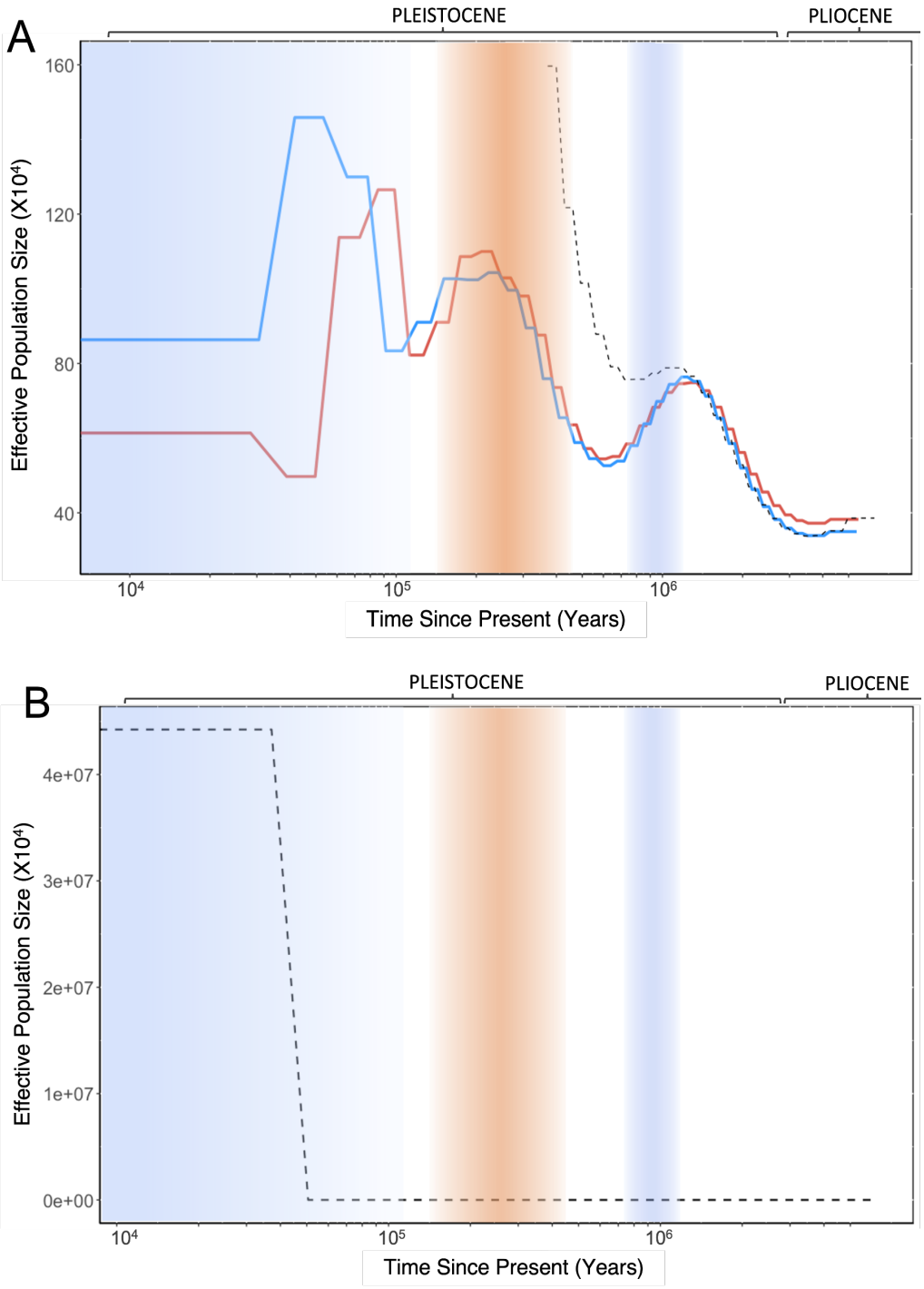


Figure 2.4 PSMC plots: A) NW Europe (Red line) and S Europe (Blue line) sampled European nightjar, as well as pseudo-diploid genome of NW/S Europe birds (dashed line), depicting demographic history (N_e change) over the last ~5 million years (bp), scaled with a mutation rate of 4.6 × 10−9 per site and generation time of 2 years. The x-axis depicts time (in years) on a log scale, with the y-axis showing effective population size. B) Estimated N_e for pseudo-diploid genome only (dashed line). Approximate timings of significant periods of global climate change are shown by shading along the x-axis. Light blue shading = last glacial period (LGP), orange shading = Mid-Brunhes event (MBE), and dark blue shading = Mid-Pleistocene Revolution (MPR).

2.4.1 Demographic history of European nightjar

My analysis suggests that nightjar have experienced significant fluctuations in N_e over the last ~5 million years. Two of the most significant N_e changes occurred during the Pleistocene, with both populations (NWE and SE) increasing throughout the early Pleistocene to a maximum N_e of ~780,000 individuals, before decreasing to ~570,000 individuals by 600 Kya during the MPR (~1 Mya - 450 Kya; Fig 2.4A). As hypothesised, the N_e of both populations then increased throughout the MBE to ~ 1 million individuals by ~240 Kya (Fig 2.4A). Both populations then decreased in N_e until ~100 Kya (Fig 2.4A). At the onset of the LGP, populations exhibited a peak in N_e, followed by a steep decline as the LGP progressed (Fig 2.4A). The N_e of the two populations then diverged in size (see Fig 2.4A).

Overall, historic nightjar N_e in Europe decreased and increased during periods of cooling and warming respectively (Fig 2.4A). Nightjar are insectivorous habitat specialists requiring clear fell, heathland, or woodland edge to breed (Cleere, 1998), feeding primarily on Lepidoptera (Mitchell et al., 2022). With reductions in temperature and glacial expansion, prey and habitat availability will have been constrained to more southerly latitudes (Schmitt, 2007), likely corresponding with a reduction in nightjar distribution and thus N_e. For example, the decrease in nightjar N_e ~1.2 Mya – ~ 600 Kya (Fig 2.4A) overlapped the MPR (~1 Mya - 450 Kya), which was characterised by shortened interglacial periods and cooler average temperatures which restricted the northward resurgence of temperate animal and plant communities (Pisias and Moore, 1981; Head and Gibbard, 2015). Conversely, warmer temperatures will have likely increased the availability of suitable habitat across northerly latitudes (Schmitt, 2007; Candy et al., 2010). Indeed the stable climate of the late Pliocene and early Pleistocene (Head and Gibbard, 2015), as well as the short glacial and warm interglacial periods of the MBE (Candy et al., 2010; Barth et al., 2018) associated with increases in nightjar N_e in my study (Fig 2.4A). Similarly, the dramatic N_e increase during the late Pleistocene prior to the LGP (Fig 2.4A) coincided with the Eemian warm phase (~127 Kya; Bergoeing, 2017), which was characterised by the expansion and persistence of temperate plant communities into northerly latitudes (Van Andel and Tzedakis, 1996; Sánchez Goñi et al., 1999; Lisiecki and Raymo, 2005).

Following similar trends exhibited by other Afro-Palearctic migrants (i.e. *Ficedcula* flycatchers; (Nadachowska-Brzyska et al., 2016), N_e of both nightjar populations greatly decreased as the LGP continued, likely restricting nightjar to Southern European refugia (Schmitt, 2007; Lombardo et al., 2022) or North Africa (Thorup et al., 2021). Bootstrapping indicates caution is required regarding exact timings of N_e fluctuations (see **Appendix** 8.1.3).However, PSMC

analysis in other Caprimulgids (i.e., Chuck-will's-widow) and Afro-Palearctic migrants (i.e.: Common cuckoo, *Cuculus canorus*) (Nadachowska-Brzyska et al., 2015), have shown similar fluctuating trends in N_e over the same timeframe, suggesting that the estimated timings of N_e change with paleoclimatic events in my study are reasonable.

2.4.2 Population structure and divergence in nightjar

When applied to a pseudo-diploid genome derived from two different populations, PSMC analysis can be used to determine the timing of population divergence. This is signalled by the pseudo-diploid N_e diverging from the two parent populations and tending towards infinity (Prado-Martinez et al., 2013). This occurs because coalescence events between the two populations were severely reduced or ceased, leading to an increase in N_e as interpreted by the analysis. In my analysis, the pseudo-diploid N_e trend appeared to diverge from the NWE and SE populations ~1.2 Mya (Fig 2.4A). However, true divergence (the point at which N_e tends to infinity) does not occur until ~40 Kya (Fig 2.4B). Even taking into account for the ~35Ky error window suggested by the bootstrapping (see **Appendix** Fig 8.2), the main divergence event between the NWE and SE populations occurred within the LGP (Fig 2.4B).

The two modern populations used in this study are spatially distant and behaviourally distinct as they exhibit different migration strategies (Evens et al., 2017b). Nightjars breeding in Western Europe typically migrate through Iberia during spring migration, with Eastern breeders migrating through Italy and SE Europe (Evens et al., 2017b; Norevik, Åkesson and Hedenström, 2017). In other trans-Saharan migrants, such migratory behaviour is thought to be ancestral and 'hard wired' into populations, likely predating the Pleistocene (Thorup et al., 2021). Although the SE bird in my study was trapped during the spring migration period (Secomandi et al., 2021), it is probable that the individual's breeding population was located within Central to Eastern Europe as suggested by recent tracking studies (e.g., Norevik, Åkesson and Hedenström, 2017).

As in other migratory Palearctic birds (e.g., Lesser whitethroat *Sylvia curruca*; Olsson et al., 2013; Pied wagtail *Motacilla alba*; Li et al., 2016), I suggest that nightjar may exhibit East-West genetic structuring, but investigation is required. Results from MtDNA analysis have suggested that nightjar can be divided into Eastern and Western lineages, with divergence being deeper (c. 2.9 Mya) (Schweizer et al., 2020) than that suggested by my PSMC analysis (initial divergence ~1.2 Mya, cessation of gene flow ~40 Kya ± 35 Ky; Fig 2.4). However, the samples

contributing to Schweizer et al. (2020) work spanned a much broader latitudinal range (encompassing W Europe to Asia) than those in my study and likely represented a deeper divergence. Much of the structure in contemporary Palearctic and Nearctic animal populations are fundamentally linked to past glacial and interglacial cycles, which have led to the contraction of temperate breeding populations into Southern refugia, and subsequent northward recolonisation during warmer interglacial periods (Hewitt, 2004; Nadachowska-Brzyska et al., 2016; Yao et al., 2022; de Greef et al., 2022). Previously panmictic populations may become isolated from one another in different refugia, during periods of glaciation, leading to genetic differentiation post-interglacial expansion (Hewitt et al., 2001).

Considering the timings of gene flow reduction and divergence between the two populations it is likely that the ancestral states of both populations utilised different refugia over historic glacial periods and most recently the LGM. Owing to the apparent divide in contemporary migration routes I suggest that the NWE population will have utilised the Iberia refugium and the SE sampled population the Italian refugia during glacial periods. Whilst most Western Palearctic avifauna (117 out of 131 studied by Pârâu and Wink, 2021) show admixture among populations, my study suggests that nightjar exhibit genetic structure within their Eurasian breeding range. With the sampling information available my results tentatively reflect East-West structuring in the European nightjar population, likely diverging during isolation in different glacial refugia during periods of glaciation. However, it should be noted that the SE individual may belong to the Southern European *C.e.meridionalis* subspecies and instead my results might reflect divergence between *C.e.meridionalis* and the nominate *C.e.europaeus* (NWE individual) subspecies. Nevertheless, validity of the *C.e.meridionalis* subspecies remains questionable, with as yet no genetic evidence to support the subspecies status (Del Hoyo et al., 2014; BirdLife International, 2022). Nightjars of the nominate race *C.e.europaeus* migrate through Italy and SE Europe (Evens et al., 2017b; Norevik, Åkesson and Hedenström, 2017). Given the individual used in this study was sampled during spring migration, it is likely that the bird was migrating to breeding grounds at a higher latitude and belongs to *C.e.europaeus*. However, further work is required to resolve population genetic structure across the species range, and there is a wider need to resolve the molecular phylogeny of European nightjar and its subspecies.

2.4.3 Evolution of migratory behaviour in nightjar

Following the timeline proposed by my study (Fig 2.4), it seems unlikely that nightjar migratory behaviour developed post-LGM as suggested by Larsen et al., (2007). The dramatically fluctuating N_e prior to the LGM throughout the Pleistocene may reflect periods of significant population expansion and contraction associated with climate driven changes in temperate breeding habitat availability (Ponti et al., 2020). If nightjar had exhibited a sedentary Afrotropic distribution prior to the LGM I might expect to see less severe fluctuation in N_e relative to global climate change (Kimmitt et al., 2023; but see Speckled mousebird; *Colius striatus* in Nadachowska-Brzyska et al., 2015). Similarly, if migratory behaviour had not developed until ~20,000 ya, I would expect divergence between populations to occur exclusively post-LGM. However, my results highlight that whilst gene flow appeared to cease between the NWE and SE populations towards the LGM (Fig 2.4B), population divergence occurred as early as ~1.2 Mya (Fig 2.4A), with subsequent episodes of mixing ensuing during periods of range expansion. My results suggest that long-distance migratory behaviour in nightjar evolved prior to the LGM and was maintained throughout the Pleistocene, likely predating the initial divergence 1.2Mya recorded in my study (Fig 2.4A). This is corroborated by the deeper divergence (2.9Mya) between range-wide East-West lineages recorded by Schweizer et al., (2020), which may indicate a migratory divide. My results contribute to the growing consensus that long distance migratory behaviour in contemporary Western-Palearctic avifauna predates the LGM (i.e. Ponti et al., 2020; Ralston et al., 2021; Thorup et al., 2021; Kimmitt et al., 2023).

2.4.4 Limitations of PSMC analysis

Results of PSMC analysis are influenced by the scaling applied to plots, determined by mutation rate and generation time (Li and Durbin, 2011; Mather, Traves and Ho, 2019). However, the overall pattern of N_e change will remain the same independent of scaling parameters (Nadachowska-Brzyska et al., 2016). Data on both of these parameters are often limited for study species (e.g. see Sato et al., 2020; Chattopadhyay et al., 2019; Ericson et al., 2022), including nightjar. Thanks to the wealth of PSMC studies over a multitude of avian taxa (e.g., Nadachowska-Brzyska et al., 2015, 2016; Kozma et al., 2018; Sato et al., 2020; Brüniche-Olsen et al., 2021) parameters suited to a wide range of avifauna can be selected, such as those used in my study as per Sato et al., (2020). Therefore, whilst caution must be applied concerning the timings and magnitudes of N_e change, as highlighted by bootstrapping (see

Appendix 8.1.3), I believe that PSMC analysis provides a valuable method to associate broad N_e trends with concurrent climate cycles.

2.5 Conclusion

PSMC is a useful tool to characterise past demography and resolve timing of species and population differentiation, processes which underlie contemporary genetic and demographic patterns. Results from my PSMC analysis suggest that nightjar were highly susceptible to climatic variation, increasing in number during warm interglacials and long periods of relative climate stability. The historical context provided by my research suggests that the current climate best suits nightjar. Limitations on population size are likely primarily anthropogenic, with humans responsible for the mass deforestation and agriculturalization of Europe from 8.2 Kya (Kaplan et al. 2009). Habitat loss, fragmentation, degradation and disturbance are reported as the primary drivers of contemporary population reduction in nightjar (Langston et al., 2007; Lowe, Rogers and Durrant, 2014; Ashpole et al., 2015). Although nightjar have been shown to persist through historic climate change, contemporary anthropogenic pressures may reduce the ability of the species to adapt to the current rapidly changing climate.

As in multiple other Palearctic and Nearctic birds, my analysis suggests that restriction to different refugia during glacial cycles may have driven divergence within the European population of nightjar. My analysis suggests a complete cessation of gene flow between the two populations by ~40 Kya during the LGP, although mixing under current interglacial conditions is likely. Genetic structure within the European population has significant conservation implications, potentially delimiting the current population into smaller conservation units. My results also suggest that migratory behaviour in nightjar evolved prior to the LGM, persisting throughout the Pleistocene. However, further research is needed to understand the spatial context of this apparent range-wide genetic structure, as well as to clarify timing of long-distance migration evolution, as well as current taxonomic assertions. I recommend a range-wide molecular analysis, including population genetics, of nightjar to better understand the extent and origins of divergence within the species. Such research would also aid ongoing taxonomic uncertainties surrounding subspeciation in nightjar (Schweizer et al., 2020). Finally, while caution is needed, here PSMC analysis has provided a useful insight into the demographic past of nightjar in Europe, which has highlighted nightjar population genetics as a valuable future research direction.

Chapter 3 The genomic signature of demographic decline in a long-distance migrant in a range extreme population

3.1 Abstract

The European nightjar (*Caprimulgus europaeus*) is a long-distance migratory bird and habitat specialist. Similar to other long-distance migrants, nightjar have suffered a significant demographic decline in Britain over the last century. Despite the species' vagility, limited ringing recoveries suggest high site fidelity and little movement between breeding sites in Britain. Nightjar are hard to study using conventional methods owing to the species cryptic and nocturnal behaviour. Coupled with a lack of accurate census data prior to 1961, there is a need to quantify the extent and measurable genetic impacts of demographic decline in the British population. I applied full genome resequencing to 60 historic (1841-1980) and 36 modern individual nightjars from the British population. Nightjar exhibited a statistically significant 34.8% loss in heterozygosity over the last ~180 years in Britain, also showing a departure from panmixia among historic samples and demonstrate spatial structure in the modern population. Such fine-scale structuring in migratory birds is rare. However, the specialist resource requirements of nightjar and location of the British population at the species range limit may have contributed towards my findings. Whilst my results provide no immediate cause for conservation concern, the genomic signature of demographic decline is evident and highlights the potential for worrying genetic and demographic trends in the future. This study demonstrates the value in characterising the spatio-temporal population genomics of migratory species. This approach enables accurate quantification of the effects and extent of population decline as well as predictions of future genetic and demographic trajectories under increasing anthropogenic pressure.

3.2 Introduction

Species and populations are under threat globally owing to ongoing habitat loss, degradation, and fragmentation (Wake and Vredenburg, 2008; Barnosky et al., 2011; Ceballos et al., 2015). Migratory birds are particularly vulnerable (Bairlein, 2016), with insectivorous species subject to severe population size reductions (Nebel et al., 2010; Sauer et al., 2017; Nebel et al., 2020). Loss and fragmentation of habitat can drive population extinction risk by reducing connectivity and inhibiting dispersal (Frankham, Ballou and Briscoe, 2010), with the detrimental impacts also recorded in vagile species with a perceived high tolerance to fragmentation (i.e. migratory birds; Lindsay et al., 2008; Hallworth et al., 2021; Larison et al., 2021). Highly spatial fragmentation can lead to once continuous populations becoming genetically distinct from one another. Reductions in population size and connectivity correspond with loss of genetic variation owing to reduced gene flow and the exacerbated effects of genetic drift (Frankham,

Ballou and Briscoe, 2010). Traditionally it was thought that as populations become isolated and smaller, the opportunity to purge deleterious alleles is reduced (Crnokrak and Barrett, 2002). This can lead to the detrimental effects of inbreeding depression and a reduced ability to cope with stochastic change, with these effects often operating synergistically (Bürger and Lynch, 1995; Frankham, Ballou and Briscoe, 2010; Hohenlohe, Funk and Rajora, 2021). In cases of rapid reductions in population size, a genetic bottleneck (a significant sustained loss in genetic diversity) may occur in the populations (Frankham, Ballou and Briscoe, 2010). Such genetic signatures (evidence of a recent bottleneck, high levels of genetic structuring, and reduced variation) may reflect a reduced capacity of a species or population to cope with environmental change and ultimately a heightened extinction risk (Kempe, 2008; Frankham, Ballou and Briscoe, 2010). An understanding of the degree of differentiation among populations and levels of variation therein are important in delineating management units (Fuentes-Pardo and Ruzzante, 2017) and in determining population connectivity in difficult-to-monitor taxa, such as nocturnal and cryptic species (e.g. Crates et al., 2019; Larison et al., 2021).

3.2.1 Temporal population genetics

An inability to sample populations before and after anthropogenic land-use changes reduces the ability of contemporary genetics studies to unearth the full extent of anthropogenic impacts on contemporary population genetic patterns (Billerman and Walsh, 2019). In such cases, researchers are restricted to performing comparisons between contemporary continuous and fragmented populations (Mossman and Waser, 2001; Keller and Largiadèr, 2003). However, the temporal lag between demographic responses to fragmentation and impacts on genetic structure can make results challenging to interpret and reduce their usefulness for informing conservation measures (Epps and Keyghobadi, 2015). Museums provide a valuable resource for population geneticists to perform temporal comparisons of contemporary and historic populations to determine changes in effective population size (N_e), genetic variation, and structure to help quantify the impacts of habitat loss and fragmentation (Billerman and Walsh, 2019; Fenderson, Kovach and Llamas, 2020). Historic DNA (hDNA) can be extracted from foot or toe pads of dry avian skins, with the latter the most commonly used material (Irestedt et al., 2022). The use of museum specimens has been constrained by DNA quality and contamination, as well as the spatiotemporal availability of samples (Irestedt et al., 2022). Most avian museum specimens (skins) are stored dry at room temperature and DNA may degrade by shearing, depurination, and deamination of cytosines to uracils (Briggs et al.,

2007; Dabney, Meyer and Pääbo, 2013). These effects typically worsen over time and with the use of preservatives (i.e. formaldehyde; Raxworthy and Smith, 2021). As such, hDNA is typically highly fragmented and characterised by low yields (Irestedt et al., 2022), which can have implications when sequencing. The incorporation of erroneous bases arising from DNA degradation may lead to sequencing errors, alignment, and downstream SNP identification issues (Irestedt et al., 2022). However, the development of optimised wet lab methods (dedicated clean rooms, extraction, and library preparation protocols, PCR amplification etc.) and dedicated bioinformatics applications (e.g. damage mapping, SNP calling by genotype likelihoods etc.) have helped mitigate these issues (Irestedt et al., 2022). Nevertheless, with reducing costs and increasing accessibility, full-genome resequencing is becoming increasingly utilised in hDNA studies, having been used to reveal temporal demographic and genetic patterns in threatened taxa (Feng et al., 2019), as well as extinct species (e.g. Murray et al., 2017).

Typically, studies tracking spatio-temporal genetic structure and N_e have been restricted to model taxa, or geographically isolated highly threatened species, for which the genomic indicators of demographic change are apparent (e.g. Feng et al., 2019; Robinson et al., 2021; Cavill et al., 2022; Westbury et al., 2022). In such cases, information on historic bottlenecks and contemporary population structure are imperative for effective conservation (e.g. translocation of individuals, delineating conservation units, etc.; Frankham, Ballou and Briscoe, 2010). However, comparatively little attention has been paid to non-model taxa or species which have avoided severe bottlenecks or are distributed across a large geographical range. The majority of threatened avifauna have avoided severe population bottlenecks, and instead exhibit moderate population declines typified by local extinctions and fragmentation across large ranges (Payevsky, 2006; Cox, 2010). Consequently, the genomic footprint of a common demographic trend among threatened avifauna remains largely unknown. Indeed, in these cases inference of historic genetic diversity and structure can offer value in classifying population trends and the anthropogenic impacts on them, which may be especially pertinent in otherwise difficult-to-study species (i.e., rare, cryptic, or nocturnal taxa) which may evade monitoring.

3.2.2 Population structure in vagile birds

Vagile species, such as long-distance migratory birds, have the potential to negate the depletion of gene flow stemming from habitat loss and fragmentation because they are able to

move between spatially distant breeding populations (Pârâu and Wink, 2021). A recent phylogeographic review of Western Palearctic birds found that out of 145 species reviewed, very few migratory species displayed genetic differentiation among populations (Pârâu and Wink, 2021). However, many of these previous studies have often relied on low-resolution genetic markers when investigating population differentiation (i.e. microsatellites and mtDNA; Haase et al., 2019; Morinha et al., 2017; Pârâu et al., 2019; Väli et al., 2022). These markers are less suited to detecting fine-scale genetic structuring among populations compared with genomic data, and may consequently lead to a premature rejection of population structuring where microsatellite markers are used. A growing number of studies are applying a genomic approach when investigating population structuring (e.g. Calderón et al., 2016; von Rönn et al., 2016; Larison et al., 2021; Pârâu et al., 2022) although next generation sequencing (NGS) techniques remain rare (just seven out of 198 reviewed studies in Pârâu and Wink, 2021). High-throughput NGS produces a large amount of high-resolution genomic data (Holliday, Hallerman and Haak, 2019; Allendorf et al., 2022). This enables powerful inferences of contemporary population genetic patterns, including fine-scale genetic structuring (Allendorf et al., 2022) from relatively small sample sizes (e.g. 3 - 8 individuals per population; Nazareno et al., 2017, as opposed to 20 - 30 for microsatellite markers; Hale et al., 2012). The application of resequencing is well suited to situations where large sample sizes may be challenging or impossible to obtain, such as in threatened or difficult-to-study taxa.

Where mobile species rely on a spatial network of habitats or are habitat specialists they may be susceptible to reductions in functional connectivity (Runge et al., 2014; Crates et al., 2019). Movement and gene flow between populations may be constrained by a species' social system, leading to high site fidelity and philopatry (Morinha et al., 2017). In either case, otherwise mobile species with high dispersal capabilities may exhibit variation in population structure over small spatial scales (Morinha et al., 2017; Crates et al., 2019). Populations at the extreme limits of a species' range are likely to be subject to reduced N_e and gene flow and are thus more likely to demonstrate increased structuring and lower genetic variation than central populations (Eckert, Samis and Lougheed, 2008). Habitat fragmentation and loss within range extremes may then have significant genetic consequences for threatened taxa, even in cases where species exhibit large geographic distributions or central population sizes (Fuller, Gaston and Quine, 2007; Eckert, Samis and Lougheed, 2008; Runge et al., 2014). Despite their inherent vagility, migratory species remain vulnerable to genetic structuring among breeding populations. Apparent population admixture and panmixia shown in previous studies of migratory birds may be an artefact of using low-resolution markers. Historic conclusions of population admixture are likely to be reconsidered as the increased adoption of NGS enables

fine-scale structuring to be detected (Pârâu and Wink, 2021), even in highly mobile migratory taxa (e.g. Larison et al., 2021, but see Calderón et al., 2016; Pârâu et al., 2022).

3.2.3 European nightjar ecology and population decline

The European nightjar (*Caprimuglus europaeus*), henceforth nightjar, is a long-distance migratory bird (Norevik, Åkesson and Hedenström, 2017). Nightjar winter in sub-Saharan Africa and breed across temperate Eurasia, ranging from Eastern Russia in the East to the British Isles in the West (Cramp and Simmons, 1985). Nightjars were once a widespread breeding species across the entirety of the British Isles throughout the 1800s (Holloway, 2010). However, the species underwent a population decline throughout the 20th century, undergoing a >50% population reduction between 1966 and 1981 (Fig 3.1), and the species was declared extinct in Northern Ireland and near-extinct in the Republic of Ireland in the late 20th century (Gribble, 1983; Conway et al., 2007). Nightjar are diet and habitat specialists, feeding predominantly on moths (Lepidoptera; Mitchell et al., 2022) and breed in heathland and felled woodland (Conway et al., 2007). As such, degradation, loss, and fragmentation of these habitats are one of the primary drivers of population declines (Langston et al., 2007). Specifically, the loss and conversion of woodland and heathland to productive land uses; e.g. agriculture, plantations, housing (Moore, 1962; Blackstock et al., 1995), in the mid 20th century (during and after the Second World War) led to a significant reduction and fragmentation in valuable breeding habitats. However, the later maturation and subsequent availability of felled coniferous plantations, in areas supporting historically suitable habitat in the late 20th and early 21st centuries enabled a partial recovery in nightjar populations in the UK (Fig 3.1; Langston et al., 2007). Nevertheless, despite a partial demographic recovery, populations remain highly fragmented owing to the availability of suitable habitats (Langston et al., 2007). Furthermore, the extent of recovery has not been consistent across the species' range, with populations in the farthest range extremes in the North West seeing minimal increases in size, while populations in Northern Ireland and Ireland becoming extinct and near-extinct respectively (Conway et al., 2007). Conversely, populations in the east of Britain saw significant increases in population size with the increased availability of clear fell (Conway et al., 2007). Despite a wealth of ringing (banding) effort across breeding sites, there is little evidence of consistent long-distance dispersal or connectivity between breeding populations. Despite their vagility, nightjars may suffer from a lack of functional connectivity between breeding sites. Limited ringing data (unpublished data) suggest cases of site fidelity and

philopatry in the species which might reflect low connectivity and thus gene flow between breeding sites.

Quantifying the effects of habitat loss and fragmentation of population decline and connectivity in a hard-to-study and mobile species represents a significant challenge (Bi et al., 2013). Whilst necessary for delineation of conservation units and management strategies, quantifying population connectivity and migration in such species can be challenging and impractical (e.g. high financial and labour costs of tracking and mark and recapture techniques; Larison et al., 2021). Consequently, population connectivity remains unknown for a large number of species for which conventional methods (GPS tracking, mark and recapture) are poorly suited (Larison et al., 2021). An incomplete understanding of population connectivity and historical demographic trends can then lead to poor quantification of anthropogenic impacts. Despite standardised national surveys (e.g. Gribble, 1983; Morris et al., 1994; Conway et al., 2007), the lack of pre-1960 census data and the nocturnal nature of nightjars significantly limits our understanding of historic demographic trends and population connectivity. NGS genome-wide data when coupled with a temporal sampling strategy provides a powerful tool to understand patterns of demographic change as well as contemporary population structure. Whilst nightjars are unlikely to have been through a significant bottleneck, the pattern of population decline and fragmentation exhibited by the species parallels that of other threatened migratory species (PanEuropean Common Bird Monitoring Scheme, 2022).

3.2.4 Aims and hypotheses

Here I used full genome resequencing data across 96 individuals from both historic (n = 60 birds) and modern populations (n = 36 birds), sampled across the historic and extant range of European nightjar in the British Isles. I used this high-resolution data to investigate the genomic signature of recent demographic decline and recovery in a population of nightjar at the limit of the breeding range, to address the following two key aims;

A1) Characterise the spatio-temporal genetic structure in the British population and determine whether the historic population (1840-1960) exhibited higher connectivity than the modern population (2019-21). If genetic structure was found, I aimed to apply IBD analysis to test the effects of spatial isolation on genetic differentiation among populations.

A2) Establish whether the British nightjar population exhibited a change in global (genome-wide) heterozygosity over time, I predict that there will be an overall decrease in heterozygosity, indicative of historic demographic decline. I aimed to determine whether heterozygosity was spatially consistent within the British populations or whether heterozygosity varies across regions.

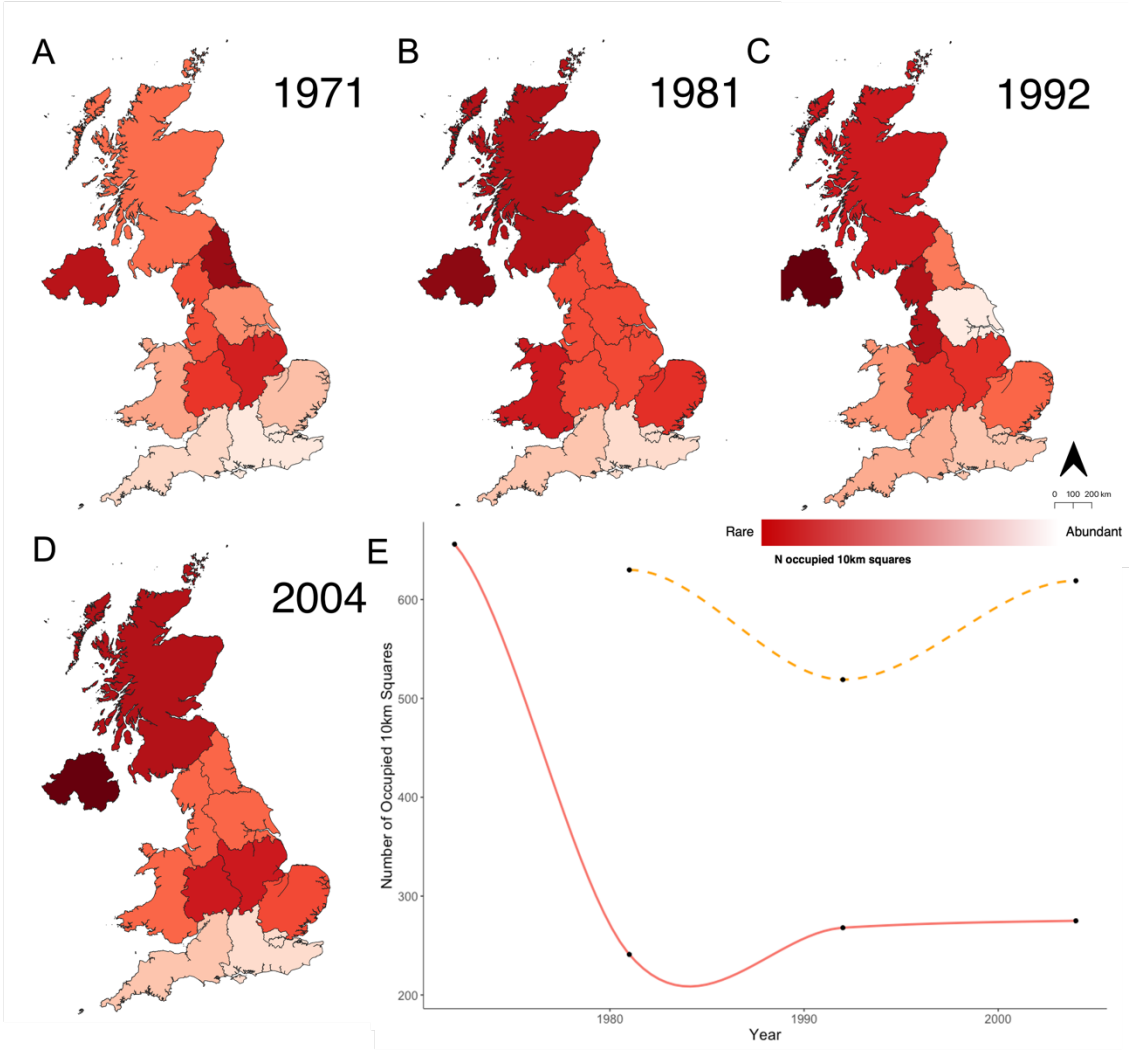


Figure 3.1 -Regional population size change in European nightjar within Britain and Northern Ireland from 1971 - 2004. Maps show population size as number of occupied squares per region. Red shading shows population size, with darker red shading representing lower abundance and lighter red higher abundance. E shows actual change in N occupied 10km squares (1981-2004) by European nightjar across the entirety of Britain and Northern Ireland. Orange dashed line shows effort (n surveyed 10km squares) and solid red line shows n occupied 10km squares. No effort data was available for the 1972 survey period. Data derived from Gribble, (1983), Morris et al., (1994), Conway et al., (2007).

3.3 Materials and methods

3.3.1 Modern sample collection and sites

Buccal swab samples were collected between 2019-21 from 32 nightjars across 13 breeding sites (Fig 3.2) throughout the extant species range with the help of citizen scientists (trained, licensed volunteer BTO bird ringers; see **Acknowledgements**). Tissue samples were also obtained from three deceased birds. In total, samples from three individuals were selected per site, with the exception of 'North Wales' (n = 1) and 'Mid Wales' (n = 2). For buccal cell sampling, nightjars were captured using mist nets within known breeding sites between June and September, this was to ensure only breeding or resident birds were included. Swab samples were taken by applying sterile rayon tipped swabs to the inside of the mouth of the nightjar for 30 seconds, rotating against the inside of the cheeks and tongue. Swabs were then frozen in the field at -20°C before being transferred to a -80 °C freezer for long term storage prior to processing. Tissue samples were taken from toe pads from deceased nightjars (n =3) and stored at -80 °C.

3.3.2 Historic DNA sample collection

Samples from nightjar skins collected between 1866 and 1948 were selected for sampling in order to span periods leading up to and encompassing the documented demographic decline throughout the 20th century. Only skins with known location of origin and dates were included. An effort was made to sample from the complete historic British and Irish range (Fig 3.2B). Samples were taken from museum specimens by scraping the toe pad. Briefly, a sterilised scalpel blade was used to remove a single 1-2 mm deep scrape of tissue from the toe pad of each nightjar skin (as per Sigurðsson and Cracraft, 2014). Samples were then placed in a sterilised 1.7 ml eppendorf and stored at room temperature prior to DNA extraction.

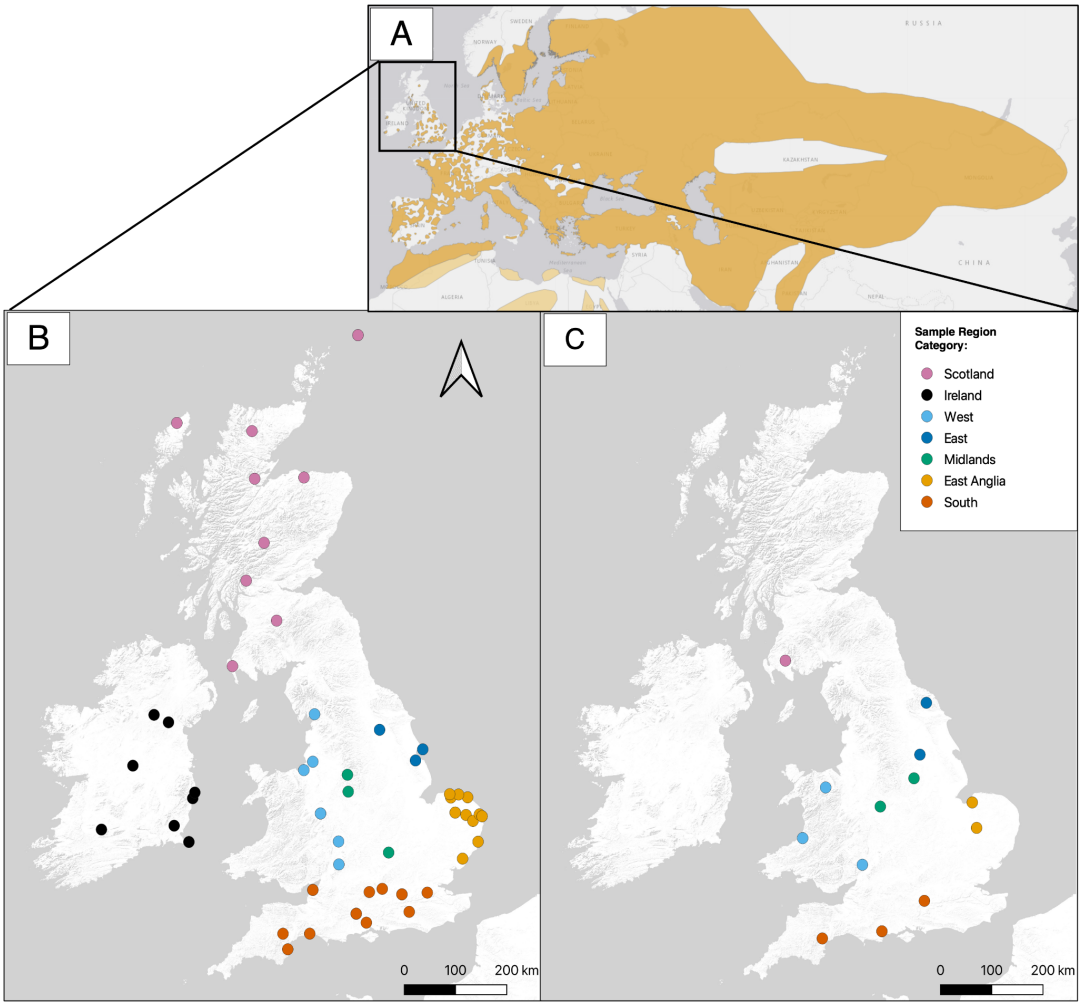


Figure 3.2 Range map (A) and sampling locations of B; historic and C; modern (n = 13 population centroids) nightjar samples. A: Eurasian range map from (IUCN, 2023), dark orange = breeding and light orange = found on passage migration only. B and C: colours reflect assigned regions to each sample. Region classifications for each sample can be found in **Appendix** Table 8.2.

3.3.3 Sample extraction and library preparation.

3.3.3.1 Sample extraction

DNA from modern samples (buccal swabs and tissue samples) was extracted using a modified ammonium acetate method as outlined by Nicholls et al., (2000) and Richardson and Burke, (2001). Briefly, the method was modified for buccal swabs by inclusion of the 5cm swab tip in the initial extraction stages, increasing the digestion stage to 24hrs and removing swabs prior to the initial centrifuge stage. The digestion stage was not increased for the tissue samples (n = 3) (see **Appendix** Section 8.2.1.1 for detailed account of extraction procedure). All historic

samples were extracted using UV sterilised equipment and under a fume hood in a PCR product-free laboratory to avoid any contamination. For each toepad sample the tissue was chopped into smaller pieces before being transferred to a 1.5ml Eppendorf. Historic samples were extracted using a modified Qiagen Blood and tissue kit protocol, with increased digestion stages (See **Appendix** Section 8.2.1.2 for full extraction procedures).

3.3.3.2 Quality analysis

To test whether the age and collection of samples (hDNA) affected sample yields a One-way ANOVA was used to test the hypothesis that both sample age and collection of origin would significantly affect the final DNA yield from hDNA samples. Analysis was conducted in R (**Appendix** Table 8.3). Contemporary samples were extracted with an average yield of 14.37 ng/µL (stdev = 23.39 ng/µL), whilst historic samples were extracted with a lower average yield of 6.58 ng/ µL (stdev = 10.27 ng/µL), and four samples failing to produce a reading suggesting very low (<0.1ng/µL) yields (see supplementary data in **Appendix** Section 8.2.3). Whilst yield declined with sample age, sample age alone did not significantly predict yield (one -way ANOVA, $F_{(1,46)} = 0.404$, $P > 0.5$) (**Appendix** Fig 8.3). However, the identity of the museum collection from which samples originated was found to significantly predict yield (one -way ANOVA, $F_{(7,46)} = 15.52$, $P < 0.001$), with three collections (BM, NMS and YMT) accounting for notably elevated yields compared to the remainder of collections (see **Appendix** Fig 8.3), even whilst controlling for sampling year (collection year * collection ID, $F_{(6,46)} = 0.135$, $P > 0.1$).

3.3.3.3 Library preparation and sequencing

All sample and library preparation post-extraction was undertaken at the University of Liverpool NERC Environmental Omics Facility. Full details of the library preparation protocol and sequencing can be found in **Appendix** Section 8.2.2

3.3.4 Read trimming and alignment

Initial read trimming was undertaken using a custom pipeline by NERC Environmental Omics Facility Centre for Genomic Research. Briefly, Cutadapt (**Appendix** Table 8.3; Martin, 2011) was used to first trim all raw Fastq reads for the presence of Illumina adapter sequences. The option -O 3 was used, so that the 3' end of any reads which matched the adapter sequence for

3 bp. or more were trimmed. The reads were trimmed further using Sickle (**Appendix** Table 8.3; Criscuolo and Brisse, 2013) with a minimum window quality score of 20, reducing erroneous reads caused through the deamination of hDNA. Any reads shorter than 15 bp. after trimming were removed. Read length and counts were characterised for both raw and trimmed reads and can be found in supplementary data in **Appendix** Section 8.2.3.

