Landscape genetics and sociobiology of Gould's long-eared bat (*Nyctophilus gouldi*) and the lesser long-eared bat (*N. geoffroyi*) in fragmented populations of south-eastern Australia

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Thesis Abstract

Habitat fragmentation represents one of the greatest threats to biodiversity, yet for the second largest mammalian order Chiroptera we have only just begun to assess the impacts of this threatening process on population connectivity and genetic diversity. Many aspects of chiropteran ecology remain unknown due to their cryptic lifestyle and difficulties in applying traditional observational and field-based techniques. At the time of this PhD project's conception there were no published studies utilising genetic techniques to address the influence of habitat fragmentation on any chiropteran species. Since that time two studies have been published, in 2009 and 2011. I add to this new body of literature by conducting genetic analyses to assess population connectivity and genetic diversity in two congeneric vespertilionids, *Nyctophilus gouldi* and *N. geoffroyi*. The study was conducted in western Victoria and south-eastern South Australia across a landscape comprising continuous and fragmented regions of native habitat. Populations within continuous forest provided a benchmark for parameters including gene flow, genetic diversity and social structure, for comparison with forest fragments. This thesis also capitalises on the underutilised potential of molecular techniques for the study of chiropterans. I applied molecular approaches to assess dispersal strategies and social structure in both species offering novel ecological insights. Four data chapters covering these topics are outlined below.

Chapter 2 describes the isolation and characterisation of 16 microsatellite markers developed to facilitate this research. I utilised next generation sequencing technology (454) to generate a microsatellite DNA library and employed Multiplex Ready Technology (MRT) as a flexible and cost effective method to test primers and design marker panels for screening. DNA was isolated from *N. gouldi* resulting in 15 loci, while cross amplification in *N. geoffroyi* produced 7 reliable loci.

Chapter 3 addresses the impact of habitat fragmentation on the forest and woodland specialist *N. gouldi*, which is listed as endangered in South Australia. Based on roosting requirements, rarity in the agricultural landscape and limited dispersal ability I predicted that *N. gouldi* populations would display reduced gene flow and signs of isolation as a result of habitat fragmentation. This prediction was confirmed by my analyses which identified reduced population connectivity, decreased genetic diversity, elevated measures of relatedness and

inbreeding, and altered demography within fragmented populations isolated by ≥27km of agricultural land. Agricultural distances <2km did not influence population connectivity providing a benchmark for habitat restoration to improve connectivity and mitigate population isolation in this species. Management recommendations include the enhancement of population connectivity between threatened SA populations, and recognition of a unique Management Unit at the Grampians National Park.

The forth chapter investigates the influence of habitat fragmentation on *N. geoffroyi* for comparison with *N. gouldi*. In contrast to *N. gouldi*, *N. geoffroyi* is a habitat generalist that occupies a diverse range of ecosystems and which is commonly recorded within agricultural landscapes. *N. geoffroyi*'s presence in modified habitat coupled with plastic ecology and roosting requirements led to the prediction that the species would display limited impacts from habitat fragmentation. My analyses again confirmed this prediction with *N. geoffroyi* displaying virtually no response to habitat fragmentation and a panmictic population structure across the study region. The comparison between *N. geoffroyi* and *N. gouldi* provided an opportunity to test the merit of several proposed predictors of bat vulnerability to habitat fragmentation, in particular wing morphology, matrix tolerance, specialisation and geographic range. The much touted predictor wing morphology failed to predict differing responses from the two species while the following three predictors listed above received further support from this study. I conclude that wing morphology may still be a useful predictor of bat vulnerability to habitat fragmentation when coupled with other indicators such as matrix tolerance and habitat specialisation.

The fifth and final data chapter utilises molecular analyses to assess several previously unknown aspects of *N. gouldi* and *N. geoffroyi* ecology, dispersal strategies, mating systems and social structure. *N. gouldi* displayed patterns consistent with female natal philopatry, male biased dispersal and a polygynous mating system, while no such evidence was found for *N. geoffroyi*. Results for *N. geoffroyi* may have been influenced by larger population sizes which, coupled with higher dispersal rates, may have masked any evidence of sex-biased dispersal. Both species displayed significant numbers of relatives at the population level, with *N. gouldi* displaying particularly high levels of related females. *N. geoffroyi* displayed higher numbers of relatives at the roost level indicating that kin selection may play an important role in social structure and cooperative roosting. Despite significant numbers of related *N. geoffroyi* at the roost level, the vast majority of pairwise comparisons indicated no

relationship between individuals suggesting that the dominant driver of sociality and cooperative behaviour may not be solely based on relatedness. Nevertheless, high incidence of related females at the population level for *N. gouldi*, and at the roost level for *N. geoffroyi*, suggests that the bonds between related females are an important aspect of *Nyctophilus* behavioural ecology and social structure.

Statement of Authorship

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Nicholas Fuller and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

This thesis represents an original and independent piece of work. All significant aspects of the work were conducted by myself including field work, laboratory work, data analysis and interpretation, the production of manuscripts and the procurement of ethics approval, research permits and all funding accrued to facilitate this project. My supervisors Steven J. B. Cooper and Susan M. Carthew contributed to the production of manuscripts and provided supervisory support and guidance. S. Cooper provided additional guidance with data analysis and interpretation.

GIS maps displayed as Figures 3.1, 4.1 and 5.1 were produced by Christopher J. Medlin and the cover image, a photograph of *N. gouldi*, was taken by Terry Reardon.

This thesis is presented as a series of manuscripts with Chapters 2-5 intended for publication in peer-reviewed journals co-authored by myself, and my supervisors Steven J. B. Cooper and Susan M. Carthew.

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This research was conducted under Animal Ethics Permits from the University of Adelaide and wildlife permits from the Department of Sustainability and Environment (DSE), the Department for Environment, Water and Natural Resources (DEWNR) and the South Australian Forestry Corporation.

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Chapter 1

General Introduction

Chiroptera is the second most speciose mammalian order following Rodentia and contains approximately 20% of described mammals (Wilson & Reeder 2005). Representing a significant contribution to biodiversity bats also provide important ecosystem services including plant pollination, seed dispersal and the suppression of insect populations (Kunz et al. 2011). Despite these roles we have limited knowledge regarding the ecology of this mega-diverse order, particularly in regards to more cryptic aspects of ecology such as social structure, dispersal strategies and mating systems (Burland & Worthington Wilmer 2001). Similarly we know little about how species within the group respond to key threatening processes such as habitat fragmentation, which has been recognised globally as one of the major threats facing terrestrial species (Baillie et al. 2004; Bennett 2003). This lack of information compromises our capacity to effectively manage and conserve chiropteran species, particularly in regards to the threat posed by habitat fragmentation.

This thesis aims to address these issues by conducting landscape and population genetic analyses on two species of *Nyctophilus*, one of the most speciose and abundant Australian genera of bats, and members of the largest chiropteran family Vespertilionidae. The study was carried out in western Victoria and south-eastern South Australia across a region comprising both small and expansive patches of fragmented remnant native forest amidst a matrix of agricultural land. This landscape facilitated an investigation into the impact of habitat fragmentation on genetic diversity and population connectivity, and assessments of dispersal patterns and social structure. We define connectivity throughout this thesis as ‘functional connectivity’ reflecting an organism’s capacity to traverse the matrix between fragmented habitat patches (Kindlmann & Burel 2008). We also use the term ‘population’ loosely to describe field sites that may be connected via continuous habitat or fragmented by agriculture. Consequently they do not necessarily represent discrete biologically defined populations.

Gould’s long-eared bat (*N. gouldi*) and the lesser long-eared bat (*N. geoffroyi*) are small insectivores that roost in tree hollows and under bark and display wing morphology

characteristic of slow manoeuvrable flight believed unsuited to long distance travel (Fullard et al. 1991). However, *N. gouldi* is a habitat specialist with a distribution limited to forest and woodland in eastern and south-western Australia and has been listed as endangered on Schedule 7 of the South Australia *National Parks and Wildlife Act 1972* (Churchill 2008). In contrast, *N. geoffroyi* is a habitat generalist that displays a ubiquitous distribution across Australia and is commonly recorded in agricultural landscapes (Churchill 2008). Comparison of the two species will provide a novel opportunity to test the validity of several proposed predictive traits for bat extinction and vulnerability to habitat fragmentation, including: wing morphology, geographic range, habitat specialisation and tolerance to the intervening matrix between habitat patches (Davies et al. 2000; Henle et al. 2004; Jones et al. 2003; Laurance 1991; Meyer et al. 2008; Safi & Kerth 2004; Viveiros de Castro & Fernandez 2004).

We are only aware of two other published studies worldwide specifically designed to investigate the impact of modern anthropogenic habitat fragmentation on bat population connectivity and genetic diversity (Meyer et al. 2009; Struebig et al. 2011). Consequently this research will significantly contribute to international knowledge regarding the conservation and management of bat populations at a landscape scale. The results from this research will also shed new light on cryptic aspects of long-eared bat ecology including dispersal strategies, social structure and mating systems. Finally, our research will assist land managers to effectively manage remnant native vegetation in south-eastern South Australia and western Victoria to maximise conservation outcomes for indigenous species.

Habitat fragmentation

Habitat fragmentation can impose barriers to dispersal between populations disrupting metapopulation dynamics and rendering populations isolated, thereby reducing their effective size (Hanski 1998; Lindenmayer & Peakall 2000; Saunders et al. 1991). Population size is the most important factor in determining population, and thus species, persistence (O'Grady et al. 2004; Reed et al. 2003; Shaffer 1981). Larger populations have been shown to contain higher levels of genetic diversity (Frankham 1996) which provides numerous benefits including greater resistance to parasites and disease (O'Brien & Evermann 1988; Spielman et al. 2004), greater adaptive plasticity to changing environmental pressures (Frankham et al. 1999; Reed & Frankham 2003) and enhanced evolutionary potential (Crandall et al. 2000; Franklin & Frankham 1998). Small populations are diminished in these respects and with

decreasing size they become increasingly vulnerable to stochastic environmental events like fire and disease, as well as genetic and demographic processes including inbreeding and genetic drift (Caughley 1994; Frankham 1995; Lacy 1997; Shaffer 1981).

Population size is primarily determined by the extent of available habitat and by connectivity between areas of suitable habitat (Fahrig & Paloheimo 1988; Saunders et al. 1991). Adequate connectivity between habitat facilitates dispersal between populations allowing them to function as larger and more robust metapopulations (Burkey 1989; Hanski 1998; Reed 2004). In addition to increasing effective population sizes, this connectivity provides a safeguard against events like fire by allowing neighbouring populations to recolonise habitat after localised extinctions occur (Hanski 1998; Wilcox & Murphy 1985). Habitat connectivity is also an important consideration for climate change as species may need to migrate with shifting environmental conditions in order to persist in suitable habitat (Hannah et al. 2002; Opdam & Wascher 2004).

The impact of habitat fragmentation on vertebrate species is varied (e.g. amphibians, Gibbs 1998; bats, Gorresen & Willig 2004; marsupials, Laurance 1990; and reptiles, Mac Nally & Brown 2001). As a consequence species-specific research is ideally required to identify the influence of habitat fragmentation on dispersal and population connectivity (Cushman 2006; Debinski & Holt 2000). Information on dispersal thresholds will allow us to manage populations in fragmented landscapes through landscape management that promotes connectivity for improved population viability. However, due to the inherent paucity of species-specific data, species level investigations can also serve as useful indications of the potential response within genera, family and higher taxonomic classifications.

The impact of habitat fragmentation on chiropterans

Despite the vagility of chiropterans, mounting research has documented the impacts of habitat fragmentation on bats, including changes to community composition and the disappearance of species from forest fragments (Cosson et al. 1999; Estrada & Coates-Estrada 2002; Estrada et al. 1993; Medina et al. 2007; Schulze et al. 2000). In Australia, the Action Plan for Bats (Duncan et al. 1999) lists habitat loss (incorporating land clearing, fragmentation and modification) as the primary threatening process for Australian bats with nearly 60% of threatened Australian chiropterans receiving their threatened status due to this cause. The

Action Plan also identifies 'the impact of forest fragmentation on bats at a landscape scale' as a priority for research. To effectively manage bat populations in fragmented landscapes data must be collected on population connectivity to identify thresholds for dispersal, and to provide recommendations for landscape management to avoid or reverse population isolation (Galindo-Gonzalez & Sosa 2003). To date, most studies investigating the impacts of habitat fragmentation on bats have used traditional field-based techniques to assess changes in species abundance and distribution, or have employed telemetry to study animal movements. However, these approaches have their limitations. Studies of abundance and distribution only document the aftermath of habitat fragmentation and fail to address the mechanisms behind the changes that occur. Telemetric approaches can provide useful insights into animal movements but they typically cannot distinguish between successful and unsuccessful migration events, while cost and labour usually result in limited datasets, analytical power and spatial scale (Hebblewhite & Haydon 2010). Genetic techniques can overcome these shortcomings and allow for the generation of broad-scale population censuses across entire landscapes and the identification of thresholds for gene flow and population connectivity.

Genetic studies have provided powerful insights into the influence of landscape features on bat population connectivity and dispersal. Many chiropteran species display largely panmictic populations across their range (McCracken et al. 1994; Sinclair et al. 1996; Webb & Tidemann 1996). However, this is not always the case as now demonstrated by numerous examples (Burland & Worthington Wilmer 2001). In particular, non-migratory species with restricted or specialised habitat requirements can display high levels of population structure (Armstrong 2009; Worthington Wilmer et al. 1999; Worthington Wilmer et al. 1994). It has also been demonstrated that landscape features, including water bodies (Castella et al. 2000; Salgueiro et al. 2008) and mountain ranges (Ruedi & Castella 2003), can act as significant barriers to dispersal and population connectivity.

Several authors have specifically proposed that poor habitat connectivity has resulted in increased population structure and reduced dispersal in bats. Campbell et al. (2009) suggested that significant F_{ST} values between neighbouring populations of *Myotis macropus* may be the result of limited dispersal due to reduced riparian habitat in the agricultural landscape. Kerth and Petit (2005) also proposed that a barrier due to habitat fragmentation could explain patterns in the population structure of *Myotis bechsteinii*. However, few

population or landscape genetic studies have been specifically designed to investigate the influence of habitat fragmentation on bat populations.

Meyer et al. (2009) examined ~340bp of the mtDNA control region (d-loop) to assess the response of two species of phyllostomid bats to habitat fragmentation caused by the creation of an artificial reservoir in Panama. The study was conducted at a microgeographic scale and compared haplotype diversity and population differentiation (F_{ST}) between isolated and continuous forest sites. The less mobile of the two study species, *Carollia perspicillata*, showed signs of genetic erosion and significant population differentiation as a result of forest fragmentation. Their findings suggest that the <2km of open water isolating fragments represented a critical threshold in population connectivity for *C. perspicillata*.

Struebig et al. (2011) employed microsatellite markers to investigate the comparative impact of habitat fragmentation on community level species richness and allelic richness in a subset of three species with varying ecology. Population differentiation (Jost's D and F_{ST}) was also assessed but only the minimum and maximum values were reported along with a series of Mantel tests to identify correlations between differentiation and three factors: community dissimilarity (Morisita-Horn index), Euclidian distance, and effective (least-cost) distance. Characterised by low population densities and limited dispersal power *Kerivoula papillosa* displayed a significant correlation between allelic richness and fragment size, where genetic diversity decreased with habitat area. There were no significant cases of population differentiation, nor was there a significant relationship between population differentiation and community dissimilarity for either distance measure.

Meyer et al. (2009) and Struebig et al. (2011) represent the only examples we are aware of that have specifically aimed to assess the impacts of habitat fragmentation on genetic diversity and population connectivity within Chiroptera. Although both studies found that the least mobile of their study species was negatively affected by habitat fragmentation both studies also had their limitations. The use of mtDNA by Meyer at al. (2009) limited the analyses that could be applied and the results only reflected female-mediated gene flow. Struebig et al. (2011) on the other hand used microsatellite markers, however, they did not endeavour to identify thresholds for dispersal. Instead their goal was more theoretical in nature as they sought to assess the relationship between declines in species and allelic richness due to habitat fragmentation.

In addition to these two studies we are aware of another multispecies investigation (Rossiter et al. 2012) utilising microsatellite markers to assess the impacts of habitat fragmentation on population genetic structure in seven codistributed microbats. Currently the results have been published on the analysis of population structure and gene flow through continuous forest while the results from the analysis of fragmented populations are yet to be published. Their initial investigation on continuous habitat suggests that roosting ecology and social structure may influence dispersal limits and that tree roosting species characterised by reduced vagility may be at greater risk to habitat fragmentation (Rossiter et al. 2012).

Identifying predictive traits associated with chiropteran vulnerability to habitat fragmentation

Conservation biologists are attracted to the prospect of identifying traits linked to vulnerability to threatening process as it permits the *a priori* identification of species at risk (Mac Nally & Bennett 1997). This issue has received much attention in terms of predictors of extinction risk and vulnerability to threatening processes. Proposed species traits include abundance, geographic range, fecundity, longevity, rarity, specialisation, body size and trophic position (Cardillo et al. 2008; Davidson et al. 2009; Henle et al. 2004; Laurance 1991; O'Grady et al. 2004; Safi & Kerth 2004). Many of these traits have been assessed in relation to habitat fragmentation, in addition to several others such as presence in the matrix and mobility, that are specific to this threatening process (Davies et al. 2000; Foufopoulos & Ives 1999; Gehring & Swihart 2003; Henle et al. 2004; Laurance 1991; Lehtinen & Ramanamanjato 2006; Mac Nally & Bennett 1997; Tscharntke et al. 2002; Viveiros de Castro & Fernandez 2004; Wang et al. 2009; Watling & Donnelly 2007).

For bats, wing morphology has been proposed as an additional predictive trait and has received some support in relation to sensitivity to habitat fragmentation (Albrecht et al. 2007; Meyer et al. 2008) and extinction risk (Jones et al. 2003; Safi & Kerth 2004). Two particular characteristics of wing morphology, low aspect ratio and low wing loading, have been linked with specialisation for closed habitat (Safi & Kerth 2004). These wing characteristics represent adaptations for slow manoeuvrable flight that are inefficient for long distance flight (Norberg & Rayner 1987), possibly reflecting a restricted capacity for movement between habitat fragments. *N. gouldi* and *N. geoffroyi* possess these wing characteristics and display near-identical wing morphology (Brigham et al. 1997; Churchill 2008; Fullard et al. 1991;

Norberg & Rayner 1987). Consequently this predictive trait would suggest that both species possess the same physical capacity for dispersal between fragmented patches of habitat. However, differing degrees of ecological plasticity indicated by contrasting geographic distributions (Churchill 2008), roosting specificity (Churchill 2008; Lunney et al. 1988; Reardon & Flavel 1987) and occurrence in agricultural landscapes (Lumsden & Bennett 2005; Lumsden et al. 2002a; Lumsden et al. 2002b) suggest that *N. geoffroyi* will possess a greater resilience to habitat fragmentation than *N. gouldi*. Consequently the comparison between the two species will test the reliability and relative influence of several proposed predictive traits for vulnerability to habitat fragmentation including wing morphology, habitat specialisation, geographic range and tolerance to the matrix between patches of remnant vegetation.

Chiropteran behavioural ecology

Chiropteran lifestyles are cryptic due to their nocturnality, flight and the fact they often shelter in difficult to access locations; consequently their behavioural ecology has proven difficult to study with traditional techniques (Burland & Worthington Wilmer 2001; Kerth 2008). As a result there is a paucity of information on chiropteran behavioural ecology compared to other social mammals (Kerth 2008). However, modern molecular techniques provide the tools to investigate these previously elusive aspects of chiropteran ecology (Burland & Worthington Wilmer 2001; Kerth et al. 2002b). Due to these developments the number of studies into chiropteran sociobiology is increasing, but the sheer size of the order means there is much work to be done. Nevertheless, many insights have been gained over the last two decades into chiropteran dispersal strategies (Arnold 2007; Kerth et al. 2002a; Petit & Mayer 1999; Weyandt et al. 2005; Worthington Wilmer et al. 1999), social structures (Furmankiewicz & Altringham 2007; Heckel et al. 1999; Kerth et al. 2000; Metheny et al. 2008; Ortega et al. 2003; Petri et al. 1997; Rivers et al. 2005; Rossiter et al. 2002; Storz et al. 2001; Veith et al. 2004; Wilkinson 1992a) and mating systems (Burland et al. 2001; Chaverri et al. 2008; Heckel et al. 1999; Ortega et al. 2003; Rossiter et al. 2000; Veith et al. 2004).

We add to this growing pool of research by investigating dispersal strategies, social structure and mating systems in two temperate vespertilionids, *N. gouldi* and *N. geoffroyi*. This aspect of our research will further complement our investigation into habitat fragmentation as dispersal patterns, social organisation and mating systems may play important roles in

chiropteran responses, as indicated by Meyer et al. (2009), Struebig et al. (2011) and Rossiter et al . (2012).

Aims

The principal aim of this thesis is to address the lack of knowledge regarding the impact of habitat fragmentation on bat population connectivity by conducting an assessment of *N. gouldi* and *N. geoffroyi* population structure and gene flow across a landscape comprising continuous and fragmented forest. We will develop a suite of microsatellite markers to facilitate the study which will be used to compare gene flow between populations connected through continuous forest and populations fragmented by agricultural land. Analyses will be used to assess population structure across the landscape and to identify dispersal events and distance thresholds for population connectivity. We will also investigate the impact of habitat fragmentation on genetic diversity, relatedness and inbreeding. These results may prove particularly important for the management of endangered South Australian populations of *N. gouldi* which are restricted to highly fragmented and limited remnant vegetation.

The comparison of the two target species will provide an opportunity to test the merit of wing morphology as a predictor of bat vulnerability to habitat fragmentation. With near-identical wing morphology the predictive trait suggests that both species will respond in the same manner. However, contrasting degrees of specialisation, varying geographic distributions and differing use of agricultural habitat indicate the species may respond quite differently to habitat fragmentation. Consequently the comparison represents a novel case to assess the relative influence of these predictive traits, and provide a more robust framework for predictions regarding chiropteran responses to habitat fragmentation.

Finally, this thesis will contribute to the growing body of research into chiropteran sociobiology by investigating dispersal patterns, mating systems, and social organisation. Modern molecular techniques represent the ideal tools to tackle many difficult questions in ecology, especially in regards to the cryptic chiropterans, yet they remain under-utilised by ecologists. The following research represents our efforts to employ these tools in order to address several important gaps in our scientific knowledge regarding chiropterans and facilitate better conservation outcomes for this important and intrinsically valuable group of mammals.

This thesis comprises four data chapters presented in a manuscript style format. We plan to submit these chapters as articles in publications such as Molecular Ecology and Conservation Genetics. However, for the purpose of this thesis we have taken the liberty of exceeding the journal word limits in order to present and discuss a greater proportion of the analyses undertaken. The specific aims of the data chapters are outlined below.

Chapter 2: Isolation and characterisation of 16 microsatellite markers for the endangered Gould's long-eared bat (*Nyctophilus gouldi*) and cross-amplification in the lesser long-eared bat (*N. geoffroyi*)

Aims:

1. Develop a suite of microsatellite markers for *N. gouldi* and *N. geoffroyi* using next generation sequencing methods.

Chapter 3: The influence of habitat fragmentation on population connectivity and genetic diversity in a microbat, Gould's long-eared bat (*Nyctophilus gouldi*)

Aims:

1. Assess the impact of habitat fragmentation on *N. gouldi* (endangered in South Australia) population structure, population differentiation and dispersal.
2. Test the hypothesis that fragmented populations of the forest specialist *N. gouldi* will display signs of reduced genetic diversity, elevated relatedness and inbreeding, and altered demography.
3. If *N. gouldi* is influenced by habitat fragmentation, identify a threshold for dispersal and population connectivity.
4. Determine whether the fragmented and endangered South Australian populations of *N. gouldi* are at risk of genetic threats associated with isolation and small population sizes.

Chapter 4: The comparative influence of habitat fragmentation on two congeneric vespertilionids with near-identical morphology and contrasting degrees of specialisation

Aims:

1. Assess the impact of habitat fragmentation on *N. geoffroyi* using comparative analyses to facilitate a direct comparison with *N. gouldi*.
2. Test the hypothesis that *N. gouldi* populations will be more impacted by habitat fragmentation than *N. geoffroyi* due to increased habitat specialisation and sensitivity to

the matrix, instead of displaying similar responses as similarities in wing morphology predict.

Chapter 5: Dispersal strategies and social structure in two species of long-eared bats, *Nyctophilus geoffroyi* and *N. gouldi*

Aims:

1. Investigate dispersal patterns in *N. gouldi* and *N. geoffroyi* to determine if either species displays a sex-bias in dispersal.
2. Infer mating systems from dispersal patterns, and assess maternity and paternity for evidence of polygyny, polyandry and multiple paternity.
3. Assess social structure in *N. gouldi* and *N. geoffroyi* by identifying relatives at the population level, and at the roost level for three roosting congregations of *N. geoffroyi.*
4. Assess the composition of long-eared bat populations to determine whether communities consist of a random assortment of individuals, or whether family groups or related pairs comprise a significant proportion of the population.
5. Assess the social composition of *N. geoffroyi* roosts for evidence of kin selection or reciprocal altruism in cooperative roosting behaviour.

Chapter 2

Isolation and characterisation of 16 microsatellite markers for the endangered Gould's long-eared bat (*Nyctophilus gouldi*) and cross-amplification in the lesser long-eared bat (*N. geoffroyi*)

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ABSTRACT

Sixteen microsatellite markers were developed for use on two species of long-eared bats (*Nyctophilus*). 454 pyrosequencing of genomic DNA was conducted on *N. gouldi* which is listed as endangered in South Australia. Fifteen loci successfully amplified on *N. gouldi* while nine cross-amplified for use on *N. geoffroyi*. Two populations from south-eastern Australia were genotyped for each species comprising 91 individuals for *N. gouldi* and 70 individuals for *N. geoffroyi*. There was no evidence of linkage disequilibrium and all loci displayed Hardy-Weinberg equilibrium except Nyg19 and Nyg39 which displayed evidence of null alleles in both *N. geoffroyi* populations. These markers will prove valuable in assessing connectivity between endangered populations of *N. gouldi*, and facilitate a comparative investigation into the impacts of habitat fragmentation on two vespertilionids.

Keywords: *Nyctophilus*, Chiroptera, microsatellites, 454 pyrosequencing

Gould's long-eared bat (*Nyctophilus gouldi*) is a forest and woodland specialist that is listed as endangered in South Australia under Schedule 7 of the *South Australian National Parks and Wildlife Act*. The South Australian distribution of the species is restricted to highly fragmented remnant habitat embedded within a matrix of pastoral land and *Pinus radiata* plantations. The nature of this landscape raises concerns about the viability of endangered *N. gouldi* populations.

To address *N. gouldi* conservation concerns we aimed to develop a suite of microsatellite markers to assess population connectivity and genetic diversity. In addition to this objective,

we have recognised an opportunity to cross-amplify these markers on the habitat generalist *N. geoffroyi* in order to compare the influence of habitat fragmentation between two congeneric, sympatric and morphologically near-identical chiropterans with contrasting degrees of specialisation. We are only aware of two published studies employing genetic techniques to investigate the impact of habitat fragmentation on chiropterans (Meyer et al. 2009; Struebig et al. 2011). Consequently this application will constitute a significant contribution towards understanding the impact of habitat fragmentation on bats.

Using methods outlined by Gardner et al. (2011) we employed a partial pyrosequencing run (½ plate) on a GS-FLX Titanium platform (Roche, 454 Life Sciences) at the Australian Genome Research Facility (AGRF, Brisbane, Australia). This approach produced a total of 21460 sequences and 752 microsatellite loci. Forty sets of primer pairs were selected for initial PCR trials on a single *Nyctophilus gouldi* individual. Nuclear DNA was extracted from wing biopsies using the Gentra Puregene extraction kit (Gentra Systems Inc.). PCR amplification was performed using “multiplex-ready technology” (MRT) developed by Hayden et al. (2008) whereby generic M13 tags are attached to the 5’ end of locus-specific primer sequences providing a flexible system for the design of locus panels for product screening.

PCR was conducted in a volume of 12µl containing ~10ng of DNA, 75nM of fluorescently labelled generic MRT forward primer (HEX) and 75nM of unlabelled reverse primer, four different concentrations of each locus-specific primer were tested (10, 20, 40 & 60nM), 0.15U Immolase DNA polymerase (Bioline, Luckenwalde, Germany) and 2.4µl of 5×ImmoBuffer (Bioline). MRT PCR-amplification is performed in two stages following a 10 minute denaturation period at 95°C. The first stage employs 5 cycles of: 60s at 92°C, 90s at 50°C, 60s at 72°C; followed by 20 cycles of: 30s at 92°C, 90s at 63°C, 60s at 72°C. The second phase comprises 40 cycles of: 15s at 92°C, 30s at 54°C, 30s at 72°C, with a final extension of 30 min at 65°C after the cycles are complete. To confirm amplification and identify unambiguous loci and optimum primer concentrations PCR products were visualised on a 6% polyacrylamide gel using a GelScan2000 instrument (Corbett Research, Sydney, Australia).

From the initial 40 loci tested 32 passed electrophoretic screening and progressed for subsequent tests of polymorphism using three individuals for each species. Loci were assigned one of four fluorescently labelled generic MRT primers (FAM, NED, PET and VIC)

for visualisation of PCR products on an ABI3730 DNA Analyser (Applied Biosystems). PCR was performed separately for each locus and products were pooled post PCR into two panels for each species using a pooling ratio of 2:3:3:6 (VIC:FAM:NED:PET). GENEMAPPER v.3.5.1 (Applied Biosystems) was used to score alleles. Fifteen loci were polymorphic and reliably scorable for *N. gouldi* and nine for *N. geoffroyi*, including one locus that only amplified in *N. geoffroyi* (Table 2.1).

GENALEX v.6 (Peakall & Smouse 2006) was used to assess allelic diversity and calculate observed and expected heterozygosity. We used GENEPOP v.3.4 (Raymond & Rousset 1995) to test populations and loci for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD), and MICROCHECKER v.2.2.3 (Van Oosterhout et al. 2004) to detect typing errors and null alleles. Sequential Bonferroni corrections were made for all tests involving multiple comparisons (Rice 1989).