Trimmed paired-end reads were aligned against the European nightjar reference genome (Secomandi et al., 2021), using BWA Mem (**Appendix** Table 8.3; Li and Durbin, 2009a). The resulting bam files were sorted using Samtools (**Appendix** Table 8.3; Li et al., 2009a) and PCR duplicates marked and removed using PICARD tools 'MarkDuplicates' (**Appendix** Table 8.3; Broad Institute, 2023). Finally, bam files were indexed using Samtools index (Li et al., 2009a). Due to the variability in depth between modern (average depth: 8.4x) and historic samples (average depth: 5.3x), down sampling was performed on the trimmed modern reads to be used in downstream analysis where all samples were included. Down sampling was performed using Picard Tools 'PositionBasedDownsampleSam' (Broad Institute, 2023). I randomly down sampled the modern reads by the proportional difference in the average number of reads between the modern and historic samples (~29%) using the 'FRACTION=0.29' command, down sampling the depth of the modern samples to 29% of their average depth. Samtools was used to collect summary statistics on read length, number, depth and map quality at each treatment stage (see supplementary data in **Appendix** Section 8.2.3).

In total, reads from all 96 samples were successfully aligned to the European nightjar reference genome, with an average coverage of 8.5x for the modern samples, 5x for the historic and 3x for the modern down sampled individuals (see **Appendix** Table 8.3 for details of read length and sample sizes).

3.3.5 Historic DNA degradation

Historic samples can be characterised by postmortem substitutions (C to T and G to A) at the terminal ends of reads, owing to degradation associated with sample age and preservation methods. These damage patterns can lead to the false identification of SNPs and thus have implications for downstream inferences. I used Mapdamage (**Appendix** Table 8.3; Jónsson et al., 2013), with the default settings, to rescale the aligned reads (bam files) of the historic samples to account for base substitution at the terminal ends of reads. The programme uses Bayesian estimation of the expected postmortem damage patterns to rescale the bam files,

resulting in adjusted quality scores to account for the degradation. The resulting rescaled files were then used for all downstream analyses.

3.3.6 Genotype likelihood calling and filtering

Owing to the low depth throughout the samples used in this study DNA, uncertainty in genotype calls was accounted for by calling genotype likelihoods. As a software package developed for working with low quality, low-coverage data, ANGSD (**Appendix** Table 8.3; Korneliussen et al., 2014) was used to produce the genotype likelihood scores for all individuals in the study. As per other studies working with low-coverage data (i.e., Çilingir et al., 2022) the GATK model ('-GL 2') was used, and major and minor alleles inferred from genotype likelihoods ('doMajorMinor 1', 'doMAF 1'). Only biallelic SNPs ('-skipTriallelic 1') from properly paired and uniquely mapped reads ('-only_proper_pairs 1' '-uniqueOnly 1') were retained. Further quality filtering was undertaken by discarding 'bad' reads ('-remove_bads 1'), as well as adjusting quality scores around indels ('-baq 1') and for excessive mismatches ('-C 50'). Sites with a map and quality less than 30 and 20 respectively ('- MinMapQ 30' '-minQ 20') were also filtered out. Finally, sites with a polymorphism significance threshold of < 1e-6 were removed (-SNP_pval 1e-6), and excess heterozygosity (> 0.5) were also filtered out to reduce potential paralogs.

Genotype likelihoods were successfully called (total n SNPS = 50,171,789, down sampled dataset = 42,413,393) for 94 individuals. Two samples, one modern and one historic, failed to produce genotype likelihoods. These were excluded from downstream analysis.

3.3.7 Population genetic analysis

3.3.7.1 Data filtering and preparation

For all population genetic structure analysis the genotype likelihoods were called as above with the addition of a minimum depth filter of one-third the average depth ('-setMinDepth'), a maximum depth filter of ~3x average depth ('-setMaxDepth'), and a maximum missingness filter of 20% also applied. Owing to the large depth variation between samples, the depth characteristics of the historic samples were chosen to inform the filters used, with the minimum depth scaled as per the average depth of the historic samples. However, so as not to

exclude a large proportion of the modern samples, the maximum was scaled as per the average modern sample depth (16x). The same filters were also applied to the down sampled dataset with the maximum depth reduced to 11x. Under the additional filters for the population genetics analysis, the full dataset accounted for a total of 1,144,436 SNPS with an average coverage of 4.2x for historic and 10x for modern samples. The down sampled dataset accounted for a total of 211,168 SNPS with an average coverage of 4.7x.

3.3.7.2 Structure analysis

To determine patterns of spatio-temporal genetic structure, first patterns of genetic similarity among individuals were assessed using Principal Components Analysis using PCAngsd (**Appendix** Table 8.3; Korneliussen et al., 2014) this was run for all samples. Where clear structure was observed by PCA biplots, structure was further characterised by Bayesian clustering, Fixation Index, with patterns of isolation by distance also tested.

PCA was run separately on 1) the full and down sampled datasets, as well as for 2) the historic and 3) the modern samples alone. Initial analysis indicated that structure of historic samples was heavily driven by inter-sample variation in missingness (**Appendix** Fig 8.4). The decision was taken to remove individuals with a high missingness from the PCA plots, (>50% missingness, n = 15 in full dataset, n =13 in down sampled dataset). PCAngsd produces a pairwise covariance matrix. This was exported to R to produce and visualise the principal components of the genotype data using the 'eigen()' command. PCA plots were then constructed using ggplot2 (**Appendix** Table 8.3; Wickham, 2016), plotted with 95% confidence ellipses to aid interpretation where appropriate. Two PCA were run for the historic samples, including all samples with < 50% missingness and a second excluding Irish samples, so to enable a direct comparison with the contemporary PCA results.

Where clear clustering of individuals was noted by PCA biplots, genetic structure was also determined using NGSAdmix (**Appendix** Table 8.3; Skotte, Korneliussen and Albrechtsen, 2013). NGSAdmix deals with genotype likelihood input files, and operates similarly to the Bayesian clustering software STRUCTURE. NGSadmix assigns individuals to clusters based on genetic similarity, aiming to minimise variation among individuals within each cluster. NGSAdmix assumes independence of loci, thus linked loci should be filtered prior to analysis. Here I used NGSld to first perform linkage analysis and secondly prune the SNP's used by NGSADMIX (**Appendix** Table 8.3; Fox et al., 2019). Linkage analysis was performed on the Beagle file generated from the full dataset after the filters applied in Section 3.3.7.1. Pairwise

LD were calculated and linked loci were pruned, allowing for a maximum among-SNP distance of 100kb and a minimum weight (LD estimate between two SNPs) of 0.5. NGSAdmix was then applied to the pruned data. NGSAdmix was run for the modern samples only, owing to little spatial structure evident among the historic samples. The software was run for each SNP set with cluster (*K*) 2 - 10, performing 10 replicates per run. The results of the analysis were then visualised in R, with the optimum *K* value determined for each SNP set using CLUMPAK (**Appendix** Table 8.3; Kopelman et al., 2015).

3.3.7.3 Fixation Index (F_{ST})

To compare levels of differentiation between regions the fixation index (F_{ST}) was calculated between each region pair. As with admixture analysis, regional differentiation was assessed in the modern samples only. Weighted pairwise F_{ST} values were calculated between regions (n = 6). F_{ST} values were calculated in ANGSD and realSFS. Firstly, site allele frequency (SAF) likelihood values were estimated for each site/region from the genotype likelihoods ('-doSAF 1') calculated as per Section 3.3.8, with the reference genome used in place of the ancestral sequence. The spectra were then used to calculate a pairwise folded site frequency spectra (SFS) between each population/region pair in realSFS. The global pairwise weighted F_{ST} values were then calculated in realSFS using '-fst stats' and exported to R for visualisation in a heat map using ggplot2.

3.3.7.4 Isolation by distance

A pattern of isolation by distance (IBD) among modern samples was tested by correlating genetic distance (F_{ST}) between regions, as calculated above, with Euclidean distance between breeding site and region centroids. Euclidean distances were calculated in QGIS (**Appendix** Table 8.3; QGIS Association, 2023). A Mantel test was used to test the correlation between the genetic and euclidean distance matrices, testing the null hypothesis that population/region F_{ST} would not increase with Euclidean distance. Analysis was conducted in R.

3.3.8 Genome wide heterozygosity

In order to investigate spatio-temporal changes in genomic diversity, genome-wide autosomal heterozygosity, hereafter global heterozygosity, was calculated per individual (n = 94) in

ANGSD using a folded SFS ('-dosaf 1', '-fold -1'), applying a minimum depth filter of 4x to reduce the effects of coverage on heterozygosity estimates (van der Valk et al., 2019). Heterozygosity analysis was conducted on all historic and down sampled modern samples, to reduce the effect of differences in sample depth. Average global heterozygosity (the number of singletons divided by the total number of sites) was calculated for each temporal category (historic and modern). Average differences in global autosomal heterozygosity between A) historic and modern samples and B) between regions within historic and modern samples separately were analysed in R using Pearson's correlation and one-way ANOVAs respectively, with results plotted using ggplot2.

3.4 Results

3.4.1 Population genetic analysis

3.4.1.1 Genetic structuring analysis of all samples

Variation in missingness (missing SNPs), likely caused by differences in DNA quality between the down sampled modern and historic samples appeared to drive the distinct clustering within each temporal group (see **Appendix** Fig 8.4). To combat this, individuals with >50% missingness were removed from the PCA plot (Fig 3.3). Outliers (possible migrants) obscured population structure in Fig 3.3. A cropped PCA biplot has then been made available to negate the effects of outliers on deciphering genetic structure patterns (Fig 3.3). Post-missingness trimming, samples remained clustered in their temporal groups (Fig 3.3), with little spatial overall structure evident. As such, the two temporal groups (Modern and Historic) were then split and analysed separately (see Section 3.4.1.2 and 3.4.1.3). However, with no other clear spatial or temporal clustering evident, further structure analysis was not applied to the full dataset.

3.4.1.2 Genetic structuring analysis of historic samples

Again, when applying the PCA to the historic samples alone, the effects of high missingness among the historic samples were mitigated through removal of individuals with >50% missingness from the PCA (Fig 3.4). Outliers which might have obscured population structure

were again negated by presenting a cropped PCA biplot (Fig 3.4), with the full biplots presented as subplots in Fig 3.4A and B. Where PCA was applied to the historic samples alone, the Irish samples formed a distinct cluster compared with the remainder of the individuals from all other regions (Fig 3.4A). However, among the remaining individuals there was little other clear spatial or temporal structure. Upon removing the Irish samples from the analysis, similar patterns of panmixia among with British samples remained (Fig 3.4B). As such, further structure analysis was not applied to the historic samples alone.

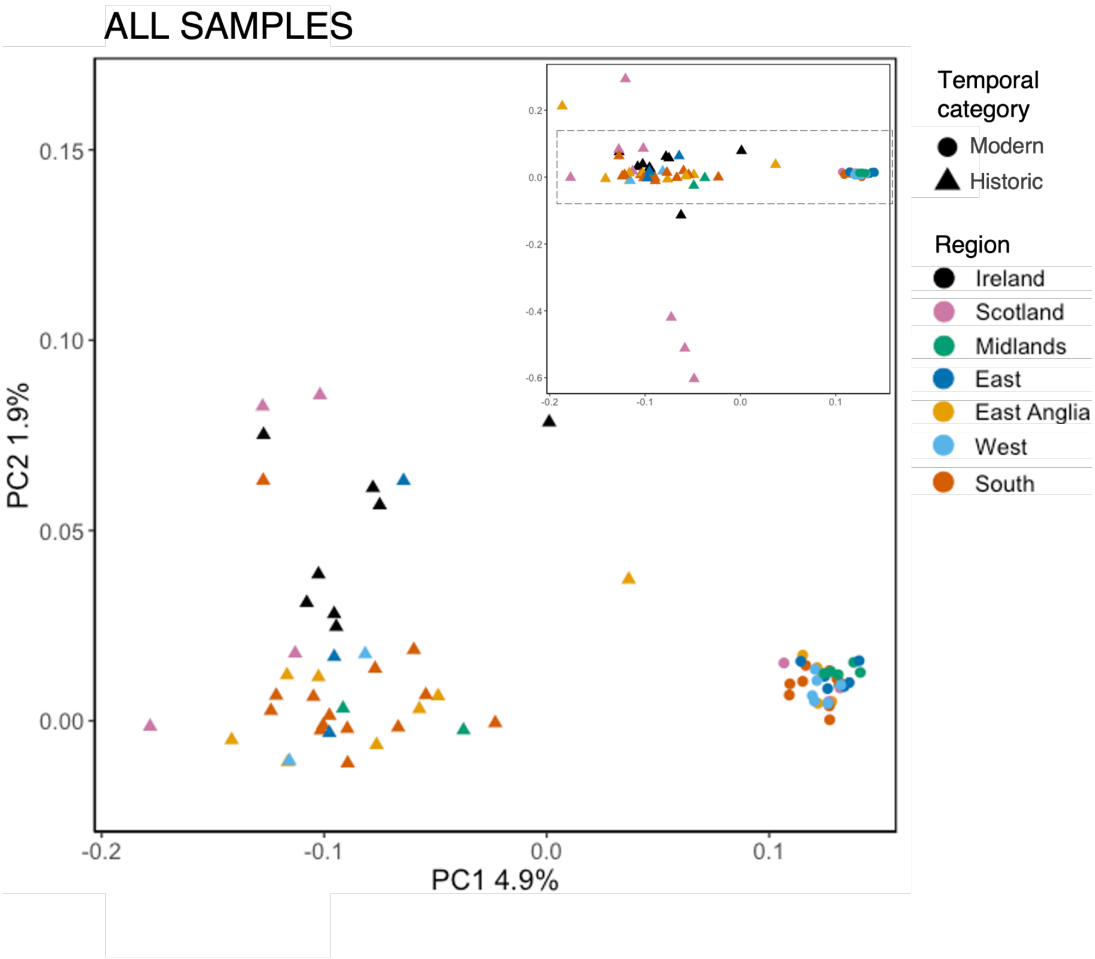


Figure 3.3 PCA biplot of genetic similarity calculated from down sampled filtered genotype likelihoods from both modern and historic samples, with individuals with >50% missingness (n = 13) removed. The main plot is a cropped subplot of the embedded plot (top right), which shows all samples. The dashed box in the embedded plot showing the cropped area presented in the main plot. Plot has been cropped to remove the effect of strongly differentiated individuals on interpreting the genetic structure.

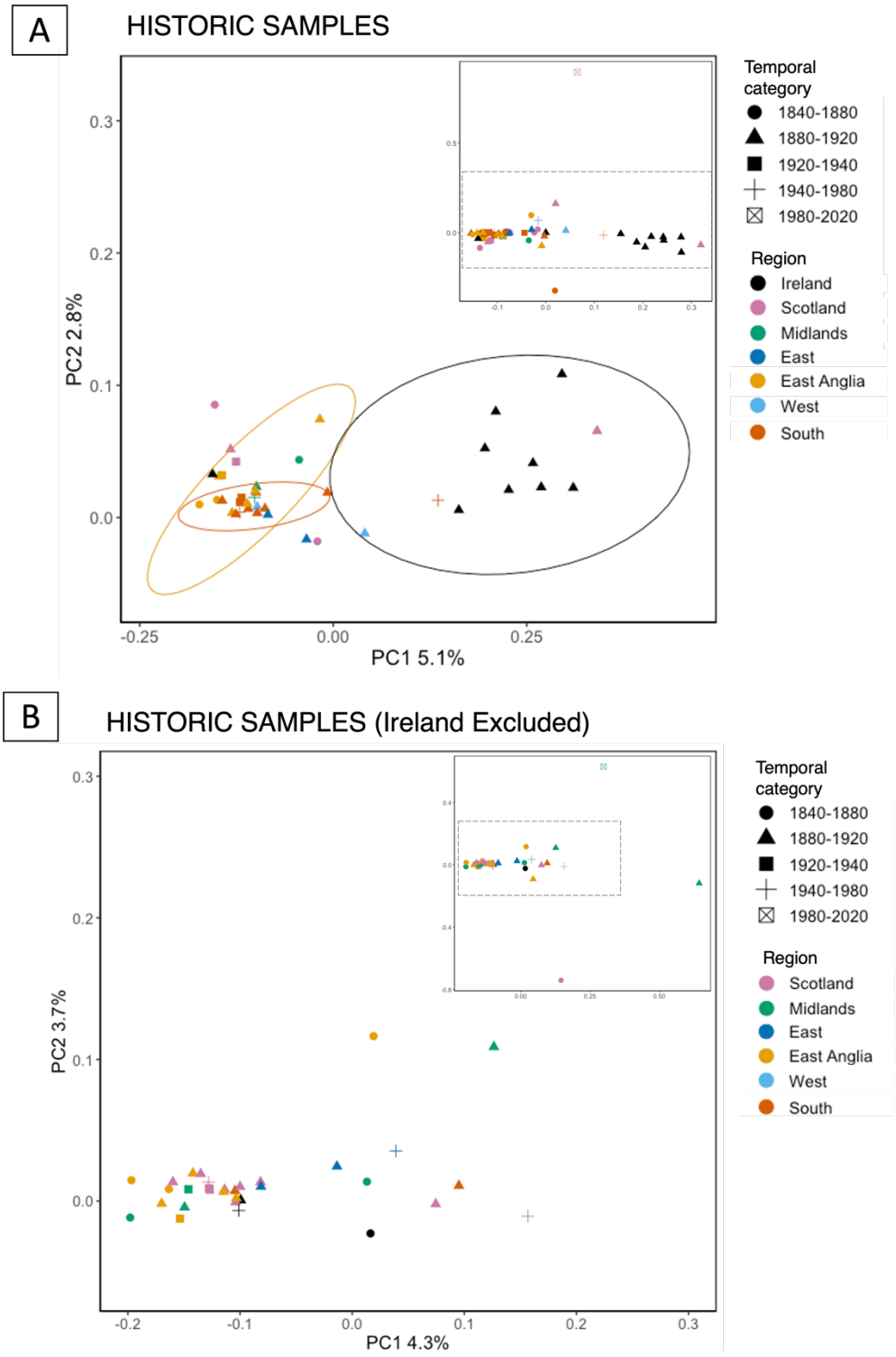


Figure 3.4 PCA biplots of genetic similarity calculated from down sampled filtered genotype likelihoods from historic samples only, with individuals with >50% missingness (n= 15) removed for all historic samples (A) and British samples only (B). The main plots are cropped subplots of the embedded plot (top right), which shows all samples. The dashed boxes in the embedded plots shows the cropped area presented in the main plots. The plot have been cropped to remove the effect of strongly differentiated individuals on interpreting the genetic structure. In fig A regional groupings (coloured circles) are presented as 95% confidence ellipses where clustering allows.

3.4.1.3 Genetic structuring analysis of modern samples

Where PCA was applied to the modern samples alone, weak spatial genetic structure was evident between regions, and samples could be broadly assigned to three main clusters (Fig 3.5). Individuals from the West, South and from Scotland (far Northwest of the species range) in the British Isles formed a tight group, with the exception of a single Scottish outlier and a bird from Wales (Western region) which appeared to group with Eastern and Midland individuals (Fig 3.5). The East Anglia birds accounted for the greatest differentiation across PC2, clustering together, although not as tightly as the West/Southern/Scottish individuals (Fig 3.5). The remainder of the birds from the East and Midlands were grouped together, more tightly clustered than the East Anglia birds but less so than the South/West/Scottish birds.

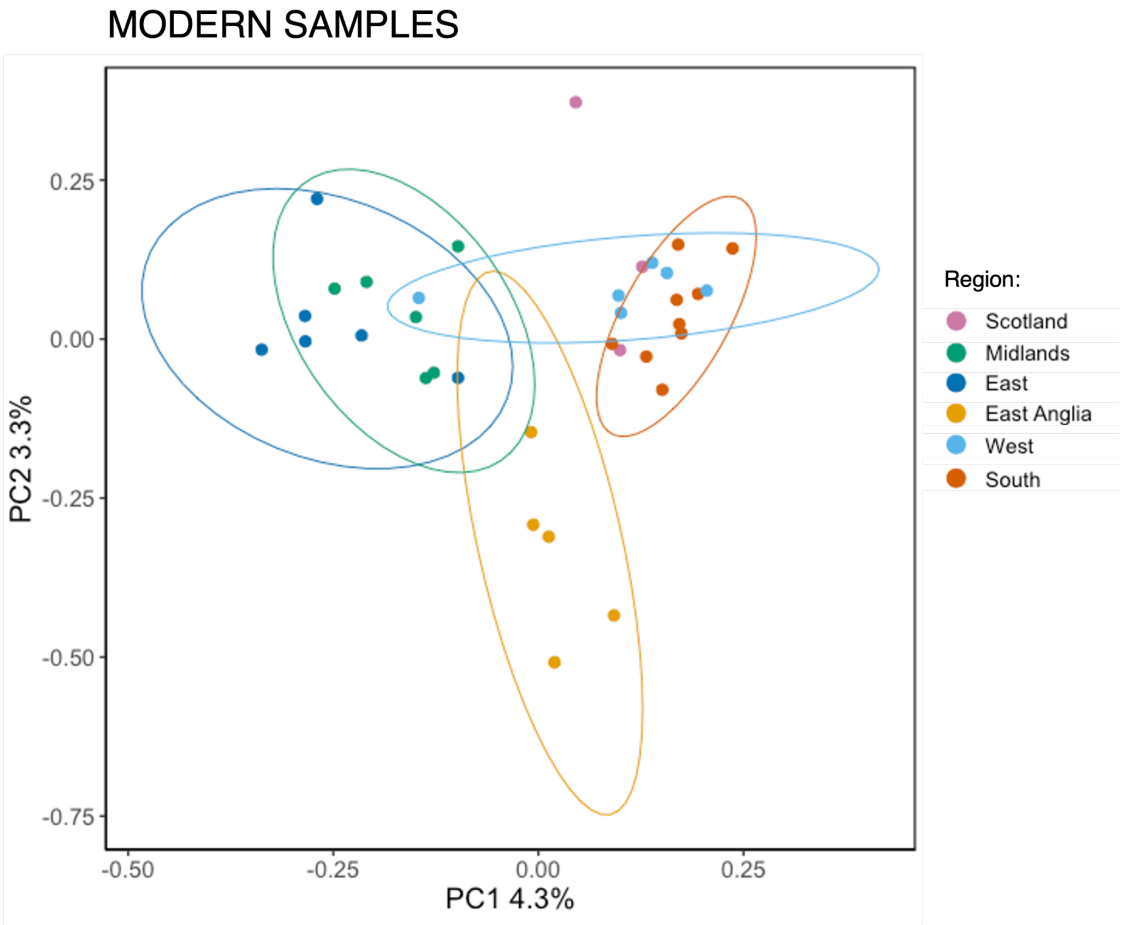


Figure 3.5 PCA biplot of genetic similarity calculated from filtered genotype likelihood for the modern samples only. Samples are differentiated by regional classification (coloured points). Regional groupings (coloured circles) are presented as 95% confidence ellipses where clustering allows.

Admixture analysis of the modern samples showed that whilst the population might be weakly structured (best fitting *K* = 5, as per CLUMPAK; **Appendix** Fig 8.5), admixture was present throughout all regions, suggesting moderately high gene flow among regions (Fig 3.6A).

Nevertheless, at *K* = 4, the proportion of shared ancestry appeared similar within the East/Midland and West/South regions respectively, with clustering sharing some congruence with that suggested by the PCA biplot (Fig 3.5; Fig 3.6A). Notably, individuals within East Anglia showed less admixture than other regional categories (Fig 3.6A), this may be congruent with the clear segregation of East Anglia birds evident from the PCA (Fig 3.5), this was particularly clear at *K* = 5 (Fig 3.6A). However, it is clear that significant admixture remained among all regions (Fig. 3.6A), as typified by the high variation in Delta K and minimal difference between the two highest delta *K* values (CLUMPAK DeltaK; *K* 3 = 1.058, *K* 5 = 1.105; **Appendix** Fig 8.5).

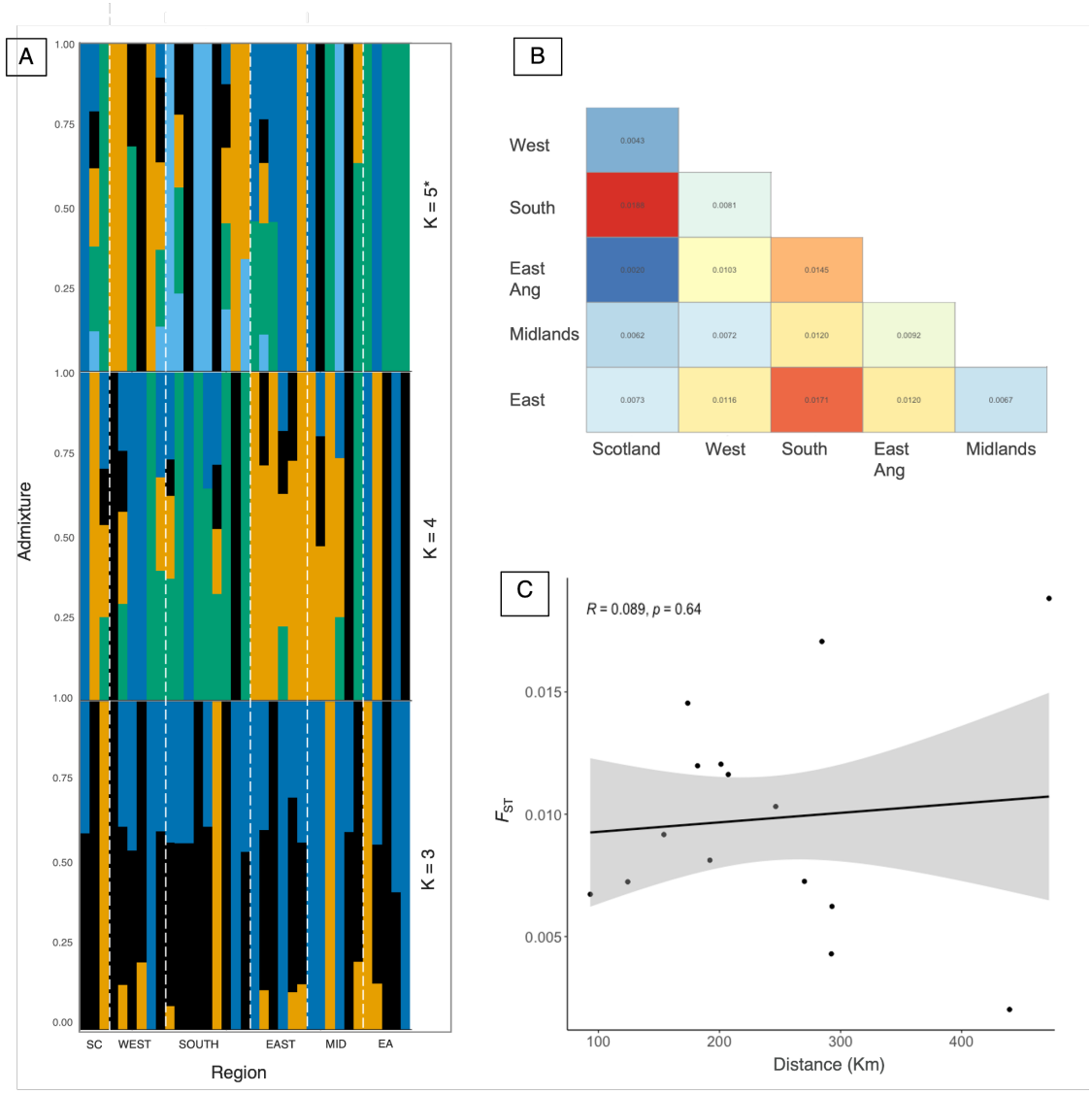


Figure 3.6 Genetic structure in modern samples. A) Admixture plot generated from NGSadmix for *K* = 3 – 5 (number of genetic clusters, coloured bars represent proportion of an individual's ancestry to *K* groups. EA = East Anglia, Mid = Midlands, Sc = Scotland. * = optimal *K* as per CLUMPAK; B) Pairwise weighted F_{ST} values for each regional grouping, colour ramp (blue – red) indicates increasing population differentiation (F_{ST}); C) Isolation by distance plot, showing correlation between pairwise F_{ST} and pairwise spatial distances between region centroids.

F_{ST} values were calculated between regions for the modern samples alone. Individuals showed low levels of differentiation across regions, with weighted pairwise F_{ST} ranging from 0.002 - 0.018 (Fig 3.6B). F_{ST} values were largely congruent with the PCA results (Fig 3.5), with the exception of the Scottish birds (Fig 3.6B). The lowest F_{ST} values occurred between the West and South and East and Midland regions respectively (Fig 3.6B), suggesting similar, although weak, clustering to that in Fig 3.5. With the exception of Scotland, the higher levels of differentiation were found mostly between East and South/West regions as per the clustering suggested in Fig 3.5. East Anglia showed moderate differentiation from all regions, again with the exception of Scotland. The only regional F_{ST} values not congruent with the PCA concerned the Scottish individuals (Fig 3.5; Fig 3.6B). The anomalous results may be due to the small sample size (n = 3 individuals from 1 site), whilst the average number of individuals contributing to the other regions was six. Small sample sizes are known to skew F_{ST} results (i.e. < 3; Willing et al., 2012), although extra steps were taken in my analysis to remove the effects of sample size (i.e. inclusion of '-which fst' command) as much as possible. The single highly differentiated Scottish individual shown in Fig 3.5 may further account for the high pairwise F_{ST} values concerning the Scottish region. However, as with the PCA, increments of differentiation presented here are low, with inter-region variation in pairwise F_{ST} values occurring within a small range. Finally, IBD analysis showed a weak but non-significant positive relationship between genetic (F_{ST}) and geographic (km) distances (Mantel test, R = 0.099, P > 0.3; Fig 3.6C).

3.4.2 Global heterozygosity

Global (genome-wide) heterozygosity was determined for 94 individuals (59 historic and 35 modern samples). Heterozygosity was found to decline significantly over time, with global heterozygosity reduced by 34.8% in modern, compared to historic samples (Fig 3.7). Notably, this decline was evident over the entirety of the timescale in which samples were collected for this study, with heterozygosity appearing to decline throughout the 20th century (Fig 3.7). Global heterozygosity varied among regional groupings of both historic and modern samples, although not significantly (One-way ANOVA, P >0.05 in both cases; Fig 3.8 A,B). Spatial variation in heterozygosity showed a similar pattern within both historic and modern groups, with global heterozygosity being greatest within East Anglia and lowest in East and West regions (Fig 3.8 A,B). However, whilst Scotland accounted for the second highest average heterozygosity in historic samples, the region exhibited the lowest heterozygosity range in modern samples (Fig 3.8 A,B).

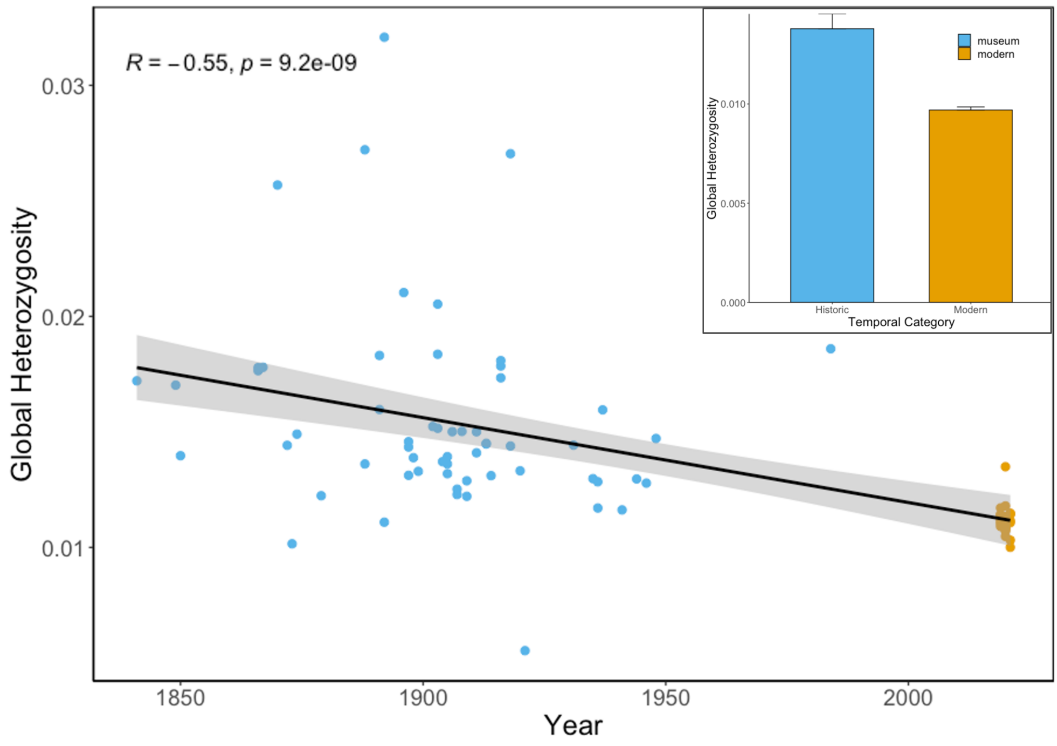


Figure 3.7 Global heterozygosity of all samples regressed against sample collection year. Regression line with 95% confidence intervals, and Pearson's product-moment correlation coefficient and P-value are presented. Inset barplot shows differences in average global heterozygosity between the modern and historic samples, with error bars reflecting standard deviation. Throughout figure blue = historic and orange = modern samples.

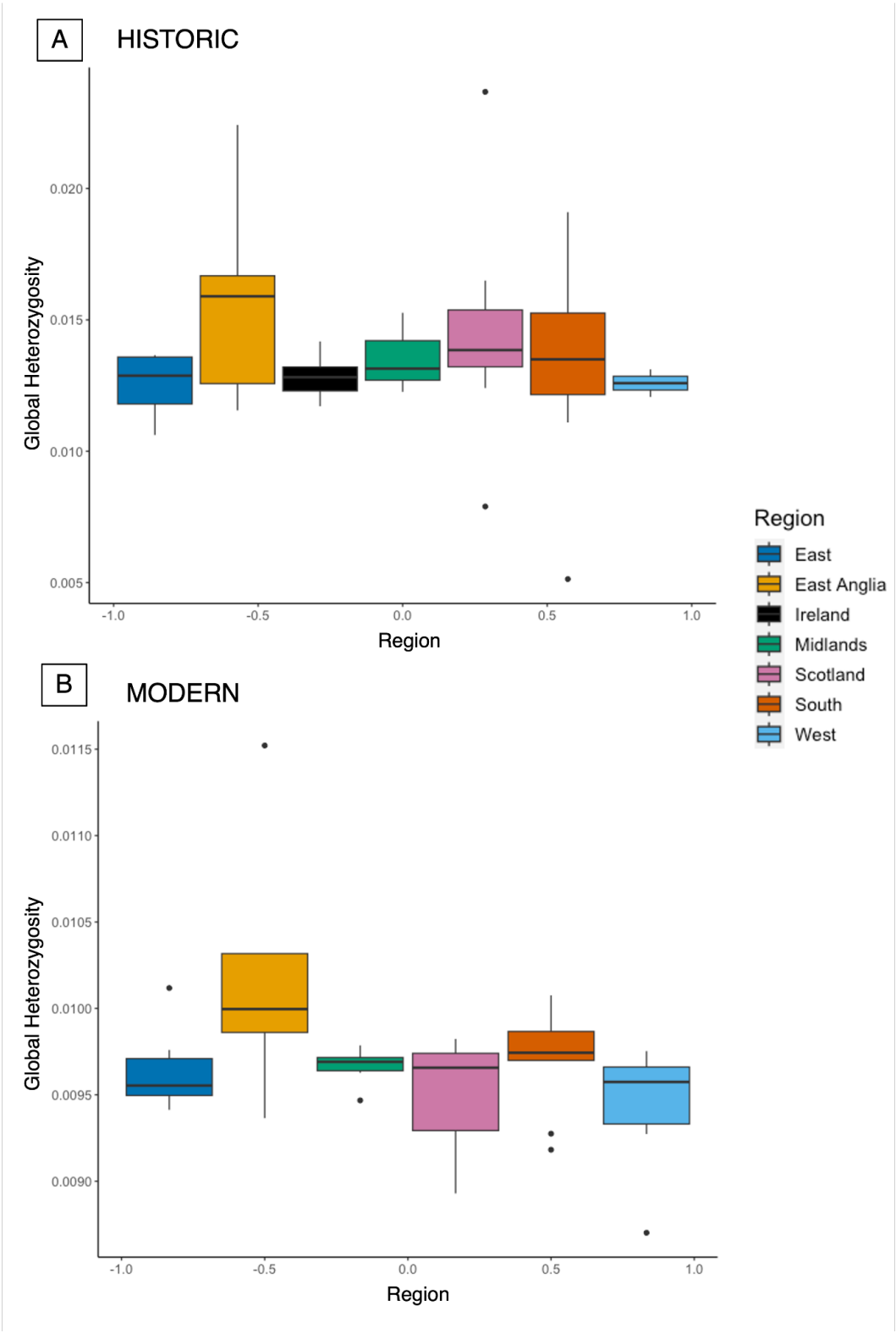


Figure 3.8 Global median heterozygosity across regions in historic (A) and modern (B) samples respectively, boxes represent median (midline) first and third quartiles and whiskers reflect range of values, with range extreme values shown as singular points.

3.5 Discussion

I successfully sequenced and aligned the genomes of 94 European nightjar from mainland Britain and Ireland, constituting 59 individuals from museum collections (1841-1980) and 35 modern samples (2019-21). Between 1841 and 2021 the British nightjar population exhibited a shift from complete panmixia among the historic samples to weak regional structure in the modern population. Modern samples showed evidence of weak spatial genetic structure, broadly clustering into three regional groups formed of the populations in 1) East Anglia, 2) East of England and Midlands and 3) South, West England, and Scottish populations. However, no strong evidence of isolation by distance could be found amongst regions. Over the same timeframe genomic diversity in the British nightjar population underwent a significant and prolonged decline. Heterozygosity varied, although not significantly, among regions in both historic and modern samples, and was consistently highest in East Anglia in both temporal groups.

3.5.1 Weak genetic structure in the British nightjar population

Between 1841 and 2021 the British nightjar population exhibited a shift from panmixia in the historic samples towards weak spatial structure in the modern mainland British nightjar population. Genetic structuring is fundamentally determined by mutation, selection and genetic drift and mediated by gene flow among populations (Frankham, Ballou and Briscoe, 2010). Much of the modern structure of temperate avifauna has been driven by paleo-climate cycles, causing species to retreat and expand to and from Southern refugia during periods of glaciation and warming respectively (Hewitt, 1999; Nadachowska-Brzyska et al., 2015; Pârâu and Wink, 2021). This has led to range-wide structuring in a number of species reflecting different refugia occupation (e.g. East-West clinal structure; Hansson et al., 2008; Lombardo et al., 2022; Väli et al., 2022), as well as panmixia in others, associated with shared refugia and mixing during interglacial periods (Pârâu and Wink, 2021; Pârâu, Wang and Wink, 2022). Results from PSMC analysis suggest that nightjar likely show significant structure across their European range (see **Chapter 2**), either reflective of an East-West latitudinal divide or the presence of two European subspecies (C. e. *europaeus* and C. e. *meridionalis*). However, on a fine scale, the vagility of birds often means that spatial structure is typically less likely than in more sedentary taxa (Coster et al., 2019; Pârâu and Wink, 2021; Pârâu, Wang and Wink, 2022; Shephard et al., 2022). Indeed, the majority of migratory birds show little fine-scale spatial genetic differentiation (reviewed by Coster et al., 2019; Pârâu and Wink, 2021; Pârâu et al.,

2022; but see Ralston et al., 2021; Shephard et al., 2022), although marker choice for the majority of studies should be considered (MtDNA, microsatellites etc.). Despite this, nightjar exhibited weak fine-scale structure in the modern population. Following the central-marginal hypothesis, the location of the British nightjar population at the species range-extreme may have driven the observed fine-scale structuring, owing to uni-directional, or lack of, gene flow (Schwartz et al., 2003; Langin et al., 2017). Moreover, long-distance migrants, such as nightjar, may exhibit a more pronounced periphery effect than short-distance or sedentary species (Ralston et al., 2021; but see Langin et al., 2017; Pârâu et al., 2022). However, if the periphery effect is the fundamental driver of structure in the modern population, I would expect to see similar spatial differentiation in the British historic samples, for which I see little evidence. Periphery effects are exacerbated where populations are subject to extreme demographic declines as well as loss and fragmentation of habitat (Eckert, Samis and Lougheed, 2008). It seems more likely here that the periphery effect represents an underlying clinal variation which may corroborate with more recent anthropogenic or environmental stressors to drive the observed contemporary population genetic structure in nightjar (Pironon et al., 2017).

As well as being at their range extreme in the UK, nightjar have specialised breeding habitat requirements of heathland or forest clear fell (Conway et al., 2007), which leaves the species particularly vulnerable to habitat loss (Correll et al., 2017, 2019). Habitat specialists typically exhibit higher genetic structure than generalists (e.g. Lindsay et al., 2008; Walsh et al., 2012; Pasinelli, 2022). Resource specialisation may lead to reduced functional connectivity for specialist species, whereby individuals will only utilise sparsely available optimum habitat, particularly at range peripheries (Devictor, Julliard and Jiguet, 2008; Mimet et al., 2013; Reino et al., 2013). Consequently, specialists may be constrained by small population sizes across a fragmented distribution, exacerbating the effects of genetic drift, driving differentiation (Li et al. 2014; Langin et al., 2017; Pasinelli, 2022). Nightjar in Britain exhibit a similarly fragmented distribution, likely exacerbated by the loss of heathland throughout the 20th century (Conway et al., 2007; Langston et al., 2007). This is corroborated by the results from this study showing a shift from panmixia in the historic population towards weak spatial structure in the modern fragmented population. Many specialists exhibit high breeding site fidelity and philopatry (Bech et al., 2009; Dolný et al., 2013; Camacho, 2014; Byer and Reid, 2022; but see Coster et al., 2019), including nightjar (Vilella, 1995; Wilkinson, 2009; Camacho, 2014; McGuire, Boyles and Brigham, 2021). Indeed, within the British nightjar population high philopatry and fidelity have been recorded at several breeding sites included in this study where long-term monitoring projects have been carried out (Lowe, Rogers and Durrant, 2014; Raymond et al.,

2020). Philopatry and site fidelity can reduce gene flow and in turn drive fine-scale genetic structuring, even in species with high dispersal capabilities (Pakanen et al., 2017; Morinha et al., 2017; Moore et al., 2020). High fidelity, particularly where local habitat availability is low, may go towards explaining the weak structure in the modern nightjar population.

My analysis suggests that the modern British nightjar population is weakly clustered into three main regional groups, 1) East Anglia, 2) East and Midlands, and 3) South, West, and Scotland. No significant evidence of isolation by distance was found and no notable geographic barriers exist between the population clusters. If individuals show high levels of site fidelity and philopatry, as suggested, the main source of gene flow into populations may be via migration. Indeed, both large (e.g. Irwin et al., 2011; von Rönn et al., 2016; Lombardo et al., 2022; Nasuelli et al., 2022) and fine-scale (e.g. Shephard et al., 2022) genetic structure can be driven by migratory connectivity. Following recent insights into nightjar migration, birds breeding in East Anglia tended to return to breeding sites via Southeast England, reducing the chance for mixing with Western or the Southern populations sampled here (Lathouwers et al., 2022). Similarly, birds breeding in Wales (West) returned to breeding sites via the South of England (Lathouwers et al., 2022), providing the opportunity for the mixing observed in this study. However, no migration tracking data currently exists for Scottish, East, or Midland populations, reducing my ability to investigate this. Whilst ringing recoveries of nightjar have been limited, few cases exist of birds moving between breeding sites, although mostly, but not exclusively, within the clusters found in this study (Robinson, Leech and Clark, 2022). The weak structure may have also been driven by asymmetric gene flow during the rapid recovery of nightjar in Britain throughout the late 20th century. Gene flow from large remnant nightjar populations in the South (Gribble, 1983; Langston et al., 2007) may have populated suitable plantation forest habitat which became rapidly available in Wales and Scotland. Similar large populations of nightjar persisted in the East (e.g. Humberhead Peatlands; Gribble, 1983) and East Anglia throughout the late 20th century and may have also aided repopulation at sites where similar plantation habitat became rapidly available (e.g. North Yorkshire Moors, Sherwood Forest etc.; Gribble, 1983). However, whilst weak structure is evident in the modern nightjar population it should be noted that high levels of admixture persisted among regions, suggesting ongoing gene flow. The current weak structure in the British population may then be a remnant of the severe demographic decline and subsequent repopulation, in which case I might expect for the population to tend towards complete admixture in the future. Conversely, the weak structure may instead reflect a trend away from the panmixia of the historic population towards an increasingly spatially structured population in the future.

3.5.2 Spatio-temporal changes in heterozygosity

The shift from panmixia towards regional genetic structuring in the British nightjar population was accompanied by a significant 34.8% loss in genome-wide heterozygosity between 1841 and present. This change coincided with a large reduction in population size over the last ~120 years (≥50%), although the population has since shown partial recovery throughout the late 20th century. My results highlight that, despite this, the population bears a signature (loss of heterozygosity) of the historic changes in population size, likely driven by habitat loss and fragmentation (Langston et al., 2007). However, the decline in heterozygosity shown here begins >100 years prior to the documented demographic decline of nightjar in Britain. Indeed, the true extent of population decline in nightjar over the last 200 years, as in most species, is unknown owing to the paucity of accurate census data. Taking global heterozygosity as a population size proxy (Grundler et al., 2019), my data suggests that decline of nightjar in Britain was likely underway prior to the documentation of significant losses during the 20th century. With industrialisation throughout the 18th century (Allen, 2004), and significant forest clearance prior to that (Simmons et al., 2021), it is likely that anthropogenic land use change has been driving historic population reduction in the species for a number of centuries. Again, the effects of habitat loss and fragmentation on population size, and ultimately heterozygosity, were probably exacerbated by the British population being at the periphery of the species' range (Pironon et al., 2017; Perrin et al., 2021; Frantz et al., 2022). Following the central-margin hypothesis, populations at a species range-limit exhibit lower genetic diversity than central populations, with gene flow reducing towards the periphery, exacerbating the effects of genetic drift (Lesica and Allendorf, 1995; Eckert, Samis and Lougheed, 2008). This pattern may also be evident at small scales, with Langin et al., (2017) finding reductions in heterozygosity in marginal populations of the Island scrub jay (*Aphelocoma insularis*) over > 20km. In my study, significant variation in heterozygosity was not observed among regions in either the historic or the modern samples. However, heterozygosity was notably highest in East Anglia in both temporal categories, and lowest in Western populations, if only marginally. Although this might be suggestive of a periphery effect on heterozygosity, with East Anglia the closest region to the range core, the regional variation in heterozygosity remains minimal. Nevertheless, on a range-wide scale the position of the British population at edge of nightjars breeding range may go towards explaining the significant loss of heterozygosity seen over the last two centuries, with the effects of fragmentation on genetic diversity more severe for range extreme populations (Eckert, Samis and Lougheed, 2008).

3.6 Implications for conservation and conclusion:

The 34.8% loss of global heterozygosity reflects the genomic impact of demographic decline and spatial fragmentation in the British nightjar population. However, whilst the loss of genomic diversity is significant, nightjar global heterozygosity in the modern British population remains high compared with threatened avifauna internationally (i.e. average global heterozygosity rate in nightjar = 0.00969, in Seychelles magpie-robin = 0.00015, see also Cavill et al., 2022; Wang et al., 2022), presenting no immediate causes for concern. Nevertheless, my results show that despite the recent partial recovery, the effects of demographic decline in the British population are not negligible. Rather, the trend in heterozygosity likely reflects a long-term, ongoing, decline in population size and genomic diversity. It is apparent that this temporal trend in heterozygosity was likely driven by habitat loss and fragmentation, with my genetic structuring results seemingly corroborating this, showing a shift from panmixia to some regional level structuring over the last ~180 years. Similar trends in heterozygosity and structure have also been found in other studies where a temporal sampling strategy has been employed, with these trends also linked to anthropogenic habitat loss and fragmentation (Feng et al., 2019; Vandergast et al., 2019; Robinson et al., 2021; Ericson, Irestedt and Qu, 2022; Westbury et al., 2022). Whilst the spatial structure in the modern nightjar population is weak, the temporal change from complete admixture towards regional differentiation is significant and highlights a potentially concerning future trajectory. The underlying causes of gene flow resistance (i.e. landscape features, habitat connectivity etc. ; Holderegger and Wagner, 2008) among regions are not immediately obvious at this time, and characterising these should form the basis of future research to better inform conservation measures for nightjar in Britain.

My study demonstrates the genomic signature of population decline in a long-distance migrant at its range extreme. I show that species with a high dispersal potential may also bear the genomic implications of population decline, and that such species should not be overlooked in future studies. The demographic decline exhibited by nightjar is not unique, with significant reductions in population size also recorded across a number of migratory birds (Bairlein, 2016). The combination of high-resolution analysis and temporal sampling enables for accurate insight into the extent and impacts of population decline on contemporary genetic and demographic patterns. This provides a valuable opportunity to quantify the effects of anthropogenic habitat destruction and fragmentation in present day populations. Moreover, this enables conservation biologists to pre-empt population genetic and demographic

trajectories in response to future perturbations, such as climate change, habitat loss and fragmentation which may alter suitable breeding habitat availability at range peripheries.

Chapter 4 Nightjar mating systems and visual communication

4.1 Abstract

The breeding biology and visual communication of Caprimulgidae have been historically neglecting owing to the inherent difficulty of studying cryptic and nocturnal taxa. The trend is not exclusive to nightjars and mating systems, sexual selection and signalling in nocturnal taxa have been overlooked, when compared with their diurnal counterparts. Constraining research to few avian phylogenetic groups ultimately biases and limits our understanding of mating systems and signal evolution. Among nocturnal avifauna Caprimulgids are well placed to address this knowledge gap. Nightjars exhibit conspicuous, but varying levels of sexual dimorphism from greatly exaggerated flight feathers to apparent monomorphism. However, most species are sexually dimorphic for conspicuous achromatic ornaments on flight feathers. Technological advancements (i.e., GPS telemetry, night vision equipment and thermal imagery) are set to alleviate most of the constraints which have until now limited breeding behaviour research on Caprimulgids. Nightjars then represent a valuable study system for studying visual communication and mating systems among nocturnal avifauna. To best place the family as a study system effort should be made to quantify secondary sexual characters, determine mating systems, and record parental care and display behaviour across more Caprimulgid species.

4.2 Introduction

4.2.1 Avian sexual selection and mating systems

Sexual selection is the process by which individual reproductive opportunities are accessed, leading to the evolution of traits which help maximise reproductive output. The mechanism of sexual selection processes have long fascinated Biologists. Mating systems, shaped by the environment and the reproductive mode, govern how reproductive opportunities become available to individuals. The mating system is maintained over time by signalling systems that allow effective communication between reproductive partners and rivals.

Sexual selection arises from differences in mating success between individuals, with the strength of selection pressure inferred by estimating variation in individual reproductive success within a population (Wade, 1979; Crow, 1989), often leading to exaggerated trait values existing beyond the optima dictated by natural selection. Such traits are typically

exaggerated in the selected-upon (constrained) sex. In most cases this is the male, due to the difference in gamete size between males and females (Parker, 1970), where female fitness is limited by the production of large, costly gametes, whilst in smaller-gamete producing males fitness is limited by mating opportunities. Exceptions do exist, notably in polyandrous shorebirds (Reynolds, 1987; Thuman and Griffith, 2005), where sex roles are reversed so that females compete for mates whilst males provide the majority of post-zygotic parental care (Erckmann, 1983). Nevertheless, under most monogamous and polygynous mating systems, selection for male traits can be very strong and males often differ from females in a number of secondary sexually selected traits including body mass or size (e.g., wing length in birds), weaponry, and most relevant here, ornamentation.

Inter- and intraspecific life history variations have led to a diversity of mating systems. Social monogamy, characterised by a stable breeding pair of two individuals of the opposite sex, is the most common mating system in birds, recorded in ~ 90% of all avian taxa. Comparatively few birds exhibit true polyandry (~1%) or polygyny (~2%). However, with the advent of molecular techniques in the late 20th century our understanding of avian mating systems rapidly transformed (Burke and Bruford, 1987; Griffith, Owens and Thuman, 2002). It is now clear that whilst birds may exhibit 'social monogamy', genetic monogamy is the exception rather than the rule (Masello et al., 2002; Taylor, Boessenkool and Jamieson, 2008; Biagolini, Westneat and Francisco, 2017; Brouwer et al., 2017; Sanchez-Donoso et al., 2018). More than 75% of studied avian taxa are socially but not genetically monogamous (Arct, Drobniak and Cichoń, 2015; Reitsma et al., 2018), engaging in mating opportunities outside the pair bond, including extra-pair paternity (EPP) (Ju et al., 2014; Wells et al., 2015; Grunst et al., 2017; Grinkov et al., 2018), intraspecific brood parasitism or quasi-parasitism (Blomqvist et al., 2002).

Inter- and intra-specific variations in avian social and genetic mating systems have received considerable attention (e.g., Griffith et al. 1999; Conrad et al. 2001; Westneat and Mays 2005; Mayer and Pasinelli 2013; Mingju et al. 2017). Knowledge of species' mating systems have enabled investigation of the evolution of sexually selected traits and how they influence mate choice (e.g., Tregenza and Wedell 2000; Balenger et al. 2009; Hasegawa 2018), as well as the interaction of ecological and environmental factors to shape breeding behaviour (e.g., Mayer and Pasinelli 2013; Biagolini, Westneat and Francisco, 2017; Brouwer et al. 2017, reviewed in Griffith, Owens and Thuman, 2002). The mechanisms underpinning female choice continue to be a topic of great interest (Darwin, 1871; Mays et al., 2008; Jones and Ratterman, 2009; Hasegawa, 2018). Whilst the fitness benefits of multiple copulations to males (the constrained sex) are seemingly obvious in terms of increased numbers of offspring; the benefits of multiple

copulations to females (the 'choosy' sex) are less clear. Female benefits can be divided into direct (e.g. fecundity, parental care, protection, resource acquisition etc.; Jones and Ratterman 2009) and indirect benefits (e.g., offspring sexual quality, offspring viability or longevity etc.; Andersson and Simmons 2006). As polyandrous mating is so common among sexually reproducing species (Hosken and Stockley, 2003), several models explaining direct and indirect fitness benefits of multiple mating to females have been developed and reviewed (Achorn and Rosenthal 2020). Finally, understanding of a species' breeding behaviour carries significant applied value. Effective population sizes (N_e) are significantly influenced by differential and skewed amounts of breeding success between individuals (Sutherland 1998) so that the productivity and viability of populations are ultimately influenced by a species' mating system (Nunney, 1993; Hogg, 2000; Schindler et al., 2013). Under different mating systems the number of individuals contributing gametes to the next generation (N_e) can vary significantly. For example, under polygyny successful reproduction will be restricted to relatively few males in the breeding population. Here, N_e and census population sizes would differ greatly, particularly if population census was determined from male display activity such as territorial singing, as it is in many birds (Gilbert, Gibbons and Evans, 1998). A lack of understanding of a species' mating system may thus lead to flawed decision-making regarding population sizes, viability, and conservation.

4.2.2 Nocturnal visual communication

Birds employ multi-modal signalling systems facilitated by vocalisations, morphological and plumage characteristics and specialised behaviours to communicate (Butcher and Rohwer, 1989; Todt and Naguib, 2000). Aside from song, the most common form of signalling mode in birds is variation in plumage pattern and colour (Andersson, 1994; Møller and Cuervo, 2000). These visual cues facilitate social (Bradbury and Vehrencamp, 1998) and defensive (e.g., crypsis and mimicry; Gluckman and Cardoso 2010; Stoddard 2012) behaviours, as well as mate choice and intra-sexual competition (Stoddard, 2012). Much of the elaborate plumage, both in colour and in structural development (e.g., ornaments such as crests, elongated tail streamers etc.), exhibited by birds are important in visual communication. A single ornament may convey information to the receiver or may act as part of a multi-modal signal when combined with other ornaments to provide information (Badyaev et al., 2001; Chaine and Lyon, 2008).

Environmental characteristics determine the effectiveness of visual communication. Structural environmental complexity and ambient light levels may constrain or facilitate visual

communication in birds (Denoël and Doellen, 2010; Penteriani and Delgado, 2017). Due to the impact of low ambient light levels on visual communication, visual signalling in nocturnal taxa was long overlooked by researchers, and the dominant hypothesis stated that nocturnal animals must primarily rely on vocalisations (Penteriani and Delgado, 2017). However, nocturnal visual signalling has gained increasing attention in recent years (Penteriani and Delgado, 2017), and the value of visual communication to nocturnal species become apparent (Aragones, Reyna and Recuerda, 1999; Jiguet, Arroyo and Bretagnolle, 2000; Penteriani and Delgado, 2017). The light environment plays a major role in the evolution of plumage colouration and visual signalling (Endler, 1993; Penteriani and Delgado, 2017) but the low light available to nocturnal species does not preclude its use. Penteriani and Delgado (2017) anticipated that visual signalling employed by nocturnal and crepuscular taxa should maximise signal attractiveness even under low light levels. Achromatic plumage enables effective communication in low light levels, with prominent high-contrast white plumage patches and ornamentation noted as important display signals in a number of crepuscular and nocturnally displaying bird species (e.g., Eurasian eagle owl *Bubo bubo*; Penteriani and Delgado 2017, Little bustard *Tetrax tetrax*; Jiguet et al. 2000). However, achromatic plumage has been largely overlooked compared to other plumage colours (e.g., carotenoid and melanin; Török, Hegyi and Garamszegi, 2003; Gladbach et al. 2011; Soravia, Aguado-Giménez and Avilés, 2020). White feathers are not as costly to produce as melanin or carotenoid plumage (Török, Hegyi and Garamszegi, 2003). White pigmentation is an outcome of internal light scattering from feather keratin structure (Tickell, 2003) and brightness is determined by barb density (Igic, D'Alba and Shawkey, 2018). However, without the strengthening effect of melanin pigments, white feathers are highly susceptible to wear, and thus require high levels of maintenance (Kose and Møller, 1999; Badyaev and Hill, 2003). Honest quality signalling via achromatic plumage may then be derived from high maintenance costs (Berglund, Bisazza and Pilastro, 1996; Qvarnström and Forsgren 1998; but see Hill and Brawner 1998; Badyaev and Hill 2000), parasite resistance costs (Berglund, Bisazza and Pilastro, 1996; Qvarnström and Forsgren, 1998; Soravia, Aguado-Giménez and Avilés, 2020) or increased conspicuousness (Andersson, 1986; Penteriani et al., 2007). Finally, some bird species are able to see ultraviolet light (wavelengths 3-400nm), with the majority of diurnal birds possessing a preponderance of cone cells expressing short wave sensitive opsin (e.g., SWS1). Whilst some nocturnal avifauna such as owls may not possess UV/V sensitive cones and expression of SWS1, they are able to detect UV light, and UV reflective plumage has been postulated as a visual signal in nocturnal birds (Camacho et al., 2019; Galván et al., 2018). As with achromatic plumage, display of UV reflective and fluorescing plumage is more effective under full-moon and twilight conditions where, relative to other wavelengths, the UV portion of light spectrum is enhanced (Kohler et

al., 2019). This correlates with recorded temporal variability in display behaviours, when many nocturnal birds increase vocal activity when atmospheric light levels are high (Reino et al., 2015), such as during the full moon.

4.3 Nightjars as a model system

Andersson and Simmons (2006) outlined the key qualities required by a species to become an effective study system for sexual selection and mate choice (See Box 4.1). However, the properties proposed by the authors are restricted to relatively few taxa (namely insects and fishes), with even many model bird species (e.g., Blue tits *Cyanistes caeruleus*; Mainwaring 2017, and Great tits, *Parus major*; van Oers et al. 2008) comparatively less amenable for sexual selection research. Indeed, whilst the criteria in Box 4.1 are preferable in study systems required for combined genotype and phenotype analyses of trait evolution by sexual and natural selection, adherence to these criteria limits our knowledge of sexual selection across many avian taxa. Despite avian mating systems constituting the most extensively studied molecular mating systems of any taxonomic group (Brouwer and Griffith, 2019), significant phylogenetic sampling bias across class Aves remains, with the majority of studies concentrated on passerines (Brouwer and Griffith, 2019). Consequently our understanding of mating systems, sexual selection and evolution of ornamentation are comparatively lacking in non-passerines and particularly nocturnal avifauna, a group which have received little attention (Brouwer and Griffith 2019; but see Müller et al. 2001; Roulin et al. 2004; Saladin et al. 2007; Horníček et al. 2017). Restricting study subjects to these criteria (Box 4.1) would mean avoiding research on whole phylogenetic groups of birds, limiting our understanding of mating systems and signal evolution, and ignoring taxa of greatly varying life histories and ecology, rendering our understanding of such questions incomplete, inadequate, and biased. Research on mating systems and visual signalling in nocturnal avifauna provide valuable opportunities to test key hypotheses across greatly differing phylogenies, life histories and under novel environmental/ecological constraints (e.g., low ambient light levels in nocturnal taxa).

Box 4.1: Desirable characteristics in a model system for analysis of sexual selection and mate choice as outlined by Andersson and Simons, (2006):

- Amenable to comprehensive study in the natural environment.
- Small body size and short generation time.
- Ideally the species, or a close phylogenetic relative, should already be genetically well known.
- Conspicuous sexual dimorphism in structure or behaviour.

Nightjar species of the family Caprimulgidae are mostly monotypic for sub-species and sexually monomorphic in body plan and for cryptic contour plumage (Cleere, 1998; Holyoak, 2001). However, the majority of Caprimulgids exhibit sexually dimorphic plumage (Cleere, 1998; Pople, 2003), ranging from achromatic ornaments (Pople, 2003) to the dramatically elongated rectrices in male Lyre- and Swallow-tailed nightjars (*Uropsalis lyra* and *U. segmentata*) and primaries in male Standard- and Pennant-winged nightjars (*Camprimulgus lonipennis* and C. *vexillarius*; Holyoak 2001) (Fig 4.1). The inter-specific variation in sexual dimorphism and dichromatism from none to extreme ornamentation, makes nightjars an interesting model for understanding the evolution of sexually selected traits in nocturnal avifauna. Recent phylogenetic studies have provided us with an excellent evolutionary history and phylogeny of Caprimulgidae, with the family mostly phenotypically distinguished by their sexually dimorphic plumage characters. With information of plumage characteristics and ornamentation available for the majority of the family, Caprimulgids present a unique and fruitful opportunity among Strisores (but see Temeles et al. 2010; Diamant et al. 2021), and more broadly nocturnal avifauna to study sexual selection and trait function evolution.

Characterisation of Caprimulgid genetic mating systems (i.e., see **Chapter 5**) would address the taxonomic bias, bridge the knowledge gap, and aid future interspecific research within the field of avian mating systems and sexual selection. Nightjars are globally distributed, breeding at both temperate and tropical latitudes, and exhibit varying degrees of migratory behaviour, enabling comparative interspecific research questions addressing latitudinal variation in mating systems. Again, amongst nocturnal taxa, nightjars provide a unique opportunity to study mating system and infidelity evolution (e.g. EPP), whilst controlling for phylogenetic influence, with ≥39% of interspecific variation in EPP occurring at the family level or above (Brouwer et al., 2017; Brouwer and Griffith, 2019). Being ground nesters, often in dry habitat,

Caprimulgid nests are typically more accessible than other nocturnal birds (e.g., Owls nesting at height in trees or hillside crags; Papageorgiou et al. 1993; LaHaye and Gutiérrez 1999; but see nest box nesting species Sacchi et al. 2004; Wendt and Johnson 2017), which would more easily enable genetic sampling and nest monitoring for mating systems and sexual selection studies. Nests of Red-necked nightjars (*Caprimulgus ruficollis),* for example, breeding in Orange grove plantations can be easily accessed and adults trapped routinely at nest sites (Zamora pers comm.). Similarly, Common nighthawks (*Chordeiles minor*) may nest on rooftops making them easily accessible and observable (Viel et al., 2020). Information on social parental associations to broods is a pre-requisite for mating system studies, and the necessary DNA sampling from putative parents and chicks can be easily collected in such scenarios.

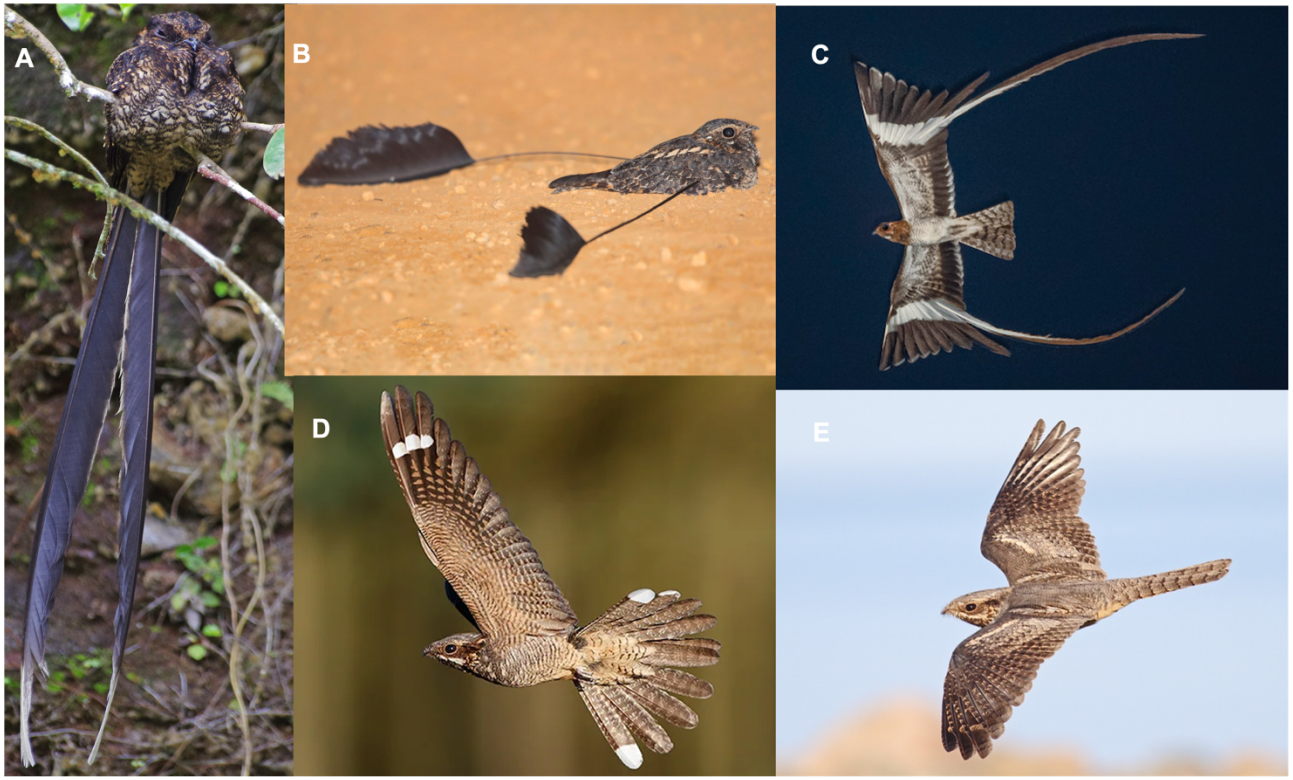


Figure 4.1 Nightjar species showing sexually dimorphic plumage ornamentation. A) Male Lyre-tailed nightjar exhibiting exaggerated outer rectrices, photo by (downloaded from https://shorturl.at/clwC5 on 20/05/23); B) Male Standard-winged nightjar showing exaggerated inner primary feathers, photo by Niall Perrins (downloaded from https://shorturl.at/EGSX6 on 20/05/23); C) Male Pennant-winged nightjar displaying exaggerated inner primaries and achromatic panel, photo by Juan van den Heever (downloaded from https://tinyurl.com/bdenev85 on 23/08/23); D and E) Male and female (left to right) European nightjar showing sexual dichromatism typical of many Caprimulgids (achromatic white spots on male flight feathers and reduced buff equivalents on female), photos by by Helge Sorensen (downloaded on 10/08/2023 from http://tiny.cc/ses9vz) and Ove Ferling (downloaded on 10/08/2021 from https://www.birdforum.net/opus/File:Nightjar15.jpg).

However, not all nightjars are as accessible as Red-necked nightjar or Common nighthawks, and poor habitat accessibility, low nesting density and cryptic plumage/behaviour have until now limited comprehensive studies of nightjar genetic mating systems. Nevertheless, at the time of writing research on EPP rates, mating systems and visual signalling are underway in multiple Caprimulgid species (e.g., see **Chapters 5 and 6**). Recent technological, and methodological advances mean nightjars provide a valuable study system for investigating visual signalling and mating systems, although significant knowledge gaps remain in our understanding of individual species.

4.4 Current understanding of nightjar mating systems, sexual selection and visual communication

4.4.1 Sexual selection and visual signalling

Nightjars are typified by their crepuscular-nocturnal habits and monomorphic cryptic plumage (Cleere, 1998; Holyoak, 2001), but many species exhibit sexually dimorphic plumage traits (Cleere, 1998; Pople, 2003). Sexually dimorphic achromatic patches on flight feathers, throat patches and moustachial stripes represent the most common forms of dimorphism in the family (Cleere, 1998; Holyoak, 2001) and 79% of species have at least one of these ornaments (Pople, 2003). Achromatic bars on the wings and tail of Caprimulgids are usually made up of a number of white patches on individual feathers. Primary feather patches are mostly located midway along the feather and are typically found on P7 – P10, although the number of primaries with white patches, patch size and location vary greatly inter- and intraspecifically (see White-winged nightjar *Eleothreptus candicans*, and Short-tailed nighthawk *Lurocalis semitorquatus*; Holyoak 2001). Similarly, tail feather white patches are mostly confined to the tips of the outer rectrices, but in some species are missing entirely (e.g., Todd's nightjar *Setopagis heterura*) or may extend to encompass the entirety of the feathers in extreme examples (e.g., White-tailed nightjar *Hydropsalis cayennensis*). Female nightjars often exhibit little or no white plumage. Pople (2003), in a comprehensive review of nightjar achromatic plumage, found that females in 38% of Caprimulgid species possess no white markings at all. In the remaining 62% of species, females usually display reduced versions of the white spots exhibited by males (Cleere 1998; Holyoak 2001, but see multiple monotypic *Nyctiphrynus* species; Cleere 1998). Pople (2003) also highlighted that Caprimulgidae overall show more dimorphism for tail than wing white patches, with females more often exhibiting white

plumage on the wing than the tail. The sexual dichromatism exhibited by many species of Caprimulgids suggests that achromatic plumage are sexually selected ornaments in males (Forero, Tella and García, 1995; Aragones, Reyna and Recuerda, 1999; Roth, Argyros and Browning, 2003), although work to determine trait function is needed to confirm this (see **Chapter 6**). Sexual, and social selection have been hypothesised to have driven much of the speciation within Caprimulgidae and particularly in *Caprimulgus* (Han, Robbins and Braun, 2010) and may explain the high levels of inter-specific variation in sexual dichromatism in the family, when compared with other mostly monochromatic families within clade Strisores (but see Temeles et al. 2010; Diamant et al. 2021).

The achromatic plumage displayed by members of Caprimulgidae are likely important in visual communication (Aragones, Reyna and Recuerda, 1999; Pople 2003; Roth, Argyros and Browning, 2003; **Chapter 6**). As seen in other nocturnal species, the bright white ornamentation in nightjars would enable effective communication in low light (Penteriani and Delgado, 2017). Nightjar eyesight is adapted for effective nocturnal vision (Tate, 1989), and they have very large eyes relative to head size. Nightjar retinas are populated by an abundance of specially adapted rod cells, which enables low light vision. Most nightjars also have a tapetum, a reflective layer behind the retina which reflects the light back through the eye and allows light photons that were not captured as they passed through the retina the first time a second chance to be picked up by photoreceptors (Martin, 2017). This physiological trait greatly enhances the bird's ability to detect low levels of light (Gondo and Ando, 1995; Cleere, 1998), but possibly at the cost of spatial resolution (Martin, 2017). Whilst colour differentiation is probably poor, nightjars may be sensitive to high contrast patterns in low light, such as white patches contrasted against largely darker plumage, to engage in visual communication at close range (Martin, 2017). Nightjars often engage in display and territorial behaviour during twilight or bright moonlight where atmospheric light levels are typically at their greatest (Jackson, 2015; Reino et al., 2015). Birds extend their wings or fan their tails during territorial or display behaviour, whether in flight (e.g. European nightjar *Caprimulgus europaeus*; Cramp and Simmons 1985) or from a perch or the ground (e.g. White-winged nightjar; Pople 2003) apparently exhibiting achromatic plumage features to conspecifics (Cramp and Simmons, 1985; Aragones, Reyna and Recuerda, 1999; Roth, Argyros and Browning, 2003). During periods of singing, throat badges can also be accentuated, a behaviour that has also been noted in other crepuscular and nocturnally displaying species (e.g., Eagle owls; Yamamoto et al. 2008, Great snipe *Gallinago media*; Höglund et al. 1990). Nightjars likely adjust display behaviour to levels of available atmospheric light to produce a more effective visual display of ornamentation (Penteriani and Delgado, 2017). Nightjar activity has been found to strongly

correlate with moon phases, with the impact of moon phase on migration, call frequency, foraging behaviour, clutch laying and hatch date noted in a number of Caprimulgids (Reino et al., 2015; Mills, 1986; Kronfeld-Schor et al., 2013; English, Nocera and Green, 2018). Whilst little work has explored the direct link between nocturnal light levels and display behaviour (but see Jackson 2015; Reino et al. 2015), it is likely that Caprimulgids are able to take advantage of full moon phases, as has been documented in other achromatically ornamented nocturnal species (e.g., Eagle owls; Yamamoto et al. 2008, Latham's snipe *Gallinago hardwickii*; Iida 1995). Given their behavioural sensitivity to light conditions and conspicuous display behaviours, nightjars represent a suitable group for investigating the effects of atmospheric light, including that from anthropogenic light pollution, on display, territorial behaviour and activity patterns for nocturnal avifauna.

As observed in other nocturnal taxa, nightjars emit porphyrin-based pink fluorescence under ultra-violet light (Stachel, Stockwell and Van Vranken, 1999; Goutte et al., 2019; Blythman, Sansom and Lohr, 2016; Galván et al., 2018), although the ability of nightjars to perceive fluorescence is currently unknown. However, whilst, in an initial investigation in Red-necked nightjar, Camacho et al. (2019) showed no significant sexual dimorphism or age variation in fluorescence, they showed that juvenile body condition was correlated with higher fluorescence, as has been found in other nocturnal taxa (e.g. Eagle owls; Galván et al. 2018). In this case fluorescence may aid reproductive-status signalling for post-hatch year breeding. At present there is little evidence to suggest that fluorescence acts as a visual signal used in sexual selection and mate choice in nightjars. However, more work here is needed before conclusions can be drawn. Caprimulgids therefore present a valuable research opportunity to understand the visual signalling roles of UV fluorescence and reflectance in nocturnal birds. Work is needed here to investigate i) the ability of nightjars to perceive UV fluorescence and reflectance ii) physiological production of UV fluorescence and reflectance and iii) its role in quality signalling over a wider range of nightjar species.

The value of visual signals in nocturnal communication (Aragones, Reyna and Recuerda, 1999; Jiguet, Arroyo and Bretagnolle, 2000; Penteriani and Delgado, 2017) coupled with the conspicuousness and ubiquity of ornamentation across species, leave nightjars ideally placed for contributing to the study of nocturnal visual communication. As a field which has yet received little attention (but see **Chapter 6**), multiple aspects remain to be investigated. Little is known regarding the evolutionary origin of visual communication systems in nocturnal birds. Detailed studies of the relationship between sexual dimorphism, age, body condition and achromatic ornamentation plus porphyrin-based fluorescence in nightjars would provide a

valuable contribution here. There is a paucity of experimental research concerning nocturnal visual signalling behaviour (Penteriani and Delgado, 2017). Nightjars may be amenable to such experimental studies as breeding males are highly responsive to audio and visual stimuli. Anecdotally, European nightjars have been found to show elevated territorial behaviour towards achromatically manipulated decoys. Nightjars are suitable for studies testing responses to experimentally manipulated signals, whether visual (e.g., white patch size, brightness manipulations) or vocal (e.g., song rate, pitch, duration etc.). Manipulated territory intruder studies, used successfully for Eagle owls (Penteriani et al., 2007), whereby territory intrusions are simulated using decoys with manipulated signal characteristics (e.g., white patch size, shape or brightness), could help determine signalling function of achromatic patches at night.

Signalling environment (e.g., ambient light levels, habitat structure and complexity) also plays a major role in communication and signalling strategies should be investigated in the context of relevant environmental conditions (Denoël and Doellen, 2010; Penteriani and Delgado, 2017). Nightjars present a useful study system for the influence of light on visual communication at night, with the influence of lunar cycles on other aspects Caprimulgid behaviour and life history well documented (Mills, 1986; Kronfeld-Schor et al., 2013; English, Nocera and Green, 2018). Display behaviour, which typically involves exhibition of achromatic patches and ornaments, are easily observed in Caprimulgids during twilight and new tracking technology enables quantitative measures of display of greater precision than ever before (Norevik, 2018). Field recordings may also document territorial behaviour, especially multimodal displays involving both song and plumage display, which in multiple Caprimulgids is thought to involve exhibition of achromatic features (Cleere, 1998; Holyoak, 2001; Roth, Argyros and Browning, 2003). Such techniques enable the influence of ambient light on visual display behaviour to be investigated. This is particularly urgent considering the increase of anthropogenic light pollution on nightjar breeding grounds (Sierro and Erhardt, 2019). Notably, a number of Caprimulgids breed in close proximity to, or even within, dense urban populations (e.g. Common nighthawk; Viel et al. 2020). Although Viel (2014) found a positive correlation between nighthawk presence and artificial light sources, currently the implications of increasing anthropogenic light pollution for intraspecific signalling is lacking in nightjars and other nocturnal taxa (Penteriani and Delgado, 2017).

4.4.2 Mating systems

Compared to other bird groups little is known regarding nightjar mating systems, with the nocturnal habits and cryptic plumage of nightjars partly responsible for the paucity of knowledge by limiting the use of traditional observational methods. Where mating systems have been described, the majority of nightjar species have been noted as 'socially monogamous' (see **Chapter 5**), with few records of polygyny. In most cases where breeding behaviour has been described, records are typically limited to anecdotal evidence and small sample sizes (Holyoak, 2001; Pople, 2014). However, Pople (2014) performed a comprehensive study of the breeding biology of White-winged nightjar*,* finding that the species had a polygynous mating system with uniparental care and a lek-like mate choice system, where multiple males display to females in 'arenas'. Similarly, Pennant and Standard-winged nightjars (*Macrodipteryx longipennis* and *M. vexillarius*) also exhibit forms of polygyny, both demonstrating uniparental care and lek-like mate choice systems (Jackson, 2004; Holyoak, 2001). European nightjar represents perhaps the most intensively studied Caprimulgid. The species has long been characterised as monogamous due to their biparental care and high levels of inter- and intra-seasonal pair fidelity, although mate switching has been recorded (Cramp and Simmons 1985; Cresswell and Alexander 1990; but see **Chapter 5**). Limited observations of polyandry (Padget et al. 2019; **Chapters 5 and 6**) and polygyny (Jensen, 2013) from single cases and anecdotal records have been noted, although the extent of and drivers for this intraspecific variation are unknown. Despite a wealth of literature on other aspects of European nightjar biology, a clear account of its genetic mating system is lacking (but see **Chapter 5**). The lack of mating system data on European nightjar is typical of most Caprimulgids and demonstrates the need to address this knowledge gap in their natural history. Researchers should look to characterise Caprimulgid mating systems where possible, with a need for both social as well as genetic parentage data if the group is to have value in future inter-specific meta-analyses (Brouwer and Griffith, 2019). Ongoing research on European nightjar (see **Chapter 5 and 6**) will address some of these gaps and inform species-specific conservation (Sutherland, 1998; Frankham, Ballou and Briscoe, 2010).

4.5 Technological solutions to research limitations

Historically, research on Caprimulgid mating systems and sexual selection have been constrained by the cryptic and nocturnal nature of the family, as it has in other difficult-to-study taxa (e.g., owls and nocturnal seabirds; Penteriani and Delgado 2017; Soravia, Aguado-

Giménez and Avilés, 2020). To study a species' mating system, nests must be located so that associations can be made between putative parents and offspring and DNA sampled. Compared to other avian model species (e.g., Blue tits and Great tits; van Oers et al. 2008; Mainwaring 2017), some nightjar nests can be challenging to locate and monitor for the inexperienced worker. However, some species provide highly accessible options such as the Red-necked nightjar and Common nighthawk. To generate robust behavioural data on parental-offspring associations, copulation and provisioning, researchers should ideally be able to individually identify birds. Whilst many nightjar species are banded, the morphology of Caprimulgids (short legs that are obscured in flight and on the ground) limits the use of individually coloured leg bands, which are perhaps the most cost-effective field-readable identification method (Gowaty and Karlin, 1984; Burley, 1986; Westneat, 1993). Passive integrated transponders (PIT tags) also provide a viable method (Mutzel et al., 2013; Schlicht, Valcu and Kempenaers, 2015), but may be challenging to use on some Caprimulgidae, as birds are known to move nestlings post-hatching, sometimes re-locating them metres from their original nest site and outside the range of the tag readers (Jackson, 2009). Wing tags may provide another option for field-readable individual identification. However, modifications to nightjars' wing morphology may have significant welfare implications by reducing hunting efficacy, camouflage and ultimately survival (Calvo and Furness, 1992; Trefry, Diamond and Jesson, 2013; Curk et al., 2021). The impact of wing tags on intraspecific communication in a species which typically possesses wing ornamentation should also be considered (Southern and Southern, 1985). Lightweight tail-mounted radio frequency tags, which are cheaper than GPS-enabled tags, have been successfully used on European nightjar to identify individuals (Feather, 2015). They require training to fit correctly but are not much more technically difficult than fitting a leg band correctly.

Regardless of individual marking methods, low light levels during times of peak activity limit the efficacy of traditional observational techniques and novel solutions need to be developed. At present nest-site capture of putative parents presents a viable method for ascertaining social and genetic parental associations (e.g., Soukup and Thompson 1997; Müller et al. 2001; Li et al. 2009b; Henry et al. 2013; see **Chapter 5 and 6**). However, due to the risk of disturbance, netting at nest sites should be limited and this may mean cases of cooperative or extra-pair behaviour (e.g., multiple individuals provisioning chicks) are under-recorded. This method does not enable recording of behaviours away from the nest site (e.g., copulations). Therefore, EPP in species where extra-pair sires do not provision offspring may go unnoticed unless genetic paternity analysis is conducted. However, despite limitations, nest capture remains currently the most appropriate and most cost-effective method of determining

individual nest associations in Caprimulgids, and has been used successfully in other avian taxa (Soukup and Thompson 1997; Müller et al. 2001; Li et al. 2009; Henry et al. 2013; **Chapter 5 and 6**).

The cryptic and nocturnal nature of nightjars creates a challenge when studying breeding behaviour, but these issues are being addressed by technological advances in affordable night-vision equipment, high precision, lightweight geospatial (GPS) recording tags and other remote data loggers and recording devices that collect localised movement, acoustic and incubation data. Satellite-linked data loggers have enabled researchers to gather real time information on animal behaviour, movement, and energy expenditure. Rapid advancements and reduction in size and cost of geospatial loggers enables less labour-intensive methods of determining nest associations in birds. GPS tracking data has revolutionised the study of animal movement and habitat use, and none more so than in cryptic and nocturnal taxa which had previously evaded researchers (Mitchell et al., 2020; Mirski et al., 2021; Wood et al., 2021). As GPS tags become lighter, cheaper and more accessible to researchers, they are being used in behavioural biology to explore a wider range of questions (Wegge, Rolstad and Storaunet, 2013; Riou and Combreau, 2014; Hernandez-Blanco et al., 2015; Silva, Moreira and Palmeirim, 2017; Wann et al., 2019). Fine-scale GPS telemetry have been used to generate data characterising the mating systems of various species of grouse by tracking lek attendance in males (Wegge, Rolstad and Storaunet, 2013; Wann et al., 2019; Milligan, Berkeley and McNew, 2020). Temperature sensitive data loggers (e.g., iButton temperature loggers, originally designed for shipping perishable goods; O'Connor and Ritchison 2013) provide researchers with the ability to remotely monitor nest attendance and incubation behaviour in birds, as well as the effect of disturbance on nest success in European nightjar (Feather, 2015). Alongside remote camera traps (or other methods of ascertaining individual nest association), temperature loggers provide an opportunity to remotely record incubation and nest visitation, having been readily applied to otherwise cryptic ground nesting species (e.g., Weston and Elgar 2005; Marchi et al. 2008; St Clair et al. 2010). Targeted (i.e., known territory holding males) or mass deployment of GPS tags will enable researchers to determine individual nest associations, without the need for invasive nest site capture methods. Whilst this method remains expensive, costs of suitable tags are decreasing rapidly, with the Argos global satellite system dedicated to ecological studies also available to researchers (Scarpignato et al., 2016; Ng et al., 2018). Wearable accelerometers enable characterisation of different behavioural states (i.e.: foraging, resting, singing etc.) and when partnered with GPS telemetry enables classification and quantification of Caprimulgid display behaviours (e.g., singing, wing clapping, display flights etc). Accelerometers have successfully been used to quantify vocal display behaviour in relation to

temperature in Little bustards (Gudka et al., 2019) and nocturnal vocal display behaviour in Houbara bustards (*Chlamydotis undulata*) in relation to lunar luminosity (Alonso, Abril-Colón and Palacín, 2021). Application of combined accelerometers and GPS tags provides a viable method of quantifying otherwise hard-to-measure display behaviour in Caprimulgids, enabling these behaviours to be studied in the context of ecological and environmental characteristics (i.e., breeding density, habitat structure and ambient light Gudka et al. 2019; Alonso, Abril-Colón and Palacín, 2021).