Individuals were genotyped from Hotspur and Annya State Forests in Victoria and Dry Creek Native Forest Reserve in South Australia (Table 2.1). The number of alleles for *N. gouldi* loci ranged from 3 to 10 (mean=6), and from 6 to 21 for *N. geoffroyi* loci (mean=11). Observed and expected heterozygosity ranged in *N. gouldi* from 0.323-0.839 and 0.377-0.842 respectively, and from 0.324-0.971 and 0.671-0.932 for *N. geoffroyi*. There was no evidence of deviation from HWE or LD with the exception of Nyg19 and Nyg39 which significantly deviated from HWE for both *N. geoffroyi* populations. This deviation was most likely due to the presence of null alleles (Hotspur, Nyg19 r=0.212, Nyg39 r=0.170; Annya, Nyg19 r=0.221, Nyg39 r=0.294). Assessment of heterozygosity revealed no evidence of sex-linked loci.

The 16 microsatellite markers presented here will facilitate an assessment of genetic diversity and population structure for endangered South Australian populations of *N. gouldi*. These markers will also provide a valuable insight into the comparative influence of habitat fragmentation on two congeneric vespertilionids.

Table 2.1: Sixteen microsatellite primer sequences isolated from *N. gouldi* and their characteristics in two species of *Nyctophilus*.

Locus	Repeat motif	Primer sequences (5'-3')*	GenBank accession number	Species	Size range (bp)#	Panel	Primer concentration (nM)	MRT generic primer label	Population	N	N_A	H_O/H_E	HWE
Nyg5	(AC)12	F:GCTTACAGGCAAGGGTGTTC	KC688295	*N. gouldi*	140-148	1	40	PET	Dry Creek	66	4	0.35/0.38	0.390
		R:ACCCAGCCAACTCTAACCCT							Hotspur	31	3	0.32/0.45	0.074
Nyg7	(AC)11	F:TTTCTGCTTATTACTGACATCACCA	KC688296	*N. gouldi*	108-122	2	40	FAM	Dry Creek	66	8	0.68/0.76	0.013
		R:CTGTGCTTGTGCATTGTGTTT							Hotspur	31	6	0.84/0.75	0.111
Nyg8	(TTTA)10	F:GGGACGGACAGATGAGAAAA	KC688297	*N. gouldi*	165-185	2	40	NED	Dry Creek	66	6	0.46/0.53	0.295
		R:GTTGCTATAAAGACATGGGCA							Hotspur	31	6	0.48/0.47	0.243
				N. geoffroyi	155-191	2	40	PET	Annya	34	9	0.85/0.80	0.707
									Hotspur	36	9	0.80/0.79	0.526
Nyg11	(AC)13	F:CCACAGAATGAAAGAATGGGA	KC688298	*N. gouldi*	215-231	1	40	PET	Dry Creek	66	7	0.73/0.76	0.328
		R:AATGCAAGAAGAGTTTCCATGA							Hotspur	31	6	0.68/0.75	0.168
Nyg13	(GA)12	F:CCATTGCTAAACTCATTTATTGG	KC688299	*N. gouldi*	149-183	2	40	PET	Dry Creek	66	7	0.76/0.75	0.270
		R:CCCTGATTAAAAGGAAGATGC							Hotspur	31	7	0.80/0.75	0.651
Nyg17	(TTAT)13	F:GCTGCAAACAGGTGTAACGA	KC688300	*N. gouldi*	308-392	2	20	PET	Dry Creek	66	8	0.76/0.78	0.289
		R:TCTCCCTAGCATCCTCTGCT							Hotspur	31	6	0.77/0.79	0.331
Nyg19	(ATCC)9	F:CCGGTTTCGGCTATTTGTAA	KC688301	*N. geoffroyi*	134-158	1	20	FAM	Annya	34	6	0.38/0.70	0.000 ***
		R:AATGCCTGGCACAAAGTAGG							Hotspur	36	7	0.36/0.67	0.000 **
Nyg20	(ATC)14	F:TTCAGTTGGAGCTACCTGGG	KC688302	*N. gouldi*	211-223	1	20	NED	Dry Creek	66	5	0.70/0.74	0.364
		R:TGGCTCAATTAGTTGCCTGA							Hotspur	31	5	0.71/0.72	0.437
				N. geoffroyi	196-226	2	20	VIC	Annya	34	7	0.79/0.80	0.179
									Hotspur	36	9	0.86/0.82	0.914
Nyg21	(GT)9	F:GGATAATGAAATTATGCTGTCTTAGAA	KC688303	*N. gouldi*	114-132	1	20	VIC	Dry Creek	66	5	0.55/0.57	0.690
		R:CAGCTTTGTCAGAACAACTGG							Hotspur	31	5	0.58/0.57	0.344
				N. geoffroyi	110-144	1	20	VIC	Annya	34	18	0.82/0.92	0.107
									Hotspur	36	16	0.80/0.89	0.233

Number of individuals screened (N), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity, Hardy-Weinberg equilibrium (HWE) *p* values with significance post sequential Bonferroni correction (*<0.05, **<0.01 and ***<0.001)

Table 2.1: continued

Locus	Repeat motif	Primer sequences (5'-3')*	GenBank accession number	Species	Size range (bp)#	Panel	Primer concentration (nM)	MRT generic primer label	Population	N	N_A	H_O/H_E	HWE
Nyg23	(TAAAA)13	F:TTGTTGCTGTTCATATGTGTTAGG	KC688304	*N. gouldi*	135-190	2	20	VIC	Dry Creek	66	10	0.82/0.84	0.187
		R:GAAAACAGAGGTTGTTTGTTGG							Hotspur	31	9	0.77/0.79	0.899
				N. geoffroyi	135-215	2	20	FAM	Annya	34	15	0.88/0.86	0.359
									Hotspur	36	11	0.78/0.85	0.289
Nyg25	(ATA)8	F:GCACAGATAATATGGTGTCCCTG	KC688305	*N. gouldi*	200-212	1	20	VIC	Dry Creek	66	5	0.60/0.69	0.507
		R:ATGGACAGGGGTTGTGTTTT							Hotspur	31	4	0.65/0.66	0.536
				N. geoffroyi	193-217	1	20	VIC	Annya	34	7	0.77/0.79	0.035
									Hotspur	36	6	0.72/0.75	0.467
Nyg29	(ATT)13	F:CTTTGCCAGGACCCAACTG	KC688306	*N. gouldi*	222-234	2	20	FAM	Dry Creek	66	5	0.70/0.74	0.446
		R:AAACGGGTTATTTCGTGCTG							Hotspur	31	5	0.80/0.73	0.973
				N. geoffroyi	206-251	1	20	NED	Annya	34	13	0.82/0.87	0.297
									Hotspur	36	12	0.94/0.88	0.887
Nyg31	(AT)9	F:TCATTCCAACCAAAATAAAATAAATG	KC688307	*N. gouldi*	107-129	2	20	VIC	Dry Creek	66	6	0.58/0.66	0.424
		R:ACTGGTCATCCTGATTGCTG							Hotspur	31	5	0.80/0.74	0.950
Nyg33	(AG)9	F:GCAGGGTACAGCTGGAGAAT	KC688308	*N. gouldi*	112-118	1	20	NED	Dry Creek	66	4	0.54/0.55	0.513
		R:AGTCACGTGTCTCATTTCCC							Hotspur	31	4	0.58/0.57	0.242
Nyg37	(TTCT)8	F:GAAATGTTTGGGAGGGGATT	KC688309	*N. gouldi*	180-232	1	20	FAM	Dry Creek	66	9	0.67/0.74	0.581
		R:TCTTCAGTGAATAGCAAGTGAAGTAA							Hotspur	31	8	0.80/0.69	0.944
				N. geoffroyi	187-295	1	20	PET	Annya	34	21	0.97/0.93	0.533
									Hotspur	36	20	0.92/0.92	0.258
Nyg39	(CAT)12	F:AATCAGCACCACTGTTGTCG	KC688310	*N. gouldi*	107-116	2	40	NED	Dry Creek	66	4	0.56/0.58	0.073
		R:CCCAGAATAAGGAGTTGTGACC							Hotspur	31	4	0.61/0.57	0.276
				N. geoffroyi		2	40	PET	Annya	34	8	0.32/0.83	0.000 ***
									Hotspur	36	10	0.53/0.82	0.000 ***

*Forward and reverse primers were tagged with a 5'M13 universal sequence (F:5'-ACGACGTTGTAAAA-3', R:5'-CATTAAGTTCCCATTA-3')
#Size range includes universal 5'M13 sequences

Chapter 3

The influence of habitat fragmentation on population connectivity and genetic diversity in a microbat, Gould's long-eared bat (*Nyctophilus gouldi*)

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ABSTRACT

Habitat fragmentation has been recognised globally as one of the major threats facing biodiversity. Chiropterans represent approximately 20% of described mammal species yet we know little about how habitat fragmentation influences population connectivity or genetic diversity in this mammalian Order. We address this issue by examining the impact of habitat fragmentation on a vespertilionid (*Nyctophilus gouldi*) in south-eastern Australia. Two hundred and fifty-nine individuals were sampled across 12 populations. We employed 15 microsatellite markers designed for this study, to assess population structure and genetic diversity in fragmented and continuous forest. We found that distances ≤27km across agricultural land may represent a barrier to dispersal for this forest specialist. In contrast, populations connected through continuous habitat revealed no structure over distances up to 80km and gene flow appears unimpeded by agricultural distances <2km. Fragmented populations displayed signs of reduced genetic diversity, inbreeding, higher numbers of relatives and skewed sex ratios. We make recommendations for the management of endangered South Australian populations and raise concerns about a proposed Management Unit at the Grampians. Despite the vagility of bat species we conclude that agricultural land can impede gene-flow and impair population connectivity raising concerns about the long-term viability and persistence of isolated populations.

INTRODUCTION

Land clearance and the subsequent fragmentation of native vegetation is recognised globally as one of the major threats facing terrestrial species (Baillie et al. 2004; Bennett 2003). Habitat fragmentation can impose a barrier to dispersal between populations disrupting metapopulation dynamics and rendering populations isolated, causing a reduction in their effective size and viability (Hanski 1998; Lindenmayer & Peakall 2000; Saunders et al. 1991). Consequently, the effective management of species in fragmented habitat requires data on dispersal thresholds and gene-flow across the intervening matrix between remnant habitat patches to ensure sufficient dispersal is maintained.

Bats are highly speciose and abundant representing approximately 20% of described global mammal species (Wilson & Reeder 2005). These species play vital roles in ecosystem function providing services such as plant pollination, seed dispersal, and the control or suppression of insect numbers (Kunz et al. 2011). Despite their importance, the influence of habitat fragmentation on bat population connectivity and dispersal thresholds has received limited attention. Although bats are extremely vagile by nature they are not necessarily immune to the impacts of habitat fragmentation and may be prone to population isolation like other less mobile taxa. Indeed, numerous studies have documented changes in the presence and abundance of bats in fragmented habitat and the disappearance of species from forest remnants (Cosson et al. 1999; Estrada & Coates-Estrada 2002; Estrada et al. 1993; Medina et al. 2007; Schulze et al. 2000).

Several bat studies have proposed that poor habitat connectivity has resulted in increased population differentiation and reduced dispersal. Campbell et al. (2009) suggested that significant F_{ST} values between nearby populations of *Myotis macropus* may be the result of limited dispersal due to reduced riparian habitat in the agricultural landscape. Kerth and Petit (2005) also proposed that a barrier due to habitat fragmentation could explain patterns in the population structure of *Myotis bechsteinii*. However, we are only aware of two studies, Meyer et al. (2009) and Struebig et al. (2011), specifically designed to investigate the impacts of anthropogenic habitat fragmentation on genetic diversity and population differentiation in bats. While both studies identified negative genetic impacts on the least mobile of their study species, both studies also had their limitations. Meyer et al. (2009) acknowledge that marker choice (mtDNA) limited the power and findings of their study reflecting only female-

mediated gene-flow. Struebig et al. (2011) on the other hand did not seek to identify thresholds for population connectivity and dispersal; instead their purpose was to investigate the relationship between declines in species richness and allelic richness due to habitat fragmentation.

The study species: Gould's long-eared bat (*Nyctophilus gouldi*)

Nyctophilus gouldi (Tomes, 1858) is a small (<16.5g) insectivorous (Grant 1991) vespertilionid that roosts in hollows produced by mature eucalypts (Lunney et al. 1988). The species distribution is limited to forest and woodland in eastern and south-western Australia (Churchill 2008; Ellis et al. 1989; Hall & Richards 1979). Wing morphology suggests that *N. gouldi* is suited to slow highly manoeuvrable flight in cluttered environments (Brigham et al. 1997; Fullard et al. 1991). Lunney et al. (1988) found that radio tracked individuals travelled <2km from roosting sites supporting indications from wing morphology that the species may be unsuited to sustained long-distance flight. Law et al. (1999) proposed that *Nyctophilus spp.* have limited dispersal abilities and are sensitive to habitat fragmentation based on the detection of a negative correlation between activity and habitat isolation, and a positive association with large or continuous habitat. Lumsden and Bennett (2005) trapped *N. gouldi* in a rural landscape across a gradient of tree densities from dense to sparse and found that the species only persisted in densely treed conditions. Collectively this evidence supports the hypothesis that *N. gouldi* is a forest habitat specialist that is sensitive to habitat fragmentation. Coupled with a threatened status in South Australia (SA), where the remaining habitat is both limited and highly fragmented, *N. gouldi* represents an ideal candidate to investigate the influence of habitat fragmentation on bat population connectivity.

Aims

This study aims to address the lack of knowledge regarding the impact of habitat fragmentation on bat population connectivity by conducting an assessment of *N. gouldi* population structure and gene flow across a landscape comprising continuous and fragmented forest. To facilitate this study we have developed a suite of microsatellite markers. These markers will be used to test the hypothesis that gene flow will be higher between sites connected by continuous native forest than between sites separated by agricultural land. Analyses will be used to assess population structure across the landscape and to identify

dispersal events and distance thresholds for population connectivity. We will also investigate the impact of habitat fragmentation on genetic diversity, relatedness and inbreeding. The study will provide insights into the impact of habitat fragmentation on microbats and make recommendations to promote *N. gouldi* metapopulation dynamics to improve population persistence in fragmented landscapes. These results will be of particular importance for the management of endangered SA populations of *N. gouldi* which are restricted to highly fragmented remnant vegetation.

METHODS

Study sites and sample collection

Fieldwork was conducted at 12 sites across south-eastern Australia (Figure 3.1). Four sites, Strathdownie, Hotspur and Annya State Forests (SF) and Mt Eccles National Park (NP), comprised an 80km transect through continuous forest in Victoria providing a comparison to distances between our fragmented sites. Although Mt Eccles is not directly connected to Annya due to several small breaks in the forest collectively spanning ~1.6km of agriculture (the largest spanning 800m) we felt it was permissible to include the site in this context given the scale of this study. This decision was later supported through genetic analyses. Two additional 'unfragmented' forest sites, the Grampians and Great Otway NPs, were sampled as possible sources of gene-flow to fragments isolated in the agricultural matrix. The remaining six sites represent forest fragments of varying size and degrees of isolation. Embedded in *Pinus radiata* plantations Nangwarry, Dry Creek and Honan's Native Forest Reserves (NFR) are located in south-eastern SA and respectively cover 2218ha, 396ha and1041ha. These sites represent three of the largest and most significant stands of remaining *N. gouldi* habitat in SA where the species is listed as endangered. The three remaining fragments are located in western Victoria amidst a vast region of agricultural land between Mt Eccles, the Grampians and the Otways. Mt Napier encompasses 2800ha and our two most isolated fragments, Woolsthorpe Nature Conservation Reserve and Framlingham Native Title Reserve, span 60ha and 1180ha respectively.

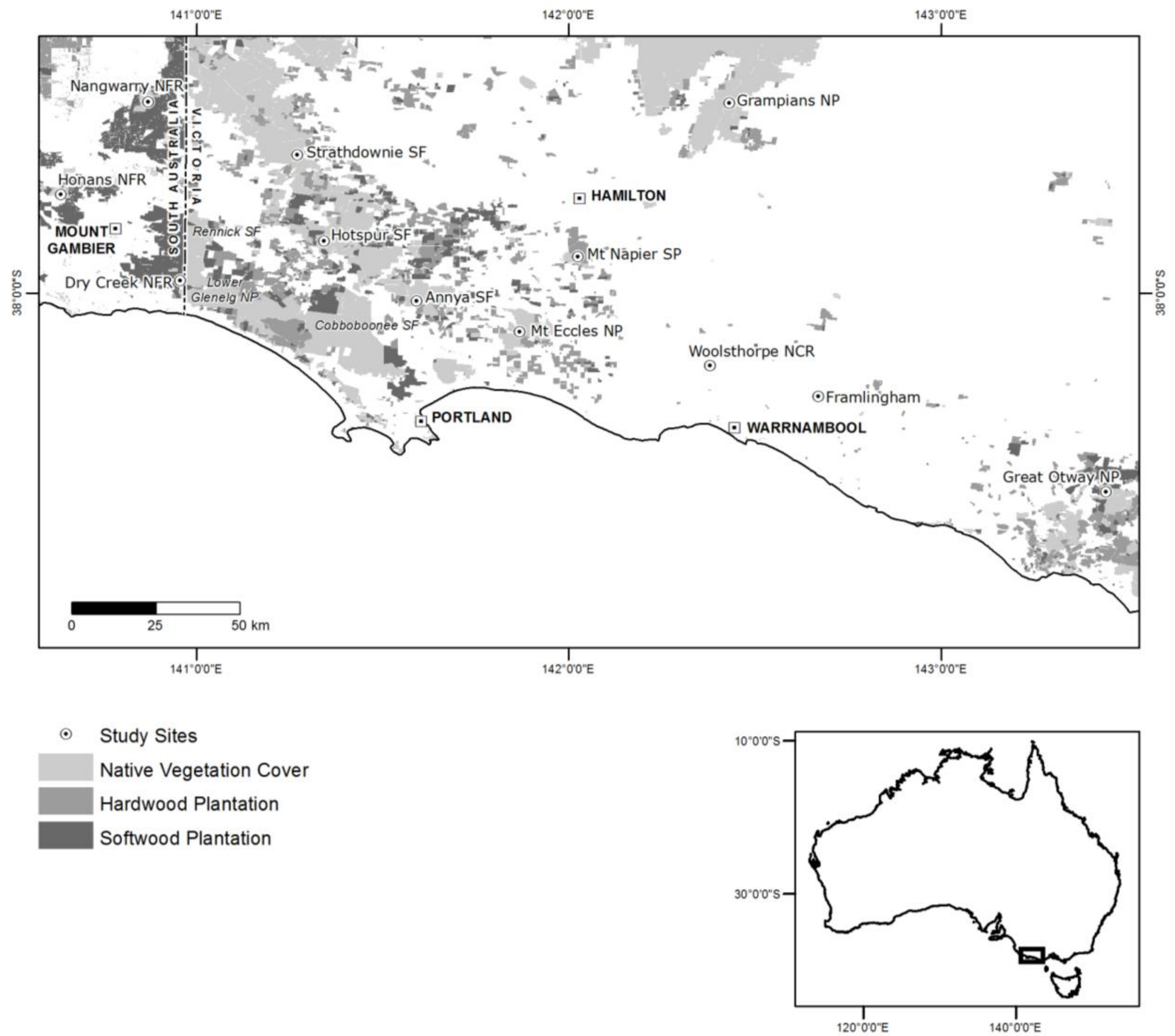

Figure 3.1: The distribution of 12 *N. gouldi* study sites across Victoria and South Australia. *N. gouldi* were sampled in native vegetation (light grey) embedded within a matrix of hardwood (mid grey) and softwood plantations (dark grey) and agricultural land (white).

The study region has been extensively cleared for agriculture since European settlement of Victoria in 1834 creating a landscape mosaic of habitat islands within an agricultural matrix. The history of the Grampians differs in this respect as it was naturally isolated from the rest of the study sites by native grassland at the time of European settlement and it is likely that this isolation dates back to the late Pleistocene or early Holocene when these grasslands emerged (DSE 2004a, b, 2011; Jones 1999). Throughout this manuscript we will refer to the study sites as fragmented or unfragmented sites, further distinguishing the latter by referring to the four sites connected through native forest as the continuous sites.

Bats were trapped between November and April over field seasons in 2008-2009 and 2009-2010 using eight harp traps for a total of 1252 trap nights. Traps were placed at locations where tree hollows were present and where the vegetation provided a funnel to increase trap success. All trap locations were recorded with GPS for spatial genetic analyses. Trapping was conducted in the central core of each site and to avoid the influence of the size of the sampling area on genetic diversity we trapped over a similar area within each site (1-2km). The exception to this approach was at the Grampians and the Otways where trapping was conducted over a larger area due to difficulties locating the target species. Trapping was also conducted in peripheral regions of these two parks so as to sample the most proximal location to neighbouring study sites in otherwise vast stretches of continuous forest.

Traps were set at dusk and checked before midnight and again before sunrise when they were closed and non-target species released under the cover of darkness. Target animals were held for processing during the day in individual hold bags kept in a cool dark quiet location and released at the point of capture the following evening. We recorded standard morphometric measurements and animals were sexed, with female reproductive condition assessed by examining teat and abdominal development. Bats were also aged and categorised as either adults or sub-adults/juveniles based on the calcification of wing bones (Tidemann 1993). Tissue samples for DNA analysis were collected via two 3.5mm wing membrane biopsies taken from each bat (one from each wing) with a sterile biopsy punch and were stored in a 50-50 ethanol-saline solution.

DNA extraction and microsatellite genotyping

Nuclear DNA was extracted from 128 biopsies using the Gentra Puregene extraction kit (Gentra Systems Inc) and the remaining 151 samples were extracted by the AGRF (Australian Genome Research Facility, Waite Campus, Adelaide). All DNA was subsequently quantified using a Nanovue spectrophotometer (General Electric) and concentrations were standardised to 10ng/µL. Individuals were screened at 15 microsatellite loci developed for this study utilising 454 sequence data (Chapter 2) and Multiplex Ready Technology (MRT) (Hayden et al. 2008). PCRs were performed according to methods outlined in Chapter 2 on a Corbett Palm Cycler (model CG1-96) utilising BIOMEK 3000 robots (Beckman Coulter) to set up PCRs and to pool products post PCR into two panels. These products were cleaned using a Millipore vacuum plate (Multi Screen PCR µ96 Plate) and manifold (Multi Screen$_{HTS}$

Vacuum Manifold), and diluted before being sent to AGRF for electrophoresis and visualisation on an ABI 3730 DNA Analyser.

Genotypes were scored using GENEMAPPER v.3.5.1 (Applied Biosystems) software and tested with the program MICROCHECKER v.2.2.3 (Van Oosterhout et al. 2004) for typing errors and the presence of null alleles before undertaking subsequent analyses. We used GENEPOP v.3.4 (Raymond & Rousset 1995) to test populations and loci for deviations from Hardy-Weinberg equilibrium (HWE), heterozygosity excess and deficiency, and linkage disequilibrium (LD), with sequential Bonferroni corrections made for these and all subsequent tests involving multiple comparisons (Rice 1989). Markov chain parameters in GENEPOP were applied using the default settings.

Population differentiation: comparing continuous and fragmented sites

To assess population structure across the study region and compare structure between sites connected by continuous habitat and sites fragmented by agricultural land we calculated several measures of population differentiation. As a well-established measure of population differentiation we calculated F_{ST} using ARLEQUIN v. 3.5 (Excoffier & Lischer 2010). Additionally, and in response to recent articles discussing the use of F_{ST} and its relatives (Gerlach et al. 2010; Heller & Siegismund 2009; Jost 2008, 2009; Meirmans & Hedrick 2011; Ryman & Leimar 2009; Whitlock 2011), we calculated Jost's D_{est} (Jost 2008) using the package *DEMEtics* (Gerlach et al. 2010) for the program R v. 2.1.3.1 (R Core Development Team 2011). Due to low capture rates (≤2 individuals) at three fragmented sites (Mt Napier, Framlingham and Woolsthorpe) these, and all subsequent, analyses were restricted to 256 individuals across nine populations.

Identifying genetic clusters across the landscape

To further investigate population structure we employed several Bayesian approaches to identify genetic clusters across the landscape. Recent reviews and comparative tests on the use of Bayesian clustering software have highlighted the advantages of concurrently employing multiple programs to verify the number of clusters (K) within a dataset (Chen et al. 2007; Francois & Durand 2010; Guillot et al. 2009; Latch et al. 2006; Rowe & Beebee 2007). Consequently we implemented four Bayesian clustering packages to estimate K across our

study region. Two of these analyses, STRUCTURE v. 2.2 (Pritchard et al. 2000) and BAPS v. 5.2 (Corander et al. 2003), were utilised to infer clusters based on genotypic data alone, whilst the remaining packages, GENELAND v. 3.3 (Guillot et al. 2005) and TESS v. 2.3 (Chen et al. 2007) incorporated both genotypic and spatial (geographic coordinates of sampling locations) data to calculate K. For further information on the differences between these packages model assumptions and algorithms refer to the recent review by Francois & Durand (2010).

Latch et al. (2006) illustrated that STRUCTURE and BAPS may have difficulty identifying the correct K and accurately assigning individuals to clusters when F_{ST} values are low (<0.03). In this scenario Latch et al. (2006) recommend that the parallel use of BAPS and STRUCTURE can increase the confidence of the results when K is inferred independently and there is a consensus between the approaches. We ran STRUCTURE to test for K between 1 to 9 with 10 iterations of each K with no priors, admixed ancestry and correlated allele frequencies with burnin and run lengths of 100 000 and 1 million respectively. STRUCTURE HARVESTER v. 0.6.8 (Earl & vonHoldt 2012) was used to employ the Evanno method (Evanno et al. 2005) to select K from our STRUCTURE results. BAPS was similarly run with 10 iterations of each K from 1 to 9 using the admixture model based on mixture clustering of individuals with 100 000 iterations and, following the recommendation of Corander and Marttinen (2006), we ignored clusters with fewer than five individuals.

Combining genotypic and spatial data, we conducted 15 independent runs of GENELAND with K set from 1 to 9. Using the correlated allele and null allele models we set the coordinate uncertainty to 500 and performed 1 million repetitions with thinning set to 100. Once the value of K was determined we used this value to rerun the analysis 10 times with the same parameters and K fixed to assess the stability of cluster locations and variation in the assignment of individuals to particular clusters. TESS was performed with 10 000 sweeps and a burnin of 5000 and we set multiple Ks from 2 to 9 with 10 iterations of each. We selected the conditional autoregression (CAR) admixture model and did not elect to set the initial CAR variance or to infer CAR variance from the data, nor did we continue with the lowest deviance information criterion (DIC) from a previous run or start from a clustering pattern obtained by a neighbour-Joining algorithm.

Prior to conducting Bayesian analyses all parent-offspring and full sibling relationships were established in KINGROUP v. 2 (Konovalov et al. 2004) using the likelihood method of Queller & Goodnight (1989). To avoid any bias from sampling family groups we removed one individual from each identified pair of relatives from the dataset. This process reduced the number of samples included in the Bayesian analyses to 229 across nine populations.

Isolation by distance: global test and comparison between continuous and fragmented sites

To identify patterns of isolation by distance (IBD) across the study region we used GENALEX v. 6 (Peakall & Smouse 2006) to perform three Mantel tests on our dataset using individual pairwise geographic coordinates and genetic distance as defined by Smouse & Peakall (1999). The first test analysed the study region as a whole, while consecutive tests investigated the influence of matrix type (forest versus agricultural land) by independently analysing sites connected through continuous forest (Strathdownie, Hotspur, Annya and Mt Eccles) and sites separated by agriculture (Nangwarry, Dry Creek, Honans, Grampians and the Otways). Mantel tests can be sensitive to missing data and with twelve individuals missing data for at least one locus we utilised the 'Interpolate Missing' data option to fill in blanks with the average genetic distance for the respective locus and population.

The influence of geographic distance, agricultural distance and intervening matrix type (forest Vs agriculture) on population differentiation

To further examine the underlying causes of genetic differentiation between sites we used IBD v. 1.52 (Bohonak 2002) to carry out Mantel and partial Mantel tests at the site level based on pairwise population F_{ST} and D_{est} values. This approach was employed using a third indicator matrix in two varying ways. For our first test the indicator matrix represented the intervening matrix type between sites represented by a '1' for agricultural land and a '0' for continuous native forest. Secondly we used the indicator matrix to input a proposed least-cost-path distance between each site measured as the route spanning the shortest accumulative distance across agricultural land which we will refer to as agricultural distance. The partial Mantel tests permitted analysis of the relationship between genetic and geographic distance when controlling for the indicator factor and conversely the relationship between genetic distance and the indicator factor when controlling for geographic distance.

Identification of dispersal events and thresholds for gene-flow

To investigate whether dispersal is occurring across agricultural land or if it is restricted to continuous forest we attempted to identify dispersal events by conducting first-generation migrant detection (F_0) in GeneClass v. 2 (Piry et al. 2004). Tests were performed according to the Bayesian method of Rannla & Mountain (1997) using the Monte Carlo resampling approach of Paetkau et al. (2004) with 10 000 simulated individuals and a significance level of 0.05. Due to the size of the study region several populations were not sampled and we implemented the appropriate model ('L=home') for migrant detection which assumes that not all possible source populations have been sampled.

We performed spatial autocorrelations within GENALEX to test for patterns associated with positive local neighbourhood structuring and negative relationships indicating distance thresholds for dispersal. These tests were performed in the same manner as our GENALEX Mantel tests utilising individual pairwise geographic coordinates and genetic distances. We independently assessed sites separated by agriculture and continuous sites over a distance of 80km. We used variable distance classes which allowed us to obtain a resolution of 5km distance classes where data permitted. Spatial autocorrelations utilised the entire dataset including six additional individuals from Warreanga NFR and Weecurra SF (see Chapter 4 for locations). These additional individuals were not reported elsewhere in this chapter as the sample sizes were too small to utilise for population level analyses and, unlike Mt Napier, Framlingham and Woolsthorpe, insufficient trapping was conducted to draw any conclusions about the presence or abundance of *N. gouldi* at these locations.