UV-enabled recording equipment (e.g., lights or cameras) provide researchers with the opportunity to record and quantify UV reflectance and fluorescence (Zampiga, Gaibani and Csermely, 2008). A promising area of research in nocturnal avifauna is use of UV reflectance and fluorescence in visual signalling (Zampiga, Gaibani and Csermely, 2008; Rajchard, 2009) and use of sensitive UV-enabled cameras will enable documentation of this signalling during display behaviour by Caprimulgids. Remote cameras (e.g., trail cameras) have been used extensively in behavioural research (O'Brien and Kinnaird, 2008; Caravaggi et al., 2017). However, their use has been limited in nocturnal and highly vagile species such as nightjars, owing to cost, equipment portability and efficacy in low light levels. The recent advancements in portable nocturnal 'night vision' recording and observation equipment (e.g., infrared, and thermal scopes) provide promising tools for nightjar researchers. Infrared remote cameras have long been used in wildlife research. When placed at nest sites these cameras enable parental and offspring behaviour to be documented at night as well as during the day. Infrared nest cameras enabled the study of Common potoo (*Nyctibius griseus*) chick feeding behaviour and parental provisioning (Cestari, Guaraldo and Gussoni, 2011), and nest visitation in European nightjar (Padget et al., 2019). Whilst thermal scopes/cameras are only beginning to be widely affordable and available, infrared thermography has been applied in behavioural studies of other nocturnal taxa such as bats and owls (see McCafferty 2013 for review). Infrared thermography has also been used to efficiently locate nightjar nest and roost sites (Shewring and Vafidis, 2021). Whilst initial costs of thermal imaging are currently high, the reductions in labour costs may mean that future breeding behavioural work in Caprimulgidae need not be constrained by difficulties in nest detectability for some species (Nunney 1993; Holyoak 2001, but see Viel 2014). Thermal and night vision recording equipment provides a relatively inexpensive method (compared to mass deployment of data loggers) to observe and quantify behaviour in nocturnal animals .

Finally, improvements in molecular methods enabled the rapid advancement in our understanding of avian mating systems, EPP rates, and sexual selection within the last 30

years. Increasingly, genetic, and genomic methods are valuable and commonly utilised tools in conservation and behavioural ecology. As molecular methods continue to become more accessible (e.g., reduced financial, labour or expertise costs), nightjar researchers are better placed to investigate mating systems and sexual selection. Less-invasive methods of sourcing DNA from buccal swabs or plucked feathers etc., are being used in genetic/genomic research to great effect (Handel et al. 2006; Eiben et al. 2017; Vilstrup et al. 2018; **Chapter 3, 6 and 7**), reducing both costs and ethical concerns when compared to blood or tissue sampling. Non-invasive genetic methods have been applied to characterise mating systems of otherwise difficult-to-study species (e.g. waterfowl), whereby family units may be reconstructed by DNA sampled from feathers and eggshell/membrane left at the nest site (Kreisinger et al., 2010). Whilst social parental associations cannot be ascertained with this technique alone, the methods provide researchers with a proven low-cost and low-labour field method of investigating genetic mating systems (Miño et al., 2011; Turjeman et al., 2016; Indykiewicz, Podlaszczuk and Minias, 2017).

The Caprimulgid researcher has an array of technology at their disposal, providing opportunities for remote methods and less labour-intensive and non-invasive studies. GPS tags enable remote nest associations to be determined and when used in combination with accelerometers for individual display behaviour to be quantified. 'Night vision' cameras and scopes allow display behaviours to be easily observed and nest visitation and paternal care to be documented. Ultimately, technological advancements now enable researchers to overcome the key constraints which have previously limited nightjar breeding behaviour research, presenting caprimulgids as a valuable study system to understand mating systems and visual signalling in nocturnal birds.

4.6 Conclusion

Multiple characteristics make Caprimulgids a valuable study system for nocturnal visual communication. Nightjars are speciated largely by sexually dimorphic characters, and both ornamentation and display behaviours are highly conspicuous comparative to other nocturnal avifauna. Caprimulgids are well placed among avifauna to investigate nocturnal visual communication and signal trait evolution. Further, the ubiquity and interspecific variation in achromatic ornamentation makes the family suited to study the role of achromatic plumage as an indicator of quality. With the emergence of the role of achromatic ornamentations in visual signalling and mate choice (Doucet et al., 2005; Gladbach, Gladbach and Quillfeldt, 2011;

Guindre-Parker et al., 2013; Soravia, Aguado-Giménez and Avilés, 2020), nightjars can contribute to this growing field, particularly within the context of nocturnal taxa. Finally, nightjars are ideal to study the role of ambient light on visual signalling in nocturnal animals. As anthropogenic light pollution increases (Gaston et al., 2013), it is fundamental for conservation biology that we understand the role of ambient light in all aspects of nocturnal ecology, including signalling and mate choice.

Caprimulgidae are defined in the minds of researchers by their cryptic and nocturnal nature, which has historically limited research on sexual selection, mating systems and visual communication in nightjars and other nocturnal taxa alike. However, advances in technology and accessibility of molecular methods provide nightjar researchers with the tools to overcome these limitations. Specifically, a combination of GPS telemetry, accelerometers and 'night vision' cameras will allow researchers to overcome the major barriers to field work. Nightjars present a genuine opportunity to study the role of sexually dimorphic ornaments in mate choice and EPP rates in nocturnal taxa providing a valuable and currently lacking perspective on nocturnal behaviour, ecology, and conservation.

I recommend a program of immediate work that should be undertaken to improve basic knowledge of this taxa, with a focus on caprimulgid species breeding in southern latitudes: 1) quantification of secondary sexual characters in more species, 2) characterisation of more species mating systems, and 3) better records of parental care and display behaviour across the family. This acceleration of data collection requires funding, as most science does, but there is a need to address inequities in taxonomic, geographic, and temporal bias if we are to develop our understanding of fundamental evolutionary processes with a view of solving practical conservation problems. Human bias towards diurnal activity has left nocturnal conservation and behavioural ecology lagging behind, while at the same time nocturnal species are negatively affected by anthropogenic disturbance, pollution and habitat destruction or alteration in ways that may not be apparent during daylight hours. We must address this imbalance urgently and nightjars are uniquely suited to allow us to do it.

Chapter 5 Intraspecific variation in the mating system of European nightjar *Caprimulgus europaeus* among British breeding sites.

5.1 Abstract

Socially monogamous but genetically polyandrous mating systems are common in birds. Variations in life history (e.g., lifespan, clutch size, and reliance on paternal care) are thought to drive differences in extra-pair paternity (EPP) between species. Population-level variation in EPP has been attributed to differences in environmental and ecological factors, such as breeding density. European nightjar, (*Caprimulgus europaeus*), hereafter nightjar, was long thought to be monogamous, being long-lived, producing small clutch sizes, and heavily reliant on biparental care. Here, for the first time, I have characterised the social and genetic mating system exhibited by nightjar, testing the effect of breeding density on EPP at two breeding sites in the United Kingdom. Nightjar displayed moderate levels of EPP (23% extra-pair offspring and 26% extra-pair broods), whilst breeding density had little overall relationship with the presence of extra-pair offspring in nests. However, when accounting for sampling year, the number of males within the average home range radius of nests positively predicted EPP in years of high male density. My results provide the first EPP rates for any species of Caprimulgiform. Nightjar displayed moderate levels of EPP, representing an anomaly among other species with similar life histories (long-lived, small clutch sizes, biparental caring). My data suggest that breeding density had little effect on EPP in nightjar until a high male density threshold was reached within the studied populations. My research gives an important initial insight into EPP and its ecological drivers in Caprimulgiformes and more broadly nocturnal taxa, providing important data toward future interspecific comparative studies. Further research is needed in nightjar to understand the underlying drivers of EPP in the species, particularly focusing on the role of male age and quality in EPP acquisition.

5.2 Introduction

Social monogamy, characterised by a stable breeding pair, is the most common mating system in birds, recorded in ~90% of all avian taxa (Griffith, Owens and Thuman, 2002). However, a wealth of molecular parentage studies have highlighted that the majority of monogamous birds exhibit some level of extra-pair copulations, resulting in extra-pair paternity (EPP; Ju et al., 2014; Wells et al., 2015; Grunst et al., 2017; Grinkov et al., 2018) and rarely intraspecific brood (IBP) or quasi-parasitism (QP; Blomqvist et al., 2002). Ultimately, life history traits influence a species' mating system. Truly monogamous species are typically sexually monomorphic and rely on biparental care of offspring. Extreme sexual dimorphisms in size and ornamentation, on the other hand, are often associated with polyandrous mating systems

(Owens and Hartley, 1998; Jones and Ratterman, 2009). However, a high proportion of otherwise monogamous species exhibit striking sexual dimorphisms (Owens and Hartley, 1998; Jones and Ratterman, 2009). High EPP in otherwise monogamous taxa have helped explain this discrepancy (Albrecht et al., 2007; Webster et al., 2007).

Parental care and mating systems are fundamentally linked (Kempenaers, 2022), with the amount of parental care invested by each sex constraining the capacity for individuals to breed. For example, male biased parental care enables multiple mating in female polyandrous shorebirds (Székely, Thomas and Cuthill, 2006). In species where bipaternal care is essential for offspring survival, females may refrain from extra-pair copulations (EPC) owing to risk of loss of paternal care from their social partner (Birkhead and Møller, 1995), with this effect compounded in species with long reproductive lifespans (Mauck, Marschall and Parker, 1999). Indeed, many birds exhibiting 'slow' life histories (i.e. long-lived, small clutch sizes with biparental care) display very low EPP rates (Arnold and Owens, 2002) or are genetically monogamous (Anderson and Boag, 2006; Shealer, Devbhandari and Garcia-Mendoza, 2014).

EPP rates in birds varies among species and populations, as well as individuals as well as over time (Griffith, Owens and Thuman, 2002; Taff et al., 2013; Mayer and Pasinelli, 2013; Brouwer et al., 2017). Ecological factors such as breeding synchrony and density can influence population EPP rates (Westneat, 1990). The breeding density hypothesis postulates that increased encounter rates between male and females under high densities increases EPP (Westneat, 1990). However, support for the hypothesis is mixed (Griffith, Owens and Thuman, 2002; Brouwer et al., 2017; Arrieta et al., 2022; but see Mayer and Pasinelli, 2013), with the effect possibly confounded by other factors (e.g. mate quality, male territoriality, habitat complexity; Griffith, Owens and Thuman, 2002). It is challenging to reliably assess the relationship between density and EPP in wild populations and the metrics used to investigate the hypotheses (e.g., NN: nearest neighbour distance, NoN: number of neighbours within a given radius, or numbers of neighbours per unit area) may not best reflect opportunities for EPCs, particularly where only a single metric is used. NN, for example, may not reflect the availability of local mating opportunities if the nearest neighbour reflects the only 'local' conspecific territory and instead the focal nest and nearest neighbour are an isolated sub-population. Therefore, incorporation of multiple population density metrics is required to accurately reflect variation in EPC opportunities amongst broods (Griffith, Owens and Thuman, 2002; Mayer and Pasinelli, 2013; Brouwer et al., 2017; Arrieta et al., 2022).

Whilst avian EPP research currently constitutes the most extensive study of molecular mating systems for any taxonomic group (Brouwer and Griffith, 2019), up until now the genetic mating system has not been determined for any species within the family Caprimulgidae (Brouwer and Griffith, 2019). European nightjar (*Caprimulgus europaeus*), henceforth nightjar, represents perhaps the most intensively studied member of Caprimulgidae or indeed Caprimulgiformes. Nightjar are long-lived (~12 years; Robinson et al., 2022), sub-Saharan migrants (Lathouwers et al., 2022). Nightjar are a ground nesting species and typically have one to two nesting attempts per annum, laying two eggs (Cramp and Simmons, 1985). Nightjar exhibits biparental care of offspring (Cramp and Simmons, 1985; Cresswell and Alexander, 1990; Holyoak, 2001), although studies on nightjar breeding behaviour are notably lacking (Cresswell and Alexander, 1990). Observational and ringing studies have long classified the species as socially monogamous (Cramp and Simmons, 1985; Holyoak, 2001). Nightjar have sexually dichromatic, achromatic ornamentation, with males exhibiting prominent white panels on primaries and rectrices, suggesting a role in sexual selection (Cleere, 1998; Holyoak, 2001). Indeed, suspected cases of social polyandry (N cases = 3 cases, Padget et al., 2019) and a single case of polygyny (Jensen, 2013) have been recorded in individuals, although evidence for this are restricted to small sample sizes from ringing studies (e.g. Jensen, 2013; Padget et al., 2019). The mating system of nightjar is therefore difficult to predict owing to 'contradictory' life history traits (i.e., biparental care and social monogamy conflicting with sexual dichromatism) and the paucity of information available in the literature.

5.2.1 Aims and hypotheses

In this chapter, I aimed to characterise the genetic mating system and extra-pair paternity rates in nightjar for the first time, also testing the effect of nest and male density on extra-pair paternity rates at two breeding sites. I combined behavioural (nest site parental associations and breeding density data) and genetic (molecular parentage analysis using microsatellite markers) information from two British nightjar breeding populations (Humberhead Peatlands and Thetford Forest) over three years (2019-21) to address the following aims and hypotheses;

A1) Characterise the social and genetic mating system and infidelity rates in nightjar for the first time, testing the hypothesis that, in line with reports of social monogamy and biparental care, nightjar will exhibit genetic monogamy;

A2) Determine whether infidelity rates vary between and within sites over sampled years;

A3) On an individual level test the effect of male and nest density on EPP in nightjar, testing the hypothesis that EPP rates will be higher under increased nest and male density.

5.3 Methods:

5.3.1 Study sites

Nightjar breeding populations were studied from mid-May through to September in 2019, 2020, and 2021 at two sites in the UK, representing a sample of the different habitat types (heathland and forest) and management strategies encountered by the species across its Northern and Western European range. Humberhead Peatlands (Lat. 53.5444, Long. -0.9448) is formed of a lowland raised bog, now classified a degraded lowland raised mire. The site was comprised of two spatial distinct 'sub-sites'; Thorne and Hatfield moors located 10 km apart. Habitat composition across the two sub-sites comprises open water, lowland raised bog, as well as Birch (*Betula pendula*) stands and restricted areas of *Calluna* rich heathland, providing nesting habitat for breeding nightjar. Thetford Forest (Lat. 52.4209, Long. 0.6625) is a lowland plantation forest in Eastern England, composed of a mosaic of discrete blocks of different age conifer tree stands, with nightjar typically utilising blocks from the point of clear-felling and replanting up to 15 years of age. The site is internationally important for its breeding population of nightjar (Sharps et al., 2015). Fieldwork was carried out at Thetford Forest during each sampling year (2019, 2020, 2021). Conversely, fieldwork at Humberhead Peatlands was limited to 2019 and 2021 due to access restrictions imposed by the COVID-19 pandemic in 2020.

5.3.2 Field methods

5.3.2.1 Nightjar capture, biometrics and DNA sampling

Nightjar were captured using 30 x 30 mm mist nets, set within 100m of known male churring (song) posts. Call playback of churring nightjars was used to attract birds to mist nets for capture, with best practice guidelines as per Redfern and Clark (2001) followed (see also Section 5.3.3). At the start of the season effort was made to trap within 100m of each male

song post twice and search for nests in areas. However, this was not possible for all territories owing to poor habitat accessibility. British Trust for Ornithology (BTO) metal rings with individually unique ID codes, were applied to each bird captured (unless already ringed). Additionally for juvenile and adult birds, biometric measures (flattened chord wing length, weight and muscle scores as per Redfern and Clark (2001) were collected. For males, scaled photographs of wing and tail achromatic spots were taken for work presented in **Chapter 6**. Nuclear DNA was sampled from all captured individuals using buccal swabbing, which provided a quick and relatively non-invasive technique for collecting genetic material (Handel et al., 2006). Sterile 4 mm Rayon tipped plastic swabs were inserted into the mouth of the nightjar and rotated 3-5 times against the inside of the bird's cheeks and tongue. The swab was removed from the mouth and air dried for 1 – 2 minutes before being stored in a sterile container. Swabs were initially stored at -20°C in the field before being transferred to -80°C for long term storage.

5.3.2.2 Nest location

Nightjar nests were found through a combination of techniques across study sites, with all nest searching undertaken following best practice guidelines in Ferguson-Lees et al., (2011). First, territories were searched for by listening for male churring at dusk and returning in the day to search for nests within ~100m radius of male 'song posts' where an individual male was observed singing, with location of these territories used to inform trapping locations (Section 5.3.2.1). Second, over 3 visits between May and August each year cold searching was conducted for nests in habitats deemed suitable for nightjar and areas where nests had been found in previous years. Third, during routine capture (see Section 5.3.2.1) females were radio tracked to find nests. Females were captured in the locality of churring (territorial) males and fitted with tail mounted Biotrack AG392 VHF tags. Female nightjars were then tracked using a Yagi antenna the following day to ascertain nest site location. This also enabled maternal social associations to nests to be made (see Section 5.3.2.3). Nest searching effort could not be fully standardised across sites owing to differences in habitat accessibility and availability of volunteer fieldworkers.

5.3.2.3 Social parental association

Maternal and paternal associations of birds at nests were determined using one of two methods (I; VHF tracking, II; capture at nest site);

I) VHF tracking was used primarily to determine social maternity whilst simultaneously enabling for nest site location (see Section 5.3.2.2). Female nightjars that were VHF tagged during routine capture were tracked to their nest sites during the day post-capture. If tagged females did not have a nest one day post-capture, then females were re-located twice weekly throughout the season to locate nests.

II) Capture at nest sites was the primary method used to ascertain paternal associations and maternal associations where females had not been VHF tagged. Nest site capture was attempted once chicks were ≥ 10 days old. Mist nets were set in a triangle formation around the nest site in order to capture individuals provisioning chicks and thus establish social parentage. A distance of > 1 m was maintained between the nest site and any one net in order to minimise risk of captured birds in the net causing damage to chicks or the nest site, whilst reducing the risk of capturing unassociated individuals. Nets were set approximately 15 minutes before dusk. Nightjar exhibit biparental care meaning both the provisioning male and female may be caught using the technique. No playback was used at the nets to minimise the capture of unassociated individuals. Trapping continued until both putative parents were caught or after 60 minutes, after which trapping ceased in order to minimise disturbance. An interval of at least 48 hours was left before trapping again at a specific nest. If the identity of one parent was already known, then nets were removed once a bird of the opposite sex had been captured, i.e., the other candidate parent. If >1 individual of the same sex was caught on the same night at the same nest, both individuals were noted as being associated with the focal nest and where possible tail mounted Biotrack AG392 VHF tags were deployed on the birds, allowing for individuals to be tracked to the nest later to either confirm or refute association. Presence of prey bolus stored in an individual's mouth, usually associated with chick provisioning (Cleere, 1998; Holyoak, 2001), was also noted where VHF tagging could not be undertaken.

5.3.2.4 Nightjar breeding density

To assess the opportunity for extra-pair copulations, intra-specific brood parasitism and quasi parasitism, both nest and male density were calculated as follows; 1) Nest density was determined using global positioning system (GPS) coordinates (Garmin eTrex 10) for nests sites, found as per the techniques outlined in Section 5.3.2.2, with suitable areas surveyed up to three times within each season, leaving me with reasonable confidence that the majority of

nests within surveyed areas had been detected. However, nightjar are cryptic ground-nesting, nocturnal birds and nest finding can be difficult even when birds nest at higher densities (Shewring and Vafidis, 2021). Consequently, nest density may not completely convey breeding density or EPC opportunities. 2) Churring male locations were ascertained following the standardised methods outlined in Conway et al., (2007) over two visits per site. Visit dates were not standardised across sites owing to spatial variability in weather. Visits were conducted between early-June and late-July. Transects through suitable habitat were walked from 20 minutes after sunset, or 90 minutes before dawn, at a steady pace (4 – 5.5 kph), with stops taken every few minutes to listen for churring nightjars. To minimise duplicate recording of the same individual, following the recommendations outlined in Gilbert, Gibbons and Evans, (1998) if non simultaneous churring was recorded <30 seconds apart within 400m of each song post this was recorded as the same individual. If non-simultaneous churring was recorded >30 seconds apart and/or at a distance >400m between song posts then this was recorded as two separate individuals. Finally, numbers of transects per site varied with the availability of accessible terrain and suitable breeding habitat.

Table 5.1 Summary of nest outcomes and social associations of parents made at nests. Total number of nests found at each site over each season is shown as well as the number of failed broods. Excluded Nests refers to broods not included in parentage analysis owing to I) lack of maternal and paternal associations, or II) poor genotyping success for offspring and/or both parents. Nests with incomplete (M = Maternal association only, P = Paternal association only) and both maternal and paternal (M + P) associations are shown. The proportion (%) of the total nests found for each category is shown. Values highlighted in grey are those included in the parentage analysis.

Site	*Year*	*N Nests*	*Failed Nests*	*Excluded Nests*	*Nests With Parental Associations*			*Total Nests Included in Parentage Analysis*
					M	P	M+P	
Humberhead	2019	27	11 41%	3 11%	1 4%	0 -	12 44%	13 48%
	2021	23	8 48%	1 4%	6 26%	0 -	8 35%	14 61%
Thetford Forest	2019	24	7 28%	4 14%	5 20%	2 8%	6 24%	13 52%
	2020	17	2 12%	3 12%	4 24%	1 6%	7 41%	12 71%
	2021	16	2 12.5%	0	4 25%	2 12.5%	8 50%	14 88%
Total **%**		**107**	**30** **28%**	**11** **10%**	**20** **19%**	**5** **5%**	**41** **38%**	**66** **62%**

5.3.3 Ethical review

The research was granted full ethical approval by the University of York's animal and welfare ethical review body. DNA collection via buccal swabs and use of VHF tags were reviewed and approved by the British Trust for Ornithology (BTO) special methods technical panel, with accredited agents trained appropriately and possessing C or A class ringing permits from the BTO. Nest disturbance was minimised (see Section 5.3.2.2) where possible and best practice guidance when using song lures was followed by fieldworkers (as per Redfern and Clark, 2001).

5.3.4 Laboratory work and Primer design

5.3.4.1 DNA extraction and quantification

Nuclear DNA was extracted from buccal swabs using a modified ammonium acetate extraction method. Briefly, the method outlined by Burke and Bruford, (1987) and Richardson and Burke, (2001) was modified for buccal swabs by inclusion of the 5cm swab tip in the initial extraction stages, increasing the digestion stage to 24hrs and removing swabs prior to the initial centrifuge stage (see **Appendix** Section 8.2.1.1 for detailed account of extraction procedure). Post-extraction, samples were suspended in 40 µl of LowTE and stored at -20°C prior to quantification and genotyping. DNA quantification was performed on a subset of samples using a FLUOstar Optima Spectrophotometer (BMG Labtech; see **Appendix** Section 8.3.2 for full methods).

5.3.4.2 Primer design and microsatellite evaluation

As there were no published microsatellite markers for nightjar, I first prepared a library of markers (see **Appendix** Section 8.3.3 for full details). Briefly, six out of 21 microsatellite markers tested were retained for genotyping owing to the excluded markers failing to amplify (n =10) or being out of Hardy-Weinberg equilibrium (HWE) and displayed high (>20%) null allele frequencies (n = 4). Further, four cross-species markers were included from Dawson et al., (2010, 2013) for genotyping (see **Appendix**, Table 8.4). PCR conditions for the final marker set can be found in **Appendix** Section 8.3.3. All markers were amplified singly, owing to poor amplification success in duplex reactions. PCR products were then separated on an ABI 3730

DNA Analyser (ThermoFisher, USA) using ROX 500 (GeneScan™, USA) as size standard. Alleles were scored using GeneMapper software V3.7 (Applied biosystems). The software program CERVUS v3.0.3 (Tristan Marshal Field Genetics Ltd) was used to calculate the number of alleles per locus, the observed and expected heterozygosity, null allele frequencies, polymorphic information content (PIC) and to test assumptions of HWE, whilst linkage disequilibrium was tested in GENEPOP on the web (Rousset, 2008). The population allele frequencies summary statistics were carried out using a randomly selected pool of adults (n = 50) from across the two breeding populations included in this study.

5.3.4.3 Parentage analysis

A two-stage approach was taken to determine genetic parentage, by first manually comparing alleles between offspring and putative parents. Where offspring mismatched with social parents at ≥2 loci, the offspring were deemed to not have been sired by that putative parent. Second, associations were confirmed using CERVUS (Marshall et al., 1998). The program used a two-step likelihood-based approach, screening candidate parents from the population and ranked them by likelihood of being the genetic parent to chicks (Marshall et al., 1998). Taking into account population allele frequencies, CERVUS used allele frequencies generated from the pool of genotyped adults across the two populations to generate logarithm of odds (LOD) scores. LOD scores are a natural logarithm of the product across all loci of likelihood ratios comparing the potential parents against all other individuals included in the population at random. Individuals with the highest LOD scores represent the most likely parents of the offspring in question. LOD statistical significance was then determined as ΔLOD, the difference in LOD between top candidate parents (Marshall et al., 1998), with critical and relaxed Δ values set at 95% and 80% respectively (Marshall et al., 1998). Parents were assigned at an 80% confidence level, which is sufficient for determining genetic parentage even in cases where putative parents may be closely related (Slate et al., 2000). Parentage determination using CERVUS was run independently for each site and year. Per year per population all putative family units as well as 'unassociated' males and females (birds not assigned to a brood) captured on site were included in the analysis.

Maternity analysis was run first so that the genetic mother identity could be included in the CERVUS paternity analysis to increase power when determining genetic paternity. Sixty-one broods for which social mothers could be assigned were included in the maternity analysis (Table 5.1). Simulations of maternity were run in CERVUS. For all site/year combinations a

typing error rate of 1% was assumed and 90% of potential mothers were sampled within each population/year. Social mothers were selected as genetic mothers where females had a confidence level of ≥80% and associations were congruent with those determined manually. In five cases the candidate social mother failed to meet the 80% LOD confidence threshold. In these cases, both female and offspring extracts were genotyped again to reduce the chance of typing errors leading to false rejection of maternity. Offspring maternity was then reanalysed on CERVUS and assessed by eye. If the social mother genotype matched offspring genotype at ≥7 loci she was accepted as the genetic mother of the offspring.

A total of 46 broods for which social fathers could be assigned were included in the paternity analysis (Table 5.1). Simulations were run for paternity analysis following the criteria used in the maternity simulations, but a reduced proportion of potential fathers were sampled (70%). Where genetic maternity was determined, Mother ID was included as the 'Known Parent' in the paternity analyses (n = 73 offspring). Genetic paternity was assigned at a confidence level of >80% either at pair LOD scores, where female ID had not been included in analysis, or at trio LOD scores where a maternal ID had been included. In four cases the social father was determined as the most likely candidate but failed to meet the 80% confidence threshold at either pair or trio LOD scores. In these cases the raw genotype scores were reassessed on Genemapper and any required corrections (e.g. re-scoring where required) made before being visually reassessed against putative offspring. In all four cases putative social fathers were accepted as the genetic father if they mismatched at < 2 loci. Where putative social fathers were rejected as the most likely candidate parent by CERVUS, original extractions from both the father and offspring were re-run and genotyped again to reduce the chance of typing errors leading to false rejection of paternity.

5.3.4.4 Relatedness analysis

Where multiple individuals of the same sex were recorded provisioning offspring at a nest site (n = 6 cases) relatedness measures were generated to determine relationships between the genetic parents and the extra-pair helper. Relatedness analysis was carried out in ML-Relate. The program estimates kinship categories (U = unrelated, HS = half-sib, FS = full-sib, PO = parent offspring) based on a maximum likelihood approach (Kalinowski, Wagner and Taper, 2006). Where FS, HS or PO categories were suggested, initial ringing dates and individual ages were first checked. If an individual's ringing date or age meant that the suggested relationship was impossible the relationship was rejected. Where suggested relationships were deemed

possible, the programs pairwise hypothesis test function was used, testing the hypothesis of the suggested category against the null of the individuals being unrelated at a 95% confidence level with 10,000 permutations. When the P-value for the test was < 0.05 the null hypothesis of the two individuals being unrelated was rejected (Kalinowski et al. 2006).

5.3.5 Data handling and analysis

5.3.5.1 Calculation of density metrics

Density metrics were calculated in QGIS (V3.16). The number of nests (NoN) and number of churring males (NoCM) within known home range size for nesting nightjar (115ha; Mitchell, 2019) of focal broods were determined using the polygon count tool using a radii of 605m, with 605m being the radii derived from the 115ha home range size in Mitchell (2019). In the case of NoN, multiple broods belonging to the same focal female were removed from this analysis. Distances from the focal broods nearest nest (NN) and churring male (NCM) were calculated using distance matrix tool. When calculating NCM the distance to the second nearest churring male was used in all instances to avoid inclusion of the focal male. In cases where the NN was a brood belonging to the same female, the second nearest nest was used to calculate NN distance.

5.3.5.2 Statistical analysis

Sixty-six broods were included in the parentage analysis in total. Sixty-one broods were included in the maternity analysis and 46 broods in paternity analysis (Table 5.1). The broods included in the paternity analysis were used in the downstream analysis detailed below. A Fisher's exact test was used to determine whether nightjar mating system departed from genetic monogamy, testing the null hypothesis that nightjar would exhibit complete genetic monogamy, with all offspring related to the assigned 'social parents'. To determine whether the proportion of extra-pair offspring (EPO) differed significantly between sites and years, a Chi-squared test for independence was used, treating each site/year combination as independent.

Finally, to determine whether nightjar breeding density influenced the number of EPO in broods, multiple generalised linear models (GLMs), with a binomial error distribution and logit

link function were used. EPO presence/absence within each brood acted as the binary response variable. Parameters shown in Table 5.2 were included in each model. Because nest and male density was not consistent between sites and years (Table 5.3), both site and year were included as interaction terms with density metrics in both models. Multicollinearity of predictor variables was determined by visually assessing correlation matrices including all numeric predictors. NoN and NN (Pearson's R = -0.54, P < 0.001) and NoCM and NCM (Pearson's R = -0.5, P < 0.001) were highly correlated with each other. However, neither NoCM and NoN and NN and NCM were found to significantly correlate with one another respectively (in both cases Pearson's R = <0.25, P > 0.05). As male density and nest density reflect different demographic factors which may influence EPP (i.e., availability of paired males and females vs availability of males irrespective or paired-status), two models including NoN and NoCM (Model 1) and NN and NCM (Model 2) were run to determine the effect of breeding density on EPP in nightjar (see Table 5.2 for model configurations). Stepwise selection for AIC was conducted for both models, with the final best-fitting models selected and presented. Mixed effects models were originally used for the analysis, with female ID included as a random effect to account for multiple broods by the same female over all study years (N = 18) in the dataset. However, in all cases mixed models converged to a singularity suggesting that female ID did not account for the large proportion of the variation in EPP within the dataset. For all models female ID was then excluded as a random effect and multiple GLMs used. All statistical analyses were carried out in R Studio (V 3.5.0; 5) using program R (V 4.0.1; 6) (R Core Team, 2020).

Table 5.2 Definitions of parameters included in each model. (*) indicates that the factors are also included as an interaction term with density metrics in each model.

Parameter	*Model*	*Definition*
Site (*)	1, 2	ID of female associated with nest to control for second nest included in analysis.
Year (*)	1, 2	Sampling year
NoCM	1	Number of churring males within a 605m radius of the focal nest
NoN	1	Number of nests within a 605m radius of the focal nest.
NCM	2	Distance to the nearest churring male from the focal nest (m).
NN	2	Distance to the nearest nest from the focal nest (m).

5.4 Results

5.4.1 Nightjar genetic mating system, EPP rates and relatedness

Congruence between social and genetic maternity was confirmed at all broods (N = 61) included in the maternity analysis, with no incidences of IBP or QP detected. Genetic paternity was unambiguously assigned to the putative social fathers at 34 out of the 46 broods included in the analysis. For the remaining 12 broods, social fathers could not be confirmed as the genetic father for one or both offspring. Genetic fathers mismatched between siblings within broods in only 2 of the 12 broods that had EPO. In all other cases (N = 10) both chicks mismatched from the social male. Across all sites and years, nightjar did not exhibit genetic monogamy (genetic monogamy: EPO = 0: 12, Fisher's exact test, $P < 0.001$), with 23% of offspring being EPO and 26% of nests containing EPO. EPP was recorded across all sites and sampling years, although the percentage of EPO differed significantly between sites, where sampling years were treated independently (Chi-squared = 20.491, df = 5, $P < 0.001$; Fig 5.1). The percentage of EPO (40%) and EPB (50%) was highest within Humberhead in 2021 and lowest at both Humberhead and Thetford in 2019 (Fig 5.1).

Finally, out of the five cases where two males were captured at nest site provisioning chicks, none of helper individuals were found to be related to either the genetic or social father or mother (ML-Relate relationship category = U in all cases).

5.4.2 Nightjar breeding density and presenceabsence of EPO

Nightjar nest and male density varied between sites and sampling years (Table 5.3). Nest and male density were higher in Thetford Forest than Humberhead Peatlands, across all metrics other than NN (Table 5.3), with density across most metrics (excluding NN) being highest in Thetford in 2021 (Table 5.3). With the exception of Humberhead in 2021, the NoCM were consistently higher than NoN within a 605m radius of focal broods across sites and years (Table 5.3). Consistent with this finding, churring males were also located closer to focal nests than the nearest (known) nests (Table 5.3).

Table 5.3 Average breeding density surrounding focal nests at both sites between 2019 and 2021 for all nests used in paternity analysis (n = 46 nests). NoCM = Number of churring males and NoN = number of nests within 605m of the focal nest, NN = nearest nest to the focal nest, NCM = nearest churring male.

Site	*Year*	*Median NoCM (range)*	*Median NoN (range)*	*Mean NN (stdev) (m)*	*Mean NCM (stdev) (m)*
Humberhead Peatlands	2019	3 (1 – 7)	2 (0 – 3)	464 (614)	338 (115)
	2021	3 (2 – 4)	5 (0 – 6)	543 (757)	284 (83)
Thetford Forest	2019	3 (2 – 5)	1 (1- 5)	271 (212)	135 (57)
	2020	3 (1 – 5)	2 (1 – 3)	330 (338)	187 (98)
	2021	5 (1 – 6)	2 (0 – 5)	276 (320)	151 (97)

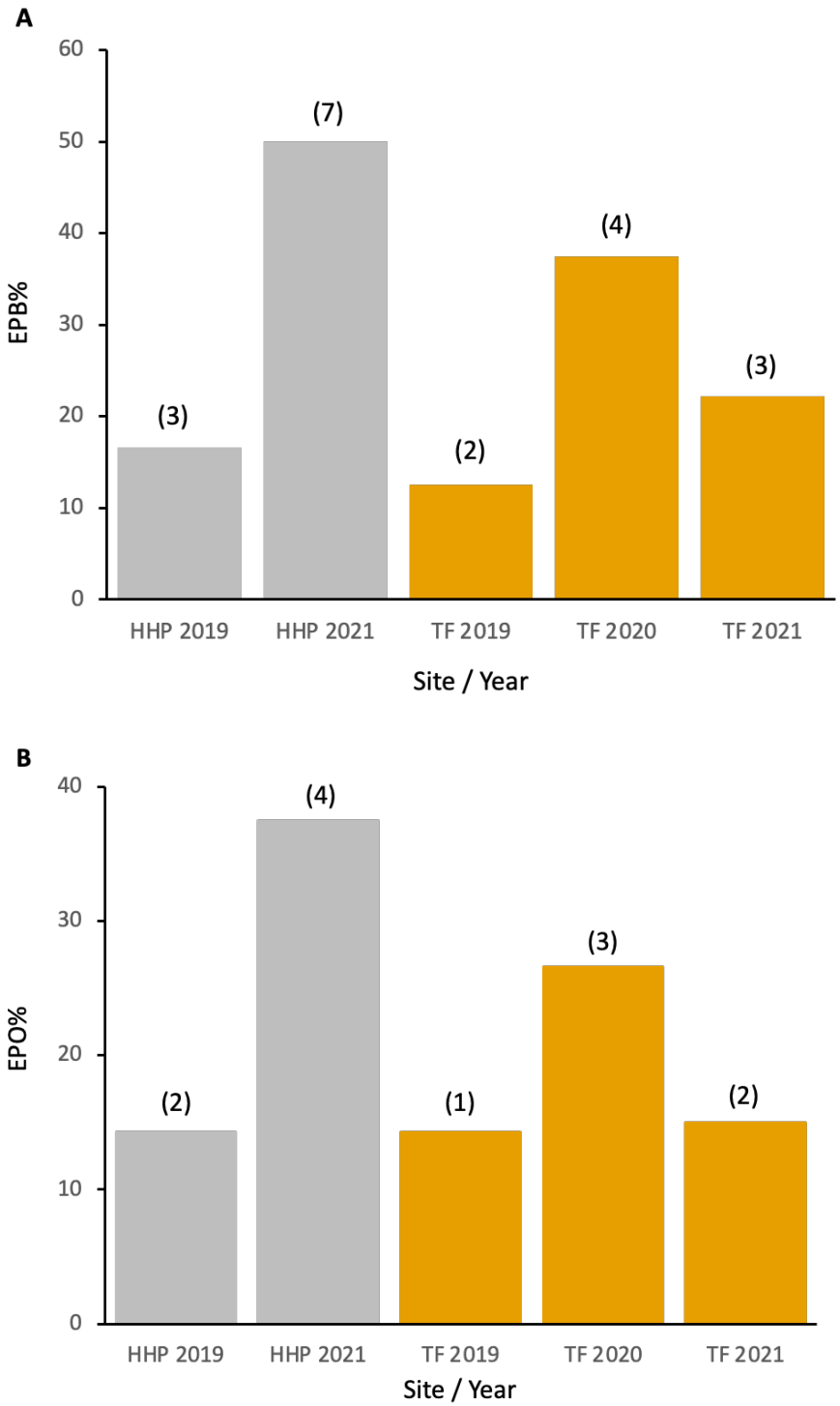


Figure 5.1 Percentage of extra-pair broods (EPB) (A) and extra-pair offspring (EPO) (B) sired within each site and sampling year. Humberhead Peatlands (HHP) = grey, Thetford Forest (TF) = Orange. Numbers of broods (A) and offspring (B) are given in brackets.

After stepwise selection for AIC was applied to Model 1, NoN (the number of nests within average home range size of focal nests) was removed from the final model (Table 5.4). The number of churring males within average home range size of focal nests (NoCM) alone was not found to be a significant predictor of EPP. Despite EPP varying between the two study sites (Fig 5.1), study site alone was also not a significant predictor of EPO presence. However, the sampling year of 2021 alone had a near significant (P = 0.054) effect on EPO presence in nightjar broods (Fig 5.1). The number of EPB was highest in 2021 at both sites, with breeding density also being highest at both sites across the majority of metrics in 2021 (Table 5.3).

The interaction between NoCM and year (2021) was a significant predictor of EPO presence within nightjar broods (Table 5.4), with the difference in NoCM between nests with and without EPO most pronounced in 2021 (Fig 5.2A). Notably, a similar, although marginally not significant (P = 0.052)., trend also occurred in 2020, with NoCM higher for nests with EPO than without (Table 5.4; Fig 5.2A). However, in 2019, little difference between average NoCM between nests with and without EPO was found in either study population, showing the effect of NoCM on EPO presence was not consistent across years. Conversely, no significant effect of the interaction between NoN and EPO presence was found across sites (Table 5.4), with the frequency of EPO presence only marginally higher at elevated NoN densities in 2019 and 2020 (Fig 5.3A).

The interaction of NoN with site was also found to be an important, marginally significant (P = 0.052), predictor of EPO presence/absence (Table 5.4), with an increased NoN around focal nests likely leading to a higher likelihood of EPO presence in Humberhead, but not Thetford. Indeed, nest density (NoN) was marginally higher at Humberhead (averaged across all years = 3; Table 5.3) than Thetford (averaged across all years = 2; Table 5.3) (see Fig 5.3B). EPO also consistently occurred at a higher frequency in nests surrounded by a higher NoCM at both Humberhead and Thetford (Fig 5.3B), although the interaction between site and NoCM was not found to be a significant predictor or EPO presence (Table 5.4). Finally, none of the predictors or interaction terms included in Model 2 (NN, NCM, Year, Site) were found to significantly predict the presence of EPO in nightjar nests (**Appendix** Table 8.5), suggesting that the distance to the nearest male or nest has little impact on EPP in nightjar.

Table 5.4 Results of multiple GLM Model 1, examining ability of the number of churring males (NoCM) and nests (NoN), site and year to predict EPO presence/absence within broods. Site and year are included as interaction terms with NoCM, and site alone as an interaction term with NoN after backwards and forwards stepwise selection for AIC. Significant P-values (<0.05) of the Wald test are highlighted in **bold**.

Predictors	*Estimates*	*CI*	*z*	*P*
(Intercept)	- 1.456	-7.41 – 2.45	-0.633	0.526
NoCM	-0.549	-2.37 – 0.71	-0.761	0.446
Site (Thetford)	5.235	-4.28 – 16.18	1.096	0.272
Year (2020)	-7.965	-20.12 – 1.92	-1.548	0.121
Year (2021)	-22.339	-51.87 – -5.88	-1.927	0.054
NoN	0.743	-0.20 – 2.16	1.323	0.176
NoCM * Site (Thetford)	0.177	-2.96 – 2.80	0.133	0.894
NoCM * Year (2020)	3.922	0.67 – 9.34	1.951	0.051
NoCM * Year (2021)	7.141	2.27 – 16.08	2.079	**0.037**
NoN * Site (Thetford)	-5.145	-11.00 – - 1.24	-1.936	0.052
Observations	46			
Tjur R^2	0.481			
AIC	48.958			

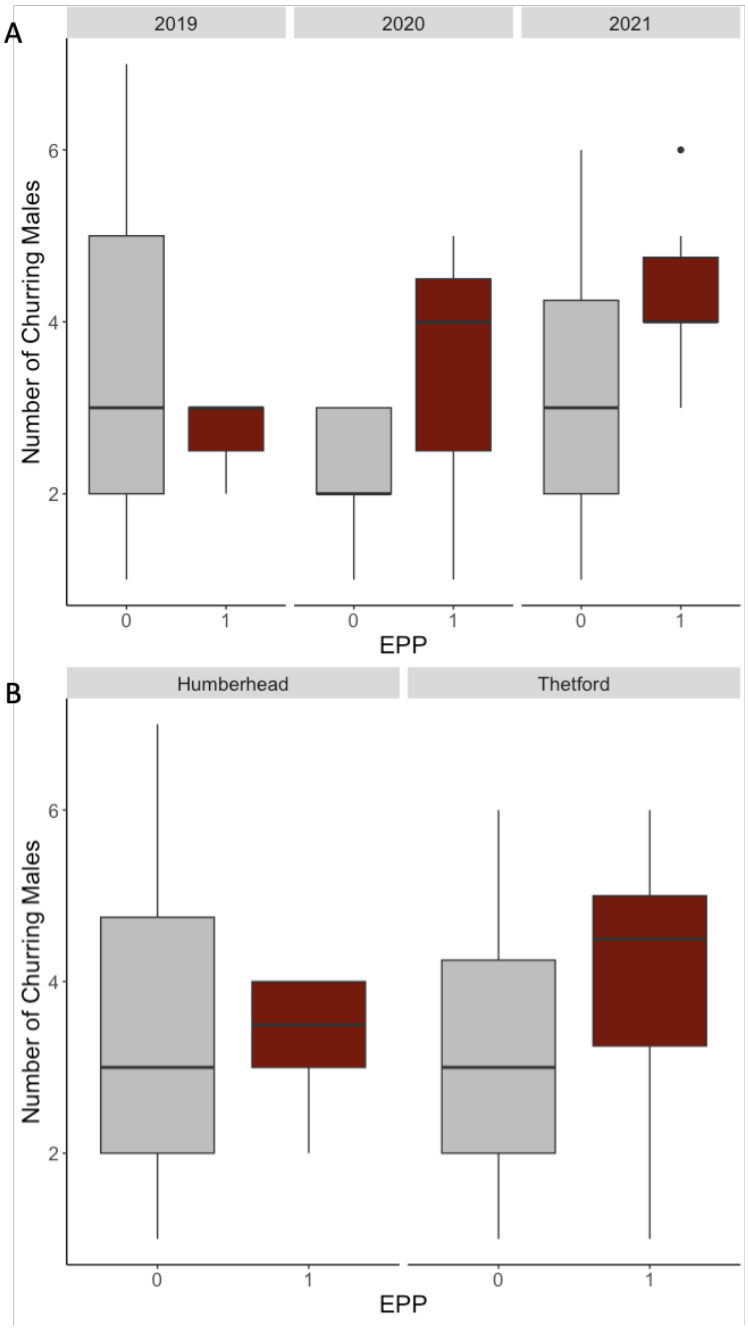


Figure 5.2 Boxplot showing variation in the number of churring males (NoCM) within the average home range (605m radius) of focal nightjar nests, between broods with (1; red) and without (0; grey) EPO. A) presents results separately for each site and B) for each sampled year. First and third quartiles are displayed as the extent of the rectangle in boxplots, with the median given as a central line, whiskers represent spread of values 1.5x greater than the 75th percentile and less than the 25th percentiles respectively.