Genetic and demographic consequences of habitat fragmentation: comparing fragmented and unfragmented sites

To investigate the genetic consequences of habitat fragmentation on small or isolated sites we assessed a range of measures reflecting genetic diversity, relatedness and inbreeding, sex ratios and bottlenecks. To assess genetic diversity across the study region we calculated standard measures of genetic diversity (private alleles, H_O & H_E) using GENALEX, and allelic richness (A_R) as a standardised measure of allelic diversity based on sample size in FSTAT v. 2.9.3 (Goudet 2001). As indicators of inbreeding we calculated the inbreeding coefficient F_{IS} in FSTAT and two additional measures reflecting inbreeding using the package

Rhh (Alho et al. 2010) in the program R; standardised heterozygosity (SH: Coltman et al. 1999) and internal relatedness (IR: Amos et al. 2001). IR is a multilocus estimator of parental relatedness centred around zero with positive values suggesting inbreeding and negative values suggesting outbreeding (Amos et al. 2001). Sex ratios were also assessed for differences between populations and between fragmented and unfragmented sites. Bottleneck v1.2.02 (Cornuet & Luikart 1996) was used to identify recent bottleneck events in fragmented sites. Wilcoxon's test was used to determine the significance of heterozygosity excess calculated with 10 000 permutations. The analysis was performed using the two-phase-mutation model (TPM) (Di Rienzo et al. 1994) and following recommendations from Piry et al. (1999) we weighted the TPM with 5% infinite-alleles model (IAM) and 95% stepwise-mutation model (SMM). Allele frequencies within each fragmented population were also assessed for signs of a mode shift from the normal L-shape distribution (Luikart et al. 1998). Finally, KINGROUP was employed to identify related individuals and determine whether fragmented sites contain a higher proportion of relatives than other sites. The analysis was performed in accordance with the methods outlined above to identify four types of relationships: parent-offspring, full siblings, half siblings and cousins.

RESULTS

Distribution of *N. gouldi* across the landscape and sample collection

We sampled a total of 259 *N. gouldi* across 11 of our 12 sites with variable trap success in response to the time of year and minimum overnight temperatures. The conduciveness of the vegetation structure for corralling bats towards traps also played a role in trap success, particularly in taller forest such as the Otways where the canopy was well beyond the reach of harp traps. Due to these factors we trapped for different durations at each site and produced variable sample numbers. However, at several sites the species was in such low densities (or absent) that we either caught no individuals or too few to utilise for population genetic analyses despite thorough trapping effort. No individuals were caught at the small and highly isolated Woolsthorpe, only one *N. gouldi* was caught at the equally isolated Framlingham and just two individuals were caught at Mt Napier. Sufficient samples for analysis were obtained from the remaining nine populations including the three fragmented sites in SA.

Tests for Hardy-Weinberg equilibrium, linkage disequilibrium and null alleles

All fifteen microsatellite loci were polymorphic displaying between five and fourteen alleles with an average of nine per locus. The Grampians was the only population to deviate from HWE at the population level ($p<0.01$) or for a particular locus (NyGo31, $p<0.05$). Heterozygote excess was detected at the Grampians for locus NyGo31 ($p<0.05$) and there were no cases of heterozygote deficiency. The Grampians was also the only site to display linkage disequilibrium (LD) which occurred between locus NyGo17 and NyGo21 ($p<0.001$). MICROCHECKER revealed no evidence of null alleles, large allele drop out or scoring errors with one exception: the Grampians displayed signs of homozygote excess or possible null alleles at four loci NyGo11, NyGo17, NyGo23 and NyGo33. With only single instances of LD and deviation from HWE all 15 loci were retained for further analyses.

Population differentiation: comparing continuous and fragment sites

Both F_{ST} and Jost's D_{est} revealed numerous cases of significant differentiation between populations (Table 3.1). F_{ST} values ranged from extremely low ($F_{ST} = 0.000$) to high ($F_{ST} = 0.270$). The four continuous sites displayed no differentiation ($F_{ST} = 0.000$) with one non-significant exception between Annya and Hotspur ($F_{ST} = 0.005$, $p = 0.303$), indicating high rates of gene flow through the continuous forest. The SA fragment Dry Creek displayed similarly low rates of F_{ST} with these four sites suggesting gene-flow is freely occurring between these locations. In stark contrast the levels of differentiation between the Grampians and other locations were both extremely high and significant ($F_{ST} = 0.229\text{-}0.270$, $p<0.001$) indicating population isolation with severely restricted or absent gene-flow to other sites. Only one other significant F_{ST} value was recorded between the two most distal study sites, Honans and the Otways ($F_{ST}=0.053$, $p<0.001$). The remaining measures of F_{ST} were low to moderate ($F_{ST} = 0.002\text{-}0.033$) and not significant ($p>0.05$).

D_{est} values also ranged from low to extremely high ($D_{est} = 0.000\text{-}0.386$) but when compared to F_{ST} many more significant cases of differentiation were revealed. Again the Grampians was significantly differentiated from all other sites ($D_{est} = 0.331\text{-}0.386$, $p<0.05$). However, all the other study sites isolated by agriculture also displayed consistent significant differentiation. The Otways and Nangwarry were significantly differentiated from all but two other study sites, the latter only showing non-significant relationships with two proximal neighbours

Table 3.1: Population differentiation calculated from 15 loci across nine populations of *N. gouldi*. F_{ST} (Arlequin) below the diagonal and D_{est} (DEMEtics) above with p values provided before ($_{*,**,***}$) and after (*,**,***) sequential Bonferroni correction respectively indicating 0.05, 0.01 and 0.001 levels of significance. Nine populations are defined as: Nan = Nangwarry, Hon = Honans, Dry = Dry Creek, Ann = Annya, Otw = Otways, Gra = Grampians, MtE = Mt Eccles, Hot = Hotpur, Str = Strathdownie.

	Nan	Dry	Hon	Ann	Otw	Gra	MtE	Hot	Str
Nan	--	0.027*$_{***}$	0.033*$_{**}$	0.045*$_{**}$	0.069*$_{***}$	0.358*$_{***}$	0.050*$_{***}$	0.023$_{**}$	0.028$_{*}$
Dry	0.011	--	0.030*$_{***}$	0.005	0.049*$_{***}$	0.386*$_{***}$	0.011	0.000	0.000
Hon	0.022$_{*}$	0.016$_{**}$	--	0.025$_{*}$	0.098*$_{***}$	0.331*$_{***}$	0.035*$_{***}$	0.024$_{**}$	0.031$_{*}$
Ann	0.033$_{**}$	0.000	0.021$_{*}$	--	0.048*$_{**}$	0.369*$_{***}$	0.016	0.001	0.000
Otw	0.018	0.011	0.053***$_{***}$	0.033$_{*}$	--	0.345*$_{***}$	0.039$_{**}$	0.050*$_{**}$	0.028$_{*}$
Gra	0.247***$_{***}$	0.258***$_{***}$	0.243***$_{***}$	0.270***$_{***}$	0.229***$_{***}$	--	0.333*$_{***}$	0.346*$_{***}$	0.339*$_{***}$
MtE	0.023$_{*}$	0.002	0.009	0.000	0.019	0.250***$_{***}$	--	0.004	0.000
Hot	0.009	0.000	0.016$_{*}$	0.005	0.012	0.252***$_{***}$	0.000	--	0.000
Str	0.008	0.000	0.024	0.000	0.000	0.269***$_{***}$	0.000	0.000	--

(Strathdownie and Hotspur). The remaining two SA fragments, Honans and Dry Creek, were also characterised by multiple cases of significant differentiation. Only sites isolated by agriculture were distinguished by significant D_{est} values suggesting an association between agricultural isolation and population differentiation. This trend is made all the more evident when considering D_{est} values prior to Bonferroni correction which revealed that all pairwise comparisons were significant with the exception of those between the four continuous forest sites (Annya, Mt Eccles, Hotspur and Strathdownie) and Dry Creek, which all recorded low measures of D_{est}.

Identifying genetic clusters across the landscape

Similar to the findings of Rowe & Beebee (2007) the various Bayesian clustering packages we employed found different solutions to estimating K and assigning individuals, although broad similarities were also evident. STRUCTURE identified two genetic clusters (K=2) that were well defined geographically, with Cluster 2 representing the Grampians and Cluster 1 encompassing the remaining eight sites (Figure 3.2a). There was very little admixture between the two clusters which was reflected in the mean probability of membership (Q) for individuals assigned to each cluster (Cluster 1, Q = 0.966; Cluster 2, Q = 0.992). There were a handful of exceptions with two putative migrants from the Grampians detected in the

Otways (individuals Ngo157 & Ngo167) and another at Nangwarry (Ngo9). Two additional individuals were detected at Nangwarry with large proportions of their genotype assigned to Cluster 2 (Q = 0.584 & 0.313).

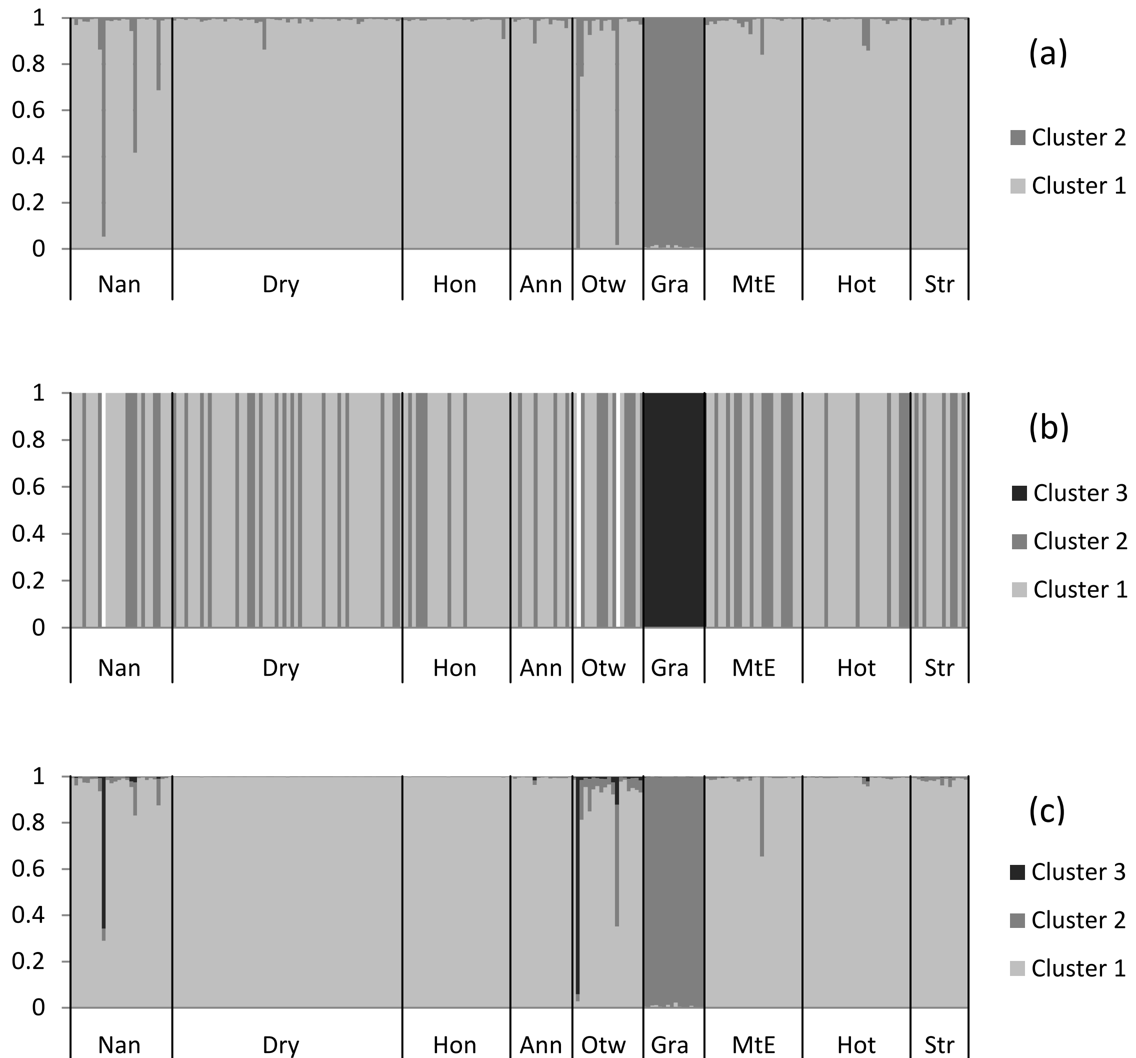

Figure 3.2: Individual assignment plots from STRUCTURE (a), BAPS (b) and TESS (c). Two hundred and twenty-nine *N. gouldi* individuals are represented along the *x*-axis by a vertical line representing the posterior probability of membership (Q), indicated along the *y*-axis, to genetic clusters (K) defined in the respective legend adjacent to each plot. Three white lines in Figure 3.2c indicate additional clusters that have been ignored due to underrepresentation (see text regarding BAPS results). Nine sampled populations are defined below the *x*-axis: Nan = Nangwarry, Hon = Honans, Dry = Dry Creek, Ann = Annya, Otw = Otways, Gra = Grampians, MtE = Mt Eccles, Hot = Hotpur, Str = Strathdownie.

BAPS found an optimal partition of six clusters (K=6) (Figure 3.2b). However, three of these clusters (Cluster 4, 5 & 6) were represented by sole individuals and in accordance with Corander & Marttinen (2006) they were disregarded as true clusters reducing the estimate to K=3. These three individuals were the same bats identified by STRUCTURE as potential migrants from the Grampians to Nangwarry and the Otways (Ngo9, Ngo157 & Ngo167). As found by STRUCTURE, one cluster discretely defined the Grampians (Cluster 3), however, the remaining sites all had similar numbers of individuals assigned to each of the two additional clusters (Clusters 1 & 2) which as a result were not geographically defined.

TESS found the highest DIC support for $K_{MAX} = 9$. However, after K_{MAX} was plotted against DIC and individual assignment probabilities were assessed, as prescribed in the TESS manual, it was evident that 3 clusters were present in the dataset (K=3) (Figure 3.2c). Most individuals were assigned to Cluster 1 and the Grampians was again distinguished as a unique cluster (Cluster 2). A third cluster (Cluster 3) was represented by the same three bats highlighted in previous analyses (Ngo9, Ngo157 & Ngo167) with two of these individuals (Ngo9 & Ngo157) assigned to this cluster. The third individual (Ngo167) displayed admixed proportions to all three clusters but was modally assigned to the Grampians (Cluster 2) ($Q = 0.515$). As Cluster 3 received the strongest representation in the Otways region the spatial plot of TESS assignment probabilities placed the cluster along the north-western edge of the Otways (Figure 3.3).

As with the previous Bayesian clustering analyses the results from GENELAND bore both similarities and differences with other packages. Four clusters (K=4) were identified in 13 of the 15 independent runs assessing variable Ks from 1-9, and this included the run with the highest overall posterior probability. Eight of the 10 subsequent runs with fixed K=4 produced consistent results for the geographic placement of clusters and individual assignment probabilities illustrated in Figure 3.4. The Grampians were again characterised by a unique cluster (Cluster 1; Figure 3.4a). Individuals from the remaining populations were all assigned to Cluster 2, although the probability of assignment to this cluster was weaker for individuals from the Otways as indicated in Figure 3.4b. The third and fourth clusters were only present in admixed proportions which received elevated representation at Annya and the Otways. Overall the distribution of posterior probabilities placed Cluster 3 along the north-eastern edge of the Otways (Figure 3.4c) and Cluster 4 was located south of Annya (Figure 3.4d).

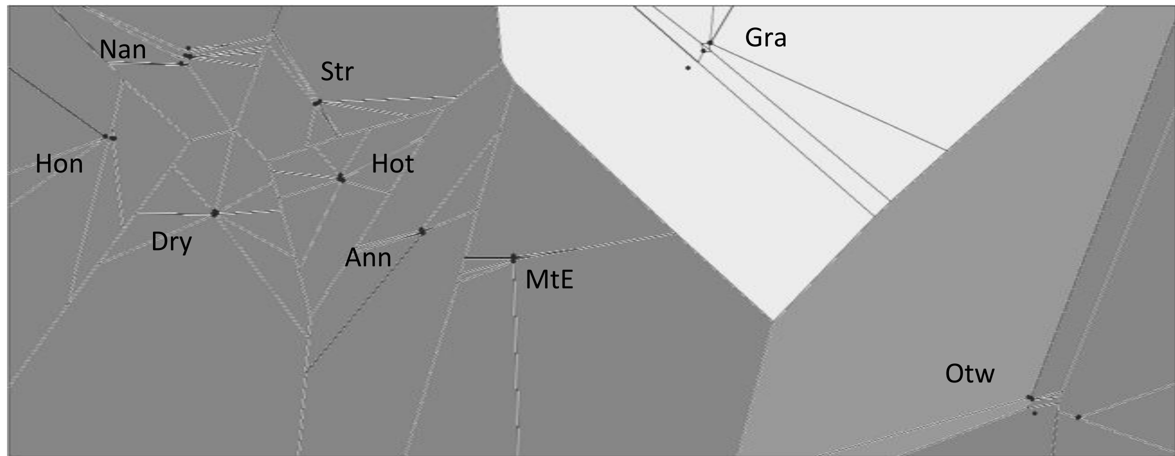

Figure 3.3: Spatial plot displaying the membership of *N. gouldi* individuals to three genetic clusters identified in TESS. Clusters are mapped via a Voronoi tessellation with black circles representing sampling locations and clusters defined as: Cluster 1 (dark grey), Cluster 2 (light grey) and Cluster 3 (mid grey). Nine sampled populations are represented: Nan = Nangwarry, Hon = Honans, Dry = Dry Creek, Ann = Annya, Otw = Otways, Gra = Grampians, MtE = Mt Eccles, Hot = Hotpur, Str = Strathdownie.

We re-ran STRUCTURE and GENELAND analyses with the Grampians removed to test whether this highly differentiated population was masking any weaker structure across the study region, but found no evidence of such structure. Similarly we separately analysed the continuous sites to test for weaker structure between populations connected by forest and again found no such signal.

Isolation by distance: global test and comparison between continuous and fragmented sites

Mantel tests assessing individual data (not populations) revealed a significant (P=0.001) yet mild correlation between geographic and genetic distance (R^2=0.057) indicating a weak pattern of isolation by distance (IBD) across the study region. Analysis of the four continuous sites (Strathdownie, Hotspur, Annya and Mt Eccles) revealed no IBD (R^2=0.0003, P=0.226). Separate analysis of the sites separated by agriculture (Nangwarry, Dry Creek, Honans, Grampians and the Otways) revealed IBD was marginally stronger than the initial correlation (R^2=0.078, P=0.001).

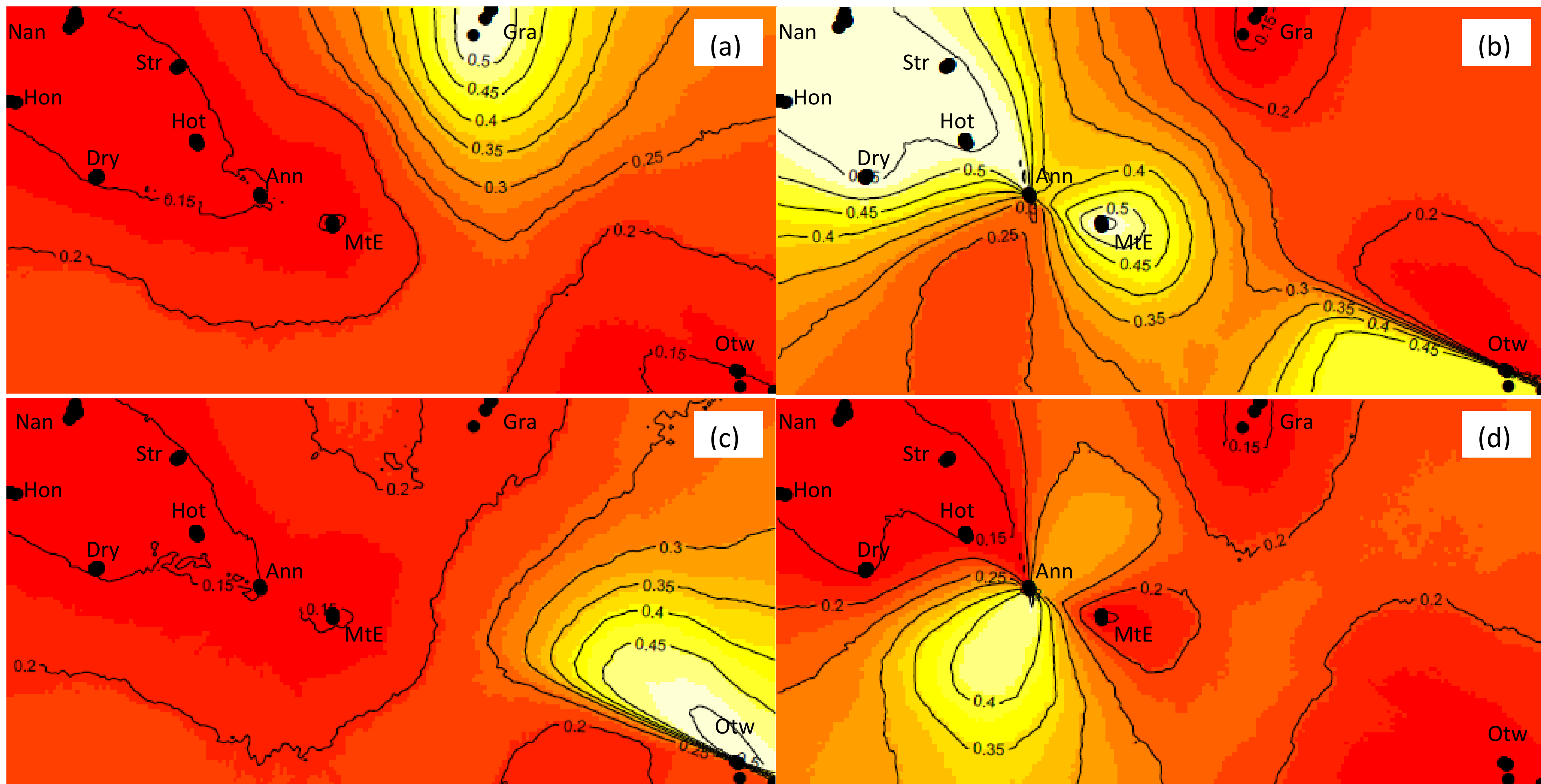

Figure 3.4: GENELAND results for *N. gouldi* illustrating the geographic distribution of four identified genetic clusters based on the posterior probability of individuals belonging to Cluster 1 (a), Cluster 2 (b), Cluster 3 (c) and Cluster 4 (d). White indicates a high probability of assignment to a given cluster while dark orange indicates low probability of assignment. Nine sampled populations are represented: Nan = Nangwarry, Hon = Honans, Dry = Dry Creek, Ann = Annya, Otw = Otways, Gra = Grampians, MtE = Mt Eccles, Hot = Hotpur, Str = Strathdownie.

The influence of geographic distance, intervening matrix type (forest Vs agriculture) and agricultural distance on population differentiation

In contrast to the previous Mantel tests, performed on individual genetic and geographic distance (above), there was no evidence of IBD between sites assessed at the population level based on pairwise F_{ST} (r=0.2171, p=0.132) or D_{est} (r=0.2859, p=0.108). However, the relationship between population differentiation and the matrix type was significant for both F_{ST} (r=0.2884, p=0.025) and D_{est} (r=0.3144, p=0.028) indicating that the presence of agricultural land between populations is positively correlated with increased genetic differentiation. The agricultural distance between sites was not correlated with either measure of differentiation (p>0.05) and partial Mantel tests produced no significant results (p>0.05).

Identification of dispersal events and thresholds for gene-flow

Twenty-four putative dispersal events were identified across the study region with the source population identified for 15 (Table 3.2). For the remaining nine events the most likely source population was the same site in which the individuals were trapped, possibly indicating that the true source population was not represented in our study sample. Of the 15 established dispersal events 11 occurred across agricultural land and four occurred between the continuous sites. Linear dispersal distances ranged from 26-258km (average = 81km) and agricultural distances ranged from 0-124km (average = 24km). Three putative dispersal events occurred between the fragmented SA sites (Nangwarry, Dry Creek and Honans). Two long-range dispersal events were proposed from the Otways to Nangwarry and Strathdownie spanning respective linear distances of 258km and 219km, and agricultural distances of 124km and 114km.

Spatial autocorrelation of the four continuous sites spanning a distance of 80km revealed no positive or negative correlations (Figure 3.5a). This contrasted with the analysis of fragmented sites spanning the same distance, where the association oscillated from positive to neutral up to 40km and then became negative beyond 60km (Figure 3.5b). This finding suggests that agricultural land limits dispersal causing a positive association with neighbouring sites and a negative association with populations >60km away. In continuous habitat neighbouring populations are no more related than sites 80kms away. In a

Table 3.2: Summary of dispersal events detected in GENECLASS displaying resident and source populations. Distance (km) of dispersal events are given as the amount of agricultural land crossed (agricultural distance) and total linear distance. Dispersal events were determined with a significance threshold of $p<0.05$.

Resident population	Source population	Agricultural distance (kms)	Linear distance (kms)
Nangwarry	Otways	124	258
Nangwarry	Dry Creek	27	53
Dry Creek	Nangwarry	27	53
Dry Creek	Strathdownie	2	47
Dry Creek	Hotspur	2	36
Dry Creek	Mt Eccles	4	82
Honans	Dry Creek	32	39
Mt Eccles	Hotspur	2	54
Mt Eccles	Annya	2	26
Mt Eccles	Dry Creek	4	82
Mt Eccles	Nangwarry	12	111
Hotspur	Annya	0	28
Strathdownie	Dry Creek	2	47
Strathdownie	Mt Eccles	2	75
Strathdownie	Otways	114	219

conservative approach we removed the Grampians from the fragmented dataset due to its possible long-term historic separation, and Mt Eccles from the continuous habitat sites as it's not strictly connected (see methods). This reduced our comparative distance to 55km which revealed the same result for the continuous sites, however, the fragmented dataset revealed a positive association within 5kms and a negative association beyond 35km. The overall story remained the same: under the influence of habitat fragmentation proximal sites are more genetically similar than distal sites, whereas there is no such pattern in continuous habitat.

Genetic and demographic consequences of habitat fragmentation: comparing fragmented and unfragmented sites

Measures of genetic diversity across the study region revealed some contrasting patterns between fragmented and unfragmented sites (Table 3.3). Allelic richness (A_R) did not significantly differ between sites (ANOVA, $p=0.86$) or between fragmented and unfragmented sites when samples were pooled (ANOVA, $p=0.68$). Nangwarry, the Otways and the Grampians all displayed notable numbers of private alleles (A_P) suggesting some level

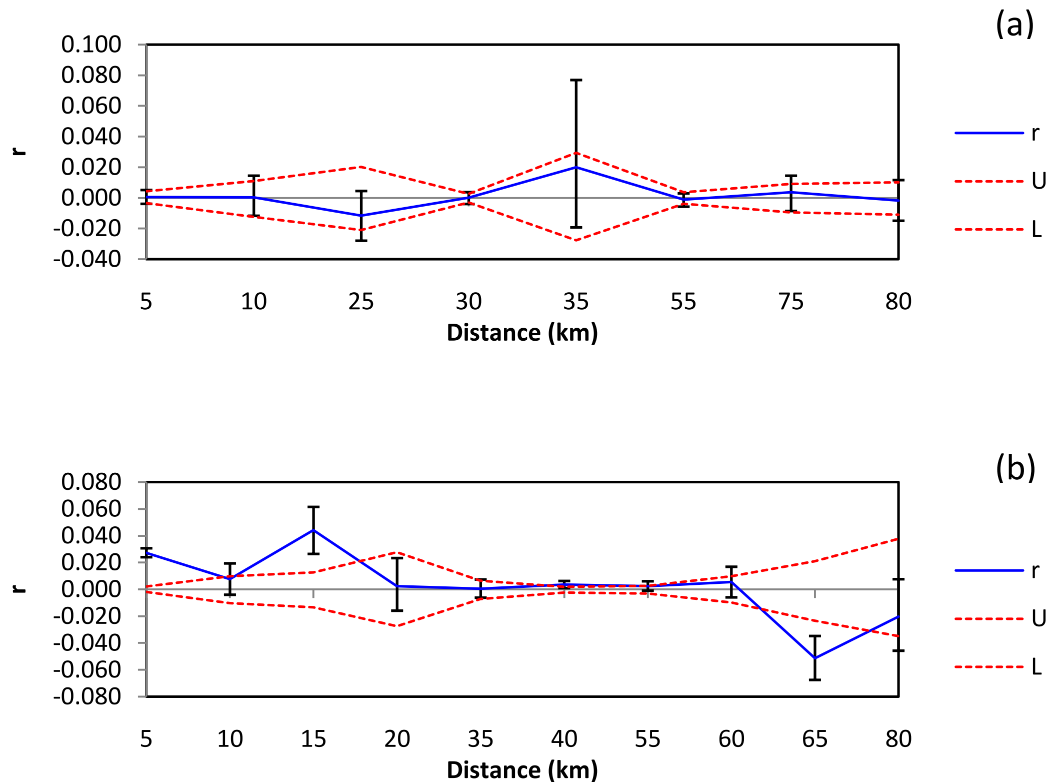

Figure 3.5: Results of spatial autocorrelations performed in GENALEX illustrating mean r (——) with 95% upper and lower confidence levels (······). Distance classes are displayed along the *x*-axis in km. Figures represent: (a) sites connected by continuous habitat, and (b) sites fragmented by agricultural land.

of independence from other sites. H_O and H_E did not significantly differ between populations (ANOVA, H_O p=0.80; H_E p=0.96), nor were differences significant between fragmented and unfragmented sites (ANOVA, H_O p=0.11; H_E p=0.91). However, H_O was consistently less than H_E in the fragmented SA populations and at the Grampians in contrast to the remaining unfragmented sites where the opposite was true. Standardised heterozygosity (SH) reflected elevated levels of heterozygosity at Nangwarry, three of the continuous sites (Annya, Mt Eccles and Hotspur) and the Otways, whereas Honans, Dry Creek, the Grampians and Strathdownie all displayed comparatively lower SH. SH differed significantly between populations (ANOVA, p=0.037) owing to differences between fragmented and unfragmented sites which, when pooled, were significantly different (ANOVA, p=0.0019).

By all measures of genetic diversity the lowest values were recorded in the most isolated fragment Honans (A_R = 4.895, H_O = 0.610, H_E = 0.631, HS = 0.933) while the highest levels

were all recorded in the largest forest sampled at the Otways (A_R = 5.889, H_O = 0.715, H_E = 0.694, HS = 1.087). Internal relatedness (IR) was higher in the three SA fragments, the Grampians and one of the continuous sites, Strathdownie (Table 3.3). IR for the Grampians (IR = 0.174) was particularly high with Honans (IR = 0.101) and Dry Creek (IR = 0.88) also recording higher values for this measure. It should be noted that IR was calculated using the entire dataset with allele frequencies pooled across populations. Consequently genetic subdivision and high numbers of private alleles, as characterised by the Grampians, can artificially elevate IR as a result of allelic partitioning rather than inbreeding per se. The remaining continuous forest sites (Annya, Mt Eccles and Hotspur) coupled with the Otways all had low IR values. IR differed significantly between populations (ANOVA, p=0.0021) and between fragmented and unfragmented populations (ANOVA, p=0.0017). The inbreeding coefficient (F_{IS}) was not significantly different between populations (ANOVA, p=0.66), but it was positive in all the fragmented SA populations, the Grampians, and just one of the continuous sites (Strathdownie), whereas the remaining three continuous populations recorded negative F_{IS} values as did the expansive Otways. This trend was reflected by the near-significant difference between pooled fragmented and unfragmented populations (ANOVA, p=0.057).

Table 3.3: Summary of population genetic measures and sample numbers across nine *N. gouldi* populations. N = number of samples, A_R = allelic richness, A_P = private alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, SH = standardised heterozygosity, IR = internal relatedness, F_{IS} = the inbreeding coefficient, and the number of male and female individuals. Fragmented sites are indicated in parentheses (f).

Population	N	A_R	A_P	H_O	H_E	SH	IR	F_{IS}	Males	Females
Nangwarry (f)	27	5.496	5	0.668	0.679	1.023	0.048	0.036	6	21
Dry Creek (f)	66	5.191	0	0.629	0.671	0.961	0.088	0.070	37	29
Honans (f)	36	4.895	0	0.610	0.631	0.933	0.101	0.046	12	24
Annya	17	5.242	0	0.684	0.644	1.041	0.005	-0.030	9	8
Otways	18	5.889	6	0.715	0.694	1.087	0.004	-0.002	13	5
Grampians	23	5.130	12	0.641	0.665	0.986	0.174	0.058	7	16
Mt Eccles	24	5.234	1	0.688	0.670	1.052	0.011	-0.006	17	7
Hotspur	31	4.965	1	0.682	0.666	1.042	0.009	-0.008	15	16
Strathdownie	14	5.267	0	0.643	0.641	0.983	0.057	0.034	13	1

Table 3.4: Summary of relationship classes detected in KINGROUP and the number of dyads for each class at nine *N. gouldi* populations. Fragmented sites are indicated in parentheses (f). Relationships were established with a confidence level of p<0.05.

Population	Parent-offspring	Full siblings	Half siblings	Cousins	Total
Nangwarry (f)		2		3	5
Dry Creek (f)	8	2	1		11
Honans (f)	5	8	1		14
Annya	1				1
Otways					
Grampians	1	16	10	2	29
Mt Eccles					
Hotspur		2			2
Strathdownie					
Total	15	30	12	5	62

Sex ratios differed significantly between populations (Pearson's x^2=34.91, df=8, p<0.0001) and between fragmented and unfragmented sites (Pearson's x^2=10.96, df=1, p<0.001) with fragmented populations and the Grampians recording more females than males. All other unfragmented populations recorded more males than females. We did not detect any signs of a genetic bottleneck in any of the fragmented populations or the Grampians which also displayed signs of genetic erosion and elevated relatedness. As the Grampians did not conform to the genetic patterns characterising the other unfragmented populations we decided that the site was somewhat unusual and may not be representative of typical unfragmented populations. Consequently the Grampians was not included in any of the fragmented versus unfragmented comparisons reported above.

The identification of relatives in KINGROUP revealed 62 related pairs (Table 3.4). Nearly half of these occurred in the Grampians which displayed particularly high numbers of full siblings. It should be noted that *N. gouldi* produce twins although the twinning rates are not known (Churchill 2008; Hosken 1998). Most of the remaining relatives were recorded in the fragmented SA populations with the exception of three pairs collectively identified at Annya and Hotspur. No relatives were detected in the Otways, Mt Eccles or Strathdownie. Overall there were low densities of relatives in large tracts of habitat, with the exception of the Grampians, and high numbers of relatives in fragmented populations.

DISCUSSION

Reduced gene-flow across agricultural land

Trapping data revealed the relative absence of *N. gouldi* from three of our six fragmented sites which included the two most isolated locations of Framlingham and Woolsthorpe. This in itself was an important finding suggesting *N. gouldi* may be unable to maintain viable populations under such degrees of isolation. Woolsthorpe is particularly small (60ha) and may not be large enough to support a viable population size. Framlingham is larger than several of our other fragmented sites supporting populations of *N. gouldi* (e.g. Honans and Dry Creek) suggesting that isolation may be a key factor determining the species near-absence. However, both Honans and Dry Creek have neighbouring networks of additional habitat patches which may collectively facilitate population persistence. Furthermore, a bushfire swept through Framlingham in 2007 affecting nearly the entire site (Geoff Clarke Jr. "Possum", Forest Manager, Framlingham Aboriginal Trust, *pers.comm.*). Consequently the absence of *N. gouldi* from the site could be due to a localised extinction or eviction caused by this fire event. In this scenario the absence may reflect the species inability to recolonise the site due to low numbers, or absence, in the surrounding agricultural matrix and lack of metapopulation dynamics with neighbouring populations to facilitate such a recolonisation.

Our genetic analyses provided multiple lines of evidence to suggest high rates of gene flow for *N. gouldi* between populations well connected by native forest, and through continuous forest, whilst indicating restricted gene flow between locations separated by larger stretches of agricultural land. This trend is supported by measures of population differentiation (F_{ST} and D_{est}), Mantel tests assessing the influence of the matrix type (agriculture versus forest), spatial autocorrelations comparing continuous and fragmented populations, and average dispersal distances of migration events proposed by GENECLASS analysis. The exception to the consensus in our results was the characterisation of population structure via Bayesian clustering analyses. Although the Grampians was consistently recognised as a unique cluster, lending support to isolation from agricultural land, this story was not reflected in other isolated sites. We suspect this phenomenon is due to the difficulty these analyses can have in identifying structure between populations when F_{ST} is <0.02-0.03 (Francois & Durand 2010; Latch et al. 2006). Excluding the Grampians from the calculation the average F_{ST} values for our populations were: Nangwarry 0.018, Dry Creek 0.006, Honans 0.023, Annya 0.013,

Otways 0.021, Mt Eccles 0.007, Hotspur 0.006 and Strathdownie 0.004. It is therefore not surprising that these approaches were unable to detect population structure elsewhere across the study region.

Despite the lack of support from these Bayesian clustering approaches for the population differentiation indicated through alternative analyses, there were nevertheless several interesting findings. Given the absence of samples in our study from populations along the Great Dividing Range, eastern Australia, and the Cobboboonee/Glenelg region in western Victoria it is possible that Clusters 3 (supported by GENELAND & TESS) and 4 (GENELAND) respectively represent the genetic influence of these potentially significant gene-pools, an interpretation supported by the proposed location of these clusters (Figures 3.3 & 4). Also of note was the lower probability of assignment for individuals from the Otways to Cluster 3.2 (GENELAND) compared to the westerly populations assigned to this cluster (see Figure 3.4b) suggesting some level of differentiation between these regions as indicated by D_{est}, and to a lesser extent F_{ST}.

As a more recently introduced measure of population differentiation, Jost's D_{est} was described by Callens et al. (2011) as being 'increasingly considered more reliable than traditional F_{ST} and related measures in assessing allelic differences'. In our study D_{est} proved highly informative, particularly in our comparison between continuous and fragmented sites. Prior to Bonferroni correction, D_{est} was significant between all locations with the exception of pairwise comparisons between the four continuous sites and Dry Creek, indicating high rates of gene flow between these five populations. Dry Creek is separated from the continuous sites by more than 30km. However, only two small agricultural crossings (~1.25km and 0.5km) separate the two via Lower Glenelg NP and Cobboboonee SF which represents the most likely path for gene flow. The agricultural distance isolating Dry Creek is similar to that separating Mt Eccles from the other continuous sites (~1.6km) and these two cases suggest that high rates of gene flow, indicated by low F_{ST} and D_{est}, can be maintained across agricultural distances <2km. Conversely, all sites separated by >2km of agricultural land displayed significant D_{est} prior to Bonferroni correction, with most cases retaining significance post correction. In addition to low measures of population differentiation, our decision to include Mt Eccles in the continuous forest transect is further supported by spatial autocorrelation suggesting unimpeded gene flow between the continuous sites.

Overall D_{est} and F_{ST} results suggested a strong positive association between habitat fragmentation and population differentiation. Mantel tests examining the correlation between population differentiation (F_{ST} and D_{est}) and the intervening landscape type (agriculture or forest habitat) confirmed a significant correlation between the presence of agricultural land and increased genetic differentiation. Meyer et al. (2009) similarly found that populations of *Carollia perspicillata* inhabiting fragmented habitat islands in an artificial lake were significantly more differentiated than populations sampled in surrounding continuous forest. Likewise, Kerth and Petit (2005) found that population differentiation in *Myotis bechsteinii* was more influenced by co-occurrence within continuous forest than by geographic distance alone. This mirrors our own findings in that populations separated by <2km of agricultural land are less differentiated than more fragmented populations regardless of the geographic distance between them. For example, Mt Eccles and Strathdownie (linear distance = 75km, agricultural distance = 1.6km) are less differentiated than Nangwarry and Strathdownie (linear distance = 38km, agricultural distance = 10km) despite being nearly twice as far away from one another.

Dispersal events proposed by GENECLASS also suggested preferential dispersal through suitable habitat. This was suggested by the respective average linear and agricultural dispersal distances of 81km and 24km. In addition, nine of the fifteen proposed dispersal events required crossing no more than 4km of agricultural land. As a further consideration, dispersal estimates in GENECLASS are probability based, and with low F_{ST} values between most sites these results should be treated with caution (Berry et al. 2004). In particular, the two long-range dispersal events are outliers to the general trend and should not be interpreted as confirmation of the species ability to traverse vast stretches of agricultural land. In fact, the individual proposed as a migrant from the Otways to Nangwarry (Ngo9) was highlighted as a migrant in STRUCTURE, BAPS and TESS, and each of these Bayesian approaches proposed a different source population. This example highlights the inaccuracy of such Bayesian techniques when population differentiation is too weak. The other proposed long-range migrant (Ngo263) did not have multiple solutions proposed by the different approaches, however, pairwise F_{ST} and D_{est} between the resident (Otways) and source (Strathdownie) populations was even lower than in the case of Ngo9. GENECLASS is also restricted to selecting the most likely candidate source population from the selection of sites provided. We acknowledge that not all locations within the study region were sampled and that the true source population may not be represented in the dataset. This seems likely given nine

migrants identified by the analysis were assigned no alternative source population. Our study was conducted across a vast region and there are several obvious potential source populations not represented in our study due to time constraints on field work. These locations include Rennick SF and Lower Glenelg NP adjoining Cobboboonee SF, in addition to forest outside of the study region along the Great Dividing Range which comprises most of *N. gouldi*'s distribution. A final consideration is that homoplasious allele sizes at distant sites have contributed to inferred long-range movements. If the two dubious long-range migrants are removed from the analysis the average linear and agricultural distances are revised down to 56km and 9km.

Distance thresholds for dispersal across agricultural land

As discussed, we found evidence for maintained gene flow across small agricultural distances <2km. Our data also sheds light upon dispersal thresholds for *N. gouldi* across agricultural land. Significant D_{est} between the three SA fragments illustrates that ≤27km of agricultural land (pastoral land and plantation pine) can result in significant population differentiation. Spatial autocorrelation of these three sites indicated a negative relationship between locations separated by a linear distance >35km, a distance derived from the comparison of Nangwarry and Honans which again corresponds to an agricultural distance of 27km (the remaining 8km comprising native habitat).

Significant differentiation was also detected between Nangwarry and the four continuous forest sites separated by an agricultural distance of just 10km. Prior to Bonferroni correction differentiation between Nangwarry and all four continuous sites was significant by D_{est}, while F_{ST} revealed significant differentiation between Nangwarry and the two more distal continuous sites of Annya and Mt Eccles. Post Bonferroni correction only the more distant Annya and Mt Eccles were significantly differentiated from Nangwarry by D_{est}. However, this example suggests that a barrier to gene flow may be imposed by as little as 10km of agricultural land.

The identification of dispersal events in GENECLASS supports preferential migration across more limited agricultural distances as indicated by the average dispersal distance of 9km (excluding the two suspect long-range events). Despite these examples, the most robust case we have to support a distance threshold for population connectivity is the collective evidence

indicating isolation between the three SA fragments. Consequently we propose a dispersal threshold of ≤27km for *N. gouldi* across agriculture. However, with high levels of gene flow only evident across agricultural distances <2km, future studies may determine that far less agricultural land can constitute a sufficient barrier to *N. gouldi* dispersal so as to cause significant population differentiation.

The identification of a ≤27km dispersal threshold across agriculture for *N. gouldi* is similar to that detected for the greater mouse-eared bat in regards to the 14km Gibraltar Strait which represents a significant barrier between populations in Europe and North Africa (Castella et al. 2000). This distance is also comparable to a threshold identified for the Azorean bat which was found to be restricted by more than 40km of open water between islands in the Azores (Salgueiro et al. 2008). Our data also suggested that gene-flow was maintained between habitat separated by <2km of agriculture. Bernard & Fenton (2003) found that this distance was also readily crossed by bats in Brazil where 23 individuals from 8 species were radio-tracked across 0.5-2.5km of open savannah in a naturally fragmented system. Lunney et al. (1988) radio-tracked 18 *N. gouldi* and found no evidence of movements beyond 2km, consequently the retention of gene-flow across <2km of agriculture in our study may reflect a limitation dictated or influenced by the species foraging range where greater distances may be energetically prohibitive. The identification of agriculture as a barrier to gene-flow supports indications from previous studies that *N. gouldi* may be sensitive to habitat fragmentation (Law et al. 1999; Lumsden & Bennett 2005).

Altered population genetics and demography in fragmented populations

The reduction in standardised heterozygosity (SH) within fragmented sites indicates a loss of genetic diversity within populations isolated by agriculture. Loss of genetic diversity in fragmented populations has been frequently recorded in a range of taxa including several recent chiropteran studies (Meyer et al. 2009; Struebig et al. 2011). *Carollia perspicillata* displayed signs of reduced genetic diversity in response to habitat fragmentation and is, like *N. gouldi*, a forest dwelling microbat of similar size (~18g) (Meyer et al. 2009). More akin to *N. gouldi* is *Kerivoula papillosa* which Struebig et al. (2011) found displays reduced genetic diversity with decreasing habitat area. This species is also a vespertilionid forest specialist that roosts communally in hollows and which was previously reported to show signs of sensitivity to habitat fragmentation (Struebig et al. 2008). In their study comparing bat

species richness and genetic diversity in fragmented habitat, Struebig et al. (2011) found that significantly more area was required to maintain genetic diversity (10 000ha) compared to species richness for forest specialists (2500ha). If such an area was required by *N. gouldi* to maintain genetic diversity it would have important implications for the threatened SA populations which are currently confined to much smaller habitat areas. Even following the completion of an initiative to enhance habitat connectivity within the region, the Lower South-East Biodiversity Corridors Project, which will increase the effective habitat area for the populations at Honans and Dry Creek, the combined area of the linked habitat patches will still be 2752ha and 1667ha respectively (ForestrySA 2003). Unless connectivity is further improved between the SA populations we may witness further reductions with long-term consequences for the viability of these threatened populations.

We found multiple lines of evidence to indicate that inbreeding may be occurring within the fragmented SA sites and the Grampians. This scenario was supported by lower observed heterozygosity than expected, reduced SH, elevated IR and F_{IS}, and the almost exclusive identification of related pairs within these four populations. Collectively these findings suggest that a barrier effect caused by agricultural land has resulted in elevated levels of inbreeding and increased numbers of related individuals. Elevated relatedness in habitat patches as a consequence of habitat fragmentation has been documented in numerous studies (Banks et al. 2005b; Delaney et al. 2010; Lancaster et al. 2011; Stow & Sunnucks 2004; Stow et al. 2001). We also detected a significant difference in sex ratios between fragmented and unfragmented populations. This trend was identical to that detected by Banks et al. (2005a) for *Antechinus agilis* where greater numbers of females were recorded in fragmented habitat and males were more abundant in unfragmented populations. Both the increase in relatedness and alterations to sex ratios could have profound impacts on the sociobiology of fragmented populations of *N. gouldi*.

Conclusion

Determining patterns of genetic loss and population connectivity in fragmented landscapes is vital to predicting population persistence and viability, and planning effective management. We have found evidence to suggest that although *N. gouldi* dispersal may occur across agricultural land it is significantly reduced, producing a range of measurable effects including significant population differentiation, localised genetic neighbourhoods, elevated relatedness,

altered sex ratios and reduced genetic diversity (SH). These effects may have significant implications for the viability of fragmented SA populations which are listed as endangered within the state.

We have proposed a dispersal threshold for *N. gouldi* across agricultural land of 27km at which point populations become isolated leading to genetic drift and erosion. More importantly for effective conservation management we have found that sufficient gene-flow can be maintained across a collective agricultural distance of 1.75km comprising multiple gaps with the largest not exceeding 1.25km. We recommend that this landscape configuration be used as a guideline for revegetation efforts to improve *N. gouldi* population connectivity and viability. Given the threatened status of *N. gouldi* within SA, such revegetation should be considered as a future addition to the Lower South-East Biodiversity Corridors Project (ForestrySA 2003). The current proposed network will link remnant habitat patches around Honans, Nangwarry and Dry Creek, and we recommend establishing connectivity between these three regions as the next step in a regional conservation management plan. This would help mitigate the genetic consequences of isolation this study has revealed for *N. gouldi* and provide conservation benefits for additional taxa.

Our analyses consistently identified the Grampians as a unique and isolated population. This was supported by Bayesian clustering methods, measures of population differentiation, high numbers of private alleles, elevated numbers of relatives, high Internal Relatedness (IR) and F_{IS} and reduced Standardised Heterozygosity (SH). The genetic distinctiveness of the population prompts us to recognise the site as a unique Management Unit (MU) based on the criteria of Moritz (1994). We recommend further investigation to determine whether the Grampians MU warrants recognition as an Evolutionarily Significant Unit (ESU) (Moritz 1994), and to confirm whether all populations of *N. gouldi* throughout the park are at risk of genetic erosion and inbreeding as detected in the sampled southern region.

This study has revealed that even robust unfragmented sites are being impacted by habitat fragmentation through increasing population differentiation as seen between the Otways and our continuous sites. We found no structure or differentiation between populations connected by suitable habitat suggesting that the once continuous forest across the study region probably supported a largely panmictic population. Consequently recent anthropogenic habitat fragmentation is artificially driving regional population differentiation that may ultimately

result in divergent populations, thus altering the evolutionary trajectory of the species. With the looming prospect of drifting species ranges due to climate change, land managers will increasingly need to consider improving regional habitat connectivity to facilitate distribution shifts in response to altering climatic conditions. Our study illustrates that even flying mammals may be limited in their capacity to adapt through migration due to barriers imposed by habitat fragmentation, thus highlighting the magnitude of risk for less vagile terrestrial and arboreal species.

Chapter 4

The comparative influence of habitat fragmentation on two congeneric vespertilionids with near-identical morphology and contrasting degrees of specialisation

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ABSTRACT

Few studies have investigated the influence of habitat fragmentation on population connectivity and genetic diversity in bats. We address this paucity of research by conducting a landscape scale investigation of population connectivity through continuous and fragmented habitats. Comparison of a habitat specialist and a habitat generalist with near-identical morphology provides a unique opportunity to test the reliability of several proposed predictors of bat sensitivity to habitat fragmentation. We developed 16 microsatellite markers to facilitate the study and sampled 502 *Nyctophilus geoffroyi* and 259 *N. gouldi* at 14 sites across south-eastern Australia. Fragmented populations of *N. gouldi* displayed reduced population connectivity, reduced genetic diversity, elevated relatedness and inbreeding (F_{IS}), and altered sex ratios. In contrast, *N. geoffroyi* displayed virtually no response to habitat fragmentation with fragmented populations showing the same levels of genetic diversity and population connectivity as populations within continuous forest. Our data suggest that *N. geoffroyi* is resilient to landscape change and is readily able to disperse across large tracts of agricultural land. Contrasting responses between these two species with near-identical morphology questions the reliability of wing morphology as a proposed predictor of bat sensitivity to habitat fragmentation. At the same time our results lend further support to other predictive traits of bat sensitivity to habitat fragmentation, most notably habitat specialisation and tolerance to the intervening matrix between fragmented habitats. We conclude that species with plastic ecology and behaviour are more likely to cope with fragmented habitat as are species commonly detected within the matrix.

INTRODUCTION

Habitat fragmentation is a principal cause of population declines and localised extinctions (Baillie et al. 2004; Burkey 1989; Reed 2004). Baillie et al. (2004) stated that habitat destruction, degradation and fragmentation represent the greatest threat to terrestrial species impacting 86% of threatened birds, 86% of threatened mammals and 88% of threatened amphibians worldwide. By restricting wildlife to small or isolated habitat islands habitat fragmentation can reduce effective population sizes exposing resident populations to a range of genetic, demographic and environmental threats (Caughley 1994; Frankham 1995; Lacy 1997; Shaffer 1981).

The ability of a species to utilise or traverse the intervening matrix between fragmented habitat patches determines population connectivity and thus whether a population is isolated, restricted in size and accessible for recolonisation following a localised extinction event (Brown & Kodric-Brown 1977; Burkey 1989; Fahrig & Merriam 1985; Fahrig & Paloheimo 1988; Hanski 1991, 1998). If a species is able to exploit the matrix as habitat then the matrix may simply represent a continuation of habitat and should not pose a barrier to population connectivity (Laurance 1991; Laurance et al. 2011). Similarly, if the matrix can facilitate dispersal then populations will not become isolated and may instead maintain connectivity as more robust metapopulations (Hanski 1991, 1998). Sufficient transfer of individuals between fragmented populations can buffer them against such threats as genetic drift, inbreeding and demographic stochasticity through the introduction of new individuals and genetic diversity (Brown & Kodric-Brown 1977; Burkey 1989). Immigration also facilitates the re-establishment of populations following reductions or localised extinctions due to stochastic environmental events such as fire or disease (Fahrig & Merriam 1985; Hanski 1991, 1998). Consequently, determining the influence of habitat fragmentation on population connectivity and dispersal is vital to identifying which species are vulnerable to this threatening process and how landscapes can be managed to encourage dispersal and mitigate negative effects, thereby improving conservation outcomes.

Studies of animal movements are a valuable approach for assessing dispersal in fragmented landscapes although telemetric methods and mark-recapture generally result in limited datasets due to cost, labour intensity and restrictive spatial scale (Hebblewhite & Haydon 2010; Nathan et al. 2003). Furthermore, these approaches fail to determine whether a

dispersal event has led to successful establishment and reproduction (Broquet & Petit 2009). An alternate approach that avoids these shortcomings is landscape genetics which provides the ideal means by which to investigate population connectivity and gene flow in relation to landscape configuration (Schlosser et al. 2009; Sork & Waits 2010).

Genetic studies investigating the influence of habitat fragmentation on gene flow and population connectivity are increasing (Storfer et al. 2010), but data are still lacking for the vast majority of taxa. This is particularly true for bats (Burland & Worthington Wilmer 2001). Although several studies have examined bat gene flow amongst sites naturally fragmented by water bodies (Castella et al. 2000; Pumo et al. 1988; Salgueiro et al. 2008), we are only aware of two studies (Meyer et al. 2009; Struebig et al. 2011) specifically designed to investigate bat gene flow in habitat fragmented by human activity, the scenario of concern and relevance to conservation biologists.

Meyer et al. (2009) compared two microbats with contrasting ecology, one more mobile than the other, utilising mtDNA to assess haplotype diversity and population differentiation (F_{ST}) amongst fragmented sites and sites within continuous forest. They found that the less mobile *Carollia perspicillata* had significantly lower haplotype diversity in fragmented sites compared with continuous forest sites and that F_{ST} was higher between fragmented sites than between sites connected through continuous forest. This contrasted with the more mobile *Uroderma bilobatum* which displayed no such effects, supporting the notion that mobility is a key factor determining bat responses to fragmentation. The study by Struebig et al. (2011) was more theoretical in nature investigating the correlation between changes to allelic diversity and species richness, and between genetic differentiation and species assemblage dissimilarity, under the influence of habitat fragmentation.

The lack of research in this area is surprising given that Chiroptera is the second most speciose order of mammals containing more than 20% of mammal species, and with nearly 24% of bat species listed as threatened (Critically Endangered, Endangered, Vulnerable) by the IUCN (Mickleburgh et al. 2002). To inform management decisions and improve conservation outcomes we require more empirical studies that target the direct impacts of habitat fragmentation on population processes such as dispersal, mating systems and demography, so that negative impacts can be identified and mitigated.

In this chapter we endeavour to further our understanding of how human induced habitat fragmentation influences the key process of dispersal and population connectivity in bats. We will build upon our companion study (Chapter 3) which explored the consequences of habitat fragmentation on the habitat specialist *Nyctophilus gouldi* by assessing a congeneric habitat generalist, *N. geoffroyi*, across the same landscape. We have selected our target species based on several considerations; Vespertilionidae is the largest of all bat families (Mickleburgh et al. 2002) and in Australia *Nyctophilus* represents one of the most species rich chiropteran genera (Churchill 2008). The genus also contains two species that provide an ideal model to compare bat responses to habitat fragmentation.

N. gouldi is listed as threatened in South Australia, and has a distribution restricted to native forest and woodland in eastern and south-western Australia (Churchill 2008; Lunney et al. 1988). This distribution suggests the species is somewhat of a habitat specialist and records within disturbed agricultural settings are rare (Lumsden & Bennett 2005) indicating that it may be vulnerable to habitat fragmentation. Indeed our investigation of *N. gouldi* found that habitat fragmentation had led to reduced population connectivity, significantly lower genetic diversity, significantly elevated levels of inbreeding and relatedness, and significantly altered sex ratios (Chapter 3). Contrasting with *N. gouldi*, *N. geoffroyi* displays a near-ubiquitous distribution across the Australian continent and is readily recorded in disturbed agricultural landscapes, suggesting the species is a habitat generalist with more plastic ecology (Churchill 2008; Lumsden & Bennett 2005; Lumsden et al. 2002a). However, the two species have much in common as both are tree roosting (hollows, cavities and under bark) (Churchill 2008; Lumsden et al. 2002b; Lunney et al. 1988) insectivores with similar diets (Fullard et al. 1991; Lumsden & Bennett 2005; Vestjens & Hall 1977), foraging behaviour (Brigham et al. 1997; Grant 1991; O'neill & Taylor 1986) and near-identical morphology (Brigham et al. 1997; Churchill 2008; Fullard et al. 1991; Norberg & Rayner 1987; Rhodes 2002), although *N. gouldi* is typically larger than *N. geoffroyi* with average weights of 12.3g and 8.2g respectively (Churchill 2008). Furthermore, analysis of wing morphology indicates very little difference between the two species which both display low aspect ratio and wing loading characteristic of flycatchers adapted for slow manoeuvrable flight (Brigham et al. 1997; Fullard et al. 1991; Norberg & Rayner 1987; Rhodes 2002).

In addition to furthering our knowledge of bat responses to habitat fragmentation, the comparison between *N. gouldi* and *N. geoffroyi* will provide a unique opportunity to assess

the merit of a proposed predictor of bat vulnerability to threatening processes: wing morphology (Jones et al. 2003; Meyer et al. 2008; Safi & Kerth 2004). The prospect of identifying traits linked to vulnerability is attractive to conservation biologists as it allows for the *a priori* identification of which species are of concern and warrant attention or intervention (Mac Nally & Bennett 1997). Predictors of extinction risk and sensitivity to threatening processes have received much attention with proposed animal traits including specialisation, body size, fecundity, longevity, rarity, abundance, geographic range and trophic position, amongst others (Cardillo et al. 2008; Davidson et al. 2009; Henle et al. 2004; Laurance 1991; O'Grady et al. 2004; Safi & Kerth 2004). Many of these traits have been examined and proposed as predictors of sensitivity to habitat fragmentation along with several additional traits specific to this particular threatening process such as mobility and tolerance to the matrix (Davies et al. 2000; Foufopoulos & Ives 1999; Gehring & Swihart 2003; Henle et al. 2004; Laurance 1991; Lehtinen & Ramanamanjato 2006; Mac Nally & Bennett 1997; Tscharntke et al. 2002; Viveiros de Castro & Fernandez 2004; Wang et al. 2009; Watling & Donnelly 2007). Wing morphology has been proposed as an additional predictive chiropteran trait, receiving some support in relation to vulnerability to habitat fragmentation (Albrecht et al. 2007; Meyer et al. 2008) and extinction in general (Jones et al. 2003; Safi & Kerth 2004). Low aspect ratio and wing loading have been associated with habitat specialisation (Safi & Kerth 2004) and indicate adaptation for slow manoeuvrable flight believed energetically unsuited to long distance flight (Norberg & Rayner 1987) thus reflecting mobility and the capacity to move between habitat fragments.

Aims

Genetic analyses will be conducted to assess the impact of habitat fragmentation on *N. geoffroyi* population connectivity and genetic diversity. These analyses will mirror our work on *N. gouldi* in Chapter 3 facilitating a comparison between the two species. Due to the near-identical wing morphology of *N. gouldi* and *N. geoffroyi* this predictive trait would suggest that both species possess the same physiological capacity for dispersal across agricultural land and will share a similar response to habitat fragmentation. However, several other factors suggest that the two species will respond in contrasting ways. Firstly, the geographic distribution of the two species suggests that *N. geoffroyi* is a habitat generalist displaying ecological plasticity whereas *N. gouldi* is strictly a forest and woodland specialist. Secondly, *N. geoffroyi* is able to exploit and traverse the agricultural matrix which has not been

documented in *N. gouldi*. Collectively, habitat specialisation and tolerance to the matrix suggest that *N. gouldi* will be more greatly affected by habitat fragmentation than in its congeneric *N. geoffroyi*. Overriding predictions based on wing morphology, we predict that the habitat specialist *N. gouldi* will be more influenced by habitat fragmentation than the habitat generalist *N. geoffroyi*.