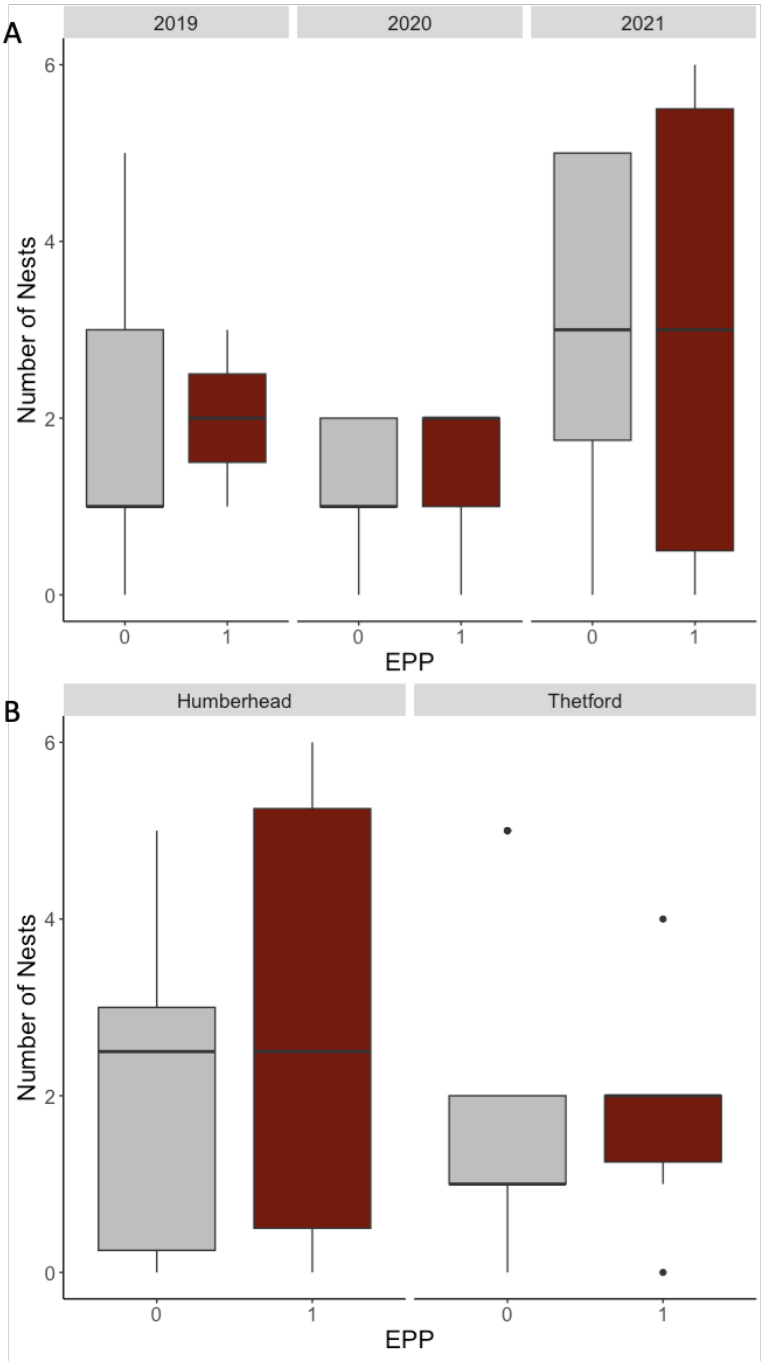


Figure 5.3 Boxplot showing variation in the number of nests (NoN) within the average home range (605m radius) of focal nightjar nests, between broods with (1; red) and without (0; grey) EPO. A) presents results separately for each site and B) for each sampled year. First and third quartiles are displayed as the extent of the rectangle in boxplots, with the median given as a central line, whiskers represent spread of values 1.5x greater than the 75th percentile and less than the 25th percentiles respectively.

5.5 Discussion

Nightjar departed from genetic monogamy in my study populations, showing moderate levels of genetic polyandry, with 26% of broods containing EPO and 23% of offspring being EPO. In the majority of broods where EPO was recorded, genetic paternity was not shared between the extra-pair and the social male observed at the nest. In these cases, the cuckolding male sired all offspring within the brood. In only two cases was genetic paternity shared within broods. EPP rates varied between sites and years, although temporal variation in the annual occurrence of EPP were consistent at both sites. Nightjar nest and churring male density also varied between sites and years, although not consistently among the different breeding density metrics. The effect of breeding density on EPP was also not consistent across years, and no density metric alone predicted EPO presence/absence in nightjar broods. However, within seasons the number of churring males had a significant effect on EPO presence/absence within nests. Nests with EPO occurred at a higher local density of churring males in 2020 and 2021, although this was statistically significant in 2021 only. Comparing the two sites, EPO occurred more frequently in nests at a higher local nest density at Thetford than at Humberhead. Finally, neither distance to the nearest churring male nor distance to the nearest nest was important in determining the presence of EPO in nests in my study.

The paternity data from my study suggests that nightjar exhibited a relatively high level of EPP compared with other long-lived biparental care-dependent species (e.g. seabirds and raptors; Valcu et al., 2021). A large proportion of seabirds and raptors display no or very low levels of EPP. e.g. Red-footed falcon, *Falco vespertinus* (2.6% EPO; Magonyi et al., 2021), 5% in Swainson's Hawk (*Buteo swainsoni*; Briggs and Collopy, 2012), and Little owl *Athena noctua* (0%; Müller et al., 2001) (see Quillfeldt et al., 2012 for a review of EPP in seabirds). However, exceptions exist (e.g., 19.3% EPO in Cooper's hawk *Accipiter cooperii*; Rosenfield et al., 2015; 20 % EPO in Black-headed gulls *Larus ridibunus*; and 21% EPO in Thin-billed prions *Pachyptila belcheri*; Quillfeldt et al., 2012), which are comparable to the proportions of EPO in my study. Comparisons with phylogenetically similar species are challenging, as no EPP data has yet been published for any species of Caprimulgiform. However, comparisons within Strisores highlight low levels of EPP in Common swifts (*Apus apus*) at 4.5% EPO (Martins et al. 2002), this species is also long-lived, relies on biparental care and exhibits long-term stable pair bonds (Carere, 1998). Conversely, in my study, where multiple broods belonging to the same female were sampled over subsequent years (N = 5 individuals; unpublished data) mate switching was observed in 4 of the 5 cases (80%). Thus, nightjar demonstrated a higher EPP and divorce rate compared with Common swifts or other species with comparable life histories (e.g. seabirds;

Quillfeldt et al., 2012; Mercier et al., 2021). Nightjar then appears to present an interesting case when considering their phylogeny and life history. High EPP rates in both populations may at least partially result from the British nightjar population at the species leading range edge (Cleere, Christie and Rasmussen, 2021), with higher EPP rates found in range edge populations in other species (e.g. Eckert, Samis and Lougheed, 2008; Corregidor-Castro et al., 2022). However, EPP characterisation at sites across the species range would be needed to test this effect.

The reasons why females might elicit EPCs has been an area of intense study and debate over the past two decades (see Griffith, Owens and Thuman, 2002; Brouwer and Griffith, 2019 for reviews). Females may partake in EPCs to obtain indirect genetic benefits from the extra-pair male. These include inbreeding avoidance (Foerster et al., 2003), producing offspring with better quality genes (i.e. good genes; Birkhead and Møller, 1995) or high-quality ornaments (i.e. sexy sons; Weatherhead and Robertson, 1979). The latter benefits usually involve females mating with better quality, well-ornamented males, and this is investigated further in **Chapter 6**. Females may derive direct benefits of EPP in the form of provisioning by extra-pair males (Tryjanowski and Hromada, 2005), which may help mediate the negative consequences of reduced paternal care following EPC. Notably, two males were captured at nest sites and suspected of provisioning chicks in six of the broods included in the paternity analysis (13%), a behaviour observed at other breeding populations in the UK (Padget et al., 2019). However, EPP was only recorded in one of the six broods, with neither of the males captured at the nest found to be the genetic father, and in no cases was paternity shared between the two males. In traditional cooperative breeding systems, helpers may often be related to the socially paired birds (i.e. offspring, kin etc., Nam et al., 2010; Barati et al., 2018; Wang et al., 2022) and derive indirect fitness gains from aiding in brood provisioning (Nam et al., 2010; Barati et al., 2018; Wang et al., 2022). However, in no cases were the extra-pair provisioning males found to be related to either the socially paired male, female or the extra-pair sires. Instead, cooperative provisioning in nightjar may be a response by extra-pair males to suspected paternity, regardless of whether males have successfully secured genetic paternity (Davies et al., 1992; Davies and Hatchwell, 1992). However, subordination and provisioning by floater males may also occur to avoid exclusion from breeding opportunities where territorial males are aggressive, as in nightjar (Moreno, 2016), or may undertake extra-pair provisioning to gain experience for future breeding attempts (Selander, 1965; Korndeur, 1996; but see Chesterton et al., 2023). Again, I am limited by my behaviour data in drawing conclusions surrounding cooperative provisioning in nightjar. The short time window imposed by the ethical constraints of nest site capturing likely leads to an underrepresentation of such cooperative behaviour in

my data. Therefore, future work would benefit from more extensive observational data documenting EPCs and individual provisioning of chicks in order to investigate this behaviour further.

High breeding densities have been found to be associated with higher rates of EPP; as it provides increased opportunities for EPCs (Griffith, Owens and Thuman, 2002; Mayer and Pasinelli, 2013). In my study, both breeding density and EPP rates varied between sites and sampling years. Corresponding with the temporal variation in breeding density, the effect of density on EPP also differed across sampling years. EPP occurred more frequently in nests at a higher local male density (NoCM) in 2021, when breeding density was highest at both sites. A near significant effect was also found in 2020, where overall annual density was the second-highest recorded. However, this effect in 2020 may have been caused by sampling being restricted to a single site (Thetford Forest) in 2020. Nevertheless, the disparity in the effects of male compared with nest density on EPP in my study may reflect a higher proportion of unpaired or floater males siring EPO than paired males (e.g. Tomotani et al., 2017), the availability of which would have been reflected by nest density. My results tentatively suggest that local breeding density had little effect on EPP in nightjar unless a high density or threshold was reached. The concept of a density threshold has been previously proposed, whereby the effects of density on EPP are negligible once density either exceeds or falls below a species or population-specific threshold (Bouwman and Komdeur, 2006; Mayer and Pasinelli, 2013). Where density exceeds this threshold, the detectable effect on EPP is lessened owing to there being a sufficient availability of extra-pair partners in the population (Bouwman and Komdeur, 2006). Conversely, if a minimum density threshold is not met within a population, potential extra-pair males will not be encountered by females regardless of the local densities recorded (Orell et al., 1997). If a density threshold is important in determining EPC opportunities in nightjar, my results suggest that a minimum threshold may need to be met before the effects of density on EPP are significant. When comparing my findings to the wealth of similar studies testing the density hypothesis, it should be noted that most studies have focused on passerines (e.g. Krokene and Lifjeld, 2000; Richardson and Burke, 2001; Charmantier and Blondel, 2003; Mayer and Pasinelli, 2013; Brouwer et al., 2017). Passerines are typically less vagile and operate at smaller spatial scales than nightjar. Indeed, analysis of non-passerines and more vagile taxa have yielded little effects of density on EPP (e.g. White stork *Ciconia ciconia* Turjeman et al. 2016; Great cormorant *Phalacarocorax carbo sinensis*; Minias et al., 2016; Little owl; Müller et al., 2001). The lack of a clear overall effect of density in my study may then be attributed to the highly vagile nature of nightjar, with birds capable of routinely commuting over large distances (> 5km in a night; Mitchell, 2019), reducing the immediate

constraints of local density on EPC opportunities. Indeed, the lack of an effect of the nearest nest or male distance on EPP in my study appears to corroborate this, suggesting that EPP in nightjar is not dedicated by the accessibility of the nearest extra-pair sire.

Finally, owing to the nocturnal nature of nightjar, behavioural data on EPCs could not be collected in my study. Therefore I have a limited understanding of whether females travelled outside of territories to elicit covert EPCs thus avoiding the negative reduced paternal care consequences (Birkhead and Møller, 1995; Tryjanowski, Antczak and Hromada, 2007), or whether EPCs occurred with intruder males at breeding territories. Understanding the spatial distribution of EPCs is important if the effects of breeding density on EPP are to be understood, with breeding density ultimately mediating the opportunities for EPCs (Mayer and Pasinelli, 2013). If females readily travel long distances to elicit EPCs the effects of local breeding density at the scales measured may be negligible. Indeed, previous studies have shown that both male and female nightjar can travel > 5km from focal nest sites when foraging (Evens et al., 2017b; Mitchell, 2019), meaning EPC opportunities may not be restricted to the radius used in this study. Further, increased mate guarding with elevated breeding density (Komdeur, 2001) can confound the effect of density on EPP (Komdeur, 2001; Kokko and Rankin, 2006). The aggressive behaviour of European nightjar males towards same-sex conspecifics (Cramp and Simmons, 1985), suggests males may respond to elevated population densities with increased mate guarding. This may reduce the positive effect of density on EPP and could go towards explaining the overall weak effect of density on EPP recorded in my study. Again, a better understanding of nightjar behaviour is required to determine the directionality of the effect of breeding density on EPP. Whilst much effort has been put into mapping nightjar habitat use and foraging behaviour, there has been little attention given to understanding other aspects of nightjar behaviour (e.g. Evens et al., 2017; Mitchell et al., 2020; Sierro and Erhardt, 2019; but see Padget et al., 2019; Eisenring et al., 2022). Consequently, large gaps remain in our understanding of nightjar ecology and breeding behaviour in particular, copulations, provisioning, and extra-territorial forays.

5.6 Conclusion

My study presents the first account of the genetic mating system and EPP rates of any species of Caprimulgiform. I have shown nightjar exhibit social monogamy with moderate EPP rates (average % of nests with EPO = 26%, average EPO % = 23%) compared with avian taxa (Average % of nests with EPO = 33%, average EPO % = 19%, n = 434 species; Brouwer and Griffith, 2019),

and high rates compared with non- and near-passerine with similar life histories (average % of nests with EPO = 5%, average EPO % = 9%, n = 145 species; Brouwer and Griffith, 2019). However, whether the EPP rates found in this study are representative across the range of nightjar is not known. Characterisation of EPP rates at other populations, particularly at those closer to the species' range centre, is needed to clarify this. Nevertheless, EPP in nightjar may drive cooperative provisioning behaviour by extra-pair males, with six cases recorded in this study, although in no cases were the extra-pair provisioning males found to father the provisioned offspring. However, whether the extra-pair males copulated with the female but failed to achieve paternity in these instances could not be ruled out, and further investigation here is needed. My results tentatively provide support for the density hypothesis. However, my work highlights the importance of including multiple metrics when investigating the density hypothesis, with only the number of males within the average home range of nests found to significantly predict EPP in years where male density was highest. The disparity of the effects on EPP between male and nest density in my study could suggest that EPP was secured primarily by unpaired or floater males in the population. However, further work to determine difference in male quality and age between within- and extra-pair males would be needed to investigate this further (see **Chapter 6**). Finally, my research provides a novel insight into EPP rates in Caprimulgiformes, adding to limited literature concerning EPP in nocturnal birds. Further research in similarly nocturnal and cryptic taxa would help provide a more complete understanding of EPP evolution in Aves with differing ecologies and life histories.

Chapter 6 Are dichromatic white spots sexually selected in European nightjar *Caprimulgus europaeus*?

6.1 Abstract

Sexually dimorphic ornaments can communicate information pertaining to individual quality. Commonly, these ornaments are subject to sexual selection via mate choice and intra-sexual competition. Visual communication and sexual selection in nocturnal taxa have been historically understudied. However, the value of achromatic ornaments and the role of visual communication in nocturnal species are becoming apparent. Male European nightjar (*Caprimulgus europaeus*), henceforth nightjar, exhibit sexually dichromatic white spots on the outer primaries and rectrices. Here, I investigate whether variations in size and asymmetry of the white spots signal individual quality by regressing spot characteristics against quality metrics (age, size, and muscle development) in male nightjars from two breeding sites in the UK. I analysed paternity (both within-pair and extra-pair genetic mating success) against relative spot size and asymmetry measures to determine the role of white spots in mate choice in nightjars. My results indicate that spot size increases with age in male nightjars, suggesting that the size of white spots signals for age and longevity, which are proxies for individual quality. However, no other quality metrics were found to predict spot size. Notably, spot size also differed independently of structural size between the two breeding sites, which may reflect spatial variation in male quality. Neither spot size nor asymmetry predicted whether male nightjars were cuckolded. However, paired males exhibited 16% larger spots than extra-pair males, suggesting that extra-pair fathers may be lower quality floater males. My research highlights the importance of achromatic ornaments in visual communication and mate choice in nocturnal taxa. Nightjars, and all Caprimulgidae, present valuable study systems for the study of crepuscular and nocturnal visual communication.

6.2 Introduction

Sexual selection (e.g., inter-sexual mate choice and intra-sexual competition) is the primary driver of ornament evolution, with ornaments often dimorphic and displayed by the selected upon sex (usually the male). Ornaments can signal individual quality, with the production of plumage pigments (e.g., carotenoid and structural pigmentation) often costly to produce for the sender (Svensson and Wong, 2011; Fromhage and Henshaw, 2022) and leading to increased conspicuity and predation risk (Andersson, 1986). White plumage typically incurs little cost to produce compared with carotenoid and structural pigments (Török, Hegyi and Garamszegi, 2003). However, white plumage has been associated with elevated parasite load (Berglund, Bisazza and Pilastro, 1996; Qvarnström and Forsgren, 1998; Soravia, Aguado-

Giménez and Avilés, 2020) and increased conspicuousness, which is particularly costly for nocturnal taxa and species reliant on crypsis for predator avoidance (Andersson, 1986; Aragonés, Reyna and Recuerda, 1999; Penteriani et al., 2006). Further, white feathers are typically subject to exaggerated wear owing to the lack of strengthening by melanin pigments (Kose and Møller, 1999). White plumage then incurs high maintenance costs for the sender, providing a secondary mechanism for honest quality signalling (Berglund, Bisazza and Pilastro, 1996; Qvarnström and Forsgren, 1998; but see; Hill and Brawner, 1998; Badyaev and Hill, 2000). Variations in signal characteristics (size, brightness, asymmetry in bilateral ornaments) can then convey differences in sender quality. For example, bilateral white tail spots of Barn swallows (*Hirundo rustica*) convey quality information, with larger spots reflective of better individual body condition (Saino et al., 2015). Likely subject to sexual selection, spot size and asymmetry in Swallows has been shown to predict lifetime reproductive success and within-season paternity respectively (Møller, 1992; Costanzo et al., 2017; but see Safran and McGraw, 2004). In *Ficedula* flycatchers (*F. hypoleuca* and *F. albicollis*), white wing and forehead patch size and 'brightness' have been shown to be sexually selected features, honestly reflecting underlying individual condition, genetic viability, and mediating intrasexual competition and mate choice (Pärt and Qvarnström, 1997; Siitari and Huhta, 2002; Török, Hegyi and Garamszegi, 2003).

Ambient light levels can impact the effectiveness of visual communication (Denoël and Doellen, 2010; Penteriani and Delgado, 2017). Avian nocturnal visual communication had long been overlooked by researchers, instead favouring a focus on vocalisations as the primary signalling method (Penteriani and Delgado, 2017). Nevertheless, it is now apparent that many nocturnal species also rely on visual communication (Aragones, Reyna and Recuerda, 1999; Jiguet, Arroyo and Bretagnolle, 2000). Visual signalling via achromatic plumage (e.g., white patches) is one form of visual signalling which may be effective under low light conditions (Soravia, Aguado-Giménez and Avilés, 2020), and conspicuous white patches are prevalent in crepuscular and nocturnal avifauna and thought to be important in display behaviours (e.g., Eurasian eagle owl *Bubo bubo*; Penteriani et al., 2006, Houbara bustard *Chlamydotis undulata*; Alonso, Abril-Colón and Palacín, 2021). More broadly, the role of achromatic ornaments in sexual selection has been largely restricted to diurnal taxa (e.g. *Ficedula* flycatchers; Pärt and Qvarnström, 1997; Siitari and Huhta, 2002; Török, Hegyi and Garamszegi, 2003). Whilst effort has been made to address the nocturnal knowledge gap surrounding avian visual communication, (e.g. Aragones, Reyna and Recuerda, 1999; Jiguet et al., 2000; reviewed by Penteriani and Delgado, 2017), the field remains under-investigated.

Sexual selection functions through within-sex variation in mating opportunities. The selected upon sex in species with highly skewed mating opportunities, such as those exhibiting polygynous mating systems, are under increased sexual selection pressure and often possess some of the most elaborate ornaments in the natural world (e.g., Indian peafowl; *Pavo cristatus*; Kirkpatrick et al., 1997). However, paradoxically, monogamous species also often exhibit sexually dimorphic ornamentation (Webster et al., 2007). Under strict genetic monogamy, variation in reproductive success should be low among breeding age individuals, and the potential for sexual selection limited. However, with ~75% of bird species characterised as socially monogamous, extra-pair copulations (EPCs) enable increased variance in reproductive success and opportunity for sexual selection (Arct, Drobniak and Cichoń, 2015; Reitsma et al., 2018). Females in multiple species have been shown to select extra-pair males displaying more exaggerated ornamentation when soliciting EPCs (Wells et al., 2015). The reasons underlying why females solicit EPCs is one of great debate (Hsu et al., 2015; Forstmeier et al., 2014), with leading hypotheses suggesting females may gain indirect fitness benefits of increased offspring survival and fitness (good genes; Hamilton and Zuk, 1982), or from offspring inheriting the attractive paternal phenotypic traits (sexy sons; Fisher, 1930), or direct benefits such as increased provisioning by the extra-pair male (Tryjanowski and Hromada, 2005). In all cases exaggerated ornaments may signal for higher sender quality, with older, larger males typically exhibiting more exuberant ornamentation (Andersson, 1994; Harris et al., 2018).

The European nightjar (*Caprimulgus europaeus*), henceforth nightjar, is a migratory nocturnal ground-nesting species, which rely heavily on cryptic plumage to avoid diurnal predation (Cramp and Simmons, 1985). The species is characterised as socially monogamous, having been found to exhibit moderate levels of extra-pair paternity (EPP; See **Chapter 5**). Nightjars display sexually dichromatic white spots on the outer primaries and rectrices, a common Caprimulgidae feature (Holyoak, 2001). Size of achromatic spots in caprimulgids are thought to reflect variations in individual quality, with larger spots associating with age (larger in older birds) and body condition in Red-necked nightjar (*Caprimulgus ruficollis*; Forero, Tella and García, 1995; Aragones, Reyna and Recuerda, 1999) and Common nighthawk (*Chordeiles minor*; Roth, Argyros and Browning, 2003). It is suspected that these features are sexually selected, being larger in males than females in both *C. ruficollis* and *C. minor* and absent in female *C. europaeus* (Aragones, Reyna and Recuerda, 1999; Holyoak, 2001; Roth, Argyros and Browning, 2003). However, as yet no attempt has been made to investigate the role of white spot characteristics in determining reproductive success variation in Caprimulgidae.

6.2.1 Aims and hypotheses

In this chapter I used behavioural (nest site parental associations), morphometric (male wing length, mass), plumage characteristics (size and asymmetry of white spots) and genetic paternity data from two British nightjar breeding populations (Humberhead Peatlands and Thetford Forest) over three years (2019-21) to establish the role of sexually dichromatic achromatic spots in nightjar in visual communication and mate choice. Specifically, I addressed the following aims;

A1) Investigate the role of sexually dichromatic white spots as visual signals and indicator of male quality in nightjar, testing the hypothesis that larger and more symmetrical white spots will be exhibited by older males with larger body mass and wing length;

A2) Establish whether dichromatic white spots were sexually selected features by determining if spot characteristics explained reproductive success variation in male nightjars. Two aspects of male mating success were explored to determine whether variance in spot, age and size characteristics influence mating success. Specifically, I investigated variation in spot characteristics between A) males who were cuckolded and those who were not cuckolded and B) between extra-pair and within-pair males. This addressed the hypotheses A) that cuckolded males will exhibit smaller spot sizes, be smaller and younger than males who secured paternity, and B) between the two categories, extra-pair males will exhibit larger spot sizes and will be older and larger than within-pair males.

6.3 Methods

6.3.1 Study sites and sampling

The study was conducted between May - September in 2019, 2020 and 2021, at two breeding sites of nightjar in England, at Thetford Forest and Humberhead Peatlands. The two sites differed in latitude, size, habitat type and breeding density. Fieldwork was carried out at Thetford Forest during each in 2019, 2020 and 2021 Fieldwork at Humberhead peatlands was conducted in 2019 and 2021. Work at Humberhead peatlands was not conducted in 2020 due to access restrictions imposed by the COVID-19 pandemic. Humberhead Peatlands (Lat. 53.5884, Long. -0.915668) was formed of lowland raised mire. The site was comprised of two

spatially distinct subsites; Thorne moor and Hatfield moor, located 10 Km apart from one another. Habitat composition across the two subsites comprised open water, lowland raised bog, Birch (*Betula pendula*) stands, and isolated areas of *Calluna*-rich heathland, providing nesting habitat for breeding nightjars. The site is designated as a SPA for the nationally important numbers of breeding nightjars it supports. Thetford forest (Lat. 52.4209, Long. 0.6625) was a lowland commercial plantation pine forest in Eastern England. A commercial plantation, Thetford Forest was composed of a mosaic of discrete blocks of different age Scots *Pinus sylvestris* and Corsican pine *P*. *nigra* stands, and nightjars typically utilised clear-felled blocks of land 2-15 years post-felling (Conway et al., 2007). The site is internationally important for its breeding population of nightjar (Sharps et al., 2015).

6.3.2 Nightjar capture, nest associations, biometrics and DNA sampling

6.3.2.1 Individual capture, biometrics and DNA sampling

All nightjars were captured using 30x30 mm mist nets. British Trust for Ornithology (BTO metal rings were applied to each bird captured (unless already ringed) and all birds, biometric measures (flattened chord wing length, mass, and muscle scores) were collected and, in the case of males, photographs of wing and tail achromatic spots taken. Spot photos were taken using an iPhone 8 (12-megapixel, Apple, USA). The bird's wing/tail was laid flat on a level A4 clipboard with a 300mm ruler placed alongside for scale. Both wing and tail feathers were spread such that the spots were unobscured (Fig 6.1). A photo was taken with the camera standard flash of each wing and tail at a distance of 300mm between the camera and the subject with the camera held parallel to the baseboard.

Figure 6.1 Example of a photograph taken for measuring male nightjar wing spot area.

All individuals were aged following EURING classifications as adults, first summer or older than first summer (EURING 4 - unknown age) based on plumage characteristics and historic ringing data in the case of recaptures. Morphometric measures were taken from all captured male nightjars. Pectoral muscle scores (muscle) were recorded based on scoring described in Redfern and Clark, (2001).

Buccal cells were sampled for DNA extraction from all individuals captured using mouth swabs, which provide a quick and relatively non-invasive technique for collecting genetic material (Handel et al., 2006). Sterile 4mm Rayon tipped plastic swabs (Copan, USA) were inserted into the mouth of the nightjar and rotated 3-5 times against the inside of the birds' cheeks and tongue. The swab was removed from the mouth and air-dried for 1 – 2 minutes before being stored in a sterile container. Swabs were initially stored at -20°C in a cool-box in the field before being transferred to a -80°C freezer for long-term storage. If birds were recaptured another swab was taken from that individual.

6.3.2.2 Social paternal association

Social paternal associations were made at nests following the methods outlined in **Chapter 5**. Social paternity was assigned at forty-six nests across both sites and all sampling seasons. To generate a sample of potential extra-pair male nightjars, birds were also captured away from nest sites at both Humberhead Peatlands and Thetford Forest. Mist nets were set in suitable habitats (woodland edge, corridors, and open heath) at least 30 minutes before civil twilight. Where possible, nets were set around territory boundaries to maximise the sample size of

local males. Tape playback of male nightjar 'churring' (song) and 'quipping' (contact calls) were used (under licence) at mist nets to aid the capture of male nightjars. Tape playback use was limited to 5 minutes at each net, repeated a maximum of three times per night, which were typically set >50m from one another. A maximum of 48 m of mist nets were set per night. An effort was made to trap across as much suitable habitat as possible within or near to known locations of churring males at both sites each year. Area covered across each site within and between years was not standardised owing to varying habitat accessibility and availability of field volunteers.

6.3.3 Male spot size measurement

A total of 158 male nightjars were captured and measured across both sites and sampling years. Eighty-nine birds had spot photographs of sufficient quality for digitization. Of the sixty-nine birds not digitised, 23 individuals were not photographed owing to constraints in the field, a further 32 had photos of insufficient quality (i.e., obscured spots, insufficient lighting, exclusion of scale), and finally 11 birds had incomplete tail feathers at the time of photography (i.e., moulting n = 10), with one bird exhibiting extreme wear of the tail feather spots.

Male nightjar wing and tail spots from the 89 birds were measured from photographs using Image-J freeware 1.52. To avoid potential bias, photographs were analysed blind with respect to individual ID and site of capture. Photographs were scaled and standardised against the 300mm rule (Fig 6.1) included in the photos. Images were viewed as grayscale to better differentiate white spots from surrounding plumage and to enable thresholding of white pixels from the surrounding dark plumage. The threshold tool was then used to measure the area of each white spot on the primaries and rectrices individually. Where spots were found to extend across the rachis, white plumage on either side of the rachis was measured separately and summed. The threshold tool interactively sets the lower and upper threshold values. Threshold values could not be standardised across images owing to variations within the lighting environment.

The representativeness of the thresholding method was tested by comparing threshold-derived 'spot area' in a subset of birds (n = 29) against measures derived using polygon area analysis, again using ImageJ. For the polygon analysis images were standardised and scaled as described above. The polygon selection tool was used to determine spot outline, and the measure function to provide area (mm^2). Each spot measurement was repeated 4 times, with

repeat number determined by asymptote calculations performed on three individuals over 42 measures repeated 10 times. Measures from the two analysis methods were assessed for equivalence visually using scatter plots and by generating Pearson's *r* values. Both quantification methods showed concordance in spot size measures (Pearson's *r*; wing = 0.96, tail = 0.77). Despite variations in lighting environment, the thresholding method employed here provides an accurate representation of primary and rectrix spot size in male nightjars.

6.3.4 Ethical review

The research was granted full ethical approval by the University of York's animal and welfare ethical review body. DNA collection via buccal swabs and use of VHF tags were reviewed and approved by the BTO special methods panel, with accredited agents trained appropriately and possessing C or A class ringing permits issued by the BTO. Species-specific licence for playback was also issued by the BTO for fieldwork. Best practice was followed by all fieldworkers to minimise nest disturbance where possible (see Section 6.3.2.2) .

6.3.5 Genetic methods and paternity analysis

DNA extraction and paternity analysis were carried out as per **Chapter 5** (see also **Appendix** Section 8.2.1.1 for detailed extraction methods). Here the identity of extra-pair sires was ascertained. In broods (n = 12; **Chapter 5**) where paternity was refuted for a broods' social male, the identity of the putative sire suggested by CERVUS with a positive LOD score with a Δ value > 80% was assumed to be the extra-pair sire of that brood (Slate, Marshall and Pemberton, 2000).

6.3.6 Data handling and analysis

6.3.6.1 Asymmetry of white spots

Eighty-nine birds with complete bilateral wing and tail spot measures were used to generate asymmetry values. Measures of left and right total wing and tail spot area were first correlated (Pearson's correlation) to test for symmetry of bilateral measures in the population. Asymmetry values for each individual were then characterised for both wing and tail spot

areas as ($X_l - X_r$), where X_l was the summed value of all spots on the individual's left side- and X_r the value on the individual's right side, for wing and tail spots, respectively (Graham et al., 2010).

6.3.6.2 Male spot characteristics as quality indicators

Only individuals with complete wing and tail spot measurements (n = 89) were included in Models 1 -6. Ten individuals were excluded for the wing dataset and 14 from the tail dataset owing to unknown ages and no muscle scores collected. A further two samples were excluded from both datasets owing to replicated measures of the same individual taken over sampling years, with one replicate chosen at random to be excluded. The final dataset consisted of 77 (wing only), 73 (tail only) and 67 individuals with complete wing and tail spot measurements for inclusion in Models 1 - 6 (for site and age sample breakdown see **Appendix** Fig 8.6). To control for the effect of body size on spot size and asymmetry all summed spot sizes (wing, tail, and total) and asymmetry values were divided against wing length for inclusion in models. Models were also run incorporating 'actual' spot sizes and asymmetry values'. However, no difference in the significant predictors were found between model runs (see **Appendix** Section 8.4.3.1).

To determine whether male spot characteristics act as quality indicators, six multiple linear regression models were employed with each spot characteristic selected as a response variable (Model 1; relative wing spot area, Model 2; relative tail spot area, Model 3; relative wing spot asymmetry, Model 4; relative tail spot asymmetry, Model 5; relative total spot area, Model 6; relative total spot asymmetry). The distribution of all response variables was assessed visually using quantile-quantile plots and histograms before inclusion in models. All response variables were normally distributed, with the exception of relative wing spot area. Relative wing spot area was normalised by square root transformation. Predictor variables (see Table 6.1) represent proxies for individual quality (age, pectoral muscle, body mass, and wing length), with site and sampling year also included in the models as fixed effects owing to wing and total spot sizes varying significantly between sites (relative wing spot area; ANOVA, $F_{(1,72)}$ = 16.267, P <0.0001, relative total spot area; ANOVA, $F_{(1,62)}$ = 13.504, P <0.001) and body mass varying significantly among sampling site and years (Site; ANOVA, $F_{(1,101)}$ = 119.53, P = 0.043, Year; ANOVA, $F_{(2,101)}$ = 134.31, P = 0.011). Scaled Mass index (SMI) was calculated (see **Appendix** Section 8.4.2) for each male nightjar as a measure of body condition following Peig and Green, (2009). However, SMI was found to be highly correlated with mass (Pearson's

correlation test, r = 0.93, P <0.0001; Fig 7.8). Body mass alone represents a good predictor of individual body condition (Labocha and Hayes, 2012). Here, I chose to use mass alone and incorporate wing length as a separate predictor of structural size in models, with the two showing no significant correlation (Pearson's correlation r = 0.17, P = 0.08).

Maximal models included all predictor variables shown in Table 6.1, with forward and backwards stepwise selection employed for each model based on AIC value. The best fitting model was then selected and presented. Each maximal model was tested for deviances of residuals from normality and evidence of heteroskedasticity visually using the plot function in R and confirmed using the Shapiro-Wilk and Breusch-Pagan test respectively. In all maximal and final models' residuals did not exhibit heteroskedasticity and could be considered normal (Shapiro-Wilk and Breusch-Pagan test, P > 0.05 in all cases). Finally, multicollinearity of predictors was assessed by applying variance inflation factor (VIF) tests to maximal Models 1 - 6. In all cases no multicollinearity was found among variables (VIF < 1.5 in all cases).

Table 6.1 Predictor variables included in Models 1 - 6.

Predictor	*Definition*
Age	Age category as per EURING classifications, first summer or adult.
Wing length	Flattened chord wing length measured in mm
Body Mass	Body mass weighed in g.
Pectoral Muscle Score	Factor of 4 levels, representing size of pectoral muscle.
Site	Sampling site - Thetford, Humberhead
Year	Sampling year - 2019, 2020, 2021

6.3.6.3 Modelling the value of male characteristics in predicting paternity

Fifty-two males could be assigned to broods either as the social (within-pair) (n = 44) or extra-pair male (n = 8), with three individuals also confirmed as both the social male for one brood and extra-pair to another. Twenty-seven males from this pool possessed complete spot (size and asymmetry) and morphometric data. A single individual accounted for two observations over two sampling years, one observation was randomly selected and removed before inclusion in the model to avoid pseudo replication. In total 26 individuals were retained for model inclusion. Again, to control for the effect of body size on spot size and asymmetry all summed spot sizes (wing, tail, and total) and asymmetry values were divided against wing length for inclusion in models. Two multiple logistic regression models (GLM with binomial error structure) using two different Bernoulli binary response variables (Model 7; within-pair breeding success, Model 8; paired status) were used to test the null hypotheses; i) within-pair paternity (gain/loss) will not be related to male spot characteristics (size and asymmetry) and ii) extra-pair males will not differ in spot characteristics (size and asymmetry) from paired males. The predictors included were as per Table 6.2, again utilising body mass and wing length over SMI. Because EPP rates, spot size and mass varied between sites and sampling years (see **Chapter 5** and Section 6.4.1.2), both site and year were included as predictors in Models 7 and 8. Forward and backward stepwise selection for AIC were again applied to both models, with the best fitting model based on lowest AIC value selected. Multicollinearity of predictors was assessed by applying variance inflation factor (VIF) tests to both models. Wing and tail spot asymmetry were both removed from Models 7 and 8 owing to high VIF values (VIF > 5 in both cases), after which no multicollinearity was detected. All statistical analyses were carried out in R (V 4.0.1) (R Core Team, 2020).

Table 6.2 Parameters included in Models 7 and 8 comparing predictors of whether a male was cuckolded and within pair birds versus extra pair males respectively.

Parameter	*Definition*
Wing length	Flattened chord wing length measured in mm
Body Mass	Body mass weighed in g.
Relative Total Wing Spot Size	Sum of wing spot area across all both wings, relative to wing length to control for size, measured in mm^2.
Relative Total Tail Spot Size	Sum of tail spot area across all both left and right sides of the tail relative to wing length to control for size variation, measured in mm^2.
Relative Wing Spot Asymmetry	Bilateral asymmetry of all wing spots summed on the left and right wing, measured in mm^2.. Spot area again presented relative to wing length to control for size
Relative Tail Spot Asymmetry	Bilateral asymmetry of all tail spots summed on both left and right sides of the tail, measured in mm^2. Spot area again presented relative to wing length to control for size.
Relative Total Spot Area	Summed area of wing and tail spots, measured in mm^2. Spot area again presented relative to wing length to control for size.
Relative Total Spot Asymmetry	Summed bilateral asymmetry of wing and tail spots, measured in mm^2. Spot area again presented relative to wing length to control for size.
Age	Age category as per EURING classifications, first summer or adult.
Site	Sampling site - Thetford, Humberhead
Year	Sampling year - 2019, 2020, 2021

6.4 Results

6.4.1 Male characteristics and quality

6.4.1.1 Fluctuating asymmetry

Left and right total wing and tail spot areas were significantly positively correlated with one-another (Pearson's *r*; wing = 0.83, tail = 0.65, df = 89 , P < 0.001) suggesting bi-lateral symmetry between ornaments. Male nightjar wing and tail spot asymmetry broadly conformed to a normal distribution (Fig 6.2), with bilateral asymmetry in both wing and tail ornaments cantering towards 0, suggesting that nightjar and wing tail spots likely conform to fluctuating bilateral asymmetry.

6.4.1.2 Male spot characteristics as quality signals

Male age significantly predicted both relative squared wing spot area and total spot area (Table 6.3). Adult male nightjars exhibited significantly larger wing and total spot areas compared with first summer males (Fig 6.3 A and C). Male nightjars in Humberhead also consistently displayed significantly larger wing and total spot area than individuals at Thetford (Fig 6.3 D and F). Relative tail spot area alone also exhibited a similar, near-significant (P = 0.056) trend to relative total and wing spot area, with adult nightjars having larger tail spots than first-summer birds (Fig 6.3B; Table 6.4). Relative tail spot area was also larger in nightjar from Humberhead than Thetford, although site was not found to be a significant predictor of relative tail spot area alone (Table 6.4). Notably, the variation in spot area between sites was not accompanied by variation in structural size (wing length; ANOVA, $F_{(1,105)} = 9.0$, P = 0.504). Finally, in Models 3, 4 and 6 none of the quality predictor variables included explained variation in wing, tail or total spot asymmetry (**Appendix** Tables 8.6 – 8.7).

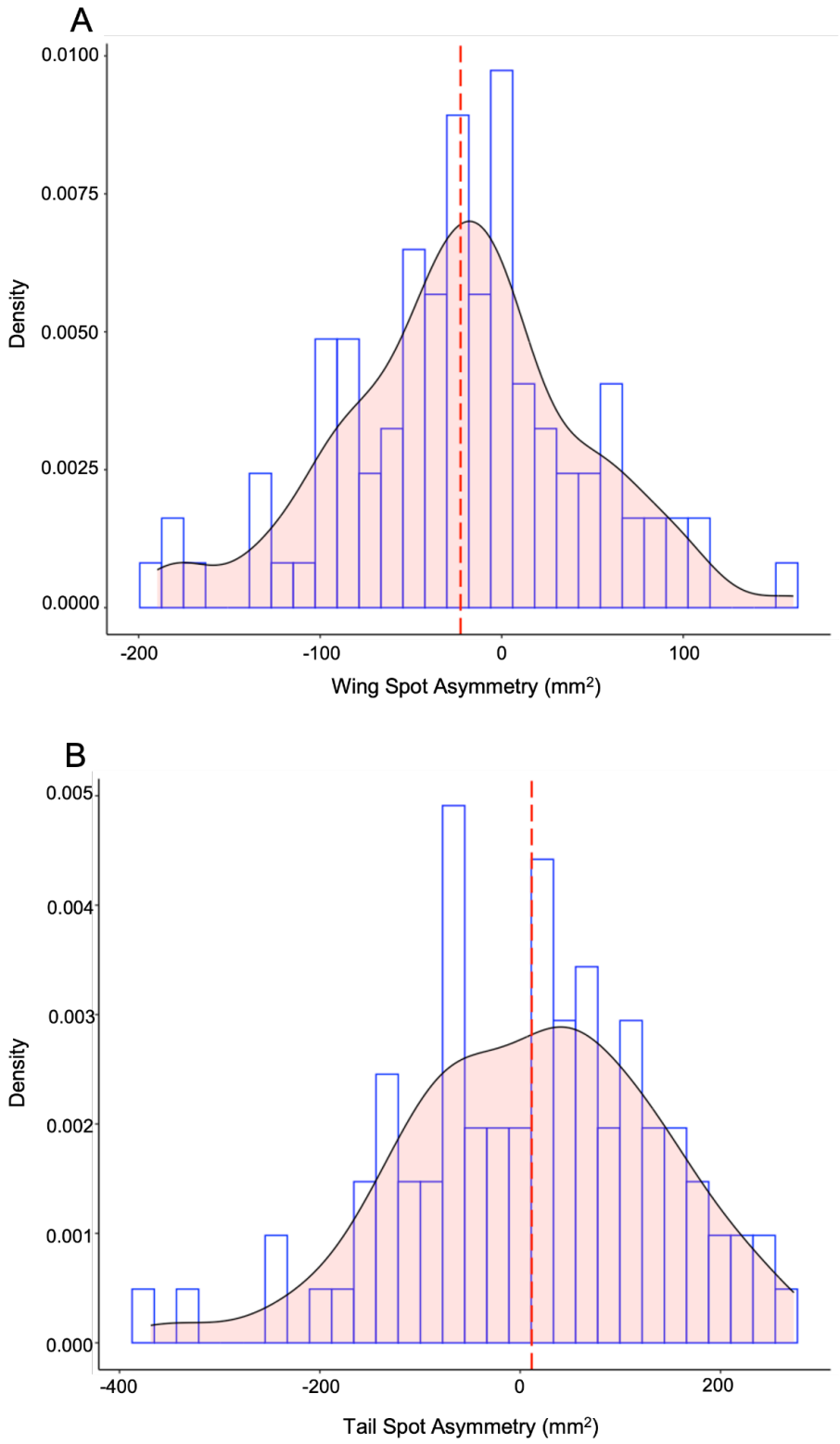


Figure 6.2 Relative (A) wing and (B) tail spot asymmetry of male nightjars. The median value is presented as a dashed red line.