METHODS

As a companion paper to our study of *N. gouldi* (Chapter 3), the methodology in this chapter mirrors that of the first. Consequently we will concisely reiterate methods but for more detailed information regarding study sites, fieldwork, laboratory work and analyses refer to Chapter 3. The only exceptions to the replication of Chapter 3 methodology is the inclusion of two additional sites (Weecurra SF and Warreanga NFR) and reduced Bayesian analyses (no TESS or BAPS).

Study sites and sample collection

Fourteen sites were sampled for *N. geoffroyi* across south-eastern South Australia and western Victoria in a region composed of native forest remnants, agriculture and plantation forestry (Figure 4.1). Five sites comprised an 80km transect through continuous forest, namely Strathdownie, Weecurra, Hotspur and Annya State Forests (SF) and Mt Eccles National Park (NP). Two additional expansive forest sites, the Grampians and Great Otway NPs, were sampled as potential sources of gene-flow to isolated fragments on the Victorian volcanic plains. Seven discrete habitat fragments were sampled in total comprising three Victorian sites surrounded by agriculture and four South Australian sites isolated by agriculture and embedded within plantation pine (*Pinus radiata*). Victorian fragments include Mt Napier State Park (SP) (2800ha), Framlingham Native Title Reserve (1180ha) and Woolsthorpe Nature Conservation Reserve (NCR) (60ha). South Australian sites represent four of the largest remnant patches of native vegetation in the south-east and include Nangwarry (2218ha), Dry Creek (396ha), Honan's (1041ha) and Warreanga (429ha including the adjoining Penambol Conservation Park) Native Forest Reserves (NFR). Over the last 150 years of European settlement the study region has been extensively cleared for agriculture creating a landscape mosaic of remnant native vegetation and plantation forestry within an

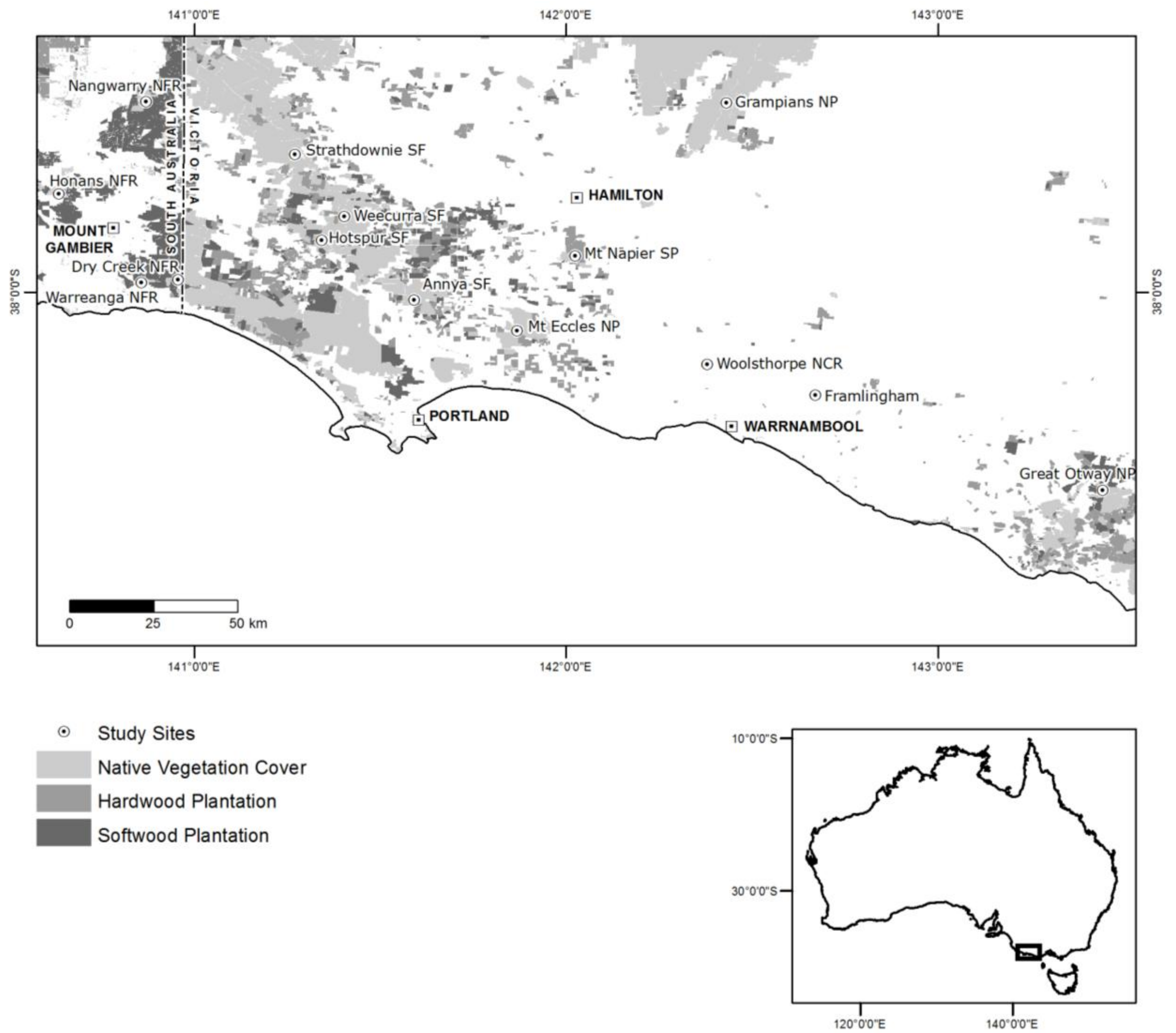

Figure 4.1: The distribution of 14 *N. geoffroyi* and *N. gouldi* study sites across Victoria and South Australia. *Nyctophilus* were sampled in native vegetation (light grey) embedded within a matrix of hardwood (mid grey) and softwood plantations (dark grey) and agricultural land (white).

agricultural matrix. The Grampians is the exception as the site may have been naturally isolated from neighbouring forest by the emergence of grasslands in the late Pleistocene or early Holocene (DSE 2004a, b, 2011; Jones 1999). Throughout this manuscript the study sites will be referred to as fragmented or unfragmented sites, with the latter further distinguished by referring to our five sites connected through native forest as the continuous sites.

We used harp traps to capture bats from November to April in 2008-2009 and 2009-2010 totalling 1252 trap nights. Traps were preferentially placed in areas containing tree hollows

and where vegetation formed a corral to funnel bats into traps, usually along tracks. All trap locations were marked using GPS for spatial genetic analyses. To maintain sampling consistency between sites we trapped in the central core of each site with all trapping conducted within 1-2km to avoid the influence of capture area on genetic diversity. Only the Otways and Grampians differed in this respect as poor trap success forced us to trap over a wide area to obtain sufficient samples. Traps were set at dusk and checked twice, once before midnight and again before dawn so that non-target animals could be released in darkness. Target animals were held for daytime processing and stored in individual cotton hold bags in a cool dark quiet location before release the following evening at the point of capture. DNA was collected by taking two 3.5mm wing membrane biopsies, one from each wing, with a sterile biopsy punch and the tissue was stored in an ethanol-saline solution for preservation. Each individual was measured and sexed, with females assessed for reproductive condition via teat and abdominal development. All bats were aged by assessing the calcification of wing joints and categorised as either adults or juveniles (Tidemann 1993).

DNA extraction and microsatellite genotyping

Nuclear DNA was extracted from 180 biopsies using the Gentra Puregene extraction kit (Gentra Systems Inc) with an additional 322 biopsies submitted for extraction by AGRF (Australian Genome Research Facility, Waite Campus, Adelaide). DNA was quantified using a Nanovue spectrophotometer (General Electric) and all concentrations were standardised to 10ng/µL. 502 *N. geoffroyi* individuals were screened at 9 microsatellite loci developed for this study utilising next generation sequencing (Roche 454 sequencing) and Multiplex Ready Technology (MRT) (Hayden et al. 2008)(see Chapter 2). PCRs were performed according to Chapter 2 on a Corbett Palm Cycler (model CG1-96) utilising BIOMEK 3000 robots (Beckman Coulter) to set up PCRs and to pool products post PCR into two panels. PCR products were cleaned using a Millipore vacuum plate (Multi Screen PCR µ96 Plate) and manifold (Multi Screen$_{HTS}$ Vacuum Manifold), and diluted before being sent to AGRF for electrophoresis and visualisation on an ABI 3730 DNA Analyser. Genotypes were scored using GENEMAPPER v.3.5.1 (Applied Biosystems) software and tested with the program MICROCHECKER v.2.2.3 (Van Oosterhout et al. 2004) for typing errors and the presence of null alleles before undertaking subsequent analyses. We used GENEPOP v.3.4 (Raymond & Rousset 1995) to test populations and loci for deviations from Hardy-Weinberg equilibrium (HWE), heterozygosity excess and deficiency, and linkage disequilibrium (LD) with

sequential Bonferroni corrections made for these and all subsequent tests involving multiple comparisons (Rice 1989). Markov chain parameters in GENEPOP were applied using the default settings.

Genetic analyses

We employed a range of genetic analyses to examine population structure across the study region and to compare population connectivity within continuous forest and between fragmented populations. We calculated two measures of population differentiation, F_{ST} using ARLEQUIN v. 3.5 (Excoffier et al. 2005) and D_{est} (equation 12: (Jost 2008) using the package *DEMEtics* (Gerlach et al. 2010) for the program R v. 2.1.3.1 (R Development Core Team 2011). Bayesian approaches were also utilised to identify genetic clusters across the landscape. We implemented STRUCTURE v. 2.2 (Pritchard et al. 2000) to infer clusters based on genotypic data alone, and GENELAND v. 3.3 (Guillot et al. 2005) to incorporate both genotypic and spatial data (geographic coordinates of sampling locations) to calculate the number of clusters (K). GENALEX v. 6 (Peakall & Smouse 2006) was used to perform Mantel tests and spatial autocorrelations across the entire dataset and to compare fragmented and continuous sites using individual pairwise geographic coordinates and genetic distance.

To further examine the underlying causes of genetic differentiation between sites we used IBD v. 1.52 (Bohonak 2002) to carryout Mantel and partial Mantel tests at the site level based on pairwise population F_{ST} and D_{est} values. This approach was employed using a third indicator matrix in two varying ways. For our first test the indicator matrix represented the intervening matrix type between sites represented by a ‘1’ for agricultural land and a ‘0’ for continuous native forest. Secondly we used the indicator matrix to input a proposed least-cost-path distance between each site measured as the route spanning the shortest accumulative distance across agricultural land which we will refer to as agricultural distance. To investigate whether dispersal is occurring across agricultural land or if it is restricted to continuous forest we attempted to identify dispersal events by conducting first-generation migrant detection (F_0) in GENECLASS v. 2 (Piry et al. 2004).

We investigated the genetic consequences of habitat fragmentation on populations by assessing a range of measures reflecting genetic diversity, relatedness and inbreeding, sex ratios and bottlenecks. We calculated standard measures of genetic diversity (private alleles,

H_O & H_E) using GENALEX, and allelic richness (A_R) as a standardised measure of allelic diversity based on sample size in FSTAT v. 2.9.3 (Goudet 2001). As indicators of inbreeding we calculated the inbreeding coefficient F_{IS} in FSTAT and two additional measures reflecting inbreeding using the R package *Rhh* (Alho et al. 2010); standardised heterozygosity (SH: Coltman et al. 1999) and internal relatedness (IR: Amos et al. 2001). Sex ratios were also assessed for differences between populations and between island and mainland sites. BOTTLENECK v1.2.02 (Piry et al. 1999) was used to identify recent bottleneck events in island sites under a two-phase-mutation model (TPM) (Di Rienzo et al. 1994). Finally, KINGROUP v. 2 (Konovalov et al. 2004) was employed to identify parent-offspring, full siblings, half siblings and cousins using the likelihood method of Queller & Goodnight (1989). This allowed us to compare the frequency of relatives in fragmented and continuous habitat.

RESULTS

Nyctophilus geoffroyi were readily caught across the study region and we generally obtained our target number of 30+ individuals from each site within 16-24 trap nights. This contrasts with *N. gouldi* which often took 80+ trap nights to reach target sample sizes. Only three sites produced fewer than the target number; Dry Creek (n=15), Weecurra (n=23) and the Otways (n=20). In total, trapping produced samples from 502 *N. geoffroyi* individuals across fourteen sites. Numbers of *N. geoffroyi* at Dry Creek were atypically lower than *N. gouldi*, perhaps reflecting differences in habitat suitability. Obtaining sufficient captures at the Otways proved difficult for *N. geoffroyi* and *N. gouldi*. We speculate that a taller canopy than other sites may have reduced the effectiveness of harp traps despite efforts to raise them into the canopy.

MICROCHECKER identified evidence of null alleles at several populations and loci including locus NyGo21 at the Grampians, NyGo20 and NyGo37 at Mt Napier, and NyGo25 and NyGo29 at Honans, Nangwarry and Woolsthorpe. In addition to these cases two loci displayed consistent signs of null alleles, NyGo19 at all 14 populations and NyGo39 at 13 populations, and were subsequently removed from further analyses, reducing the number of usable loci to seven. Following Bonferroni correction GENEPOP identified three populations that deviated from HWE, Nangwarry ($p<0.05$), Honans ($p<0.001$) and Woolsthorpe

(p<0.005). At the locus level there was only a single incidence of deviation from HWE which occurred at Honans (NyGo29, p<0.001). GENEPOP also identified loci displaying heterozygote deficiency at Annya (NyGo21, p<0.001) and Woolsthorpe (NyGo20, p<0.05 & NyGo29, p<0.001). The deviation of Nangwarry, Honans and Woolsthorpe from HWE coincides with evidence from MICROCHECKER of homozygote excess and possible null alleles at NyGo25 and NyGo29. This could be due to the cross-amplification and use of microsatellite markers originally developed for a different species (*N. gouldi*). Other possible explanations include outbreeding which could be expected in fragmented populations, however, the presence of heterozygote deficiency suggests that null alleles, inbreeding or a Wahlund effect are more likely explanations.

F_{ST} values were low and ranged from 0.000-0.013 while D_{est} values were higher ranging from 0.000-0.101 (Table 4.1). The highest F_{ST} occurred between Honans and Mt Eccles (F_{ST} = 0.0131) and the lowest between Annya and Strathdownie (F_{ST} = 0.0000). The highest D_{est} occurred between Dry Creek and Framlingham (D_{est} = 0.101) and the lowest D_{est} (0.0000) occurred between Annya and Strathdownie. Prior to Bonferroni correction there were numerous significant cases of population differentiation. With one exception, all such cases involved sites separated by agriculture: Mt Eccles displayed significant differentiation from Hotspur and Strathdownie via F_{ST} and D_{est}. It should be noted that several small gaps of agriculture separate Mt Eccles from the other continuous sites; these gaps collectively span 1.6km with the largest single gap spanning ~800m (see Chapter 3). Post Bonferroni correction there were only two cases of significant differentiation; both were via F_{ST} and indicated differentiation between Honans and two other sites, the Grampians and Mt Eccles. To allow direct comparison with the *N. gouldi* dataset, these analyses were re-run using nine common populations. This revealed that prior to Bonferroni correction *N. gouldi* displayed 16 cases of significant F_{ST} and nine post correction, while *N. geoffroyi* displayed 12 cases prior and just two cases post. This comparison was further pronounced for D_{est} where *N. gouldi* numbered 26 significant cases prior to Bonferroni correction and 19 post, while *N. geoffroyi* tallied 10 significant cases prior and no cases post.

Bayesian clustering analyses similarly revealed less structure for *N. geoffroyi* than was detected for *N. gouldi*. STRUCTURE identified a single cluster (K=1) across the landscape suggesting that the population is panmictic. GENELAND identified 2 clusters (K=2), east

Table 4.1: Population differentiation measures estimated from 7 loci across 14 populations of *N. geoffroyi*. F_{ST} (ARLEQUIN) below the diagonal and D_{est} (DEMEtics) above with p values provided before (*,**,***) and after (*,**,***) sequential Bonferroni correction respectively indicating 0.05, 0.01 and 0.001 levels of significance.

	Nangwarry	Warreanga	Dry Creek	Honans	Annya	Weecurra	Framlingham	Otways	Grampians	Woolsthorpe	Mt Napier	Mt Eccles	Hotspur	Strathdownie
Nangwarry	--	0.065***	0.047	0.035*	0.000	0.033	0.038*	0.031	0.046*	0.038*	0.052*	0.022	0.043*	0.031
Warreanga	0.011***	--	0.071*	0.035*	0.038*	0.009	0.028	0.084**	0.076***	0.045**	0.060**	0.043**	0.020	0.024
Dry Creek	0.006	0.008	--	0.035	0.038	0.017	0.101**	0.016	0.045	0.090**	0.039	0.026	0.024	0.055
Honans	0.010***	0.007**	0.005	--	0.000	0.006	0.043**	0.053*	0.052**	0.021	0.040*	0.070***	0.006	0.016
Annya	0.002	0.003	0.004	0.000	--	0.000	0.017	0.011	0.008	0.013	0.000	0.007	0.011	0.000
Weecurra	0.007	0.003	0.002	0.002	0.000	--	0.045*	0.040	0.025	0.003	0.027	0.020	0.000	0.004
Framlingham	0.006	0.002	0.011*	0.005*	0.001	0.004	--	0.070*	0.070**	0.012	0.041*	0.055*	0.050*	0.036*
Otways	0.004	0.007*	0.003	0.010*	0.001	0.005	0.006	--	0.047	0.060*	0.057*	0.039	0.021	0.025
Grampians	0.009**	0.010***	0.008	0.010*** ***	0.003	0.006	0.010**	0.009*	--	0.057**	0.024	0.032	0.060**	0.049*
Woolsthorpe	0.008*	0.008*	0.013*	0.005	0.002	0.002	0.001	0.009	0.011**	--	0.030	0.093***	0.053*	0.041*
Mt Napier	0.010**	0.009**	0.004	0.009***	0.003	0.007	0.006	0.008	0.006	0.005	--	0.011	0.063**	0.044*
Mt Eccles	0.003	0.004	0.002	0.013*** ***	0.002	0.003	0.006	0.004	0.004	0.013**	0.003	--	0.056*	0.054*
Hotspur	0.010*	0.003	0.002	0.003	0.000	0.001	0.007*	0.002	0.010***	0.011**	0.010**	0.007*	--	0.021
Strathdownie	0.007*	0.004	0.007	0.004	0.000	0.003	0.005	0.005	0.008*	0.008*	0.009**	0.009*	0.005	--

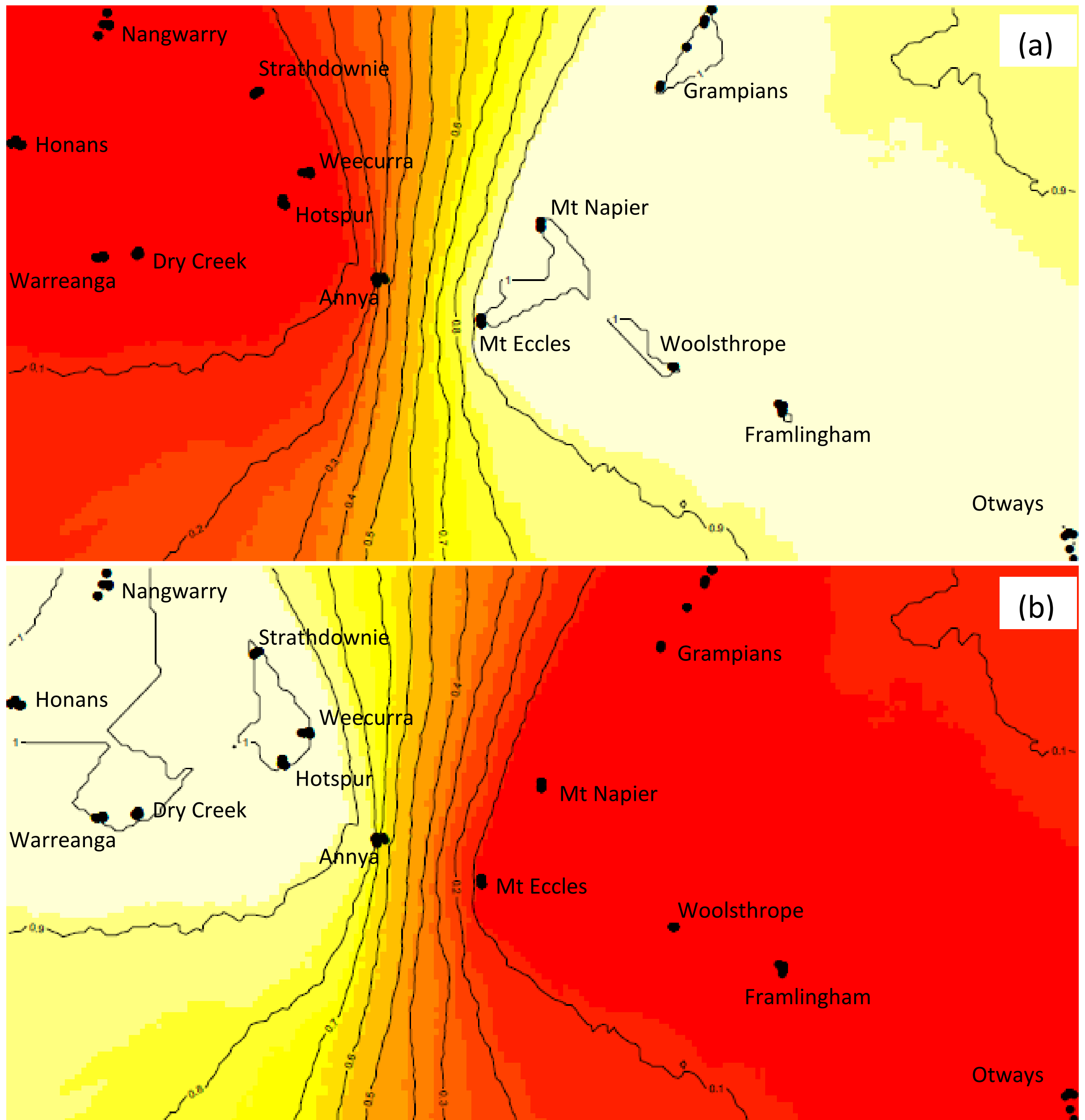

Figure 4.2: GENELAND results illustrating the geographic distribution of two identified genetic clusters based on the posterior probability (Q) of 502 *N. geoffroyi* individuals belonging to Cluster 1 (a) and Cluster 2 (b). Colours represent a gradient of proportional assignment ranging from high (white>0.9) to low (red<0.1). The sampling location of each individual is represented by a black circle at one of fourteen study sites across south-eastern South Australia and western Victoria.

and west, with a divide between Annya and Mt Eccles running due north-south (Figure 4.2). All populations were strongly assigned to either cluster (Q>0.96) with the exception of Annya which displayed some admixture characterised by an average posterior probability of assignment to Clusters 1 and 2 of 0.20 and 0.80 respectively.

Mantel tests at the individual level revealed no significant relationship between genetic and geographic distance across the study region, within continuous forest, or between fragmented sites (R^2<0.001, p>0.05). Mantel tests at the population level revealed a significant relationship between D_{est} and geographic distance (r=0.2653, p=0.019), but not F_{ST} (r=0.1964, p=0.076). Both F_{ST} (r=0.2997, p=0.016) and D_{est} (r=0.2914, p=0.027) displayed a significant relationship with the matrix type, but only D_{est} showed a significant relationship with agricultural distance (r=0.2517, p=0.043). For comparison with *N. gouldi* we again reduced the number of *N. geoffroyi* populations to nine, and this produced no significant relationships with geographic distance, matrix type or agricultural distance for either F_{ST} or D_{est}.

Global spatial autocorrelation of all sites illustrated a positive association for populations within 20km of each other after which associations were non-significant with the exception of negative relationships at 130km, 160km and 270km (Figure 4.3a). Independent analysis of the five continuous sites (Mt Eccles, Annya, Hotspur, Weecurra and Strathdownie) over a distance of 80km revealed a negative association at 75km (Figure 4.3b). Comparative analysis of sites fragmented by agricultural land over a distance of 80km uncovered a contrasting trend with a positive association for sites within 20km of each other (Figure 4.3c).

Fifty-seven dispersal events were inferred with GENECLASS and all but three events spanned agricultural land (Table 4.2). The average linear dispersal distance was 97.7km and the average agricultural dispersal distance was 41.7km. More than half of all dispersal events (n=25) spanned less than 16.5km of agricultural land while nine events covered more than 100km of agriculture. The longest inferred dispersal event was undertaken by two individuals from the Otways to Nangwarry, traversing a linear distance of 254.1km and an agricultural distance of 123.5km.

Genetic diversity measures and measures of inbreeding (Table 4.3) revealed no significant differences between populations or between island and mainland populations when samples were pooled (ANOVA, p>0.05). Allelic richness (A_R) was highest at Strathdownie (A_R=10.125) and lowest at Weecurra (A_R=9.086), both sites located within continuous forest. Observed heterozygosity (H_O) ranged from 0.857 at Dry Creek to 0.779 at Woolsthorpe, the latter representing the smallest and equal most isolated fragment. Expected heterozygosity (H_E) was highest at Warreanga (H_E=0.861) and lowest at Weecurra (H_E=0.828). Dry Creek recorded the highest standardised heterozygosity (SH=1.047) and the lowest internal

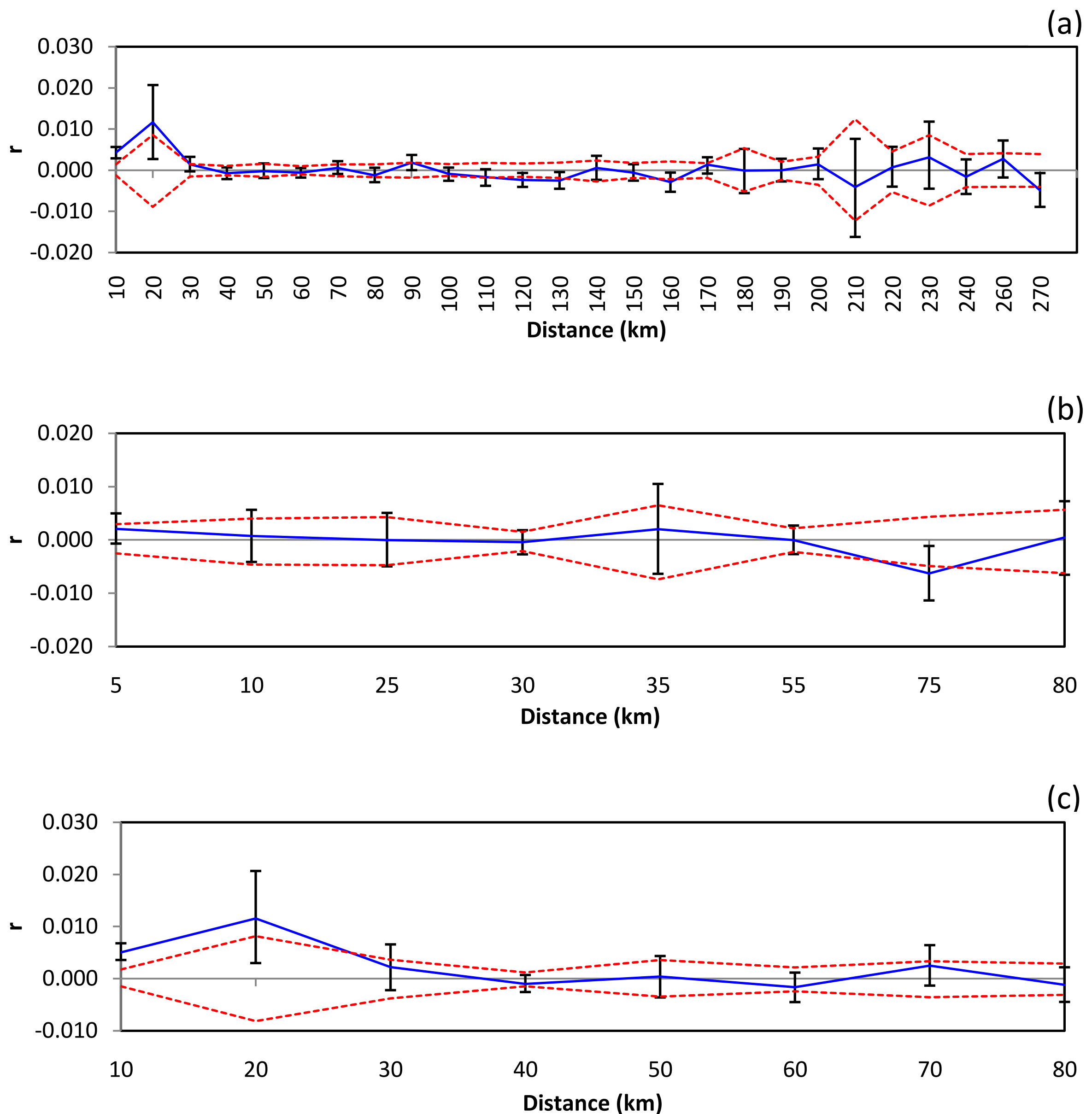

Figure 4.3: Results of spatial autocorrelations preformed in GENALEX illustrating mean r (——) along the Y axis with 95% upper and lower confidence levels (······). Distance classes are displayed along the X axis in km. Figures represent: (a) all sites, (b) sites connected by continuous habitat, and (c) sites fragmented by agricultural land.

relatedness (IR=0.014) while Woolsthorpe recorded the lowest SH (0.951) and highest IR (0.094). The inbreeding coefficient (F_{IS}) ranged from a low of 0.012 at Mt Eccles to 0.086 at Woolsthorpe. Private alleles were uncommon being recorded at Warreanga (A_P=3), Dry Creek (A_P=1), Annya (A_P=2), Hotspur (A_P=1) and Strathdownie (A_P=3). For comparison with *N. gouldi* we re-ran tests to identify significant differences between populations and

Table 4.2: Identification of dispersal events in GENECLASS determined with a significance level of $p<0.05$. The inferred source population and the population in which an individual was trapped are displayed. Fourteen sampled populations are defined: Nan = Nangwarry, War = Warreanga, Dry = Dry Creek, Hon = Honans, Ann = Annya, Wee = Weecurra, Otw = Otways, Gra = Grampians, MtN = Mt Napier, MtE = Mt Eccles, Hot = Hotpur, Str = Strathdownie.