Table 6.3 Multiple linear regressions Models 1 (M1) and 5 (M5) after forwards and backward selection with response variables squared relative wing spot (Model 1) and relative total spot size (Model 5) respectively. Models one and five were comprised of 77 and 67 observations, adjusted R^2 values of 0.206 and 0.209, and AIC values of 321.62 and 95.01 respectively. Significant P-values (<0.05) are highlighted in **bold**.

Predictors	*Estimates*		*CI*		*p*	
	M1 (wing)	M5 (total)	M1 (wing)	M5 (total)	M1 (wing)	M5 (total)
(Intercept)	25.98	13.50	23.24 – 28.73	12.75 – 14.25	**< 0.001**	**< 0.001**
Age (first summer)	-4.19	-1.13	-8.04 – -0.35	-2.19 – -0.08	**0.033**	**0.036**
Site (Thetford)	7.68	-1.87	-11.60 – -3.76	-2.94 – -0.79	**<0.001**	**0.001**

Table 6.4 Multiple linear regressions Model 2 after forwards and backward selection, with squared relative tail spot area as the response variable. Model 2 was comprised of 73 observations, with an adjusted R^2 value of 0.06, and AIC of 297.154. Significant P-values (<0.05) are highlighted in **bold**.

Predictors	*Estimates*	*CI*	*p*
(Intercept)	8.49	7.86 – 9.12	**<0.001**
age [first summer]	-0.83	-1.69 – 0.02	0.056
Site [Thetford]	-0.66	-1.51 – 0.19	0.125

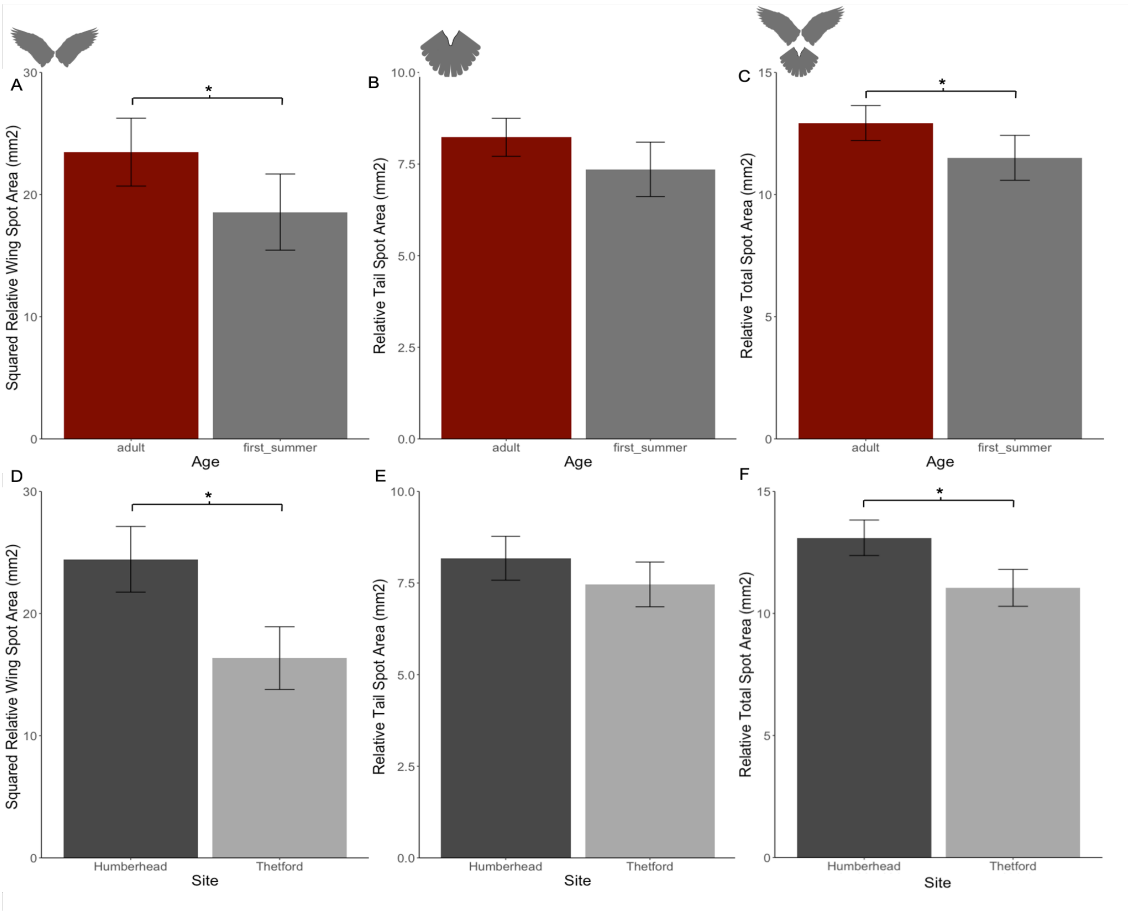


Figure 6.3 Comparison of mean (=/- 95% CI) squared relative wing spot, tail and total spot area between age classes (A-C) and Site (D-F) from Models 1, 2 and 6. Significant predators are highlighted with *.

6.4.2 Role of male spot characteristics in predicting paternity

When comparing social sires who were and were not cuckolded, no spot characteristics included in Model 7 were associated with cuckoldry in male nightjars (**Appendix**, Table 8.9). However, relative total spot area significantly predicted whether nightjars were within or extra-pair sires (Table 6.5). Paired males exhibited a larger relative total spot area than birds who achieved paternity through extra-pair copulations (Fig 6.4A). Paired males also had a larger body mass than extra-pair birds (Fig 6.4B), although mass did not significantly predict paired status (Table 6.5). Notably, despite variation in mass, paired and extra-pair males did not differ greatly in wing length (average wing length; within-pair male = 195.39 mm, SE = 0.35 extra-pair male = 196.5 mm, SE= 0.33).

Table 6.5 Multiple logistic regression Model 8, modelling male nightjar spot characteristics against male paired status as binary response variable (within pair and extra-pair male). Results presented are after stepwise forward and backward AIC selection. Model 8 consisted of 26 observations, with a Tjur R^2 of 0.350 and AIC of 32.595. Significant P-values (<0.05) are highlighted in **bold**.

Predictors	*Odds Ratios*	*CI*	*P*
(Intercept)	0.00	0.00 – 1.84	0.105
Relative wing spot size (mm^2)	0.32	0.03 –1.68	0.233
Relative total spot size (mm^2)	1.80	1.09 – 3.65	**0.045**
Mass	1.32	1.00 – 2.06	0.109
Age	0.39	0.03 - 4.36	0.442

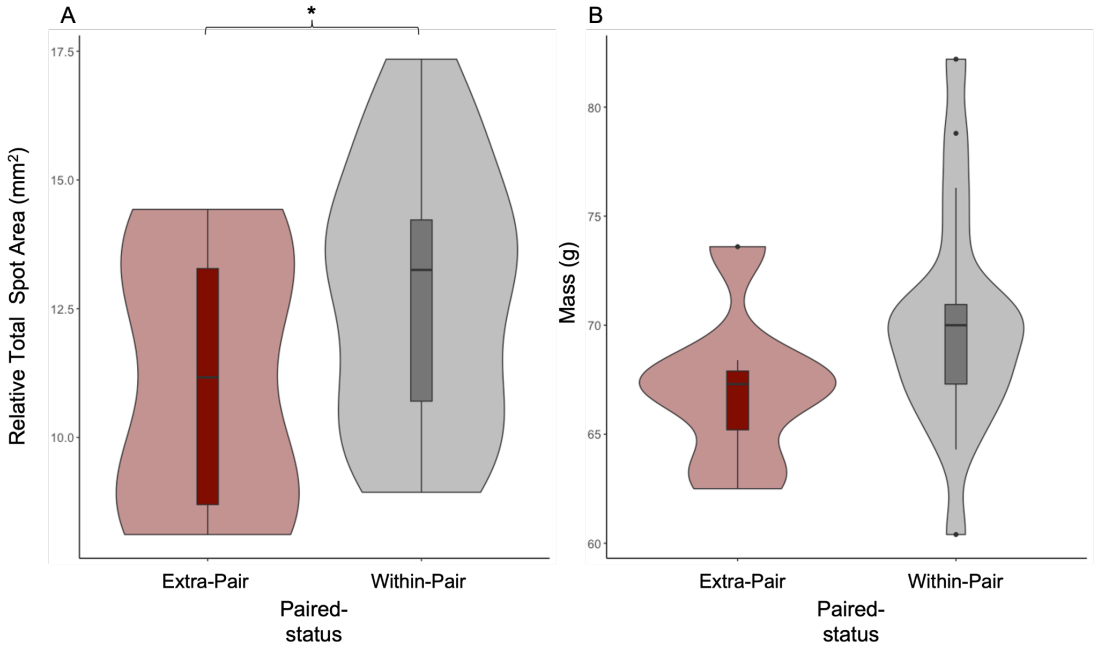


Figure 6.4 Extra-pair and within-pair male (A) Relative total spot area and (B) mass (n = 26). First and third quartiles are displayed as the extent of the rectangle in embedded boxplots, with the median given as a central line. Spread of data are represented as density curves on either side of the boxplot. Significant predictors are highlighted with *.

6.5 Discussion

Age class significantly predicted wing and total spot size, being larger in adults than first summer males. Tail spot area displayed a similar, near-significant trend, again larger in adults than first summer males. Both wing and total spot size also varied between study sites, and was consistently larger at Humberhead than Thetford, despite no significant difference between the sites in individual male size, as estimated by wing length. Whilst nightjars exhibited fluctuating asymmetry for bilateral wing and tail spot area, asymmetry for either feature did not appear to signal individual quality. Spot size, asymmetry, age, mass, and wing length showed no significant difference between cuckolded males and males who sired within-pair offspring. However, paired males exhibited a significantly larger total spot size than extra-pair males. Although not significant, paired males also tended to be heavier than males who gained paternity through EPC's, despite not differing greatly in wing length.

Similar to results from other Caprimulgids (e.g. *C. ruficollis*, *C.minor*; Forero, Tella and García, 1995; Aragones, Reyna and Recuerda, 1999; Roth, Argyros and Browning, 2003), adult male nightjars in my study exhibited larger white wing and tail spots than first summer birds. This age association in other species has been hypothesised to reflect differences in male experience, dominance, and quality (Forslund and Pärt, 1995; Brooks and Kemp, 2001; Moreno-Opo, Trujillano and Margalida, 2020) and is a common trend exhibited by ornaments under sexual and social selection (Freeman-Gallant et al., 2010). Black grouse (*Lyrurus tetrix*), for example, show a distinct age difference (larger in adults than young males) in eye comb size, with ornament size linked to individual condition and dominance (Harris et al., 2018). Age acts as a proxy for quality signifying an individual's ability to survive between breeding seasons, increasing breeding opportunities. Male nightjars exhibit inter-annual territory fidelity at both study sites, although recapture rates of male nightjars at Humberhead were anecdotally higher than at Thetford (G.Conway pers.comm). If age is a proxy for dominance and individual experience, the display of white spots may be an important signalling strategy in intrasexual aggression and competition, as well as mate choice. Male nightjars regularly engage in apparent intrasexual aggression and territorial disputes, where males will display both wing and tail spots to one another during display flights (Cramp and Simmons, 1985). White spot size may provide a method of communicating individual age and potentially dominance to rival male nightjars, limiting or avoiding costly physical contests.

In previous studies of Caprimulgids, age-related increases in spot size have been used as evidence that white spots are subject to sexual selection (e.g., Forero, Tella and García, 1995;

Aragones, Reyna and Recuerda, 1999; Roth, Argyros and Browning, 2003). However, no attempt was made to quantify paternity in relation to spot characteristics. I was unable to find any significant differences in wing and tail spot size or asymmetry, individual age, or size (mass and wing length), between paired males who had secured and lost paternity (cuckolded), although a small sample size may be responsible for the result. Other studies have also found no effect of ornament size (Akçay and Roughgarden, 2007; Hsu et al., 2017) and age (Kleven, Marthinsen and Lifjeld, 2006) on cuckoldry in birds. Differences between cuckolded and non-cuckolded males may have occurred in other ornament characteristics not considered in my study (e.g., spot shape and brightness or song repertoire). Feather ornament brightness, contrast, and shape have been shown to be important in mate choice in other species (Saino et al., 2015; Romano, Saino and Møller, 2017). Further, spot size and asymmetry may combine with other signalling channels and traits to form a multimodal display (Chaine and Lyon, 2008; Akçay and Beecher, 2019). Male nightjars produce a distinctive song (churr). Song repertoires can vary between individuals (Rebbeck et al., 2001; but see; Raymond et al., 2020) but also with paired-status (Docker, Lowe and Abrahams, 2020). Vocalisations can signal individual quality in birds (Hsu et al., 2017), and song repertoires may increase with age and change to reflect differences in individual condition in real-time (Garamszegi et al., 2007; Hsu et al., 2015). Differences in cuckoldry might then be explained by other ornaments and signalling channels not considered in this study. Future research may benefit from also considering more spot characteristics (e.g. shape and brightness) as well as vocalisations when investigating mate choice in nightjars.

I was unable to investigate differences in spot characteristics, size, or age between paired and unpaired males. This was because I could not determine that males captured away from nest sites were truly unpaired. Therefore, analysis was restricted to males for which paternity outcomes were known (cuckolded vs not cuckolded and within-pair vs extra-pair males). Whilst not important in predicting cuckoldry, spot characteristics may inform social mate choice, with a females' social mate holding a territory and providing nest-sites and paternal care (Cramp and Simmons, 1985; Holyoak, 2001). My comparison of socially paired and extra-pair males highlighted differences in total spot size and non-significant differences in body mass. Extra-pair males had smaller spot sizes and lower body mass compared with paired birds. My results conflict with well-established hypotheses explaining the female benefits of EPP (e.g. good genes and sexy sons; Fisher, 1930; Hamilton and Zuk, 1982), which combine to predict extra-pair males will be larger and more ornamented than within-pair males. However, if spot size signals quality and dominance in male nightjars it might be expected that paired birds would possess larger spots and a larger body mass. Nightjars exhibit life histories similar to other long-lived species with small clutch sizes (i.e., seabirds and some raptors), producing relatively few young (up to 2

broods of ≤ 2 offspring), and are heavily dependent on biparental care (Cramp and Simmons, 1985). In such species EPP is usually rare, partly owing to the high fitness costs of losing paternal care (Griffith, Owens and Thuman, 2002; Bried et al., 2010; Quillfeldt, Masello and Segelbacher, 2012). Nightjars present a rare case in which fecundity is low and biparental care and EPP are relatively high (see **Chapter 5**; but see Quillfeldt, Masello and Segelbacher, 2012; Sakao et al., 2019). Females may select high-quality males for which to pair, in order to secure a high-quality territory, nets site, and paternal care in the case of better quality, older, and more experienced males (Bayne and Hobson, 2001; Reitsma, Hallworth and Benham, 2008). Lower quality males may then exhibit a floater male strategy, and gain paternity through EPCs, rather than investing in territory holding (Brekke et al., 2015). Floater males are usually of lower quality than paired birds, typically less ornamented and lower in body mass (Moreno, 2016).

Floater males occur more frequently where mate and territory availability is limited, often under high breeding densities and skewed operational sex ratios (Newton, 1998; Moreno, 2016). In my study populations EPP was more frequent at increased male territory densities (see **Chapter 5**). In cases where floater males are present in the population, variation in breeding success among paired birds may not be explained by differences in male ornamentation or size, with variation instead being explained by differences between territory holding and floater males (Moreno, 2016). Floater males may intrude into paired males' territories (Veiga et al., 2013; Turrin and Watts, 2014; Wischhoff et al., 2015), and even form polyandrous trios in some cases (Carrete et al., 2006). This leads to a high level of intrasexual male aggression and territoriality by territory-holding males (Moreno, 2016). Male nightjars are highly territorial, and allocate a significant amount of energy to territorial defence against intruders (Cramp and Simmons, 1985). From my limited data, it is apparent that extra-pair males may constitute floaters in the studied populations, being lighter weight and less well ornamented than paired birds (Pitnick et al., 2009; Brekke et al., 2015). Congruent with this concept, I detected no differences in size, age or spot characteristics relating to mating success amongst paired birds, with variation in spot size restricted to differences between paired males and extra-pair sires, which may constitute floater males. However, not all cases of EPP in my study could be attributed to floater males, with known territory-holding paired males also found to obtain EPP. A single paired male at Humberhead was found to be the extra-pair sire of two broods belonging to two different females within close proximity (< 100m) of his territorial nest (unpublished data). Nevertheless, it has often been anecdotally noted that significant proportions of male nightjars at breeding sites might be un-paired and take on a floater strategy (Sharps, 2013). My results appear to align with this concept. However, further research with larger sample sizes and more comprehensive behavioural data would be needed to better explain my findings and understand the

evolutionary and fitness consequence and adaptive nature of this behaviour. Finally, floater or beta males may aid in offspring provisioning paternity is assumed (Davies et al., 1992; Davies and Hatchwell, 1992), or simply to avoid exclusion by territorial high quality males (Moreno, 2016). Although extra-pair provisioning was observed at 6 nests across sites during years included in this study (**Chapter 5**), in no cases were the extra-pair provisioning males found to be the genetic father of the provisioned offspring (**Chapter 5**). However, owing to limitations of my behavioural data I cannot rule out that provisioning males solicited extra-pair copulations and thus assumed paternity of the provisioned brood. Again, further research incorporating more comprehensive behavioural, and nest-site observational data is needed to better understand this behaviour in nightjar.

Spot size showed a distinct geographic variation. Both wing and tail spot sizes were consistently larger at Humberhead than at Thetford. Humberhead is ~160km North of Thetford, a relatively short distance in the context of geographic morphological variation (Lehtonen, Primmer and Laaksonen, 2009; Conklin et al., 2011). Nevertheless, Sáez Gómez et al., (2016) detected differences in skeletal body size in Red-necked nightjar between two populations located <20km apart. They proposed that differences in habitat and land use between the populations likely drove the observed morphological variation. The condition-dependent nature of ornaments means that variations in resource availability, habitat quality, and stress between populations may account for geographic differences in ornament expression (Soravia, Aguado-Giménez and Avilés, 2020; Jones et al., 2022). However, nightjars, as with most trans-Saharan migrants, undergo a complete moult during winter in Africa (Demongin, 2016). Individuals during the breeding season may then not reflect body condition when the ornamented feathers were grown several months prior (Saino et al., 2015). Additionally, white plumage is highly susceptible to wear (Kose and Møller, 1999; Badyaev and Hill, 2003), and inter-individual variation in spot size could reflect a male's ability to invest in maintenance (Berglund, Bisazza and Pilastro, 1996; Qvarnström and Forsgren, 1998). Therefore, although feather ornaments were originally moulted in the wintering grounds, differences in spot sizes between sites could ultimately reflect geographic variation in male quality. Variation in habitat quality and prey availability may drive geographically separated differences in spot size, either with better quality males able to hold better quality territories, or localised differences in resource availability enabling some individuals to invest more in plumage maintenance. However, my data are only able to provide limited insight into a trend likely worthy of further attention. Whilst data on prey availability and habitat use at both of my study sites exist (Sharps et al., 2015; Evens et al., 2020b; Mitchell et al., 2020, 2022), differences in field-based effort and collection dates mean that representative comparisons are not possible with existing data. However, this is a trend that likely warrants

further examination with extrapolation to more distantly separated breeding sites and investigation into differences in habitat quality.

6.6 Conclusion

Male European nightjars display prominent white wing and tail spots, contrasting with their otherwise cryptic plumage. Congruent with previous findings in other Caprimulgids, my results suggest that these spots could be reflective of individual quality as they are larger in adult than in first-summer males. I found limited evidence that the white spots may be subject to sexual selection via mate choice, with extra-pair sires possessing smaller spots than within-pair sires, conflicting with well-established hypotheses explaining the female benefits of EPP. However, these results may be explained by floater males accounting for at least some of the extra-pair sires in the studied populations. Male nightjars exhibited a geographic variation in spot size which did not coincide with a difference in structural size, possibly signifying differences in male quality between breeding sites. Overall, my results demonstrate that achromatic ornaments may convey quality signals in nocturnal taxa and be subject to sexual selection. Further work is required if the role of sexually dichromatic white spots in other nightjars is to be better understood. I recommend further investigation with larger sample sizes, also considering other signalling modes (i.e., brightness) of white spots. Other nightjar signalling mechanisms also deserve consideration, including UV fluorescence (Camacho et al., 2019), and vocalisations (Eisenring et al., 2022). The importance of visual signalling for communication in nocturnal taxa is becoming increasingly apparent, as highlighted by my results. The evolution of visual ornaments in nocturnal taxa provides an exciting avenue for future research. Indeed, understanding the role of nocturnal visual communication is now more pressing with the ubiquity of light pollution and encroachment of urban land use into remnant habitat patches.

Chapter 7 General discussion and conclusion

Spatiotemporal patterns in genetic variation can provide information on population demography, gene flow and thus population connectivity (Frankham, Ballou and Briscoe, 2010), with the amount of genetic variation in a population ultimately determining it's capacity to adapt to stochastic environmental change (Bürger and Lynch, 1995; Frankham, Ballou and Briscoe, 2010; Hohenlohe, Funk and Rajora, 2021). This is particularly pertinent under current anthropogenically driven climate change, as well as historic and continued habitat loss and fragmentation (Dirzo and Raven, 2003; Hanski, 2011; Lees et al., 2022). Species with specialist resource requirements and populations located at range peripheries are particularly vulnerable to these anthropogenic threats (Pironon et al., 2017; Perrin et al., 2021; Frantz et al., 2022). Characterising temporal and spatial patterns in genetic variation enable us to infer historical demographic change in response to such anthropogenic pressures, as well as the impacts on population connectivity and 'health' (i.e., genetic diversity) to be quantified (Frankham, Ballou and Briscoe, 2010). Such data are particularly useful in species that are difficult to monitor, in which current understanding of population dynamics are limited by a lack of available data derived from conventional, field based, methods (Crates et al., 2019; Larison et al., 2021). At the population level, demographic growth is fundamentally constrained by breeding success. Thus a detailed understanding of a species breeding behaviour is then important to ensure effective conservation and management (Quader, 2005), and has particular value in elucidating the proportion of breeding individuals and effective population size (N_e; Sutherland, 1998). Moreover, understanding the effects of ecological and environmental factors on breeding behaviour can enable the development of tailored management strategies to maximise population productivity (Nunney, 1993; Hogg, 2000; Schindler et al., 2013).

In this final chapter I outline the key findings from each data chapter presented in this thesis and put them into the theoretical framework outlined in the general introduction (**Chapter 1**; see also Fig 7.1), linking my results to the broader context of conservation of a range extreme population of a migratory habitat specialist.

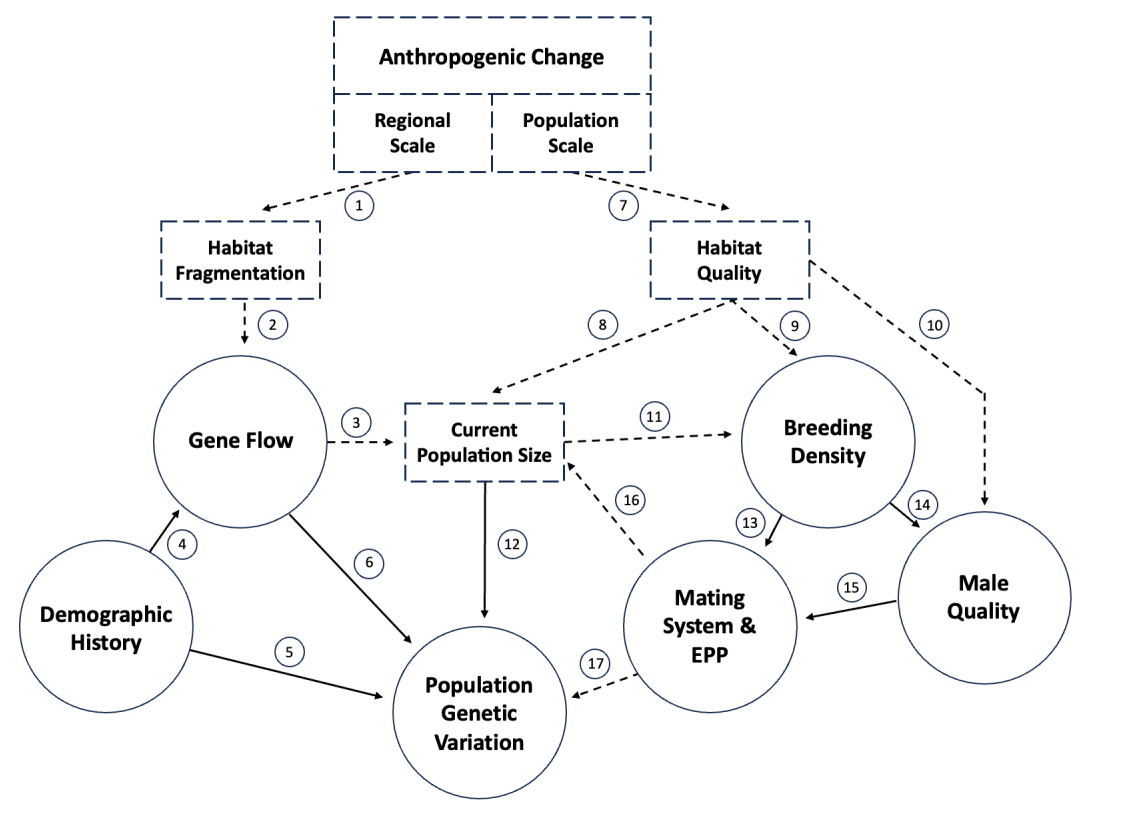


Figure 7.1 Conceptual framework summarising the relationships of ecological and evolutionary processes that drive population genetic variation studied in this thesis (solid circles) and not covered in this thesis (dashed rectangles). Arrows denote directionality of relationship between processes, with solid lines denoting relationships supported by work in this thesis and dashed support by evidence in the literature. Numbers on arrows are used to identify relationships as they are discussed in the main text.

7.1 Thesis summary

In **Chapter 2**, Pairwise Sequentially Markovian Coalescence (PSMC) analysis was applied to two nightjar genomes from Northwest and Southern Europe to elucidate temporal changes in nightjar N_e in response to ancient climate change. I found that N_e and likely the range of nightjar expanded and contracted with periods of warming and cooling respectively across temperate Europe over the last 5My (Ponti et al., 2018, 2020). PSMC analysis on a pseudo diploid genome constructed from the two nightjars suggested that the two contemporary populations are genetically distinct, having experienced slightly different N_e trends (Fig 7.1 arrow 4). Divergence appeared to begin as early as ~1.2 Mya, but I estimate that most recent point of divergence likely occurred during the last glacial maxima (110–10 Kya), when populations were probably restricted to available habitat in different glacial refugia in Southern Europe (Hewitt, 1999). My results suggest that nightjar have likely exhibited migratory behaviour since at least ~1.2 Mya when initial divergence of the two populations

probably occurred. This is significantly earlier than previously published estimations (e.g. Larsen et al., 2007). Contemporary European nightjar populations likely exhibit a paleo-climate driven migratory divide originating at least ~1.2 Mya, which I suggest may have resulted in range-wide structuring in the contemporary population, as in other Palearctic species (e.g., Olsson et al., 2013; Li et al., 2016) (Fig 7.1 arrow 4). However, range-wide population genetic analysis is needed to resolve this in *C.europaeus* (see Section 7.6.1).

In **Chapter 3**, I used full genome resequencing of historic (museum specimens) and modern nightjar samples from a range extreme population (Britain and the Republic of Ireland), to study spatio-temporal population genomics, during a period of anthropogenic land use change (1841 – 2021) (Ratcliffe, 1984). Over a period of ~180 years the British nightjar population saw a shift from panmixia to weak genetic structure, also exhibiting a significant 34.8% loss in global heterozygosity (**Chapter 3**). The shift towards spatial genetic structuring and loss of heterozygosity were likely reflective of demographic decline (Grundler et al., 2019) driven by habitat loss and fragmentation (Conway et al., 2007; Langston et al., 2007)(Fig 7.1 arrows 2, 6 and 12), with resource specialists, such as nightjar, sensitive to reductions in functional habitat connectivity (Langin et al., 2017; Pasinelli, 2022). These effects were likely further exacerbated by the situation of the British nightjar population at the species range extreme (Eckert, Samis and Lougheed, 2008). The results of **Chapter 3** likely reflect a concerning temporal trend towards increasing spatial structure and decreasing genomic diversity in the British nightjar population. Whilst current heterozygosity and genetic structure in the population do not represent an immediate cause for concern, pre-emptive conservation strategies (e.g. increasing connectivity of breeding sites) may help mitigate this trend worsening in the future (see Section 7.3 for further details).

Ultimately population growth and genetic diversity can be constrained or facilitated by a species breeding behaviour (Fig 7.1 arrows 16 and 17). An effective understanding of a species' mating system then carries substantial applied value and is needed for effective monitoring (Quader, 2005), and in some cases management (i.e. provisioning of suitable nesting sites, and lekking arenas etc.; Hovi et al., 1996; Heinrichs et al., 2018). In **Chapter 4** I synthesised the current knowledge of mating systems, sexual selection, and visual communication in Caprimulgidae. I highlighted the dearth of knowledge in this area across all Caprimulgids as well as nocturnal taxa more broadly, with the majority of extant research in the field concentrated on diurnal taxa and predominantly passerines (Brouwer and Griffith, 2019; but see Roulin et al., 2004; Saladin et al., 2007; Horníček et al., 2017). The biassing of research efforts towards certain phylogenetic groups ultimately limits our understanding of mating

systems, extra-pair paternity (EPP) and signal evolution, which are highly phylogenetically constrained (Bennett and Owens, 2002; Brouwer and Griffith, 2019). Nevertheless, technological, and methodological advancements are enabling researchers to mitigate many of the factors which historically limited work on Caprimulgid breeding behaviour (see **Chapter 4**). Nightjar and more broadly Caprimulgids may in fact may become a useful study system for testing hypotheses regarding visual signalling and the role of plumage ornamentation in nocturnal taxa because they exhibit achromatic dichromatic ornamentation of varying extents across species and demonstrating highly conspicuous display behaviour comparative to other nocturnal avifauna.

In **Chapter 5**, I used a combination of behavioural and genetic data to elucidate the genetic mating system and EPP rates in nightjar and examine the effects of breeding density on EPP occurrence in two British breeding populations. At the two study sites, nightjar exhibited a socially, but not genetically, monogamous mating system with moderate levels of EPP (26% of broods contained extra-pair offspring and 23% of offspring were extra-pair). Among Aves, this places nightjar below the average EPP rates among birds generally (Average % of nests with extra-pair offspring = 33%, average % of extra-pair offspring in broods = 19%, n = 434 species; Brouwer and Griffith, 2019), but above comparable non and near-passerines with similar slow life histories (i.e., average % of nests with extra-pair offspring = 5%, average % of extra-pair offspring in broods = 9%, n = 145 species; Brouwer and Griffith, 2019). Notably, range extreme populations, such as those studied in this thesis, have been shown to exhibit elevated EPP rates compared to those at the range centre (Eckert, Samis and Lougheed, 2008; Corregidor-Castro et al., 2022). This could go towards explaining the high EPP rates in my study populations, although further work would be needed to characterise EPP rates at comparable populations closer to the range centre to test this. EPP rates in my study varied over years and between the two study sites, with nightjar nest and male density also differing between years and sites. Breeding density is a recognised ecological driver of EPP in other species (Mayer and Pasinelli, 2013; Brouwer et al., 2017; Danner et al., 2018; but see Ramos et al., 2014; Mingju et al., 2017) (Fig 7.1 arrow 13). Although, in my study, nest density did not significantly influence EPP rates, male density did appear to positively predict EPP, with EPP being greater in years and at sites with higher local male densities (Fig 7.1 arrow 13). My results from **Chapter 6** suggest that males siring EPO might have been of lower quality than socially paired males, and thus I suggest represent floater males within the population (Fig 7.1 arrow 15). The disparity between the effects of nest and male density on EPP rates likely reflect this, with my results tentatively suggesting that a higher availability of males (as measured by churring male density), but not paired males (as measured by nest density), leads to higher rates of EPP (Fig

7.1 arrow 13). High male, but not nest, densities in socially monogamous birds can occur where there is sufficient resource availability but a low availability of nest sites (i.e. Heinsohn et al., 2007; Heinsohn, 2008) (Fig 7.1 arrow 9). In these cases breeding populations are typically also characterised by genetic polyandry and increased cooperative breeding behaviour (Heinsohn et al., 2007). Notably in six nest sites in my study two males were recorded attempting to provision young (**Chapter 5**). Although in neither case was the 'extra' male found to sire offspring in either brood, this tentatively provides evidence of cooperative provisioning in the *C. europaeus* when considered alongside recent nest site observations of similar behaviour (Padget et al., 2019; see Section 7.2 for more details).

My analysis in **Chapter 6** of the sexually dichromatic white spots displayed by nightjar showed that the size, but not symmetry, of spots increased with age of male nightjars. Thus spot size is predicted to be a signal of male quality in this species (e.g. Forero, Tella and García, 1995; Aragones, Reyna and Recuerda, 1999). Interestingly, paired male nightjars exhibited larger spots than extra-pair males. This has led me to suggest that spots are sexually selected and that the quality of extra-pair males may be lower than socially paired birds (Fig 7.1 arrow 15). Previous research in other Caprimulgids have shown similar age-related variations in spot size although my research is the first to relate this to breeding success in Caprimulgiformes. My results are perhaps not surprising considering the relatively high rates of EPP exhibited by this species. Comparative analysis suggests that the strength of sexual selection, and thus the degree of dimorphic ornamentation, in birds is seemingly driven by EPP rates (Valcu, Valcu and Kempenaers, 2023). My research ultimately helps fill a large phylogenetic knowledge gap in our understanding of mating system, ornamentation and visual signalling evolution in nocturnal taxa. Indeed my work, alongside that conducted in other Caprimulgids (e.g. Forero, Tella and García, 1995; Aragones, Reyna and Recuerda, 1999; Roth, Argyros and Browning, 2003) suggest that nightjar and more generally Caprimulgidae can provide an excellent study system in the growing field of nocturnal visual signalling (Penteriani and Delgado, 2017; see **Chapter 4**), with many of the challenges of studying species at night, locating nests etc. becoming easier with new technologies and approaches (i.e. less-invasive DNA sampling, genomic analysis etc.).

7.2 Synthesis

Figure 7.1 shows the conceptual framework, outlined in **Chapter 1**, that summarises the relationships between different ecological and evolutionary processes that can drive changes

in population genetic variation. Here, I provide a synthesis of how my thesis has provided empirical evidence to support some of the relationships between drivers of population genetic variation of European nightjar, presenting my findings in the context of species conservation (see Section 7.3 for detailed conservation recommendations).

A population's demographic history can underlie the contemporary distribution of genetic variation (Taylor, Jamieson and Wallis, 2007; Nadachowska-Brzyska et al., 2016; Sato et al., 2020) (Fig 7.1 - arrow 5). Ancient fluctuations in population size contribute towards speciation events, contemporary population structure (Fig 7.1 - arrow 4), and vulnerability to anthropogenic threats (Nadachowska-Brzyska et al., 2015, 2016). In my study, nightjar exhibited a fluctuating ancestral N_e pattern, broadly coinciding with changes in global climate which I suggest resulted in range shifts within the species (**Chapter 2**). My results suggest that restriction of the species range to glacial refugia likely led to genetic differentiation across nightjar's contemporary European range (**Chapter 2**) (Fig 7.1 - arrow 4), aligning with genetic structure seen in other Afro-Palearctic species (Hung, Drovetski and Zink, 2013; Nadachowska-Brzyska et al., 2016). Indeed, my results suggest that the British nightjar population might be distinct from those breeding in Central Europe, with gene flow likely ceasing by the LGM (**Chapter 2**). This result is corroborated by the fine scale structuring also seen in the British population (**Chapter 3**), suggesting that contemporary gene flow between continental Europe and Britain may be minimal, and that Europe-wide genetic structuring is likely in the species. If so, this would potentially delineate the British population as a separate conservation unit from that of the European population. Populations at the leading-edge of a species range, at the periphery of the species climate tolerance, can possess adaptive variation important for survival under future climate change, although this is largely reliant on range-edge populations being genetically distinct from those at the centre (Gibson, Van Der Marel and Starzomski, 2009; Hannah et al., 2014; Sexton, Hangartner and Hoffmann, 2014; Tellería, Hernández-Lambraño and Carbonell, 2021). Thus, if the British population is genetically differentiated from those on the continent, it could hold important adaptive potential under future climate change and should be considered a conservation priority (Rehm et al., 2015). However, further range wide population genetic analysis is needed here to resolve the extent of population genetic structure in the species and the relative conservation value of the British population (see Section 7.4.1).

Habitat fragmentation presents a threat to biodiversity (Haddad et al., 2015; Wilson et al., 2016), reducing population connectivity (Fig 7.1 - arrow 2), increasing differentiation and reducing genetic variation (Fig 7.1 - arrow 6) (Nguyen et al., 2022). The effects of

fragmentation on gene flow in Aves are mixed (Amos et al., 2014), likely owing to the vagility of most birds (Pârâu and Wink, 2021). The effects vary among species, with resource specialists especially vulnerable to the loss of functional connectivity through fragmentation of suitable habitat (e.g. Graham et al., 2022; Pasinelli, 2022). Gene flow is a fundamental driver of population genetic variation (Fig 7.1 - arrow 6), and in most cases is imperative for population persistence (Song et al., 2013; Sexton, Hangartner and Hoffmann, 2014). Unfortunately, I was unable to explicitly investigate the effects of habitat availability and fragmentation on gene flow in nightjar (see Section 7.4.2 for study limitations). However, I applied a temporal sampling strategy which allowed me to determine changes in genomic diversity and structure before and after much of the documented land use change in Britain in the 19th and 20th centuries (Ratcliffe, 1984), which included the destruction and conversion of ~78% of nightjar breeding habitat (Ratcliffe, 1984). Thus, I have been able to infer implications of habitat loss and fragmentation on the spatio-temporal population genomic trends observed in **Chapter 3**. My results suggest that gene flow within the British nightjar population has reduced between 1841 and present, likely driven by habitat loss and fragmentation over the same timespan (Fig 7.1 - arrow 2) (Ratcliffe, 1984). Over the same time period I found that heterozygosity declined significantly by 34.8% in the British nightjar population, likely reflective of a corresponding large population decline (Fig 7.1 - arrow 12). This loss in heterozygosity might have in part been caused by the observed reduction in gene flow within the population (Song et al., 2013; Klinga et al., 2020)(Fig 7.1 - arrow 6), or might simply reflect the suspected large decline in population size (Fig 7.1 - arrow 12), with both likely driven by habitat loss and fragmentation over the same time period (Ratcliffe, 1984) (Fig 7.1 - arrows 2 and 6). However, I am unable to make clear conclusions surrounding the drivers of these patterns and implications of changes in gene flow on heterozygosity in the population, and further work here may be required (see Section 7.4). Nevertheless, the change in nightjar population heterozygosity and differentiation in Britain over the last two centuries is clear. Changes in habitat availability and fragmentation are likely the broad drivers of this change, with the effects likely more severe owing to species specialist resource requirements (Fig 7.1 - arrows 2 and 6). In addition, the situation of the British population at the specie's range edge probably exacerbates the recorded pattern of reduced gene flow and declining heterozygosity (Schwartz et al., 2003; Langin et al., 2017). My results show that despite their vagility, long-distance migratory birds, such as nightjar, can exhibit fine scale genetic structuring and significant losses of heterozygosity likely driven by centuries of habitat loss and fragmentation (Ratcliffe, 1984). Moreover, my work highlights the importance of functional connectivity in ensuring gene flow and the maintenance of genetic variation in resource specialist species, regardless of vagility and dispersal potential (e.g. Pasinelli, 2022); see also similar patterns in non-resource specialists; Adams and Burg, 2015).

However, work is needed to explicitly link habitat distribution and availability with the spatio-temporal genomic data from my research (see Section 7.4.2).

My work has provided limited evidence to support the breeding density hypothesis (**Chapter 5**), which postulates that higher breeding densities can lead to increased EPP in birds (Fig 7.1 - arrow 13)(Brouwer et al., 2017; Danner et al., 2018; Mayer and Pasinelli, 2013). My results suggest that the effect of male density (number of churring males within the average home range of a nest) is more important in determining the occurrence of EPP than nest density (**Chapter 5**). Under high male densities, increased mate choice options (Fig 7.1 - arrow 14) are predicted to enable females to more easily pair with high-quality males and thus produce high quality offspring (Prokop et al., 2012; Mingju et al., 2017). Female mate choice is likely important in nightjar, owing to the high level of paternal care required (Cleere, 1998; Cramp and Simmons, 1985). Indeed, my work (**Chapter 6**) suggests that paired males are usually of high quality (large white spots, older males), which may enable these individuals to hold high quality territories and effectively provision young (Wilkin, King and Sheldon, 2009; Guillod, Arlettaz and Jacot, 2016). However, high male densities may also lead opportunities for females to mate with lower quality floater or beta males (Moreno, 2016), or unsolicited copulations (Adler, 2010), potentially limiting the number of offspring sired by high-quality socially paired males (**Chapter 6**). This could result in a risk of reduced paternal care from the socially paired male (Brouwer et al., 2017). Nevertheless, in such cases EPP may provide net gains in population genetic diversity (Fig 7.1 - arrow 17) (Petrie and Kempenaers, 1998; Foerster et al., 2003; Corregidor-Castro et al., 2022), and also lead to cooperative behaviour by unpaired or floater males, particularly if paternity is assumed (Davies et al., 1992; Davies and Hatchwell, 1992; Krams et al., 2022). Indeed, limited availability of optimal resources (habitat and prey), is thought to leave specialist species, such as nightjar, more likely to exhibit cooperative breeding behaviour (Arnold and Owens, 1999), whereby additional provisioning by extra-pair males may aid female reproductive success (Davies and Hatchwell, 1992). Provisioning at nest sites by more than one male has been observed at nightjar breeding sites (e.g. Padget et al., 2019), including those in this study (**Chapter 5**), although the inability to trap at nests throughout the night may have led to an under-recording of this behaviour in this thesis (see Section 7.5.2). Cooperative provisioning then does not appear to be an anomaly in nightjar, but instead a readily displayed behaviour, and may increase the likelihood of offspring fledging (e.g. Davies and Hatchwell, 1992; Nam et al., 2010), providing benefits for females and in turn population productivity (Fig 7.1 - arrow 16) (Hatchwell et al., 2004; Ribeiro et al., 2012). Nightjar may exhibit a system more similar to Dunnocks whereby beta, or lower quality, extra-pair males aid provisioning when paternity is assumed (Davies et al., 1992; Davies and

Hatchwell, 1992; Krams et al., 2022), providing fitness benefits for females and contributing towards population productivity (Fig 7.1 - arrow 16). However, whether extra-pair matings are unsolicited or sought by the female cannot be determined from my limited behavioural data (see Section 7.5.2), and further work is needed to determine the true extent and the ecological factors driving this behaviour in nightjar.

The drivers of the disparity in nest and male density (i.e. average male density (number of churring males) was 34.4% higher than nest density (number of nests) across both sites) at both sites are not clear from my research. However, I suggest that a patchy availability of suitable nesting habitat (as is the case at both study sites; Sharps, 2013; Mitchell, 2019) leads to aggregations of males within the few suitable areas of nesting habitat (Fig 7.1 - arrow 9). This is typified at my study sites by a low nest density and seemingly male bias adult sex ratio at Humberhead (2.25 males per female averaged across years), with the data not available for Thetford. In these areas, high quality males will likely occupy the best quality territories (Lampe and Espmark, 2003; Potti et al., 2018), but may lose paternity to nearby lower quality floater males (Fig 7.1 - arrows 14 and 15) (**Chapter 6**), males occupying nearby lower value territories or floater males who range large distances across the site (Sharps, 2013). Similarly, a low availability of females in the population and a male bias adult sex ratio may also drive the differences in nest and male density recorded in **Chapter 5**. Indeed, female nightjar appear to show a lower site fidelity than males (Silvano and Boano, 2012), although survival only differs slightly between sexes in nightjars (i.e. male = 0.74, females = 0.64; Forero et al., 2001; Silvano and Boano, 2012). However, the low detectability of female nightjar might mean that the actual adult sex ratio is closer to 1:1. Therefore, although I used multiple methods to locate female nightjars and nests in the thesis (i.e., mist-netting, targeted nest site capture, VHF tracking females, cold nest searching etc.), caution is needed when extrapolating from these results. Nevertheless, it is clear that current male-biased methods for ascertaining population size and viability (i.e., churring male counts; Gilbert, Gibbons and Evans, 1998; TBH Partnership, 2023) are likely unrepresentative of the number or distribution of nests at a given site (see Section 7.3.2). My results indicate that paternity is not distributed evenly among males (**Chapter 5 and 6**) which could have implications for population productivity (Quader, 2005). My results also tentatively show that male density does not necessarily reflect nest density at nightjar breeding sites. Therefore, land managers and conservation bodies should apply caution when using this data to make decisions pertaining to conservation status, population viability and habitat management.

Referring back to my theoretical framework (Fig 7.1), my results highlight that a species demographic history can drive contemporary genetic variation and population structure over large spatial scales (**Chapter 2**). Furthermore, my results provide evidence that habitat loss and fragmentation can lead to long term declines in genetic variation and gene flow in highly vagile species (i.e. migratory birds), with the effects likely more severe in resource specialist species and range extreme populations (**Chapter 3**). My work provides evidence that male density can increase EPP in birds (**Chapter 5**), increasing the opportunity for paternity in low quality floater males (**Chapter 6**), which may be linked to male biased adult sex ratios or low and patchy availability of nesting habitat. However, further work is needed here to clarify the drivers of this behaviour. Within nightjar specifically, my research has provided novel insight into the species ancient and recent demographic history, elucidated likely drivers for population change, and has described the species genetic mating system and role of achromatic ornaments in mate choice. Following my theoretical framework (Fig 7.1) and my results, I have been able to outline conservation recommendations for nightjar, and these are discussed in Section 7.3.

7.3 Conservation recommendations

Conserving range edge populations, such as the British nightjar population, is important in maintaining genetic diversity, and thus adaptive potential important in ensuring future range shifts and expansion under climate change (Rehm et al., 2015). Whilst my thesis has not explicitly tested the effects of habitat on nightjar population genomics or breeding behaviour, following the theoretical framework and synthesis presented above I am able to outline key conservation recommendations at a regional and breeding site scale.

7.3.1 Regional scale conservation recommendations

Nightjar habitat has undergone a ~78% decline and fragmentation in Britain over the last two centuries (Ratcliffe, 1984). My results suggest that the loss and fragmentation of this habitat has led to a trend towards increasing regional isolation of populations which may be due to reduced immigration and local population declines. I suggest that an effort is made to increase availability of suitable habitat (heathland, cleared woodland etc.; Cleere, 1998) between known nightjar breeding sites in Britain, with a particular effort to ensure connectivity between East and West clustered populations (**Chapter 3**). Increased availability of suitable

nesting habitat from felled coniferous plantations increased nightjar populations in Britain significantly in the late 20th and early 21st century (Conway et al., 2007; Langston et al., 2007). Similarly, increasing the availability of cleared woodland may similarly provide opportunity for previously locally extinct populations of nightjar to recolonise and reduce population spatial fragmentation. The British government have committed to planting 30,000 hectares of new woodland each year by the year 2025 (DEFRA, 2020), which may go towards providing suitable breeding habitat for nightjar over the next century where felling rotational practices are followed, and new woodland does not encroach on currently cleared or heathland habitat. Potential also exists for heathland habitat to be created and restored across Britain, with successful restoration schemes recorded (e.g. Wilton-Jones and Ausden, 2005a, 2005b), although significant intervention may be required when restoring from nutrient rich agricultural land uses (Pywell et al., 2011). Nevertheless, current anthropogenic land uses such as disused quarries provide viable heathland creation opportunities (CEMEX, 2022; but see Lane, 2020). Covering ~64,000 ha of England, quarries have been highlighted as valuable sites for wader habitat restoration, reducing spatial fragmentation of available breeding and foraging sites (Day et al., 2017), and have similarly been suggested as viable sources of heathland habitat to reduce the spatial fragmentation of heathland in Britain (CEMEX, 2022). Reduced fragmentation of such habitats will likely aid gene flow in other heathland specialist species (i.e. Woodlark; Langston et al., 2007, Adder; François et al., 2021), which have likely suffered similar losses of gene flow, or more severe in cases where dispersal potential is comparatively a lot lower (e.g. Adder; François et al., 2021). However, careful decisions should be made when converting habitats (i.e., through heathland restoration or afforestation), so that equally rare habitats are not destroyed in the process, with biodiversity maintenance kept at the forefront of decision making. Nevertheless, I recommend that an increased availability of suitable habitat either through heathland restoration and creation schemes or felling of plantation woodland would enable increased breeding habitat and thus connectivity for nightjar in Britain.

7.3.2 Breeding site scale conservation recommendations

My results suggest that reproductive success is not evenly distributed among male nightjars and that male and nest density do not correlate with one another (**Chapter 5**). The number of males present within a population then does not necessarily reflect the number of breeding pairs or population productivity. With survey methods highly biased towards male detection, care must be taken when interpreting results from population census data which records the

number of singing males present at a site (Gilbert, Gibbons and Evans, 1998; Docker, Lowe and Abrahams, 2020). Land managers and conservation bodies should be realistic that this data simply reflects the number of singing males present in the population. Paired male nightjars are said to produce a different and distinctive, shorter churr type than unpaired males (Ferguson-Lees et al., 2011; Lowe, Rogers and Durrant, 2014; Docker, Lowe and Abrahams, 2020), which may be discerned by surveyors when collecting male occupancy data. In such cases, this would provide information on the proportion of paired and unpaired birds in the population, which will produce a better reflection of population productivity than the number of males alone. Indeed, advancements in remote audio recording may enable land managers and conservation bodies to more easily monitor and classify paired-status in male nightjars (Zwart et al., 2014; Docker, Lowe and Abrahams, 2020), although the evidence that individuals can be discerned by their churr alone is mixed (Rebbeck et al., 2001; Raymond et al., 2020). Moreover, as new technologies such as thermal imaging and specifically drones carrying thermal cameras become available they will enable nightjar nests to be easily found (Shewring and Vafidis, 2021) and could provide a valuable method, alongside acoustic monitoring to quantify nest and male distribution and density at nightjar breeding sites.

Finally, an increased availability of nesting habitat and management of disturbance at nightjar breeding sites may increase female uptake of nest sites and help reduce the disparity seen between nest and male density seen in this study (**Chapter 5**), which may be driving EPP in nightjar (**Chapter 5 and 6**). Although little is known about nest site microhabitat requirements for nightjar (Sharps, 2013), increasing the availability of suitable nesting habitat (i.e. bare ground, brash, heathland; Cramp and Simmons, 1985) in close proximity to suitable foraging areas may help increase the number of nests at breeding sites. A heterogeneous mix of habitat types (e.g. heath, woodland/scrub edge, grassland) at breeding sites will provide suitable foraging and nesting habitat (Sharps, 2013; Evens et al., 2017a; Mitchell, 2019), although a greater emphasis should perhaps be placed on suitable nesting habitat, as nightjar show little territoriality over foraging habitat (i.e. large overlaps in home ranges found at both site; Humberhead; 61 – 78%, Thetford Forest; 78%; Sharps, 2013; Mitchell, 2019) and are able to forage large distances from nest sites (i.e. > 5km; Mitchell, 2019). Furthermore, disturbance should be limited (i.e. diverting and fencing of paths to stop dog disturbance) where nesting habitat is created in order to insure increased female uptake (Lowe, Rogers and Durrant, 2014). The implementation of measures to increase nesting habitat and thus nests, could lead to lower EPP rates through a reduction in density of unpaired or floater males and reduce the apparent bias ratio of males to nests at both sites (**Chapter 5**). This may have small negative effects on population genetic diversity (i.e. through loss of genetic diversity gains via EPP;

Corregidor-Castro et al., 2022), although this loss will likely be negated by an overall increase in breeding pairs and thus effective population size (Frankham, Ballou and Briscoe, 2010).

7.4 Knowledge gaps and future research

Here I outline the key remaining knowledge gaps identified by the work presented in this thesis and valuable future direction for research. Below I split this section into four key themes.

7.4.1 Range-wide structuring analysis in nightjar

The European-wide population genetic differentiation suggested by the PSMC analysis in **Chapter 2** and fine scale genetic structure in the British nightjar population found in **Chapter 3**, are indicative of European wide genetic structure in nightjar. If structuring exists across the species European range, this could result in isolated breeding populations and separate the wider population into smaller conservation units (Funk et al., 2012), increasing the conservation value for populations at the range edges (Gibson, Van Der Marel and Starzomski, 2009; Rehm et al., 2015), such as the British nightjar population. This work would be achievable through large scale sample collection from the specie's extant range. Using less-invasive methods (i.e. buccal swabs) should provide suitable DNA yields needed for genomics methods (i.e. full genome resequencing), with the nightjar reference genome already available (Secomandi et al., 2021). Furthermore, the application of genomic over genetic (i.e. microsatellite genotyping) methods would enable for smaller sample sizes to be taken from each region or candidate population (Nazareno et al., 2017). The directionality of gene flow can then be studied to better test the central-marginal hypothesis (Schwartz et al., 2003; Langin et al., 2017). In addition to likely drivers of range-wide genomic structuring such as migratory divides, as suggested by my work in **Chapter 2**, could also be investigated.

7.4.2 Nightjar spatio-temporal landscape genomics

My analysis in **Chapter 3** suggests that changes in habitat availability and fragmentation over the last ~200 years have led to a reduced geomic diversity and increased structure in the British nightjar population. However, I was unable to explicitly test the effects of changes in

habitat availability on the genomic patterns observed. Incorporation of historic land use data and landscape genomics approaches would enable for the influence of anthropogenic habitat loss on nightjar genomic structure and diversity to be directly investigated (Holderegger and Wagner, 2008; Amos et al., 2012). This may enable information pertaining to current and historic barriers to fine-scale gene flow to be derived, providing useful information for future conservation measures and habitat creation and management (Holderegger and Wagner, 2008; Epps and Keyghobadi, 2015). Furthermore, this research would have wider-reaching implications in our understanding of the effects of long-term anthropogenic habitat change on highly vagile and resource specialist species, such as migratory birds.

7.4.3 Applications of genomics across less threatened taxa

My work has also highlighted the need for the application of population genomic analysis outside of highly threatened or isolated populations (i.e. Cortes-Rodriguez et al., 2019; Feng et al., 2019; Dussex et al., 2021; Cavill et al., 2022). My research has shown that application of these methods to vulnerable species (i.e., resource specialists and migratory species; Bergamini et al., 2009; Clavel, Julliard and Devictor, 2011; Bowler et al., 2019; Correll et al., 2019) experiencing moderate prolonged population declines can provide valuable insight into anthropogenic impacts on contemporary population structure and genomic diversity (**Chapter 3**). I recommend that spatio-temporal population genomics analysis should be applied to more migratory and habitat specialist species, with populations at range centres and extremes included to understand the range-wide variation. The increasing accessibility and affordability of sequencing and bioinformatics (Narum et al., 2017) and ability to sequence from low yield or poor quality samples (Raxworthy and Smith, 2021; **Chapter 3**) will hopefully allow for a wider application of genomic analysis across a wide range of taxa. Indeed, the use of non-invasive and less-invasive (e.g., buccal swabs and skins scrapes from museum specimens, see **Chapters 3**, **5 and 7**) samples in such studies will allow for large sample sizes (i.e., collected over large spatial scales) in non-model and otherwise cryptic or difficult to study species to be collected, as has been demonstrated in this thesis.

7.4.4 Mating systems and visual signalling in nightjar

In **Chapter 4** I highlighted the value that Caprimulgidae provide as a study system for testing hypotheses in the fields of visual signalling, mate choice and to a lesser extent mating systems.

Moreover, my research in **Chapters 5 and 6** shows that detailed species-specific research can be applied to Caprimulgids (but see limitations; **Chapter 4** and Section 7.5.2). Nightjar and more broadly Caprimulgidae present a potentially valuable study system for researching visual signalling evolution in nocturnal taxa. However, more studies detailing the extent, role and signalling value in achromatic ornamentation are needed across more species of Caprimulgids, building on the work already conducted (e.g. Forero, Tella and García, 1995; Aragones, Reyna and Recuerda, 1999; Roth, Argyros and Browning, 2003; **Chapter 6**). My research has shown that white spots exhibited by male nightjar are likely sexually selected, and future research in other species should also aim to study the effects of male and female spot characteristics on breeding success. Indeed, further research in this area concerning nightjar as well as other Caprimulgids should consider other spot characteristics, namely brightness (Penteriani et al., 2007; Romano, Saino and Møller, 2017) as well as porphyrin-based fluorescence (Camacho et al., 2019), alongside size and structure, which may also act as a signalling mode (Camacho et al., 2019). More generally within Caprimulgidae, if the value of the family as a study system for nocturnal visual signalling is to be realised, future research must focus on extending the study and quantification of secondary sexual characteristics and behaviours to more species.

Finally, further research classifying the mating systems and infidelity rates in other Caprimulgidae would provide valuable data towards future interspecific analyses, with Caprimulgidae demonstrating varying life history characteristics which are thought to be important in mating system evolution, exhibiting varying extents of migratory behaviour (Stutchbury and Morton, 1995; Spottiswoode and Møller, 2004), biparental care (Arnold and Owens, 2002; Brouwer and Griffith, 2019), moderately long lived (Liu, Wu and Liao, 2023), as well exhibiting a wide latitudinal distribution (Valcu, Valcu and Kempenaers, 2021). Characterisation of Caprimulgid genetic mating systems may provide valuable phylogenetic contributions to future comparative analysis of mate choice and EPP, helping address the taxonomic bias and provide valuable data towards future interspecific research within the field of avian mating systems and sexual selection (Brouwer and Griffith, 2019).

7.5 Study limitations

This study has suffered a number of limitations, imposed by resource and data availability, methodological constraints as well as the inherent difficulty of studying a nocturnal and cryptic species of bird, with the COVID-19 pandemic exacerbating many of these issues (See COVID Impact Statement in **Appendix** Section 8.5).

7.5.1 Resource and data constraints

Uncertainty surrounding the breeding population of the individual from SW Europe in **Chapter 2** (Secomandi et al., 2021), which was the only published genome of *C.europaeus* outside of this study, ultimately limited interpretation of the PSMC analysis. Due to the bird in question being sampled on migration (Secomandi et al., 2021), a known breeding site could not be ascertained. Whilst available migration tracking data allowed me to hypothesise likely breeding locations, membership of the individuals to the subspecies *C.e.meridionalis* could not be conclusively ruled out, limiting certainty in my final conclusions surrounding the population genetic structure of nightjar in Europe (**Chapter 2**). However, regardless of the sample collection data availability, my conclusions in **Chapter 2** were ultimately constrained by the sample size used in this study (n = 2). Whilst PSMC analysis enables reliable insight into ancient N_e patterns from single genomes (Li and Durbin, 2011; Mather, Traves and Ho, 2019; Sato et al., 2020), a range-wide population genomics study would be needed to solidify the conclusions drawn in **Chapter 2** pertaining to genetic structure of nightjar across Europe (See Section 7.4.1).

As is common in such studies, my population genomics analysis in **Chapter 3** was ultimately constrained by the quality of hDNA samples and spatio-temporal distribution of samples (Billerman and Walsh, 2019). Whilst the latter can not be compensated for as is determined by the availability of collected specimens over time, significant advancements are being made in overcoming the limitation imposed by hDNA quality in genomics studies (Billerman and Walsh, 2019; Raxworthy and Smith, 2021). By applying methods to control for hDNA quality including damage profiling and rescaling, stringent filtering and down sampling of high quality samples (see **Chapter 3** methods), I was still able to present spatio-temporal genomic trends from my data. As wet lab and bioinformatics techniques continue to improve for inclusion of hNDA, the application of a temporal genomics will become increasingly viable and valuable (Raxworthy and Smith, 2021).

7.5.2 Methodological and species ecology constraints

The use of citizen scientists (Licensed British Trust for Ornithology bird ringers) for data collection for **Chapter 3** enabled sampling of contemporary nightjar populations across the

majority of the species extant range. Without the help of these citizen scientists (see **Acknowledgements**) this data could not have been collected. Similarly, data collection over two sites for **Chapters 5 and 6** would not have been possible without citizen scientists, with the use of easily-learned DNA sampling methods (buccal swabs) enabling the large scale data collection seen in this thesis. Nevertheless, inconsistencies in quality of data, including low-reliability in nest-site associations for EPP estimation (**Chapter 5**) and varying quality in the photography of male nightjar wing and tail spots (**Chapter 6**), meant that ultimately the number of sites included in final analysis for **Chapter 5** and the number of photographed birds able to be included in analysis for **Chapter 6** were reduced. Looking forward, these issues may be addressed by increasing in-the-field training of citizen scientists (not possible during the COVID pandemic), rather than reliance on documentation and remote training (Frigerio et al., 2018). However, researchers should be realistic about the reliability of citizen science data, particularly where reliable behavioural data are required, if detailed in-the-field training can not be provided (Frigerio et al., 2018). Nevertheless, my work has shown the high value in citizen science data collection for population genetic studies. Indeed, with the use of less-invasive sampling methods, citizen science data collection may help alleviate the constraint of sample availability, which can limit population genetic studies (e.g. Willing et al., 2012; Nazareno et al., 2017; Davis et al., 2021; Londono-Gaviria, 2022; Adams et al., 2023).

Finally, my research on nightjar mating system and EPP (**Chapter 5**) were primarily constrained by the difficulties in nest finding and confirming paternal nest site associations, which limited sample sizes. I mitigated these constraints through intensive nest searching and use of VHF tracking to locate brooding or incubating females. However, ultimately the cryptic nesting behaviour of nightjar will have limited the detection of nests at my study sites. Parental associations to nests were achieved by trapping birds at the nest site during provisioning. However, for welfare reasons trapping could not be conducted throughout the night or over multiple occasions. Therefore, provisioning by multiple individuals was probably under-recorded. Furthermore, an inability to collect behavioural data away from nests meant that behaviours such as extra-pair copulations could not be recorded in my study, limiting my ability to assess assumed paternity by provisioning males. Future studies might be able to mitigate this through use of nest cameras and individual marking of birds (see **Chapter 4**) to record provisioning behaviour and parental associations with specific nests. Mass deployment of GPS tags or use of PIT tags (e.g., Mutzel et al., 2013; Schlicht, Valcu and Kempenaers, 2015) may also provide a solution, although such approaches were outside of the financial constraints of my study.

7.6 Concluding remarks

My thesis has provided a valuable overview of the population genomics, mating system and mate choice in a migratory, resource specialist at its range extreme. Such an overview is useful in providing context to contemporary genetic variation, understanding the implications of demographic decline and habitat fragmentation, as well as implications of site level demography on mating systems and mate choice (Quader, 2005; Taylor, Jamieson and Wallis, 2007; Frankham, Ballou and Briscoe, 2010; Allendorf et al., 2022). Over the last five million years ancient climate change has driven fluctuations in nightjar population size and has likely led to genetic structure in the European populations. The species has shown an increase in genetic structure and significant decline in genomic diversity over the last ~200 years in a range edge population, likely as a result of anthropogenic habitat loss and fragmentation. Nightjar join 76% of studied avifauna, being biparental caring, socially monogamous and demonstrating moderate levels of extra-pair paternity, which are at least partially influenced by male density at breeding sites. My research has shown that achromatic plumage ornaments are likely sexually selected in nightjar and that the species along with Caprimulgidae likely present a valuable study system in the field of nocturnal visual communication and mate choice.

From my research I make three key recommendations for nightjar conservation and habitat management in Britain in order to reverse the trends in genomic diversity and structure and ensure high productivity. First, regional scale habitat fragmentation should be addressed and efforts made to restore nightjar breeding habitat outside of known breeding sites to facilitate regional scale landscape connectivity and thus gene flow. Second, current population census methods should be changed to quantify the number of active nests, female nightjar or paired males at breeding sites in order to more accurately reflect population size. Thirdly, the expansion of suitable nesting habitat should be a priority at nightjar breeding sites, particularly where there is already an adequate availability of foraging habitat. This could reduce the density of unpaired males whilst increasing population productivity, although the effect on breeding behaviour should be monitored. Previously, attention has been paid to understanding the foraging ecology and habitat use in order to conserve nightjar (e.g. Lowe, Rogers and Durrant, 2014; Sharps et al., 2015; Evens et al., 2017; Mitchell et al., 2020; Polakowski et al., 2020). However, there is now a need to effectively understand the species breeding behaviour, dispersal, and population connectivity, which I suggest could be limiting species recovery. My research has provided a vital starting point from which future efforts can be built, and also highlights the prospects that genomics provides as a tool to gain valuable

insights into the demography, ecology and breeding behaviour of a cryptic and traditionally 'hard-to-study' species.

Finally, my work has highlighted that the impacts of anthropogenic land use change on population genomic patterns are not reserved to highly threatened, isolated and low vagility taxa, but are also present in vagile, long-distance migratory species. Resource specialist species and range extreme populations are likely particularly vulnerable and future population genomics research should include analysis of similar populations, to understand whether the genomic patterns unearthed in this thesis are ubiquitous across other species with similar life histories. More attention should be paid to the mating systems, mate choice and visual communication of nocturnal taxa, with technological and methodological advancements (i.e. use of less-invasive sampling, nest site remote capture, thermal imaging; McCafferty, 2013; Shewring and Vafidis, 2021), opening up new avenues of research in such nocturnal and cryptic species. A resource specialist, nightjar distribution and abundance over time has likely been driven by habitat availability, shaping contemporary patterns in genomic diversity, both over continental and regional spatial scales, with the species specialist nesting habitat requirement also likely influencing breeding site demography and mating system. It is important that future research concerning avian population genomics or mating systems consider specialist resource requirements, with special attention paid to range-extreme populations, which may provide vital adaptive variation under future climate change.

Chapter 8 Appendix

8.1 Appendix for Chapter 2

8.1.1 Blood sample extraction

The 40 µl blood sample was initially stored in 500 µl SET buffer and frozen. Pre-extraction the sample was then thawed at 37° before being spun down, before adding 10 µl RNase (100 mg/ml), and after resting the sample for 2 minutes at room temperature (RT). After which, 13µl of SDS (20%) was added to the sample and then let to rest 30 min at 37° for continued RNase treatment. 7.5µl of proteinase K (~20mg/ml) was then added and mixed well before being spun down. The sample was then transferred to a water bath (55°C) and left overnight, mixing the sample after one hour. The sample was then spun down again using a centrifuge before adding 50µl of 5M NaCl Spin down, which was mixed and again spun down using a centrifuge. 540µl of phenol was added to the sample mixing well until the solution forms a homogenous mix. The sample was then left to rest for 40-60 minutes at RT under a fume hood and mixed every 5 minutes. Samples were then transferred to a centrifuge and spun down at 10,000 rpm for 15 minutes. 500µl of chloroform/isoamyl alcohol (24:1) was added to a new set of 3mL glass tubes. The supernatant from the phenol samples was then removed and transferred to the tubes containing the chloroform, this was mixed well and centrifuged at 10,000 rpm for 15 minutes. 50µl of NaAc (3M) was added to a new 1.5mL tube before transferring the supernatant from the chloroform stage tubes to the new tubes containing the NaAc. 100µl of 95% ethanol chilled on ice was then added to the sample before mixing well. Mixing was continued until precipitation of DNA was observed. A pipette was used to remove the precipitated DNA from the sample. The precipitated DNA was then rinsed in ice-cold 70% ethanol three times. The DNA was then air dried for 3-minutes until all of the ethanol had evaporated. The DNA was then dissolved in 100µl of 0.1x TE (pH7.8), the sample was kept at 4°C overnight prior to quantification using a Qubit fluorometer (Invitrogen), subsequent dilution and size determination using 0.8% agarose gel at low voltage (max 50V for 1.5h) against lambda DNA. The sample was then sorted at -80°C until PacBio library preparation was required.

8.1.2 Sample yields and summary statistics

Table 8.1 summary statistics from sample extraction and HiFi sequencing.

ID	*Yield (ng/µl)*	*Mean HiFi read length (bp)*	*Number of HiFi Reads*	*Number of HiFi Bases*	*Hifi Median Accuracy*	*Median Number of HiFi passes distribution*
NWE	96	14918	1386719	35605959186	Q31	9

8.1.3 Bootstrapped PSMC plots

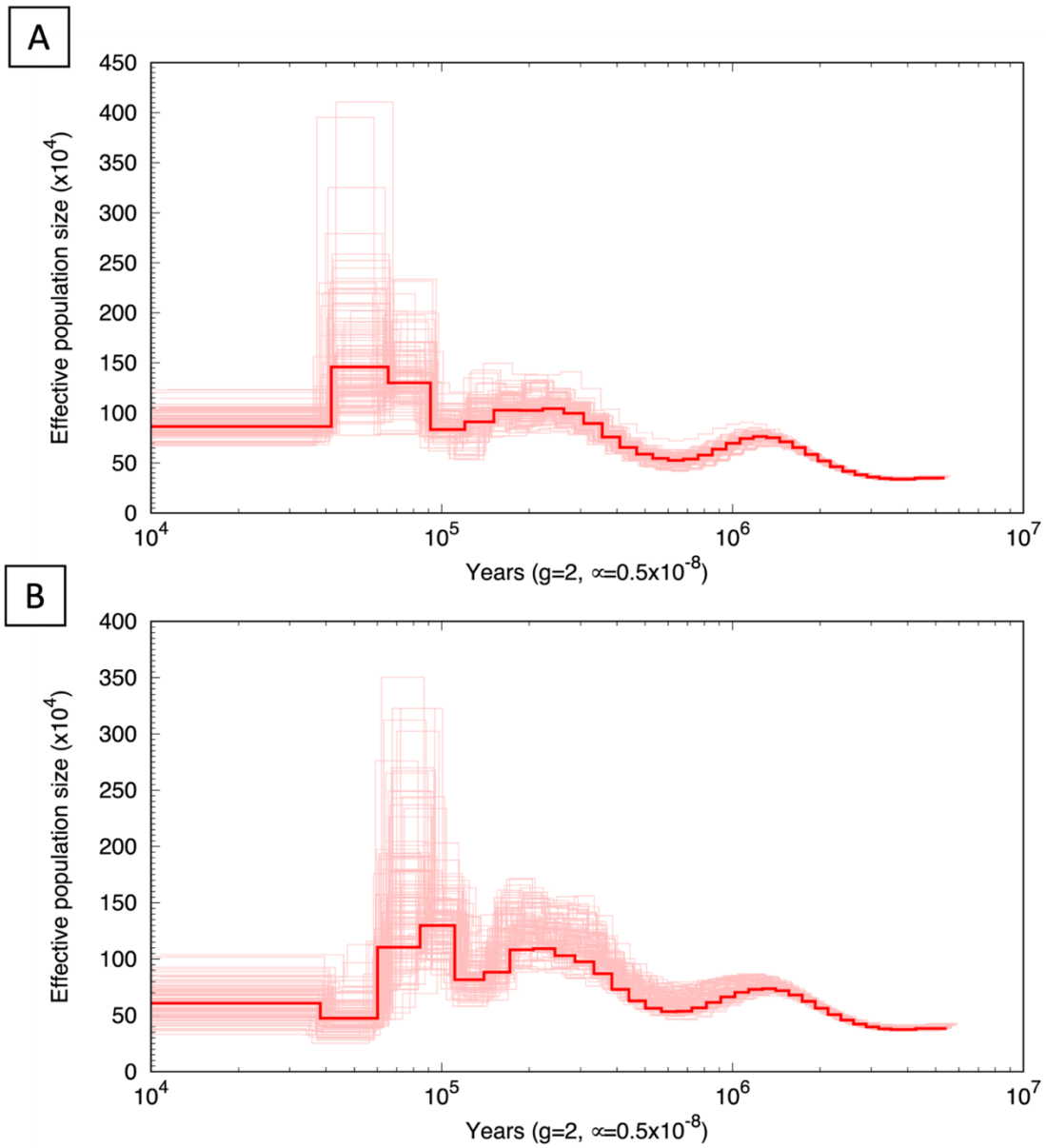


Figure 8.1 Bootstrapped PSMC plot for SE (A) and NWE (B) sampled European nightjar depicting demographic historic (Ne change) over the last ~5 million years (bp), scaled with a mutation rate of 4.6 × 10−9 per site and generation time of 2 years. The X-axis depicts time (in years) on a log scale, with the Y-axis showing effective population size. The red line shows effective population size estimate, with the light pink lines depicting PSMC estimates for 100 bootstrapped sequences.

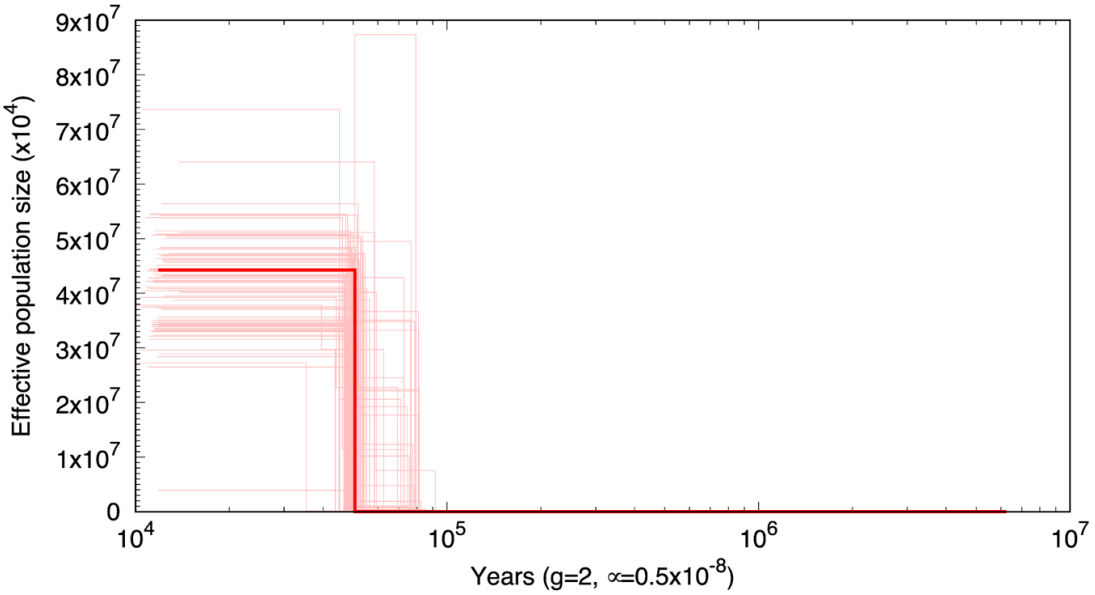


Figure 8.2 bootstrapped PSMC plot for pseudo-diploid genome of the NW and S European sampled populations. The X-axis depicts time (in years) on a log scale, with the Y-axis showing effective population size. The red line shows the effective population size estimate, with the light pink lines depicting PSMC estimates for 100 bootstrapped sequences.

8.2 Appendix for Chapter 3

8.2.1 DNA extraction and quantification

8.2.1.1 Modern samples (buccal swab) extraction

Buccal swabs were removed from storage tubes and placed into 1.5ml microcentrifuge tubes before being cut down using sterile scissors, leaving ~5cm of swab stalk remaining above the tip. Tissue samples were chopped into smaller pieces (2mm^2 cubes) before being transferred to a 1.5ml microcentrifuge tube. Per tube 250µL of Digsol buffer and 10mg/ml 10µL of Proteinase-K (20mg/ml for tissue samples) were added before vortexing of 2.5 minutes and a 24 hr digestion stage at 55°C in a rotating oven (12hr for tissue samples). After incubation a 300µL of Ammonium acetate was added before vortexing for 20 minutes in order to precipitate the proteins. Buccal swabs were then removed from the microcentrifuge tubes and discarded. The remaining solution in the Eppendorfs was then centrifuged for 10 minutes at 13,000, and the supernatant aspirated into a clean 15ml microcentrifuge tube. 1ml of 100% ethanol was added to the solution and Eppendorfs inverted 12 times to precipitate the DNA. Samples were further centrifuged for 10 minutes at 13,000 rpm and ethanol poured off. To rinse the pellet 500µL of 70% ethanol was added to the sample and Eppendorfs again inverted 12 times. A final centrifuge step (5 minutes at 13,000rpm) was used to ensure no DNA loss when pouring off the ethanol. Samples were finally left to air dry for 1 hour and 40µL of LowTE added. Samples were then placed in a water bath for 30 minutes (37 °C) to ensure pellet resuspension, before being stored at -20°C prior to quantification.

8.2.1.2 Historic sample (toepad) DNA extraction

A small amount of Buffer ATL was added to a sterile microscope slide before carefully cutting up the toepad sample in small (≤ 1mm^2) chunks. The sample was then pipetted into 1.5ml Eppendorf, before adding 180µL of Buffer ATL and 20µL of 1M DTT and 20µL 10mg/ml Proteinase-K. The solution was then vortexed thoroughly before being placed in a rotatory incubator at 56 °C overnight. The sample was then pipetted into 1.5ml Eppendorf, before adding 180µL of Buffer ATL and 20µLof 1M DTT and 20µL 10mg/ml Proteinase-K. The solution was then vortexed thoroughly before being placed in a rotatory incubator at 56 °C overnight. Upon removing the sample from the incubator samples were vortexed again for 15 seconds. At

this stage if samples had not appeared to be digested, more Proteinase-K (10mg/ml) was added and the incubation stage performed again. Buffer AL (200µL) was then added before vertexing again and further incubated at 70°C for 10 minutes. Then 200 µl of 100% ethanol was added to the solution before vertexing thoroughly. A spin-column was then inserted into a 2ml collection tube before pipetting the entire mixture through the spin column and centrifuges at 9000 rpm for 1 minute. The spin column was then placed in a new collection tube and 500 µl of Buffer AW2 added through the column before centrifuging again at 13000 rpm for 4 minutes. The spin column is then places in a 1.5ml eppendorf before eluting the DNA by washing with 40 µl Buffer AW, being careful to ensure the buffer makes direct contact with the spin column membrane. The sample is centrifuged again at 8000rpm for 1 minute. Finally, the previous step was repeated by running the 40µL resultant solution back through the column. This helped to maximise the final DNA yield. The final DNA, which was suspended in 40µLof buffer AW was then frozen at -20°C prior to quantification, library preparation and sequencing.

8.2.1.3 DNA quantification

All modern samples (n = 30) were quantified using a using a FLUROstar Optima Spectrophotometer (BMG Labtech). BMG black plate wells were loaded with 2µL of each sample and 7 calf thymus quantification standards of 0, 3.24, 6.49, 12.98, 25.95, 51.9 and 103.8ng/µL. Hoesct dye (200 µl) was added to each well before being run on the fluorometer and readings taken. All samples which exceeded 10ng/µl were diluted to 10ng/µL concentration with Low T.E prior to genotyping. Samples with a DNA concentration below 10ng/µL samples were not dehydrated to increase DNA concentration owing to the low elute (30ul). Historic samples (n = 60) were quantified individually using a Qubit fluorometer.

8.2.2 Library preparation for sequencing

All samples were normalised for a total library input of 20ng, where possible although many of the samples were below this amount. The standard in-house protocol for 1/10th miniaturised NEB Ultra II FS for Mosquito platform was followed, with a fragmentation time of 6 mins and 12 cycles of PCR. Libraries were indexed using unique dual indexes (IDT) and purified with a final 0.6x AMPure XP bead clean. Completed libraries were quantified using Qubit (Thermo Fisher) and fragment sizes assessed using the Fragment Analyzer (Agilent) It was noted that the museum samples produced very low outputs with small insert lengths which would not have sequenced well. The modern samples produced much larger fragment insert lengths

which were suitable for sequencing. The museum samples which were suitable for sequencing, were equimolar pooled and purified with a 0.7x AMPure XP bead purification and eluted in 20ul. The final concentration and library size was assessed using the Agilent Fragment Analyzer. The protocol below was then followed for the museum samples.Museum samples were repeated without fragmentation using the NEB Ultra II DNA Kit. Up to 100ng per sample was used as input, where available. The protocol was followed according to the kit manual, without the optional size selection step due to the small insert size. The samples were indexed using 10 cycles of PCR to match the library prep for samples prepared using the mosquito, using unique dual indexes (IDT) and purified with a 0.9x AMPure XP bead clean. The concentration of the final libraries was assessed using Qubit and the sized using the Fragment Analyzer (Agilent) and equimolar pooled. The final pool was size selected using the Pippin Prep, selecting for 180-600bp on a 2% gel. The size selected pool was purified with a 1:1 AMPure XP bead purification and eluted in 20 µl nuclease free water. The final concentration and library size was assessed using the Agilent Bioanalyzer.

The quantity and quality of the pool was assessed by the Bioanalyzer and subsequently by qPCR using the Illumina Library Quantification Kit from Kapa on a Roche Light Cycler LC480II according to manufacturer's instructions. Briefly, a 10 µl PCR reaction (performed in triplicate for each pooled library) was prepared on ice with 8 µl SYBR Green I Master Mix and 2 µl diluted pooled DNA (1:1000 to 1:100,000 depending on the initial concentration determined by the Qubit® dsDNA HS Assay Kit). PCR thermal cycling conditions consisted of initial denaturation at 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds (denaturation) and 60°C for 45 seconds (annealing and extension), melt curve analysis to 95°C (continuous) and cooling at 37°C (LightCycler® LC48011, Roche Diagnostics Ltd, Burgess Hill, UK).

Following calculation of the molarity using qPCR data, template DNA was diluted to 300pM and denatured for 8 minutes at room temperature using freshly diluted 0.2 N sodium hydroxide (NaOH) and the reaction was subsequently terminated by the addition of 400mM TrisCl pH=8. To improve sequencing quality control 1% PhiX was spiked-in.

The libraries were sequenced on the Illumina® NovaSeq 6000 platform (Illumina®, San Diego, USA) following the standard workflow over 1 lane of an S4 flow cell, generating 2 x 150 bp paired-end reads.

8.2.3 Historic and modern sample information

Table 8.2 Sample details (sample type, collection date and location) for all samples sequenced for Chapter 3. Both museum and modern specimens are presented below. Accession numbers are given for all museum samples, and individual sample ID's given for all modern specimens. Yields are given for each sample as ng/µL, No Reading (NR) is given for samples from which I was unable to take a reading owing to low yields. Museum Collection pertains to the museum from which the samples are stored, abbreviations refer to as follows; BM: Birmingham Museum, LWM: Liverpool World Museum, NHM: Natural History Museum (London), NMINH: National Museum of Ireland, NMS: National Museum of Scotland, RAMM: Royal Albert Memorial Museum, UMZC; Cambridge University Museum of Zoology, YMT: York Museum Trust.