Population	Source population														
Trapped	Nan	War	Dry	Hon	Ann	Wee	Fra	Otw	Gra	Woo	MtN	MtE	Hot	Str	Total
Nan			1	1	1			2			1	1			7
War				2			1					2		1	6
Dry		1		2											3
Hon	1				2								1		4
Ann				1				2	1						4
Wee		1											1		2
Fra	1			2	1			1		1		1			7
Otw		1			1										2
Gra	1		1			1				1		1			5
Woo		1		1										1	3
MtN					1							1		1	3
MtE		1						1					1	1	4
Hot		1						1			1				3
Str		1			1			1		1					4
total	3	7	2	9	7	1	1	8	1	3	2	6	3	4	57

Table 4.3: Summary of population genetic measures and sample numbers across 14 *N. geoffroyi* populations. N = number of samples, A_R = allelic richness, A_P = private alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, SH = standardised heterozygosity, IR = internal relatedness, F_{IS} = the inbreeding coefficient.

Population	N	A_R	A_P	H_O	H_E	SH	IR	F_{IS}
Nangwarry	40	9.577	0	0.786	0.842	0.959	0.087	0.079
Warreanga	53	9.922	3	0.836	0.861	1.020	0.037	0.039
Dry Creek	15	9.714	1	0.857	0.842	1.047	0.014	0.016
Honans	69	9.541	0	0.810	0.839	0.988	0.056	0.042
Annya	34	10.111	2	0.845	0.853	1.031	0.021	0.025
Weecurra	23	9.086	0	0.783	0.828	0.956	0.084	0.077
Framlingham	34	9.522	0	0.836	0.845	1.021	0.031	0.026
Otways	20	10.094	0	0.829	0.857	1.012	0.050	0.059
Grampians	39	9.420	0	0.799	0.832	0.975	0.069	0.053
Woolsthorpe	33	9.114	0	0.779	0.839	0.951	0.094	0.086
Mt Napier	36	9.750	0	0.810	0.843	0.988	0.060	0.054
Mt Eccles	34	9.361	0	0.845	0.842	1.031	0.019	0.012
Hotspur	36	9.369	1	0.833	0.843	1.018	0.032	0.025
Strathdownie	36	10.125	3	0.841	0.860	1.027	0.032	0.035

between island and mainland sites for *N. geoffroyi* with a reduced nine population dataset, and again no significant differences were detected (ANOVA, $p>0.05$).

The detection of relatives in KINGROUP identified 38 related pairs with a significance level of $p<0.05$ (Table 4.4). With seven sites representing both fragmented and unfragmented site categories less than 30% of relatives were detected in unfragmented sites. Honans was particularly noteworthy containing 10 related pairs, more than a quarter of those detected across the whole study region, and nearly a third of all identified full siblings. Warreanga also displayed an elevated number of relatives with 7 pairs identified representing nearly 20% of established relatives.

Table 4.4: Pairs of relatives identified using KINGROUP. Four types of relationships were examined: parent-offspring, full siblings, half siblings and cousins. Results are presented for 14 populations across south-eastern South Australia and western Victoria. Relationships were established with a confidence level of $p<0.05$.

Population	Parent-offspring	Full siblings	Half siblings	Cousins	Total
Nangwarry		1			1
Warreanga	1	6			7
Dry Creek					
Honans	1	9			10
Annya					
Weecurra					
Framlingham	2	1			3
Otways		1			1
Grampians		1			1
Woolsthorpe	2	2			4
Mt Napier		2			2
Mt Eccles		2			2
Hotspur	2	1			3
Strathdownie		3		1	4
Total	8	29		1	38

DISCUSSION

The impact of habitat fragmentation on *N. geoffroyi*

As predicted *N. geoffroyi* displayed little response to habitat fragmentation. However there were several analyses that produced results indicating some degree of population structuring across the study. For example, GENELAND identified an east and west cluster which may indicate a geographic cline in allele frequencies across the study region. This result differed from STRUCTURE which identified a single cluster suggesting that the population is panmictic. Importantly, the clusters in GENELAND did not reflect the configuration of forest due to habitat fragmentation suggesting that the presence of agricultural land is unlikely to be the factor driving population differentiation. Spatial autocorrelation of fragmented sites did reveal a positive neighbourhood effect within a radius of 20km, a trend not detected within the continuous forest. This finding suggests a barrier effect where individuals may be influenced to display more philopatric behaviour than those within continuous forest simply due to the imposition of a barrier formed by agricultural land. However, this positive neighbourhood effect was not coupled with a negative association with distal sites indicating that *N. geoffroyi* is able to successfully disperse to distant populations. The identification of dispersal events supports this ability as the average agricultural distance traversed by dispersing individuals was over 40km and nine events spanned more than 100km of agriculture. Despite this support, putative dispersal events should be treated with caution as population differentiation between sites was low (Berry et al. 2004).

N. geoffroyi displayed few significant cases of pairwise population differentiation with only two F_{ST} values retaining significance following Bonferroni correction. Although these two cases occurred between sites separated by agriculture they did not represent the most isolated or distal populations and there was no consistent pattern of population differentiation between fragmented sites. We did however detect significant correlations between *N. geoffroyi* population differentiation (F_{ST} and D_{est}) and the presence of agricultural land, and between D_{est} and the agricultural distance between sites. These findings were determined with the complete *N. geoffroyi* 14 population dataset while the reduced nine population dataset, used for direct comparison with the *N. gouldi* dataset, revealed no such correlations.

Comparing the influence of habitat fragmentation on *N. geoffroyi* and *N. gouldi*

As we hypothesised *N. geoffroyi* did indeed contrast *N. gouldi* in its response to habitat fragmentation displaying little impact from the fragmentation of habitat across our study region. The difference between the two species is perhaps best illustrated by the example given above regarding the correlation between population differentiation and the presence of agricultural land. While this assessment did reveal a significant correlation for both *N. geoffroyi* and *N. gouldi*, significance for *N. geoffroyi* was only produced with the larger 14 population dataset. This dataset included the addition of three highly isolated sites (Mt Napier, Framlingham and Woolsthorpe), which were not represented in *N. gouldi*. The reduced nine population *N. geoffroyi* dataset, used for comparison with *N. gouldi*, revealed no significant correlation. This difference suggests that while habitat fragmentation may increase population differentiation between *N. geoffroyi* populations, the effect is not of the same magnitude as that detected for *N. gouldi*.

In our companion paper (Chapter 3) we documented multiple lines of evidence to consistently indicate that agricultural land acts as a barrier to *N. gouldi* gene flow. This evidence included significant population differentiation between all sites separated by agriculture (prior to Bonferroni correction), contrasting spatial autocorrelations between continuous and fragmented sites, and the identification of reduced genetic diversity, skewed sex ratios, increased relatedness and evidence of inbreeding within fragmented populations. In stark contrast *N. geoffroyi* appears more resilient to habitat fragmentation with only two cases of significant population differentiation and no evidence of reduced genetic diversity, elevated relatedness or altered demography as a consequence of isolation. *N. gouldi* dispersal events proposed by GENECLASS also contrasted with *N. geoffroyi*. The average agricultural dispersal distance for *N. gouldi* was 24km, almost half that proposed for *N. geoffroyi*. Furthermore this result was heavily influenced by two suspected outliers (see Chapter 3) which, when removed, reduced the average *N. gouldi* agricultural dispersal distance to just 9km.

While we proposed a dispersal threshold of ≤27km across agriculture for *N. gouldi* and found evidence of maintained gene flow across agricultural distances <2km (Chapter 3), we did not find any evidence of a threshold for *N. geoffroyi* population connectivity. This case is well illustrated by our identification of a unique *N. gouldi* management unit (Moritz 1994) for the

Grampians (see Chapter 3), despite the fact that the Grampians are less than 35km away from neighbouring forest. We explored this issue in Chapter 3 where we considered the possibility that *N. gouldi* in the Grampians had been naturally isolated from the rest of the study region by the emergence of grassland during the late Pleistocene or early Holocene. However, contrasting *N. gouldi*, *N. geoffroyi* displayed no genetic differentiation between the Grampians and populations to the south indicating that this population has remained connected to the rest of the study region. Similarly, *N. geoffroyi* populations have persisted at the three most isolated habitat patches in Victoria (Mt Napier, Framlingham and Woolsthorpe) where *N. gouldi* was in low densities or missing altogether. *Nyctophilus geoffroyi*'s presence at these three sites suggests either: a direct capacity to cope with high degrees of isolation; the ability to readily supplement sink populations or recolonise sites after localised extinctions, or; that agricultural land represents a continuation of the species habitat as opposed to an intervening matrix. It should also be noted that larger *N. geoffroyi* population sizes, indicated by trapping, would result in slower rates of genetic drift and thus lower levels of population differentiation than *N. gouldi*. Consequently we cannot exclude the possibility that *N. geoffroyi* populations may still be affected by habitat fragmentation, but that the impacts may take longer to manifest than for *N. gouldi*.

Why do the two species respond differently to habitat fragmentation?

Coupled with information on *N. geoffroyi* and *N. gouldi* distributions and occurrence in agricultural land, our results suggest that differences in habitat specialisation and tolerance to the matrix may explain the different responses to habitat fragmentation between the two species. For example, *N. geoffroyi* displays an extensive geographic range spanning diverse ecosystems from desert to tropical rainforest, while *N. gouldi* is restricted to native forest and woodland (Churchill 2008; Ellis et al. 1989; Hall & Richards 1979). The ecological flexibility of *N. geoffroyi* potentially bestows a greater capacity to exploit modified or disturbed landscapes. Differences in the use of agricultural land between the two species have also been reported. Lumsden & Bennett (2005) found that *N. geoffroyi* persisted in agricultural land almost devoid of trees (<1 tree per ha), while *N. gouldi* was only recorded twice in the agricultural study area, and both cases were confined to densely treed paddocks (10-34 trees per ha). In addition, *N. geoffroyi* has been reported commuting, foraging and roosting within agricultural land, proving the species capacity to occupy and utilise the agricultural matrix (Churchill 2008; Lumsden et al. 2002a). Collectively these examples

support the notion that *N. geoffroyi* is a habitat generalist contrasting with *N. gouldi* which is a forest and woodland specialist.

Differences in mobility between *N. geoffroyi* and *N. gouldi* could also explain their differential responses to habitat fragmentation. Although the two species possess near-identical wing morphology, this trait may not necessarily be able to discern between certain flight capabilities (Jones et al. 2003; Meyer et al. 2008; Safi & Kerth 2004), and there is limited evidence to suggest there are differences in vagility between *N. geoffroyi* and *N. gouldi*. For example, Lunney (1988) employed radio tracking and found that *N. gouldi* confined all of their activity within 2km of their roosts, whereas Lumsden et al. (2002a) reported that radio tracked *N. geoffroyi* regularly traversed up to 12km of agricultural land on daily foraging expeditions. Lumsden et al. (2002a) also observed *N. geoffroyi* employing two modes of flight, one slow manoeuvrable mode when foraging and another faster more direct mode of flight when commuting, which has not been reported for *N. gouldi*. This capacity alone, if not shared by *N. gouldi*, could potentially explain the differences in population connectivity we have recorded.

Roosting behaviour is a key aspect of chiropteran ecology and requires consideration as a possible factor behind the two species differing responses to habitat fragmentation. Differences in the characteristics of roost trees between the two species have been documented. Lunney (1988) found that *N. gouldi* forest roosts were restricted to riparian zones and occurred within large (DBH >80cm) mature hollow bearing trees. *N. geoffroyi* displays a preference for dead hollow bearing trees, both small and large, and is known to roost within agricultural land (Churchill 2008; Lumsden et al. 2002a). Differences in the availability of the two species preferred roost trees within the matrix, and abiotic conditions at potential roost trees (eg. exposure, temperature and humidity), could influence their capacity to utilise modified agricultural landscapes. Both species have also been recorded roosting in manmade structures such as buildings, fence posts and other opportunistic locations (Churchill 2008; Ellis et al. 1989; Hall & Richards 1979). However, despite both species capacity to exploit opportunistic roosting locations, it is possible that they differ in their willingness to do so. For example, we sampled three roosts in farm buildings in south-eastern South Australia (Chapter 5) and captured a total of 157 *N. geoffroyi* compared to just one *N. gouldi*.

Finally, the different responses to habitat fragmentation between *N. geoffroyi* and *N. gouldi* may come down to more cryptic differences in behaviour that are more difficult to assess. *N. gouldi* may simply display an avoidance of open spaces for evolutionary reasons such as predator avoidance. Laurance et al. (2002) suggested that understorey species may lack historic evolutionary exposure to open spaces resulting in innate behaviour to avoid exposed areas. Similarly, Greenberg (1989) proposed that selection for reduced exploratory behaviour away from preferred habitat could explain species avoidance of open or novel areas. However, for these behavioural explanations we can only speculate.

Insights into chiropteran responses to habitat fragmentation

Although wing morphology has received support as an indicator of chiropteran sensitivity to habitat fragmentation (Albrecht et al. 2007; Meyer et al. 2008), it did not predict the contrasting responses of *N. geoffroyi* and *N. gouldi* which possess near-identical wing morphology. Wing morphology may still have merit as a predictor of chiropteran responses to habitat fragmentation, but its influence may not be as great as other factors such as habitat specialisation and tolerance to the matrix which may supersede its effects. Safi & Kerth (2004) acknowledged that although wing morphology does, on average, correlate with higher extinction risk 'exceptions exist on the level of single species'. Safi & Kerth (2004) also acknowledged that wing morphology alone cannot explain differences between species foraging behaviour or habitat adaptations, both representing factors that could influence chiropteran responses to habitat fragmentation. Our study provides direct evidence that wing morphology alone cannot predict the response of chiropterans to habitat fragmentation. The assessment of wing morphology may still be useful as a first step in identifying chiropterans sensitive to habitat fragmentation. However, this should be followed by consideration of habitat specialisation (geographic range and critical resources), and tolerance to the matrix (presence in the matrix), in order to make more robust predictions about chiropteran species at risk to habitat fragmentation.

As a final note we wish to raise an important issue of scale regarding the study of bats and habitat fragmentation which we do not believe has been addressed within the literature. Virtually all of the studies we have examined regarding chiropteran responses to habitat fragmentation have assessed landscapes where fragments are separated by <2km of modified or cleared habitat (Bernard & Fenton 2003, 2007; Cosson et al. 1999; Estrada & Coates-

Estrada 2002; Estrada et al. 1993; Faria 2006; Galindo-Gonzalez & Sosa 2003; Gorresen & Willig 2004; Johansson & Jong 1996; Klingbeil & Willig 2009; Law et al. 1999; Meyer et al. 2008; Meyer et al. 2009; Schulze et al. 2000; Struebig et al. 2008). The few exceptions include Struebig et al. (2011) who included several sites isolated by 3-5km, and Montiel et al. (2006) who included two sites categorised as 'far' which were located 10.2km and 11.5km from the nearest forest. In our companion paper focussing on the response of *N. gouldi* to habitat fragmentation (Chapter 3) we proposed a dispersal threshold across agriculture of ≤27km, a result that would not have been detected at the scale adopted by any of the above studies.

Other landscape genetic studies assessing barriers to bat dispersal have identified distance thresholds of a similar magnitude to that proposed for *N. gouldi*. Castella et al. (2000) found that the 14km wide Gibraltar Strait represents a significant barrier to *Myotis myotis* between populations in Europe and North Africa. Similarly, Salgueiro et al. (2008) found that *Nyctalus azoreum* was restricted by more than 40km of open water between islands in the Azores. Furthermore, we found evidence to suggest that *N. gouldi* gene flow was maintained between habitat separated by <2km of agriculture. Collectively these findings indicate that the identification of barriers to bat population connectivity and distance thresholds for dispersal will require a scale that considers 10s of kilometres rather than several kilometres. Microgeographic studies provide valuable insights into the effects of habitat fragmentation at a fine scale, but ultimately they may prove misleading in definitively determining species vulnerability to habitat fragmentation. We propose that studies investigating the impacts of habitat fragmentation on chiropteran population connectivity, or changes to community composition, will benefit from adopting a larger scale more appropriate for this highly vagile group of mammals. If we are to effectively manage species at a landscape or regional level we believe it is more constructive to assess scales that inform our capacity to do so, and that means considering larger distances that often characterise distances between significant conservation areas. In addition to habitat fragmentation *per se* this information may prove increasingly valuable as pressure from climate change increases our need to manage regional connectivity to facilitate range shifts in response to drifting environmental conditions (Hannah et al. 2002; Opdam & Wascher 2004).

Chapter 5

Dispersal strategies, mating systems and social structure in two species of long-eared bats, *Nyctophilus geoffroyi* and *N. gouldi*

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ABSTRACT

Chiroptera is a mega-diverse order with species providing a range of essential ecosystem services such as plant pollination and the regulation of insect populations. However, despite their importance we know little about the life history of many bat species particularly in regards to social structure, mating systems and dispersal. To address this knowledge gap we utilised 16 microsatellite markers to investigate dispersal strategies and social structure in two species of long-eared bats, *Nyctophilus geoffroyi* and *N. gouldi*. We sampled 502 *N. geoffroyi* and 265 *N. gouldi* across 14 sites in south-eastern Australia, and 157 *N. geoffroyi* in three roosts in farm buildings. We provide evidence of male biased dispersal, female philopatry and polygynous mating in *N. gouldi*, but detected no such patterns for *N. geoffroyi*. Analysis of social structure at the population level revealed that nearly twice as many *N. gouldi* (26.5%) possessed a relative as *N. geoffroyi* (13.9%), although this figure was higher for *N. geoffroyi* roosts (43.9%). Populations of both species, and *N. geoffroyi* roosts, contained significantly more female relatives than males or mixed-sex relatives. We hypothesise that matrilineal social groups may play a significant role in the social structure and behaviour of both species. Despite the high proportion of individuals with relatives within *N. geoffroyi* roosts, the vast majority of pairwise comparisons indicated no relationship between roosting individuals. This finding suggests that reciprocal altruism, not kin selection, is the principal mechanism behind cooperative roosting behaviour for *N. geoffroyi*.

INTRODUCTION

Chiroptera is a mega-diverse order that represents approximately 20% of mammalian diversity and plays key roles in ecosystem function including the regulation of insect populations and the propagation of plant communities via pollination and seed dispersal (Kunz et al. 2011). Despite these facts we have limited knowledge regarding the ecology of many chiropterans, particularly cryptic aspects of ecology such as dispersal strategies, social structure and mating systems (Burland & Worthington Wilmer 2001; Kerth 2008). Bats show a propensity to form social groups and, like the majority of mammals, they are typically polygynous (Clutton-Brock 1989; McCracken & Wilkinson 2000). Chiroptera also contains a great diversity of social structures, dispersal patterns and mating systems providing a novel window into how these mechanisms evolve in mammals (Kunz et al. 2011). It is perhaps surprising then that, in comparison to other social mammals like primates, ungulates and rodents, chiropterans are highly underrepresented in terms of study into behavioural ecology (Kerth 2008).

In the past this lack of chiropteran research has been largely due to the cryptic nature of bats which make them difficult to study with traditional field based techniques (Burland & Worthington Wilmer 2001; Kerth et al. 2002b). However, modern molecular techniques have made these previously elusive aspects of bat ecology accessible for study and as a result the number of such studies is increasing (Burland & Worthington Wilmer 2001; Kerth et al. 2002b). Despite this progress we have only begun to understand chiropteran sociobiology, a point illustrated by our knowledge of mating systems which had only been determined for 6.9% of >1000 species by the year 2000 (McCracken & Wilkinson 2000). Nevertheless, genetic research over the last two decades has begun to reveal a diverse range of dispersal strategies and social structures within Chiroptera. These include the typical mammalian male biased dispersal and female philopatry (Arnold 2007; Kerth et al. 2002a; Petit & Mayer 1999; Weyandt et al. 2005; Worthington Wilmer et al. 1999), natal philopatry in both sexes (Burland et al. 1999), dispersal in both sexes (Dechmann et al. 2007), and colonies with varying degrees of relatedness (Furmankiewicz & Altringham 2007; Heckel et al. 1999; Kerth et al. 2000; Metheny et al. 2008; Ortega et al. 2003; Petri et al. 1997; Rivers et al. 2005; Rossiter et al. 2002; Storz et al. 2001; Veith et al. 2004; Wilkinson 1992a) and differing compositions of relatives (Bryja et al. 2009; Burland et al. 2001; Kerth et al. 2002b). Genetic investigations have also provided probing insights into a range of chiropteran mating systems

from swarming sites to harem structures and mating success (Burland et al. 2001; Chaverri et al. 2008; Heckel et al. 1999; Ortega et al. 2003; Rossiter et al. 2000; Veith et al. 2004).

In this paper we employ a combination of molecular techniques to investigate philopatry, dispersal patterns and mating systems in two endemic Australian species of *Nyctophilus* (Vespertilionidae), *N. gouldi* and *N. geoffroyi*. We also assess social structure at the population level in both species, and at the roost level for *N. geoffroyi*, by calculating measures of relatedness and identifying putative relatives. The two species appear to differ in ecological plasticity as *N. gouldi* displays a distribution limited to forest and woodland in eastern and south-western Australia, apparently specialising in such habitat, while *N. geoffroyi* is a habitat generalist with a continent-wide distribution spanning a diverse range of ecosystems (Churchill 2008). Both species are small insectivores that roost in tree cavities, however, they are also known to form colonies in manmade structures (Reardon & Flavel 1987). *N. geoffroyi* sexes are reported to roost separately throughout most of the year either alone or in small groups, however, maternity colonies of up to 30 females are known to occur often accompanied by a single male (Churchill 2008; Lumsden et al. 2002a; Lumsden et al. 2002b; Reardon & Flavel 1987). Mixed-sex colonies of up to 200 individuals have been reported in buildings elsewhere (Reardon & Flavel 1987) and it is possible that these artificial spaces facilitate year-round co-roosting behaviour between the sexes. *N. gouldi* females are reported to form colonies of 20 or more individuals while males generally roost alone or in small groups comprising fewer than six individuals (Churchill 2008).

Little is known about the mating systems of *N. gouldi* or *N. geoffroyi*. More than 90% of mammals display some form of polygynous mating system, and the majority of assessed bat species conform to this trend (McCracken & Wilkinson 2000). Both species mate in autumn and females store sperm until spring when ovulation and fertilisation take place (Churchill 2008; Hosken 1997). Male *N. gouldi* have also been reported to mate sporadically throughout winter with torpid females (Churchill 2008). This behaviour has been recorded in another Australian vespertilionid, *Vespadelus vulturnus* (Tidemann 1993), and is likely to occur in *N. geoffroyi*. Hosken (1998) conducted consecutive isolation experiments with opposite sex pairs of *N. geoffroyi* and found that both sexes mated with multiple individuals. Females stored viable sperm for up to 93 days which, coupled with the formation of copulatory plugs by males, provided strong evidence of sperm competition (Hosken 1998). Hosken (1998)

also assessed paternity using electrophoresis of blood enzymes, and although all females mated with two males, all offspring were sired by the same male.

Evidence of multiple matings in captivity, and reproductive biology conducive to sperm competition, is strong evidence of polygynous and polyandrous behaviour in wild populations of *N. geoffroyi*. However it is unclear whether the species forms single-male multiple-female groups for breeding or multiple-male multiple-female groups (McCracken & Wilkinson 2000). As discussed above, Churchill (2008) states that *N. geoffroyi* maternity colonies are often accompanied by a single male suggesting single-male multiple-female group formations. However, Reardon and Flavel (1987) reported a large mixed-sex colony of *N. geoffroyi* supporting the occurrence of multiple-male multiple-female congregations. It is possible that the composition of colonies in manmade structures is not representative of typical behaviour. Consequently, in the case of polyandry, it is not clear whether males 'invade' single-male multiple-female colonies to mate with females, or whether polyandry occurs freely within mixed sexed colonies. Both species also give birth to twins, although the twinning rate is believed to be higher for *N. geoffroyi* (Churchill 2008). This raises the possibility of multiple paternity. Only one *N. geoffroyi* female gave birth to twins in the captive study by Hosken (1998), and as stated above, all offspring were fathered by the same individual. Consequently, the question of multiple paternity remains unanswered.

We aim to test the hypothesis that *N. gouldi* and *N. geoffroyi* display female natal philopatry and male biased dispersal. Male biased dispersal is highly correlated with a polygynous mating system and would provide further support for polygyny in wild populations (Dobson 1982). Evidence of polygyny, and polyandry, may be provided by the identification of half siblings indicating males and/or females had mated with different individuals over multiple seasons. Similarly, the identification of juvenile half siblings born in the same season would provide evidence of multiple paternity, provided the half siblings share the same mother. Given the relatedness of the two species we expect them to display similar dispersal patterns and social structure. Female philopatry should result in higher numbers of female relatives within populations than males. Consequently we expect to find more female relatives at the population level than male relatives, or female-male relatives, and we expect this trend will be more pronounced at the roost level for *N. geoffroyi*. The hypothesis further predicts that female relatives will comprise a significant component of social structure at the population level for both species, and at the roost level for *N. geoffroyi*. High numbers of relatives at the

roost level for *N. geoffroyi* will provide an insight into the role of kin selection and reciprocal altruism in cooperative roosting. In particular, high numbers of female relatives at the roost level may suggest that female relatives play an important role in group formation and social behaviour.

METHODS

Fieldwork was conducted at 14 sites across western Victoria and south-eastern South Australia comprising a mixture of expansive forest regions and smaller fragmented patches of remnant vegetation (Figure 5.1). Further details regarding these sites are provided in Chapter 3. No *N. gouldi* were caught at the small (60ha) and highly isolated Woolsthorpe fragment, with low numbers obtained at four other locations; Warreanga (n=3), Weecurra (n=3), Mt Napier (n=2) and Framlingham (n=1). *N. gouldi* was captured in higher numbers (n=14-66) at the remaining nine sites and *N. geoffroyi* was readily caught at all 14 locations (n=15-69). In addition to these sites, three roosting groups of *N. geoffroyi* were sampled in farm buildings south of Mt Gambier in South Australia. Two of the roosts were located within wall and roof cavities of two separate houses (Telford House and Feast House), while the third was located within a shearing shed adjacent to one of the houses (Telford Shed) (Figure 5.1).

Eight harp traps were used to capture the target species over two field seasons from November to April 2008-2009 and 2009-2010, totalling 1252 trap nights. The three roost sites were sampled over two days in March 2009. We set up netting around emergence points to funnel bats into harp traps and as a consequence we likely sampled most individuals within each roost. Genetic samples were obtained by taking a 3.5mm biopsy from the wing membrane. Further details regarding field methods are provided in Chapter 3. Sixteen microsatellite markers developed for the study (see Chapter 2) were used to genotype 265 *N. gouldi* at 15 loci and 659 *N. geoffroyi* at 9 loci, the latter including 157 individuals from the three sampled roosts. PCR products were sent to AGRF for electrophoresis and visualisation on an ABI 3730 DNA Analyser (Applied biosystems) with further laboratory methods provided in Chapters 2 and 3. Genotypes were scored using GENEMAPPER v.3.5.1 (Applied Biosystems) and MICROCHECKER v.2.2.3 (Van Oosterhout et al. 2004) was used to check data for scoring errors and the presence of null alleles. We used GENEPOP v.3.4 (Raymond & Rousset 1995)

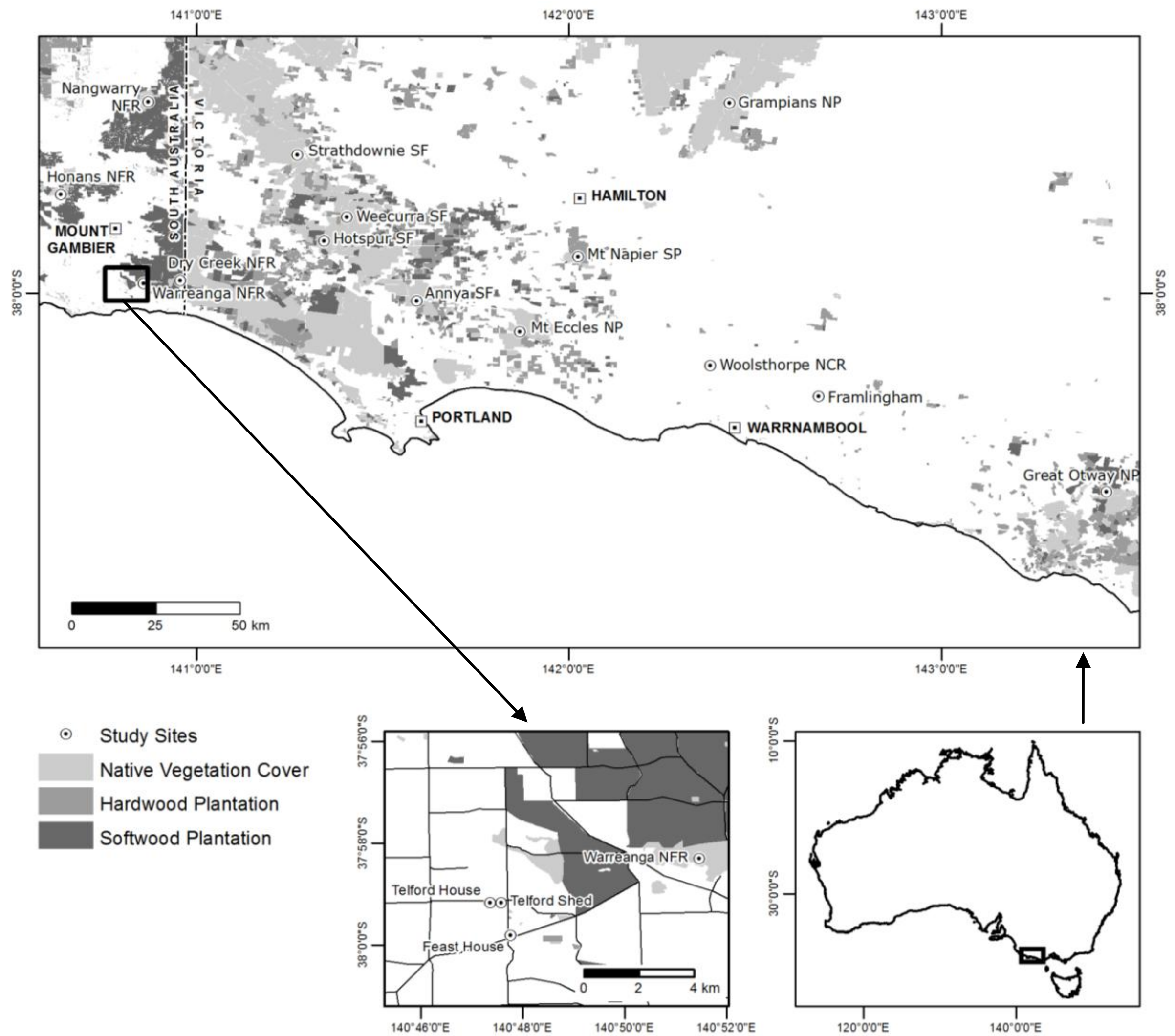


Figure 5.1: The distribution of 14 *N. geoffroyi* and *N. gouldi* study sites across Victoria and South Australia. *Nyctophilus* were sampled in native vegetation (light grey) embedded within a matrix of hardwood (mid grey) and softwood plantations (dark grey) and agricultural land (white). The location of three *N. geoffroyi* roosts in farm buildings is displayed in the lower expansion showing their proximity to Warreanga NFR.

to test populations and loci for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) with sequential Bonferroni corrections made for multiple comparisons (Rice 1989).