						Collection Location		Yield (ng/µL)
Accession no / Sample ID	*Sample type*	*Museum Collection / Breeding Site*	*Temporal Classification*	*Collection Year*	*Collection Region*	Latitude	Longitude	
1898.4.188	Toe pad	NMS	Historic	1866	East Anglia	52.628869	1.2933072	6.60
YORYM_B815	Toe pad	YMT	Historic	1866	East Anglia	52.901044	0.8828071	12.30
T2863	Toe pad	LWM	Historic	1867	East Anglia	52.90533	1.3370569	0.14
1954 z1.780	Toe pad	BM	Historic	1891	East Anglia	52.666667	1	12.70
1954 z1.778	Toe pad	BM	Historic	1891	East Anglia	52.666667	1	18.30
1919.12.10.1184	Toe pad	NHM	Historic	1892	East Anglia	52.207146	1.6194206	0.48
1934.1.1.3223	Toe pad	NHM	Historic	1896	East Anglia	52.535437	1.4812472	1.63
1934.61.2040	Toe pad	NMS	Historic	1906	East Anglia	52.638138	-1.6573486	21.00
1934.61.2041	Toe pad	NMS	Historic	1907	East Anglia	52.608193	1.7271394	17.90

1934.61.2042	Toe pad	NMS	Historic	1909	East Anglia	52.608193	1.7271394	48.60
1953.76.106	Toe pad	NHM	Historic	1935	East Anglia	52.949639	1.0902069	0.65
1970.76.237	Toe pad	NMS	Historic	1911	Midlands	53.255229	-1.9151678	17.90
NMINH: 1916.126.196	Toe pad	NMINH	Historic	1897	Ireland	54.059531	-6.7594287	7.18
NMINH: 1916.128.276	Toe pad	NMINH	Historic	1902	Ireland	52.890911	-6.1039447	2.78
NMINH: 1916.128.273	Toe pad	NMINH	Historic	1904	Ireland	52.890911	-6.1039447	1.39
NMINH: 1916.128.275	Toe pad	NMINH	Historic	1905	Ireland	52.202844	-6.207274	4.60
NMINH: 1905.127.1	Toe pad	NMINH	Historic	1905	Ireland	52.399303	-8.5744751	16.10
NMINH: 1909.157.1	Toe pad	NMINH	Historic	1907	Ireland	53.396201	-7.7240549	4.32
NMINH: 1916.126.198	Toe pad	NMINH	Historic	1908	Ireland	52.202844	-6.207274	0.95
NMINH: 1911.257.1	Toe pad	NMINH	Historic	1911	Ireland	54.173333	-7.151111	3.78
NMINH: 1916.126.199	Toe pad	NMINH	Historic	1913	Ireland	52.460187	-6.6065155	5.26
NMINH: 1916.126.197	Toe pad	NMINH	Historic	1914	Ireland	52.980502	-6.0470191	3.80
D1861	Toe pad	LWM	Historic	1850	East	53.456963	-2.8531422	NR
2010.11.29	Toe pad	NHM	Historic	1898	West	53.32737	-3.0963764	0.29
1934.61.2043	Toe pad	NMS	Historic	1909	West	54.18204	-2.8066213	17.70
1898.12.2.387	Toe pad	NHM	Historic	1870	Scotland	56.156196	-4.6562105	NR
UMZC 21/CAP/1 k/2	Toe pad	UMZC	Historic	1872	Scotland	58.2501	-4.5000946	0.84
2013.3.132	Toe pad	NHM	Historic	1873	Scotland	55.575297	-3.833333	NR
UMZC 21/CAP/1 k/1	Toe pad	UMZC	Historic	1874	Scotland	57.595819	-4.4274167	0.15

1907.31.2	Toe pad	NMS	Historic	1902	Scotland	57.613034	-3.0935703	32.80
1965.38.336	Toe pad	NMS	Historic	1916	Scotland	58.360927	-6.533796	20.80
1918.8.19.1	Toe pad	NHM	Historic	1918	Scotland	54.903641	-5.0275822	NR
1910.187	Toe pad	NMS	Historic	1920	Scotland	59.532591	-1.6310434	10.70
1931.127.2	Toe pad	NMS	Historic	1931	Scotland	59.532591	-1.6310434	43.80
YORYM_B814	Toe pad	YMT	Historic	1984	Scotland	56.696636	-4.1735172	7.00
1885.7.16.7	Toe pad	NHM	Historic	1879	South	51.081463	-0.24428481	0.18
1890.12.16.55	Toe pad	NHM	Historic	1888	South	52.034603	-0.80136937	0.18
1983.929	Toe pad	LWM	Historic	1888	South	50.904891	-1.4043127	0.75
1903.8.4.1	Toe pad	NHM	Historic	1903	South	51.392217	0.24508251	1.09
1951.13.718	Toe pad	NHM	Historic	1936	South	51.453802	-0.97375977	0.47
1951.13.719	Toe pad	NHM	Historic	1936	South	51.050636	-1.6786005	1.48
1951.13.721	Toe pad	NHM	Historic	1937	South	51.050636	-1.6786005	3.38
1951.13.722	Toe pad	NHM	Historic	1941	South	51.050636	-1.6786005	1.17
LIV.2010.1.15	Toe pad	LWM	Historic	1946	South	55.040596	-1.5664713	1.30
UM2C 21/CAP/1/12/7	Toe pad	UMZC	Historic	1849	South	51.845065	-2.1481836	3.06
NML-V2 1.12.08.197	Toe pad	LWM	Historic	1897	South	51.436062	-2.8526531	1.91
NML-V2 1.12.08.198	Toe pad	LWM	Historic	1899	South	51.436062	-2.8526531	1.51
1914.9.30.544	Toe pad	NHM	Historic	1903	South	50.725152	-2.9365094	0.10
NML-V2 1.12.08.199	Toe pad	LWM	Historic	1903	South	51.364874	-0.44154574	11.10

NML-V2 1.12.08.200	Toe pad	LWM	Historic	1905	South	51.364874	-0.44154574	1.34
1934.1.1.3225	Toe pad	NHM	Historic	1913	South	50.724141	-3.6607788	0.20
1920 z19.1	Toe pad	BM	Historic	1916	South	52.652339	-2.6435641	1.35
1920 z19.3	Toe pad	BM	Historic	1916	South	52.652339	-2.6435641	18.50
12/1921/75 RAMM	Toe pad	RAMM	Historic	1921	South	50.468826	-3.5314071	NR
NMINH: 1913.34.20	Toe pad	NMINH	Historic	1897	South	51.93802	1.2004609	3.52
UM2C 21/CAP/1/12/6	Toe pad	UMZC	Historic	1841	Midlands	52.210949	-2.1569555	1.65
1951.13.724	Toe pad	NHM	Historic	1944	Midlands	52.993319	-1.8949124	1.15
1934.1.1.3222	Toe pad	NHM	Historic	1892	East	54.056893	-2.8228925	1.60
1938.3.19.39	Toe pad	NHM	Historic	1918	East	53.476472	-0.074366978	0.15
NML-V2 1983.928	Toe pad	LWM	Historic	1948	East	53.945752	-1.045865	1.62
778	Buccal swab	Canford Heath	Modern	2019	South	50.764259	-1.9646645	2.23
7747	Buccal swab	Canford Heath	Modern	2019	South	50.764259	-1.9646645	1.84
780	Buccal swab	Canford Heath	Modern	2019	South	50.764259	-1.9646645	91.59
3422	Buccal swab	Cannock Chase	Modern	2019	Midlands	52.760503	-2.0077515	5.86
6392	Buccal swab	Cannock Chase	Modern	2019	Midlands	52.760503	-2.0077515	3.70
3423	Buccal swab	Cannock Chase	Modern	2019	Midlands	52.760503	-2.0077515	2.51
3120	Buccal swab	Dersingham Bog	Modern	2019	East Anglia	52.828048	0.47693808	11.33
3132	Buccal swab	Dersingham Bog	Modern	2020	East Anglia	52.827272	0.47717571	4.22
3126	Buccal swab	Dersingham Bog	Modern	2019	East Anglia	52.825311	0.48144342	9.70

S3A	Tissue	Dumfries & Galloway	Modern	2021	Scotland	54.983751	-4.5859506	88.30
8806	Buccal swab	Dumfries & Galloway	Modern	2021	Scotland	54.983742	-4.58595	9.97
8805	Buccal swab	Dumfries & Galloway	Modern	2021	Scotland	54.985869	-4.5767701	3.38
S2_A	Tissue	Humberhead	Modern	2021	East	53.614252	-0.89345455	73.32
3314	Buccal swab	Humberhead	Modern	2021	East	53.532632	-0.948302	3.53
S1_C	Tissue	Humerbhead	Modern	2021	East	53.565904	-0.93585491	50.97
5707	Buccal swab	B&LV	Modern	2020	South	50.842757	-1.6319529	5.78
8969	Buccal swab	B&LV	Modern	2020	South	51.239058	-0.82401967	3.67
5841	Buccal swab	B&LV	Modern	2020	South	51.259916	-0.81475963	2.62
6213	Buccal swab	Sherwood	Modern	2020	Midlands	53.145123	-1.0800934	4.35
281	Buccal swab	Sherwood	Modern	2020	Midlands	53.145123	-1.0800934	4.07
1211	Buccal swab	Sherwood	Modern	2020	Midlands	53.200819	-1.0956388	2.76
2936	Buccal swab	Thetford	Modern	2020	East Anglia	52.414062	0.68553602	8.10
988	Buccal swab	Thetford	Modern	2020	East Anglia	52.434888	0.71546405	6.72
9632	Buccal swab	Thetford	Modern	2020	East Anglia	52.425835	0.59981557	11.46
6715	Buccal swab	Wales	Modern	2020	West	52.263988	-4.1088833	28.66
7033	Buccal swab	Wales	Modern	2020	West	52.263988	-4.1088833	5.30
7099	Buccal swab	Wales	Modern	2021	West	53.058291	-3.4864888	2.27
294	Buccal swab	NYMR	Modern	2020	East	54.423406	-1.1009127	10.48
213	Buccal swab	NYMR	Modern	2020	East	54.319329	-0.83627721	20.17

211	Buccal swab	NYMR	Modern	2020	East	54.35512	-0.76074874	8.35
364	Buccal swab	FoD	Modern	2020	West	51.840253	-2.506384	12.31
365	Buccal swab	FoD	Modern	2020	West	51.840253	-2.506384	4.86
367	Buccal swab	FoD	Modern	2020	West	51.839601	-2.492598	6.14
338	Buccal swab	Haldon	Modern	2020	South	50.567608	-3.6456695	1.34
337	Buccal swab	Haldon	Modern	2020	South	50.646793	-3.5777113	4.41
336	Buccal swab	Haldon	Modern	2020	South	50.646793	-3.5777113	1.26

8.2.4 Supplementary data

Below is a link to the supplementary data sheet containing summary statistics for all samples after every major trimming and filtering steps.

Summary statistics datasheet can be found as Supplementary data sheet (Day_202034089_C3_Summary_Stats.xlsx) attached with this thesis or alternatively at this link: https://rb.gy/lzk9k

8.2.4.1 Software packages Used

Table 8.3 Details of software and packages used in Chapter 3.

Package / Software name	*Version*	*Citation*	*Link to software repository*
QGIS	V 3.30.0	QGIS Association, (2023)	http://www.qgis.org
Cutadapt	V 1.2.1	Martin, (2011)	https://rb.gy/wnpgi
Sickle	V 1.33	Criscuolo and Brisse, (2013)	https://rb.gy/wit1w
BWA	V 0.7.17	Li and Durbin, (2009)	https://rb.gy/9aan4
Samtools	V 1.17	Li et al., (2009a)	http://www.htslib.org/
Picard toolkit	V 3.0	Broad Institute, (2023)	https://rb.gy/tqgf5
Mapdamage	V 2.2.1	Jónsson et al., (2013)	https://rb.gy/a56y5
ANGSD	V 0.938	Korneliussen et al., (2014)	https://rb.gy/o95e3
VCFTOOLS	V 0.1.17	Danecek et al., (2011)	https://rb.gy/175dy
PCAngsd	V 0.938	Korneliussen et al., (2014)	https://rb.gy/8pcdc
NGSld	V 1.1.0	Fox et al., (2019)	https://rb.gy/2p2gv
NGSadmix	V 3.2	Skotte et al., (2013)	https://rb.gy/mkcvm
CLUMPAK	beta	Kopelman et al., (2015)	https://rb.gy/kogeq
R	V 4.1.2	R Core Team, (2020)	https://ropensci.org/
ggplot2	V 3.4.1	Wickham, (2016)	https://rb.gy/7cu7c

8.2.5 Sample DNA yields

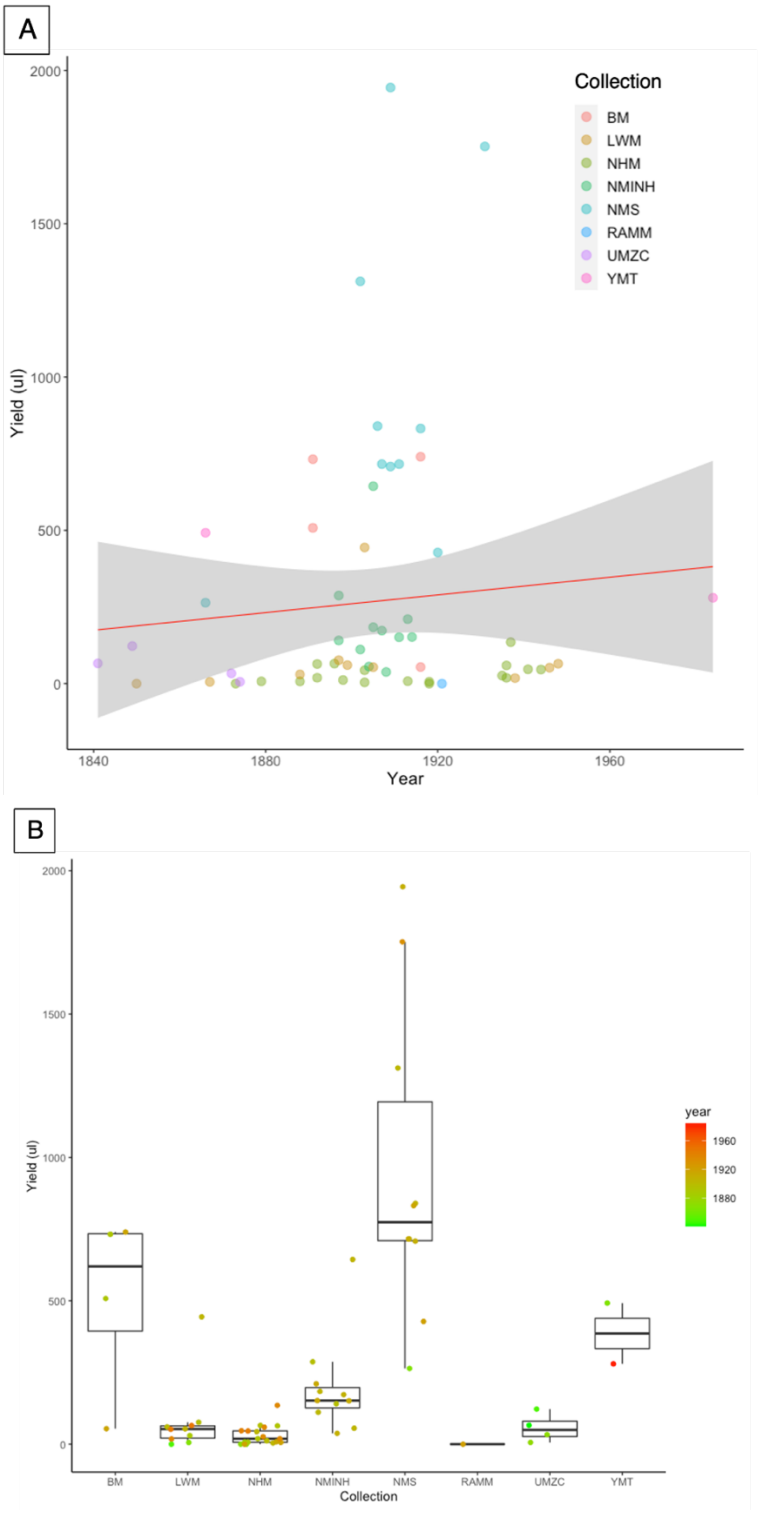


Figure 8.3 relationship between yield and year (A) and collection of origin (B) of museum specimens included in the Illumina sequencing. Red line in plot A represents the trend line with the grey area shading representing 95% confidence intervals, with museum collections represented by different colour points. In plot B collection year is presented as colour gradient on overlain data point for context. Museum collection codes on both figures are as per Table 8.2.

8.2.6 Structuring analysis

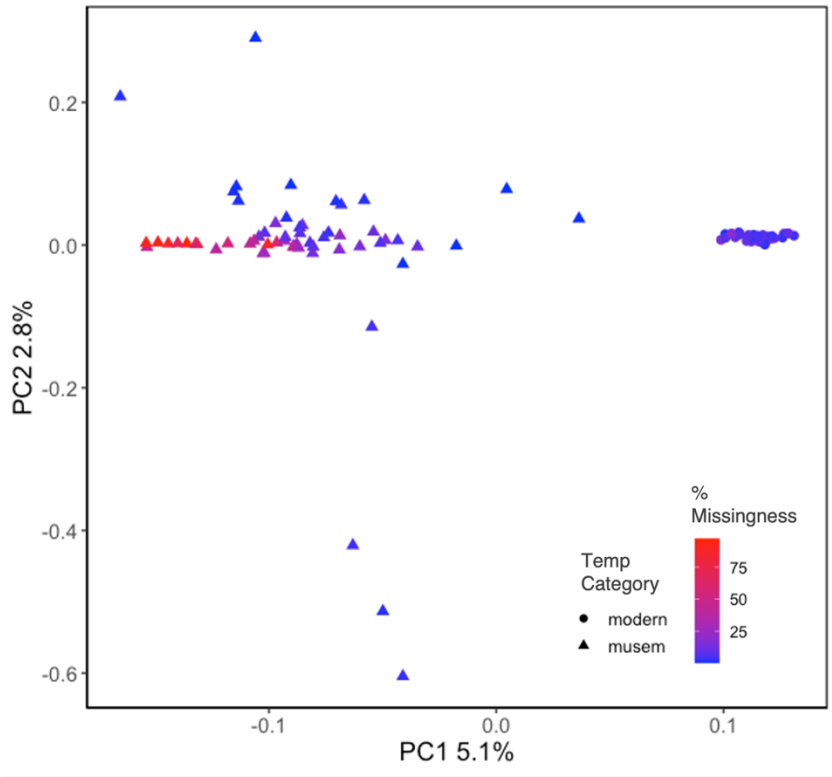


Figure 8.4 PCA of all samples (historic and modern) from the down sampled data set with relative missingness (%) presented as colour ramp.

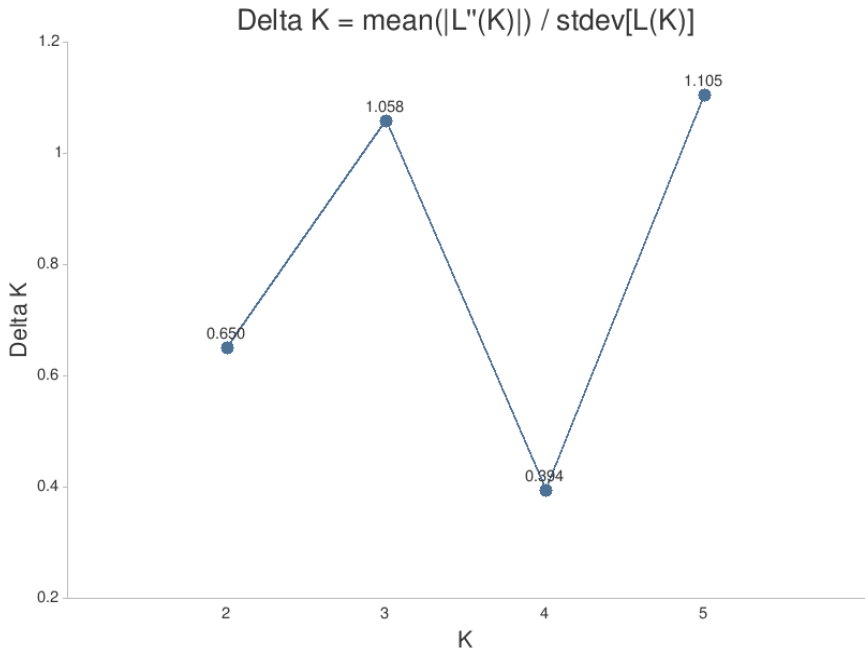


Figure 8.5 optimal (Delta) *K* for admixture analysis, where *K* = the number of sub or ancestral populations from which the total population is comprised. Figure generated from CLUMPAK optimal *K* analysis.

8.3 Appendix for Chapter 5

8.3.1 Churring male survey methods

As per Gilbert, Gibbons and Evans (1998) visits were conducted in early-mid June and the mid-late July respectively. Transects through suitable habitat were walked from 20 minutes after sunset, or 90 minutes before dawn, at a steady pace (4 – 5.5 kph), with stops taken every few minutes to listen for churring (singing) nightjar. When heard, the position of churring males were marked on a map and GPS locations taken. If 'two birds' were heard simultaneously, this was noted so to avoid under-representation, with both individuals' locations recorded. A second individual was recorded when a bird was heard to call from two separate locations ≥400m apart < 30 seconds apart. Otherwise, the second call was attributed to the initial male and the direction to which it moved was recorded. Data from the two visits were consolidated, taking centroids between call locations for individual calling birds.

8.3.2 DNA quantification and sample preparation

DNA samples were quantified using a FLUROstar Optima Spectrophotometer (BMG Labtech). BMG black plate wells were loaded with 2 µl of each sample and 7 calf thymus quantification standards of 0, 3.24, 6.49, 12.98, 25.95, 51.9 and 103.8ng/µl. Hoesct dye (200 µl) was added to each well before being run on the fluorometer and readings taken. All samples which exceeded 10ng/µl were diluted to 10ng/µl concentration with Low T.E prior to genotyping. Samples with a DNA concentration below 10ng/µl samples were not dehydrated to increase DNA concentration owing to the low elute (30 µl).

8.3.3 Microsatellite marker library preparation and testing

The species-specific microsatellite library (CAE markers) was constructed by GenoScreen (Hasselt University, Belgium) using nuclear DNA from 14 birds from a breeding population of nightjar in Belgium. Twenty-one microsatellites from the 860 loci sequenced were used to design primer sets. However, I retained only 6 from initial testing of allele frequencies and genotyping (Table 8.4), with 10 failing to amplify across an initial test of 20 individuals from a single breeding population in the UK and a further 4 displaying high null allele frequencies (>20%) and out of HWE. Markers were selected with preference for tri and tetra-nucleotide

repeats, with motif repeats ≥10 in most instances (Table 8.4). Primer 3 v.0.4.0 was used to design primer pairs (product size range 100 – 193bp), with primers selected to have an optimum melting temperature of 60 °C, an optimum length of 20 bp, presence of a G/C clamp and all other parameters set to default. Forward primers were 5'-labelled with a fluorescent dye (either 6-FAM or HEX). Finally, primer sequences were then assessed using BLAST software to ensure selection of unique sequences (Altschul et al., 1997). Further to the 6 species-specific loci, five cross-species microsatellite markers were selected from (Dawson et al., 2010, 2013) to be amplified. Both 'CAM' and 'TG' markers have been shown to amplify in both passerines and non-passerines alike (Dawson et al., 2010, 2013), although none have been amplified at any nightjar species at the time of writing. Polymerase chain reaction (PCR) amplification for all markers was performed in a singleplex format at 2 µl reaction volumes (1-20ng air-dried DNA, 1 µl of 0.2 µM primer mix and 1 µl QIAGEN Maser Mix; QIAGEN Inc) with 20 µl mineral oil overlaid and were conducted using a DNA Engine Tetrad2, Peltier Thermal Cycler (MJ Research). PCR steps were as follows; initial denaturisation at 95 °C for 15 min, followed by 35 cycles of 94°C for 30 seconds, 56°C for 20 for 90 seconds and 72°C for 60 seconds, with a final 15 minutes at 60°C after cycling.

8.3.4 Results of DNA yields and microsatellite loci testing

DNA concentrations ranged from 0.3ng/µl to 61ng/µl in a 30 µl elution (average yield = 7.6ng/µl, stdev = 7.86). Of the 10 microsatellites tested all amplified successfully across the majority of individuals (see Table 8.4). Two markers (cae_11666 and cae_18432) did not conform to Hardy-Weinberg equilibrium. All remaining markers exhibited null allele frequencies well-within the threshold acceptable for accurate parentage analysis (20%; Table 8.4; Dakin and Avise, 2004). Within the remaining 8 loci, no significant linkage disequilibrium was found. The average number of alleles among the 8 loci was 10.5, ranging from 2 to 20 (Table 8.4), with the polymorphism information content (PIC) being highly informative (>0.7) in 75% of markers, with the average PIC across loci being 0.69 (Table 8.4). Observed heterozygosity across populations ranged from 0.229 to 0.911, being lower than the expected heterozygosity across the majority of markers (Table 8.4). The combined non-exclusion probability was sufficient in reliably determining genetic parentage, being 0.00339 for the first parent, and 0.000109 and 0.0000001 for the second parent and parent pairs respectively. The combined non-exclusion probability for sib-identity across the 8 loci was 0.000.

8.3.5 DNA yields and microsatellite loci

DNA concentrations ranged from 0.3ng/µl to 61ng/µl in a 30 µl elution (average yield = 7.6ng/µl, stdev = 7.86). Of the eight loci selected for genotyping, all exhibited null allele frequencies well-within the threshold acceptable for accurate parentage analysis (20%; Table 5.2; Dakin and Avise, 2004). Observed heterozygosity across populations ranged from 0.229 to 0.911, being lower than the expected heterozygosity across the majority of markers (Table 8.4). The combined non-exclusion probability was sufficient in reliably determining genetic parentage, being 0.00339 for the first parent, and 0.000109 and 0.0000001 for the second parent and parent pairs respectively.

8.3.6 Microsatellite loci Information

Table 8.4 microsatellite loci attributes of novel European nightjar loci (CAE) and cross-species markers (CAM and TG) as per Dawson et al., (2013)

Locus	*Primer sequences (5' – 3'), (F = forward, R = reverse)*	*Repeat motif*	*n*	*No of alleles*	*H_O/H_E*	*Est. null allele freq*	*NE-1p*	*NE-2p*	*NE-SI*	*PIC*
CAE _11666 ***	F : [6-FAM]CCCTGATGTTACCAACAAACTTC R:ACATAACTTAAATTGGCTAGAATCCAC	$[ATTT]_{12}$	36	12	0.528 /0.565	0.2387	0.453	0.291	0.038	0.837
CAE _18432 ***	F: [6-FAM]ACTGCAGCCGGTTAGAGTG R: TGGTACGCTGCAAAACTCTC	$[AGC]_{17}$	50	13	0.620 / 0.830	0.1479	0.510	0.339	0.352	0.818
CAE _6447	F: [HEX]CATTGCTGCCTGTCGTTATG R: CCTGGGCTGTACTTTGACAAG	$AAAC_7$	45	11	0.556 / 0.555	-0.0111	0.817	0.635	0.531	0.531
CAE _17145	F: [6-FAM]ACATCAGGCTTGGCGAGTC R: GCTGTGCAGAGGTTTCGTTC	CAG_{13}	48	12	0.854 /0.870	0.0046	0.423	0.277	0.328	0.846
CAE _16217	F: [HEX]AGGAATGTGGGTTGCTGTG R: GTCAGACCAGCCTTCTCCAG	GAT_{11}	49	20	0.857 / 0.871	0.0081	0.415	0.261	0.327	0.851

CAE _23564	F: [6-FAM]CAGAAACTGGTAAAGCACAAAGC R: TTCCACCTCATTAAACTCTCTTCC	AG_{15}	49	20	0.878 / 0.936	0.0276	0.257	0.148	0.010	0.929
CAM-06	F: [HEX]GTGATGGTCCAGGTCTTGC R: CAAGAGGAACAGATGAGGGTC	AT_8 (ZF) AT_{11} (CH)	48	2	0.375 / 0.399	0.0259	0.922	0.842	0.664	0.317
CAM-18	F: [HEX]TTAAGAAGTTTACACCCAGCG R: GCTAAATAACAGAGCCAGGAAG	TA_{11} (ZF) TG_{11} (CH)	47	5	0.723 / 0.646	-0.0573	0.785	0.628	0.478	0.577
TG01-000	F: [6-FAM]TTGCTACCA**R**AATGGAATGT R: TCCTAACCATGAGAAGCAGA	AT_8 (ZF) AT_9 (CH)	44	6	0.818 / 0.726	-0.0736	0.674	0.489	0.418	0.687
TG02-012	F: [6-FAM]-TTGGGCAAAGATGATATGAATG R: AGCCAGGTCCAGTTTCTAAGC	$AT_{4,7}$ (ZF) $AT_{4,10}$ (CH)	43	4	0.628 / 0.631	-0.0110	0.791	0.635	0.465	0.564

**** Loci with significant departures from Hardy-Weinberg Equilibrium.*

In cross-species markers (CAM and TG): forward and reverse primer sequences match 100% to zebra finch and 86–100% to chicken Gallus gallus when the degenerate bases are accounted for. The degenerate bases used in the primer sequences shown in bold and underlined, ***R*** *= A or G.*

ZF / CH – in cross-species markers (CAM and TG) where repeat motifs with greatest number of repeats differ between chicken Gallus gallus (CH) and zebra finch Taeniopygia guttata (ZF), the corresponding species is shown (Dawson et al., 2010, 2013).

8.3.7 Breeding density model results

Table 8.5 Results of multiple GLM Model 2, examining ability of the distance to the nearest nest (NN) and nearest churring male (NCM), site and year as well as the interaction of density metrics with site and year to predict EPP as a binary response. Model presented after backwards and forwards stepwise selection for AIC. P-values of the Wald test are also presented.

Predictors	*Estimates*	*CI*	*Z*	*P*
(Intercept)	- 1.010	-2.95 – 1.202	-1.049	0.294
NCM	-0.001	-0.01 – <0.01	-0.578	0.564
Site (Thetford)	1.282	-1.61 – 4.49	0.731	0.464
Year (2020)	1.188	-5.19 – 2.36	-0.631	0.528
Year (2021)	-0.605	-3.74 – 1.92	-0.417	0.676
NN * Site (Thetford)	-0.011	-0.04 – < 0.01	-1.193	0.233
NN * Year (2020)	0.014	0.001 – 0.04	1.501	0.133
NN * Year (2021)	0.008	<0.01 – 0.02	1.031	0.303
Observations	46			
Tjur R^2	0.235			
AIC	57.107			

8.4 Appendix for Chapter 6

8.4.1 Data distribution

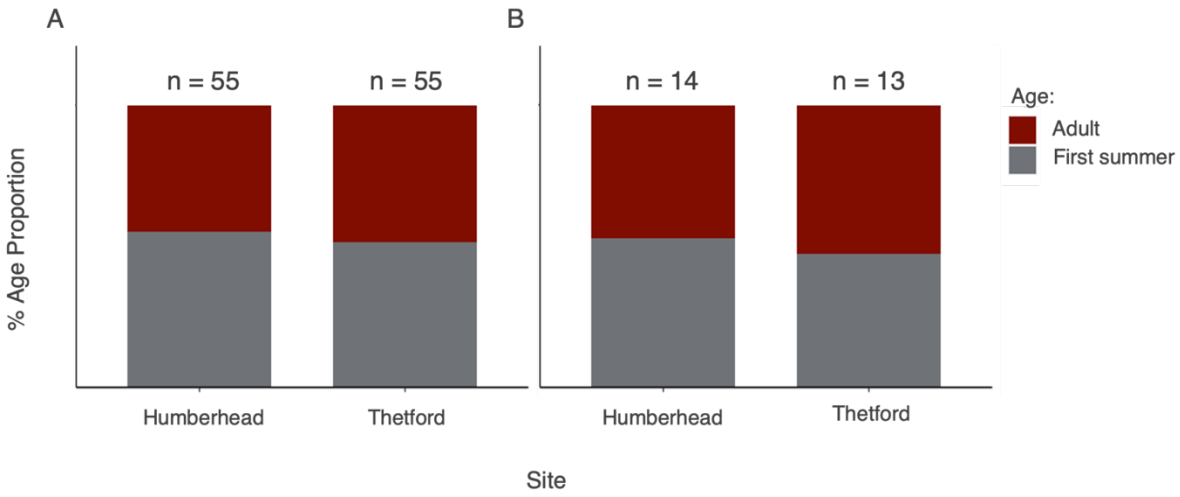


Figure 8.6 proportional age composition at both study sites for datasets used for Models 1 - 6 (A) and Models 7 and 8 (B).

8.4.2 Scaled mass Index

Scaled mass index (SMI) was calculated as per Peig and Green, (2009), following the calculation below, where SMI = Scaled Mass Index, M = absolute body mass, L0 = average structural size (wing length) for the population, Li = individual structural size (wing length) and finally BSMA = the slope of the relationship between TBM (mass) and L (wing length) for all male nightjar calculated as per standardised major axis regression in R. Metrics were calculated across all age classes, with average wing length exhibiting little variation between the two age classes wing length (mm); (adult = 195.40 , first summer = 195.75).

$$SMI_i = M_i \left[\frac{L_0}{L_i}\right]^{bSMA}$$

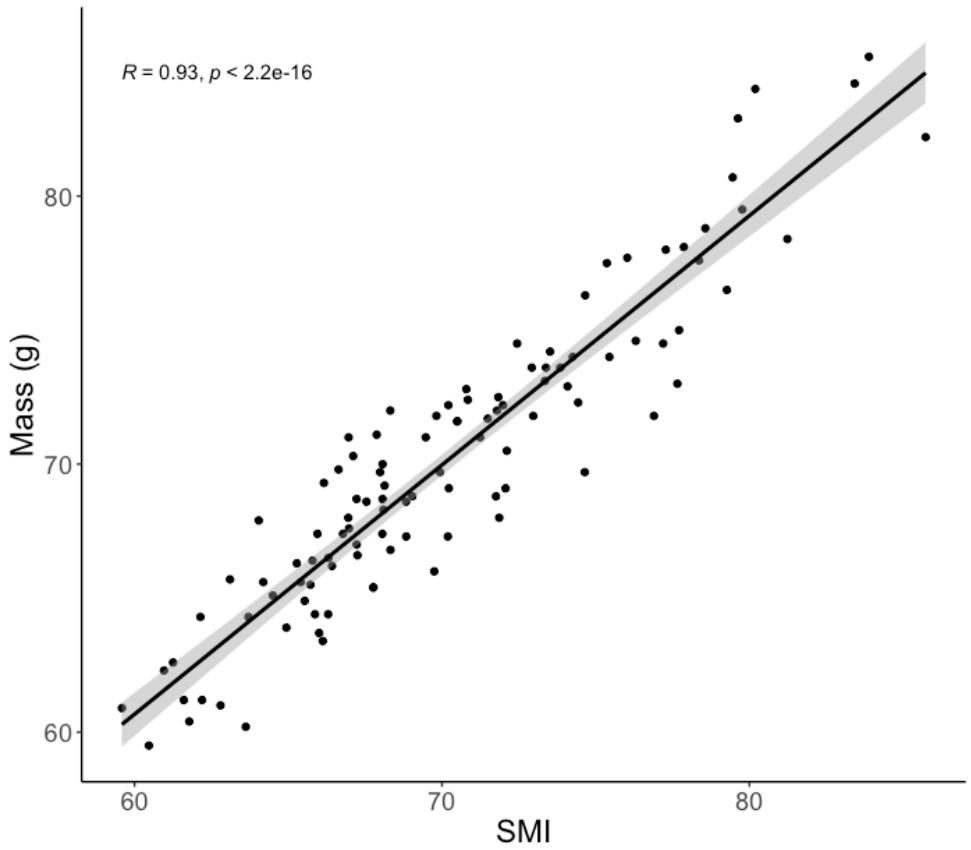


Figure 8.7 Regression of Scaled Mass Index (SMI) and mass (g) of male nightjars across all age categories and sites. Results of Pearson's correlation test are presented.

8.4.3 Model outputs

Table 8.6 Model 3 results of maximal multiple linear regression with relative wing spot asymmetry as the response variable. Maximal model presented, with all predictors excluded by stepwise selection.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	1.02	-2.64 – 4.68	0.580
Age (first summer)	0.07	-0.10 – 0.23	0.409
Muscle Score	-0.07	-0.29 – 0.14	0.508
Wing	-0.00	-0.02 – 0.01	0.609
Mass	-0.00	-0.01 – 0.01	0.969
Year (2020)	-0.12	-0.47 – 0.24	0.511
Year (2021)	-0.01	-0.22 – 0.19	0.894
Site (Thetford)	0.01	-0.24 – 0.26	0.913
Observations	75		
R^2 / R^2 adjusted	0.025 /-0.077		
AIC	61.350		

Table 8.7 Model 4 results of final multiple linear regression after backwards and forwards stepwise selection for AIC, modelling relative tail spot asymmetry against male quality metrics, site, and year.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	0.07	-0.17 – 0.32	0.555
Site (Thetford)	-0.36	-0.74 – 0.02	0.061
Year (2020)	0.58	-0.01 – 1.18	0.055
Year (2021)	-0.02	-0.38 – 0.33	0.891
Observations	73		
R^2 / R^2 adjusted	0.090 / 0.050		
AIC	151.675		

Table 8.8 Model 6 results of final multiple linear regression after backwards and forwards stepwise selection for AIC, modelling relative total spot asymmetry against male quality metrics, site, and year.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	0.03	-0.22 – 0.29	0.788
Year (2020)	0.15	-0.39 – 0.69	0.581
Year (2021)	-0.35	-0.73 – 0.04	0.075
Observations	67		
R^2 / R^2 adjusted	0.086 / 0.043		
AIC	141.889		

Table 8.9 Results of final multiple logistic regression Model 7 after backwards and forwards stepwise selection for AIC, modelling male nightjar spot characteristics against within-pair paternity success.

Predictors	*Odds Ratio*	*CI*	*P*
(Intercept)	2.00	0.34 – 2.29	1.000
Site (Thetford)	0.15	0.09 – 2.09	0.313
Observations	27		
R^2 Tjur	0.007		
AIC	39.456		

8.4.3.1 Actual Spot Size Models Outputs

Table 8.10 Model 1 multiple linear regression with wing spot area as the response variable. Results presented are the best fitting (AIC) model after forwards and backwards stepwise selection.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	0.56	-33.51 – 34.63	0.974
Age (first summer)	-2.00	-3.58 –-0.42	**0.014**
Site (Thetford)	-3.31	-4.94 –-1.67	**<0.001**
Wing	0.16	-0.02 – 0.33	0.076
Observations	77		
R^2 / R^2 adjusted	0.254 / 0.224		
AIC	412.131		

Table 8.11 Model 2 multiple linear regression with tail spot area as the response variable. Results presented are the best fitting (AIC) model after forwards and backwards stepwise selection.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	1585.40	1460.62 – 1710.18	**<0.001**
Age (first summer)	-128.29	-299.79 – 43.22	0.140
Site (Thetford)	-85.51	-254.17 – 83.15	0.316
Observations	73		
R^2 / R^2 adjusted	0.042 / 0.017		
AIC	1179.317		

Table 8.12 Model 3 maximal multiple linear regression with wing spot asymmetry as the response variable. Results presented are the maximal model, with backwards and forwards stepwise selection for AI presenting intercept as the best fitting model.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	187.66	-527.64 – 902.97	0.602
Age (first summer)	13.72	-18.52 – 45.97	0.399
Muscle score	-13.76	-55.85 – 28.33	0.516
Wing	-0.92	-4.64 – 2.80	0.624
Mass	-0.00	-2.87 – 2.87	1.000
Year (2020)	-22.52	-91.26 – 46.22	0.515
Year (2021)	-2.67	-43.21 – 37.87	0.896
Site (Thetford)	2.59	-46.27 – 51.44	0.916
Observations	73		
R^2 / R^2 adjusted	0.024 /-0.078		
AIC	852.652		

Table 8.13 Model 4 maximal multiple linear regression with tail spot asymmetry as the response variable. Results presented are the maximal model, with backwards and forwards stepwise selection for AI presenting intercept as the best fitting model.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	-598.71	-2555.12 – 1357.70	0.549
Age (first summer)	13.54	-73.55 – 100.63	0.761
Muscle score	26.83	-85.96 – 139.62	0.641
Wing	3.51	-6.55 – 13.56	0.494
Mass	-1.71	-9.18 – 5.76	0.654
Year (2020)	63.01	-101.98 – 228.01	0.454
Year (2021)	26.31	-75.92 – 128.53	0.614
Site (Thetford)	-24.06	-148.51 – 100.38	0.705
Observations	73		
R^2	0.023		
AIC	1031.396		

Table 8.14 Model 5 multiple linear regression with total spot area as the response variable. Results presented are the best fitting (AIC) model after forwards and backwards stepwise selection.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	100.96	-4430.63 – 4632.56	0.965
Age (first summer)	-218.14	-427.73 –-8.56	0.042
Site (Thetford)	-364.25	-580.56 –-147.94	**0.001**
Wing	12.97	-10.34 – 36.29	0.270
Observations	67		
R^2 / R^2 adjusted	0.221 / 0.184		
AIC	1003.988		

Table 8.15 Model 6 maximal multiple linear regression with total spot asymmetry as the response variable. Results presented are the maximal model, with backwards and forwards stepwise selection for AI presenting intercept as the best fitting model.

Predictors	*Estimates*	*CI*	*P*
(Intercept)	215.90	-1404.83 – 1836.63	0.791
Age (first summer)	18.06	-53.94 – 90.05	0.617
Muscle score	-22.52	-113.79 – 68.75	0.623
Wing	-1.03	-9.42 – 7.36	0.806
Mass	0.06	-6.11 – 6.23	0.985
year (2020)	83.82	-65.69 – 233.33	0.266
Year (2021)	-12.94	-102.58 – 76.69	0.774
Site (Thetford)	-62.30	-169.69 – 45.10	0.250
Observations	67		
R^2 / R^2 adjusted	0.068 /-0.047		
AIC	835.158		

Table 8.16 Results of final mixed effects logistic regression Model 7 including actual spot size characteristics after backwards and forwards stepwise selection for AIC, modelling male nightjar spot characteristics against within-pair paternity success.

Predictors	*Odds Ratio*	*CI*	*P*
(Intercept)	2.00	0.34 – 2.29	1.000
Site (Thetford)	0.15	0.09 – 2.09	0.313
Observations	27		
R^2 Tjur	0.007		
AIC	39.456		

Table 8.17 Results of final multiple logistic regression Model 8 including actual spot size characteristics after backwards and forwards stepwise selection for AIC, modelling male nightjar spot characteristics against male paired status as binary response variable (paired / extra-pair).

Predictors	*Odds Ratios*	*CI*	*P*
(Intercept)	0.00	0.00 – 2.24	0.107
Wing spot total area	0.99	0.98 – 1.00	0.219
Total spot area	1.00	1.00 – 1.01	**0.044**
Mass	1.32	1.00 – 2.05	0.111
Age (first summer)	0.39	0.03 – 4.38	0.442
Observations	27		
R^2 Tjur	0.352		
AIC	32.567		

8.5 Covid impact statement

Owing to facility access restrictions (i.e., access to lab facilities), seasonality of field data collection and closure and disruption of museums, my project was severely impacted by the COVID-19 pandemic. All data chapters in the thesis are dependent on field-collected data. My study species (European nightjar; *Caprimulgus europaeus*) is a migratory bird and is only present in the UK between May –September. Fieldwork is seasonal, with daily work required in this time period. Two full field seasons were required to meet the aims of my data chapters. Sampling took place over 13 sites in the UK, with 3 sampled by me and 10 by volunteers. Owing to access restrictions between 03/20 – 09/20 caused by the COVID-19 pandemic, data collection in 2020 was drastically reduced and was largely postponed until 2021, meaning that subsequent lab work, analysis and writing was also delayed by a year. Furthermore, initial closure of facilities during the first nation-wide lockdown and subsequent reduced capacity access significantly delayed my lab work, with lab facilities (NERC Environmental Omics Facility (NEOF) Sheffield) not reopening until November 2020. Consequently, lab work (testing of microsatellite markers and sample extraction) was delayed. I mitigated this by accessing facilities at the University of York from September until November 2020 to conduct DNA extraction, enabling me to continue with the microsatellite testing in December 2020 once the NBAF facility reopened. However, despite these mitigatory measures the lab closure led to my lab work being delayed by six months.

Chapter 3 was reliant on samples from museum specimens. Owing to national restrictions, museum closures, and furloughing of staff, access to museum collections was hugely delayed. Museum specimen sampling should have taken place between 09/20 and 12/20. However, owing to access restrictions the last samples were not received until 02/22 and sequencing could not be undertaken until all samples were received. This led to the dependent sequencing work for **Chapter 3** being delayed by 12 months. Furthermore, owing to an increased workload at the NERC Environmental Omics Facility imposed by COVID-19 sequencing and job backlog, delivery of sequence data was delayed by a further ~4 months (see **Appendix** Section 8.5; Covid Impact Statement).

I mitigated the delays faced to my project as best as possible through adjustment of aims and scope (reducing data collection for **Chapters 5-6,** from 4 to 2 sites), and by working on a peer-reviewed book chapter (**Chapter 4**) when field and data collection were not possible. I was also able to utilise published genomic data (nightjar reference genome) and a single genome sequenced at an earlier date to produce **Chapter 2** whilst waiting for the delayed Illumina

sequence data for **Chapter 3**. Ultimately, the pandemic constrained sample size (**Chapters 5 and 6**) and the time I was able to allot to analysis of genomic data (**Chapter 3**), for which I have mitigated to the best of my ability.

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