To assess dispersal patterns and compare trends between males and females we used GENALEX v. 6 (Peakall & Smouse 2006) to conduct Mantel tests and spatial autocorrelations using individual pairwise geographic coordinates (GPS trap locations) and genetic distance as defined by Smouse & Peakall (1999). Our dataset contained 12 *N. gouldi*

with missing data for at least one locus so we utilised the 'Interpolate Missing' data option to fill in blanks as Mantel tests can be sensitive to missing data. To further assess sexual differences in dispersal patterns we performed first-generation migrant detection (F_0) in GENECLASS v. 2 (Piry et al. 2004) to identify putative dispersal events. Migrant detection was performed using the Bayesian method of Rannla & Mountain (1997) and the Monte Carlo re-sampling approach of Paetkau et al. (2004) with 10 000 simulated individuals and a significance level of 0.05. As several forests were not sampled across the study region we utilised a model that assumes not all potential source populations have been sampled ('L=home').

To assess social composition and the frequency of relatives at the population and roost level we used KINGROUP v. 2 (Konovalov et al. 2004) to identify parent-offspring, full siblings, half siblings and cousins using the likelihood method of Queller & Goodnight (1989). To further assess relatedness at the roost and population level we calculated pairwise relatedness (*r*) in GENALEX using the method of Queller & Goodnight (1989). We used the 'Pop Means' function to calculate mean pairwise *r* for each population and roost with 95% confidence bounds (9999 bootstraps), and to test whether mean *r* was significantly higher or lower than a mean permuted value (9999 permutations). To further assess background levels of relatedness we also assessed the distribution and frequency of pairwise *r* values at the population level for *N. gouldi*, and the population and roost level for *N. geoffroyi*. To achieve this we separately calculated the pairwise *r* values for each population and roost and then pooled results for *N. gouldi* populations, *N. geoffroyi* populations and *N. geoffroyi* roosts. We then calculated the proportion of *r* values within each 0.1 increment where, for example, pairwise *r* values between -0.05 and 0.05 were classed as '*r*=0' and values between 0.05 and 0.15 were classed as '*r*=0.1'. This process was repeated by independently assessing males and females so that the distribution and frequency of *r* values could be assessed at the population and roost level, with comparisons made between males, females and both sexes combined. For *N. gouldi* the analysis of relatives and relatedness excluded four sites with insufficient samples (3 or fewer: Warreanga, Wecurra, Mt Napier and Framlingham), reducing the number of sites to nine.

RESULTS

Dispersal strategies in long-eared bats

Spatial autocorrelations revealed a similar trend for male and female *N. gouldi* with significant positive *r* values for proximal individuals within 10km of each other and incidents of significant negative *r* values beyond 100km (Figures 5.2a and 5.2b). However, this trend was more pronounced for females which displayed three significant negative correlations compared to just one case for males. Mantel tests independently assessing *N. gouldi* sexes indicated a clear contrast between males and females. The correlation between genetic and geographic distance was not significant for males (R^2 = 0.0083, p = 0.056) but was highly significant for females (R^2 = 0.1295, p = 0.001). The identification of dispersal events in GENECLASS indicated a male bias in dispersal with 12 of the 15 established dispersal events attributed to males.

Spatial autocorrelations detected little structure for *N. geoffroyi* sexes with few significant associations and little difference between the two (Figures 5.2c and 5.2d). Females displayed a significant positive correlation between individuals within 10km of each other and males revealed a significant negative correlation for individuals at a distance of 160km. Mantel tests also revealed little difference between the sexes with neither males (R^2 = 0.0017, p = 0.054) nor females (R^2 = 0.0005, p = 0.143) displaying a significant association between genetic and geographic distance. The identification of dispersal events in GENECLASS revealed no sexual bias in dispersal with an even number of cases attributed to males (n=27) and females (n=30).

Social structure in long-eared bats

Two hundred and fifty-six *N. gouldi* from nine populations were analysed for relatives and 26.5% (n=68) possessed a relative comprising 62 related pairs categorised as parent-offspring, full siblings, half siblings or cousins. Ten and a half percent of individuals had a parent or offspring (n=27), 14.8% of individuals had a full sibling (n=38), 6.3% had a half sibling

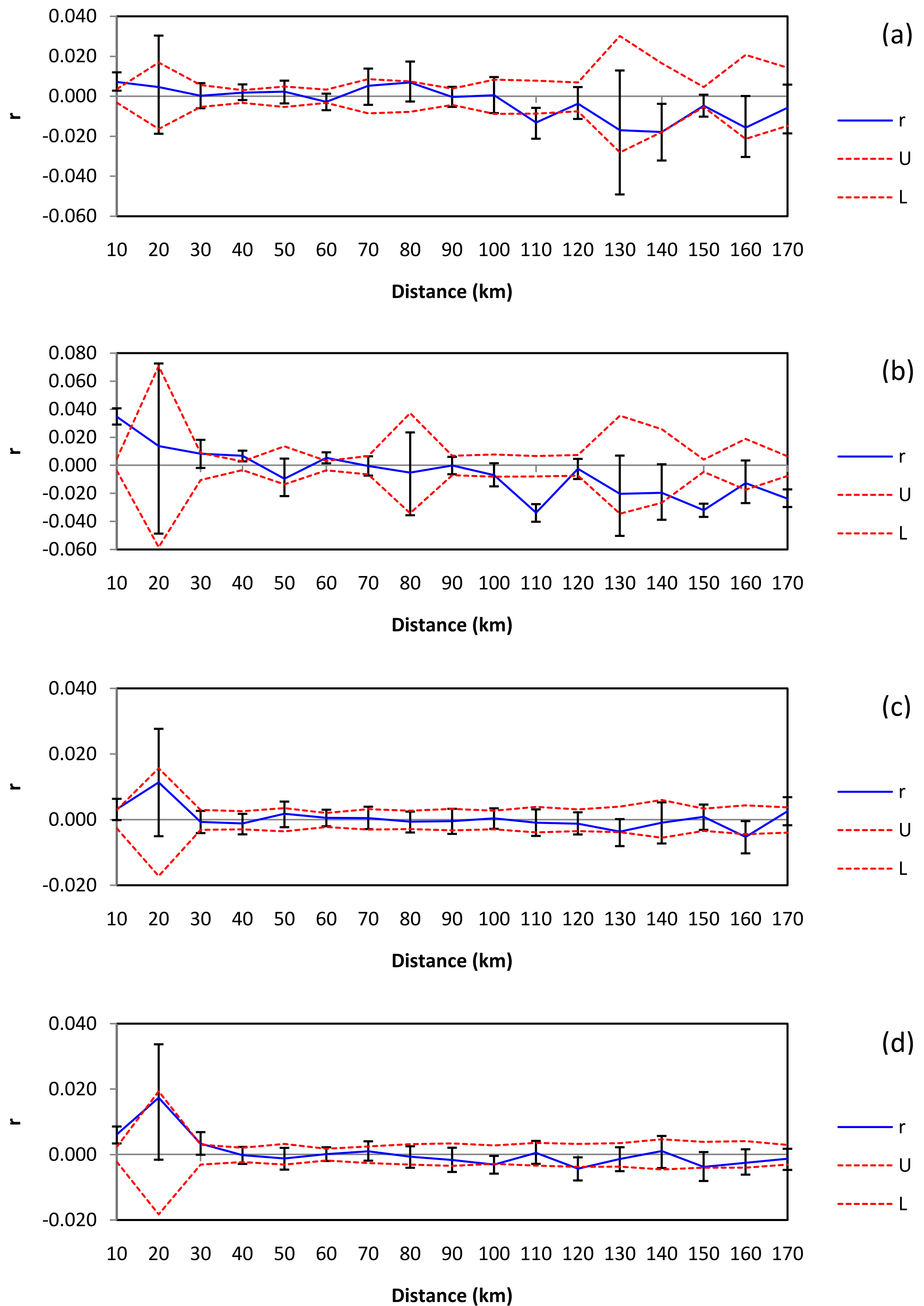


Figure 5.2: Results of spatial autocorrelations preformed in GENALEX illustrating mean r (——) with 95% upper and lower confidence levels (······). Distance classes are displayed along the *x*-axis in km. Figures represent: (a) *N. gouldi* males, (b) *N. gouldi* females, (c) *N. geoffroyi* males, and (d) *N. geoffroyi* females.

(n=16) and just 3.5% had a cousin (n=9) identified (Figure 5.3). The sexes were evenly represented in the total sample with 130 males and 126 females. Comparison of the sexes revealed that 75% (n=51) of relatives were females and 25% (n=17) were males, and that 40.5% of females possessed a relative compared to 13% of males. To compare social structure between males and females we calculated the number of female-female (FF), female-male (FM) and male-male (MM) dyads (Figure 5.4). There were five times more FF dyads (n=35) than MM dyads (n=7) while the number of FM dyads was intermediate (n=20) (Figure 5.4a). Females were significantly more likely to be related than males, or females and males (X^2=66.595, df=2, p=0). Full siblings were the most common association for FF and FM dyads, while parent-offspring associations were the most common MM dyads (Figure 5.4b).

The *N. geoffroyi* dataset at the population level comprised 502 individuals from 14 populations. 13.9% (n=70) of individuals possessed a relative comprising 38 related pairs. Out of 502 individuals 3.2% had a parent or offspring (n=16), 10.6% had a full sibling (n=53), no half siblings were detected and 0.4% of individuals had a cousin (n=2) (Figure 5.3). The sexes were evenly represented with 237 males and 265 females. Comparison of the sexes revealed that 57% (n=40) of relatives were females and 43% (n=30) were males, and that 12.7% of males and 15% of females possessed a relative. We indentified a similar number of

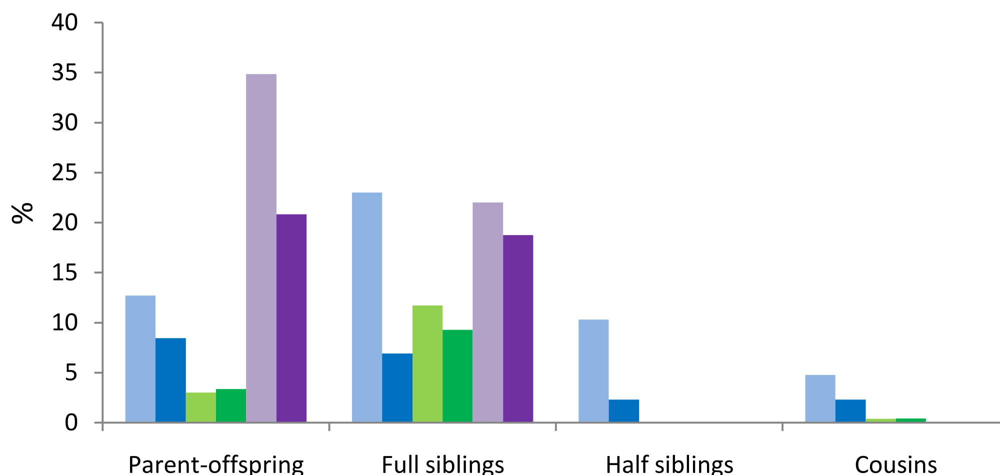


Figure 5.3: Social structure based on parent-offspring, full siblings, half siblings and cousins identified using KINGROUP for populations of *N. gouldi* (■) and *N. geoffroyi* (■), and within three artificial *N. geoffroyi* roosts (■). Females (■■■) and males (■■■) are compared in terms of the percentage of individuals with a relative in each category.

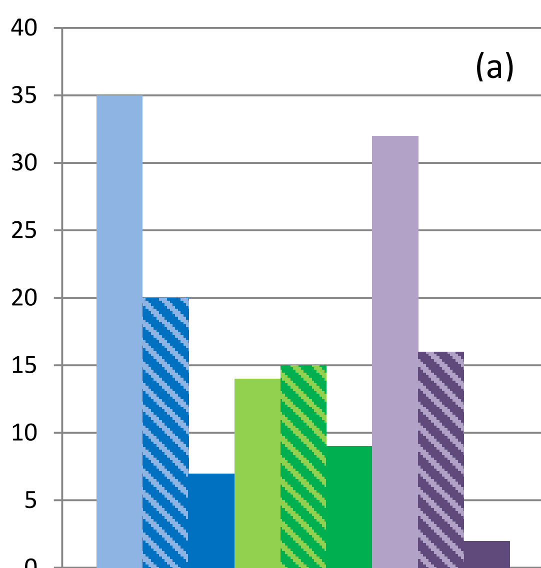
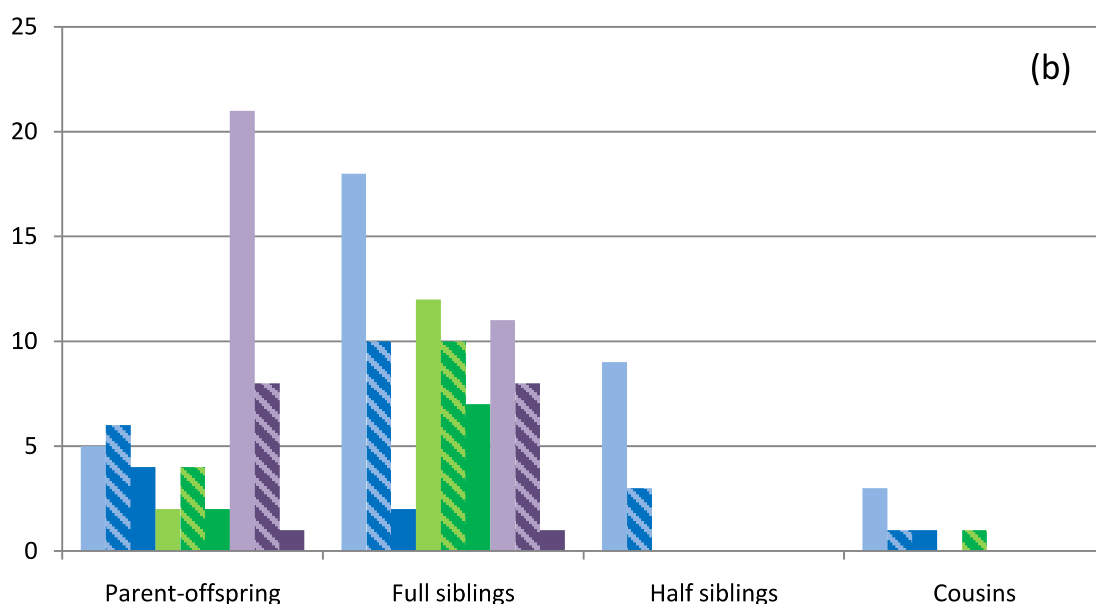


Figure 5.4: Comparison of social structure based on parent-offspring, full siblings, half siblings and cousins identified using KINGROUP. Three groups are compared: populations of *N. gouldi* (■) (n=256) and *N. geoffroyi* (■) (n=502), and three *N. geoffroyi* roosts (■) (n=157). Light shades indicate female-female relatives (■■■), dark shades male-male (■■■), and striation of light and dark shades indicates female-male relatives (■■■). Figure (a) compares the total number of female-female, male-male, and female-male related pairs. Figure (b) compares the number of related pairs assigned to each class of sexual dyad and within each of the four relative categories.



FF (n=14), MM (n=9) and FM (n=15) dyads, however, females were significantly more likely to be related than males, or females and males (X^2=16.833, df=2, p=0.0002) (Figure 5.4a). Full siblings were the most common type of relative for FF, MM and FM dyads (Figure 5.4b).

Analysis of 157 *N. geoffroyi* individuals sampled at the three roost sites revealed that 43.9% (n=69) possessed a relative comprising 50 related pairs. Overall 30.6% of the roosting individuals had a parent or offspring (n=48), 21% had a full sibling (n=33) and no half siblings or cousins were detected (Figure 5.3). The roosts were comprised of approximately twice as many females (n=109) as males (n=48). Comparison of the sexes revealed that 75%

(n=52) of relatives were females and 25% (n=17) were males, and that 47.7% of females and 35.4% of males possessed a relative. There were twice as many FF dyads (n=32) as FM dyads (n=16) and few MM dyads (n=2) (Figure 5.4a). Females were significantly more likely to be related than males, or females and males (X^2=25.015, df=2, p=0.0000037) (Figure 5.4a). Parent-offspring associations were the most common FF dyads and full sibling associations were the most common MM and FM dyads (Figure 5.4b). It should be noted that we had low juvenile capture rates for both species and parent-offspring dyads usually comprised two adults suggesting long-term site or group fidelity.

Overall the number of relatives in *N. gouldi* populations was significantly higher than the number of relatives in *N. geoffroyi* populations (X^2=20.689, df=1, p=0.0000054). *N. geoffroyi* also displayed a significantly higher number of relatives at the roost level than at the population level (X^2=7.697, df=1, p=0.00553).

Analysis of population and roost mean relatedness (*r*) revealed several significant differences (Figure 5.5). *N. gouldi* population mean *r* ranged from -0.05 at the Otways to 0.214 at the Grampians and four of the nine populations displayed a mean *r* significantly higher than the mean permuted value (Dry Creek, *r*=0.034, p=0.004; Honans, *r*=0.093, p=0.000; Annya, *r*=0.056, p=0.03; Grampians, *r*=0.214, p=0.000) (Figure 5.5a). The average population mean *r* for *N. gouldi* was 0.056 and 0.036 excluding the Grampians (previous research suggested the Grampians may be somewhat unusual; see Chapter 3). *N. geoffroyi* population and roost mean *r* ranged from -0.018 at the Otways to 0.039 at Telford House with three populations and two roosts displaying a mean *r* significantly higher than permuted (Nangwarry, *r*=0.016, p=0.037; Honans, *r*=0.022, p=0.001; Grampians, *r*=0.029, p=0.003; Telford House, *r*=0.039, p=0.001; Feast House, *r*=0.019, p=0.003) (Figure 5.5b). Average population mean *r* for *N. geoffroyi* was 0.006, and the roost average was 0.018. Consequently, average *r* for both species at the population level, and at the roost level for *N. geoffroyi*, was close to zero indicating that the background relatedness amongst individuals was low. This pattern was further reflected by the distribution of *r* values at the population and roost level (Figure 5.6). *r* values between females, males and both sexes all displayed a normal distribution with a peak frequency bounding zero within the -0.05 to 0.05 range (Figure 5.6). Therefore, despite the identification of relatives indicating females were significantly more likely to be related than males, or females and males, there was little difference in overall background relatedness.

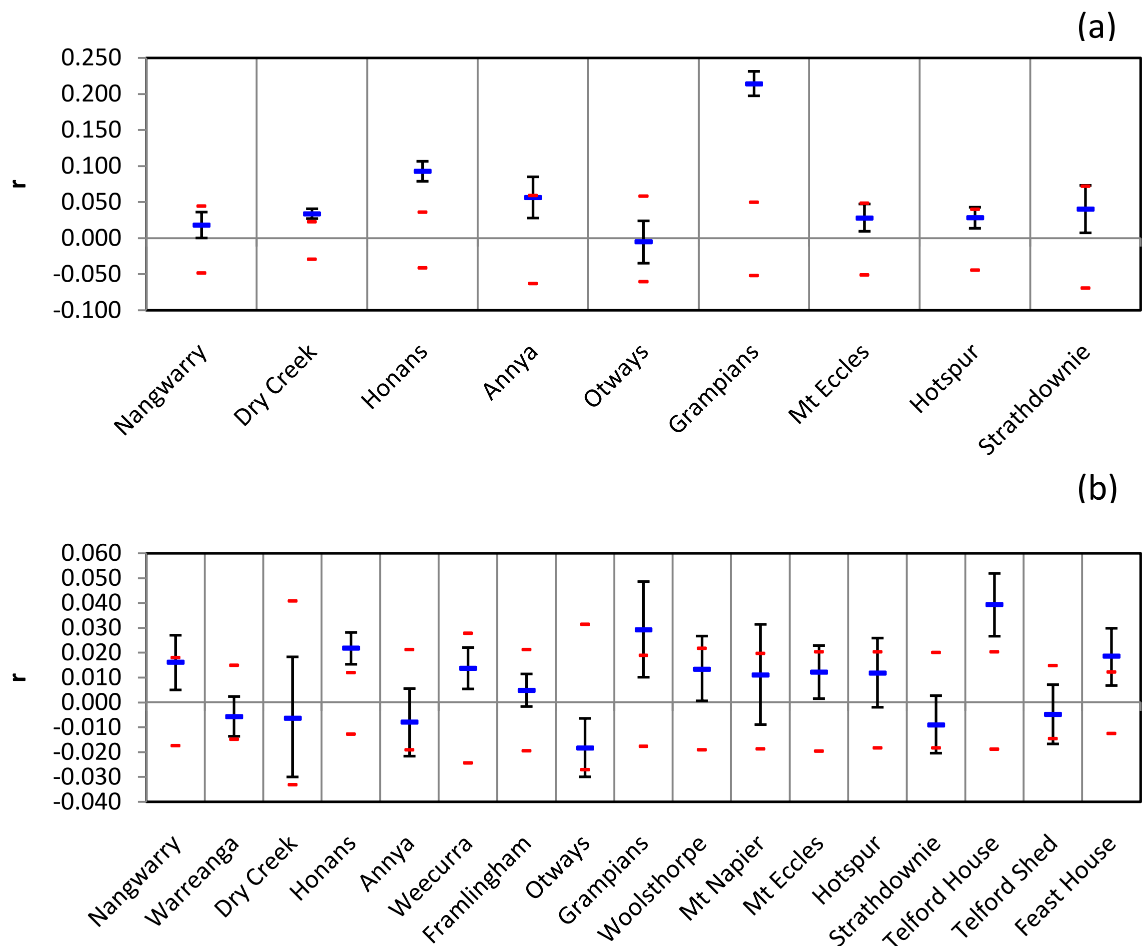


Figure 5.5: Mean (—) population and roost relatedness (*r*) for (a) *N. gouldi*, and (b) *N. geoffroyi*. Upper and lower confidence limits (95%) (—) that there is no difference between the populations and roosts based on 9999 permutations. Error bars based on bootstrap re-sampling (9999 bootstraps).

DISCUSSION

Dispersal patterns

As predicted, our genetic analyses of *N. gouldi* provide evidence of a male bias in dispersal and female philopatry within this species. Consistent with this strategy Mantel tests revealed a significant pattern of isolation by distance (IBD) for females indicating that proximal females were more closely related than distal females. Female philopatry was also support by the identification of relatives which indicated that females were significantly more likely to be related than males, or males and females. No IBD was detected for males which accounted for 80% of the dispersal events proposed by GENECLASS. Common amongst mammals,

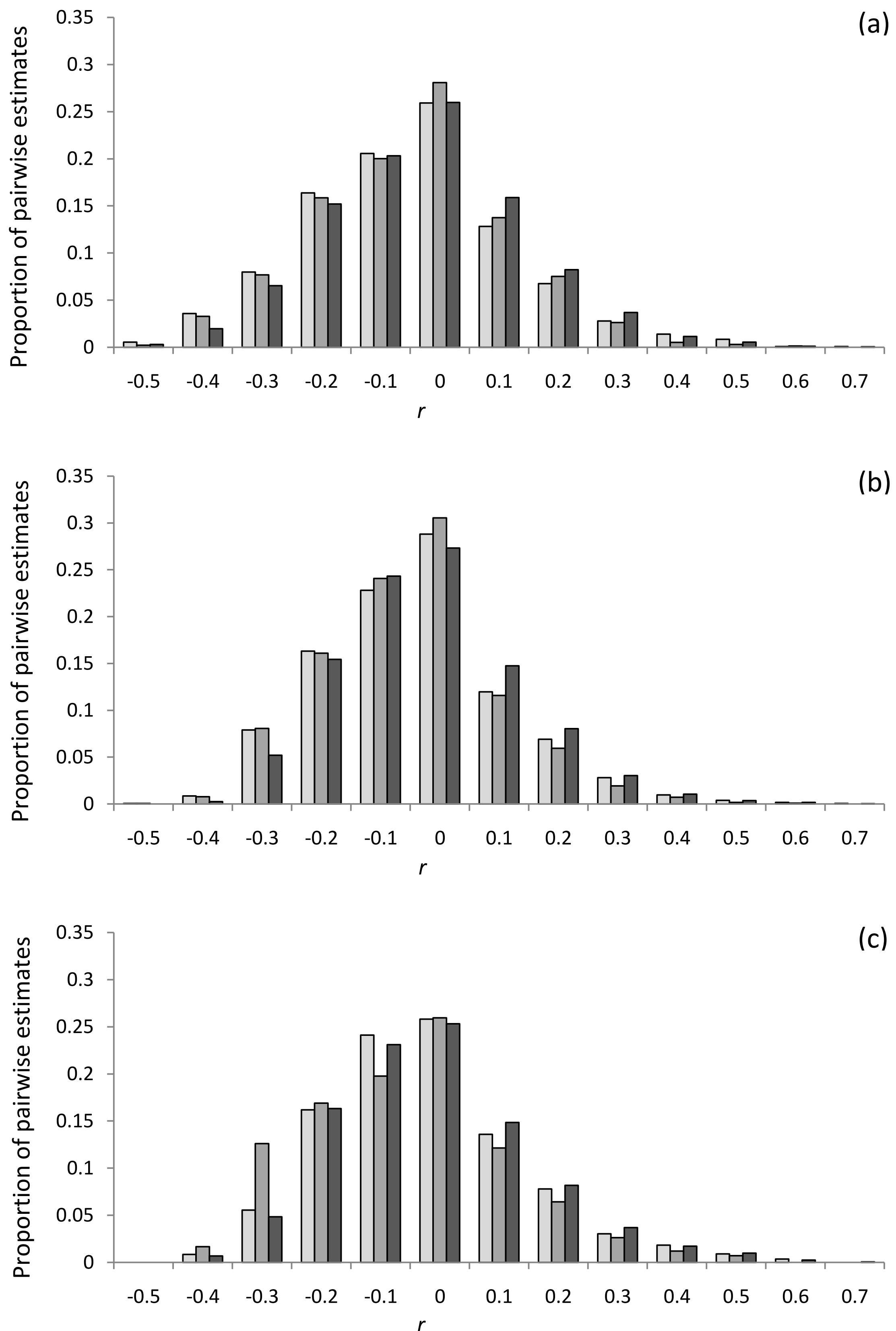


Figure 5.6: Distribution and frequency of pairwise relatedness (*r*) values comparing females (■), males (■) and both sexes (■) for (a) *N. gouldi* populations (females 1288 comparisons, males 1368 comparisons, both sexes 4756 comparisons), (b) *N. geoffroyi* populations (females 2342 comparisons, males 3175 comparisons, both sexes 10396 comparisons), and (c) *N. geoffroyi* roosts (females 2236 comparisons, males 420 comparisons, both sexes 4476 comparisons).

male biased dispersal has been identified in numerous chiropterans including *Rhinolophus monoceros* (Chen et al. 2008), *M. bechsteinii* (Kerth et al. 2002a), *Nyctalus noctula* (Petit et al. 2001), *Myotis myotis* (Petri et al. 1997), *Macroderma gigas* (Worthington Wilmer et al. 1999), *Myotis septentrionalis* (Arnold 2007) and *Corynorhinus townsendii ingens* (Weyandt et al. 2005). Although this appears to be the most common dispersal strategy within Chiroptera, other strategies are also employed. For example, both sexes are recruited into *Plecotus auritus* colonies (Burland et al. 1999), while in *Lophostoma silvicolum* both offspring disperse (Dechmann et al. 2007).

In contrast to *N. gouldi*, we found no evidence to suggest a sexual bias in dispersal for *N. geoffroyi*. This finding conflicted with our hypothesis that the two closely related species would display similar dispersal strategies. We did detect a significant female bias in the number of related *N. geoffroyi* consistent with female philopatry and male biased dispersal. However, male and female *N. geoffroyi* are known to roost separately (Churchill 2008; Lumsden et al. 2002a; Lumsden et al. 2002b; Reardon & Flavel 1987) and a localised bias in female relatives could reflect a pattern of social structure that occurs independently of dispersal strategies. We propose that large population sizes and prolific male dispersal may have masked evidence of male biased dispersal that could be detected with nuclear markers. Consequently, we recommend the use of a sex-linked marker, such as mtDNA, coupled with more intensive sampling at fewer sites to resolve *N. geoffroyi* dispersal strategies.

Social structure

Our hypothesis that *N. gouldi* and *N. geoffroyi* would display similar social structure was only partially supported. In both species females were significantly more likely to be related than males, or males and females. However, this relationship was of greater significance for *N. gouldi*, and over 40% of *N. gouldi* females possessed a relative with females comprising 75% of relatives. This contrasted with *N. geoffroyi* where only 15% of females possessed a relative and females accounted for 57% of relatives. *N. gouldi* also displayed significantly more relatives than *N. geoffroyi* at the population level with approximately twice as many *N. gouldi* (26.5%) possessing a relative than *N. geoffroyi* (13.9%). This suggests that social bonds between relatives may play a more significant role in the social structure of *N. gouldi* populations. However, as acknowledged regarding dispersal patterns, we cannot rule out that these differences are purely due to larger *N. geoffroyi* population sizes. Had we sampled a

larger proportion of *N. geoffroyi* populations we may have identified a similar ratio of relatives in both species. Nevertheless, *N. gouldi* populations contained a substantial number of related individuals suggesting that relatives comprise a significant component of social structure. This trend was most pronounced for female relatives, especially sisters, suggesting that the bonds between female full siblings may be of particular social significance. Although fewer relatives were detected within *N. geoffroyi* populations the same pattern was observed: female full siblings were the most common type of relatives detected.

The social structure of *N. geoffroyi* roosts differed significantly from that detected at the population level for the species. *N. geoffroyi* roosts displayed more similarity to *N. gouldi* populations with 47.7% of females possessing a relative and females comprising 75.4% of relatives. However, the similarities ended there as 43.9% of roosting individuals possessed a relative, the roosts contained fewer males (30.6%) than females (69.4%), and males frequently possessed relatives (35.4%). While full siblings were the most common relatives for both species at the population level, parent-offspring dyads were the most common associations within the roosts. The high number of parent-offspring was driven by female-female (FF) associations and may be due to female philopatry and recruitment into the colonies. Nevertheless, male-male (MM) and female-male (FM) dyads within roosts displayed similar numbers of parent-offspring and full siblings contrasting *N. geoffroyi* populations which contained more full siblings. The skew towards parent-offspring dyads may be a consequence of recent breeding activity at the sites, discussed below.

N. geoffroyi parturition occurs in October and November and lactation generally ceases by February when juveniles can no longer be distinguished from adults and when dispersal occurs (Churchill 2008; Hosken 1997). Mating typically commences in Autumn between March and May (Churchill 2008; Hosken 1997). Due to the time of roost sampling (March), and the mixed sex composition of the colonies, it is unclear whether the composition of the roosts represents relictual maternity colonies or congregations forming in anticipation of mating. The landowner who alerted us to the colonies confirmed that the roosts were permanent year-round colonies and that juveniles were readily observed at the Telford Shed site several months earlier (A.Telford. *pers.comm.*). This confirmed the use of the Telford Shed site as a maternity roost, and given the permanent status of all three colonies, it is highly likely that all three sites contained maternity colonies several months prior to sampling.

As discussed, female and male *N. geoffroyi* typically roost apart, alone or in small groups, with maternity colonies of up to 30 females forming often accompanied by a male (Churchill 2008; Lumsden et al. 2002a; Lumsden et al. 2002b; Reardon & Flavel 1987). Other large mixed-sex colonies, such as ours, have been reported in buildings elsewhere (Churchill 2008; Lumsden et al. 2002a; Lumsden et al. 2002b; Reardon & Flavel 1987), and it is possible that these artificial spaces facilitate year-round co-roosting behaviour between the sexes. Such atypical mixing of the sexes could potentially be sustained through the provision of varied thermal conditions to suit both sexes (Lumsden et al. 2002b; Turbill 2006; Turbill & Geiser 2006), or through internal compartmentalisation of the space to maintain strict social structures.

If the hypothesis is true that the composition of the roosts represents a relictual maternity colony, it is not clear whether this scenario is typical of *N. geoffroyi* roosts at this time of year, or whether it is a consequence of artificial roosting sites. Lumsden et al. (2002a) found that *N. geoffroyi*, including breeding females, would travel up to 12km to forage within agricultural land suggesting that agricultural land may represent an optimal foraging habitat for the species. Combined with thermally diverse and abundant roost sites within manmade structures, it is possible that farms may facilitate increased philopatry or prolonged parental care.

In contrast to the identification of relatives, the calculation of pairwise relatedness (*r*) revealed little difference in the distribution and frequency of *r* values between the two species, or between *N. geoffroyi* at the roost and population level. This seems counter intuitive given the differences identified through the assessment of related dyads. However given the pairwise comparisons for *N. gouldi* and *N. geoffroyi* populations and *N. geoffroyi* roosts tally 4756, 10396 and 4476 respectively, the respective identification of 62, 70 and 50 related pairs gives context to the small proportion of pairwise comparisons constituting relatives. Despite this apparent contradiction there was some agreement between the two approaches. Female *N. geoffroyi* within roosts appear to display a greater proportion of positive *r* values compared to males which concurs with the higher incidence of female relatives compared to males. However, *N. gouldi* displayed a higher proportion of positive *r* values for males compared to females, conflicting with the analysis of relatives which showed a clear female bias in the frequency of relatives.

There were also some seemingly conflicting results between the identification of relatives and the calculation of mean population and roost relatedness (*r*). An increase in the number of related dyads did not always coincide with an increase in population or roost mean *r*. For example, the two roosts with a mean *r* significantly higher than permuted, Telford House (mean *r*= 0.039) and Feast House (mean *r*=0.019) contained lower percentages of relatives (34% and 38% respectively) compared to Telford Shed (58%) which had the lowest mean *r* amongst the roosts (mean *r*= -0.05). This again highlights the fact that related pairs actually comprised a small proportion of pairwise comparisons. In reality the populations and roosts were predominantly comprised of unrelated individuals, thus the background mean *r* was low. Kerth et al. (2002b) obtained a similar result for *Myotis bechsteinii* colonies where despite 75% of individuals possessing a relative mean *r* was close to zero (*r* = 0.02). They concluded that average *r* is a poor predictor of kin selection as it fails to recognise family groups amidst the background noise of unrelated individuals. Rossiter et al. (2002) made a similar discovery regarding *Rhinolophus ferrumequinum* which displayed low background mean *r* (0.03) amongst colony females despite the presence of matriline groups within the colony with average relatedness levels of 0.17-0.64.

Our results suggest that while bonds between related females may represent an important aspect of social structure in *N. gouldi* and *N. geoffroyi*, they are not necessarily the principal factor driving sociality. Both Rossiter et al. (2002) and Kerth et al. (2002b) discuss similar scenarios for *R. ferrumequinum* and *M. bechsteinii* and propose that reciprocal altruism , not kin selection, is the dominant mechanism behind sociality and colonialism. For example, basic cooperative behaviour such as clustering may be shared between conspecifics while higher order cooperation such as cooperative breeding or information transfer may be restricted to kin. Rossiter et al. (2002) found that cooperative foraging behaviour in *R. ferrumequinum* was indeed biased towards kin indicating that individuals did discriminate between relatives and non-relatives to engage in cooperative behaviour. Similar discrimination in cooperative behaviour has been proposed in *Desmodus rotundus* where food sharing and grooming is positively correlated with relatedness (Wilkinson 1984, 1986). We concur with conclusions made by Kerth et al. (2002b) regarding *M. bechsteinii*, that low background *r* suggests that kin selection does not constitute the principal factor driving social systems for *N. gouldi* and *N. geoffroyi*. Instead, reciprocal altruism may be the dominant mechanism driving sociality within these two species, but without behavioural observations we can only speculate. Nevertheless, kin selection does represent a significant component of

social structure and may play an important role in cooperative behaviour. This study also supports findings by Kerth et al. (2002b) that average *r* is a poor indicator of the prevalence of kin selection and the significance of social bonds between relatives and fails to detect biases in kin selection between the sexes.

Cooperative roosting provides numerous benefits to bats including a reduction in thermoregulatory costs (Racey & Swift 1981; Wilde et al. 1995), reduced predation risks through clustered emergence (Kalcounis & Brigham 1994; Speakman et al. 1999), information transfer regarding foraging (Wilkinson 1992b) or roosting sites (Kerth et al. 2001), and cooperative breeding (Kerth et al. 2001). Related individuals have been found to represent a significant proportion of roosting groups or colonies in other bat species, as have high numbers of female relatives in particular (Kerth et al. 2000; Metheny et al. 2008). The practice of kin selection provides individuals with a genetic benefit by preferentially increasing the fitness of their own gene-pool. However, given the proportion of unrelated individuals in *N. geoffroyi* roosts reciprocal altruism may be equally important, a strategy that also provides inclusive fitness and a behaviour that has been reported elsewhere within Chiroptera (Wilkinson 1988).

Mating systems

The identification of male biased dispersal and female philopatry in *N. gouldi* provides support for a polygynous mating system in wild populations (Dobson 1982). *N. gouldi* displayed a high number of half siblings providing direct evidence of polygamy, at least across different mating seasons. We identified a single case for *N. gouldi* at the Grampians where a parent was assigned to a half sibling. A juvenile male (Ngo192) possessed an adult female half sibling (Ngo182) which did not share the same mother (Ngo185). This case suggests that males will mate with multiple females across different seasons. No juvenile half siblings were identified to indicate that either sex bred with multiple mates within the same breeding season. We identified full siblings born in different seasons (eg. an adult and a juvenile) for both species indicating that mating pairs may mate across multiple years. We did not identify any half siblings that shared the same mother and consequently we could not provide any direct evidence of polyandry in wild populations. However, we maintain that polyandry is likely in both *N. gouldi* and *N. geoffroyi* based on evidence of sperm competition, copulatory plugs, and reports of males mating with torpid females over winter

(Churchill 2008; Hosken 1998). We did not identify any juvenile half siblings to provide evidence of multiple paternity and consequently this question remains unanswered. Furthermore, we did not identify any half siblings or any direct evidence of male biased dispersal for *N. geoffroyi* providing no support for polygamy in wild populations. However, we did find that related females were significantly more common than male relatives, or male and female relatives. This finding provided some support for female philopatry and consequently indirect evidence of male biased dispersal. As a final observation, long-term female philopatry should lead to high numbers of female half siblings, unless males retain their position as dominant breeders across breeding seasons. We identified no half siblings for *N. geoffroyi* providing support for this mating strategy.

Conclusion

The application of molecular techniques is a powerful tool for probing cryptic aspects of chiropteran ecology. We have shed light on dispersal patterns, social structure and mating systems within *N. gouldi* and *N. geoffroyi*. Our hypotheses that *N. gouldi* would display male biased dispersal, female philopatry and evidence of a polygynous mating system were confirmed. Similarly, our prediction that female relatives would comprise a significant component of social structure within populations was established and, as hypothesised, the trend for *N. geoffroyi* was more pronounced at the roost level. The bias towards female relatives in both species suggests that matrilineal social groups may play an important role in the behavioural and social ecology of these species. This pattern has been identified in other chiropterans such as *R. ferrumequinum* (Rossiter et al. 2002). Social structure within *N. geoffroyi* roosts suggests that female relatives may play an important role in sociality or group formation for roosting colonies. However, reciprocal altruism rather than kin selection appears to be the principal mechanism behind cooperative roosting.

Although we identified different patterns in some aspects of the two species sociobiology, we cannot exclude the possibility that patterns evident within *N. gouldi* populations were masked in *N. geoffroyi* by larger population sizes and prolific male dispersal. However, the lack of clear evidence to support male biased dispersal or polygamy for *N. geoffroyi* was unexpected. We predict that further research into *N. geoffroyi* sociobiology will reveal similar patterns to those we have identified for *N. gouldi*, although, the bias in philopatry and dispersal may be weaker. We recommend that future research into *N. geoffroyi* sociobiology obtains larger

sample sizes and utilises sex-linked markers, such as mtDNA and Y-chromosome markers, for greater resolving power to determine dispersal patterns.

Chapter 6

General Discussion

The following general discussion focuses on further management implications derived from this thesis and considers future prospects for research. This thesis has made a significant contribution in its objectives to bridge the knowledge gap in several aspects of chiropteran conservation and ecology. Prior to the commencement of this thesis there were no published studies investigating the influence of habitat fragmentation on gene flow or genetic diversity for any chiropteran species. This was cause for concern given two important facts: habitat fragmentation represents one of the key threats facing global biodiversity and chiropterans comprise more than 20% of described mammal species (Baillie et al. 2004; Bennett 2003; (Wilson & Reeder 2005). Nearly a quarter of all chiropterans are listed on the IUCN Red List as threatened (Critically Endangered, Endangered or Vulnerable) and habitat destruction, degradation and fragmentation are identified as impacting 86% of threatened mammal species (Baillie et al. 2004; Mickleburgh et al. 2002). At a national level, the primary cause for listing 60% of threatened Australian chiropterans is habitat loss, incorporating land clearing, fragmentation and modification (Duncan et al. 1999). Furthermore, the Australian Action Plan for Bats identifies 'the impact of forest fragmentation on bats at a landscape scale' as a priority for research efforts (Duncan et al. 1999). Presence and abundance studies have documented changes to the composition of chiropteran communities due to habitat fragmentation, including the loss of some species from isolated fragments (Cosson et al. 1999; Estrada & Coates-Estrada 2002; Estrada et al. 1993; Medina et al. 2007; Schulze et al. 2000). Therefore despite their vagility, it appears that not all chiropterans are immune to impacts on dispersal and population connectivity due to habitat fragmentation. Clearly the impact of habitat fragmentation on bat population connectivity warrants urgent critical assessment.

Insights into chiropteran responses to habitat fragmentation

Adding to published studies by Struebig et al. (2011) and Meyer et al. (2009), this study used genetic data to provide a significant insight into chiropteran responses to habitat fragmentation. The study has documented changes to population structure and connectivity,

genetic diversity, inbreeding and relatedness, and sex ratios. In doing so, this research has shed further light on the range of potential impacts facing chiropterans. The comparative influence of habitat fragmentation on *N. gouldi* and *N. geoffroyi* also serves as an interesting example into how two morphologically, ecologically and taxonomically similar species can respond in contrasting ways to this threatening process. The investigation confirmed the prediction that *N. gouldi* would be more significantly influenced by habitat fragmentation than *N. geoffroyi*. As hypothesised, the differing responses are most likely driven by the fact that *N. geoffroyi* displays greater habitat and roosting plasticity, and is recorded commuting, foraging and roosting within agricultural land (Churchill 2008; Lumsden & Bennett 2005; Lumsden et al. 2002a). In contrast, *N. gouldi* appears to display a distribution limited to forest and woodland, more selective roosting requirements, and rarity in agricultural land (Churchill 2008; Lumsden & Bennett 2005; Lunney et al. 1988).

The contrasting response of the two species indicates that wing morphology alone may be an unreliable predictor of chiropteran vulnerability to habitat fragmentation. The use of wing morphology for such predictive purposes is based on two characteristics, low aspect ratio and low wing loading, which are adaptations for slow manoeuvrable flight indicating specialisation for cluttered habitat, and reduced energetic efficiency for long distance flight suggesting limited dispersal capacity. Although these morphological traits have been linked with extinction risk (Jones et al. 2003; Safi & Kerth 2004) and vulnerability to habitat fragmentation (Albrecht et al. 2007; Meyer et al. 2008), our study clearly demonstrates its limitations for identifying chiropterans of conservation concern. This point was also acknowledged by Safi & Kerth (2004) who recognised that wing morphology alone cannot explain differences between species foraging behaviour or habitat adaptations, and that bats are also influenced by the availability of critical resources such as roosting sites. This study supports these considerations, and in the case of *N. gouldi* and *N. geoffroyi* it appears that tolerance to the matrix and differences in ecological plasticity, and possibly roosting requirements, are the likely determinants of vulnerability to habitat fragmentation, not wing morphology.

Tolerance to the matrix is a well known determinant of species responses to habitat fragmentation (Antongiovanni & Metzger 2005; Laurance 1991; Laurance et al. 2011). Species will only be affected by habitat fragmentation and subject to isolation if they perceive the matrix as a hostile or suboptimal landscape. Consequently, evidence of a species ability

to readily traverse the matrix, utilise resources within the matrix, or permanently reside within the matrix are the strongest indicators that a species will be resilient to habitat fragmentation. The opposite is also true; species that do not display these characteristics may be vulnerable and prone to population isolation following habitat fragmentation. An additional indirect measure of tolerance to the matrix is a species' degree of specialisation. Species with specialist habitat or resource needs are less likely to find these resources within modified landscapes than generalist species able to exploit a variety of habitats and resources. Consequently, indicators of specialisation can be useful predictors of vulnerability to habitat fragmentation, as illustrated by support for chiropteran wing morphology as a predictive trait (Albrecht et al. 2007; Meyer et al. 2008). Limited geographic range suggesting narrow ecological tolerances, and evidence of specialised dietary or habitat requirements may also serve as informative predictors of species responses to habitat fragmentation.

Evidence of tolerance to the matrix and habitat specialisation proved valuable predictors of vulnerability to habitat fragmentation in this study, overriding predictions derived from wing morphology alone. With several studies supporting the use of wing morphology as a predictor of chiropteran vulnerability to habitat fragmentation (Albrecht et al. 2007; Meyer et al. 2008) I recommend that researchers use caution when using this approach. Instead, I propose that the predictive framework for chiropteran responses to habitat fragmentation be refined to include consideration of habitat and roosting specialisation, and tolerance to the matrix, in conjunction with this meritorious morphological trait. This refinement will improve the accuracy and reliability of efforts to predict chiropterans at risk to habitat fragmentation.

Candidates for future chiropteran studies assessing the impact of habitat fragmentation

Until the impact of habitat fragmentation on population connectivity is assessed in additional bat species we will not have a clear idea how prevalent vulnerability to habitat fragmentation is within Chiroptera. The results of this research suggest that future studies should start by assessing species with traits similar to *N. gouldi*. Key traits to consider include wing morphology (low aspect ratio and low wing loading), habitat specialisation, a distribution limited to regions of forest or woodland, and direct evidence of matrix avoidance or a positive association with tree density. Based on these considerations several Australian bat species

may serve as good candidates for future studies assessing the impact of habitat fragmentation on chiropteran population connectivity.

Law et al. (1999) used ultrasonic detectors to assess bat activity across a range of habitat categories including continuous forest, fragmented forest and open areas. The activity of three vespertilionids, *Chalinolobus morio*, *Vespadelus regulus* and *Falsistrellus tasmaniensis*, was positively associated with habitat area and habitat diversity and negatively associated with habitat isolation, suggesting sensitivity to habitat fragmentation (Law et al. 1999). *C. morio* activity was significantly greater in continuous forest than small forests and the species displayed a low detection rate within open spaces. *V. regulus* activity was lowest within small remnants, corridors and open spaces. *F. tasmaniensis* also displayed the greatest activity within continuous forest and was absent from small forest fragments and corridors, however, it was recorded moving through cleared landscapes. Lumsden and Bennett (2005) provided further evidence of possible sensitivity to habitat fragmentation in *C. morio* and *V. regulus* in their study assessing bat activity across a gradient of tree cover using sonic detectors and harp trapping. Via both sampling techniques *C. morio* activity displayed a significant positive correlation with tree cover. *V. regulus* activity was not significantly correlated with tree cover, but both sampling methods revealed the highest activity within densely treed paddocks and the lowest activity within open paddock.

Chalinolobus morio and *V. regulus* display geographic distributions concentrated in forest, woodland and mallee across southern and south-eastern Australia, however, both species distributions also include some regions of shrubland (Churchill 2008). *F. tasmaniensis* appears to display a higher degree of habitat specialisation with a distribution strictly limited to forest, woodland and mallee in south-eastern Australia (Churchill 2008). *C. morio* and *V. regulus* both display wing morphology similar to *N. geoffroyi* and *N. gouldi* characterised by low aspect ratio and wing loading, indicating adaptation for slow manoeuvrable flight suited to cluttered environments (Fullard et al. 1991; Norberg & Rayner 1987; O'neill & Taylor 1986; Rhodes 2002). In contrast, *F. tasmaniensis* displays wing morphology adapted for fast flight with limited manoeuvrability characterised by a higher aspect ratio (Norberg & Rayner 1987; O'neill & Taylor 1986) and wing loading (Norberg & Rayner 1987). Despite its differing wing morphology, *F. tasmaniensis* displays an affinity for tall (≥20m) forest where it forages in and around the canopy (Churchill 2008). Based on the collective evidence given

above I propose that *C. morio*, *V. regulus* and *F. tasmaniensis* all represent suitable candidates for future studies investigating the impact of habitat fragmentation on Australian chiropterans.

Management implications for *N. gouldi*

Endangered South Australian populations

Having proposed a threshold for *N. gouldi* population connectivity of 27km across agricultural land comprosed of pasture and plantation pine in Chapter 3, and identifying unimpeded gene flow across small agricultural distances <2km, I suggest that the true dispersal threshold lies somewhere between the two. Lack of captures at three Victorian fragmented sites (Framlingham, Woolsthrope and Mt Napier) impeded my capacity to further refine this threshold for *N. gouldi* population connectivity. Future studies could build upon this work by assessing gene flow between sites within this distance range to further refine a threshold estimate. In the meantime I recommend applying the precautionary principal by utilising the <2km threshold, 1.75km to be precise (see below), as a known agricultural distance across which *N. gouldi* dispersal is maintained, to guide conservation and revegetation efforts. However, as a species-specific guideline, this information should be considered in conjunction with data on additional taxa for a holistic approach to regional conservation and landscape management.

The results of this research suggest that *N. gouldi* dispersal events may occur across agricultural land but that dispersal rates are significantly reduced, leading to a range of measureable impacts within fragmented populations. These impacts include significant population differentiation, elevated measures of inbreeding and relatedness, reduced genetic diversity (standardised heterozygosity) and altered sex ratios. These findings have direct implications for long-term persistence of the endangered SA populations of *N. gouldi* which are restricted to limited and highly fragmented patches of remnant habitat. However, my data indicate that sufficient gene flow to limit population differentiation can be maintained across agricultural crossings spanning a collective distance of <1.75km, with the largest single gap not exceeding 1.25km. Therefore to improve connectivity between the SA populations revegetation could be conducted to establish stepping-stones or corridors according to these guidelines to bridge the agricultural gaps between sites. This approach could mitigate the negative impacts I have identified and secure the SA populations as a more robust

metapopulation with enhanced long-term prospects for persistence. However, other considerations may also need to be addressed to achieve this management outcome. Despite the proximity of Dry Creek to the extensive Lower Glenelg and Cobboboonee NPs the site still suffers from the same symptoms characterising the other isolated SA populations. As the smallest site included in our analyses (396ha) it may be that the limited size of this remnant is also influencing the genetic and demographic composition of the population. These issues may be resolved upon completion of the South East Biodiversity Corridors Network which will connect Dry Creek with neighbouring forest remnants within the local plantation pine matrix and significantly increase the effective habitat area for the population (ForestrySA 2003). The south East Biodiversity Corridors Network provides several opportunities for future research to improve conservation outcomes for the endangered SA populations of *N. gouldi*. I recommend further sampling be conducted within sites designated for inclusion within the corridor network to establish *N. gouldi* population structure prior to corridor establishment, particularly within the Mt Burr South and Caroline groups (see (ForestrySA 2003). Subsequent post corridor sampling would then be able to measure the effectiveness of corridors for facilitating *N. gouldi* dispersal, improving genetic diversity and normalising sex ratios.

The Grampians

The Grampians was a distinctive *N. gouldi* population within this study. It was clearly identified as the most unique population by Bayesian clustering tests, genetic differentiation (F_{ST} & D_{est}) and the identification of 12 private alleles. Initially I considered the population would comprise an unfragmented 'mainland' site to compare with fragmented 'island' populations. However, the site revealed some surprising characteristics including almost half of the identified related pairs within the study, the highest Internal Relatedness (IR), the second highest F_{IS}, lower observed than expected heterozygosity and Standardised Heterozygosity (SH) below parity. These findings were in stark contrast to the other unfragmented sites and were akin to results for the small isolated fragments in SA. This was surprising given the Grampians spans 167 000ha and the SA fragments of Nangwarry, Dry Creek and Honans cover 2218ha, 396ha and 1041ha respectively.

Pre-European estimates of vegetation cover indicate that the Grampians was naturally separated from neighbouring forests by a belt comprised primarily of two ecological

vegetation classes (EVCs), Plains Grassland which is devoid of tree cover, and Plains Grassy Woodland characterised by a tree canopy cover of 20% (DSE 2004a, b, 2011). EVCs represent an approach by the State Government of Victoria for describing broad vegetation categories (Woodgate et al. 1994). EVCs are modelled using GIS based on field data (floristics and vegetation structure) and environmental spatial data (soils, rainfall and topography). Pre-European estimates of EVC distributions are modelled in consultation with historical records such as Parish plans. The canopy cover present in Plains Grassy Woodland corresponds most closely with the 'moderately scattered' tree density class used by Lumsden & Bennett (2005) in which no *N. gouldi* were caught. It is reasonable to assume that the belt of grassland complexes surrounding the Grampians prior to European settlement may have posed a barrier or filter to *N. gouldi* dispersal effectively isolating the resident population. Pollen analysis of sediment cores from lake beds throughout the region indicate that vegetation types were fairly stable during the Holocene and that these grasslands could even date back to the late Pleistocene (Jones 1999).

The possible isolation of the Grampians since the early Holocene or late Pleistocene would explain the high F_{ST} and D_{est} values, the significant structure detected in our Bayesian tests, and the high number of private alleles. However, in such a large forest, isolation alone is unlikely to have produced the signs of inbreeding and elevated relatedness. These characteristics suggest that the population is small, either permanently due to limited or marginal habitat or temporarily as a consequence of a population bottleneck. In 2006 the region did experience a significant bushfire that ravaged much of the National Park. Such an event could have caused a population bottleneck, but our analyses revealed no signs that a bottleneck took place. Consequently it appears that despite the size of the Grampians suitable *N. gouldi* habitat may be limited, supporting only small numbers of the species. This hypothesis sits well with my trapping effort as I extensively trapped over a considerable area of the southern Grampians and located all but one individual along several kilometres of the Wannon River. This region was characterised by moister conditions and a greater abundance of older hollow bearing trees, a phenomenon I recognised at many of the sites where high capture rates were recorded. However, I believe this trend was most pronounced at the Grampians where these presumably optimal areas were less common and captures elsewhere were scarce. On the south coast of NSW Lunney et al. (1988) found that conditions suitable for *N. gouldi* were only provided in gullies along water lines. I propose the same pattern is likely in the Grampians.

With a potentially restricted population size concentrated in limited regions of suitable habitat, the population of *N. gouldi* at the Grampians could be at risk of threats associated with small populations including stochastic events and genetic and demographic processes (Caughley 1994). This is of particular concern as I propose that the Grampians population warrants recognition as a unique Management Unit (MU) based on the criteria of Moritz (1994). Further analysis of this population may elevate this status to an Evolutionarily Significant Unit (ESU) (Moritz 1994). I recommend that additional research is conducted on the Grampians population with sampling undertaken in the north and west of the National Park. This additional sampling could determine whether the limited distribution and genetic impoverishment detected in the south is indicative of the entire site, and confirm whether my concerns regarding the genetic health and size of the population are warranted.

Molecular insights into chiropteran ecology

As reviewed by Burland and Worthington Wilmer (2001), molecular techniques are ideally suited to the study of chiropterans, a group whose characteristics make them difficult to study with traditional field-based techniques. As a consequence of these difficulties there are many baseline ecological factors that remain unknown for chiropterans, particularly tree dwelling microbats. This fact prompted my investigation of dispersal strategies and social structure in *N. gouldi* and *N. geoffroyi*. Male biased dispersal and female philopatry was evident within populations of *N. gouldi*, a trend not revealed for *N. geoffroyi*. I acknowledge that higher abundance resulting in potentially less representative sampling, and high rates of dispersal, may have masked the identification of male biased dispersal for *N. geoffroyi*. This strategy may still be revealed for *N. geoffroyi* if future studies conduct more intensive sampling at fewer locations and assess male- and female-mediated gene flow through the assessment of mtDNA or y-chromosome markers respectively.

The assessment of social structure in both species indicated that female relatives may form bonds that play a significant role in the species behavioural ecology. The assessment of social structure within *N. geoffroyi* roosts also raised the possible role of kin selection and reciprocal altruism in cooperative roosting behaviour. Several studies have documented kin selection in chiropteran cooperative behaviour; for example, *Desmodus rotundus* displays a correlation between relatedness and both grooming and food sharing (blood regurgitation) (Wilkinson 1984, 1986). Similarly, *Rhinolophus ferrumequinum* has been shown to display kin selection

in cooperative foraging behaviour (Rossiter et al. 2002). Tree dwelling species pose many challenges for conducting joint observational, telemetric and social studies, particularly when they reside within dead or aging trees that can be inaccessible or dangerous for researchers to access. *N. geoffroyi* represents a prime opportunity in this respect as they frequently roost within accessible manmade structures such as barns and houses, as demonstrated in this study. Although one of the roosts sampled in this study was subsequently dislocated due to building renovations the other two roosts remain, providing the ideal opportunity to conduct further sociobiology studies such as the examples given above. Due to their propensity to form social groups and the diversity of social behaviour chiropterans represent ideal model organisms for sociobiology studies exploring the evolution of social and cooperative behaviours. Future studies capitalising on the suitability of *N. geoffroyi* may yield valuable insights into the evolution of kin selection, reciprocal altruism and sociality amongst mammals.

Conclusion

This study has contributed to the growing wealth of chiropteran ecological knowledge gained through the application of molecular techniques and further promotes continued efforts to capitalise upon these tools for the study of cryptic chiropterans. Chiroptera is a vast order displaying diverse ecologies, the continued application of molecular studies will, no doubt, reveal a rich tapestry of ecological strategies and behaviour, shedding much light on the evolution of mammalian behaviour. More importantly, this study has contributed valuable information for the conservation of Chiroptera, the second largest mammalian order, by identifying a range of potential impacts that can result from habitat fragmentation. As far as I am aware it is the first chiropteran study to utilise microsatellite markers to address the influence of habitat fragmentation on a host of factors including population connectivity, genetic diversity, inbreeding and relatedness, and changes to demography. In doing so my research has drawn attention to the potential threat posed by habitat fragmentation to chiropterans, which in extreme cases may jeopardise population and species persistence. These issues are of vital importance if we are to conserve global chiropteran fauna, maintain healthy ecosystems and manage chiropteran range shifts due to climate change. This field of study is still in its infancy but I hope that this example serves to raise a flag prompting further research into the magnitude and prevalence of chiropteran vulnerability to the process of habitat fragmentation.

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